

THIRTY YEARS OF BIOSYNTHESIS

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In discussing thirty years of carotenoid biosynthesis in thirty minutes my approach will inevitably be somewhat autobiographical and anecdotal but for all that I trust it will not be too unbalanced. At the end of the Second World War I was working under Professor R. A. Morton on the site of conversion of β -carotene into vitamin A in mammals and it soon became clear to me that we should know more about how plants and microorganisms made carotenoids. After all in Nature the ultimate source of all vitamin A is the carotenoid group of pigments. About that time W. H. Schopfer published a book on Vitamins in Plants (1) and from studying that I decided that *Phycomyces blakesleeanus* would be a suitable organism with which to begin our studies. We first considered the general factors controlling synthesis and the most useful observation to emerge was that, in static cultures, growth had to be essentially complete before β -carotene synthesis took place and that assimilable carbohydrate had to be available at the end of the growth period (2,3). At this time, which was some years before mevalonic acid was discovered or isotopes became readily available to biochemists, Schopfer & Grob (4) showed that *Phycomyces blakesleeanus* could grow on acetate and produce carotenoids, thus indicating that acetate was probably a basic building block for carotenoids. There was at that time still no insight into how acetate was converted into an isoprenoid residue but we were excited when we showed that in *Phycomyces blakesleeanus* the branched chain amino acids valine and leucine, particularly leucine, greatly stimulated β -carotene synthesis and could thus provide a branched C-5 unit (5). This was taken up by the late Gordon Mackinney and C. O. Chichester and a series of papers reporting work on leucine labelled with ^{14}C in different carbon atoms revealed that the C-4 and C-5,5' atoms were more effectively incorporated than were the C-2 and C-3 atoms and that C-1 was not significantly incorporated (see 6). At about this time the conversion of leucine into β -hydroxy- β -methylglutaryl-CoA in mammals had been elucidated and shown to involve a CO_2 fixing step (7). We demonstrated that CO_2 was also fixed into β -carotene in *Phycomyces blakesleeanus* only in the presence of leucine (8), and this was confirmed by Mackinney's group (9). In order to accommodate this observation [CO_2 is fixed into C-1 of mevalonic acid which is normally lost when it is converted into isopentenyl pyrophosphate] and the labelling results of Mackinney's group the pathway outlined in Fig. 1 was proposed. The existence or otherwise of this pathway is still to be proved. The possible significance *in vivo* of leucine as a carotenoid precursor in higher plants will be considered later.

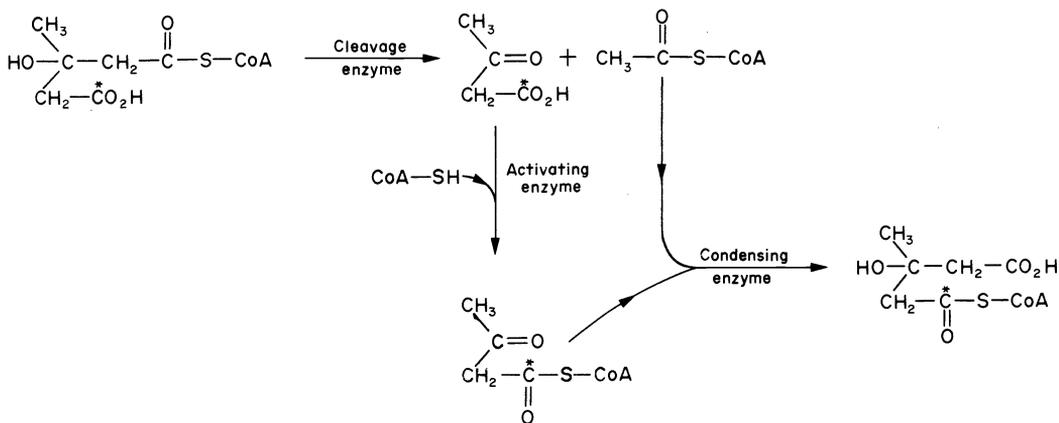


Fig. 1. Pathway by which C-1 and C-5 of HMG-CoA can be randomized.

During this period (1940-1950) Zechmeister and his colleagues had been describing the natural occurrence of the partly saturated colourless polyenes, phytoene and phytofluene (10) and Porter and Lincoln (10) after examining in detail the polyenes in a series of tomato mutants proposed a pathway from phytoene to lycopene via phytofluene ξ -carotene and neurosporene. This pathway has now become clearly established although at the time the exact chemical structures of the individual components were not known. Our contribution to this aspect of the problem at that time was based on the use of the inhibitor diphenylamine. This compound was first shown to inhibit 'chromogenesis' in certain mycobacteria (12) and this turned out to be a specific effect on carotenoid synthesis (13). In *Phycomyces blakesleeanus* (14) and *Rhodospirillum rubrum* (15) inhibition of carotenogenesis by diphenylamine was accompanied by a massive accumulation of phytoene. When the inhibitor was removed and the washed cells re-suspended in new media free from diphenylamine the pigments were synthesised at the expense of the accumulated phytoene (8, 16). This type of experiment has now been demonstrated many times in many microorganisms (see 6), although diphenylamine has no obvious effect on carotenogenesis in higher plants. A problem of interpretation in these early experiments was that the amount of phytoene which accumulated was much greater than that of the pigments present in uninhibited cultures; presumably feed-back control by the end product (e.g. β -carotene) disappears and the early precursors, uncoupled from control, are channelled into phytoene in excessive amounts.

