Pure & Appl. Chem., Vol. 51, pp. 609-622.
Pergamon Press Ltd. 1979. Printed in Great Britain.

0033-4545/79/0301-0609 $02.00/0

ENZYMATIC SYNTHESIS OF CAROTENES

John W. Porter and Sandra L. Spurgeon

William S. Middleton Memorial Veterans Administration Hospital and the Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin 53706 USA

ABSTRACT

The pathway of carotene biosynthesis from isopentenyl pyrophosphate in tomato fruit plastids has been established, except for minor details, through the conversion of radioactive substrates and intermediates to acyclic and cyclic carotenes in cell-free systems.

After the pathway of carotene biosynthesis was established attention was turned to the isolation and purification of enzymes of carotene biosynthesis. An enzyme that converts isopentenyl pyrophosphate to phytoene has been isolated from an acetone powder of tomato fruit plastids and partially purified. This enzyme behaves as a complex on Biogel filtration. It dissociates, though, into two or more components on DEAE-cellulose chromatography. One of these synthesizes geranylgeranyl and prephytoene pyrophosphates from isopentenyl pyrophosphate. This enzyme has been purified to homogeneity and some of its characteristics and the reactions it catalyzes have been determined. Since this protein yields only one band on SDS gel electrophoresis, it is a polyfunctional peptide.

Studies on the enzymes converting phytoene to lycopene have been minimal. It is known, from tracer studies, that one *cis-trans* isomerization and four dehydrogenations occur in these reactions. Whether five or fewer distinct enzymes occur is not known. Neither is it known whether the enzymes for this series of reactions are separable or whether they exist in a complex. Cofactor studies on the system converting phytoene to lycopene indicate that $NADP^+$, FAD and Mn^{++} are required for these reactions.

Studies on the conversion of lycopene to cyclic carotenes indicate that two separate enzymes are involved in the formation of α-ionone and β-ionone rings. This conclusion is based upon the finding of some tomato strains which have an enzyme system that forms only β-ionone ring-containing carotenes, whereas others have an enzyme for the formation of both of these compounds. It is not certain whether the introduction of a β-ionone ring into γ- and δ-carotenes to yield β- and α-carotenes requires two separate enzymes or whether this reaction is effected by a single enzyme. Cofactor studies have shown that FAD is necessary for the conversion of lycopene to cyclic carotenes.

INTRODUCTION

Studies on the biosynthesis of carotenes have passed through several phases over the past 20 to 30 years. These phases have been reported by Professor Goodwin in his Introductory Lecture. Hence, we will not repeat them, except to indicate their relationship to studies on the enzymatic synthesis of carotenes. Early studies established the structures and the stereochemistry of many of the carotenes that we will consider in this report. At the same time, or later, studies on the biosynthesis of carotenes were initiated. Early studies utilized the techniques common in plant physiology and genetics. Other studies made extensive use of inhibitors such as diphenylamine. Most of these studies were carried out with tomato fruits or with various microorganisms. More recent studies have utilized radioactive precursors of the carotenes; first *in vivo* and then in cell-free extracts. Through these studies the general features of the pathway of carotene biosynthesis have been established.

In the report that we are presenting we will attempt to move our understanding of carotene biosynthesis one step further. We will concern ourselves almost exclusively with the enzymology of carotene biosynthesis. Through such investigations one can identify the enzyme, or enzyme complex, that effects a particular reaction and the properties of that enzyme. In addition, when an enzyme is purified various studies on the mechanism of a reaction can be carried out which are not possible with cruder systems. It is also possible to determine unequivocally whether certain compounds are intermediates in a reaction: for example, lycopersene in the synthesis of phytoene. Finally, the relationship between genes for carotene biosynthesis and specific enzyme proteins can be established.

Unfortunately, studies on the enzymatic synthesis of carotenes are in their early stages. Therefore, many of the answers to questions on carotene biosynthesis that are attainable by this method are still not available. There have been, though, enough surprises in early studies to indicate that this method will produce many interesting results in the future.

The studies on the enzymatic synthesis of carotenes that are to be presented in this paper will be divided into three parts. In the first of these we will discuss the enzymatic conversion of isopentenyl pyrophosphate to phytoene, and in the second section the conversion of phytoene to lycopene. The last section will be concerned with the conversion of lycopene to cyclic carotenes. Our discussions will be based first on the reactions that are effected by enzymes obtained from tomato fruits, and then on the reactions effected by cell-free extracts or enzymes from other plants or microorganisms. Emphasis on the latter studies will be placed upon deviations, if any, from the tomato system.

CONVERSION OF ISOPENTENYL PYROPHOSPHATE TO PHYTOENE

A major breakthrough which has led to studies on the enzymatic synthesis of carotenes in tomato fruits was achieved over ten years ago by Jungalwala and Porter (Ref. 1). These workers demonstrated that an acetone powder could be made from tomato fruit plastids with the retention of enzyme activity for carotene biosynthesis. The enzymes could then be extracted as soluble proteins with a buffer, as evidenced by the fact that they remained in the 100,000 x g supernatant on ultracentrifugation. Protein in this supernatant solution could then be subjected to purification procedures. It was found that an enzyme system that converted isopentenyl pyrophosphate to phytoene could be precipitated with ammonium sulfate (20 to 60%) and then subjected to Bio-Gel A filtration (Ref. 1 & 2). An activity of approximately 0.2 nmole of phytoene formed, per mg of protein per hour, was observed with this preparation, Table 1.

