CAROTENOID BIOSYNTHESIS—THE EARLY STEPS

BRIAN H. DAVIES and RICHARD F. TAYLOR
Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Aberystwyth, Dyfed, SY23 3DD, UK

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 phytone 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 C₄₀ 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 chromatography, and their sensitivity to heat, light, oxygen, acids and, in some cases, to alkali, dictate that chromatography, and their sensitivity to heat, light, oxygen, acids and, in some cases, to alkali, dictate that.

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. For this purpose, it has been convenient to consider carotenoid biosynthesis as comprising four distinct phases, namely (1) the formation of the first C₄₀ 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 (C₉₀) carotenoids, our contribution also includes a report on the progress of these studies (involving both carotenes and xanthophylls).

THE VERY EARLY STEPS: UP TO C₉₀

The ready availability of ¹⁴C-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 [1-¹⁴C]- and [2-¹⁴C]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; C₁₀) 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 C₁ as CO₂, into Δ¹-isopentenyl pyrophosphate (IPP; C₁₀). The isomerization of IPP to dimethylallylpyrophosphate (DMAPP), the condensation of these two C₁₀ molecules (IPP and DMAPP) to yield geranyl pyrophosphate (GPP; C₁₀) and subsequent additions of C₁₀ IPP units result in the formation of farnesyl pyrophosphate (FPF; C₁₅) and then geranylgeranyl pyrophosphate (GGPP; C₉₀). The reactions of the biosynthesis of GGPP from MVA are summarized in Fig. 1.

THE NATURE AND FORMATION OF THE FIRST C₉₀ HYDROCARBON

The reductive dimerization of FFP (C₁₀) to yield squalene (Δ); C₁₀, the symmetrical acyclic precursor of sterols, involves a cyclopropylcarbinyl pyrophosphate intermediate, presqualene pyrophosphate (PSPP). The absolute configuration of PSPP is established and the mechanism of its formation and conversion to squalene largely understood as a result of studies with model compounds. The C₁₀ carotenes are formed by the dimerization of GGPP (C₉₀) and the first product is again an intermediate cyclopropylcarbinyl pyrophosphate,
prephytoene pyrophosphate\textsuperscript{13,14} (PPPPP, also called pre-lycopersene pyrophosphate\textsuperscript{14}), which is the C\textsubscript{20} analogue of PSPPP and has the same absolute configuration.\textsuperscript{14} The enzyme responsible for its formation in a \textit{Mycobacterium} sp. is fully photoinduced.\textsuperscript{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\textsuperscript{23}) by yeast squalene synthetase,\textsuperscript{14} by an enzyme system from \textit{Neurospora crassa}\textsuperscript{10} and by a tomato plastid extract\textsuperscript{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\textsuperscript{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\textsuperscript{24} (rather than via lycopersene) has now been tested in extracts from a \textit{Mycobacterium} sp. and from the C5 (phytoene-accumulating) mutant of \textit{P. blakesleeanus}.\textsuperscript{25} Both 15-cis and all-trans phytoene, formed from \([4-{\textsuperscript{14}C, 1-3H}_2]\)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\textsuperscript{26} and by chloroplasts\textsuperscript{27} had shown that these two hydrogens (of 15-cis phytoene) are derived exclusively from the pro-\textit{R} hydrogens of \((5R)-5-{\textsuperscript{3}H}_3\)MVA. By using \([4-{\textsuperscript{14}C, (1S)-3H}_3]\)GGPP as a substrate, it was further shown\textsuperscript{25} that
15-cis phytoene (Phycomyces) retained neither of the labelled (pro-S) hydrogens (confirming the earlier results from higher plant systems) while trans phytoene (Mycobacterium) retained one pro-R and one pro-S hydrogen at the central carbons. It has been argued 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 over whether lycopersene or phytoene is the first C9 hydrocarbon precursor has been in progress for some 15 yr. The preliminary results are now available 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; \( \psi, \psi \)-carotene) by a series of dihydrogenations, each of which forms a new double bond and brings a previously isolated double bond into conjugation. The intermediates are shown in Fig. 3 and are, in sequence, phytofluene (IV; 7,8,11,12,7',8'-hexahydro-\( \psi, \psi \)-carotene, a conjugated pentaene), a conjugated heptaene, and neurosporene (VII; 7,8-dihydro-\( \psi, \psi \)-carotene, a conjugated nonaene). Some fungi (e.g. *N. crassa*) are also capable of dehydrogenating lycopene to 3,4-dehydrolycopene (IX; 3,4-didehydro-\( \psi, \psi \)-carotene).

