

LATER REACTIONS OF CAROTENOID BIOSYNTHESIS

GEORGE BRITTON

Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, UK

Abstract—The later reactions of carotenoid biosynthesis are considered in two groups, the basic reactions occurring at the C-1,2 double bond, and the final modifications to the cyclic and acyclic structures thus produced. Recent work on these biosynthetic processes is reviewed, with the emphasis on the mechanism and stereochemistry of the reactions. The concept that carotenoid "half-molecules", rather than individual compounds are the substrates recognised by the enzymes is used to simplify the overall picture of the biosynthesis of cyclic and acyclic carotenoids, and ideas that enzyme aggregates may be involved are discussed. Attention is drawn to the difficulties of correlating details of the stereochemistry of biosynthesis of different carotenoids in different organisms.

INTRODUCTION

The early stages of carotenoid biosynthesis, as reviewed by Davies and Taylor¹ are common to the formation of all carotenoids. The wide range of structural variations encountered in natural carotenoids is due largely to differences in the later stages of biosynthesis, i.e. to events occurring after the normal desaturation sequence. This review is entitled "Later reactions of carotenoid biosynthesis" since it will deal with some of the reactions that normally follow desaturation in the formation of many carotenoids, rather than with pathways, real or postulated, for the biosynthesis of individual compounds. The later reactions will be considered as two groups, firstly the "basic reactions" involving the C-1,2 double bond, since these are involved, in some form, in the biosynthesis of almost all naturally occurring carotenoids, and secondly the final reactions, e.g. modifications introducing one specific structural feature such as a hydroxy or epoxy group into the molecule, or the reactions leading to the production of the specific carotenoids often restricted to a single species or group of species. The basic C-1,2 reactions have been studied extensively; on the later modifications there has been very little biochemical work but very much speculation about biosynthetic pathways from a consideration of the structures of the compounds involved.

It is intended to review, selectively, some recent developments in the study of the later stages of carotenoid biosynthesis, but one of the main aims is to correlate and integrate results obtained by workers in many laboratories, and to use these results to form a picture of the overall situation. Throughout, the emphasis will be on reactions and reaction sequences rather than on biosynthetic pathways.

BASIC REACTIONS AT THE C-1,2 DOUBLE BOND

Several addition reactions can occur at the C-1,2 double bond. The most obvious of these are seen in the acyclic carotenoid series. The best-known example is the addition of water characteristic of the photosynthetic and some other bacteria, but in some cases H₂ or a C₅ unit may be added. The cyclization reaction can be considered a similar case involving addition of the C-5,6 double bond. Figure 1 shows the overall similarity between the various reactions that can occur at C-1,2. Some of these reactions have been studied in some detail.

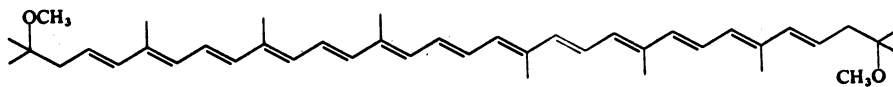
Acyclic carotenoids

The hydration reaction. Carotenoids with an acyclic end-group containing a free or substituted (e.g. glycosy-

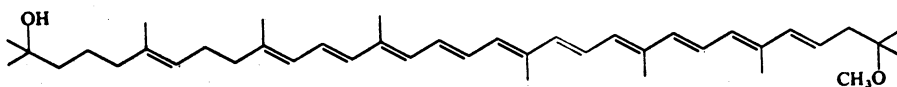
lated) tertiary hydroxy group at the C-1 position are frequently encountered in micro-organisms, especially bacteria. In particular, in the photosynthetic bacteria, especially the Rhodospirillaceae, the characteristic carotenoids are acyclic with tertiary hydroxy or methoxy groups at C-1 and often C-1', e.g. spirilloxanthin (1,1' - dimethoxy - 3,4,3',4' - tetradehydro - 1,2,1',2' - tetrahydro - ψ,ψ - carotene, I) and hydroxyspheroidene (1' - methoxy - 3',4' - didehydro - 1,2,7,8,1',2' - hexahydro - ψ,ψ - caroten - 1 - ol, II). The introduction of the hydroxy groups is an anaerobic process, and it is likely therefore that this is a hydration reaction (Fig. 1a, i).^{2,3} Recent studies⁴⁻⁶ have shown that the introduction of this hydroxy group is inhibited by nicotine and CPTA [2 - (p - chlorophenylthio)triethylammonium hydrochloride]. In *Rhodomicrobium vannielii* the normal main carotenoid, rhodopin (1,2 - dihydro - ψ,ψ - caroten - 1 - ol, III) is replaced by lycopene (ψ,ψ -carotene, IV) in the presence of these inhibitors.^{4,6} Removal of the inhibitors then allows the hydration to proceed and the lycopene is converted into rhodopin. The incorporation of labelled lycopene into rhodopin by a cell-free preparation from *Rm. vannielii* has also been demonstrated.⁶ In this case the hydroxy group is introduced after desaturation of the C-7, 11, 7' and 11' bonds is complete, although work with the inhibitor diphenylamine (DPA) has shown that the hydration can also occur if the C-7,8 double bond has not been introduced, e.g. hydroxy and methoxy derivatives of phytoene, (7,8,11,12,7',8',11',12' - octahydro - ψ,ψ - carotene), phytofluene (7,8,11,12,7',8' - hexahydro - ψ,ψ - carotene) and tetrahydrolycopene (7,8,11,12 - tetrahydro - ψ,ψ - carotene) have been isolated from DPA-inhibited cultures of *Rhodospirillum rubrum*.⁷⁻¹¹ This is also obviously the case in hydroxyspheroidene(II), a major carotenoid of some *Rhodopseudomonas* species. There is however no evidence for desaturation of the C-3,4 bond taking place before hydration of the C-1,2 double bond.

Hydrogenation. Somewhat similar to the hydration reaction is the hydrogenation of the C-1,2 double bond (Fig. 1a, ii), known only in one species, *Rhodopseudomonas viridis*.^{12,13} This reaction is also inhibited by nicotine and CPTA⁶ and can occur before desaturation at C-7,8; in the main carotenoid of this organism, 1,2 - dihydroneurosporene(1,2,7,8 - tetrahydro - ψ,ψ - carotene, V) it is the more saturated half of the molecule that undergoes hydrogenation at C-1,2.

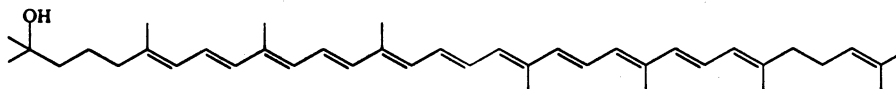
Addition of C₅ units. The biosynthesis of bacterioruberin (2,2' - bis(3 - hydroxy - 3 - methylbutyl) - 3,4,3',4' - tetradehydro - 1,2,1',2' - tetrahydro - ψ,ψ - carotene - 1,1' - diol, VI, from *Halobacterium salinarum*)¹⁴ and related



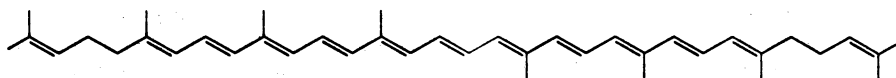
Spirilloxanthin (I)



Hydroxyspheroidene (II)



Rhodopin (III)



Lycopene (IV)

compounds has not been studied in detail, but the mechanism is likely to be similar to that of the hydration reaction, initiated by an electrophilic C_3 species instead of a proton (Fig. 1a, iii). This is an interesting case because the product has a chiral centre (C-2), and is optically active,¹⁵ thus demonstrating the stereospecificity expected of an enzymic process. Determination of the absolute configuration of bacterioruberin will enable the

stereochemistry of this addition to the C-1,2 double bond to be correlated with the stereochemistry of some of the cyclization processes to be discussed below.