TABLE 1 Partial purification of phytoene synthetase complex

Purification step	Total protein	Specific activity[a]	Total activity	Purification
	mg	nmole/mg/h	nmol incorporated/h	-fold
Plastids	600	0.004	2.4	0
Acetone powder extract	235	0.012	2.8	3
20-60% $(NH_4)_2SO_4$	104	0.11	11.4	28
Bio-Gel A-1.5m	38	1.42	54.0	354

[a] Nanomoles of isopentenyl pyrophosphate incorporated into phytoene per milligram of protein per hour.

Surprisingly, this enzyme system behaved as a complex on gel filtration (Ref. 2), Fig. 1. Its molecular weight, as determined by gel filtration, is 200,000, Fig. 2. This enzyme system requires Mn^{++} ions, Fig. 3, and its activity is increased 6- to 7-fold in the presence of ATP, Fig. 4. The function of ATP in stimulating the formation of phytoene is not clear. Since ATP is not involved in the reactions by which isopentenyl pyrophosphate is converted to phytoene, another function for this compound needs to be postulated. Possibly ATP may be important in maintaining the integrity of the complex, or it may act as an allosteric regulator of the activity of this enzyme.

The reactions effected by the phytoene synthetase complex are presented in Fig. 5. This enzyme system appears to be a remarkable complex, inasmuch as it carries out (1) the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate, (2) the condensation reactions that yield successively geranyl, farnesyl and geranylgeranyl pyrophosphates, (3) the condensation reaction that yields prephytoene (also called prelycopersene) pyrophosphate and (4) the reaction that converts the latter compound to *cis*-phytoene.

Phytoene synthetase is an unstable complex. It may be stabilized, though, in the presence of 30% glycerol, 2 m*M* dithiothreitol and -20°C for at least a month. It is unstable to standard ionic methods of purification of proteins, inasmuch as all activity for phytoene synthesis is lost in attempts to purify this enzyme by DEAE-cellulose chromatography. Hence, the problem of purification of phytoene synthetase to homogeneity has not been solved, and will not be until a new method of purification such as affinity chromatography is developed.

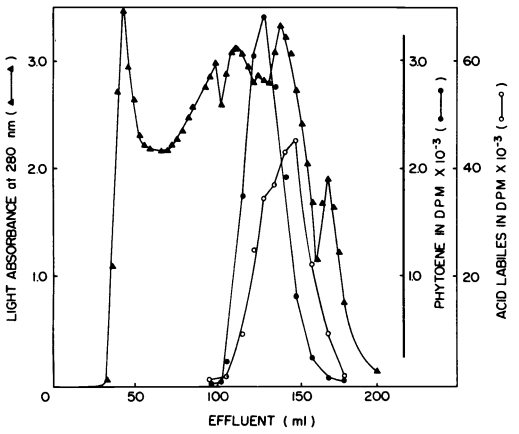

Fig. 1. Elution of phytoene synthetase complex from a Bio-Gel A-1.5m (200-400 mesh) column (2.5 x 36 cm). Absorbance at 280 nm (▲—▲), activity for phytoene formation (●—●) and acid-labile formation (o—o), (Ref. 2).

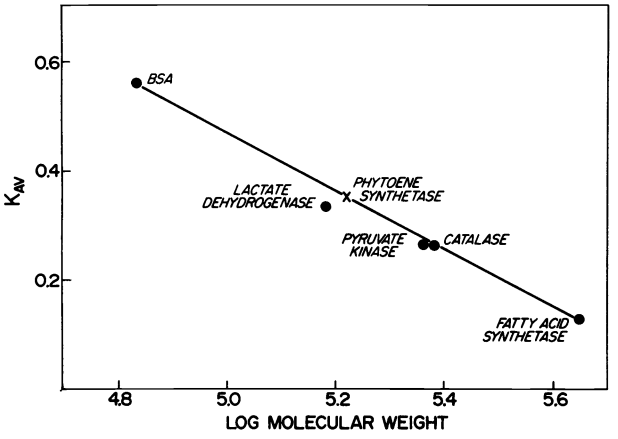

Fig. 2. Molecular weight of phytoene synthetase complex as estimated by gel filtration on a Bio-Gel A-0.5m (200-400 mesh) column (1.2 x 30 cm). BSA, bovine serum albumin (Ref. 2).

