Regulation of Carotenoid Biosynthesis

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I. Introduction[1]

The carotenoids constitute the most widespread group of pigments in nature. They are present in all photosynthetic organisms, where they form an essential part of the photosynthetic apparatus (*78, 227, 310*). They are also responsible for most of the yellow to red colors of flowers and fruits as they occur within chromoplasts. In addition, they are found in certain fungi (*128, 129*) and some non-photosynthetic bacteria (*205, 206*). Carotenoids are also found in some animals, in particular, birds, insects, and marine invertebrates. In these cases, however, the pigments originate from the diet, although they may undergo modification in different animal species (*59, 177, 345*). In recent years, carotenoids have become of increasing commercial potential as natural colorants (*182*) and also as anticancer agents (*223, 224, 254*). Consequently, there is an increasing need for obtaining high quantities of colored carotenoids from naturally occurring sources such as fungi (*244*) or marine algae (*15, 16*).

The diversity of structures, and hence of biosynthetic pathways within the carotenoid group, together with their diverse locations and functions in complex organelles such as the chloroplast or other photosynthetic membranes, clearly implies that efficient regulatory mechanisms are present within cells to control not only the rates of biosynthesis and metabolism of carotenoids in response to environmental

[1]Abbreviations: MVA, mevalonic acid; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IPP, isopentenyl pyrophosphate; MVAP, mevalonic acid 5-phosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; PPPP, prephytoene pyrophosphate; $LHCP_3$ and $LHCP_y$, light harvesting chlorophyll *a*/*b* xanthophyll proteins; CP1 and CP1a, chlorophyll proteins of photosystem I; CP_a, chlorophyll *a* protein of photosystem II; CPTA, 2-(4-chlorophenyl)triethylamine; MPTA, 2-(4-methylphenoxy)triethylamine; PCMB, *p*-chloromercuribenzoate.

and developmental changes but also the deposition at their functional locations within a cell. The aim of this article is to describe our current understanding of the control of carotenoid biosynthesis and to discuss future experimental approaches which will increase our knowledge of these regulatory mechanisms.

II. Structure and Nomenclature

Carotenoids are terpenoids that generally consist of eight isoprene units (i.e., C_{40}), joined together so that the linking of the units is reversed at the center of molecule. The most prominent feature of carotenoids is the polyene chain, which may extend from 3 to 15 conjugated double bonds. This chromophore is responsible for the characteristic absorption spectrum and color of the molecule. Cyclization of the carbon skeleton, at one or both ends, also occurs, while xanthophylls are formed from the hydrocarbon carotenes by the introduction of oxygen functions. In addition, skeletal modifications involving chain elongation (to C_{45} or C_{50}) or degradation to apocarotenoids take place, and some carotenoids are biogenetically C_{30}.

The structures of most known carotenoids are given by Straub (*324*), and updates are found in publications of the Chemical Society (e.g., *36*). Common carotenoids have well-established trivial names, which are used in this article, with the IUPAC-recommended semisystematic names (*81, 82*) given in parentheses at the first mention of each carotenoid.

III. Biosynthetic Pathways and Carotenogenic Enzymes

Since carotenoids are terpenoids they are related biosynthetically, and share a common early pathway, with other biologically important isoprenoids such as sterols, gibberellins, and terpenoid quinones (Fig. 1). In the following subsections, the carotenogenic pathway is divided into several convenient stages, with emphasis being placed on the features of the pathway and its associated enzymes which are pertinent to discussion of the regulation of carotenoid biosynthesis. Reviews are available elsewhere which include either descriptions of the entire pathway (e.g., *35, 129, 320*) or detailed accounts of specific aspects of biosynthesis (*27, 91, 92, 234, 320*).

A. Formation of Phytoene from Acetyl-CoA

The first specific precursor of all the terpenoids is mevalonic acid (MVA), which itself is formed from acetyl-CoA via 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA; Fig. 2). Most studies on these early steps in carotenogenic systems have used chloroplast preparations. HMG-

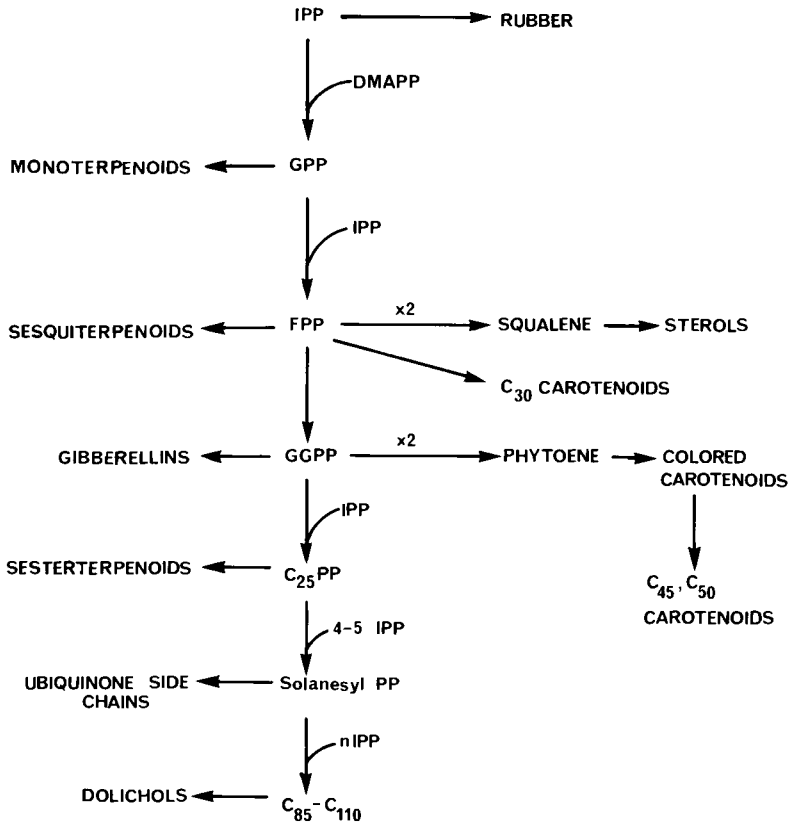

FIG. 1. Biosynthetic relationships among the isoprenoids. For abbreviations see footnote 1.

CoA reductase has been characterized from chloroplasts (*145*), and it has been shown that spinach chloroplasts are autonomous with respect to β-carotene (β,β-carotene) formation, with acetyl-CoA being formed directly from intermediates of the pentose phosphate pathway (*147*). HMG-CoA can also be synthesized from leucine in the cytoplasm of algae and plants (*146, 292*).

MVA is converted to isopentenyl pyrophosphate (IPP), the universal isoprene unit, via a three-step sequence involving the soluble enzymes MVA kinase, MVAP kinase, and pyrophosphomevalonate decarboxylase (Fig. 2). Isomerization of IPP to dimethylallyl pyrophosphate (DMAPP), catalyzed by IPP isomerase, is followed by a series of condensation reactions, resulting in the formation of geranyl pyrophos-

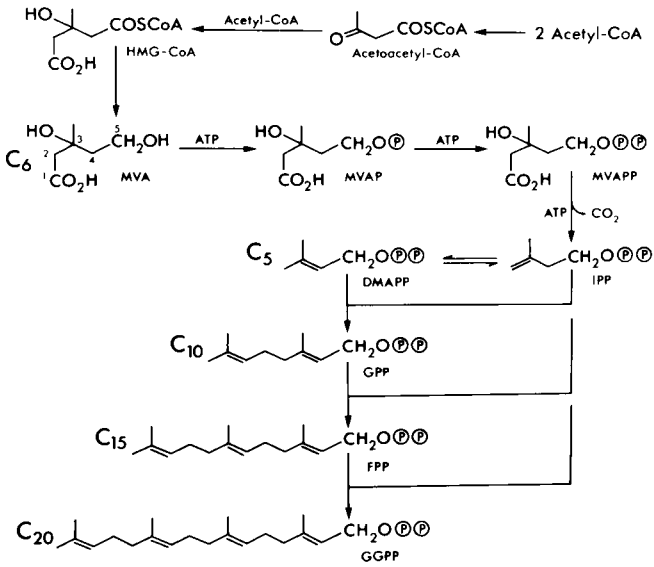

FIG. 2. Formation of geranylgeranyl pyrophosphate (GGPP) from acetyl-CoA. From Davies (*91*). Reproduced with permission.

phate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP), as shown in Fig. 2. These condensation reactions are catalyzed by prenyltransferases. The number and catalytic activities of these enzymes vary from organism to organism (see Ref *260*).

Detailed mechanistic studies on prenyltransferases have been made (*259, 260*). Thirty-carbon carotenoids are derived from the dimerization of FPP (*94*). One of the six possible metabolic fates of GGPP is to undergo tail-to-tail dimerization to form the first C_{40} carotene, phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene). This two-step conversion via prephytoene pyrophosphate (PPPP) is catalyzed by two enzymes (*321*) and forms either 15-*cis*- or all-*trans*-phytoene (Fig. 3). Phytoene synthetase, which catalyzes the second step, has not been purified to homogeneity. It is located exclusively in the plastid compartment of higher plants (but not synthesized on the 70 S ribosomes; *53*), whereas it is a peripheral membrane protein in fungi (*31, 235*) and is cytosolic in *Mycobacterium* (*138*), *Halobacterium* (*192*), and *Flavobacterium* (*39*). Cofactor requirements for these conversions are described by Bramley (*27*). The C_{30} carotenoids are derived from the dimerization of FPP.

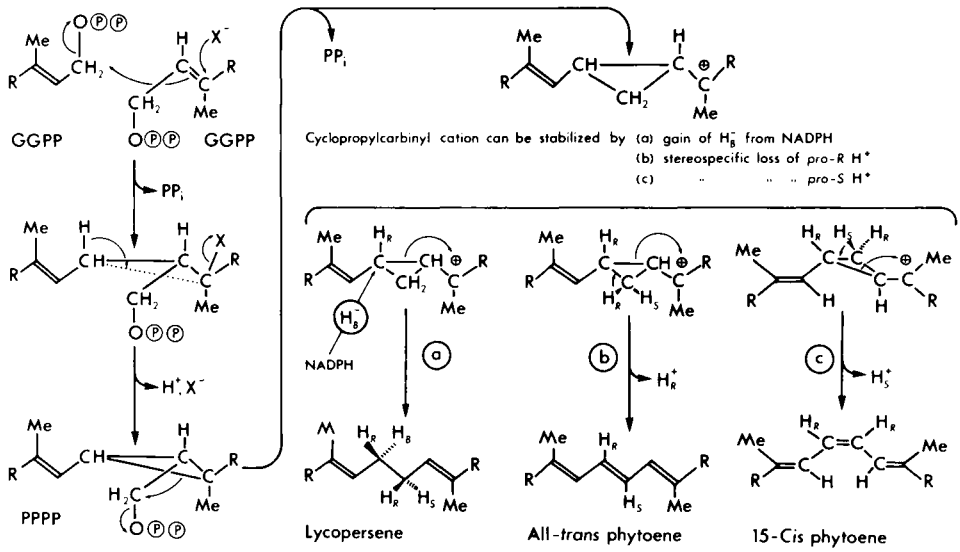

FIG. 3. Formation of prephytoene pyrophosphate (PPPP) from GGPP and its conversion to lycopersene, all-*trans*-phytoene, and 15-*cis*-phytoene. From Davies (*91*). Reproduced with permission.

B. Desaturation of Phytoene

In higher plants and algae phytoene is desaturated to lycopene (ψ,ψ-carotene) by a series of four didehydrogenations occurring alternatively at either side of the chromophore (Fig. 4), the intermediates being phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene), ζ-carotene (7,8,7′,8′-tetrahydro-ψ,ψ-carotene), and neurosporene (7,8-dihydro-ψ,ψ-carotene). Each dehydrogenation step is stereospecific (*232, 362*). In some fungi, the desaturation proceeds one stage further to yield 3,4-dehydrolycopene (3,4-didehydro-ψ,ψ-carotene; *204*). Variations in this desaturation sequence are found in some microorganisms, e.g., *Rhodospirillum rubrum* (*90*).