In higher plants, the conjugated heptaene is the symmetrical \( \zeta \)-carotene (V; 7,8,7',8'-tetrahydro-\( \psi, \psi \)-carotene) but the purple photosynthetic bacteria *Rhodopseudillum rubrum* and *Rhodopseudomonas globiformis* both form the unsymmetrical isomer, 7,8,11,12-tetrahydrolycopene (VI; 7,8,11,12-tetrahydro-\( \psi, \psi \)-carotene) and use this, apparently exclusively, as the conjugated heptaene intermediate in dehydrogenation. Other bacteria, both photosynthetic (e.g. *Rhodopseudomonas viridis* and *Rhodopseudomonas globiformis*), and fungi (e.g. *N. crassa* and *P. blakesleeanus*) appear to have both isomers (V and VI) as alternative intermediates in their biosynthesis of \( \psi, \psi \)-carotenoids.
desaturation sequences. It may be that the carotene with the unsymmetrically-placed conjugated polyene system is mechanistically preferable\textsuperscript{37} to its symmetrical isomer as a substrate for further metabolism by cyclization in fungi (e.g. \textit{P. blakesleeanus})\textsuperscript{37} or by hydration\textsuperscript{38} and methoxylation\textsuperscript{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 \textit{N. crassa}, converts lycopene into 3,4-dehydrolycopene 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-\(\gamma\)/\(\iota\)-carotene)\textsuperscript{20,36} but stronger mass spectral M-137 ions for some samples of natural lycopene than for the synthetic carotene\textsuperscript{36,40} may indicate the presence of traces of an "unsymmetrical lycopene" (3,4-didehydro-7',8'-dihydro-\(\gamma\)/\(\iota\)-carotene).\textsuperscript{38}

THE STEREOCHEMISTRY OF PHYTOENE

Although it has been recognized for some years that higher plant phytoene has the 15-cis configuration,\textsuperscript{41} it was only the recent availability of synthetic conjugated triene models\textsuperscript{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, \textit{N. crassa}, \textit{P. blakesleeanus} (CS mutant and DPA-inhibited wild type) and \textit{R. rubrum} (DPA-inhibited).\textsuperscript{44} In addition it was shown that all-trans phytoene is present as a minor natural component in all the above samples\textsuperscript{44} and accompanies 15-cis phytoene in \textit{Mucor hiemalis} (cultured in the presence and absence of 9-fluorenone),\textsuperscript{45} in 2-hydroxybiphenyl-inhibited cultures of \textit{R. rubrum} and \textit{Rhodopseudomonas sphaeroides}\textsuperscript{46} and, in the absence of inhibitors, in another \textit{Mucor} sp. and in photosynthetic mutants of \textit{R. rubrum}.\textsuperscript{47} In one of the latter (S,B), the all-trans isomer is the major component of phytoene is illuminated anaerobic cultures.\textsuperscript{47} In some non-photosynthetic bacteria, however, the all-trans isomer predominates under all conditions. Such is the case for \textit{F. dehydrogenans},\textsuperscript{42,43} \textit{Halobacterium cutirubrum}\textsuperscript{48,49} and a \textit{Mycobacterium sp.}\textsuperscript{25,30} samples of phytoene from the Roche \textit{Flavobacterium} strains, however, are predominantly 15-cis.\textsuperscript{5,52}

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 \(\xi\)-carotene, 7,8,11,12-tetrahydrolycopene, neurosporene and lycopene generally have the all-trans configuration.\textsuperscript{33,34}