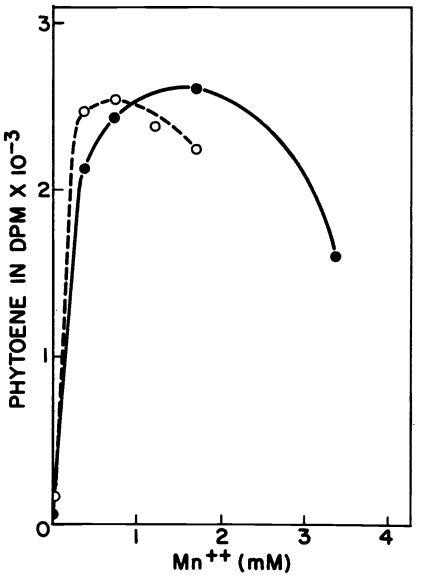

Fig. 3. The effect of manganese ion concentration on phytoene synthesis from isopentenyl pyrophosphate. No magnesium ions and varying amounts of manganese ions added to the incubation mixture, o- -o; the concentration of magnesium ions kept constant at 12.5 m*M* and varying amounts of manganese ions added to the incubation mixture, ●——● (Ref. 2).

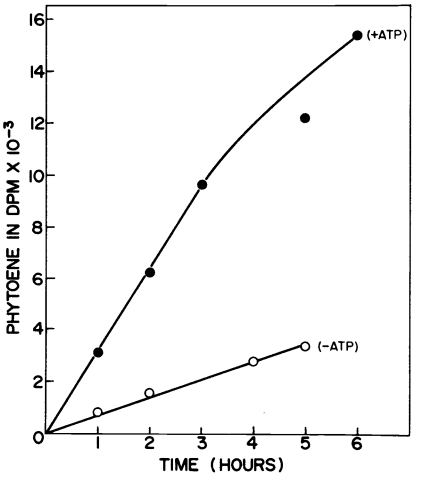

Fig. 4. The synthesis of phytoene as a function of time from isopentenyl pyrophosphate by the phytoene synthetase complex. Incubations were carried out in the presence and absence of ATP (Ref. 2)

When phytoene synthetase is subjected to DEAE-cellulose chromatography all activity for phytoene synthesis is lost, but activity for the formation of acid-labile compounds from isopentenyl pyrophosphate remains (Ref 3), Fig. 6. Presumably, this activity is dissociated from phytoene synthetase, inasmuch as the molecular weight of the acid-labile synthesizing enzyme is 40,000, Fig. 7, as compared with 200,000 for phytoene synthetase. The acid-labile synthesizing system has been purified to homogeneity, or near homogeneity (Ref. 4). A purification of approximately 60-fold was achieved, Table 2. Evidence for the homogeneity, or near homogeneity, of this enzyme system was obtained through disc gel and SDS gel electrophoresis, Fig. 8. Only one band of protein was observed. A single species of protein was also observed on isoelectric focusing (Ref. 4).

The products formed by the acid-labile synthesizing enzyme have been identified as geranylgeranyl and prephytoene pyrophosphates by several thin-layer chromatographic systems. One separation of these compounds is shown in Fig. 9. The geranylgeraniol moiety of geranylgeranyl

pyrophosphate has also been identified by gas-liquid chromatography after acid cleavage of the pyrophosphate compound.

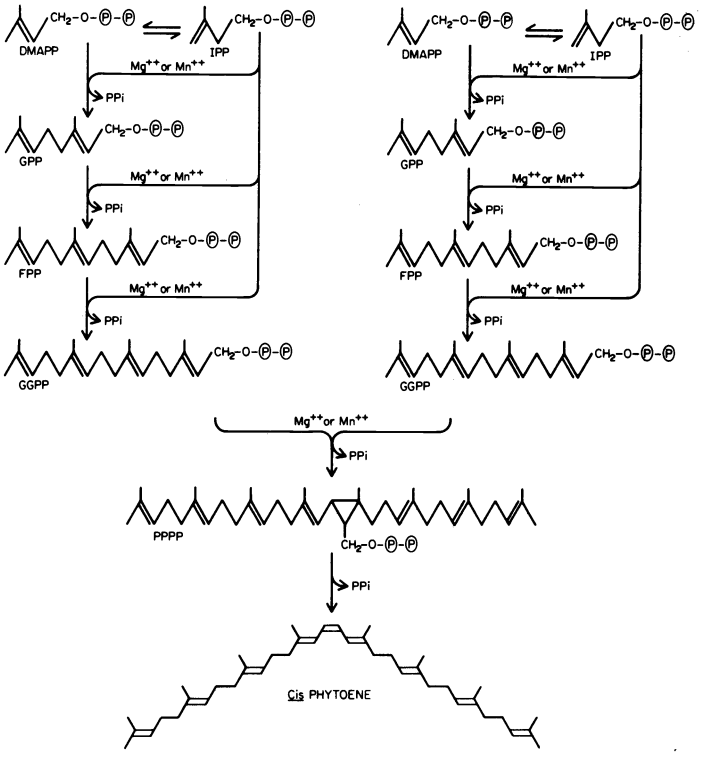

Fig. 5. The enzymatic reactions effected by the enzyme complex that converts isopentenyl pyrophosphate to phytoene.