In organisms containing 15-*cis*-phytoene, the desaturation sequence must include a cis-to-trans isomerization step, since the more unsaturated carotenes are all-trans (except for tissues containing poly-*cis*-carotenes, e.g., tangerine tomatoes; *77, 112*). In higher plants, this step has been shown to be at the phytofluene stage (*52, 56, 195, 265*), but it may occur at the phytoene level in *Flavobacterium* R1560 (*44*) and *Phycomyces* (*29, 101*). The permutations of possible isomerization reactions are discussed fully by Spurgeon and Porter (*320;* see also Section IV).

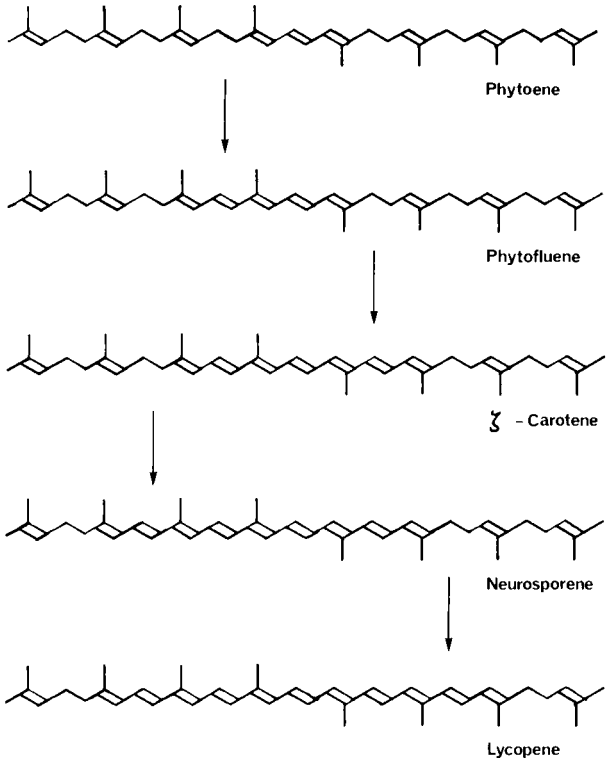

FIG. 4. General pathway for the conversion of phytoene to lycopene. For convenience, all structures are shown as the all-*trans* isomers. See Section III,B for details.

With the exception of the extreme halophile, *Halobacterium cutirubrum,* in which desaturation reactions occur in the cytoplasm (*192*), the conversion of phytoene to unsaturated acyclic carotenes takes place on membranes (reviewed by *27*). In higher plants and algae these are granal membranes or the inner chromoplast membrane (*56, 187*). The fungal enzymes are located on the endoplasmic reticulum (*235*) and/or on oil droplets of *Phycomyces blakesleeanus* (*284*). The cofactor requirements for these reactions have been recently discussed by Bramley (*27*).

Since no one has yet purified the enzymes which catalyze the desaturation or isomerization of carotenes, it is not known how many proteins are responsible for these conversions. Some indications have come from genetic studies (see Section IV), especially with *Phycomyces*

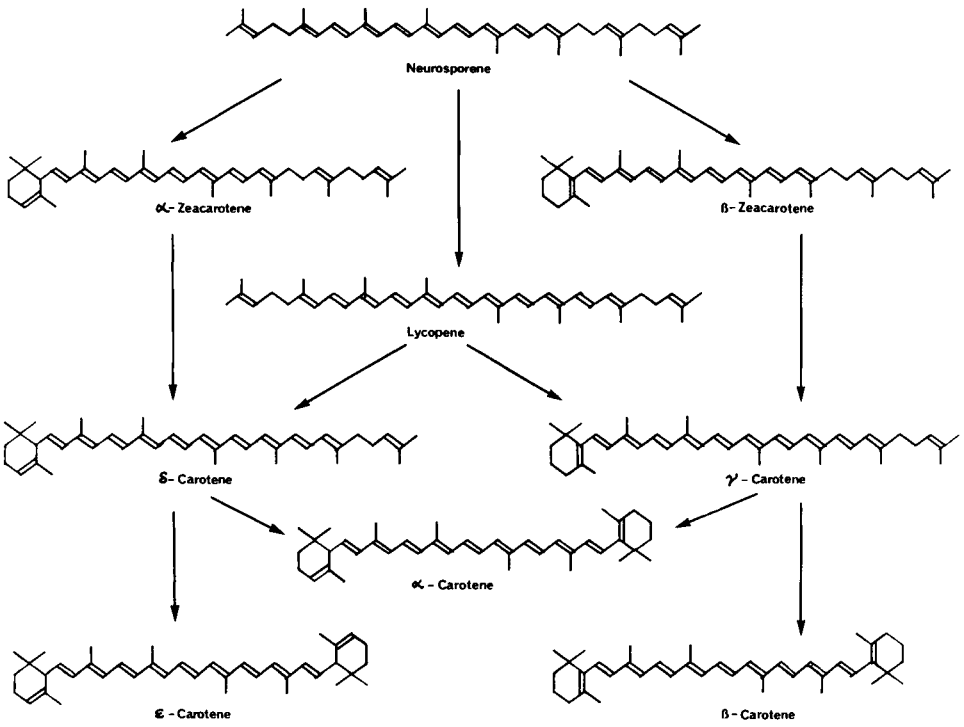

FIG. 5. An overall scheme for the biosynthesis of cyclic carotenes. See Section III,C.

(63). In higher plants, the enzymes are encoded by nuclear rather than chloroplast genes *(180;* see Sections IV and V,B).

C. Cyclization Reactions

An overall scheme for the formation of mono- and bicyclic carotenes is shown in Fig. 5. The β and ε rings are formed independently from a common carbonium ion precursor *(32, 33, 234)*. Several of these conversions have been demonstrated *in vitro* (reviewed by *27*), but little is known about the properties or number of cyclases involved, although studies of mutants have predicted the number in certain organisms (see Section IV). In all reports to date, with the exception of *H. cutirubrum (192)*, the cyclases have been found to be integral membrane proteins and are located in plastid membranes of higher plants *(54, 57, 187)*.

D. Xanthophyll Formation

Little is known about the formation of xanthophylls, even though most naturally occurring carotenoids are oxygenated. Hydroxylation occurs late in the pathway and requires molecular oxygen in a mixed-function oxidase reaction (reviewed by *32, 33, 35*). Epoxy groups are introduced directly from molecular oxygen, and an epoxidation–depoxidation sequence, between zeaxanthin (β,β-carotene-3,3′-diol) and violaxanthin (5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol), known as the violaxanthin cycle, is found in thylakoid membranes (*143, 150, 366*).

The photosynthetic bacteria, especially the Rhodospirillaceae, synthesize a range of acyclic xanthophylls, typically with tertiary hydroxy or methoxy groups. Possible pathways for the formation of spheroidene (1-methoxy-3-4-didehydro-1,2,7′,8′-tetrahydro-ψ,ψ-carotene), spheroidenone (1-methoxy-3,4-didehydro-1,2,7′,8′-tetrahydro-ψ,ψ-caroten-2-one), and related compounds have been suggested, but they have not been verified by *in vitro* experiments. These compounds can be derived from neurosporene (*233, 298*), and mutants of *Rhodopseudomonas spheroides* have been isolated that accumulate neurosporene alone or neurosporene and chloroxanthin (1,2,7′,8′-tetrahydro-ψ,ψ-caroten-1-ol; see Section IV).

E. Other Reactions

A vast range of carotenoids not described above has been structurally characterized, e.g., C_{45}, C_{50}, and aromatic carotenoids, but little is known about the biosynthetic pathways or regulatory factors involved. Details of their structures, occurrence, and speculative biosynthetic reactions can be found in reviews by Goodwin (*129*) and Spurgeon and Porter (*320*).

IV. Genes Involved in Carotenoid Biosynthesis

Mutants affected in carotenoid metabolism are normally easily recognized by their differences in color compared to the wild type. Such mutants result either from alteration of an enzyme directly involved in the carotenoid pathway (structural genes) or alteration of another cellular component with regulatory interactions with carotenogenesis (regulatory genes). These two types of mutants can be distinguished by quantitative and qualitative analysis of their carotenoids, examination of their enzymes or cell-free systems, and by their responses to known regulatory effectors (light, chemicals, etc.). Complementation and mapping studies identify the number and distribution of the genes involved.

Mutants with altered structural genes, although possessing similar

overall carotenoid levels compared to wild types, are characterized by changes in the patterns of carotenoids with an accumulation of intermediates prior to the blocked step. These mutants have been helpful in elucidating the biochemical pathways for carotenoid formation and the nature of the participating enzymes.

A. Phytoene Formation

Relatively few mutants have been positively identified with lesions prior to phytoene formation. However, mutations in steps earlier than FPP formation are likely to be lethal since FPP is a precursor for steroid synthesis as well as carotenoid synthesis. *al-3* mutants (linkage group V) of *Neurospora crassa* are reported to be blocked in GGPP synthetase (*155*). Although it was thought that the same prenyl transferase catalyzed all the steps from DMAPP to GGPP, it would seem that, at least in this organism, either there are different prenyl transferases for the synthesis of steroids and carotenoids or there is a specific transferase responsible for the FPP–GGPP conversion. A number of albino mutants of *Aspergillus giganteus* have been isolated blocked in PPPP synthetase (*110*). Phytoene synthetase itself appears to be blocked in mutants (SG4, SG75, and SG76) of *Gibberella fujikuroi* (*9, 242*) and *al-2* mutants (linkage group I) of *N. crassa (155, 193)*.

Condensation of GGPP forms either 15-*cis*-phytoene or all-*trans*-phytoene, depending on the stereochemistry of hydrogen removal (see Section III,B). Although both isomers are normally present in the same organism, 15-*cis*-phytoene predominates in plants, algae, fungi, and nonphotosynthetic bacteria while all-*trans*-phytoene predominates in photosynthetic bacteria (*320*). Evidence for specific *cis*- and *trans*-phytoene synthetases has been provided by incorporation studies with stereospecifically labeled precursors with systems from tomato, bean leaf, and *Phycomyces blakesleeanus* (*138, 362*), respectively.

Other mutants lacking phytoene have been identified, but the nature of the lesion is unknown. These include mutants of maize (cl_1; *288*), *Euglena* spp. (PBZ G4 and PR4; *142*), *Ustilago violacea* (*wA; 361*), and *Rhodopseudomonas capsulata* (*crtB* and *crtE; 220*). 4-4′-Diapophytoene is absent from type I mutants of *Pseudomonas rhodos* (*183*) and *Staphylococcus aureus* (*221*). Colored carotenoids are also absent from mutants of *Helianthus annuus* (*149*), *Cheiranthus cheirii* (*306*), *Micrococcus roseus* (*304*), *Thermus aquaticus* (*258*), *Micrococcus radiodurans* (*364*), and *Chloroflexus aurantiacus* (7-32; *255*).

B. Isomerization

Organisms containing 15-*cis*-phytoene must include a cis–trans isomerization step during the desaturation sequence from phytoene to

lycopene since the more unsaturated carotenes are all-trans isomers. Experimental evidence suggests this occurs at phytoene in *P. blakesleeanus* (*336*), *Flavobacterium* strain R1560 (*44*), *Rhodospirillum rubrum,* and *Rps. spheroides* (*94, 229*) and at a later stage, phytofluene, in tomato (*172*) and *Capsicum annuum* (*52*). The existence of isomerase enzymes is suggested by *in vitro* studies. Plastid preparations from tomato fruit (*195, 265*) and chromoplast preparations from *C. annuum* (*56*) convert 15-*cis*-phytofluene to all-*trans*-phytofluene, and cell extracts of *Flavobacterium* R1560 (*44*) and *P. blakesleeanus* (*30*) convert 15-*cis*-phytoene to all-*trans*-phytoene. Mutants for isomerase activity have not as yet been identified. Although *carB* mutants of *P. blakesleeanus* (blocked in phytoene dehydrogenase) accumulate predominantly *cis*-phytoene, their cell extracts produce mostly the all-trans isomer (*101*). Therefore dehydrogenation and isomerization appear to be separate functions in this organism. In *Mucor hiemalis,* the conversion of 15-*cis*- to all-*trans*-phytoene is at least in part photochemical (*158*).

