

THE BIOSYNTHESIS OF CAROTENOIDS

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INTRODUCTION

Considerable progress has been made in the field of carotenoid biochemistry in the last ten to fifteen years. Prior to that very little, if anything, was known about the units which formed the intermediates of the coloured 40-carbon pigments, or the structures of those that were proposed as intermediates. Like all fields of biochemistry, as knowledge is accumulated specific problems are crystallized. These generally concern key areas, which until an understanding is achieved can substantially hinder the unification of a field or problem. The biochemistry of the carotenoids has arrived at this point.

INTERMEDIATE COMPOUNDS IN CAROTENOID BIOSYNTHESIS

There is general consensus as to the identity of the intermediate compounds which form the building blocks of the carotenoids. Studies in *Phycomyces blakesleeanus*, tomatoes, corn, carrots, *Mucor hiemalis*, spinach leaves, and bean leaves all coincide to indicate that the common building block of carotenoids is mevalonic acid¹⁻⁷. Some experiments concerned with carotenoids, and many with sterols, have shown that β -methyl- β -hydroxyglutarate-CoA, and/or acetoacetic-CoA, are the normal sources of mevalonic acid^{1, 8}. In *Phycomyces blakesleeanus*, as well as tomatoes, it has been shown rather unequivocally that the first steps in the conversion of mevalonic acid to the C-40's are identical to those postulated for the formation of sterols⁹⁻¹¹. The mevalonic acid is converted via the 5-phosphomevalonic acid to the 5-pyrophosphoryl mevalonic acid¹². This compound is then decarboxylated to form the Δ^3 -isopentenol pyrophosphate. The decarboxylation requires an additional mole of ATP, but the pentenol does not exchange phosphorus, although it is postulated that the 3-phosphate is an intermediate¹³. It is then isomerized to the γ,γ -dimethylallyl pyrophosphate¹⁴. A unit of the diallyl pyrophosphate condenses with the isopentenyl pyrophosphate to give a 10-carbon unit, which then in turn is condensed with an additional isopentenol pyrophosphate to give farnesyl pyrophosphate¹⁵⁻¹⁸. It has been shown that utilizing labelled compounds the Δ^3 -isopentenol pyrophosphate can be utilized to form the C-40 carotenoids— β -carotene in mould extracts and lycopene in tomato homogenates. Since iodoacetamide has been shown to block the conversion of mevalonic acid into carotenoids, and as it blocks the isopentenol isomerase, the conclusion can be drawn that both the Δ^3 -isopentenol pyrophosphate and the

γ,γ -dimethylallyl pyrophosphate are obligatory intermediates in the synthesis of carotenoids. *Figure 1* shows these initial condensations. Further, it has been shown in both tomatoes and *Phycomyces* that farnesyl pyrophosphate is incorporated rapidly into β -carotene and lycopene²¹.

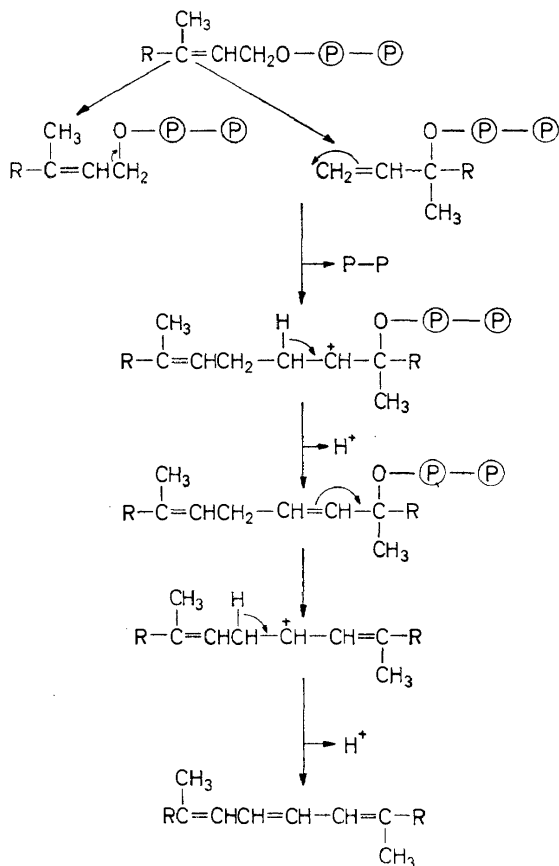


Figure 1

Goodwin, Porter, Yokoyama, Suzue and others have suggested on the basis of experimental work that a C-20 compound formed by the coupling of farnesyl pyrophosphate to a C-5 unit is an obligatory intermediate in the reaction prior to the formation of a C-40 chain¹⁹⁻²². A logical compound is geranylgeranyl pyrophosphate, the enzymatic synthesis of which has been demonstrated by Nandi and others. There has, however, been no actual proof that this compound is the condensing unit^{19, 20}.

