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CAROTENOID BIOSYNTHESIS—THE EARLY STEPS

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Abstract—This paper describes recent biosynthetic and enzymological studies from a number of laboratories and relates them to our current understanding of the pathways of carotenoid formation. A number of problems concerning phytoene biosynthesis and dehydrogenation are discussed and the work of our own laboratory on the biosynthesis of the triterpenoid carotenoids is reviewed.

INTRODUCTION

The number of different carotenoids which are recognized as being natural products continues to increase. The diversity of their structures continues to present the biochemist with many intriguing questions concerning not only the biosynthesis of the basic C_{40} skeleton, but also the origin of such features as allenic linkages, acetylenic bonds and the alicyclic and aromatic rings, and the mechanism of such processes as oxygen insertion, ring formation, opening and contraction, chain elongation and degradation. Few of these processes are, as yet, understood in detail, and it is worth posing the question as to why, in comparison with our knowledge of the formation of certain other groups of natural products, so relatively little detail is known of the mechanism of carotenoid biosynthesis.

The answer lies very largely in the nature of these pigments. Their instability in dilute solution and on chromatography, and their sensitivity to heat, light, oxygen, acids and, in some cases, to alkali, dictate that great care is required in their handling in the laboratory[1] if low overall recoveries, losses of particularly labile compounds and their conversion to other carotenoids, and *cis–trans* isomerization are to be avoided. Another major obstacle in biosynthetic studies has been the hydrophobic nature of carotenoids, which has precluded their ready use as substrates for aqueous enzyme preparations; all but the most recent experiments (in which labelled carotenes are solubilized in such detergents as Tween 80) have had to rely on the use of radioactive substrates which are very early precursors not only of carotenoids, but also of sterols and other terpenoids which may contaminate carotenoid samples. Extended purification procedures of individual carotenoids involve risks of loss, but they are essential if the results of labelling experiments are to be other than ambiguous.

In spite of these practical difficulties, considerable progress has been made in establishing the general pathway of carotenoid formation and, largely as a result of the application of stereospecifically-labelled substrates and the recent development of particularly active cell-free preparations, the mechanism and stereochemistry of a number of the individual steps are already understood. The aim of this and the following review is to highlight some of the more recent and important developments in the field against the background of our accumulated knowledge of carotenoid biosynthesis.[2] For this purpose, it has been convenient to consider carotenoid biosynthesis as comprising four distinct phases, namely (1) the formation of the first C_{40} hydrocarbon, (2) the successive dehydrogenation of this precursor to yield unsaturated acyclic (hydrocarbon) carotenes, (3) the formation of alicyclic and aromatic carotenes, and (4) the oxygenation of carotenes, sometimes accompanied by skeletal modifications, to yield xanthophylls. Recent studies on the reactions of the last two steps are the subject of another review by Dr. Britton; we shall deal here with the "early steps", the reactions leading to the formation of the unsaturated acyclic carotenes. As the work in our laboratory has concentrated almost exclusively, for the last 2–3 yr, on the novel biosynthetically-triterpenoid (C_{30}) carotenoids, our contribution also includes a report on the progress of these studies (involving both carotenes and xanthophylls).

THE VERY EARLY STEPS: UP TO C_{20}

The ready availability of ^{14}C-labelled substrates in the early 1950's led to the first experiments to yield direct and unambiguous indications of how the basic carotenoid molecule is built up from simple units. Chemical degradation of the β-carotene (β,β-carotene) formed by moulds from [1-^{14}C]- and [2-^{14}C]acetate revealed that carotenoids are biosynthesized symmetrically about the central (15,15′) linkage and that each isoprene unit has the same labelling pattern. The late 1950's saw the recognition of mevalonic acid (MVA; C_6) as a precursor of carotenoids as of other terpenoids, and in the course of the next decade it became understood how MVA is converted via its 5-phosphate (MVAP) and 5-pyrophosphate (MVAPP), and with the loss of C-1 as CO_2, into Δ^3-isopentenyl pyrophosphate (IPP; C_5). The isomerization of IPP to dimethylallylpyrophosphate (DMAPP), the condensation of these two C_5 molecules (IPP and DMAPP) to yield geranyl pyrophosphate (GPP; C_{10}) and subsequent additions of C_5 (IPP) units result in the formation of farnesyl pyrophosphate (FPP; C_{15}) and then geranylgeranyl pyrophosphate (GGPP; C_{20}). The reactions of the biosynthesis of GGPP from MVA[2] are summarized in Fig. 1.

THE NATURE AND FORMATION OF THE FIRST C_{40} HYDROCARBON

The reductive dimerization of FPP (C_{15}) to yield squalene (I; C_{30}), the symmetrical acyclic precursor of sterols, involves a cyclopropylcarbinyl pyrophosphate intermediate, presqualene pyrophosphate (PSPP).[3–7] The absolute configuration of PSPP is established[8] and the mechanism of its formation and conversion to squalene largely understood as a result of studies with model compounds.[9] The C_{40} carotenes are formed by the dimerization of GGPP (C_{20})[10–12] and the first product is again an intermediate cyclopropylcarbinyl pyrophosphate,

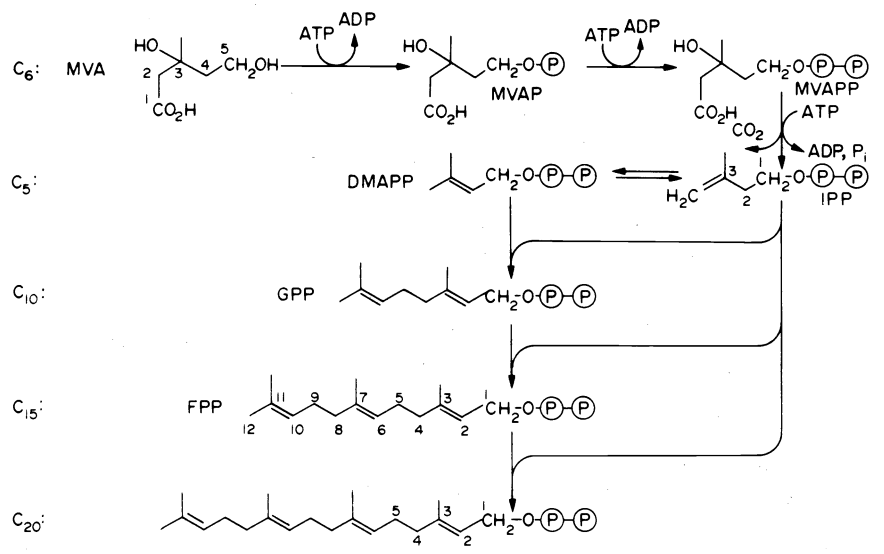
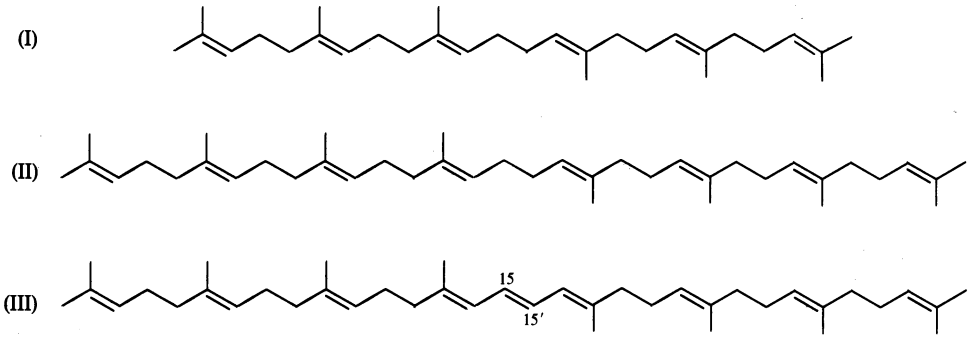