A few mutants have been identified with different carotenoid isomer patterns compared to wild types, but they are not easily explained by alterations in known enzyme functions. S_1B_4 mutant of *R. rubrum* accumulates mostly *cis*-phytoene (similar to wild type) when grown in the dark and under aerobic conditions. However, when grown in the light and anaerobic conditions, the trans isomer dominates. Another mutant, FR_1VI, accumulates the cis isomer under both growth regimes (*230*). *Scenedesmus obliquus* appears normally to convert *cis*-phytofluene to *trans*-phytofluene during lycopene synthesis (*41*), but in the PG1 mutant *cis*-phytofluene is converted to 15-*cis*-ζ-carotene before isomerization occurs (*261*). *cis*-ζ-Carotene has also been observed in some *Chlorella* mutants (*73, 96*). Poly-*cis*-carotenes (e.g., prolycopene, proneurosporene) are synthesized by the dark-grown 5/520 mutant of *Chlorella vulgaris* (*72*) and the dark-ripened tangerine tomato fruit, the tangerine trait being governed by a single recessive, nuclear gene (*77, 112, 352*). In both cases, light stimulates the interconversion of the cis to the trans forms (*74, 277*).

C. Dehydrogenation

The sequential desaturation of phytoene to lycopene involves a series of four didehydrogenations (Fig. 4 and Section III,B). This raises the possibility of four discrete dehydrogenases, one specific to each step. Mutants blocked in dehydrogenation are listed in Table I.

From the mutants isolated so far, it appears unlikely that there are specific dehydrogenases for each step. There are possibly two different

TABLE I

MUTANTS BLOCKED IN DEHYDROGENATION

Organism	*Mutant*	*Product accumulated*	*Enzyme*	*Ref.*
Maize	W_3	Phytoene	Phytoene dehydrogenase	*4*
	ζ	ζ-Carotene	ζ-Carotene dehydrogenase	*159*
Barley	*tig*-b	ζ-Carotene	ζ-Carotene dehydrogenase	*243a*
Tomato (leaves and fruit)	Ghost (*gh*)	Phytoene	Phytoene dehydrogenase	*218*
Tomato (fruit)	Virescent orange (*Vo*)	ζ-Carotene	ζ-Carotene dehydrogenase	*180*
Chlorella vulgaris	5/871	Phytoene	Phytoene dehydrogenase	*71, 72*
	5/518	ζ-Carotene	ζ-Carotene dehydrogenase	
Chlorella pyrenoidosa	G34	Phytoene	Phytoene dehydrogenase	*86*
Scenedesmus obliquus	C61, C55	Phytoene	Phytoene dehydrogenase	*41, 261*
	1L, 1K	ζ-Carotene	ζ-Carotene dehydrogenase	
Phycomyces blakesleeanus	*carB*	Phytoene	Phytoene dehydrogenase	*248*
Ustilago violacea	White (*wB*)	Phytoene	Phytoene dehydrogenase	*290, 361*
	Yellow (*y*)	No lycopene	Neurosporene dehydrogenase	
	AB278a-1 (double mutant)	*cis*-β-Zeacarotene	Neurosporene dehydrogenase and *cis*-β-zeacarotene dehydrogenase	
Neurospora crassa	*al-1*	Phytoene	Phytoene dehydrogenase	*126, 326*
Gibberella fujikuroi	SG43, SG78	Phytoene	Phytoene dehydrogenase	*9, 242*
Micrococcus luteus	93A	Colorless polyenes	Phytoene dehydrogenase?	*225, 226*
Halobacterium halobium	W5002-1	Phytoene	Phytoene dehydrogenase	*194*
Staphylococcus aureus	White	4,4'-Diapophytoene	Diapophytoene dehydrogenase	*221*
Rhodopseudomonas spheroides	Blue-green	Phytoene	Phytoene dehydrogenase	*140*
Rhodopseudomonas capsulata	Blue-green (*crtI*)	Phytoene	Phytoene dehydrogenase	*125a*

enzymes in plants and algae, phytoene dehydrogenase (phytoene → phytofluene → ζ-carotene) and ζ-carotene dehydrogenase (ζ-carotene → lycopene), since two types of mutants have been characterized that accumulate phytoene and ζ-carotene, respectively. One might have predicted a different enzyme for the first step, *cis*-phytoene to *cis*-phytofluene, on the basis of sterospecificity. For example, cell extracts from the nonphotosynthetic bacterium *Halobacterium cutirubrum* are highly specific for *trans*-phytoene and *trans*-phytofluene, using the cis isomers only poorly (*192*). Also, studies with *H. cutirubrum* (*195*) and extracts from spinach (*225*) and tomato (*195*) suggest that the conversion of phytoene to phytofluene requires NADP while phytofluene to lycopene requires FAD. As a cautionary note, Bejarano and co-workers (*13*) recently isolated a ζ-carotene-accumulating mutant (S442) of *P. blakesleeanus* which, by complementation analysis, mapped at the *carB* gene responsible for the single dehydrogenase.

It seems more likely that a single enzyme, phytoene dehydrogenase (or diapophytoene dehydrogenase in *Staphylococcus aureus*) is responsible for catalyzing all four steps (phytoene to lycopene) in fungi and bacteria. *Ustilago* is a possible exception. The patterns of carotenes accumulated by white and colored strains of *U. violacea* have been interpreted as resulting from lesions in three separate genes, each coding for different dehydrogenases, phytoene dehydrogenase (phytoene → neurosporene), neurosporene dehydrogenase (neurosporene → lycopene) and *cis*-β-zeacarotene dehydrogenase (β-zeacarotene → γ-carotene) (*361*).

D. Cyclization

Fewer mutants have been reported blocked in cyclization (Table II). It seems surprising, for example, that the extensive searches for carotenoid mutants in such organisms as *N. crassa* and *G. fujikuroi* have not produced the easily recognizable lycopene-accumulating mutants.

Only mutants accumulating lycopene and not γ-carotene have been observed. It seems likely, therefore, that in plants and microorganisms a single cyclase is responsible for the conversion of lycopene to β-carotene. The red fruits of tomato, papaya, and watermelon appear to be recessive for the genes coding for lycopene cyclase. In *Ustilago* the cyclase gene is complex and in *Phycomyces* is associated with another function (A) in the *carRA* bifunctional gene (*353*). The lycopene mutant of maize and δ-carotene variety of tomato fruit which accumulate δ-carotene lend support for the *in vivo* and *in vitro* evidence (reviewed by *27*) that different enzymes are required for the synthesis of the β and ε ring systems.

TABLE II
MUTANTS BLOCKED IN CYCLIZATION

Organism	Mutant	Product accumulated	Ref.
Maize	Lycopene	δ-Carotene	*159*
Barley	*tig*-n, *tig*-o	Lycopene	*243a*
Tomato fruit	Red (*B*$^-$, *Del*$^-$)	Lycopene	*180*
	β-Carotene (*B*$^+$)	β-Carotene	
	δ-Carotene (*Del*$^+$)	δ-Carotene	
Papaya fruit	Red	Lycopene	*365*
	Yellow	β-Carotene	
Watermelon fruit	Red	Lycopene	*249*
	Orange	β-Carotene	
Phycomyces blakesleeanus	*carR* (*carRA* complex locus)	Lycopene	*102, 114, 353*
Ustilago violacea	*O/P* complex locus	Lycopene (and some β-carotene)	*361*
Pseudomonas echinoides	—	Lycopene	*85*
Halobacterium halobium	W4002	Lycopene (and bacterioruberins)	*194*

Summarizing, genetic evidence suggests there are fewer than four different dehydrogenases (one or two) and only a single cyclase. No isomerase gene has been found. Except for *H. cutirubrum,* these enzymes are all membrane bound (reviewed by *27*). That these enzymes might be organized into a multienzyme complex has been suggested by quantitative genetic complementation studies with mutants of *P. blakesleeanus* (reviewed by *64*). This multienzyme complex is composed of four dehydrogenases (copies of the *carB* gene product) and two cyclases (copies of the *carR* gene product) arranged sequentially. Unfortunately, there are, as yet, no enzyme studies to support this model.

E. Other Reactions

Mutants blocked in a wide variety of reactions involved in further modifications of carotenoids are listed in Table III. These include defects in genes for such enzymes as mixed-function oxidases, 3,4-dehydrogenases, hydratases, methylases, and glycosyl transferases. The best characterized genes are those coding for the carotenogenic enzymes in *Rps. capsulata.* Marrs and co-workers have aligned the genetic and restriction enzyme maps of the chromosomal region carrying the carotenogenic genes (*22*). Therefore, it is now possible to isolate and study a specific carotenogenic gene from this system (*125a*).

TABLE III
MUTANTS BLOCKED IN OTHER STEPS

Organism	Mutant	Products		Defective enzyme	Ref.
		Accumulated	Absent		
Helianthus annuus	Albino	Carotenes	Xanthophylls	Mixed-function oxidase?	*216*
Capsicum annuum	Yellow	Lutein, violaxanthin	Cryptocapsin, capsanthin, capsorubin	Acylcyclopentanol formation	*315*
Euglena sp.	PBZ G1, PBZ G2, PBZ G3, PR1, PR2, PR3	β-Carotene	Xanthophylls	Mixed-function oxidase	*141, 142*
Neurospora crassa	*ylo-1*	Lycopene, α-carotene	Neurosporaxanthin, torulene, 3,4-dehydrolycopene	3,4-Dehydrogenase	*126*
Gibberella fujikuroi	SG68	Torulene	Neurosporaxanthin	Dioxygenase?	*9, 242*
Staphylococcus aureus	Yellow	4,4′-Diaponeurosporene	4,4′-Diaponeurosporanal	Mixed-function oxidase	*221*
	Orange	4,4′-Diaponeurosporanal	4,4′-Diaponeurosporenoic acid	Mixed-function oxidase	
	Deep yellow	4,4′-Diaponeurosporenoic acid	Glucosyl-diaponeurosporenoates	Glycosyl transferase	
Pseudomonas rhodos	Orange	4,4′-Diapolycopene	4,4′-Diapolycopenal, 4,4′-diapolycopendial	Mixed-function oxidase	*183*

	Red to violet	4,4′-Diapolycopenal, 4,4′-diapolycopendial	4,4′-Diapolycopenoic acid, 4,4′-diapolycopendioic acid	Mixed-function oxidase	*183*
	Bright red	4,4′-Diapolycopenoic acid, 4,4′-diapolycopendioic acid	Glycosyl-diaponeurosporenoates	Glycosyl transferase	
Halobacterium halobium	3	Lycopene (and β-carotene)	Bacterioruberins	Prenyl transferase	*194*
Rhodopseudomonas spheroides	—	Neurosporene	Chloroxanthin	Hydratase	*229, 327*
	Green	Chloroxanthin	Demethylspheroidene	3,4-Dehydrogenase	
	Brown	Spheroidene	Spheroidenone	Mixed-function oxidase	
Rhodopseudomonas capsulata	Green (*crtC*)	Neurosporene	Hydroxyneurosporene	Hydratase	*125a, 220, 305*
	Green (*crtD*)	Neurosporene, hydroxyneurosporene, methoxyneurosporene	Demethylspheroidene, spheroidene	3,4-Dehydrogenase	
	Muddy brown (*crtF*)	Hydroxyneurosporene, demethylspheroidene	Methoxyneurosporene, spheroidene	O-Methylase	
	Yellow brown (*crtA*)	Demethylspheroidene, spheroidene	Demethylspheroidenone, spheroidenone	Mixed-function oxidase	

V. Regulation of Carotenoid Biosynthesis

A. Light

The influence of light is probably the most widely studied of all the regulatory factors which control carotenoid biosynthesis, and several recent reviews have been devoted to this topic (*154, 270–273, 308*). The discussions in this article will center on the biochemical events which occur after absorption of light by the photoreceptor, using examples from higher plants, algae, fungi, and bacteria.

1. Higher Plants and Algae

The formation of carotenoids in higher plants and algae is closely associated with the development of the chloroplast. At least three photoreceptors are known to operate in plants: phytochrome, the blue/uv photoreceptor (often called "cryptochrome"), and protochlorophyll(ide). The chemical nature and properties of these receptors are discussed elsewhere (*154, 237, 271*), and the photoregulation of the composition of thylakoid membranes has been covered by Anderson (*2*).