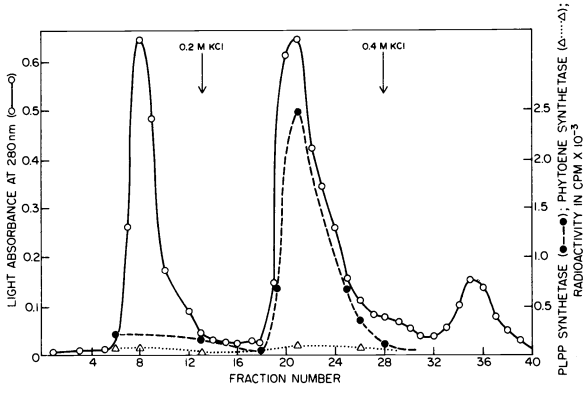

Fig. 6. Chromatography of the phytoene synthetase complex on a DEAE-cellulose column (9.5 X 1.3 cm). Light absorption at 280 nm (o——o) and enzyme activity for the formation of phytoene (Δ...Δ) and acid labiles (●--●), (Ref. 2).

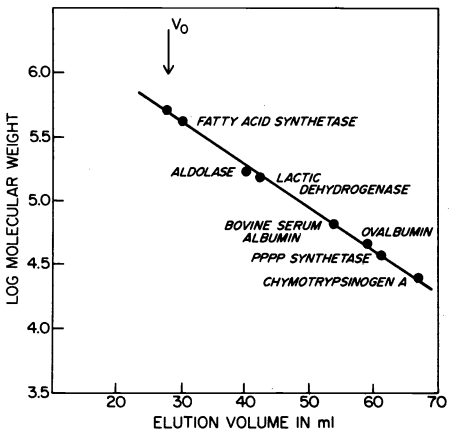

Fig. 7. Determination of molecular weight of prephytoene pyrophosphate synthetase by gel filtration on a Sephadex G-200 column,(Ref. 3).

Since the acid-labile synthesizing enzyme forms prephytoene pyrophosphate, it has been designated as prephytoene pyrophosphate synthetase. It is a remarkable enzyme in that it appears to have three separate functional groups, or active sites, in one peptide of 40,000 molecular weight. This enzyme system catalyzes all of the reactions shown in Fig. 5, except for the conversion of prephytoene pyrophosphate to phytoene. Studies relating genes for carotene biosynthesis to enzymes effecting the synthesis of these compounds have shown that none of the genetic selections of tomatoes studied thus far are lacking in phytoene synthetase.

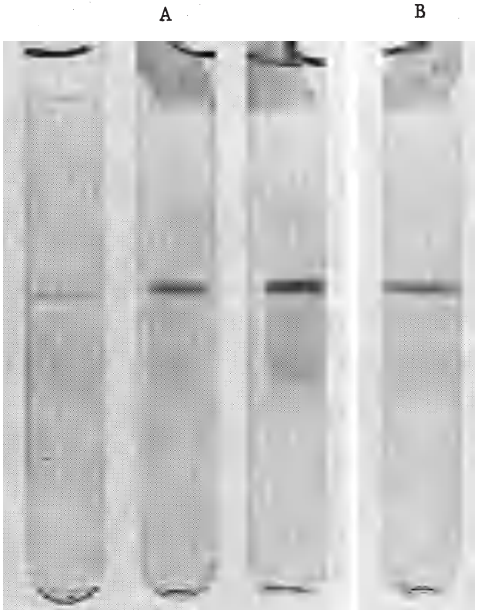

Fig. 8. Electrophoresis of prephytoene pyrophosphate synthetase on native and SDS gels. A, native gel, from left to right 50, 110 and 150 μg of protein; B, SDS gel, 110 μg of protein, (Ref.4).

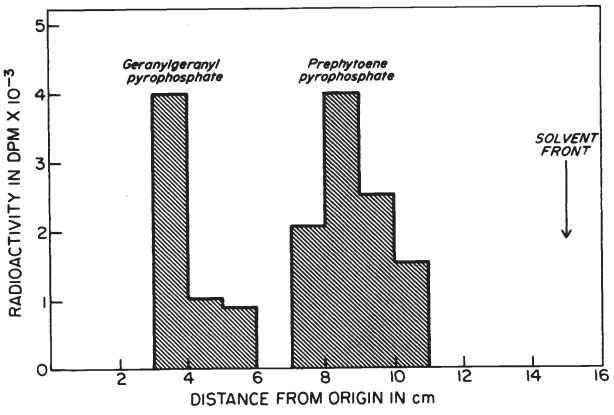

Fig. 9. Thin-layer chromatography of geranylgeranyl and prephytoene pyrophosphates on Silica gel H in a solvent system of $CHCl_3$::CH_3OH:H_2O (50:50:10). Geranylgeranyl pyrophosphate and prephytoene pyrophosphate had R_f values of 0.23 and 0.57, respectively (Ref. 4).