Fig. 1. Biosynthesis of geranylgeranyl pyrophosphate (GGPP) from mevalonic acid (MVA).

prephytoene pyrophosphate[13,14] (PPPP, also called pre-lycopersene pyrophosphate[14]), which is the C_{40} analogue of PSPP and has the same absolute configuration.[14] The enzyme responsible for its formation in a *Mycobacterium* sp. is fully photoinduced.[15]

might be concluded that lycopersene is not an obligatory intermediate in carotene formation. Its formation from GGPP (in lower yields than the formation of squalene from FPP[23]) by yeast squalene synthetase,[14] by an enzyme system from *Neurospora crassa*[10] and by a tomato plastid



In squalene formation, the cyclopropylcarbinyl cation formed by rearrangement on the loss of pyrophosphate from PSPP is stabilized by the stereospecific transfer of a hydride ion (H_B^- or 4*S*-H^-)[16] from NADPH.[3] An analogous reaction for (C_{40}) carotene biosynthesis would result in the formation from PPPP of lycopersene (II; 7,8,11,12,15,7′,8′,11′,12′,15′- decahydro-ψ,ψ- carotene), the C_{40} counterpart of squalene, which is centrally saturated (15,15′-bond). PPPP is formed from GGPP by purified yeast squalene synthetase[14] and by a soluble extract of an acetone powder from tomato fruit plastids;[17] it is converted by both systems into lycopersene as long as NADPH is present.[14,17] There is, however, no such requirement for NADPH in carotene formation by chloroplasts[18] or fungi;[19] the addition of NADPH merely stimulates squalene formation in a carotenogenic enzyme system from the C5 mutant of *Phycomyces blakesleeanus*[20] and in chloroplasts.

From this evidence, and the fact that the conjugated C_{40} triene phytoene (III; 7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene, a known precursor[2] of the coloured carotenes), rather than lycopersene, accumulates in microorganisms when dehydrogenation is blocked by mutation[20] or by inhibition with diphenylamine (DPA)[19,20] or San 6706,[21,22] it extract[17] may result from a lack of absolute specificity for FPP as a substrate on the part of the squalene synthetase. The apparent conversion of lycopersene into phytoene by tomato plastid acetone powder extracts[17] could again be rationalized in terms of non-absolute substrate specificity (of the carotene dehydrogenase system).

The proposal that phytoene may be formed directly from the cyclopropylcarbinyl cation by proton loss[24] (rather than via lycopersene) has now been tested in extracts from a *Mycobacterium* sp. and from the C5 (phytoene-accumulating) mutant of *P. blakesleeanus*.[25] Both 15-*cis* and all-*trans* phytoene, formed from [4-^{14}C, 1-$^3H_2$]GGPP by the fungal and bacterial enzymes respectively, retained two of the four labelled hydrogens. Had lycopersene been an intermediate, a portion of the hydrogens on the central (15,15′) carbons might have been anticipated to arise from NADPH; no radioactivity was incorporated into phytoene from [4-$^3H_2$]NADPH. Earlier studies on phytoene formation by tomato slices[26] and by chloroplasts[27] had shown that these two hydrogens (of 15-*cis* phytoene) are derived exclusively from the *pro-R* hydrogens of [(5*R*)-5-$^3H_1$]MVA. By using [4-^{14}C, (1*S*)-$^3H_1$]GGPP as a substrate, it was further shown[25] that

15-*cis* phytoene (*Phycomyes*) retained neither of the labelled (*pro-S*) hydrogens (confirming the earlier results from higher plant systems[26,27]) while *trans* phytoene (*Mycobacterium*) retained one *pro-R* and one *pro-S* hydrogen at the central carbons. It has been argued[25] that the loss of the *pro-S* hydrogens from *cis* phytoene makes it highly unlikely that lycopersene can be an intermediate in phytoene formation.

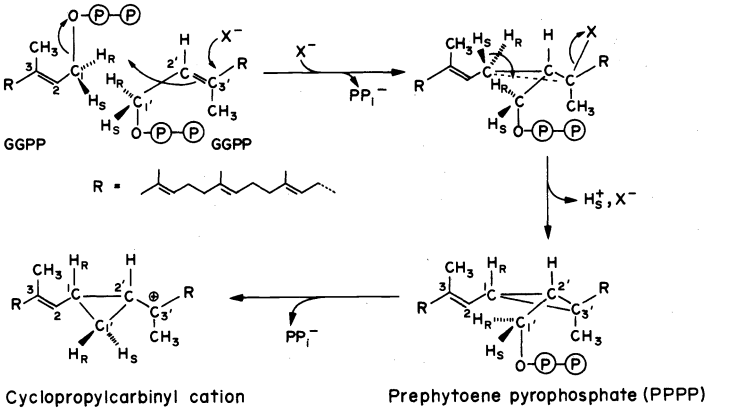
A current view of the situation is illustrated in Fig. 2. The cyclopropylcarbinyl cation formed by pyrophosphate loss from, and rearrangement of PPPP can be stabilized in any of three ways, namely (a) by gaining a hydride ion from NADPH to yield lycopersene, as in the yeast squalene synthetase system, (b) by the stereospecific loss of a *pro-R* proton to yield all-*trans* phytoene, as in *Mycobacterium*, or (c) by the stereospecific loss of the adjacent *pro-S* proton, as in *Phycomyces* and higher plants, to form 15-*cis* phytoene.

The controversy[2,17] over whether lycopersene or phytoene is the first C_{40} hydrocarbon precursor has been in progress for some 15 yr. The preliminary results are now available[28] from what is probably the most crucial study of all. A partially (500-fold) purified "phytoene synthetase" complex, prepared from an acetone powder of tomato plastids, converts IPP into phytoene with GGPP as an intermediate. It has no squalene synthetase activity and NADPH inhibits phytoene formation. The results of further studies, particularly those defining the intermediates between GGPP and phytoene, are awaited with considerable interest.