In the light the development of photosynthetically active chloroplasts during plant growth proceeds from undifferentiated proplastids. Chloroplast development involves a series of complex processes which are regulated by the cell, including the biosynthesis of carotenoids. Although neoxanthin (5′,6′-epoxy-6,7-didehydro-5,6,5′6′-tetrahydro-β,β-carotene-3,5,3′-triol) and violaxanthin are found in the etioplast, major synthesis occurs at the same time as that of chlorophyll *b* (*113, 276*). Within the thylakoids, chlorophylls and carotenoids are bound to six different pigment–proteins. The light-harvesting chlorophyll proteins ($LHCP_3$, $LHCP_y$) contain chlorophylls *a* and *b* (ratio around 1 : 1) with lutein (β,ε-carotene-3,3′-diol) and neoxanthin; CP1 and CP1a, the pigment–proteins of photosystem I, contain β-carotene, as does CP_a, the reaction center of photosystem II (*207, 210, 227, 310*). Clearly, the formation of these complexes must be precisely regulated in the plant. The amount of carotenoids within the thylakoids is also governed by the intensity of the incident light: high-light chloroplasts contain more β-carotene due to increased CP1 and CP1a, and lower LHCP levels, compared with low-light chloroplasts (*207–209*). Therefore, there must be considerable turnover of carotenoids in mature chloroplasts.

There is some evidence of differences in the kinetics of carotenoid biosynthesis in the different pigment–protein complexes (*148*), and also that carotenes and xanthophylls are made at separate sites in the

chloroplast by different enzymes (*283*). If the carotenoids are not synthesized at their functional site in the chloroplast, there may be specific carotenoid carrier proteins present which transport these pigments within the organelle.

In addition to carotenoids being present in thylakoids, they have also been found in the chloroplast envelope (*107, 211*) and in the envelope of amyloplasts (*119, 243*). The carotenoid composition of the chloroplast and amyloplast envelopes are almost identical, but they contain a higher xanthophyll : carotene ratio than the thylakoids (approximately ×3). Carotenoids are also located in plastoglobuli in photosynthetic tissues (*171*).

Any hypothesis regarding carotenoid biosynthesis and its regulation in chloroplasts must account for the involvement of envelope membranes in this process. There is an exchange of carotenoids between the envelope and thylakoids during illumination (*311*), and the spinach chloroplast envelope converts zeaxanthin to violaxanthin (*83*). The activity of stromal prenyl transferase is stimulated by isolated envelope membranes (*24*). Such a stimulation is also found with daffodil (*186, 187*) and *Capsicum* chromoplasts (*54*). These interactions among the chloroplast envelope, stroma, and thylakoid may also be important for the biosynthesis of other terpenoids (see Section VII).

One theory for the accumulation of carotenoids in greening tissues has been suggested by Frosch and Mohr (*124*). In this "push and pull" scheme, the "push" regulation is due to phytochrome-mediated regulation of enzyme levels before a pool of free carotenoids is produced, whereas the "pull" control is due to the complex-forming ability of chlorophyll which drains the pool of free carotenoids. This hypothesis necessitates an interdependence between light-dependent chlorophyll and carotenoid biosynthesis. Although carotenogenesis is stimulated by red light (*143*), the overall theory is too simple because it does not explain the various locations of different carotenoids in the pigment–protein complexes described earlier. It must be concluded, therefore, that at present we do not know how light coordinately regulates carotenoid and chlorophyll biosynthesis during chloroplast development in higher plants.

Algae form intact, carotenoid-containing chloroplasts in the dark, indicating that carotenogenesis in algae has no absolute light requirement. In wild-type strains, only *Euglena gracilis* is reported to show photoregulation. In this alga, illumination triggers a large increase in carotenoids (*106, 188, 323, 356*) and is related to chloroplast development as in higher plants. In contrast, mutants of *Euglena, Chlorella,* and *Scenedesmus* have been isolated that exhibit significant pho-

toregulation. In these cases, dark-grown cells do not form cyclic carotenoids, but light-grown cells do (reviewed by *273*). The addition of deuterated water to cultures of *Scenedesmus* PG1 during darkness and in the light has shown that during the first 5 hours of greening the accumulated ζ-carotene is converted to cyclic carotenoids, but after that time they are produced almost entirely *de novo* from CO_2 and ^2H_2O. This *de novo* synthesis is inhibited by both chloramphenicol and cycloheximide, but the conversion of accumulated ζ-carotene to cyclic carotenoids is not prevented by the inhibitors. The cyclases, therefore, are present in dark-green cultures but require light for activation (*34, 37, 42*). These enzymes may undergo a photomodulation similar to that shown with flavoproteins (reviewed by *162, 291, 372*). Since the dark-accumulated ζ-carotene is 15-cis, the light-activated reactions must also involve an isomerization step to the all-trans isomer (*261, 262*); this conversion also occurs on illumination of dark-grown cultures of *Euglena* W_3BUL (*120, 322*).

2. Fungi

Photoregulation of carotenoid biosynthesis has been reported in at least 15 species of fungi (see *272*). In some of these, light enhances the colored carotenoid content of the cells, e.g., *P. blakesleeanus* (*20, 297*), in others, e.g., *N. crassa,* only small amounts of phytoene are produced in the dark (*151, 370*), while a third category, e.g., *Aspergillus giganteus* (*110, 374*), exhibit strict photoregulation of carotenogenesis.

The kinetics of photoregulation in fungi follow two stages; a first light-dependent, but temperature-independent, phase, which varies in length from species to species but is typically of short duration (the "light phase"), followed by a light-independent stage during which carotenoid biosynthesis occurs (the "dark reactions"). Between these two phases a lag period exists, whose duration also varies among organisms. *Verticillium agaricinum* differs from other fungi, as carotenoids only accumulate during illumination (*250*).

The nature of the photoreceptor in fungi has been the subject of considerable debate over many years. The action spectra generally show a maximum at 370–380 nm and three peaks (or shoulders) between 400 and 500 nm. Light of wavelengths above 520 nm is ineffective, i.e., a uv/blue light effect. In *Verticillium,* however, red light also has an effect (*250, 357*). In both *Neurospora* (*305*) and *Phycomyces* (*165*) the fluence–response curve for β-carotene induction is biphasic. Discussions on the relative merits of flavoproteins and/or carotenoids as candidates for the photoreceptor can be found in several reviews (*97, 154, 264, 270, 271, 273, 301, 306, 335*). A range of mutants of

Phycomyces (the *mad* and *pic* strains, see Section VI) are defective in aspects of photoreception and have been studied intensively in attempts to identify the photoreceptor(s) (*62, 63, 257*). However, the situation is complicated by the other photosensory events, such as phototropism, which are characteristic of this fungus. Transduction of the light signal is thought to be mediated by redox reactions (see *270, 271, 273*).

Regardless of the nature of the photoreceptor, two questions can be asked of the other aspects of photoregulation of carotenoid biosynthesis: (a) what enzymes are photoinducible, and (b) at what metabolic level does photoregulation occur?

a. Induction of Carotenogenic Enzymes. Initial *in vivo* studies aimed at elucidating which enzymes were induced/activated during illumination, e.g., in *N. crassa* (*23, 269*), suggested that the carotenogenic enzymes were coordinately induced by light. However, in order to demonstrate unequivocally which biosynthetic enzyme activities change during illumination, it is necessary to use cell-free preparations from dark- and light-grown cultures with suitable substrates. Relatively few *in vitro* systems are available from photoregulated fungi (see *27*), but some useful information has been obtained with *N. crassa,* and, more recently in our laboratory, with *Aspergillus, Gibberella,* and *Phycomyces.*

Using [1-^{14}C]IPP, with an *in vitro* system from *N. crassa,* it has been shown that the steps to phytoene are stimulated in light-grown cultures compared with dark-grown controls (*321a*). These results have been independently confirmed with [2-^{14}C]MVA as the substrate (*236*). Investigations with the *al-2* and *al-3* mutants of *Neurospora* (see also Section IV) have demonstrated that GGPP synthetase is also photoregulated (*155*). A comparison of enzymatic activities from dark- and light-grown cultures of *P. blakesleeanus* 1+, incubated with [2-^{14}C]MVA, found that the latter contained twice the activity of the dark-grown preparation, and these data were taken as an explanation of the increased levels of β-carotene *in vivo* following illumination (*297*). A recent study in our laboratory, using wild-type *Phycomyces* (NRRL 1555), however, has revealed no differences in the specific activities of the enzymes from MVA to β-carotene in light- and dark-grown cultures, despite a 4-fold increase in β-carotene levels *in vivo* on illumination (*249a*). Similarly, *in vitro* carotenogenic activities of *madA, madB, picA,* and *picB* strains (see Section VI for gene nomenclature) were the same in light- and dark-grown mycelia.

The reason for this anomaly between the two studies has yet to be explained experimentally, and it may be due to the different wild-type

strains employed. Our own data may reflect up to three possibilities. First, the rate-limiting, light-controlled enzyme may not be between MVA and β-carotene, e.g., HMG-CoA-reductase, as found for *Rhodotorula minuta* (*333;* see below). Second, the photoaccumulation of β-carotene may reflect differences in its metabolism to retinol, etc., rather than changes in biosynthetic enzyme activities, and these are not assayed *in vitro* with our cell extract (*275*). Finally, the recent model for the regulation of carotenogenesis in *Phycomyces* (*64;* see also Section VI) can be used as an explanation, if one assumes that the pA:β-carotene:pS complex cannot be formed *in vitro*. It is of interest to note that a similar situation occurs with cell extracts of wild-type and SG22 strains of *Gibberella fujikuroi;* light- and dark-grown cultures show photoregulation, but *in vitro* activities are the same (*9, 242*). On the basis of *in vitro* data, phytoene synthetase is strictly photoregulated in mutant strain SG43 but not so in the wild type (*242*).

The use of ^{14}C-labeled MVA, GGPP, PPPP, phytoene, and lycopene with cell extracts of light- and dark-grown *A. giganteus* has demonstrated that the enzymes catalyzing the conversions from PPPP to β-carotene are totally absent in dark-grown mycelia and are photoinduced on illumination (*110, 111*). In the same organism, IPP isomerase is stimulated 2.5-fold, but prenyl transferase activity is virtually identical under both conditions. Using a cell-free system from *R. minuta,* it has been shown that HMG-CoA reductase is photoregulated (2-fold increase in activity on illumination). The enzymes which convert phytoene to colored carotenoids are not photoinducible in the yeast (*333*).

b. The Site of Photoregulation. In vivo studies with inhibitors of transcription and translation (reviewed by *272*) have generally led to the conclusion that regulation by light affects transcription, e.g., *Fusarium aquaeductuum* (*271*), *A. giganteus* (*110*). Evidence for the involvement of *de novo* mRNA synthesis has also been obtained from studies with *Fusarium* (*302, 303*). More recently, the Munich group has extracted mRNAs from light- and dark-grown mycelia of *Neurospora* and *Fusarium* and compared their translational capacities *in vitro*. The levels of poly(A) RNA were higher in illuminated mycelia, and differences in the *in vitro* translated polypeptides were also detected by two-dimensional electrophoresis (*236*).

The derepression of carotenoid gene transcription in *N. crassa* is controlled by the intracellular level of cAMP. A series of experiments with dark- and light-grown cultures and with mutants deleted in adenylate cyclase or phosphodiesterase have demonstrated that light causes a decrease in cAMP levels, which in turn alleviates carotenoid gene repression. An additional controlling mechanism is thought to be

the activity of NAD kinase, i.e., the cellular levels of NADP. In submerged, vegetative mycelia, light causes the photooxidation of NADH (an inhibitor of NAD kinase), whereas, in maturing conidia, activation of NAD kinase occurs. These mechanisms work in parallel with the cAMP-mediated control of gene expression (*1, 189–191, 318*).