In 1957 came the discovery of mevalonic acid as a precursor of cholesterol in animals (17) and soon after it was shown to be a precursor of β -carotene in *Phycomyces blakesleeanus* (18). The pattern of incorporation was the same as in the triterpenes because the C-3' atoms of mevalonic acid became specifically incorporated into the lateral methyls and the C-2 atoms were located only in the chain (18). Eventually incorporation of mevalonic acid into many other carotenogenic systems have been reported (see 6). However, an important development was the demonstration that in shoots of greening etiolated seedlings, in which carotenoids were being rapidly synthesized in the developing chloroplasts, mevalonic acid was incorporated very poorly into the carotenoids although, on the other hand, CO_2 was rapidly incorporated (19). There is no doubt that chloroplasts have the biosynthetic machinery to make carotenoids from mevalonic acid (20) and isopentenyl pyrophosphate (21) but it is still not clear what is the immediate source of the precursors in the chloroplast (22). Some of the possibilities are indicated in Fig. 2. A real possibility which is not indicated in Fig. 1 is that

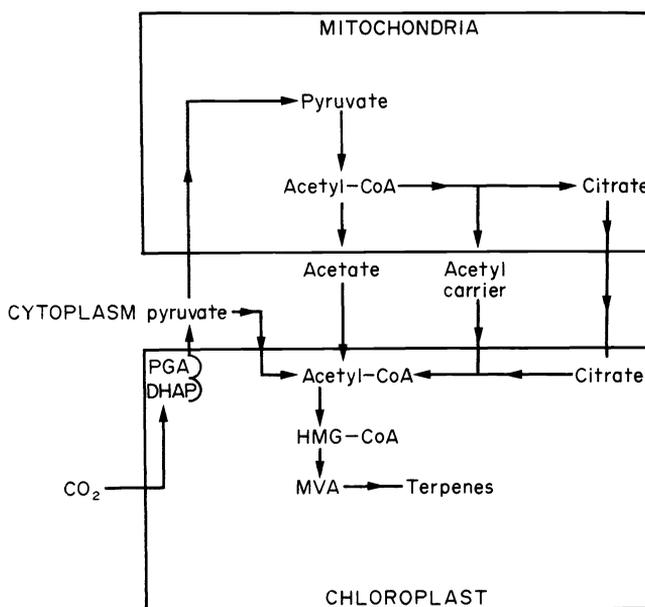


Fig. 2. Some possible sources of mevalonic acid in chloroplasts.

leucine provides HMG-CoA which can then be reduced in the chloroplast to mevalonic acid (23). Whatever turns out to be the main pathway it seems clear that the chloroplast cannot itself make mevalonic acid directly by the fixation of CO_2 but that there must be a collaboration between this organelle and other parts of the cell. The collaboration must be extremely efficient because the uptake of $^{14}\text{CO}_2$ into carotenoids in developing chloroplasts is very rapid (24).

The next significant breakthrough in the study of terpenoid biosynthesis in general was the synthesis of the six chiral species of mevalonic acid in which each prochiral hydrogen at C-2 and -5 was stereospecifically replaced by either tritium or deuterium (25). The generosity of Sir John Cornforth in making these compounds available to us before they became commercially available allowed us to solve many problems of carotenoid biosynthesis which were previously not amenable to study. These results have been reviewed in detail (see e.g. 6, 26) but in summary the significant observations were: (i) the pro-R-hydrogen is lost from C-4 of MVA in the chain-elongating process which produces the C₂₀ precursor geranylgeranyl pyrophosphate (GGPP); (ii) in the condensation of two molecules of GGPP the hydrogens at C-1 of each molecule, which arise from the pro-R-hydrogens at C-5 of mevalonic acid, are lost in synthesizing the central (15, 15') cis double bond of phytoene; (iii) the stereochemistry of the formation of the other double bonds the carotenoid molecules is the same in each case and involves loss of hydrogens arising from the pro-R and pro-S-positions. These results are illustrated in Fig. 3.