A tomato enzyme system capable of synthesizing phytoene from isopentenol pyrophosphate also synthesizes acid labile phosphorylated compounds. Upon gas chromatographic analysis of these compounds, Porter has reported that an appreciable amount of radioactivity from labelled

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farnesyl pyrophosphate was found associated with geranylinallool. Radioactivity was also found associated with geranylgeraniol²⁰. He, as well as ourselves, was unable to show that this compound was incorporated to any appreciable extent into carotenoids.

Thompson isolated a C-20 pentaene from tomatoes after the injection of radioactive mevalonate into tomato fruit. This compound increased rapidly after the addition of mevalonate and decreased upon subsequent pigment formation. However, when the radioactive pentaene was injected, very little, if any, incorporation was found in the carotenoids²³. Kandutsch isolated C-14-labelled geranylgeraniol from *Micrococcus* incubated with various pyrophosphates²⁴. However, numerous other compounds were found equivalently labelled, and properties of these compounds differed only very slightly from geranylgeraniol. Goodwin also suggests that geranylgeranyl pyrophosphate is the condensing unit to form the first C-40, based on indirect evidence¹⁹. In short, there have been many experiments which show that the compound is formed in systems capable of synthesizing carotenoids. *Figure 2* illustrates a possible mode of formation of geranylgeraniol pyrophosphate. There is some circumstantial evidence that indicates a C-20 unit is the condensing unit, but no direct proof of the fact that geranylgeranyl pyrophosphate is the condensing unit has been forthcoming.

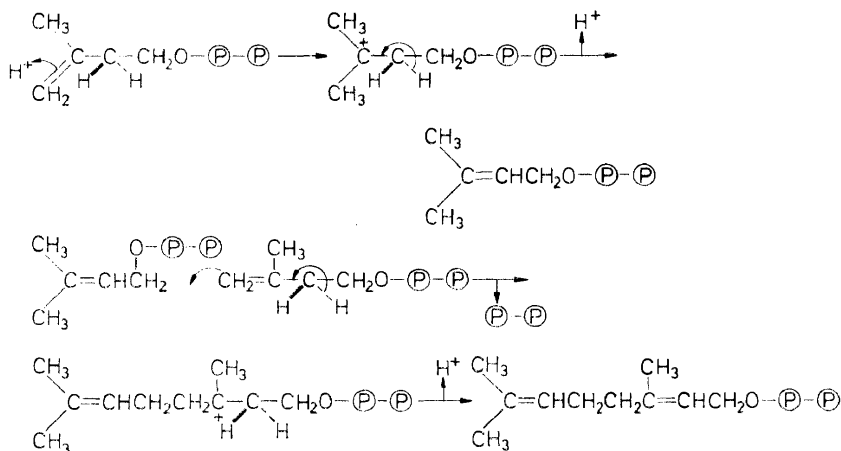


Figure 2

An interesting suggestion is that of Quackenbush, who has postulated recently that rather than a single C-20 compound being the general precursor for all C-40 compounds, the possibility exists that different structures, alicyclic and aliphatic, may be formed prior to condensation at C-20 level to form the known carotenoids. In this respect a series of five C-20 units could conceivably serve as the basis for most of the hydrocarbons known²⁵. *Figure 3* illustrates the five possible C-20 precursors and the methods of linking these to give a particular carotenoid.