Cyclic carotenoids—the cyclization reaction

General aspects. The characteristic carotenoids of higher plants and algae, and of many fungi and bacteria, are cyclic, i.e. contain one or two rings, usually of the β -

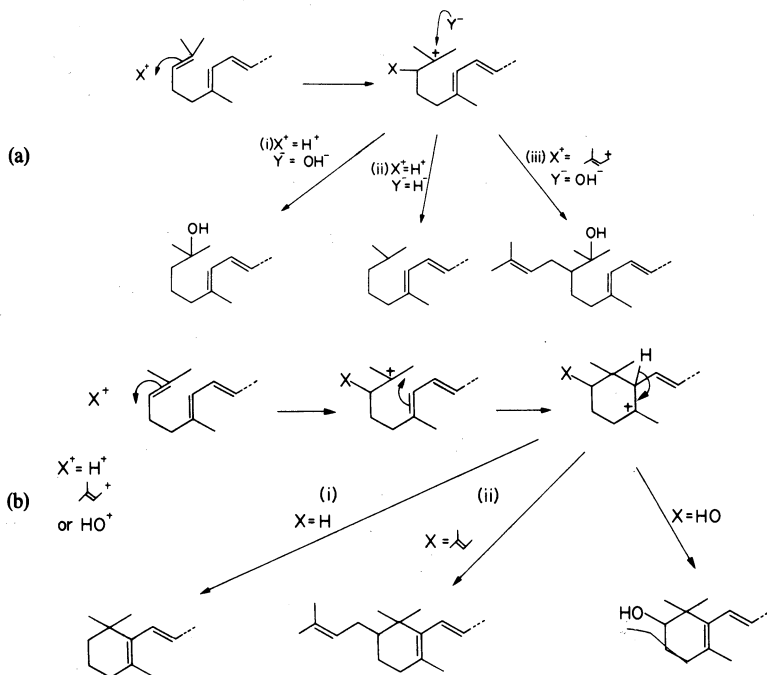
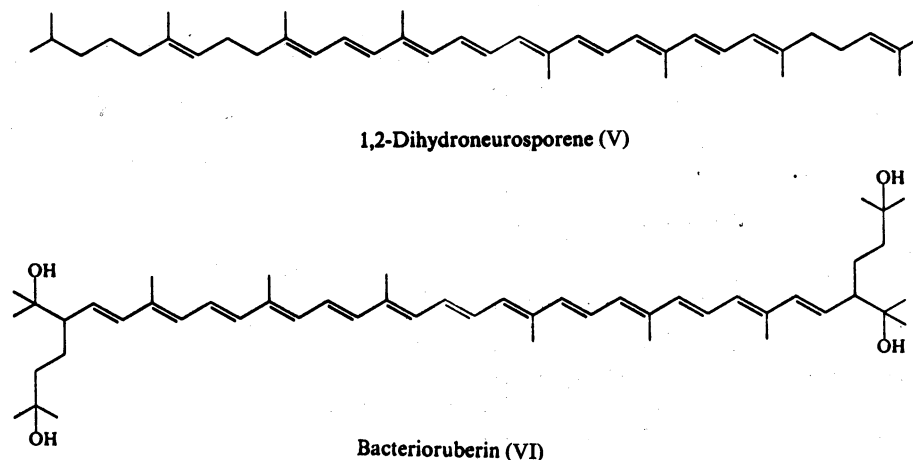


Fig. 1. Basic reactions occurring at the C-1,2 double bond: (a) to produce substituted acyclic carotenoids; (b) to produce cyclic carotenoids.



or ϵ -type (VII, VIII). The mechanism generally accepted for the cyclization reaction (Fig. 1b, i), though without any real experimental support, involves proton attack at C-2 of an acyclic precursor followed by ring closure to give a "carbonium ion" intermediate (IX) which can be stabilized in three ways by proton loss from different positions to give the β -, ϵ - or in one case, the discomycete *Caloscypha fulgens*,¹⁶ a γ -ring (X) (Fig. 2). This implies that synthesis of the different ring types occurs independently, though from the same precursor. This theory of independent synthesis is supported by inheritance studies with tomatoes,¹⁷ which suggest that the ability to form the different ring types, β and ϵ , is controlled by different genes. Confirmation has been obtained by isotopic labelling studies. Thus (Fig. 3a) α -carotene (β , ϵ -carotene) biosynthesized from [2-¹⁴C, (4R)-4-³H₁] mevalonate retains the tritium at C-6.¹⁸ This tritium is lost in the formation of the β -ring^{19,20} and would thus be absent in the ϵ -ring if this were formed from a β -ring. Similar

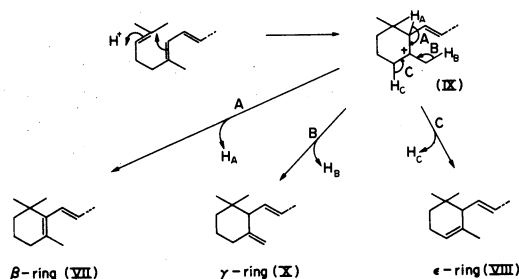


Fig. 2. Formation of β -, γ - and ϵ -rings from a common carbonium ion intermediate.

labelling experiments with [2-¹⁴C, 2-³H₂] mevalonate have proved that the β -ring is not formed by isomerization of an ϵ -ring (Fig. 3b).²⁰

A general overall scheme for the biosynthesis of cyclic carotenes is often presented (Fig. 4). According to this scheme there are two major points at which cyclization

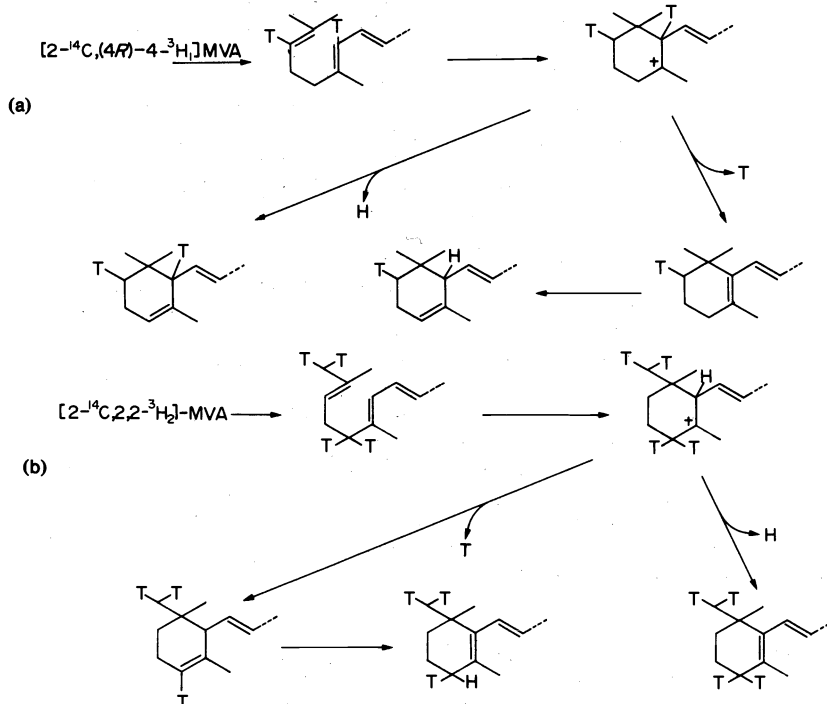


Fig. 3. Verification of the independent formation of β - and ϵ -rings: (a) by use of [2-¹⁴C, (4R)-4-³H₁] mevalonate; (b) by use of [2-¹⁴C, 2,2-³H₂] mevalonate.