THE GENERAL PATHWAY OF CAROTENE DESATURATION

Phytoene (III) is converted into the acyclic conjugated undecaene, lycopene (VIII; ψ,ψ-carotene) by a series of didehydrogenations, each of which forms a new double bond and brings a previously isolated double bond into conjugation.[29,30] The intermediates are shown in Fig. 3 and are, in sequence, phytofluene (IV; 7,8,11,12,7',8'-hexahydro-ψ,ψ-carotene, a conjugated pentaene), a conjugated heptaene, and neurosporene (VII; 7,8-dihydro-ψ,ψ-carotene, a conjugated nonaene). Some fungi (e.g. *N. crassa*[20]) are also capable of dehydrogenating lycopene to 3,4-dehydrolycopene (IX; 3,4-didehydro-ψ,ψ-carotene).

In higher plants, the conjugated heptaene is the symmetrical ζ-carotene (V; 7,8,7',8'-tetrahydro-ψ,ψ-carotene) but the purple photosynthetic bacteria *Rhodospirillum rubrum*[31] and *Rhodopseudomonas globiformis*[32] both form the unsymmetrical isomer, 7,8,11,12-tetrahydrolycopene (VI; 7,8,11,12-tetrahydro-ψ,ψ-carotene) and use this, apparently exclusively, as the conjugated heptaene intermediate in dehydrogenation. Other bacteria, both photosynthetic (e.g. *Rhodopseudomonas viridis*[33,34]) and non-photosynthetic (e.g. *Flavobacterium dehydrogenans*[35]), and fungi (e.g. *N. crassa*[36] and *P. blakesleeanus*[37]) appear to have both isomers (V and VI) as alternative intermediates in their



The three possible mechanisms for stabilizing the cyclopropylcarbinyl cation formed from prephytoene pyrophosphate are: (a) gain of hydride ion from NADPH to yield lycopersene, (b) loss of *pro*-R proton to yield all-*trans* phytoene and (c) loss of *pro*-S proton to yield 15-*cis* phytoene.

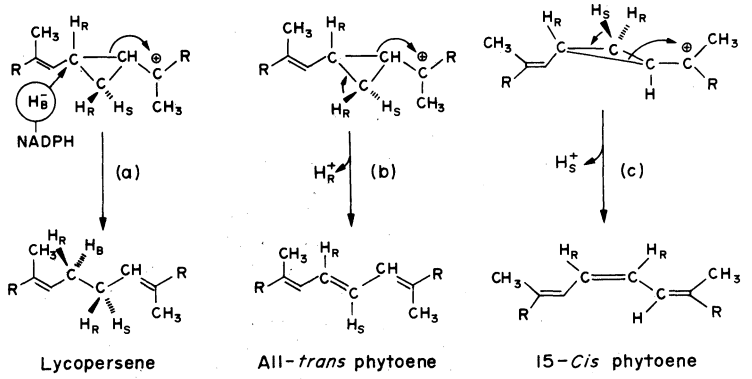


Fig. 2. The role of prephytoene pyrophosphate in carotene biosynthesis. Illustrated are the formation of *trans* phytoene (as in *Mycobacterium*[25]) and 15-*cis* phytoene (as in *Phycomyces*[25] and higher plant systems[26,27]), and the formation of lycopersene as by a non-specific squalene synthetase system.

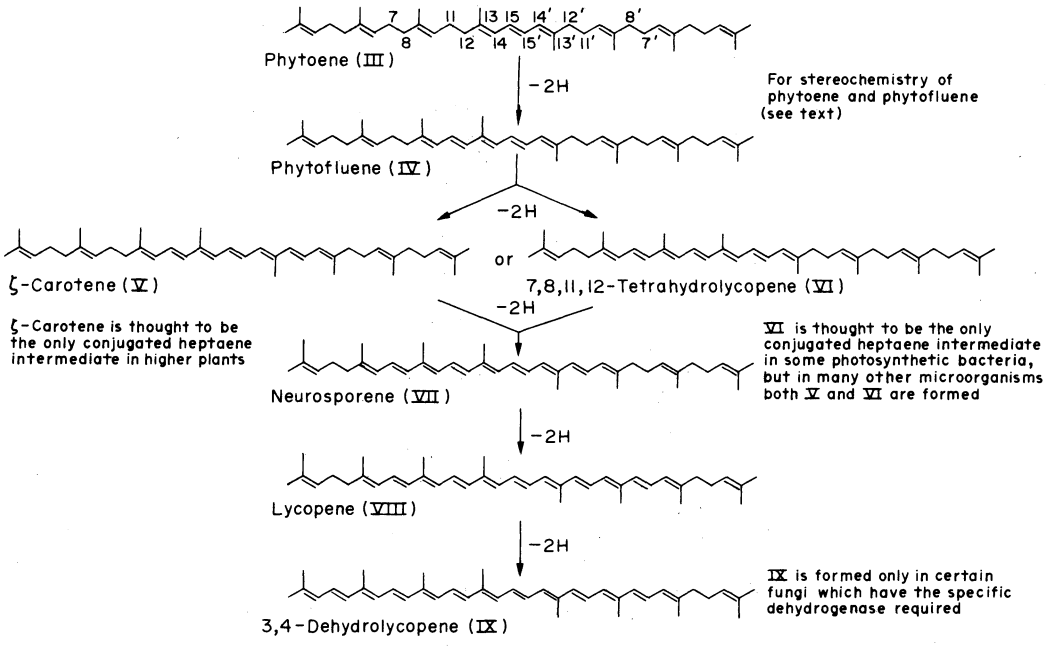


Fig. 3. The general pathway of carotene desaturation.

desaturation sequences. It may be that the carotene with the unsymmetrically-placed conjugated polyene system is mechanistically preferable[37] to its symmetrical isomer as a substrate for further metabolism by cyclization in fungi (e.g. *P. blakesleeanus*)[37] or by hydration[38] and methoxylation[32,39] in Rhodospirillaceae. The possibility of alternative intermediates participating at later stages of the desaturation sequence has also been considered. The dehydrogenase which, in *N. crassa*, converts lycopene into 3,4-dehydroycopene appears to be too substrate-specific to desaturate 7,8,11,12-tetrahydrolycopene to an "unsymmetrical neurosporene" (3,4-didehydro-7′,8′,11′,12′-tetrahydro-ψ,ψ-carotene)[20,36] but stronger mass spectral M-137 ions for some samples of natural lycopene than for the synthetic carotene[36,40] may indicate the presence of traces of an "unsymmetrical lycopene" (3,4-didehydro-7′,8′-dihydro-ψ,ψ-carotene).