3. Bacteria

Certain species of nonphotosynthetic bacteria contain negligible or no phytoene in the dark, but on illumination a large synthesis of colored carotenoids occurs, e.g., *Mycobacterium marinum* (*12, 222*), *Mycobacterium* sp. (*285, 286*), *Myxococcus xanthus* (*50*), *Micrococcus roseus* (*340*), *Flavobacterium dehydrogenans* (*359*), *Brevibacterium sulfureum* (*185*), and certain *Micromonospora* spp. (*174, 175*). In a similar fashion to fungi, two phases can be recognized; an initial photochemical reaction (temperature-independent), followed by a series of dark metabolic reactions (temperature-dependent steps). *In vitro* studies with the *Mycobacterium* sp. have established that PPPP synthetase is fully photoinduced, GGPP synthetase is increased severalfold by light, but IPP isomerase is not photoinduced (*138*).

Inhibition experiments with chloramphenicol have shown that the dark reactions require *de novo* protein synthesis (e.g., *176, 185*), but this does not exclude the possibility of regulation of gene expression. Indeed, a recent report on photoinduction in *Mycobacterium smegmatis* suggests that light causes the complete derepression of carotenoid genes and that constitutive (dark) carotenoid formation is due to incomplete derepression (*175;* see Section VI).

Finally, it should be noted that certain chemicals are able to mimic light-induced carotenogenesis, e.g., antimycin A (*10, 11*). Such effects are also reported for fungi, e.g., *p*-chloro- or *p*-hydroxymercuribenzoate (*251, 268, 338*). However, the effect of chemoinduction is additive to that of photoinduction in *M. marinum* (*11*) and *Fusarium aquaeductuum* (*339*), suggesting a different regulatory site for each (cf. *Phycomyces,* Section VI).

B. Developmental Processes

Several developmental changes, such as chloroplast development, chloroplast-to-chromoplast transitions (often associated with fruit ripening), and the aging of microbial cells in culture (and associated effects of media) are known to trigger changes in the carotenoid contents of tissues and cells. Since chloroplast development is directly influenced by light, it has been discussed in Section V,A.

1. Ripening

In many fruits ripening is accompanied by a massive synthesis of carotenoids, as the chloroplasts change into chromoplasts. Most fruits are autonomous with respect to ripening, as it continues after the fruits are detached from the plant. Light is not essential for ripening, but maximum carotenoid formation in ripening fruits is stimulated by red light and inhibited by far-red light, suggesting the participation of phytochrome in this process (*166, 167, 343, 344*). Typically, the immature fruits contain the usual foliar carotenoids such as β-carotene, lutein, and violaxanthin, but at the onset of ripening there is a loss of chlorophyll and the formation of chromoplast-specific carotenoids occurs, e.g., lycopene in tomatoes (*127, 313*), capsorubin (3,3′-dihydroxy-κ,κ-carotene-6,6′-dione) and capsanthin (3,3′-dihydroxy-β,κ-carotene-6′-one) in red peppers (*55, 56, 68*), and auroxanthin (3,3′-dihydroxy-5,8,5′,8′-diepoxy-β-carotene) in the mango (*169*). Some other fruits, however, e.g., yellow peppers (*93*) and Cox's Orange Pippin apple (*184*), undergo chloroplast-to-chromoplast transformations but retain their chloroplastidic carotenoids, while a third group shows greatly enhanced synthesis of one chloroplast carotenoid, without concomitant formation of the others, e.g., zeaxanthin in *Physalis alkekengi* (*373*).

The changing pattern of carotenoid synthesis and the morphological and biochemical changes accompanying chromoplast development have been intensively studied in tomatoes and red peppers. Consequently, the regulatory mechanisms underlying these changes are, to some extent, understood. In both plants, it is apparent that the control of carotenogenesis is different in chloroplasts and chromoplasts.

The characteristic accumulation of lycopene in red tomato fruits results from the drastic reduction in its cyclization to β-carotene, although the latter compound is still present in small amounts. This composition is altered, however, if fruits are ripened at 30° rather than 20°C, since at the higher temperature lycopene formation is inhibited but β-carotene synthesis is not (*131, 348, 350*). This effect is reversed on lowering the temperature to 20°C. However, in "high-β" tomatoes, which contain the *B* gene, and hence produce large quantities of β-carotene rather than lycopene (see Section IV), β-carotene formation is also temperature sensitive (*348, 350*). Although this inhibitory effect was reported to be reversed by prior application of CPTA (*266;* see also Section V,D), a later study claimed that this is incorrect (*66*).

The apparent anomaly, of high temperatures inhibiting lycopene biosynthesis but not that of its metabolite β-carotene, can be explained

by the presence of two populations of chromoplast in the fruit. Electron microscopy has shown that one type of chromoplast, found in the jelly of the pericarp, contains β-carotene in osmiophilic granules, whereas the other, found in the outer part of the fruit, contains numerous crystalloids of lycopene as well as osmiophilic globules (e.g., *156, 197*); these differences have been verified by carotenoid analyses (*200*). It has been suggested that the lycopene-accumulating chromoplasts are regulated differently than those containing β-carotene, in that lycopene synthesis in the former is temperature-sensitive, and that the protein (cyclase?) coded by the *B* gene of high-β varieties is transported into these chromoplasts, thus making β-carotene formation also sensitive to elevated temperatures. In this hypothesis, the β-carotene chromoplasts in red phenotypes are analogous to chloroplasts, in that carotenogenesis to β-carotene is temperature insensitive (*277, 279*). The sensitivity of lycopene, but not β-carotene, synthesis to dimethylsulfoxide (*278*) can also be explained by its inhibiting carotenogenesis in the lycopene-containing chromoplasts.

An alternative view has recently been expressed by Premachandra (*203*), who suggests that the dominant alleles *B* and m_{OB} (which are specific for fruits), when present together, control the balance between β-carotene and lycopene synthesis by activating or inhibiting the two alternative routes (via β-zeacarotene or lycopene) to β-carotene. Whichever theory is correct, a further consideration must be the absence of xanthophylls (with the exception of carotene epoxides (*17, 38*) in the fruit. This may be a reflection of the repression of gene expression in the chromoplast.

The situation in red peppers is rather more straightforward, since it involves only the induction or activation of an enzyme responsible for the conversion of the 3-hydroxy-5,6-epoxy end group to the acylcyclopentanol structure of capsanthin and capsorubin (*93*). The mutant yellow variety presumably does not contain this enzyme (see Section IV). During the first stage of ripening of red peppers, there is a lag period with respect to carotenoid content as chlorophyll degradation continues, during which time the induction of carotenogenic enzymes presumably occurs. It coincides with the development of a peripheral reticulum inside the plastid (*55, 56*) which may facilitate the cooperation between plastid and cytoplasm and, therefore, enable enhanced carotenoid biosynthesis to occur by the increased permeability of the plastid membrane toward acetate and MVA (*298a*).

Studies on mango fruits have shown that the ripened fruit contains increased levels of phosphatase activity and of nonphosphorylated carotene precursors, e.g., geraniol. It has been suggested that increased β-

carotene levels stimulate phosphatases and hence regulate the fate of carotenogenesis in the fruit (*228*).

2. Developmental Changes in Microbial Cultures

There are numerous reports in the literature which show that changes in growth rate, age of culture, media constituents (especially carbon and nitrogen sources), aeration, etc. affect the carotenoid content of microbial cells. In many cases, any connection may only be tenuous and simply a reflection of the metabolic state of the microorganism under certain cultural conditions. Stimulation of carotenogenesis can be achieved by including specific precursors in the medium, e.g., valine or leucine with *P. blakesleeanus* (*67, 132*). There are, however, some general observations which can be made, especially for fungi.

Carotenoids in fungi are generally classified as secondary metabolites, i.e., they are produced during conditions of growth limitation (*47*). Early studies with *P. blakesleeanus* (*125, 132*), *Epicoccum nigrum* (*121*), and *Sporobolomyces roseus* (*25*) demonstrated that carotenoids are mainly accumulated after the cessation of growth, or when growth rates are limited by nutrient supplies, e.g., *Mortierella ramanniana* (*37*). The final carotene level, e.g., in *Phycomyces,* is dependent on the C : N ratio in the medium (*134*). However, this pattern was not observed with *car* mutants of *Phycomyces (29)* nor with *A. giganteus* (*110*). In both of these fungi, carotenogenic enzymatic activities were detected from the onset of growth and decreased during stationary phase. The reason for this apparent anomaly may relate to the different growth conditions adopted; the earlier studies were conducted with static or agar cultures, whereas the later investigations used shake cultures.

The pH of the fungal culture, which in closed systems typically falls to around 3.0 during growth, may also have an important regulatory effect. Buffering the medium of *Phycomyces* almost completely inhibits carotenoid formation, although growth is unaffected (*134*). Although medium pH is known to control extracellular enzyme synthesis in several microorganisms (*51, 80, 213*), it is not known whether it also regulates the production of intracellular, membrane-bound enzymes.

Conidiation is often associated with the appearance of carotenoids, e.g., *N. crassa* (*154*). The regulatory mechanism controlling carotenoid formation appears to be mediated by cAMP, since at high concentrations (10^{-3} *M*), both conidiation and carotenogenesis are inhibited, but below 10^{-4} *M* both are stimulated in *N. crassa* (*355*). Athrosporulation of *Trichophyton mentagrophytes* is also associated with the initiation

of carotenoid biosynthesis (*151*), while the stimulation of synthesis caused by mating in the Mucorales and mediated by trisporic acids, is well documented (discussed in Section V,C).

C. Bioinducers

When the two mating types [designated (+) and (−)] of the Mucorales (Zygomycetes) are cultured together, they produce much higher levels of carotenoids than do either mating type cultured alone. For example, in *Blakeslea trispora* mated strains synthesize up to 850 μg carotene/g dry weight compared with 30–50 μg/g dry weight of unmated strains (*341*). This massive increase in carotenoid formation is due to the synthesis of trisporic acids, and especially trisporic acid C, by the mixed strains. The trisporic acids also mediate sexual differentiation (*49*) as well as an increased synthesis of sterols and ubiquinone-9 (*367*). The trisporic acids themselves are formed from β-carotene (*7*) via sex-specific precursors, the prohormones P^+ and P^- (*328, 330, 331*). Methyl trisporic acids, produced by (+) cultures, are in turn demethylated by the (−) strain (*332*), and so the addition of either methyl trisporates or trisporates to the culture medium of (−) cultures of *P. blakesleeanus* increases the β-carotene content significantly (*136*). A similar effect is shown in intersexual heterokaryons (*239*).

Although this system represents a good example of positive feedback regulation, the precise level of control is not fully understood. Early studies demonstrated that *de novo* synthesis of protein was necessary for such an increase in carotenogenesis (*117, 342*), and it has been suggested that control is at the translational level (*129*). It seems more feasible, however, that the trisporic acids, either directly or indirectly, repress a considerable number of genes, including those responsible for carotenogenic enzymes.

A stimulation of RNA synthesis on addition of trisporic acids to *Blakeslea* has been observed (*49*), and it was also shown that a concomitant rise in the cAMP levels of mated mycelia occurs (*48, 49*). This increase of intracellular cAMP levels has been confirmed in an independent study (*88*). Whether the effects of trisporic acids are directly on a cAMP-mediated system or are only indirect awaits further investigations. The precise enzymes which are induced following trisporic acid production are reported to be those responsible for the conversion of MVAP to DMAPP (*267*). It should also be noted that sexual stimulation of carotenogenesis is additive to the stimulations induced by light, retinol, dimethyl phthalate, and mutation of the *carS* gene (*136;* see Sections V,A and V,D), but not β-ionone (*267*).

β-Ionone stimulates carotenoid biosynthesis in both mating types of

Phycomyces (*114, 217, 282*) and in heterothallic cultures of *B. trispora* (e.g., *70, 282*). Other terpenes (*60*) and derivatives of β-ionone (*245*) are also stimulatory. Retinol is a particularly effective stimulator of carotenogenesis in wild-type, *carA, carB,* and *carR* strains of *Phycomyces* (*114*) and in *Blakeslea* (*116*). The effects of both β-ionone and retinol are inhibited by cycloheximide (*114*), although the significance of this has recently been challenged (*64;* see Section VI).