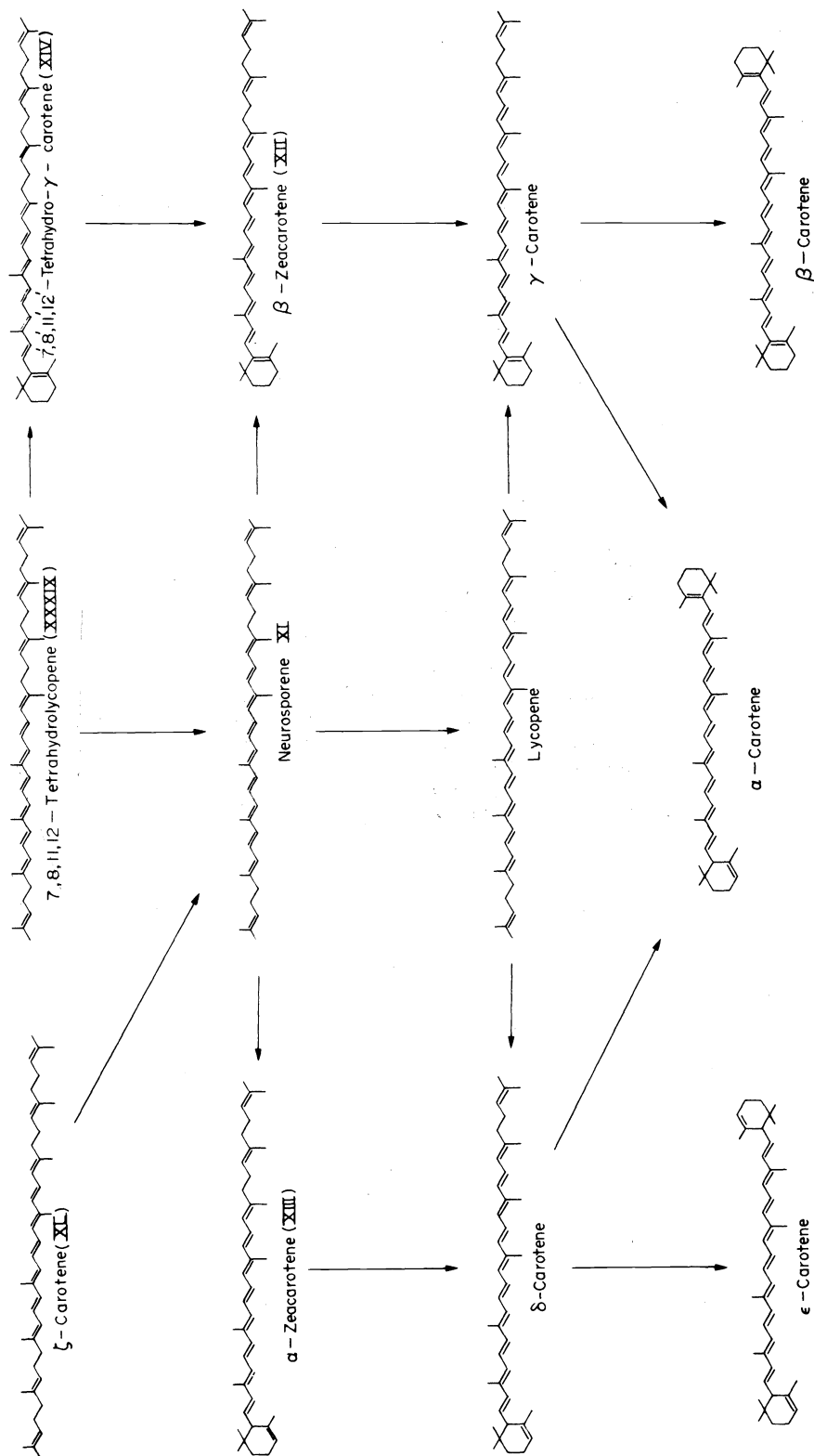


Fig. 4. Overall scheme for biosynthesis of the cyclic carotenes.

may occur. If normal desaturation goes to completion before cyclization, then lycopene is the key immediate precursor of the cyclic carotenes. Alternatively, if cyclization occurs before desaturation is complete, neurosporene (7,8 - dihydro - ψ,ψ - carotene, XI) and the zeacarotenes (7',8' - dihydro - β,ψ - carotene, XII and 7',8' - dihydro - ϵ,ψ - carotene, XIII) are the key intermediates. That cyclization can also occur at the conjugated heptaene level is shown by the isolation of a "cyclic ζ -carotene" (7',8',11',12' - tetrahydro - β,ψ - carotene, XIV) from a DPA inhibited culture of a mutant of *Phycomyces blakesleeanus*.²¹

A great deal of energy has been expended in an effort to determine whether in various systems lycopene or neurosporene fills the important key role. A full discussion of the evidence and arguments used to support the rival claims is presented in the review by Goodwin,²² but as a result of more recent work, especially with inhibitors, the argument has been taken up again with renewed vigor.

Inhibitor studies. Great use has been made recently of substances that inhibit, specifically, the cyclization reaction. Two compounds, nicotine and CPTA, have been particularly widely used.^{4,23-43} In many organisms in the presence of one of these inhibitors, formation of the normal cyclic carotenoids is prevented and acyclic compounds, usually lycopene, accumulate. Frequently removal of the inhibitor has allowed formation of the normal cyclic carotenoids to proceed at the expense of the accumulated lycopene.^{4,23-26,28,30,32,37,39} This has often been interpreted as indicating that lycopene is the immediate precursor of the cyclic carotenes, but other interpretations are possible. Cyclization normally proceeding via neurosporene and β -zeacarotene would also be inhibited by nicotine or CPTA but further desaturation would not be prevented, so lycopene would again accumulate. This situation will be discussed again later.

The work of Kleinig and Reichenbach with *Myxococcus fulvus* has shown^{28,29} that cyclization is more easily inhibited (i.e. by lower concentrations) by nicotine and CPTA than is hydration of the C-1,2 double bond. This conclusion is supported by work with *Rhodospirillum rubrum*; formation of β -carotene is inhibited by lower nicotine concentrations than is rhodopin formation.⁶ Almost all the inhibitor work with nicotine and CPTA has concentrated on the β -ring.

Nothing is known about the mode of action of these inhibitors except that they seem to inhibit the cyclization process itself and not synthesis of the cyclase enzymes.^{23,24,37} Some attempt has been made to correlate the effectiveness of various nitrogenous bases as inhibitors of the carotenoid cyclization reaction with their pK_a values,⁴⁴ and nicotine has been shown to be a more effective inhibitor of the cyclization reaction in *Cucurbita*

ficifolia cotyledons in alkaline than in neutral conditions.³¹

Cell-free systems. Work with crude cell-free systems has provided evidence for the direct incorporation of lycopene and neurosporene into β -carotene and other carotenes, and evidence for the formation of β -carotene from neurosporene via β -zeacarotene.

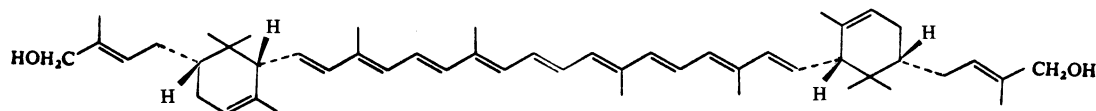
Cell-free preparations from spinach chloroplasts and from chromoplasts of tomato fruit, especially the "hi- β " and "hi- δ " strains, have been used to demonstrate the direct incorporation of labelled lycopene into α -, β -, γ - and δ -carotenes (β,ϵ -, β,β -, β,ψ - and ϵ,ψ -carotenes) and some of their *cis* isomers.⁴⁵⁻⁴⁷ The incorporation was enhanced by NADP⁺ and FAD. The conversion of lycopene into cyclic carotenoids, particularly β -carotene, has also been demonstrated with a preparation of plastids from bean leaves or tomato fruit.⁴⁸ Labelled lycopene has been incorporated into β -carotene and zeaxanthin by a cell-free preparation from *Flavobacterium* R1560,⁴⁹ and lycopene and neurosporene have been converted into β -carotene by a crude preparation of *Phycomyces blakesleeanus*.²⁷

The incorporation of [2-¹⁴C]mevalonate and [¹⁴C] neurosporene into β -carotene by the crude *P. blakesleeanus* preparation can be diluted out by the addition of unlabelled carotenes, which are then found to be radioactive on reisolation from the incubation mixtures. The trapping efficiencies, especially of lycopene and β -zeacarotene are considered to be consistent with the operation of alternative cyclization routes, in which either of these compounds may be bypassed.²⁷

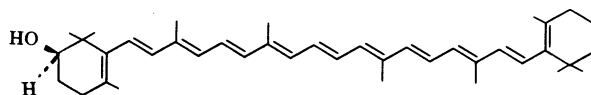
As yet this early work with cell-free systems has provided little information about the mechanisms of the cyclization reaction.

Addition of C₅ units. A modification of the cyclization reaction could give rise to the C₄₅ and C₅₀ carotenoids such as decaprenoxanthin (2,2' - bis(4 - hydroxy - 3 - methyl - 2 - butenyl) - ϵ,ϵ - carotene, XV). In *Flavobacterium dehydrogenans* all acyclic carotenoids than can be detected (phytoene $\rightarrow$ lycopene) are the normal C₄₀ intermediates⁵⁰ and it is suggested that the extra C₅ units are introduced during the cyclization step. This could occur if an electrophilic C₅ species replaced the proton as the cyclization-initiating species. The formation of the substituted β - or ϵ -ring would thus be analogous to the normal cyclization reaction (Fig. 1b, ii).