THE STEREOCHEMISTRY OF PHYTOENE

Although it has been recognized for some years that higher plant phytoene has the 15-*cis* configuration,[41] it was only the recent availability of synthetic conjugated triene models[42,43] which permitted the unambiguous assignment of 13-*trans*,15-*cis*,13′-*trans* stereochemistry to the chromophore of the major component of samples of phytoene from tomato fruit, carrot oil, *N. crassa*, *P. blakesleeanus* (C5 mutant and DPA-inhibited wild type) and *R. rubrum* (DPA-inhibited).[44] In addition it was shown that all-*trans* phytoene is present as a minor natural component in all the above samples[44] and accompanies 15-*cis* phytoene in *Mucor hiemalis* (cultured in the presence and absence of 9-fluorenone),[45] in 2-hydroxybiphenyl-inhibited cultures of *R. rubrum* and *Rhodopseudomonas sphaeroides*[46] and, in the absence of inhibitors, in another *Mucor* sp. and in photosynthetic mutants of *R. rubrum*.[47] In one of the latter (S_1B_4), the all-*trans* isomer is the major component of phytoene is illuminated anaerobic cultures.[47] In some non-photosynthetic bacteria, however, the all-*trans* isomer predominates under all conditions. Such is the case for *F. dehydrogenans*,[42–43] *Halobacterium cutirubrum*[48,49] and a *Mycobacterium* sp.;[25,50] samples of phytoene from the Roche *Flavobacterium* strains, however, are predominantly 15-*cis*.[51,52]

In higher plants, fungi and photosynthetic bacteria at least, isomerization of the polyenes from 15-*cis* to all-*trans* must occur at an early stage in the dehydrogenation sequence because ζ-carotene, 7,8,11,12-tetrahydrolycopene, neurosporene and lycopene generally have the all-*trans* configuration.[30,31]

ALTERNATIVE PATHWAYS OF PHYTOFLUENE FORMATION FROM PHYTOENE

Phytoene, predominantly 15-*cis*, accumulates in fungi when dehydrogenation is inhibited by DPA. Removal of the inhibitor results in a resumption of unsaturated carotene synthesis, but not at the expense of all the accumulated phytoene. Individual carotene levels in the C9 (lycopene-accumulating) mutant of *P. blakesleeanus* at various times after DPA removal are consistent with all-*trans* phytoene being converted to phytofluene.[20] Total phytoene (containing the all-*trans* isomer) is more effective than pure 15-*cis* phytoene in diluting out the incorporation of [^{14}C]MVA into β-carotene by an enzyme system[53] from the C115 (high β-carotene) mutant. In *Phycomyces*, the immediate precursor of phytofluene appears to be all-*trans* phytoene; the available evidence shows that 15-*cis* phytoene is formed first[25] and is then isomerized to the all-*trans* isomer prior to dehydrogenation.[20]

The situation in *R. rubrum* and *Rps. sphaeroides* is similar. Samples of phytoene (with the exception of those from the S_1B_4 mutant of *R. rubrum*)[47] are predominantly 15-*cis*[44,46,47] while the phytofluene which accumulates under conditions of 2-hydroxybiphenyl[46] or DPA inhibition (in *R. rubrum*)[54] is predominantly all-*trans*. The removal of DPA from inhibited cultures of *R. rubrum* results in an increase in the level of all-*trans* phytoene at the expense of the 15-*cis* isomer.[55]

Thus in those fungi and photosynthetic bacteria which

have been examined, and in the non-photosynthetic *Flavobacterium* R1560, a cell-free preparation of which converts labelled 15-*cis* into all-*trans* phytoene,[51] 15-*cis* phytoene is formed first, isomerized to all-*trans* (a step which is at least in part photochemical in *M. hiemalis*[45]) and then dehydrogenated to all-*trans* phytofluene. An alternative pathway must operate, however, in higher plants, where dehydrogenation precedes isomerization. Tomato phytofluene is largely 15-*cis*[41] and the dehydrogenation of labelled 15-*cis* phytoene to yield 15-*cis* phytofluene (red, "hi-β", "hi-δ" and Golden Jubilee tomatoes)[56] and the isomerization (in the dark) of labelled 15-*cis* to all-*trans* phytofluene (red[57] and tangerine[58] tomatoes) have been demonstrated using plastid extracts. The intermediates in phytofluene formation are different again in *H. cutirubrum*, for 15-*cis* isomers do not participate at all.[48,49] The reactions involved in phytofluene formation in these different organisms are shown in Fig. 4.

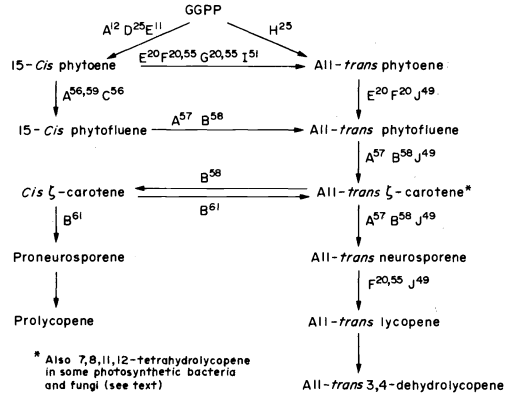


Fig. 4. Biogenetic transformations of acyclic carotenes. The letters (A–J) indicate those organisms and systems in which *individual reactions* have been either demonstrated *directly* (solubilized labelled substrate) or *specifically* inferred. (A) Red tomato fruit (soluble enzyme from plastids); (B) Tangerine tomato fruit (same system); (C) "Hi-β", "hi-δ" and Golden Jubilee tomato fruit (same system); (D) *P. blakesleeanus*, C5 mutant (cell-free preparation); (E) *P. blakesleeanus*, C9 mutant; (F) *P. blakesleeanus*, C115 mutant (cell-free preparation); (G) *R. rubrum*; (H) *Mycobacterium* sp. (soluble enzyme system); (I) *Flavobacterium* R1560 (cell-free preparation); (J) *H. Cutirubrum* (cell-free preparation).

THE INDIVIDUAL REACTIONS OF CAROTENE DEHYDROGENATION

Virtually all the individual steps of the carotene dehydrogenation sequence have now been demonstrated by direct incorporation experiments in which pure labelled carotenes, solubilized with the aid of Tween 80, were used as substrates. The conversion of 15-*cis* [^{14}C]phytoene into the complete series of unsaturated acyclic and cyclized carotenes has been accomplished both in soluble extracts of plastids from the fruits of different genetic selections of the tomato[56,59] and in *P. blakesleeanus* mutant enzyme systems.[20,55] Extracts of lyophilized plastids from red tomato fruit convert not only *cis* into *trans* phytofluene (see above) but also *trans* phytofluene into *trans* ζ-carotene and *trans* ζ-carotene into neurosporene and lycopene.[57] A similar system from tangerine (t^t and t^o) tomatoes is capable of both (a) converting *trans* ζ-carotene into more unsaturated all-*trans* carotenes and their poly-*cis* isomers (proneurosporene and prolycopene)[58] and (b) converting *cis* ζ-carotene[60] into poly-*cis* and all-*trans* carotenes.[61] ζ-Carotene is clearly the branch point for poly-*cis* carotene formation in the tangerine tomato (Fig. 4). A recent report[49] shows that a bacterial preparation, from *H. cutirubrum*, is also able to effect, among others, the conversions of *trans* phytofluene to *trans* ζ-carotene and of ζ-carotene to neurosporene. The final dehydrogenation of the general pathway, that of neurosporene to lycopene,has been demonstrated directly only in extracts from *H. cutirubrum*[49] and from the C115 mutant of *P. blakesleeanus*.[20,55] For the latter experiments, the radioactive substrate was prepared by taking advantage of the inhibition by nicotine of the further metabolism of neurosporene in *Rps. sphaeroides*.[20,62] The *in vitro* systems in which each individual step of the carotene desaturation sequence has been demonstrated directly are indicated in Fig. 4.