Plant hormones such as abscisic acid, cytokinins, indoleacetic acid, and ethylene generally reduce the carotenoid levels of plant seedlings and plant cell cultures (reviewed by *129*). The mechanisms of action in these cases are unknown.

D. Synthetic Chemicals

A large number of organic compounds are known to inhibit and/or stimulate carotenoid biosynthesis. These can be broadly divided into two categories: (1) those causing the accumulation of more saturated carotenoids, typically phytoene and phytofluene, and (2) compounds which inhibit cyclization, usually with a large buildup of lycopene. It should be remembered, however, that not all of the compounds are effective in all organisms, and there are apparent variations in their modes of action in those organisms in which they are effective. Comprehensive lists of inhibitors can be found in the review by Spurgeon and Porter (*320*). For convenience, only selected chemicals from each group will be discussed in this article.

1. Compounds Causing the Accumulation of More Saturated Carotenoids

The group of compounds causing saturated carotenoid accumulation is typified by the classic inhibitor of carotenogenesis, diphenylamine, whose effect was first demonstrated in 1936 (*178*). It is effective against a range of organisms *in vivo* (reviewed by *129* and *320*) and is also inhibitory *in vitro* in *Phycomyces* cell extracts (*75*). The *in vivo* accumulation of phytoene in *P. blakesleeanus* is greater than expected on the basis of the decrease in β-carotene (*246*). This may be due to a loss of feedback inhibition by β-carotene on the step(s) before phytoene (mediated by the *carS* gene? see Section VI). The *in vitro* effect is reversible, suggesting that diphenylamine inhibits the activity of phytoene dehydrogenase. A range of other biphenyl compounds are also inhibitory *in vitro* (*28*) and may act as competitive inhibitors of the fungal enzyme (*296*). In contrast, diphenylamine is ineffective in a tomato plastid system, leading to the suggestion that it acts at the gene level.

A range of bleaching herbicides also inhibit the phytoene dehydrogenase activity of *Aphanocapsa* thylakoids, with the alkylbenzamide S3422 exhibiting noncompetitive inhibition kinetics (*76*). The recent discovery that *cis*-dihydropyrone herbicides inhibit *in vitro* at the level of ζ-carotene dehydrogenase, but far less so at phytoene dehydrogenation, suggests that at least two enzymes are present in this cyanobacterium (*76;* compare mutants lacking carotene dehydrogenases, Section IV). Studies with pyridazinone herbicides have led to the suggestion that there are two separate sets of enzymes in higher plants: one for the synthesis of carotenes and another for xanthophylls (*283*). If this is correct, it implies that carotenoid biosynthesis in higher plants is fundamentally different from that in microorganisms, and must also reflect a major difference in the regulation of the pathway(s).

2. Cyclase Inhibitors

The best known cyclase inhibitors are nicotine and 2-(4-chlorophenyl)triethylamine (CPTA). Their uptake by many microorganisms and higher plants causes the accumulation of lycopene, sometimes with small amounts of monocyclic carotenes. In photosynthetic bacteria, which contain no cyclic carotenoids (see *298*), these two compounds inhibit the hydroxylation of neurosporene to spheroidene in *Rps. spheroides* (*233, 316*) or lycopene hydroxylation in *Rhodomicrobium vanielli* (*40*), probably because the reaction mechanism for cyclization is similar to that of hydroxylation.

A large range of onium compounds structurally related to CPTA has also been tested, usually with the flavedo of citrus fruits (*367*). These compounds regulate the formation of either *cis*- or *trans*-carotenoids. Inhibitors of the latter pathway have the general formula $(C_2H_5)_2NCH_2R$, and the magnitude of the stimulation of lycopene synthesis is dependent on the nature of the R moiety (*256*). They are effective in citrus fruits (*267*) and in fungi (*79*). Literature reports on the mode of action of these bioregulators are somewhat equivocal. The induction of lycopene formation by CPTA is prevented by cycloheximide in several tissues (*53, 123, 161*), indicating that protein synthesis is necessary. A recent study, using the tertiary amine MPTA [2-(4-methylphenoxy)triethylamine] with the flavedo of lemons, has demonstrated that the stimulatory action of this compound requires gene expression and translation of the poly(A)$^+$ RNA on 80 S ribosomes, clearly indicating that CPTA-like compounds act directly at the level of gene expression, perhaps by binding to a regulatory protein and entering the nucleus (*180*). This would mimic the induction of carotenoid biosynthesis which occurs during ripening of fruits (see Section V,B,1).

In contrast with this hypothesis, Murillo (*238*), using a *carA* mutant of *Phycomyces* with CPTA, has suggested that the stimulation of lycopene biosynthesis is due to the release of a negative-feedback control, normally mediated by β-carotene, in this fungus, i.e., CPTA has only an indirect effect on gene expression.

The *in vitro* activity of cyclase inhibitors has been shown for CPTA with *Aphanocapsa* membranes (*295*) and for AMO 1618, octyldiethylamine, and dodecyldiethylamine with *Capsicum* chromoplast membranes (*57*). In contrast, however, little or no inhibition of lycopene cyclization by CPTA was demonstrated with a tomato plastid system (*45*), a *Narcissus* chromoplast preparation (*21*), or with nicotine and *Capsicum* chromoplasts (*57*). In conclusion, therefore, compounds which affect the cyclization of *trans*-carotenoids have dual modes of action; however, the precise molecular mechanisms of these activities cannot be ascertained from data currently available.

The bioregulators which cause the accumulation of poly-*cis*-carotenoids have not been studied in as much detail. They all induce the biosynthesis of poly-*cis*-carotenes in the flavedo of Marsh white seedless grapefruit and include secondary and tertiary amines such as 4-chlorodibenzylamine (*256a*) and *N*-(4-bromobenzyl)furfurylamine (*256b*). None of these compounds inhibits lycopene cyclization, and their sole mode of action is thought to involve gene derepression, probably of a recessive gene which controls the biosynthesis of poly-*cis*-carotenoids (*256a, 256b*). Although there is such a gene in tomato fruit (see Section IV,B), insufficient experimental evidence is currently available to verify this hypothesis.

3. Other Stimulators of Carotenogenesis

Several compounds mimic the photoinduction of carotenoid formation in fungi, e.g., *p*-hydroxymercuribenzoate, *p*-chloromercuribenzoate (*251, 268*), antimycin A (*10*), and hydrogen peroxide (*338*). These are fully discussed in Section V,A.

Dimethyl phthalate activates carotenogenesis in *Phycomyces* wild type and mutants of genes *carA, carB, carR,* and *carS* but not *carI* (*64, 65*). This effect is fully discussed in the context of the regulation of carotenogenesis in *Phycomyces* (Section VI).

E. Miscellaneous

A range of widely differing effectors have been reported to alter carotenoid biosynthesis (Table IV). In most cases no mode of action has been ascertained, and such effects may only be of an indirect nature. In contrast, increases in carotenogenesis in *B. trispora,* caused by phos-

TABLE IV

MISCELLANEOUS EFFECTORS OF *in Vivo* CAROTENOID BIOSYNTHESIS

Effector	Tissue/organism	Effect on carotenogenesis	Mode of action	Ref.
Mo^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}	Roots, leaves, fruits, micro-organisms	Increase	Unknown	Reviewed by *129*
Inorganic	*Blakeslea trispora*	4.5-Fold increase	Decrease of phosphatase levels	*105*
F^-	*Blakeslea trispora*	5-Fold increase	Inhibition of phosphatases	*103*
Cu^{2+} (10^{-8} *M*)	*Blakeslea trispora*	Increase	Derepression of trisporic acids; increase in MVA kinase	*135*
Penicillin	*Blakeslea trispora*	Increase	Increase in MVA kinase	*104*
High pressure	*Blakeslea trispora*	Decrease	Unknown	*118*
Oxygen	*Rhodotorula gracilis*	Increase	Unknown	*14*
Surfactants	Fungi	Increase	Unknown	*3*
Urea	Maize and millet	Decrease	Unknown	*173*
Growth factors	Various	Varied	Unknown	Reviewed by *129*
pH	Fungi	—	See Section V,B	
Temperature[a]	Algae, fungi, bacteria	Increase at higher temperatures	Thermodynamics (?)	Reviewed by *129*
	Fruits	Increase	During ripening	See Section V,B

[a] See Section V,A for the effect of temperature on photoinduced carotenogenesis.

phate, Cu^{2+}, fluoride, or penicillin, all cause an increase of intracellular prenyl pyrophosphate levels, either by inhibiting phosphatases or by increasing the MVA kinase activity of the cells (*103, 105*). However, no evidence has yet been reported to show that the levels of these intermediates are normally rate limiting. The involvement of trisporic acids in carotenogenesis in *Blakeslea* (*135*) has been discussed in Section V,C, and that of pH on fungal carotenoid biosynthesis in Section V,B.

VI. Regulatory Genes

Regulatory mutants produce either reduced or increased levels of carotenoids or fail to respond to external effectors. Mutants with altered levels of carotenoids compared to the wild type are listed in Table V.

In higher plants, increased carotenoid biosynthesis is an integral part of the light-stimulated formation of chloroplasts from etioplasts and the conversion of chloroplasts to chromoplasts during fruit ripening (see Sections V,A and V,B). The regulation of carotenogenesis is tightly coupled to chlorophyll metabolism and the reorganization of membrane structures. Unfortunately, very few mutants have been recognized with altered carotenoid levels in chloroplasts (e.g., maize, *Helianthus,* and tomato). This is not surprising since mutations which involve deletion in the synthesis of carotenoids in chloroplasts are normally lethal unless the plants can be grown in dim light or heterotrophically in the dark. Oxygen and light cause photodestruction of chlorophylls in chloroplasts lacking β-carotene (*180*). However, mutations affecting chromoplast carotenogenesis have been observed. It would appear that, at least to some extent, the carotenogenic systems act independently in chloroplasts and chromoplasts. For example, in red fruit tomatoes chromoplasts produce predominantly lycopene (lacking lycopene cyclase) while chloroplasts accumulate β-carotene. In tomato, except for the ghost (*gh*) and yellow fruit (*v*) genes which affect both chloroplast and chromoplast carotenoids, the other recognized structural genes (Tables I and II) and regulatory genes (Table V) are exclusive to the chromoplast. All the genes show normal Mendelian inheritance and are assumed to be nuclear in location. Therefore, it seems likely that some of the carotenogenic genes are different for the two systems.

Yellow (*r*) and apricot (*at*) fruit varieties of tomato produce greatly reduced levels of carotenoids, but spraying the mutant fruit with CPTA overcomes this block (*180*). As an explanation, it was proposed that the two mutant tomatoes produce a defective regulatory protein

TABLE V
MUTANTS WITH ALTERED CAROTENOID LEVELS

Organism	Effect on carotenogenesis	Ref.
Antirrhinum	Decrease	*371*
Arachis hypogaea	Decrease	*334*
Corchor olitorus	Decrease	*89*
Eschscholtzia californica	Decrease	*108*
Lycopersicon	Decrease	*202, 203*
Tomato		*123, 180*
Yellow fruit (*v*)	Decrease (affects both leaves and fruit)	
Yellow fruit (*r*)	Decrease	
Apricot fruit (*at*)	Decrease	
High-pigment fruit (*hp*)	Increase	
Capsicum annuum fruit	Decrease	*179*
Watermelon crimson fruit	Increase	*346*
Chlamydomonas reinhardi	Decrease	*293*
Rhodoturula luteus	Increase	*196*
Epicoccum nigrum	Increase	*139*
Phycomyces blakesleeanus	Increase and decrease	*63*
Gibberella fujikuroi	Increase	*9*
Micrococcus luteus	Decrease	*225, 226*
Mycobacterium phlei	Increase	*358*
Mycobacterium smegmatis	Decrease	*174, 175*
Pseudomonas rhodos	Increase and decrease	*183*
Staphylococcus aureus	Increase (type VII) and decrease (type VI)	*221*

which binds effectively to CPTA but not the endogenous natural inducer. In turn, the regulatory protein–CPTA complex permits transcription of the genes for carotenoid biosynthesis. However, recent studies with tomato mutant callus suggest a more complicated action for CPTA and these regulatory genes. Treatment of green, light-grown *at* mutant callus with CPTA not only induced and inhibited the formation of chromoplast and chloroplast carotenoids, respectively, but also induced chromoplast differentiation from chloroplasts. Thus CPTA may, through the *at* gene product, activate the entire chromoplast differentiation pathway of which carotenoid biosynthesis is only one part.