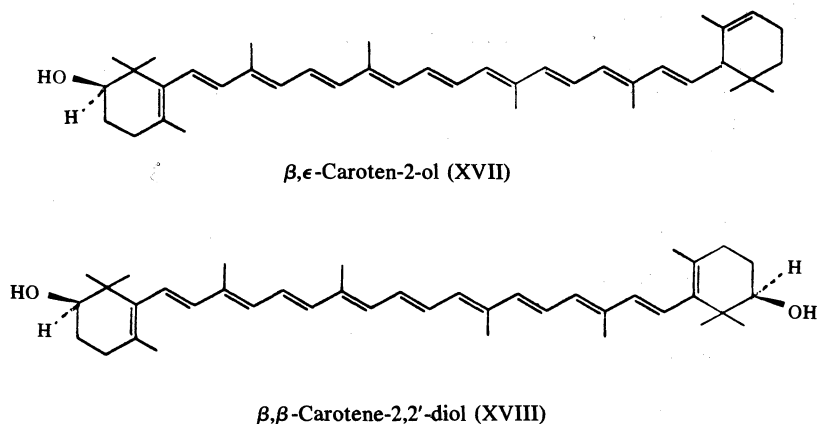
Cyclization to form caroten-2-ols. Several carotenoids with β -rings hydroxylated in the 2-position have recently been isolated, notably β,β -caroten-2-ol (XVI), β,ϵ -caroten-2-ol (XVII) and β,β -carotene-2,2'-diol (XVIII) from the alga *Trentepohlia liolithus*.⁵¹ It has been suggested that these could be produced by a modification of the cyclization reaction involving either an initial attack by a species such as HO⁺ instead of a proton (Fig. 1b, iii)



Decaprenoxanthin (XV)



β,β -Caroten-2-ol (XVI)



or by proton-initiated cyclization of an acyclic 1,2-epoxide (Fig. 5) in a manner similar to the cyclization of squalene epoxide in triterpenoid biosynthesis. Such 1,2-epoxycarotenoids have been reported,⁵² though not from *Trentpohlia*.

Stereochemistry of cyclization. Knowledge of the stereochemistry of cyclization is essential for an understanding of the mechanism(s) involved. In particular, questions arise about the manner of folding of the acyclic precursor before and during the cyclization process, e.g. is the carbon skeleton folded in an approximate chair or boat conformation, and on which faces of the C-1,2 and C-5,6 double bonds does electrophilic attack occur? Diagrams of several possibilities have been presented elsewhere.³ In order for these questions to be answered it is necessary to determine factors such as the stereochemistry of proton attack at C-2, the orientation and behaviour of the methyl substituents at C-1, and, in the case of ϵ -ring formation, the absolute configuration at the chiral C-6 position and the stereochemistry of hydrogen loss from C-4, (Fig. 6).

The little evidence available for the stereochemistry of β -ring formation has come from studies of natural products related to (abscisic acid) or derived from (trisporic acid XIX) β -carotene. Bu'Lock and co-workers^{53,54} have determined the distribution of radioactive label from [2-¹⁴C]mevalonate incorporated into

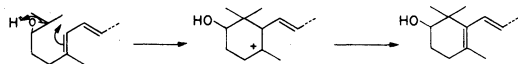


Fig. 5. Formation of caroten-2-ol by cyclization of a 1,2-epoxide.

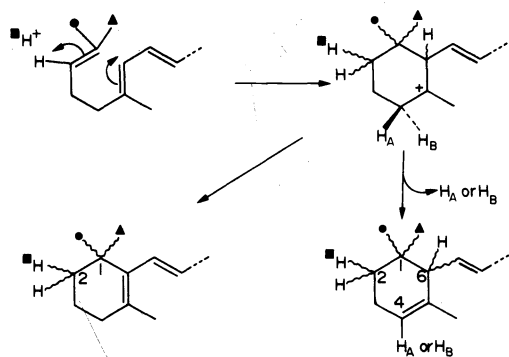


Fig. 6. Stereochemical details that need to be determined in order to define the mechanism of cyclization.

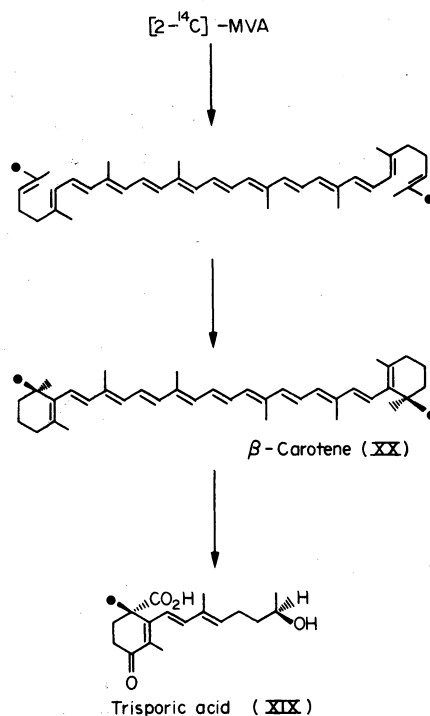
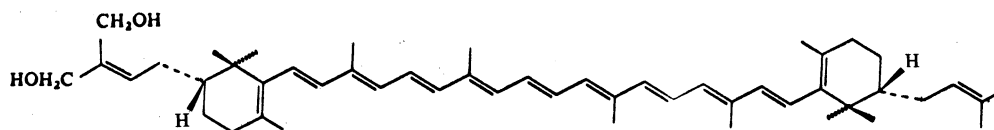


Fig. 7. Determination of the labelling pattern of the C-1 methyl groups in β -carotene biosynthesized from [2-¹⁴C]mevalonate, by correlation with trisporic acid.

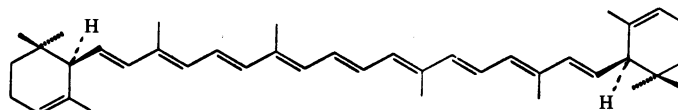
trisporic acid by *Blakeslea trispora*. Of the C-1 substituents (carotene numbering) only the methyl group was labelled. Since β -carotene (XX) was also shown to be a precursor of trisporic acid it was inferred that the label from [2-¹⁴C]mevalonate must be located in the *pro-R* methyl substituent at C-1 of β -carotene (Fig. 7). Similar stereochemistry has been demonstrated for abscisic acid biosynthesis.⁵⁵

The stereochemistry of proton attack at C-2 during β -ring formation is obviously very difficult to determine experimentally and it is not possible to correlate it with the stereochemistry of attack by other electrophiles, such as HO⁺ or a C₅ species (see below). The mechanism of formation of the unsubstituted β -ring therefore cannot be fully defined.

In all examples of carotenoids so far studied containing a non-isoprenylated ϵ -ring, the *R*-configuration has been established at C-6.^{56,57} It has also been demonstrated⁵⁸ by



C.P. 450 (XXI)



ε-Carotene (XXII)

incorporation of geranylgeranyl pyrophosphate labelled from $[2-^{14}\text{C}, (2R)-2-^3\text{H}_1]\text{mevalonate}$ and $[2-^{14}\text{C}, (2S)-2-^3\text{H}_1]\text{mevalonate}$ into tomato slices that it is the 2-*pro-S* hydrogen atom of mevalonate that is lost from C-4 during formation of the ϵ -ring. No information is available about the stereochemistry of events at C-1 and C-2 during formation of the ϵ -ring, so the mechanism cannot be fully defined.

Determination of the absolute configuration at C-2 of substituted carotenoids might be expected to help solve the problem of the stereochemistry of electrophilic attack at C-2 during cyclization. However, the stereochemistry at C-2 of the isoprenylated carotenoids, e.g. "C.p. 450" [2-(4-hydroxy-3-hydroxymethyl-2-butenyl)-2'-(3-methyl-2-butenyl)- β,β -carotene, XXI]⁵⁹ is opposite to that of the caroten-2-ols, XVI-XVIII.⁶⁰ If these two types are formed, as has been suggested, by cyclization initiated by a C_3 electrophile and $\text{HO}^\cdot$ respectively, then clearly the stereochemistry of the cyclization is different in the two cases. It is not possible to relate the mechanism of formation of the unsubstituted β -ring to either of these cases.

The absolute configuration at C-2 of decaprenoxanthin is the same (*R*) as in "C.p. 450,"⁶¹ but this cannot be correlated with the stereochemistry of proton attack at C-2 during formation of the unsubstituted ϵ -ring, especially in view of the recent report⁶¹ that the absolute configuration at C-6 of decaprenoxanthin (XV) is opposite to that determined for the unsubstituted ϵ -rings, e.g. in ϵ -carotene (ϵ,ϵ -carotene, XXII).