THE STEREOCHEMISTRY OF CAROTENE DEHYDROGENATION

The development of a crude preparation from *Flavobacterium* R1519, capable of rapidly incorporating [^{14}C]MVA into zeaxanthin (β,β-carotene-3,3′-diol) and phytoene (and, when nicotine is present, also into lycopene), has recently provided a unique opportunity for completing investigations of the stereochemistry of hydrogen loss during the desaturation of phytoene to lycopene.[63] The four double bonds inserted in the dehydrogenation sequence (Fig. 3) are those linking C-7 and C-8, C-11 and C-12, C-7′ and C-8′ and C-11′ and C-12′. Although earlier work,[26] in which [2-^{14}C, (5*R*)-5-^{3}H$_1$]- and [2-^{14}C, 5-^{3}H$_2$]MVA were incorporated into phytoene, phytofluene, ζ-carotene, neurosporene and lycopene using slices of *delta* and *tangerine* mutant tomato fruit, was able to demonstrate that the hydrogens lost from C-7, C-11, C-7′ and C-11′ were those that arose from the 5-*pro*-*R* hydrogen of MVA (the 5-*pro*-*S* hydrogen being retained at each stage), subsequent attempts to establish the stereochemistry of hydrogen loss from the other carbons involved (C-8, C-12, C-8′ and C-12′) were hampered because the IPP/DMAPP equilibration[64] led to a scrambling of label and loss of labelling stereospecificity during the long incubation periods (30–60 hr) required by the tomato slice systems. The results of rapidly incorporating [2-^{14}C, (2*R*)-2-^{3}H$_1$]- and [2-^{14}C, (2*S*)-2-^{3}H$_1$]MVA into phytoene, lycopene and zeaxanthin using the *Flavobacterium* preparation (incubation time 90 min) show that the 2-*pro*-*S* hydrogen from MVA is lost from, and the 2-*pro*-*R* retained at, each of the carbon atoms C-8, C-12, C-8′ and C-12′ in the conversion of phytoene to lycopene. An overall *trans* elimination of hydrogens therefore occurs at each dehydrogenation step.

ASPECTS OF THE CONTROL OF CAROTENE FORMATION

Although carotene cyclization is to be one of the subjects of Dr. Britton's contribution, it is not possible to discuss the control of phytoene formation and desaturation without referring, but to a deliberately limited extent, to the formation of cyclized carotenes.

It is now becoming clear which of the steps of carotene formation are influenced by light. In a *Mycobacterium* sp., GGPP formation is enhanced by illumination and PPPP formation is fully photoinduced.[15] There is evidence that the formation of *trans* phytoene in both *Mucor* spp. and mutants of *R. rubrum* is, at least in part, photochemical.[45,47] In *N. crassa*, the enzymes for both phytoene formation[65] and desaturation[66] appear to be photoinducible, and the proportion of cyclized carotenes

is increased by illumination.[20] There are both constitutive and photoinduced carotenogenic isoenzymes in *Fusarium aquaeductuum*[67] where, as in other fungi,[47] a requirement for oxygen in photoinduction may be related to the protective role of the pigments.

While evidence from *F. aquaeductuum*[68] and *Myxococcus fulvus*[69] tends to militate against the operation of a multienzyme carotenogenic complex in either organism (although an assembly line of carotenogenic enzymes associated with the cytoplasmic membrane has been suggested for the latter[70]), the situation in *Phycomyces* is different. Quantitative genetic complementation studies on the C2 (carotenoidless) and C9 (lycopene-accumulating) mutants of *P. blakesleeanus* suggest[71] that the conversion of phytoene into β-carotene is catalyzed by a multienzyme aggregate containing four dehydrogenases and two cyclases. A similar conclusion arose from studies of the haploid fungal species, *Ustilago violacea*.[72] The operation of such an aggregate in *Phycomyces* (which may be the case only during the exponential phase of growth[73]) is consistent with the low conversions (compared with those of MVA) into β-carotene obtained even using efficiently solubilized high specific activity carotene substrates.[20,74] Further genetic studies on *P. blakesleeanus* mutants[75] showed that only three genes are involved in normal carotenogenesis, *carA*, *carB* and *carR*. While *carA* and *carB* mutants are all white, only the *carB* mutants (e.g. C5) accumulate phytoene; the *carA* mutants (e.g. C2) are carotenoidless and the red *carR* mutants (e.g. C9) accumulate lycopene. The derivation from a red lycopene-producing strain of a leaky *carB* mutant (S86) which forms less phytoene than other *carB* mutants but also forms (in decreasing amounts in order of desaturation) phytofluene, ζ-carotene, neurosporene and lycopene showed that the dehydrogenases of the aggregate are four copies of the same enzyme coded by the *carB* gene.[76] Although it is also suggested that the two cyclases are two copies of the product of the *carR* gene,[76] they show different sensitivities to the cyclization inhibitors 2-(4-chlorophenylthio)triethylamine hydrochloride (CPTA)[77] and nicotine.[37,78]

Apart from the effects of chemical inhibitors (DPA, CPTA, nicotine etc.), the production of carotenes by *P. blakesleeanus* is influenced by other factors such as light and sexual activity. While the stimulatory effects of β-ionone on β-carotene formation[2] have been recognized for some time, it was only recently shown that both retinol (vitamin A) and β-ionone, presumably acting as trisporic acid mimics, stimulate the formation of phytoene in *carB*, and lycopene in *carR*, mutants.[79] The *carB* mutants contain more phytoene, and the *carR* mutants more lycopene, than the wild-type *Phycomyces* contains β-carotene. This suggests that β-carotene exerts end-product regulation so that a lack of β-carotene induces a greater carotenogenic activity with an increased accumulation of intermediates.[76] Indeed, studies using cycloheximide and CPTA indicate that β-carotene regulates its own production through regulating enzyme synthesis.[80] End-product regulation and photoinduction may exert their effects through a common mechanism (operating before phytoene), for *carB* and *carR* mutants do not significantly increase carotenogenesis as a result of illumination.[76]

Evidence has appeared from our own biosynthetic studies which indicates an element of negative feedback control (probably in addition to any end-product regulation of enzyme synthesis) in *P. blakesleeanus*. Individual carotenes were added to an enzyme system (from the C115 high β-carotene mutant)[53] actively forming β-carotene from [2-^{14}C]MVA.[20,55,74] While the addition of β-carotene itself specifically inhibited carotene cyclase activity, lycopene, γ-carotene (β,ψ-carotene), neurosporene or β-zeacarotene (7′,8′-dihydro-β,ψ-carotene), but not β-carotene, each inhibited phytoene dehydrogenation. The addition of phytoene or squalene reduced terpenoid formation in general. The ability of lycopene, and the failure of β-carotene, to inhibit phytoene desaturation in *Phycomyces* is confirmed by the growth characteristics of two mutant strains.[53] In the high β-carotene C115 strain, no phytoene but only β-carotene accumulates in the late stages of growth, in contrast to the situation in the lycopene-accumulating C9 strain where phytoene accumulates and radioactivity from [2-^{14}C]MVA appears in phytoene rather than in lycopene as soon as high lycopene levels are reached.