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 \textit{P. blakesleeanus} at various times after DPA removal are consistent with all-trans phytoene being converted to phytofluene.\textsuperscript{29} Total phytoene (containing the all-trans isomer) is more effective than pure 15-cis phytoene in diluting out the incorporation of [\textsuperscript{14}C]MVA into \(\beta\)-carotene by an enzyme system\textsuperscript{53} from the Cl15 (high \(\beta\)-carotene) mutant. In \textit{Phycomyces}, the immediate precursor of phytofluene appears to be all-trans phytoene; the available evidence shows that 15-cis phytoene is formed first\textsuperscript{25} and is then isomerized to the all-trans isomer prior to dehydrogenation.\textsuperscript{20}

The situation in \textit{R. rubrum} and \textit{Rps. sphaeroides} is similar. Samples of phytoene (with the exception of those from the S,B\textsubscript{a} mutant of \textit{R. rubrum})\textsuperscript{67} are predominantly 15-cis\textsuperscript{64,67} while the phytofluene which accumulates under conditions of 2-hydroxybiphenyl\textsuperscript{46} or DPA inhibition (in \textit{R. rubrum})\textsuperscript{54} is predominantly all-trans. The removal of DPA from inhibited cultures of \textit{R. rubrum} results in an increase in the level of all-trans phytoene at the expense of the 15-cis isomer.\textsuperscript{55}

Thus in those fungi and photosynthetic bacteria which
have been examined, and in the non-photosynthetic \textit{Flavobacterium} R1560, a cell-free preparation of which converts labelled 15-cis into all-trans phytoene,\textsuperscript{51} 15-cis phytoene is formed first, isomerized to all-trans (a step which is at least in part photochemical in \textit{M. hiemalis})\textsuperscript{48} 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\textsuperscript{52} and the dehydrogenation of labelled 15-cis phytoene to yield 15-cis phytofluene (red, "hi-β", "hi-8" and Golden Jubilee tomatoes)\textsuperscript{9} and the isomerization (in the dark) of labelled 15-cis to all-trans phytofluene (red\textsuperscript{53} and tangerine\textsuperscript{8} tomatoes) have been demonstrated using plastid extracts. The intermediates in phytofluene formation are different again in \textit{H. cutirubrum}, for 15-cis isomers do not participate at all.\textsuperscript{49,50} The reactions involved in phytofluene formation in these different organisms are shown in Fig. 4.

The intermediates in phytofluene formation are different again in \textit{H. cutirubrum}, for 15-cis isomers do not participate at all.\textsuperscript{49,50} The reactions involved in phytofluene formation in these different organisms are shown in Fig. 4.

The intermediates in phytofluene formation are different again in \textit{H. cutirubrum}, for 15-cis isomers do not participate at all.\textsuperscript{49,50} The reactions involved in phytofluene formation in these different organisms are shown in Fig. 4.

\textbf{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-8" and Golden Jubilee tomato fruit (same system); (D) \textit{P. blakesleeanus}, C5 mutant (cell-free preparation); (E) \textit{P. blakesleeanus}, C9 mutant; (F) \textit{P. blakesleeanus}, C11 mutant (cell-free preparation); (G) \textit{R. rubrum}; (H) \textit{Mycobacterium} sp. (soluble enzyme system); (I) \textit{Flavobacterium} R1560 (cell-free preparation); (J) \textit{H. Cutirubrum} (cell-free preparation).

\textbf{THE INDIVIDUAL REACTIONS OF CAROTENE DEHYDROGENATION}

\begin{itemize}
  \item 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 \textit{[14}C\textit{]}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,\textsuperscript{52,53} and in \textit{P. blakesleeanus} mutant enzyme systems.\textsuperscript{30,35} Extracts of lyophilized plastids from red tomato fruit convert not only cis into \textit{trans} phytofluene (see above) but also \textit{trans} phytofluene into \textit{trans} ζ-carotene and \textit{trans} ζ-carotene into neurosporene and lycopene.\textsuperscript{31} A similar system from tangerine (t' and t") tomatoes is capable of both (a) converting \textit{trans} ζ-carotene into more unsaturated all-

\textit{trans} carotenes and their poly-cis isomers (proneuros-

\textit{porene} and \textit{lycopene}) and (b) converting \textit{cis} ζ-

\textit{carotene}\textsuperscript{32} into poly-cis and all-

\textit{trans} carotenes.\textsuperscript{61} ζ-

\textit{Carotene} is clearly the branch point for poly-cis carotene formation in the tangerine tomato (Fig. 4). A recent report\textsuperscript{49} shows that a bacterial preparation, from \textit{H. cutirubrum}, is also able to effect, among others, the conversions of \textit{trans} phytofluene to \textit{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 \textit{H. cutirubrum}\textsuperscript{64} and from the C115 mutant of \textit{P. blakesleeanus}.