Cyclization—comments and conclusions

Although much work has gone into attempts to determine whether lycopene or neurosporene is the key cyclization intermediate, and the conversion of these compounds into cyclic carotenoids has been demonstrated, very little is known about the cyclization reaction itself. Because of the difference in absolute configuration at C-2 and C-6 in the various carotenoids, as discussed above, it is not possible to define the stereochemistry and mechanism of cyclization by correlating stereochemical details for the several variations of the cyclization process. It is likely that the mechanisms of all the cyclization processes are similar, but the stereochemistry, e.g. of folding or of initial electrophilic attack, is different in different cases, and may depend on the cyclization-initiating species. The question of the stage at which formation of the β - and ϵ -rings diverges must also remain unanswered. They may be formed by loss of different

protons from the same intermediate (Fig. 2) or the point of divergence may be much earlier, e.g. there may be differences in the stereochemistry of folding of the pre-cyclization intermediate before proton-initiated cyclization begins.

The mechanism usually quoted for cyclization (Figs. 1b, 2) involves carbonium ions as intermediates. The fact that in all known cases the reactions are stereospecific indicates that no free carbonium ions are involved. The reactions could be concerted processes, or may involve actual enzyme-bound intermediates (Fig. 8). If enzyme-bound intermediates such as XXIII or XXIV are involved rather than carbonium ions, then inversions of configuration are likely as the enzyme groups leave. All the stereochemical details for the biosynthesis of one compound must be determined in the same system before the possible involvement of enzyme groups in the reaction can be established.

LATER MODIFICATIONS

Acyclic carotenoids

O-Methylation and further desaturation. In those organisms that normally accumulate mainly or entirely acyclic carotenoids, especially the photosynthetic bacteria, the characteristic reactions occurring after hydration of the C-1,2 double bond are methylation of the C-1 tertiary hydroxy group so introduced, and desaturation of the C-3,4 bond (Fig. 9). This can give rise to a considerable range of carotenoids, culminating in spirilloxanthin (I) the normal main pigment of *Rhodospirillum rubrum*. Very many hydroxy and methoxy compounds at all levels of desaturation have been isolated, often in very small amounts, from cultures of photosynthetic bacteria, especially *R. rubrum*, grown under normal conditions or in the presence of inhibitors.⁷⁻¹¹ Although it has often been claimed that these compounds may be intermediates

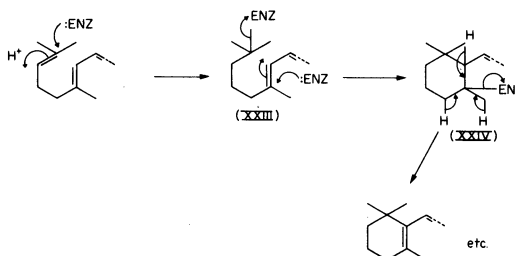


Fig. 8. Mechanism for cyclization involving enzyme-bound intermediates.

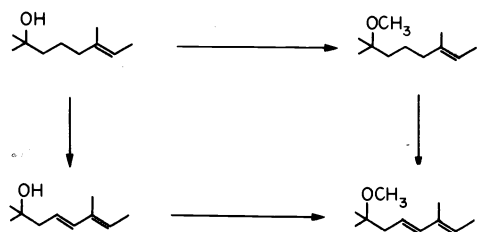


Fig. 9. *O*-Methylation and 3,4-desaturation in the biosynthesis of acyclic carotenoids.

in the normal biosynthetic pathway, it is likely that they are formed because the enzymes responsible for C-1 hydroxylation and *O*-methylation can accommodate a range of substrates at different saturation levels.

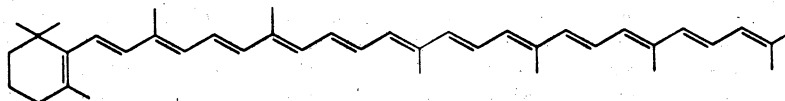
It has been demonstrated that *S*-adenosylmethionine is the methyl donor in the *O*-methylation reaction⁶² but nothing is known about the introduction of the C-3,4 double bond in photosynthetic bacteria, i.e. whether or not this reaction is similar to the desaturations at C-7,8 and C-11,12. In torulene (3',4'-didehydro- β,ψ -carotene, XXV) biosynthesis in *Rhodotorula glutinis* desaturation at C-3',4' like the basic C-7,8 and C-11,12 desaturations is inhibited by diphenylamine.³⁹

Introduction of carbonyl groups. It is also typical of many photosynthetic bacteria to produce carotenoids containing carbonyl functions. The best known, e.g.

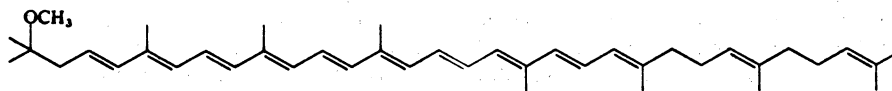
spheroidenone (1-methoxy-3,4-didehydro-1,2,7',8'-tetrahydro- ψ,ψ -caroten-2-one, XXVI) have an oxo group in the C-2 position. These 2-oxo compounds are formed in the presence of oxygen from the parent carotenoids which accumulate under anaerobic conditions, e.g. spheroidenone is formed from spheroidene (1-methoxy-3,4-didehydro-1,2,7',8'-tetrahydro- ψ,ψ -carotene, XXVII) by *Rhodospseudomonas spheroides*.^{63,65} A double bond at C-3,4 appears to be necessary for the oxo function to be introduced at C-2. The oxo group introduced originates from molecular oxygen.⁶⁶

The introduction of two other carbonyl functions, an aldehyde group at C-20 as in rhodopinal (13-*cis*-1-hydroxy-1,2-dihydro- ψ,ψ -caroten-20-al, XXVIII) in *Chromatium warmingii*^{67,68} and an oxo group at C-4 in the carotenoids of *Rhodospseudomonas globiformis*⁶⁹ and in okenone (1'-methoxy-1',2'-dihydro- χ,ψ -caroten-4'-one, XXIX) from *C. okenii*⁷⁰ occurs under strictly anaerobic conditions and may involve a hydration and dehydrogenation via the intermediate alcohols (Fig. 10).² Rhodopin-20-ol (13-*cis*-1,2-dihydro- ψ,ψ -carotene-1,20-diol, XXX) and similar compounds have been detected in small amounts in *C. warmingii*.⁶⁸ No biochemical evidence is available for any of these reactions.

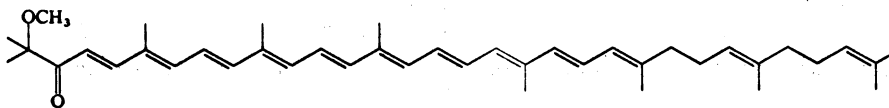
Cyclic carotenoids. The most abundant and widespread carotenoids, especially in higher plants and algae, are the cyclic xanthophylls, of which there are many structural modifications, e.g. oxygen substituents in the rings and skeletal variations such as cyclopentane rings, allene and



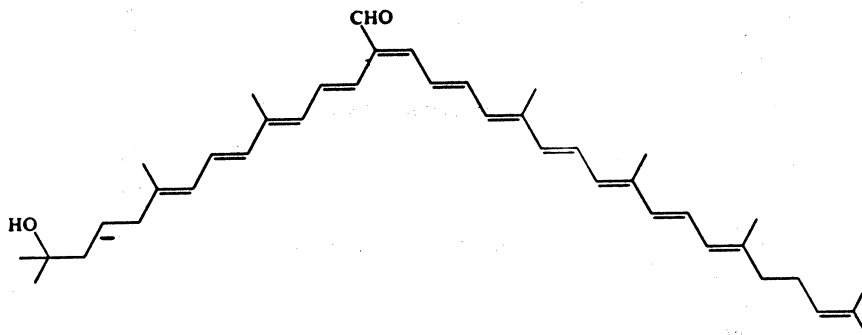
Torulene (XXV)