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If, on the other hand, a C-40 compound is formed which is in turn modified to give a series of compounds which we know as the hydrocarbon carotenoids, the question arises, what compound is the initial unit. This is certainly an area of dispute in the field. Grobe and Boschetti, Nusbaum and Villoutreix suggest that the first C-40 compound is lycopersene, containing a central single bond^{26, 27}. Other investigators have not been able to detect lycopersene in a number of carotenoidgenic systems. Porter was able to demonstrate a solubilized enzyme system from tomato plastids that uses

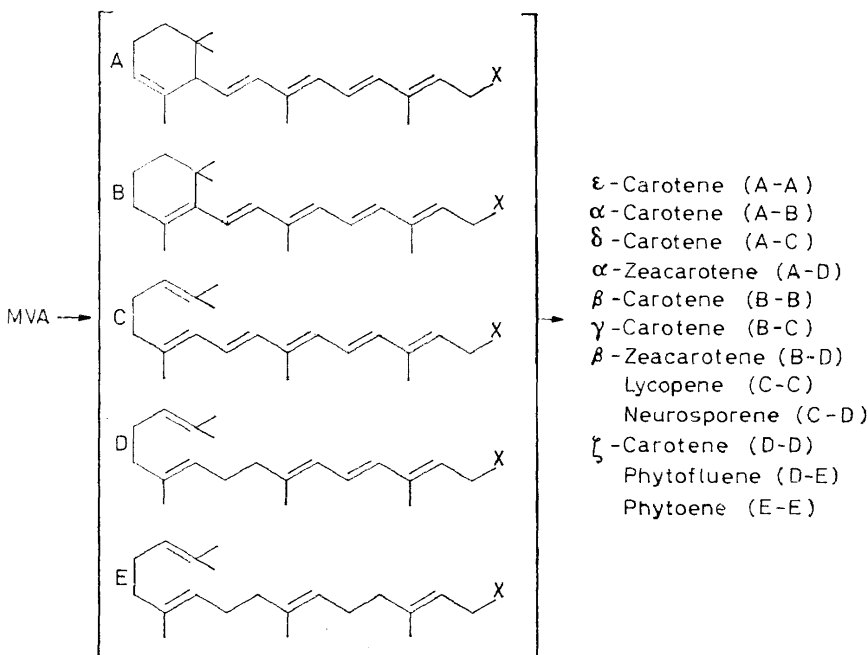


Figure 3

isopentenol pyrophosphate as a substrate for the formation of phytoene. Further studies indicated that an equivalently active substrate for the formation of phytoene was farnesyl pyrophosphate. The enzyme system was partially purified by precipitation in ammonium sulphate, absorption on DEA cellulose, and subsequent elution using a phosphate buffer. In this system (capable of synthesizing phytoene) a search was made for lycopersene, and it is reported that no evidence has been found which suggests that lycopersene is an intermediate in phytoene formation²⁰. The mechanism for the pyrophosphate and proton elimination was not reported.

The conversion of mevalonic acid to squalene during sterol synthesis is a net reductive process. The utilization of mevalonate for carotenoid synthesis in the overall sense, however, is oxidative. If the first compound synthesized is lycopersene, or dihydrophytoene, the initial step is a reductive

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one. The conversion, however, to phytoene, which has been suggested as the initial intermediate in the formation of other carotenoids, occurs essentially without change in oxidation state. Phytoene contains a central double bond in conjugation with two adjacent double bonds. The placement of these double bonds fixes the central portion of the molecules, leaving only the end of the chain containing the three isolated double bonds on either side of the centre free to rotate. The absence of a rigidity in the centre of lycopersene could allow it to fold in a manner similar to squalene, and thus undergo cyclization into C-40 analogues of the sterols. These have not been extensively reported.

It would appear that a majority of investigators in this field would favour phytoene as the initial C-40 compound formed, but simultaneously they are at a loss to explain the reported formation of lycopersene.

The stepwise conversion of phytoene or lycopersene to the more unsaturated compound was postulated 15 years ago, before the structures of the supposed intermediates were known, the sequential synthesis requiring the stepwise removal of hydrogen and subsequent cyclization. A kinetic study by Jensen *et al.* using *Rhodospirillum rubrum*, in which the carotenoid synthesis was inhibited by the addition of diphenylamine, is a strong supporting evidence for this scheme²⁸. The diphenylamine was added initially to cultures which produced phytoene in large amounts. The inhibitor was then removed by washing, and the rate of synthesis of the unsaturated carotenoid determined. The results of these experiments showed that there is a stoichiometric relation between the loss of phytofluene and ζ -carotene and the production of unsaturated carotenoids. This certainly would support the conversion of at least phytofluene to the unsaturated, or highly coloured, carotenoids. The problems in these experiments, as well as many others, centre about phytoene itself, which has been postulated to be an obligatory intermediate in the formation of the carotenoids from 5-carbon units. This particular problem has been recently investigated by Quackenbush in *Neurospora crassa*²⁵. Initially radioactive mevalonic acid was fed to cultures grown under conditions in which they would accumulate phytoene. They were allowed to metabolize the radioactive material, and after a period, the supply of medium to the culture was stopped, and non-labelled mevalonic acid was added. Samples were then taken at intervals. The specific activity of the phytoene remained constant after removal of the radioactive substrate, whereas pigments formed during this period had specific activities 10-100 times that of the phytoene. The total activity of the pigments also exceeded that of the phytoene. It was concluded that little, if any, of the phytoene which accumulates in colourless growing *Neurospora* is utilized for pigment synthesis.