TABLE 2 Purification of prephytoene pyrophosphate synthetase from tomato fruit plastids

Purification step	Total protein	Specific activity	Total units of activity	Purification	Yield
	mg	dpm/hr/mg protein[a]	dpm/hr	-fold	%
Plastids, homogenized	662.4	12,112	8,023,320	-	100
Ammonium sulfate, 20 to 60%	124.3	31,311	3,891,946	2.6	48.5
Bio-Bel A 1.5m chromatography	47.8	100,796	4,822,064	8.3	60.1
DEAE-cellulose chromatography	8.16	310,142	2,530,756	25.6	31.5
CM-cellulose chromatography	1.96	715,562	1,402,502	59.1	17.5

[a] One nanomole of isopentenyl pyrophosphate = 6,000 dpm.

CONVERSION OF ISOPENTENYL PYROPHOSPHATE TO PHYTOENE BY OTHER SYSTEMS

Phytoene synthesis has been studied in other systems, but not as extensively as in tomato preparations. Cell-free extracts have been prepared from pea fruits (Ref. 5), Mycobacteria (Ref. 6), Phycomyces (Ref. 7,8 & 9), Flavobacteria (Ref. 10), Neurospora (Ref. 11 & 12) and Halobacteria (Ref. 13). In Flavobacteria, Phycomyces, Halobacteria and pea fruits the phytoene-synthesizing system is found in a fraction that does not sediment at 100,000 x g. It is evident, therefore, that the enzyme system for the formation of phytoene in these species is soluble and it may also be a complex. However, this point has not yet been investigated. In Neurospora it is reported (Ref. 12) that there is a soluble system for the formation of geranylgeranyl pyrophosphate and a particulate one for the conversion of this compound to phytoene. This result suggests that the enzyme systems for the formation of phytoene exist in different forms in different organisms. In the tomato the genes coding for the enzymes converting isopentenyl pyrophosphate to prephytoene pyrophosphate appear to be fused, whereas in some other organisms they are not.

It has been found, too that the product of phytoene synthetase action is not the same in all organisms. In the tomato and in many other organisms, the product is *cis*-phytoene. In *Neurospora crassa, Phycomyces blakesleeanus* and *Rhodospirillum rubrum,* mainly *cis*-phytoene (Ref. 14) is found. In contrast, Mycobacteria (Ref. 9), Halobacteria (Ref. 13) and Flavobacteria (Ref. 15 & 16) synthesize *trans*-phytoene.

The synthesis of *cis*- and *trans*-phytoene by different organisms has led Gregonis and Rilling (Ref. 9) to propose a mechanism for the formation of these compounds from prephytoene pyrophosphate, Fig. 10. In this mechanism a pro-R or pro-S hydrogen is removed to form, respectively, *trans*-phytoene or *cis*-phytoene. From the studies that have been made on phytoene synthetase it appears that these extractions of hydrogens from prephytoene pyrophosphate are effected by the enzyme in the absence of a cofactor or a prosthetic group, since no evidence for the requirement of either has been obtained.

It appears fairly obvious that the enzymes that form *cis*- and *trans*-phytoenes must differ significantly in the spatial geometry of their active sites, inasmuch as the hydrogen removed by each has a different position relative to the remainder of the prephytoene pyrophosphate structure. It will be of interest, therefore, to isolate, purify and compare the properties of phytoene synthetases synthesizing *cis*-and *trans*-phytoenes.

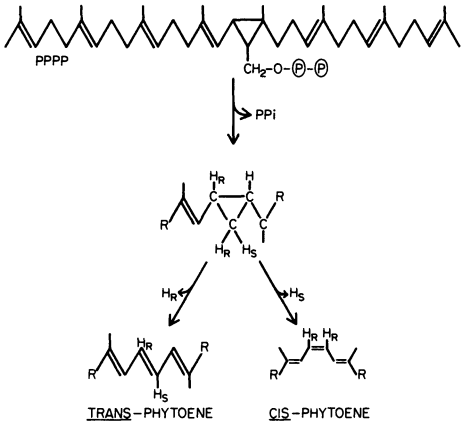

Fig. 10. Proposed mechanism for the enzymatic synthesis of *cis*- and *trans*-phytoene from prephytoene pyrophosphate (Ref. 9).

CONVERSION OF PHYTOENE TO LYCOPENE

An enzyme system capable of converting phytoene to lycopene was isolated from an acetone powder of tomato fruit plastids and then partially purified by ammonium sulfate precipitation by Kushwaha *et al.* (Ref. 17). When the enzyme system was obtained from red tomato fruit plastids radioactive phytoene was converted to *cis*-phytofluene, *trans*-phytofluene, ζ-carotene, neurosporene, lycopene and γ-carotene, Table 3. Similar conversions, but with the added synthesis of radioactive carotenes peculiar to that genetic selection were obtained with enzyme from Hi β, Hi ὺ and Golden Jubilee (tangerine variety) tomato fruit plastids. Later studies by Qureshi *et al.* (Ref. 18) demonstrated the conversion of *cis*-[^{14}C]phytofluene, *trans*-[^{14}C]-phytofluene and *trans*-ζ-[^{14}C]carotenes to neurosporene and lycopene, Table 4. These conversions demonstrated the sequence of reactions by which phytoene is converted to lycopene in red tomato fruits, Fig. 11. In this set of conversions the pathway is *cis*-phytoene → *cis*-phytofluene → *trans*-phytofluene → *trans*-ζ-carotene → neurosporene → lycopene.