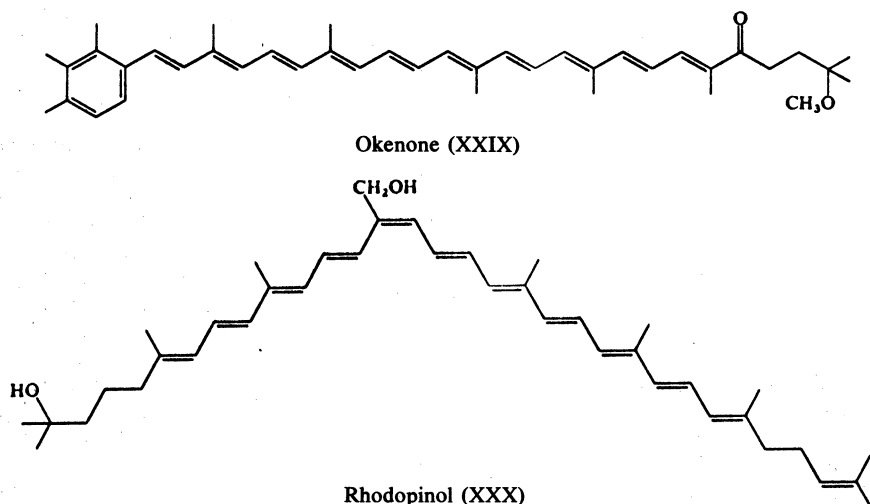
Spheroidene (XXVI)



Spheroidenone (XXVII)



Rhodopinal (XXVIII)



acetylene groups and the *retro* arrangement of conjugated double bonds. In most cases very little or no biochemical information is available about the elaboration of these structural features. This discussion will concentrate on the introduction of a structural feature common to a large number of carotenoids, a hydroxy group, especially common at C-3 but also found at C-2 and C-4.

Hydroxylation at C-2. The possible formation of β,β -caroten-2-ol and related compounds by HO^+ -initiated cyclization (Fig. 1b, iii) or by proton-initiated cyclization of an acyclic 1,2-epoxide (Fig. 5) has been mentioned previously. A third possibility, direct hydroxylation at C-2 of the β -ring similar to that at C-3 discussed below must also be considered. No biochemical work on these caroten-2-ols has been reported.

Introduction of oxygen functions at C-4. Carotenoids containing oxygen functions at C-4 (hydroxy or especially oxo) are often encountered in flowers and micro-organisms and are well known as the secondary carotenoids produced by many green algae under strained cultural conditions.⁷¹ Inhibitor studies with the alga *Dictyococcus cinnabarinus* (DPA)⁷² and with a strain of

Brevibacterium (glutathione)³² suggest that the corresponding caroten-4-ols, isocryptoxanthin (β,β -caroten-4-ol, XXXI) and isozeaxanthin (β,β -carotene-4,4'-diol, XXXII) are intermediates in the formation of the normal caroten-4-ones, echinenone (β,β -caroten-4-one, XXXIII) and canthaxanthin (β,β -carotene-4,4'-dione, XXXIV). No other biochemical information has been obtained.

Hydroxylation at C-3. The most extensively studied of these later reactions is the introduction of the hydroxy group at C-3 in the common xanthophylls such as lutein (β,ϵ -carotene-3,3'-diol, XXXV) and zeaxanthin (β,β -carotene-3,3'-diol, XXXVI). Such carotenoids occur universally as chloroplast constituents in plants, but their biosynthesis has been investigated largely in strains of a zeaxanthin-producing *Flavobacterium*.

Inhibitor studies²⁶ with nicotine have demonstrated that the hydroxy group is introduced after cyclization, thus confirming the work of Claes⁷³ with mutants of *Chlorella*. If a *Flavobacterium* strain, R1519, is grown in the presence of nicotine the cyclization reaction is inhibited and lycopene accumulates in place of the normal main pigment zeaxanthin. If the inhibitor is removed, the

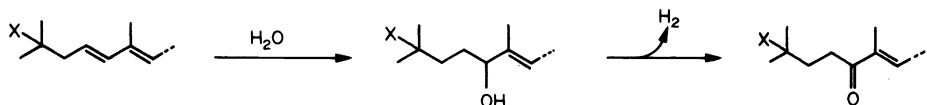
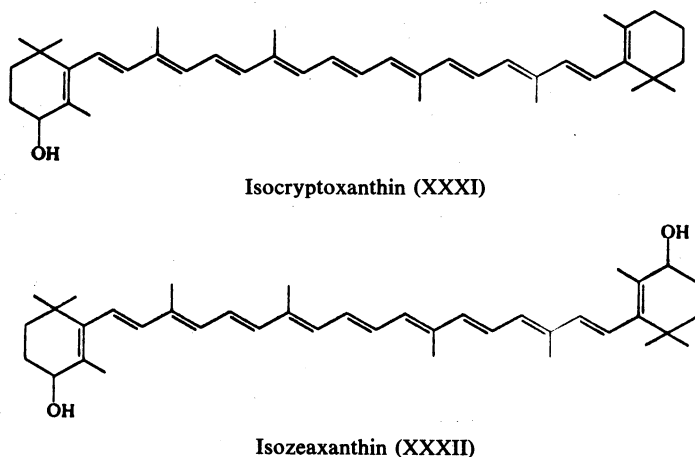
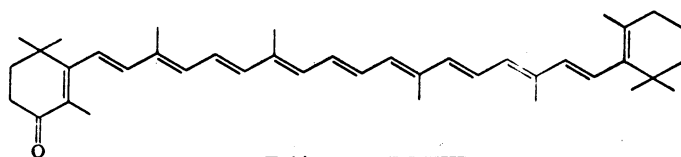
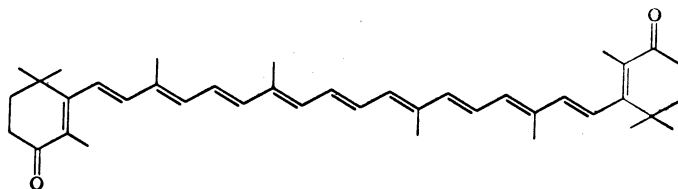


Fig. 10. A mechanism for the anaerobic introduction of carbonyl groups at C-4 in carotenoids of photosynthetic bacteria.

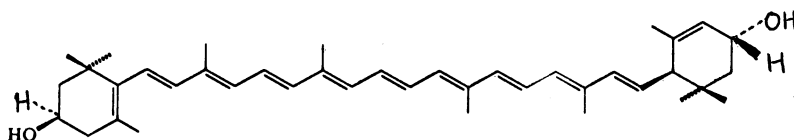




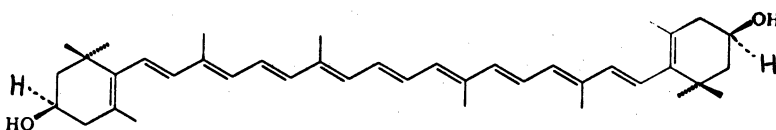
Echinenone (XXXIII)



Canthaxanthin (XXXIV)



Lutein (XXXV)



Zeaxanthin (XXXVI)

accumulated lycopene is cyclized to β -carotene under anaerobic conditions. In the presence of oxygen, this β -carotene is converted into zeaxanthin. The fact that lycopene and not a hydroxy derivative of lycopene accumulates if cyclization is prevented, and the successive conversions of lycopene into β -carotene and β -carotene into zeaxanthin clearly show that cyclization occurs before hydroxylation. It is also clear that hydroxylation at C-3 requires the presence of oxygen, thus confirming the work of Yamamoto *et al.*⁷⁴ who showed that the hydroxy groups in several leaf xanthophylls come from molecular oxygen, not from water.

An interesting effect is seen when zeaxanthin formation in *Flavobacterium* is inhibited by lower concentrations of nicotine. In this case cyclization of one half of the molecule is prevented and rubixanthin (β,ψ -caroten-1-ol, XXXVII) becomes the main pigment; the conversion of the accumulated rubixanthin into zeaxanthin can be demonstrated. This would seem to suggest that alternative pathways of zeaxanthin biosynthesis, via β -carotene or via rubixanthin operate in *Flavobacterium* (Fig. 11). (This situation will be discussed later). In both cases, however, cyclization precedes hydroxylation. Very recently examination of the minor carotenoids of cultures of *Flavobacterium* R1560 grown in the presence of nicotine has revealed a rubixanthin derivative with a second hydroxy group situated in the terminal isoprene unit of the acyclic half of the molecule.⁷⁵ The position of this hydroxy group has not yet been established but it would be interesting if it proved to be at C-3'.