There are other evidences for phytoene being non-active in sequential pigment synthesis. Yokoyama observed that *Phycomyces* fed various labelled substrates produced phytoene and β -carotene of specific activity, which would tend to negate the hypothesis of sequential formation²⁹. Purcell concluded in the studies of mevalonate incorporation of lycopene into tomatoes, that little conversion of phytoene to other carotenoids occurs. Lowry studied the conversion of mevalonate to α - and β -carotenes in a carrot system, and concluded from the relation of specific activities and

total activity that little, if any, conversion of phytoene to α - and β -carotenes occurred³⁰. Suzue, in studying enzymatic conversions using a cell-free extract from *Sporobolomyces shibatanus*, was able to recover five per cent of the label of phytoene in a β -carotene fraction³¹. Porter attempted the same conversion utilizing highly purified labelled phytoene, and was able to show a five per cent conversion to phytofluene²⁰. Although these results may be cited as an argument against sequential synthesis, due to the very poor conversions found other explanations are possible. One can postulate that only a portion of the phytoene is active for sequential conversion. The active phytoene could be bound to an enzyme surface upon which its conversion takes place. The observed conversions, then, from phytoene to phytofluene could occur on the same surface. As a consequence, in cases where phytoene is accumulated, its accumulation may result in its displacement from an active site. Its accumulation in large amounts would suggest that it entered a pool, after its initial formation. The relative inactivity of the accumulated phytoene could also be due to a stereospecific effect, in that isomerization of the active form could occur, as it is removed from the metabolic pathway. The isomeric form would thus be inactive with respect to further conversions. If the new-formed phytoene has a single *cis* (central) double bond as has been reported, it is possible that it or other double bonds are isomerized in the inactive form³².

The place of phytoene in carotenoid biosynthesis is thus of major importance, and one which still awaits a definitive answer.

The alicyclic non-substituted carotenoids more de-saturated than phytofluene appear to be well in hand. Considerable evidence from kinetic work would indicate that phytofluene under normal circumstances is transformed to γ -carotene, and hence to neurosporene, and beyond this, more likely into lycopene. *Figure 4* illustrates these conversions. The difficulty of sequential formation of carotenoids revolves about the formation of the cyclic compounds. Whether or not ζ -carotene or neurosporene is sequentially transformed into lycopene and this in turn cyclized to β - or α -carotene is a point of long argument. A number of intermediates in this conversion can be envisioned, such as β -zeacarotene, γ -carotene, etc. Numerous investigations have built up proponents of cyclization of the completely unsaturated carotenoids, and others that have supported the cyclization of the unsaturated with subsequent hydrogen removal of the already cyclized units. Decker and Uehleke reported that chloroplasts could convert lycopene to β -carotene³³. Goodwin, however, reported that he has not been able to confirm this observation¹⁹. Yet a fairly recent report by Wells *et al.* suggests that lycopene is converted to γ -, Δ -, and β -carotenes by tomato fruit plastids³⁴. On the other hand, kinetic studies in *Phycomyces* indicate that β -carotene is produced from β -zeacarotene. (C. O. Chichester, unpublished data.) In *Chlorella vulgaris* it appears that β -carotene is derived from partially saturated carotenoids³⁵. A telling experiment appears to be that of Davies, in which labelled mevalonic acid was added to a culture of *Rhizophlyctis rosea*, which produces both γ -carotene and lycopene³⁶. Lycopene is synthesized under experimental conditions between 7 and 14 days, while γ -carotene synthesis is initiated only when lycopene reaches a steady-state level. When labelled mevalonate was present during