THE BIOSYNTHESIS OF TRITERPENOID CAROTENOIDS IN BACTERIA

On investigating the carotenes of the non-photosynthetic Gram-positive *Streptococcus faecium* UNH 564P (*Enterococcus* 564[81,82]), we made the surprising observation that although these compounds might have been identified by their absorption spectra and chromatographic behaviour on columns of alumina as normal acyclic carotenes (III–VII above), each was demonstrably less polar on thin-layer chromatography than the corresponding authentic carotene isolated from *P. blakesleeanus* or *Rps. sphaeroides*.[83] We have recently shown the value of gas–liquid chromatography of catalytically-hydrogenated derivatives in indicating such fundamental structural characteristics of carotenoids as the acyclic, monocyclic or bicyclic nature, and the size and manner of substitution of the carbon skeleton.[84] Nowhere in our studies has this method proved of more value than in establishing that the *Strep. faecium* carotenes are all triterpenoid (C_{30}) analogues of the C_{40} acyclic carotenes.[83] Mass spectrometry readily confirmed this.[83]

The triterpenoid pigments can be accommodated by the current carotenoid nomenclature system[85,86] by designating them as 4,4′-diapocarotenoids. The carotenes occurring in *Strep. faecium*[82,83] are: 4,4′-diapophytoene (X; 4,4′-diapo-7,8,11,12,7′,8′,11′,12′- octahydro-ψ,ψ-carotene; predominantly 13-*trans*, 15-*cis*, 13′-*trans*), 4,4′-diapophytofluene (XI; 4,4′-diapo-7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene), 4,4′-diapo-ζ-carotene (XII; 4,4′-diapo-7,8,7′,8′-tetrahydro-ψ,ψ-carotene) and its unsymmetrical isomer, 4,4′-diapo-7,8,11,12-tetrahydrolycopene (XIII; 4,4′-diapo-7,8,11,12-tetrahydro-ψ,ψ-carotene), and 4,4′-diaponeurosporene (XIV; 4,4′-diapo-7,8-dihydro-ψ,ψ-carotene; all-*trans*, neo B and neo C isomers). It is our practice to include the fully systematic name of each in its original description.[83]

Of these C_{30} carotenes, only diapophytoene has been reported previously, as "bacterial phytoene" in *Staphylococcus aureus* 209P[87] and as "dehydrosqualene" in *H. cutirubrum*.[48] It has been postulated that it might act as a precursor of C_{40}-phytoene in *H. cutirubrum* (which also forms C_{40}-phytoene and other tetraterpenoid carotenoids)[48] and in *Staph. aureus*[87] (which was thought to form C_{40} carotenoids[88,89]). Perhaps it was the alternative suggestion,[56,87] that C_{30}-phytoene might be the precursor of a then-hypothetical series of C_{30}-carotenes, which prompted our reinvestigation of the carotenoids of

Staph. aureus 209P. The carotenes present[90] are 4,4′-diapophytoene (X; again predominantly 13-*trans*, 15-*cis*, 13′-*trans*), all-*trans* 4,4′-diapophytofluene (XI), all-*trans* 4,4′-diapo-ζ-carotene (XII; but no isomeric unsymmetrical conjugated heptaene) and all-*trans* 4,4′-diaponeurosporene (XIV); all are also formed by Strep. *faecium*.

A cell-free preparation of *Strep. faecium* is capable of incorporating radioactivity from [2-^{14}C]MVA or [1-^{14}C]IPP into all the terpenoid components (total incorporations of up to 20 and 50%, respectively, may be achieved). The effects on these incorporations of ATP, nicotinamide coenzyme and metal ion (Mg^{2+} and Mn^{2+}) concentrations, and of different incubation conditions have already been established.[91]

Our early studies of triterpenoid carotene biosynthesis concentrated on the formation of 4,4′-diapophytoene and squalene (I; also present in *Strep. faecium*) from labelled MVA, IPP and FPP. The latter was conveniently prepared, together with labelled GGPP, from labelled MVA using a barley seed embryo extract[92] which we have found to be more reliable than systems from other organisms[93–97] for the preparation of substrate quantities of labelled terpenyl pyrophosphates. That diapophytoene arises in *Strep. faecium*, like squalene, from the dimerization of FPP rather than by the degradative loss of the terminal C_5 units from C_{40}-phytoene, has been demonstrated[74,91] by following the simultaneous incorporations of [1-^{14}C]IPP and [4,8,12-^{3}H]FPP (Fig. 5). All six isoprene units of both squalene and diapophytoene became labelled with ^{3}H. When C_{40}-phytoene was formed from the same combination of labelled substrates by the *P. blakesleeanus* (C5 mutant) enzyme system, the ratio of isoprene units labelled with ^{3}H to those labelled with ^{14}C approximated (6:1.71) to the expected value (6:2; Fig. 5). Traces of ^{14}C were incorporated into diapophytoene by the *Strep. faecium* system, presumably because some of the [^{14}C] was converted into [^{14}C]FPP which was then incorporated, but this did not interfere with the main experiment; it did permit, however, the demonstration that the decreasing ^{3}H:^{14}C ratios of the C_{30}-carotenes (and xanthophylls) were consistent with their structural formulations and their postulated route of biosynthesis (Fig. 6).

The crucial question, which should be resolved by our continuing studies, is whether the 15-*cis* 4,4′-diapophytoene of these bacteria is formed via squalene or directly from presqualene pyrophosphate. Since squalene is present in both *Strep. faecium* and *Staph. aureus*, this problem is even more intriguing than that concerning lycopersene and C_{40}-phytoene formation (see above).