TABLE 3 Enzymatic conversion of [^{14}C]phytoene to more unsaturated carotenes by a soluble enzyme system obtained from red tomato fruit plastids

Carotene	Total radioactivity
	dpm
Cis-Phytofluene	9943
Trans-Phytofluene	990
ζ-Carotene	2866
γ-Carotene	1900
Neurosporene	2536
Neolycopene	2788
Lycopene	3893

Incubation was carried out for 5 hours in the dark at 25°C under nitrogen. The incubation system contained [^{14}C]phytoene, 2.39 nmoles (250,000 dpm) as substrate, enzyme and other cofactors as noted in Ref. 17.

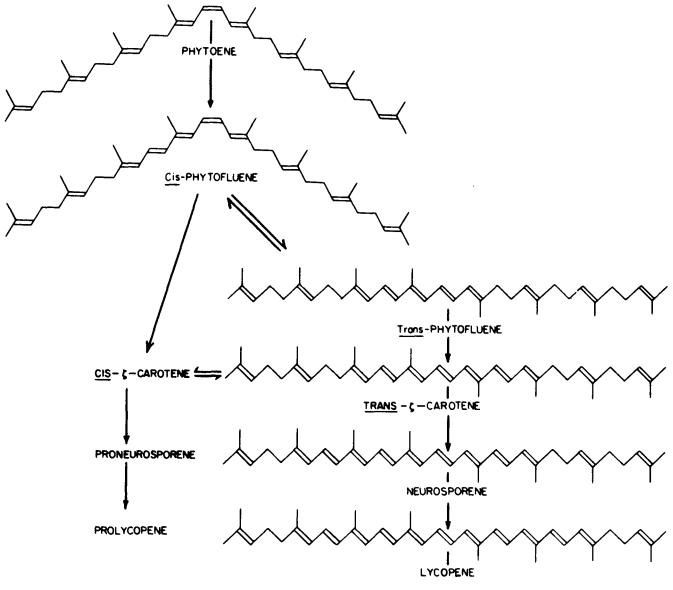

Fig. 11. The pathways of lycopene and prolycopene formation from *cis*-phytoene by cell-free extracts from tomato fruit plastids

Additional studies have been carried out using cell-free extracts of tangerine tomato fruit plastids (Ref. 19). In this genetic strain, the predominant pigments which occur are the poly-*cis*-compounds, proneurosporene and prolycopene. A soluble enzyme system of plastids from these tomato fruits incorporates radioactivity from *cis*-[^{14}C]phytofluene, *trans*-[^{14}C]phytofluene, and *trans*-ζ-[^{14}C]carotene into both proneurosporene and prolycopene (Ref. 19). It was concluded from this study that the branch point in the conversion of the poly-*cis*-compounds is *trans*-ζ-carotene, with the intermediate formation of *cis*-ζ-carotene. However, the possibility of the direct conversion of *cis*-phytofluene to *cis*-ζ-carotene could not be eliminated.

TABLE 4 Enzymatic conversion of *cis*-[^{14}C]phytofluene, *trans*-[^{14}C]-phytofluene, and *trans*-ζ-carotene to more unsaturated acyclic carotenes

Carotene	*Cis*-[^{14}C]-Phytofluene	*Trans*-[^{14}C]-Phytofluene	*Trans*-ζ-[^{14}C]-Carotene
	dpm	dpm	dpm
Trans-Phytofluene	8780	-	-
Trans-ζ-Carotene	735	6220	-
Neurosporene	999	5640	6703
Lycopene	1147	14380	21810

Enzyme extracts were prepared from an acetone powder. Incubations with enzyme, substrate and cofactors were then carried out for 8 hours in the dark at 25°C under nitrogen. Substrate concentrations were as follows: *cis*-[^{14}C]phytofluene, 0.284 nmole (27,120 dpm) and *cis*-[^{12}C]phytofluene, 20 nmoles; *trans*-[^{14}C]phytofluene, 1.39 nmoles (132,500 dpm) and *trans*-[^{12}C]phytofluene, 20 nmoles; *trans*-ζ-[^{14}C]carotene, 1.35 nmoles (127,920 dpm) and *trans*-ζ-[^{12}C]-carotene, 20 nmoles (Ref. 18).

In a companion study (Ref. 20) *cis*-ζ-carotene was isolated from tangerine tomato fruits and purified. This compound was characterized by its light absorption spectrum before and after iodine catalysis, Fig. 12. Radioactive *cis*-ζ-carotene was then prepared and its conversion to proneurosporene, prolycopene, *trans*-ζ-carotene, neurosporene and lycopene was demonstrated with an extract of lyophilized tomato fruit plastids, Table 5. This compound is also converted in small amounts to γ- and β-carotenes by the same enzyme system. These studies show that *cis*-ζ-carotene is an intermediate in the formation of poly-*cis*-carotenes. The conversion of this compound to *trans*-ζ-carotene has also been demonstrated. Whether *cis*-ζ-carotene can be formed directly from *cis*-phytofluene has not been determined. However, this conversion appears to be a reasonable possibility, Fig. 11.