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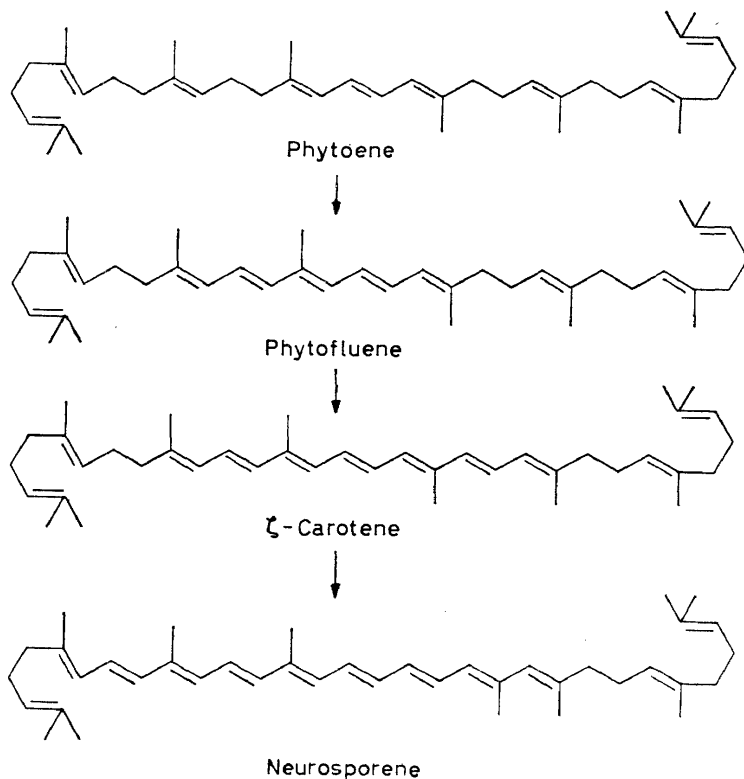


Figure 4

the entire growth period, both the lycopene and γ -carotene were equally labelled. If the addition of mevalonate was delayed until lycopene reached maximum concentration, only the γ -carotene became labelled. If the inverse experiment is performed, mevalonic acid being added at the initiation of the experiment and removed before the lycopene concentration reaches a maximum, the lycopene is labelled, and the γ -carotene is essentially unlabelled. This would certainly be indicative of non-interconvertibility between γ -carotene and lycopene. Porter has recently suggested that cyclization may take place at the neurosporene step²⁰. The existence of the monocyclic β -zeacarotene argues in this favour. The possibility of two pathways to β -carotene exists, but the weight of evidence seems to be away from the formation of β -carotene via lycopene.

The mechanism of cyclization has been explored by Goodwin and Williams, utilizing mevalonic acid labelled with both carbon-14 and tritium^{37, 38}. The ratio of tritium to carbon-14 in phytoene with eight isoprene units in an open ring should be 8:8. If the ring closure of phytoene to give β -carotene were to occur by the removal of one tritium per end-group, the ratios should amount to 8:6 in β -carotene. Their results indicate that such is the case, and the ring closure proceeds in a manner similar to that of

squalene-lanosterol, although in the carotenoids this would involve a proton attack on carbon-one rather than an OH^+ attack.

One of the problems in the formation of cyclic compounds, aside from whether they are formed from lycopene or from more unsaturated precursors, is that of the isomeric forms, α - and β -carotene. Whether or not α -carotene is formed from β -carotene by isomerization, or from a parent compound possessing α -ionone ring such as α -zeacarotene has been a point of argument. Utilizing the stereospecific labelled mevalonic acid, Goodwin and Williams were able to show that the ratio of tritium to carbon-14 in α -carotene was substantially different from that in β -carotene³⁹. If one were formed from the other after cyclization, i.e. α -carotene formed from β -carotene, the ratio of carbon-14 to tritium should be the same in both compounds. As it was not, it would appear that the hydrogen must be eliminated from a different position, rather than an isomerization of the pre-formed double bond taking place. *Figure 5* illustrates the possible formation via an independent pathway. This would then rule out the formation of the α -ionone ring from a β -carotene ring.