The stereochemistry of the hydroxylation in zeaxanthin biosynthesis in *Flavobacterium* has been determined⁷⁶ through the use of $[2-^{14}\text{C}, (5R)-5-^3\text{H}_1]\text{mevalonate}$ (Fig. 12). This direct hydroxylation with retention of configuration is characteristic of a mixed-function oxidase reaction.⁷⁷

The absolute configuration at C-3' of lutein is *R*, i.e. opposite to that at C-3, and at C-3 and C-3' of zeaxanthin.⁷⁸ Preliminary results⁷⁹ however, indicate that the stereochemistry of hydrogen loss from this position during hydroxylation is the same as for the 3-hydroxy- β -ring, a situation difficult to explain.

Other modifications. The introduction of other structural modifications will not be considered. Very many biosynthetic schemes have been postulated for a range of carotenoid structural types. Most of these schemes are, on paper, very reasonable, but in most cases biochemical evidence to support them is completely lacking.

FINAL COMMENTS

Much of the work on carotenoid biosynthesis has been concerned with deciding between a number of possible alternative pathways for the formation of a particular compound. The prime example of this is the long-maintained argument over whether it is neurosporene or lycopene that is the immediate precursor of the cyclic carotenoids (Fig. 4). Perhaps the time is now ripe for a reassessment of this and related situations. Carotenoid biosynthesis immediately becomes simpler and more rational if it is considered that the substrate of each enzyme is a carotenoid half-molecule rather than a specific compound. Then although to the chemist and biochemist neurosporene and lycopene are distinct compounds with different chemical and physical properties, the cyclizing enzyme would not distinguish between them, since each contains the required half-molecule substrate (XXXVIII). The unsymmetrical isomer of ζ -carotene, 7,8,11,12 - tetrahydro - ψ,ψ - carotene (XXXIX) would also be a substrate (shown by the formation of 7',8',11',12' - tetrahydro - β,ψ - carotene, XIV), but ζ - carotene (7,8,7',8' - tetrahydro - ψ,ψ -

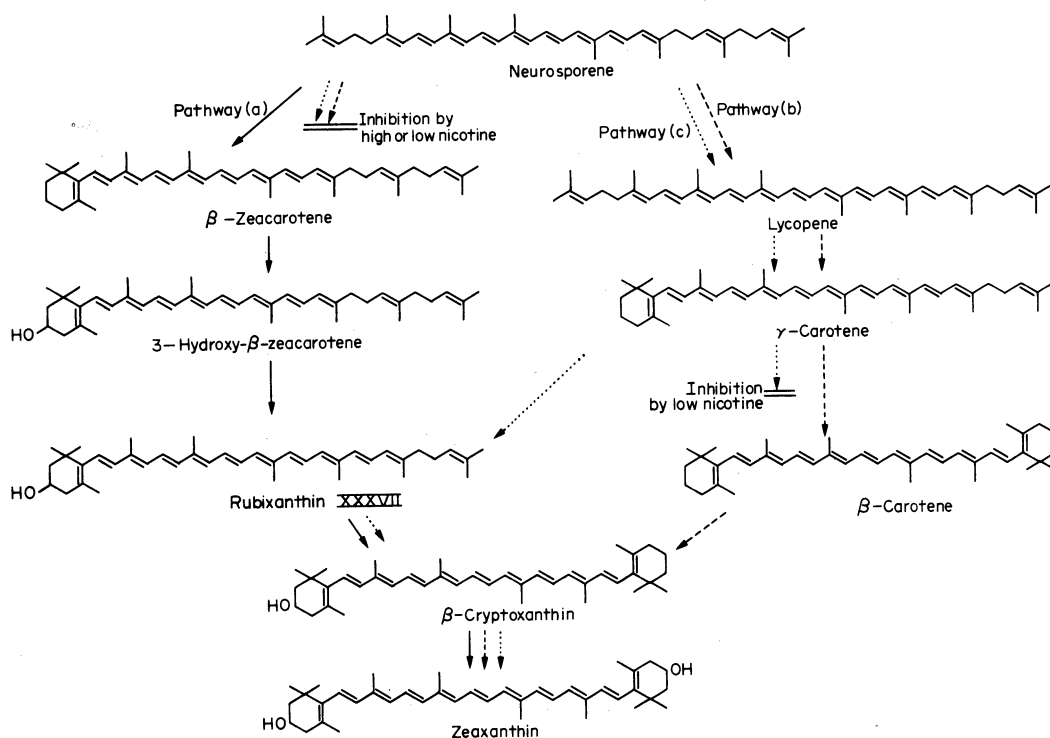


Fig. 11. Alternative pathways of zeaxanthin biosynthesis from neurosporene in *Flavobacterium*.

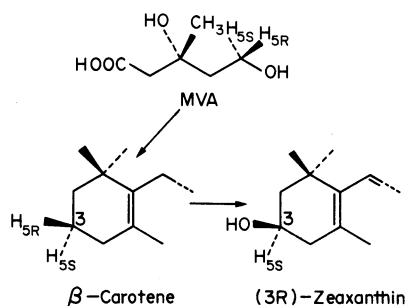


Fig. 12. Stereochemistry of hydroxylation in zeaxanthin biosynthesis.

carotene, XL), and phytoene (7,8,11,12,7',8',11',12' - octahydro - ψ,ψ - carotene) and phytofluene (7,8,11,12,7',8' - hexahydro - ψ,ψ - carotene) would not be cyclized. The important distinction is therefore *not* between neurosporene and lycopene, but between ζ -carotene on the one hand and neurosporene and lycopene on the other. Consideration of all available data shows that desaturation at C-7,8 is essential before cyclization of that half-molecule can occur. The sequence of events, C-7,8 desaturation followed by cyclization is clearly seen several times in the scheme depicted in Fig. 4.

Work on zeaxanthin biosynthesis in *Flavobacterium* species, especially with inhibitors, gives results that can be interpreted in terms of several alternative pathways (Fig. 11). However, if carotenoid half-molecules are considered to be the substrates of the enzymes involved, then the sequence of reactions, desaturation-cyclization-hydroxylation is always observed. This work has led to the suggestion²⁷ that there may be two sites of enzyme activity to account for the two half-molecule substrates, since differences have been observed in the

susceptibility of the two sites to inhibitors such as nicotine (Fig. 13).

This idea of half-molecule substrates also simplifies the picture of carotenoid biosynthesis in the photosynthetic bacteria. A scheme for the biosynthesis of spirilloxanthin from lycopene has been proposed² (Fig. 14). Examination of this reveals that it involves one reaction sequence, C-1,2 hydration, *O*-methylation, 3,4-desaturation, occurring firstly in one half-molecule, and then repeated in the second half-molecule. Variations in the relative rates at which the processes occur at the two sites could result in other compounds ("intermediates") being produced. It is also apparent that hydration at C-1,2 can occur before desaturation at C-7,8 and C-11,12 is complete, i.e. the enzyme responsible for hydration is able to accommodate a wider range of half-molecule substrates than are the cyclizing enzymes.

In other photosynthetic bacteria, e.g. *Rhodospseudomonas spheroides*, the same sequence of reactions, hydration, *O*-methylation and 3,4-desaturation occurs in one half-molecule, but the repetition of these events in the second half-molecule cannot take place because C-7,8 desaturation in this half-molecule is prevented. Consequently spheroidene accumulates as the main pigment (Fig. 15). The blockage of C-7',8' desaturation is confirmed by the action of nicotine and CPTA, which results in the accumulation of neurosporene and not lycopene as in *R. rubrum*.^{5,6} In addition to the "half-molecule substrate" hypothesis, it is also necessary to consider that an enzyme "assembly line" situation may be involved, with all the enzymes responsible for a particular sequence of reactions being located in some kind of enzyme aggregate. Evidence for this has been provided by studies of the genetic aspects of carotenoid biosynthesis in fungi, especially *Phycomyces blakesleeana*.^{80,81} From this work it has been proposed that β -carotene synthesis takes