Red tomato fruits produce two types of chromoplast, one rich in lycopene and the other in β-carotene (*200*). The chromoplasts respond differently in that temperatures above 30°C inhibit lycopene but not β-carotene synthesis (*87*). Evidence that these chromoplast types have different carotenogenic systems governed by different nuclear genes

has come from studies with β-carotene (*B*) and high pigment (*hp*) tomato fruits. Both the dominant *B* and recessive *hp* genes result in elevated β-carotene levels but by different mechanisms. The *B* gene (coding for lycopene cyclase, Table II) is responsible for a temperature-sensitive increase in β-carotene synthesis at the expense of lycopene. On the other hand, *hp* (a regulatory gene) results in a temperature-insensitive increase in β-carotene (*348*). It may be that the two chromoplast types differ with respect to their cyclase genes and their response to the *hp* gene product. Since chloroplasts and chromoplasts appear to have a common phytoene dehydrogenase (*gh* mutations affect both chloroplasts and chromoplasts, Table II), it seems likely that the carotenogenic systems diverge after this step. The *v* mutations (yellow fruit) also affect both chloroplast and chromoplast carotenoid synthesis, but the site of action is unknown.

A number of genes have been identified which modify the expression of another gene. A dominant modifier gene cl_M can partly or completely suppress albinism in the maize mutant homozygous for the gene cl_1 (*287*). Another gene, mo_B, modifies the effect of the *B* gene in high β-carotene tomato fruits (homozygous or heterozygous for the dominant *B* allele). The presence of the dominant allele of the mo_B gene, in either the homozygous or heterozygous form, reduces the level of β-carotene by 50% and increases that of lycopene by the same amount (*351*). mo_B has no effect on lycopene levels in red tomato fruits (homozygous for the recessive *B* allele). Therefore, the dominant alleles of the *B* and mo_B genes, when present together, control the balance between β-carotene and lycopene synthesis. The simplest explanation is that the product of the mo_B gene interferes in some way with the *B* gene lycopene cyclase. An alternative explanation (*263*) is that both *B* and mo_B are regulatory genes, the *B* gene exerting its influence by activating β-carotene synthesis by the β-zeacarotene and lycopene routes, and the mo_B gene by activating neurosporene-to-lycopene and/or inhibiting lycopene-to-β-carotene conversions (see also Section V). The wide variations in pigmentation in carrot roots and *N. crassa* mycelia also suggests the influence of modifier genes (*163, 198, 307*).

Light regulates carotenoid biosynthesis in a large number of microorganisms and plants (Section V,A). Mutants in a variety of organisms have been isolated with altered responses to photoregulation. *Myxococcus xanthus* normally requires blue light illumination for carotenoid synthesis but two classes of regulatory mutants, *carA* and *carR*, are able to synthesize carotenoids in the dark. Other carotenoid mutants, *carB* and *carC*, fail to produce carotenoids in either light or dark but, as yet, have not been characterized as structural or regulatory mutants (*9a, 221a*). Algae, except for *Euglena*, do not normally require

light for β-carotene synthesis, but certain mutant strains of *Chlorella vulgaris* (5/520) and *Scenedesmus obliquus* (PG1) accumulate ζ-carotene in the dark and only produce β-carotene in the light. Other ζ-carotene mutant strains are killed on illumination (*41, 71, 72*). Only oxygen is required for xanophyll formation. Since β-carotene appears immediately and only during illumination, some mechanism involving photomodulation rather than photoinduction is assumed (*273, 299*).

In those fungi showing photoregulation, light either is required for (photoinduction) or stimulates carotenogenesis (photostimulation) (see Section V,A). *Fusarium aquaeductuum* is normally strictly photoinduced, but a number of mutants have been described which lack photoregulation and which synthesize carotenoids in the dark (*164, 204*). *p*-Chloromercuribenzoate (PCMB) simulates photoinduction in that it induces carotenoid synthesis in the dark (*338*). The PCMB effect is additive to that of light and, unlike photoinduction, is inhibited by thiols (*199, 339*). Addition of PCMB to the mutants which fail to respond to light but have constitutive carotenoid levels in the dark still stimulated additional carotene synthesis (*251, 338*). The authors conclude that in *Fusarium* spp. two separate (isoenzyme) carotenogenic systems exist, one responsive to light and the other to PCMB. Both effects are blocked by protein and RNA synthesis inhibitors, and induction of *de novo* synthesis of carotenogenic enzymes is suggested (*338*).

Recently, two interesting phytoene-accumulating mutants of *Gibberella fujikuroi* (blocked in phytoene dehydrogenase) have been identified which have different responses in the dark. Mutant SG78 synthesizes phytoene in the dark and more in the light. In contrast, SG43 synthesizes phytoene only in the dark (*9, 242*). This also suggests the possibility for two isoenzymatic systems for phytoene synthesis in *Gibberella*. In this respect, the recent report of mutants of *Mycobacterium smegmatis* defective in either photoregulation or dark-grown carotenoid levels, or both, may be of interest (*174*).

Carotenoid biosynthesis is constitutive in *Neurospora crassa* conidia but is induced by blue light in mycelia (*78, 370*). That photoinduction involves the *de novo* synthesis of some enzymes is supported by protein synthesis inhibitor studies (*153, 274*) and by the increases in phytoene synthetase and GGPP synthetase activities in cell-free extracts by *in vivo* light treatment (*155*). A group of white collar (*wc*) mutants have been isolated in which the mycelium remains white on illumination although, unlike the *al* mutants (discussed earlier), their conidia show normal constitutive carotenoid synthesis (*252*). In addition, the phytoene synthetase and GGPP synthetase activities which are increased by *in vivo* light treatment in the wild type remain unchanged in a *wc*

mutant (*155*). The structural genes for carotenogenesis appear to be functional but are blocked in photoinduction. All the *wc* mutants map at two loci, *wc*-1 in linkage group VII and *wc*-2 in linkage group I (*99, 213*). Observations that other blue light effects, such as phototropism of perithecial beaks and photoinduction of protoperithecia formation, are defective in *wc* mutants but not in *al* mutants (*99, 152*) help to substantiate the hypothesis that the *wc* genes regulate the initial photoresponse steps for all the blue light effects in *Neurospora* (*154*).

Phycomyces blakesleeanus has been extensively examined with respect to carotenogenesis and its regulation by light and various chemicals (see Section V). Studies with carotenogenic mutants have been particularly helpful in trying to understand the regulatory mechanisms involved (review, *63, 64*). The carotenogenic mutants and some of their characteristics are summarized in Table VI.

Gene *carB* codes for phytoene dehydrogenase (*101, 115, 248*). Genetic analysis (*353*) has shown that gene *carRA* contains two segments, the proximal segment, *carR* (5′ end of the mRNA), coding for lycopene cyclase (*101, 248*) and the distal segment, *carA*, responsible for substrate transfer (*241*). The *carRA* gene is thought to code for a single polypeptide which is processed into the cyclase and A proteins. Nonsense or frameshift mutations in the R segment would result in defective or missing R and A proteins (*carRA* phenotype), while missense mutations in the R segment and any mutations in the A segment would produce the *carR* and *carA* phenotypes, respectively. *carA* mutants produce virtually no carotenes, including phytoene (*248*).

Four copies of the carotene dehydrogenase and two copies of the carotene cyclase are sequentially arranged in an enzyme complex (*5, 102, 115, 241*). This proposal is based on quantitative analyses of the nuclear proportions and carotenes produced in heterokaryons containing nuclei with the appropriate wild-type and mutant (*B* or *R*) alleles. A mathematical relationship between nuclear proportions and carotene levels was predicted if such an enzyme complex existed and mutant and wild-type enzymes mixed randomly into aggregates. Another assumption was that the carotene intermediate was released at the first inactive subunit and accumulated unaltered. The experimental results obtained with *carR***carA* heterokaryons agreed with those predicted by the model but not those with *carR***carS* and *carR***imb-2* heterokaryons, the latter producing much higher relative amounts of β-carotene than *carR***carA* mycelia carrying the same proportion of *carR* nuclei (*102, 241*). The conclusion was that the *carA* segment is responsible for substrate transfer in and between enzyme complexes. As stated earlier, the model assumes that transfer between complexes

TABLE VI

CAROTENOGENEIC MUTANTS FOR *Phycomyces blakesleeanus*[a]

Mutant gene(s)	Linkage[b] group	Proposed function	Major carotene	Dark levels (g^{-1} dry weight)	Response to[c]	
					Light	Retinol
Wild type	—	—	β-Carotene	52	+	+
carB	IV	Phytoene dehydrogenase	Phytoene	1800	−	+
carR	IV	Lycopene cyclase	Lycopene	1600	−	+
carA	IV	Substrate transfer (pA)	β-Carotene	2	−	+
carRA	IV	R and A functions	Lycopene	2	−	−
carS	IV	End-product control (pS)	β-Carotene	2700	−	+
carC	III	Photocarotenogenesis	β-Carotene	13	−	+
carI	?	Chemostimulation	β-Carotene	29	+	−
madA	II	Photoresponses	β-Carotene	56	−	+
madB	IX	Photoresponses	β-Carotene	54	−	+
picA	?	Photocarotenogenesis	β-Carotene	44	−	+
picB	?	Photocarotenogenesis	β-Carotene	46	−	−
carAcarS			β-Carotene	2	−	+
carBcarS			Phytoene	2153		+
carIcarS			β-Carotene	5595		−
carRAcarS			Lycopene	5		−
carBcarR			Phytoene	1430	−	
carAcarC			β-Carotene	3		
carBcarC			Phytoene	1900		
carRcarC			Lycopene	1650		
carScarC			β-Carotene	1460		

[a] Table compiled from Refs. *114, 136, 215, 239, 240,* and *280.*

[b] Mapping data from Ref. *249.*

[c] +, Stimulation; −, no or partial stimulation.

does not occur, and this requirement is fulfilled in heterokaryons with mutant *carA* nuclei. However, similar experiments with *carB***carA*, *carB***carR*, and *carB***carS* heterokaryons all showed excellent agreement between experimental and predicted (based on the above model) results, irrespective of the presence of *carA* mutations (*5*). This questions the validity of the proposed enzyme model or *carA* function, unless the *carA* product is not necessary for substrate transfer between dehydrogenases. In this connection Hsu *et al.* (*160*) failed to observe the predicted relationship between nuclear and carotene proportions (*102*), using the same *carR***carA* heterokaryon, albeit grown in liquid culture. It is also difficult to explain the observed absence of phytoene in *carA* mutants, both *in vivo* (*248*) and *in vitro* (*101*), simply by their lack of substrate transfer ability.

Several lines of evidence support the conclusion that β-carotene regulates its own synthesis. For example, when β-carotene synthesis is inhibited either genetically (by *carB* or *carR* mutation (*114*) or chemically (by diphenylamine (*246*) or CPTA (*161*)), total carotene synthesis is greatly increased. Also, in partially inhibited situations, β-carotene levels remained constant even though the levels of intermediates were increased (*5, 241*). Another group of mutants, recessive to the *carS* gene, accumulate large quantities of β-carotene compared to wild type (*240*). The *carS* gene product (pS) is implicated in control by β-carotene based on observations that introduction of the *carS* gene into *carB* mutants failed to stimulate phytoene synthesis (*240*) and the inability of chemical inhibitors to increase overall carotene production in *carS* mutants (*75, 201*). Superproducer mutants of *G. fujikuroi* have been isolated which accumulate not only carotenoids but also gibberellins and sterols. *Gibberella* differs from *Phycomyces* in that end-product regulation appears not to operate (*9, 240*). As stated earlier, *carRA* mutants are unable to produce carotenes under any conditions which suggests that the A segment gene product (pA), whatever its function, is essential for operation of the pathway. Recently, Cerda-Olmedo (*64*) proposed a scheme which attempts to explain β-carotene regulation and its involvement in photo- and chemo-stimulation. The essential tenets are that formation of an inactive pA : β-carotene : pS complex determines the availability of pA and the flux of the pathway, any situation which reduced the levels of either β-carotene (*carB* and *carR* mutation) or pS (*carS* mutation) would increase carotenogenesis. He also suggests that *carA* mutants (i.e., those stimulated by retinol) have greatly reduced threshold levels for β-carotene inhibition (*238*). However, this hypothesis fails to explain other experimental observations. For example, double *carAcarS* mutants produce the same β-carotene

levels as single *carA* mutants (*240*). Also, within the limits of the efficiencies of such systems, the characteristics of the *in vitro* carotene-synthesizing systems from wild-type *carA* and *carS* cultures parallel those observed *in vivo*, suggesting control at the enzyme level rather than flux of the pathway (*101*). Finally, protein synthesis inhibitors block the stimulation of β-carotene synthesis by light and chemicals (*114, 238*).