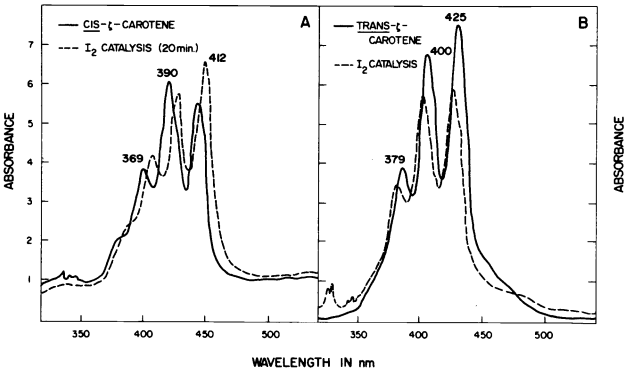

Fig. 12. (A) Light absorption curves of *cis*-ζ-carotene (——) and the mixture of stereoisomers formed by catalysis with iodine (---). (B) Light absorption curves of *trans*-ζ-carotene and the mixture of stereoisomers formed by catalysis with iodine (---), Ref. 20.

At present the number of enzymes involved in the biosynthesis of carotenes and whether they are organized in a complex is not known. Some observations can be made, though, on this point on the basis of the above and other investigations on the pathway of carotene biosynthesis in tomatoes. In higher plants the predominant isomer of phytoene is the *cis*-compound. However, ζ-carotene, neurosporene, lycopene and the cyclic carotenes are present as *trans*-compounds. This means that in an early stage in the pathway of phytoene desaturation there is an isomerization reaction at the central *cis* double bond to form the *trans*-isomer. Only traces of *trans*-phytoene have been found in tomato fruits and it is not believed that this compound is an intermediate in the synthesis of the more unsaturated carotenes. *Cis*-phytoene is instead converted to *cis*-phytofluene, which is then isomerized to *trans*-phytofluene in the red tomato. Presumably, one isomerase exists in tomato fruits which effectively isomerizes *cis*-phytofluene to *trans*-phytofluene, Fig. 11.

In the tangerine variety of tomatoes poly-*cis*-carotenes replace the *trans*-carotenes present in red tomatoes. The tangerine variety of tomato also differs from the normal red variety in carotene biosynthesis by a single gene. Unfortunately, the exact *cis-trans* structures of the poly-*cis*-compounds, proneurosporene and prolycopene, are not presently known. This is obviously an important question to an understanding of the identity of the enzyme change that has given rise to this genetic strain and one that needs to be answered relatively soon. Since there is a single gene difference in the genes for carotene biosynthesis in red and tangerine tomatoes, it appears probable that the defective gene in the tangerine variety codes for an isomerase that very inefficiently isomerizes *cis*-phytofluene to *trans*-phytofluene. Under these circumstances desaturation would continue at the *cis*-phytofluene stage with the formation of poly-*cis*-carotenes. This would suggest that the specificity of the dehydrogenases is broad enough to desaturate *cis*- or *trans*-carotenes when they are present in plastids. This point could be verified by determining whether *cis*-ζ-carotene is converted to more unsaturated carotenes by an extract of plastids of red tomato fruits. (See Addendum.)

TABLE 5 Enzymatic conversion of *cis*-ζ-[^{14}C]carotene to more unsaturated carotenes by a soluble enzyme system obtained from tangerine (t^t and t^v) tomato fruit plastids

Carotenes	Lyophilized powder			
	t^t		t^v	
	dpm	%	dpm	%
Trans-ζ-Carotene	7892	24.66	8830	25.71
Proneurosporene	1101	3.15	1190	3.71
Prolycopene	6370	19.90	7050	22.03
Neurosporene	780	2.43	867	2.70
Lycopene	1323	4.13	1423	4.44
γ-Carotene	447	1.39	511	1.59
β-Carotene	602	1.88	635	1.98

Extracts were prepared from a lyophilized powder of tomato fruit plastids. Incubation was carried out under nitrogen in the dark for 8 hours at 25°C with 0.330 nmole *cis*-ζ-[^{14}C]carotene (32,000 dpm) and 20 nmoles nonradioactive *cis*-ζ-carotene as substrate (Ref. 20).

In the conversion of phytoene to lycopene four dehydrogenation steps are required, Fig. 11. These dehydrogenations introduce *trans*-double bonds. In the first of these dehydrogenations *cis*-phytoene is converted to *cis*-phytofluene. This compound is then isomerized to *trans*-phytofluene. Three successive dehydrogenations then occur with the formation of ζ-carotene, neurosporene and lycopene, respectively. Whether these dehydrogenations are effected by one or more dehydrogenases is not known. However, it seems probable that more than one dehydrogenase exists. It is also not known whether the dehydrogenases exist as a complex, as individual enzymes or as a single polyfunctional protein. It seems reasonably clear, though, that the dehydrogenases are separate from the isomerase in that different genes code for these enzymes. It is quite possible, though, that the dehydrogenases and the isomerase exist in an enzyme complex. Obviously, the enzymes for conversion of phytoene to lycopene need to be isolated and purified to establish which of the above possibilities is the correct one.