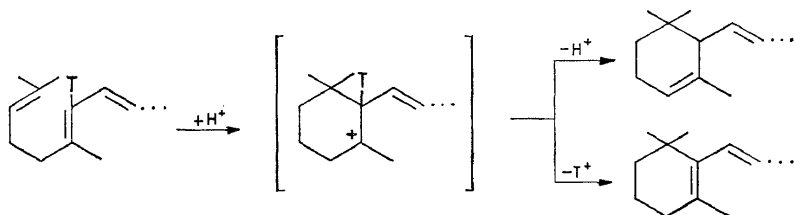
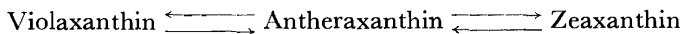


Figure 5

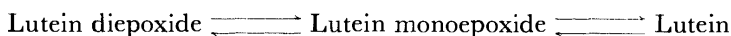
I should like to leave a major portion of the discussion of the formation of xanthophylls to Dr. Jensen, who has contributed enormously to the identification and the possible sequences in their formation. Rather, it might be worthwhile to discuss the problems of the conversions of xanthophylls in photosynthetic tissue. There is increasing evidence that the xanthophylls participate in photosynthetic reactions in a manner which at the present time is still open to question.

Sapozhnikov suggested that violaxanthin was in equilibrium with lutein, and under anaerobiosis and light the epoxide was converted to the hydroxide, the reverse reaction taking place in the dark^{40, 41}. Numerous other investigations by Sapozhnikov *et al.* have reported this conversion in isolated chloroplast suspensions containing labelled violaxanthin, and in model systems of protein lipid carrier and pigment⁴²⁻⁴⁵. Yamamoto *et al.* suggested that in leaves, violaxanthin epoxidation produced antheraxanthin, which in turn was in equilibrium with the zeaxanthin⁴⁶. This pathway was consistent with other observations such as those by Liaaen and Sorensen, and Krinsky, as shown below⁴⁷⁻⁴⁹.

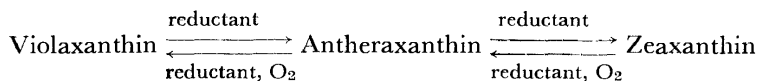


Similarly the same scheme could serve in the case of the xanthophylls with a configuration

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Krinsky showed that the de-epoxidation of antheraxanthin in *Euglena* was a dark reaction, requiring a reductant, and has suggested that the reverse reaction, i.e. the epoxidation of zeaxanthin, was a non-enzymatic reaction requiring light and oxygen⁵⁰. Yamamoto *et al.* have shown that oxygen addition to the hydrocarbons to give the hydroxylated xanthophylls requires molecular oxygen, and further, that aerobic oxygen was utilized in the formation of antheraxanthin or violaxanthin⁵¹⁻⁵⁴. He also has recently shown that de-epoxidation in the leaf is a dark reaction which proceeds in the light, and has suggested that in addition to oxygen, a light-generated product (possibly a reductant) was required for the de-epoxidation⁵⁵. The following scheme was suggested to account for the observations



It is interesting to note that in contrast to Krinsky's scheme, these reactions would not go in leaves which had been inactivated by heat. These data would apparently explain some conflicting results on the dark incorporation of O¹⁸ from oxygen into antheraxanthin, and the light-stimulated incorporation of O¹⁸ from water into violaxanthin of algae. Light-stimulated O¹⁸ incorporation from water may have resulted from a re-incorporation of O₂¹⁸ produced photosynthetically from water labelled H₂O¹⁸.

BIOSYNTHETIC OXIDATION-REDUCTIONS IN CAROTENOIDS

Although some of these studies are still partially in conflict, the oxidation-reduction activities of the carotenoids may actually be closer together than has been imagined. In isolated chloroplasts it can be demonstrated that there is a net de-epoxidation over a long period of illumination. The chloroplasts act in the same way as the leaves, in that de-epoxidation occurs after exposure to light for some period of time. Over a short period, however, there is initially a photo de-epoxidation followed by an epoxidation occurring in the light. The magnitude of the oscillatory behaviour of the epoxides is dampened until it reaches a steady-state equilibrium value at some point lower than the initial value. The equilibrium is dependent upon the level of illumination. Various compounds modify these effects greatly.

The light-induced changes in the concentrations in epoxides must of necessity be linked intimately with photosynthesis. Our results would indicate that the observed changes are red light-dependent, and can be inhibited by AMP-2-phosphate, DCMU, TCMB, and FMN. All of these would indicate that the epoxides are connected with the photosynthetic cycle in a manner similar to ferridoxin and NADP. Some of the reactions, such as the inhibition with hydroxylamine, are reversible. Incubation of chloroplasts in hydroxylamine results in an extremely rapid decrease in epoxides with a stoichiometric increase in the hydroxylated compounds. Removal of the hydroxylamine by dialysis inverts the reaction and results in a re-epoxidation concurrently with a loss of the hydroxylated compounds, as shown in *Figure 6*. This reaction is completely inhibited by freezing and

thawing. We would thus suggest that both epoxidation and de-epoxidation are enzymatically mediated in the chloroplasts and were connected directly to the photosynthetic mechanisms in a manner which could explain the protective effect of the carotenoids in some cases. It could further suggest that their role is that of an electron buffer system capable of accepting electrons or releasing them, depending upon the requirement of the photosynthetic system.