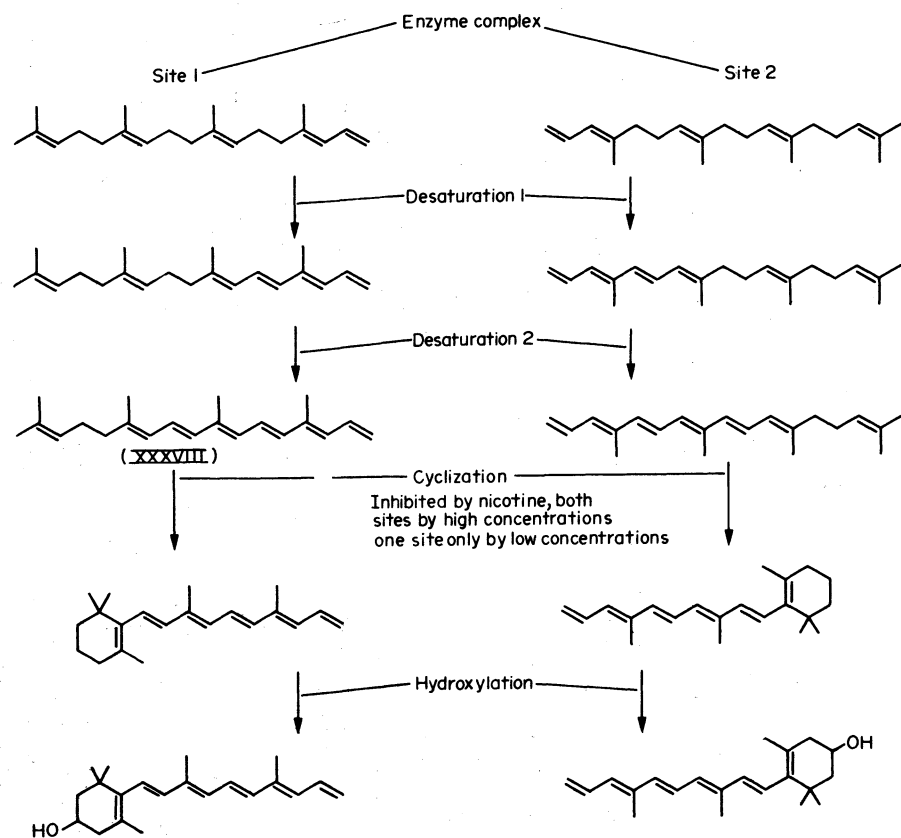


Fig. 13. Sequence of reactions occurring in the carotenoid "half-molecules" at the two sites of an enzyme complex involved in the biosynthesis of zeaxanthin.

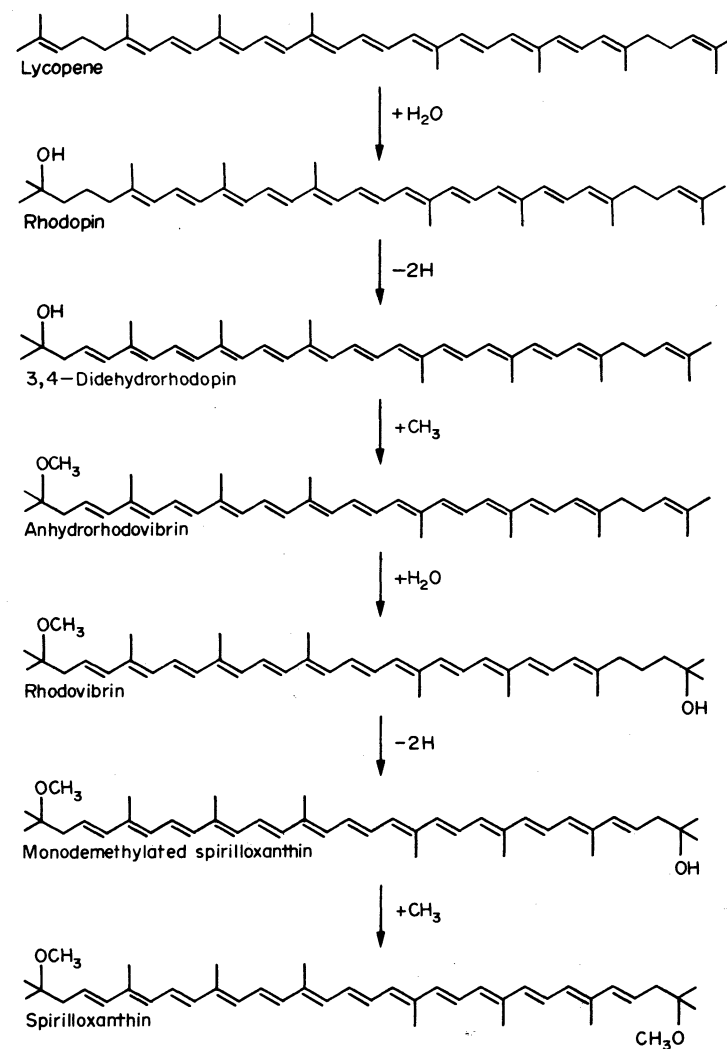


Fig. 14. Postulated scheme for biosynthesis of spirilloxanthin from lycopene in *Rhodospirillum rubrum*.

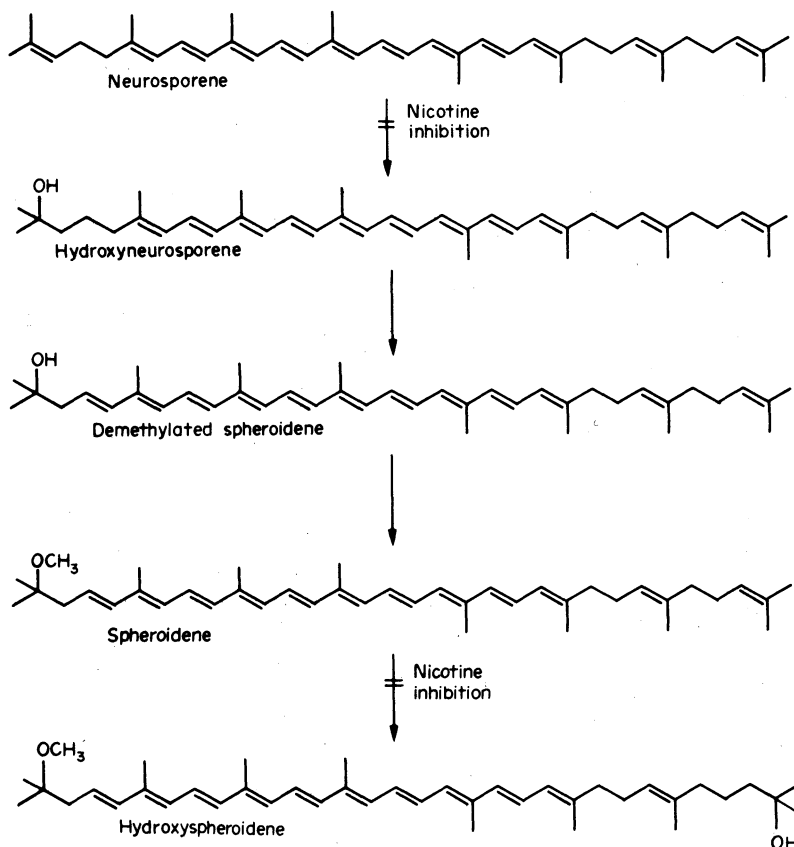


Fig. 15. Postulated scheme for biosynthesis of spheroidene and hydroxyspheroidene from neurosporene in *Rhodopseudomonas spheroides*.

place on an enzyme complex consisting of phytoene synthetase and then two desaturases and a cyclase for each half-molecule. Another interesting observation was made by Kleinig,⁸² who showed that the lycopene which can be accumulated in *Myxococcus fulvus* can be converted into the normal main carotenoids of this organism only if *de novo* synthesis from phytoene is prevented. He suggests that this indicates an arrangement of the carotenogenic enzymes in an "assembly line", with access at a late point (e.g. lycopene) not being possible unless the supply of early substrates (e.g. phytoene) is removed.

Stereochemical studies on carotenoids have revealed differences in chirality at several positions. Carotenoids with different absolute configurations at C-2 and C-6, and the opposite chirality of the C-3 and C-3' positions in lutein have already been mentioned. In trying to determine the mechanism of a reaction in the biosynthesis of a carotenoid it is therefore essential to consider the chirality of the compound concerned and the stereochemical details of its biosynthesis, and not to assume that these are the same as in compounds previously studied in other systems. It is possible that as yet undetected differences in the stereochemistry of biosynthesis may be revealed, i.e. differences in the stereochemistry of hydrogen loss in some reactions. The loss of different hydrogen atoms in the formation of all-*trans* and 15-*cis* phytoene is a case in point.⁸³ Thus, especially if no degradation is undertaken to establish the position of labelling, results on the stereochemistry of biosynthesis must be interpreted with great caution.

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