Photocarotenogenesis is blocked in the single mutants *carB, carR, carA, carRA,* and *carS,* and the double mutants, *carBcarR* and *carA-carS,* suggesting this response also requires β-carotene and the gene products, pA and pS (*62, 114, 215*). According to Cerda-Olmedo's hypothesis (*64*), light in some way destabilizes the pA : β-carotene : pS complex permitting the pathway to operate. Other mutants have reduced photocarotenogenesis. *CarC, picA,* and *picB* mutants are specifically defective in photocarotenogenesis (*215, 280*). In contrast, the *madA* and *madB* mutants are also defective in other blue-light responses, such as phototropism and photomorphogenesis (*20, 257, 248*), suggesting a common requirement for these gene products (reviews, *61, 62, 214*). Typically, the β-carotene content of the wild type increases with light intensity after a certain threshold value. The intensity–response curves of the *mad* and *picA* mutants are similar to the wild type but displaced to higher light intensities (*215, 275*). However, the *picB* and *carC* mutants produce reduced β-carotene levels at all light intensities (*215, 280*). These types of behavior are believed to reflect lesions in different stages in the photocarotenogenic pathway, the *mad* and *picA* mutants being defective in an "early" stage and the *picB* and *carC* mutants in a "late" stage (*62, 215*). The *madA* and *madB* genes are comparable to the white collar genes in *Neurospora* (*99*). So far, the precise identities of the *pic* genes have not been verified by mapping experiments. *CarC* mutants produce lower dark levels of β-carotene than wild type, but this negative effect is not expressed in the double mutants, *carCcarB, carCcarR,* and *carCcarS* (*280*). In addition, studies with heterokaryons, *carC*carA* and *carC*carS,* showed that all three normal gene products were required for maximum photocarotenogenic effect (*281*). Thus, β-carotene, pA, and pS appear to be necessary for *carC* gene action. Cerda-Olmedo (*64*) suggests the mutant *carC* gene product in some way stabilizes the pA : β-carotene : pS complex, counteracting the proposed light effect.

A new light response, different from that on β-carotene synthesis, has been observed in the double mutant, *carScarE*. The *carE* gene behaves as an extranuclear genetic factor. This mutant accumulates a red β-carotene–protein complex in the dark and the yellow free form

of β-carotene in the presence of light or retinol. Light has no effect *in vitro* (*100*).

Various seemingly unrelated chemicals stimulate β-carotene synthesis in *Phycomyces* (Section V). Their effects on the different *car* mutants have been investigated to determine which gene products are involved in this process. Retinol, dimethyl phthalate, and veronal stimulate β-carotene synthesis in wild types and mutants, *carB*, *carR*, *carS*, and *carA* (retinol also in *carBcarS* and *carAcarS* double mutants) indicating that neither β-carotene itself nor the normal *carS* gene product is required for the chemostimulatory effect (*65, 114, 240*). It seems likely that the chemicals increase β-carotene synthesis by interacting with the *carA* gene product, counteracting to some extent the effect of the mutation. A class of chemoinsensitive mutants, *carI*, have been isolated which are normal with respect to β-carotene levels and photocarotenogenesis but fail to be stimulated by chemicals (*64, 289*). The chemo- and photo-sensory pathways are linked since *picB* mutants are defective in both responses (*215*). Therefore, chemoregulation requires, at least, functional *carA*, *carI*, and *picB* gene products. Cerda-Olmedo (*64*) has recently reinterpreted the data from his earlier experiments with retinol (*114, 240*) as affecting only wild-type and *carA* strains. His suggestion is that retinol increases the flux of the carotenogenic pathway by combining with pA and therefore reducing the levels of the pA : β-carotene : pS complex. Sexual interaction (or simulation by treatment of homokaryans with trisporates and intersexual heterokaryon formation) increases β-carotene synthesis in wild-type *carA* and *carS* mutants and is additive with the effects of retinol and light (*114, 136, 240*). Stimulation by trisporates also requires the presence of β-carotene in the receiving cells (*136*). β-carotene levels are important in sexual development since mutants either lacking or overproducing β-carotene are sexually incompetent (*329*). *carD* mutants, with β-carotene levels intermediate between wild-type and *carS* mutants, cross normally (*294*). *CarI* mutants are also insensitive to trisporates (*136*), thus linking the stimulatory pathways of trisporates and other chemicals. In conclusion, regulatory genes have been identified in a wide variety of organisms and, in some cases, even particular functions have been proposed. The lack of appropriate biochemical information, however, has made it impossible to assign any precise functions.

VII. Conclusions and Future Studies

The regulation of carotenoid biosynthesis is clearly a complex phenomenon, which is often associated with important developmental pro-

cesses such as fruit ripening and chloroplast development in higher plants (see Section V,B) or sexual mating in fungi (see Section V,C). Consequently, it is often difficult to distinguish the control of carotenogenesis *per se* from the overall regulation of cell or organelle differentiation. In addition, the synthesis of carotenoids is only one part of terpenoid formation as a whole (see Fig. 1). The cell must have, therefore, the ability to coordinate the biosynthesis of these compounds and thus independently regulate their formation.

In higher plants compartmentation of the terpenoid pathways is thought to be important in the regulation of biosynthesis; i.e., the chloroplast and extrachloroplast sites of synthesis have a common sequence of reactions (acetyl-CoA to GGPP), but the former location converts GGPP to phytoene while at the latter squalene is formed from FPP (*133, 354*). The importance of the polyprenyltransferase complex in terpenoid biosynthesis has recently been discussed by Douce and Joyard (*107a*). Additional controls, via membrane permeability and feedback regulation at branch points, are also mechanisms to be taken into account. Although there is no evidence for such compartmentation in fungi, the levels of terpenoids in *Gibberella fujikuroi* are altered with respect to each other by external factors such as light and media, as well as by age and mutation (*242*).

At the present time, no single regulatory mechanism is fully understood, whether it is a "natural" effect, e.g., light (see Section V,A), or is mediated by "unnatural" effectors such as CPTA (see Section V,D). This distinction between "natural" and "unnatural" effectors is blurred in some cases, e.g., chemicals which mimic photoregulation.

Obviously, many intriguing questions remain to be answered. In order to understand the molecular mechanisms associated with the regulation of carotenoid formation it is necessary to isolate and characterize the structural and regulatory genes involved in these processes, as well as the gene products, i.e., the levels of carotenogenic enzymes and the regulatory gene products. Two, parallel experimental approaches should be pursued in order to achieve these goals.

A. Biochemistry/Enzymology

In order to estimate the levels of carotenogenic enzymes, and any changes which occur as a result of regulatory processes, it is necessary to develop *in vitro* assays which accurately reflect the physiological activities of these enzymes. Unfortunately, there are only a limited number of suitable cell-free systems currently available. Chloroplast preparations are notoriously low in carotenogenic activities, producing, at best, phytoene but not unsaturated carotenoids (see *27*). This

precludes an assessment of these enzymes during chloroplast development. Similarly, cell extracts of photosynthetic bacteria have not been reported, despite the detailed genetic studies on *Rps. capsulata* (see Sections IV and V). Indeed, it is noticeable that many of the organisms which are well characterized with respect to the number and types of carotenoid genes, e.g., *Ustilago, M. smegmatis, Ps. rhodos,* have not been used for *in vitro* studies. The development of cell-free systems from these organisms, and especially from chloroplasts, would be of considerable value in the context of relating regulatory phenomena to changes in enzymatic activities.

The cell-free preparations that are available from higher plants are derived from chromoplasts, e.g., *Capsicum* fruits (*52–56*) and red and tangerine tomatoes (*195, 265, 363*). It is possible, therefore, to monitor carotenogenic activities during the ripening process, although this has not been reported. In addition, *in vitro* preparations of other tomato varieties (Tables I, II, and V) would help to characterize the products of the genes implicated in carotenoid formation. Fractionation of mature chromoplasts in order to isolate those containing β-carotene from the lycopene-rich type (see Section V,B) and subsequent enzyme assays would also be of interest with respect to the enzymatic complement of these different chromoplast types.

Several cell extracts of fungi have been used in studies on the carotenoid pathway, subcellular location of the enzymes, and their associated cofactor requirements, e.g., *Phycomyces* (*29–31*), *Neurospora* (*235*), *Aspergillus* (*111*), and *Gibberella* (*242*). In the case of *Phycomyces, Neurospora,* and *Gibberella,* a range of carotenoid mutants have been isolated (see Section IV), and so the *in vitro* preparations have considerable potential in studies on the regulation of carotenogenesis. It is important to note, however, that none of the cell-free systems synthesize the same levels of carotenoids as those found *in vivo;* in all cases and especially in *Neurospora* there is a significant accumulation of phytoene *in vitro,* which is not found in whole cells. Consequently, comparisons of *in vivo* carotenoid synthesis with *in vitro* activities may be misleading and should be viewed with some caution until the cell-free activities mirror physiological levels. This may be achieved on purification of the enzymes; certainly, purified proteins would be most valuable for the production of antibodies, both for enzymatic studies and for the isolation of carotenoid genes (see below).

B. Genetics/Molecular Biology

The large numbers of mutants obtained from fungi, photosynthetic, and nonphotosynthetic bacteria (see Sections IV and VI) have been

valuable in establishing steps of the carotenoid biosynthetic pathway and in establishing the complexity of the regulatory processes within individual organisms (e.g., *Phycomyces*) as well as showing that no single, common regulatory mechanism exists among carotenogenic organisms. The diversity in the numbers of structural and regulatory genes reported to date argues in favor of expanding these studies to other organisms and tissues, especially bacteria which have yielded carotenogenic cell extracts, e.g., *Halobacterium* (*192*) and *Mycobacterium* (*138*). The isolation of mutants from photosynthetic organisms such as *Aphanocapsa* presents difficulties due to the masking of carotenoids by chlorophylls and because they cannot be cultured heterotrophically. Higher plant cells, maintained as callus cultures, may be more suitable experimental tissues for this purpose. The carotenoid genes identified within an organism in this way should be mapped on the chromosome(s) in order to establish them as separate entities. This has been achieved with *Neurospora, Phycomyces,* and *Rps. capsulata* (see Section IV).

The ultimate aim in using molecular biology techniques is the isolation and characterization of the carotenoid genes. Since antibodies are not yet available, gene isolation must use transformation techniques with suitable vectors. With the notable exception of studies by Marrs and co-workers with *Rps. capsulata* (*220*), such experiments have yet to be reported. However, the increasing availability of transformation systems and vectors for filamentous fungi such as *Neurospora, Aspergillus,* and *Penicillium* (reviewed by *19*) now makes it feasible to isolate carotenoid genes from fungi. Once isolated, the regulatory sequences of the gene can be identified by *in vitro* mutagenesis, while the genes themselves can be used as probes for monitoring changes in mRNA levels following treatment of the cells with light, trisporic acids, CPTA, etc. (see Section V). This approach will also provide absolute proof of transcriptional control of carotenoid biosynthesis by such effectors.

To date, the main emphasis of research has been on the structures and distribution of carotenoids, their chemical and biosynthetic pathways, and the regulatory roles of environmental and developmental factors. However, with the use of modern biochemical and molecular biological techniques, it should now be possible to isolate and characterize carotenogenic enzymes and regulatory proteins and to elucidate the molecular mechanisms involved in regulation.

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