The xanthophylls of *Strep. faecium* are also triterpenoids. The two most abundant are a primary alcohol formally derived from 4,4′-diaponeurosporene, namely 4-hydroxy-4,4-diaponeurosporene (XV; 4,4′-diapo-7′,8′-dihydro- ψ,ψ-carotene-4-ol), and its glucoside, 4-D-glucopyranosyloxy-4,4′- diaponeurosporene (XVI; 4-D-glucopyranosyloxy-4,4′- diapo-7′,8′-dihydro-ψ,ψ-carotene).[98] The minor xanthophylls are both aldehydes; the first is that corresponding to the above primary alcohol (XVII; 4,4′-diaponeurosporen-4-al or 4,4′-diapo-7′,8′-dihydro- ψ,ψ-caroten-4-al) while the second (XVIII; 4,4′-diapolycopen-4-al or 4,4′-diapo-ψ,ψ-caroten-4-al) is formally derived from the first by a further dehydrogenation to complete the conjugation of the C_{30} molecule.[99]

A logical biosynthetic sequence for the carotenoids of *Strep. faecium* can easily be postulated (Fig. 6).[99] The carotene dehydrogenation steps, with alternative intermediates (XII and XIII) at the conjugated heptaene level, terminate in the formation of 4,4′-diaponeurosporene. The oxidation of a methyl group (C-4) to form 4-hydroxy-4,4′-diaponeurosporene (XV) takes place in a manner analogous to that of the ω-hydroxylation of *n*-alkanes by bacterial enzymes. This would appear to be the only route available for the oxidation of the carotene since its postulated dehydrogenation product, 4,4′-diapolycopene

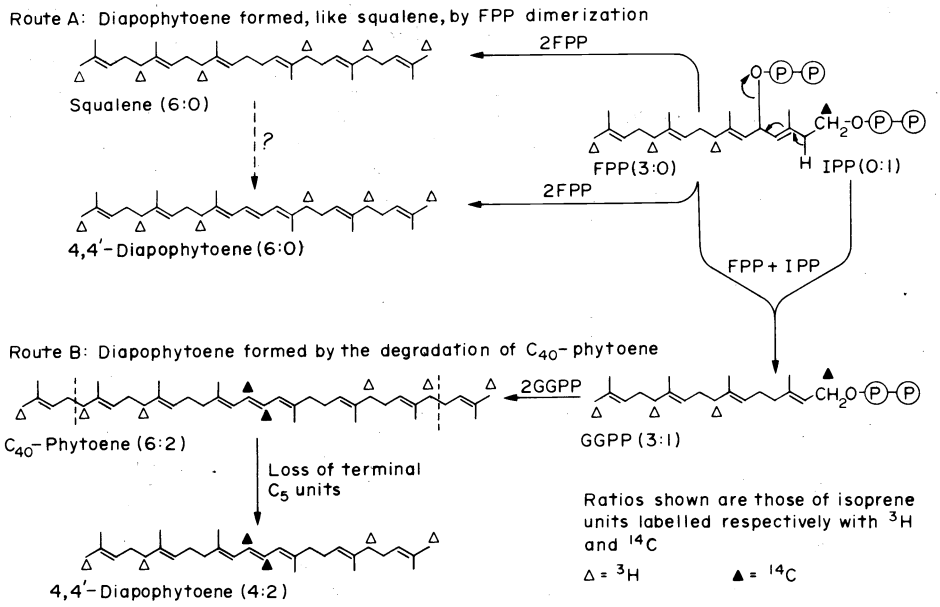


Fig. 5. Determination of the route of 4,4′-diapophytoene formation from [1-^{14}C]IPP and [4,8,12-^{3}H]FPP, If C_{30}-carotenes are formed by FPP dimerization (Route A), both diapophytoene and squalene will each have 6 isoprene units labelled with ^{3}H and none with ^{14}C (6:0). If diapophytoene is formed by degradation of C_{40}-phytoene, it will have a corresponding ratio of 4:2. The ratios obtained by experiment were: In *Strep. faecium*, squalene 6:0, diapophytoene 6:0; in *P. blakesleeanus*, squalene 6:0, C_{40}-phytoene, 6:2. Therefore route A operates in *Strep. faecium*.

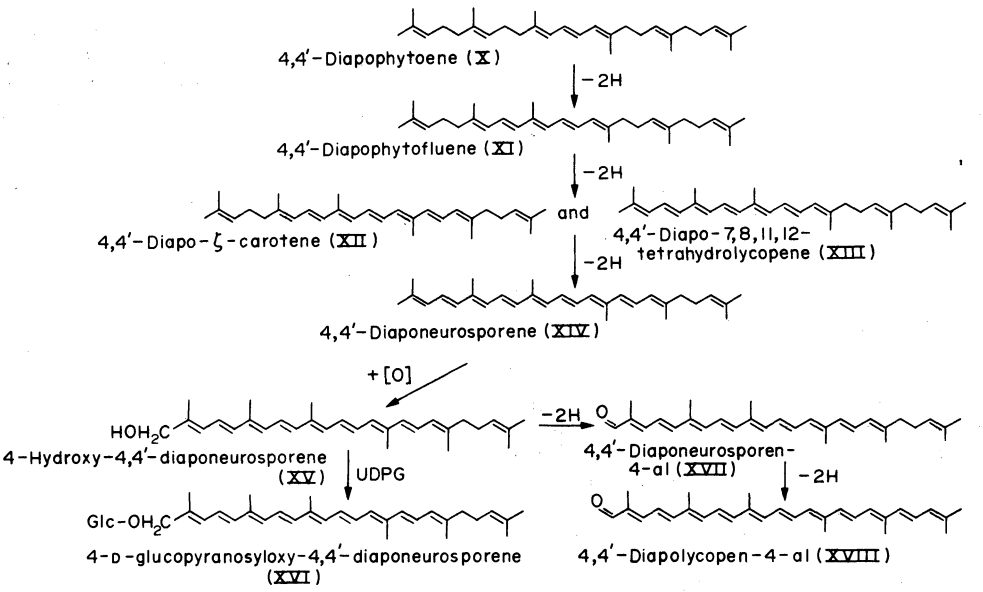


Fig. 6. Triterpenoid carotene and xanthophyll biosynthesis in *Streptococcus faecium* UNH 564P.[83,98–100]

(4,4'-diapo-ψ,ψ-carotene) has not been detected. Two alternative routes are then available for the further metabolism of the primary alcohol (XV). In the presence of glucose it could be converted to its glucoside (XVI), while otherwise it would be oxidized to its corresponding aldehyde (XVII). The latter reaction and the subsequent dehydrogenation of the aldehyde (XVII) to a fully conjugated triterpenal (XVIII) might represent the first stages of the oxidative degradation of the *Strep. faecium* carotenoids.

Our studies of the formation of xanthophylls in *Strep. faecium* have used two different, but complementary, approaches. In the first, a detailed investigation was made of the effects of varying the culture conditions on the nature of the carotenoids formed.[100] Increasing the glucose concentration of 24 hr static cultures from 0.1 to 0.5% changed the proportions of diapophytoene (X) and all-*trans* diaponeurosporene (XIV) from 85 and 2% respectively to 30 and 41% respectively of the total carotenoid. The effect of aerating cultures was such that virtually all the carotenoid of a 24 hr shake culture was in the form of unidentified degradation products, but the provision of glucose (0.5%) in the medium had a sparing effect on degradation and 75% of the total carotenoid could be recovered as the glucoside, 4-D-glucopyranosyloxy-4,4'-diaponeurosporene (XVI) after 24 hr growth.