\textbf{THE STEREOCHEMISTRY OF CAROTENE DEHYDROGENATION}

The development of a crude preparation from \textit{Flavobacterium} R1519, capable of rapidly incorporating \textit{[14}C\textit{]}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.\textsuperscript{65} 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,\textsuperscript{26} in which \textit{[2-14}C\textit{, (5R)-5-3H_1]} and \textit{[2-14}C\textit{, 5-3H_2]}MVA were incorporated into phytoene, phytofluene, ζ-carotene, neurosporene and lycopene using slices of \textit{delta} and \textit{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 \textit{5-pro-R} hydrogen of MVA (the \textit{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/DAPP equilibration\textsuperscript{66} 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 \textit{[2-13}C\textit{, (2R)-2-3H_1]} and \textit{[2-13}C\textit{, (2S)-2-3H_2]}MVA into phytoene, lycopene and zeaxanthin using the \textit{Flavobacterium} preparation (incubation time 90 min) show that the \textit{2-pro-R} hydrogen from MVA is lost from, and the \textit{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 \textit{trans} elimination of hydrogens therefore occurs at each dehydrogenation step.

\textbf{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 \textit{Mycobacterium} sp., GGPP formation is enhanced by illumination and PPPP formation is fully photoinduced.\textsuperscript{19} There is evidence that the formation of \textit{trans} phytoene in both \textit{Mucor} spp. and mutants of \textit{R. rubrum} is, at least in part, photochemical.\textsuperscript{67} In \textit{N. crassa}, the enzymes for both phytoene formation\textsuperscript{14,15} and desaturation\textsuperscript{14} appear to be photoinducible, and the proportion of cyclized carotenes

\textbf{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-8" and Golden Jubilee tomato fruit (same system); (D) \textit{P. blakesleeanus}, C5 mutant (cell-free preparation); (E) \textit{P. blakesleeanus}, C9 mutant; (F) \textit{P. blakesleeanus}, C11 mutant (cell-free preparation); (G) \textit{R. rubrum}; (H) \textit{Mycobacterium} sp. (soluble enzyme system); (I) \textit{Flavobacterium} R1560 (cell-free preparation); (J) \textit{H. Cutirubrum} (cell-free preparation).}
is increased by illumination. There are both constitutive and photoinduced carotenogen isoenzymes in Fusarium aquaeductuum where, as in other fungi, a requirement for oxygen in photoinduction may be related to the protective role of the pigments.

While evidence from *F. aquaeductuum* and *Myxococcus fulus* tends to support 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), the situation in *Phycymyces* is different. Quantitative genetic complementation studies on the C2 (carotenoidless) and C9 (lycopene-accumulating) mutants of *P. blakesleeanus* suggest 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, *Usilago violacea*.

The operation of such an aggregate in *Phycymyces* (which may be the case only during the exponential phase of growth) is consistent with the low conversions (compared with those of MVA) into 3-carotene obtained even at growth73) is consistent with the low conversions (comparable to those of MVA) into 3-carotene obtained even at growth.

Evidence from *F. aquaeductuum* and *Myxococcus fulus* tends to support 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). The situation in *Phycymyces* is different. Quantitative genetic complementation studies on the C2 (carotenoidless) and C9 (lycopene-accumulating) mutants of *P. blakesleeanus* suggest 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, *Usilago violacea*.