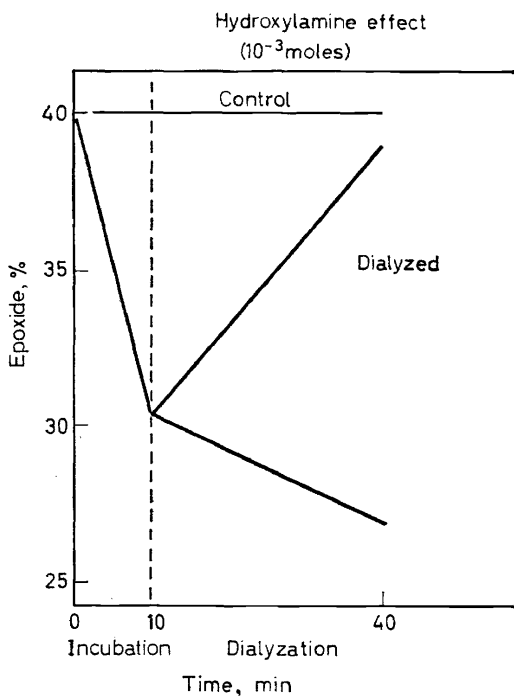


Figure 6

CONTROL MECHANISMS

An area which has received comparatively little attention in carotenoid biosynthesis is that of control mechanisms. Several years ago, in a study of the effects of ionone on carotenogenesis, it was found that this compound apparently stimulated carotene synthesis by blocking a regulatory pathway between the synthesized cyclic compounds and the formation of the diallyl or isopentenol intermediates⁵⁶. The presence of β -ionone did not induce the formation of enzymes, but did increase the turn-over rate comparatively far back in the synthetic chain. It was concluded that β -carotene would attach itself to an enzyme surface so as to competitively inhibit the formation of intermediates required for its synthesis. This inhibition required the presence of the chain, but the attaching point was that of the ionone ring. If β -ionone was substituted, it competitively inhibited the attachment of β -carotene and since it possesses no central bridging chain, the enzyme system was not inhibited. In the sterols, regulation at a similar level has

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been noted, and ionone shows the same stimulation. These observations, however, would not explain the difference between carotenoid and sterol synthesis. Diphenylamine, a competitive inhibitor of the dehydrogenase reaction, bears a steric resemblance to the saturated portion of the carotenoid, and may control in a similar manner.

Some time ago our laboratory investigated a cell-free system capable of synthesizing β -carotene from mevalonate. This system was not particularly efficient, and was one which in reproducibility was rather limited. In the system, however, it was found that NAD was required for ergosterol synthesis, but not for β -carotene synthesis. This would suggest a point of control for the diversion of intermediates to either type of compound. Goodwin has suggested regulation by compartmentalization, which supposes that the chloroplast synthesizes the photosynthetic terpenoids, while the non-chloroplast systems synthesize the terpenoids and sterols⁵⁷. We, as well as others, seem to find that the chloroplast is impermeable to mevalonic acid, *in situ* or isolated in aqueous media, and must of necessity synthesize this compound from fixed CO₂. The cytoplasm, on the other hand (being the site of sterol synthesis), would synthesize its materials from pre-formed mevalonate. In an investigation of the formation of terpenoids in *Eucalyptis*, it was found that radioactivity from mevalonic acid, isopentenol pyrophosphate, and farnesyl pyrophosphate were incorporated rapidly into terpenoid compounds in a growing seedling; but little or none was incorporated into the carotenoids⁵⁸.

It is interesting to note that while the ionone effect can be explained nicely in *Phycomyces*, the lack of effect in other organisms, or its inverse effect in other organisms, is at the moment a problem.

In any case, this is another area in which biochemically we have very little knowledge. The control of the synthesis of very similar compounds is not established, and although theories have been postulated, little or no data are available.

While at present we have a vast knowledge of the types of carotenoids which exist in Nature, we still are not in a position to do more than generally describe the pathway of their synthesis—and have even less knowledge of the mechanisms of synthesis or the enzymes concerned with their alteration.

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