For the second approach, advantage was taken of the results of the first and, by using the cell-free preparation from *Strep. faecium* to incorporate [2-$^3H_3$]MVA into the alcohol (XV), the aldehydes (XVII and XVIII) and the glucoside (XVI), it was shown how glucose or UDPG influenced the extent to which the alcohol was either glucosylated or oxidized.[91] A preparation from cells grown in static culture showed little increase in glucoside synthesis in the presence of glucose but glucosylation (at the apparent expense of aldehyde formation) was significantly increased by UDPG. In preparations from shake cultures, both glucose and UDPG stimulated glucosylation, thus confirming the sparing effect of glucose on oxidative degradation, and the highest glucoside radioactivity achieved in all the incubations was that recorded in the presence of UDPG. The conclusion that UDPG acts as a glucosylating agent for carotenoid glucoside synthesis was confirmed by the simultaneous incorporation of radioactivity of both [2-$^3H_2$]MVA and uridine diphospho-D-[U-^{14}C]glucose into 4-D-glucopyranosyloxy-4,4'-diaponeurosporene.[91]

Studies on the biosynthesis of the carotenoids of *Staph. aureus* are less well advanced, but a pathway can be postulated (Fig. 7) as a result of our recent determinations of the structures of the carotenes[90] and xanthophylls[101] present in normal cultures. As described above, the C_{30}-carotene dehydrogenation sequence terminates in 4,4'-diaponeurosporene (XIV). As in *Strep. faecium*, the major xanthophyll is an oxidation product of this, not the primary alcohol (XV) or its aldehyde (XVII), neither of which has been detected in our analyses, but the corresponding acid, 4,4'-diaponeurosporen-4-oic acid (XIX; 4,4'-diapo-7',8'-dihydro-ψ,ψ- caroten-4-oic acid; a small amount of a *cis* isomer but mainly all-*trans*). In the course of detailed chemical studies on this acid,[101] it was shown that treatment with 5% methanolic KOH caused a partial methylation. It must be admitted that a combination of this reaction and a transesterification reaction between methanol and another carotenoid present (XXII) caused us to think at first that the methyl ester was the major xanthophyll; 5% methanolic KOH had been used as the original extractant! As we have since carried out partial methylations of a number of authentic carotenoic acids using 5% methanolic KOH, it is now our opinion that some methyl carotenoates[102–105] reportedly extracted with, or recorded after saponification with, this mixture may not be true products of biosynthesis. It should be noted, however, that under slightly more rigorous conditions (e.g. 10% methanolic KOH) the balance of the reaction is in favour of saponification rather than methylation, so that isolation of carotenoic acids[106–108] results.

The structures of other xanthophylls present[109] indicate that there is also oxidation, but less drastic, at the other, more saturated end of the molecule. The C-4' methyl group of 4,4'-diaponeurosporen-4-oic acid is apparently oxidized to a primary alcohol, with 4'-hydroxy-4,4'-

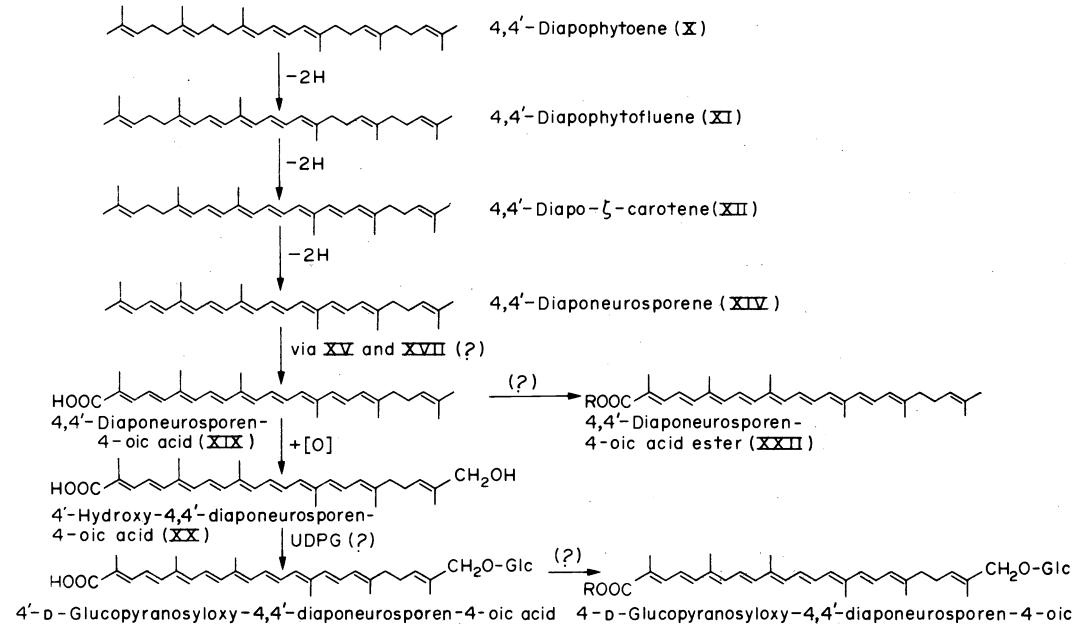


Fig. 7. Structures of the carotenoids isolated from *Staphylococcus aureus* 209P[90,91,104,105] and their postulated pathway of biosynthesis.

diaponeurosporen-4-oic acid (XX; 4'-hydroxy-4,4'-diapo- 7',8'-dihydro-ψ,ψ- caroten-4-oic acid) as the product. Like the primary alcohol (XV) of *Strep. faecium*, this can be converted to its glucoside, 4'-D-glucopyranosyloxy-4,4'- diaponeurosporen-4-oic acid (XXI; 4'-D-glucopyranosyloxy-4,4'- diapo-7',8'-dihydro-ψ,ψ-caroten-4-oic acid). Both the major xanthophyll (XIX) and this acidic glucoside (XXI) can apparently also exist as natural esters (XXII and XXIII respectively).

One of the interesting biosynthetic features arising from this structural work on the carotenoids of *Staph. aureus* is the rapid and complete oxidation to a carboxyl group of one of the terminal methyl groups (that at the more unsaturated end) of 4,4'-diaponeurosporene. Although neither of the presumed intermediates, the primary alcohol and the aldehyde, were detected, there is already indirect evidence that the initial oxidation of the carotene is by a mixed-function hydroxylation. This is the observation[109] that the formation of the major xanthophyll is inhibited and carotenes accumulate when *Staph. aureus* is treated with known mixed-function oxidase inhibitors.

CONCLUSION

The last 5 years' studies, carried out in many laboratories, have collectively achieved considerable progress in increasing the extent and improving the clarity of our understanding of the "early steps" of C_{40}-carotenoid biosynthesis. One of the main contributory factors has been the development of a number of high-activity cell-free preparations capable not only of rapidly incorporating stereospecifically labelled precursors without risk of randomization of label or loss of labile pigments, but also of efficiently utilizing artificially-solubilized labelled carotenes as substrates. The availability of ranges of carotenogenic mutants of a number of organisms for study by biochemical and genetic methods, and the use of a more enzymological approach to biosynthesis have also contributed. Further investigations on the biosynthesis of the triterpenoid (C_{30}) carotenoids will continue to take considerable advantage of the hard-won background of knowledge and experience gained from studies of their tetraterpenoid analogues.

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