The operation of such an aggregate in *Phycymyces* (which may be the case only during the exponential phase of growth) is consistent with the low conversions (compared with those of MVA) into 3-carotene obtained even at growth.
The carotenes present are 4,4'-diapophytoene (X; again predominantly 13-trans, 15-cis, 13'-trans), all-trans 4,4'-diapophytofluene (XI), all-trans 4,4'-diapophyto-ζ-carotene (XII; but no isomeric 4,4'-diapophytoene (X; again predominantly 13-trans, 15-cis, 13'-trans), all-trans 4,4'-diapophytofluene (XIII). All-trans 4,4'-diapophytofluene (XII) is formalized from the first by a further dehydrogenation, with alternative interconversions of the C30 molecule to that corresponding to the above primary alcohol (XVII). 4-n-glucopyranosyloxy-4,4'-diaponeurosporen-4-ol, the minor xanthophylls of Strep. faecium are both aldehydes; the first is that corresponding to the above primary alcohol (XVII; 4,4'-diaponeurosporene-4-al or 4,4'-diapophytofluene-7',8'-dihydro-4'-α,β-carotene-4-al) while the second (XVIII; 4,4'-diapolyisoprene-4-al) is formally derived from the first by a further dehydrogenation to complete the conjugation of the C30 molecule.

A biological synthetic sequence for the carotenoids of Strep. faecium can easily be postulated (Fig. 6). The carotene dehydrogenation steps, with alternative intermediates (XII and XIII) at the conjugated heptane 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'-diapolyisoprene

Fig. 5. Determination of the route of 4,4'-diapophytoene formation from [1-14C]IPP and [4,8,12-3H]FPP. If C40-carotenes are formed by FPP dimerization (Route A), both diapophytoene and squalene will each have 6 isoprene units labelled with H and none with 14C (6:0). If diapophytoene is formed by degradation of C50-carotene, 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, C50-carotene, 5:2. Therefore route A operates in Strep. faecium.
(4,4'-diapo-\(\phi,\phi\)-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.\textsuperscript{109} 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-\(\alpha\)-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 \textit{Strep. faecium} to incorporate [2-3\textsuperscript{H}]MVA into the alcohol (XV), the aldehydes (XVII and XVIII) and the glucoside (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-\(\alpha\)-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 \textit{Strep. faecium} to incorporate [2-3\textsuperscript{H}]MVA into the alcohol (XV), the aldehydes (XVII and XVIII) and the glucoside (XIV), it was shown how glucose or UDPG influenced the extent to which the alcohol was either glucosylated or oxidized.\textsuperscript{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 [2-3\textsuperscript{H}]MVA and uridine diphospho-D-[U-\(\textsuperscript{14}C\)]glucose into 4-\(\alpha\)-d-glucopyranosyloxy-4,4'-diaponeurosporene.\textsuperscript{91}

Studies on the biosynthesis of the carotenoids of \textit{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\textsuperscript{90} and xanthophylls\textsuperscript{91} present in normal cultures. As described above, the C\textsubscript{30}-carotene dehydrogenation sequence terminates in 4,4'-diaponeurosporene (XIV). As in \textit{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-\(\phi,\phi\)-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,\textsuperscript{109} 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 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 carotenotes\textsuperscript{109}--\textsuperscript{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\textsuperscript{106}--\textsuperscript{108} results.

The structures of other xanthophylls present\textsuperscript{109} indicate that there is also oxidation, but less drastic, at the other, more saturated end of the molecule. The C\textsubscript{4}-methyl group of 4,4'-diaponeurosporen-4-oic acid is apparently oxidized to a primary alcohol, with 4-hydroxy-4,4'-
diaponeurosporene-4-oic acid (XX; 4'-hydroxy-4,4'-diap-7',8'-dihydro-\(\cdot\)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'-diaponeurosporene-4-oic acid (XXI; 4'-D-glucopyranosyloxy-4,4'-diap-7',8'-dihydro-\(\cdot\)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\(^{10}\) 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 \(\text{C}_{40}\)-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 carotenoids 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 \(\text{C}_{30}\) carotenoids will continue to take considerable advantage of the hard-won background of knowledge and experience gained from studies of their tetraterpenoid analogues.

**Acknowledgements**—In including in this review references to work carried out since 1970 in this laboratory, we would like to acknowledge with gratitude the contributions of former collaborators of B.H.D., particularly Drs. P. M. Bramley and Aung Than and Mrs. A. F. Rees. R. F. T. held a NATO Postdoctoral Fellowship in Science (1972-3) and is currently completing his tenure of an S.R.C. Senior Research Associateship. The Science Research Council has also provided further financial support.

**REFERENCES**

Carotenoid biosynthesis—the early steps