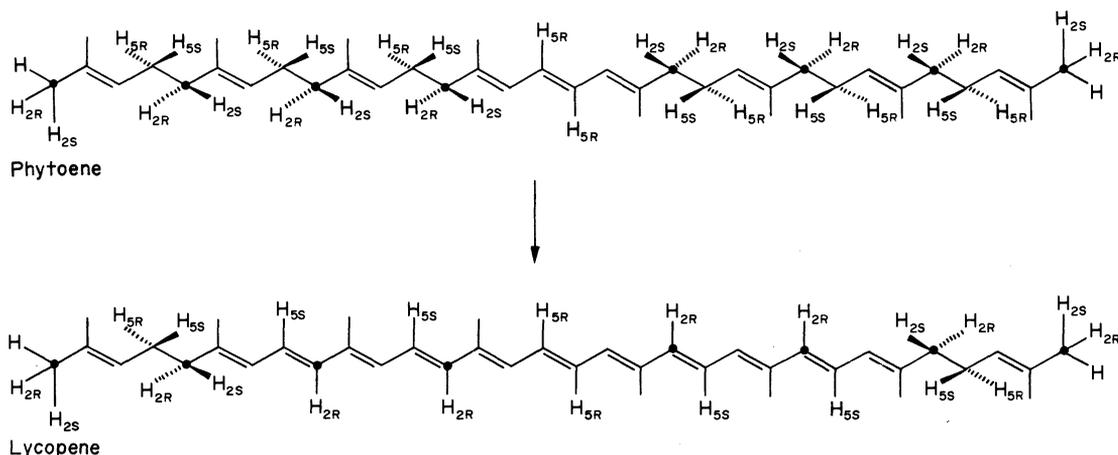


Fig. 3. The stereochemistry of the desaturation of phytoene to lycopene. Hydrogens indicated 2 arise from hydrogens at C-2 of mevalonic acid and those indicated 5 from hydrogens at C-5 of mevalonic acid.

Our latest interest has been in the mechanism and stereochemistry of ring closure in carotenoid biosynthesis. On the assumption of initial proton attack at C-2 it has been possible to show that the β - and ϵ -rings of carotenoids are formed independently and that the pro-S-hydrogen at C-4 is eliminated in formation of the ϵ -ring (see 8, 26).

The most recent work on ring formation is the result of the combined use of two powerful tools in modern biochemical research; the first, biological, the isolation of appropriate mutants, and the second physico-organic 270 MHz p.m.r. and ¹³C-m.r.t.

The isolation by Dr. R. Powls of a mutant of *Scenedesmus obliquus* which synthesized mainly β -carotene in the dark: on illumination it rapidly develops functional chloroplasts which contain the expected mixture of cyclic carotenoids, which are synthesized at the expense of the β -carotene produced in the dark. If the darkgrown cells are illuminated in D₂O instead of H₂O, then the β -carotene isolated after illumination, contain as shown by mass spectrometry, two deuterium atoms, one at C-2 and one at C-2' (27). This constitutes the first experimental proof that cyclization involves proton attack at C-2.

The same situation was demonstrated in a *Flavobacterium* species obtained from Hoffmann-La Roche. In this case the cells were grown in the presence of nicotine, which inhibits the cyclase enzymes and causes the accumulation of lycopene in place of zeaxanthin (3,3'-dihydroxy β -carotene) (28). The cells are washed free from nicotine and resuspended in D₂O. The absence of the inhibitor allows the accumulated lycopene to be converted into zeaxanthin which contains one deuterium atom at C-2 and one at C-2'. Because the assignments of the FMR signals of the two hydrogens at C-2 in zeaxanthin are known we could demonstrate that the incoming proton attacks the re re face of the terminal double bond (Fig. 4) (29,30). The stereo-

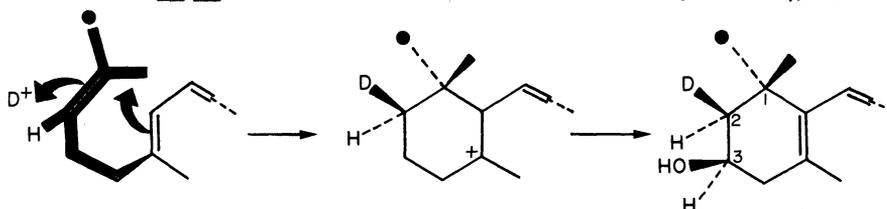


Fig. 4. The stereochemistry of the formation of the ring of zeaxanthin.

chemical behaviour of the C-1 methyl groups during cyclization has also been settled by using Flavobacterium growing in the presence of [$2-^{13}\text{C}$] mevalonic acid (31). The methyl group arising from C-2 of mevalonic acid takes up the pro-S (axial α) position (Fig. 4). As the stereochemistry of hydroxylation had previously been determined (see 6) the complete stereochemistry of cyclization in zeaxanthin biosynthesis in Flavobacterium can now be described in detail (Fig. 4). The conformation (chair or boat) leading to the cyclization has not yet been established.

To look for a moment to the future one would expect considerable advances at the enzymic level of carotenogenesis. Carotenoid enzymology is extremely difficult but we now have the pioneering efforts of Dr. Porter's group to build on and elucidation of detailed mechanisms involving active sites of purified enzymes should be feasible within the next few years. Such investigations would be expected to settle problems concerning the participation of cis and trans phytoene in the biosynthetic pathway and the way in which both ends of acyclic precursors are treated separately. For the first time in a carotenoid symposium we have an analysis of very recent work on carotenoid biosynthesis explored by the classical methods of microbial genetics; already impressive results are emerging and one anticipates much new insight into carotenogenesis, particularly its control, within the next few years. There are still some subtle stereochemical problems to be settled and I am confident that a combined attack by biochemists and chemists will have dealt with most of them by the time of our next symposium.

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