Some studies on the cofactor requirements for carotene biosynthesis have been carried out which suggest that more than one dehydrogenase is involved in the conversion of phytoene to lycopene. In this process it is evident that the removal of hydrogen is effected by a dehydrogenase and not by a mixed function oxidase, inasmuch as oxygen is not required in the conversion of phytoene to lycopene. In the early studies of Kushwaha *et al.* (Ref. 17) it was found that the conversion of phytoene to phytofluene was dependent on $NADP^+$, whereas the reactions from phytofluene to lycopene appeared to require FAD and Mn^{++}, Table 6. These results suggest the possibility that the dehydrogenase for the conversion of phytoene to phytofluene might be different from the ones converting phytofluene to lycopene. In the studies by Qureshi *et al.* (Ref. 18) on the conversion of *cis*-[^{14}C]phytofluene to other carotenes it was found that the omission of FAD, $NADP^+$ or Mn^{++} resulted in diminished incorporation of radioactivity into more unsaturated carotenes, Table 7. These results are in agreement with those of Kushwaha *et al.* (Ref. 17) and they suggest the possibility of two separate types of dehydrogenation, or possibly the involvement of $NADP^+$ in the *cis-trans* isomerization reaction.

CONVERSION OF PHYTOENE TO LYCOPENE IN OTHER SYSTEMS

The enzymatic conversion of [^{14}C]phytoene to acyclic carotenes has also been demonstrated with an ammonium sulfate precipitated spinach enzyme system (Ref. 21). In this system the

incorporation of radioactivity into phytofluene and lycopene required both light and a boiled extract. Neither of these is required by the tomato enzyme system. FAD was also required for the conversion of isopentenyl pyrophosphate to lycopene and $NADP^+$ was required for the formation of phytofluene. The enzymes for the conversion of phytoene to lycopene are located in the plastids, as is found with the tomato system, but the enzymes for the conversion of isopentenyl pyrophosphate to phytoene appear to be located outside the plastids.

TABLE 6 Effect of various factors on enzymatic conversion of [^{14}C]phytoene to more unsaturated carotenes

System	Phytofluene		ζ-Carotene	Lycopene
	cis-	*trans*-		
	dpm	dpm	dpm	dpm
Complete	2612	144	1100	8807
− FAD	2321	198	0	0
− $NADP^+$	746	58	0	0
− $MnCl_2$	2801	-	14	22
− $MgCl_2$	3046	-	0	2866
− DTT	1966	-	334	1561

Incubations were carried out for 5 hours in the dark at 25°C under nitrogen, with 0.354 nmoles (37,050 dpm) of [^{14}C]phytoene (Ref. 17).

TABLE 7 Effect of various factors on the enzymatic conversion of *cis*-[^{14}C]-phytofluene to more unsaturated carotenes by a soluble enzyme system prepared from plastids of red tomato fruits

System	*Trans*-Phytofluene	*Trans*-ζ-Carotene	Neurosporene	Lycopene	α-Carotene	β-Carotene
	dpm	dpm	dpm	dpm	dpm	dpm
Complete	6901	1118	501	651	374	309
− FAD	4378	452	201	352	110	95
− $NADP^+$	5872	617	312	492	332	297
− $MnCl_2$	7298	1415	216	157	112	97
− $MgCl_2$	4882	904	207	710	370	289
− DTT	7547	1263	702	812	402	480
− Tween 80	3772	791	274	414	81	98
− FAD, $NADP^+$, $MnCl_2$ and $MgCl_2$	202	56	42	34	35	30

Incubations were carried out at 25°C under nitrogen in the dark for 4 hours with *cis*-[^{14}C]-phytofluene, 0.213 nmole (20,340 dpm) and *cis*-[^{12}C]phytofluene, 25 nmoles, as substrate.

Crude extracts from mutants of *Phycomyces blakesleeanus* have also been shown to incorporate radioactivity from [2-^{14}C]mevalonate into lycopene as well as other carotenes (Ref. 22). It has been reported, too, that these extracts can convert [^{14}C]phytoene to lycopene (Ref. 23) but the cofactor requirements for these reactions have not been determined. Whether or not these are soluble enzymes as are found in higher plants or membrane-bound enzymes is unknown since the extracts used were obtained by low-speed centrifugation. The enzyme system from *Phycomyces* is of particular interest since there is evidence from quantitative genetic complementation studies for a multienzyme complex carrying out the desaturation of phytoene to lycopene in this organism (Ref. 24). In the *Phycomyces* pathway, as in higher plants, the more unsaturated acyclic and cyclic carotenes are *trans* whereas phytoene is predominantly *cis*. However, in *Phycomyces*, *cis*-phytoene appears to be converted to the *trans*-isomer before dehydrogenation (Ref. 23). The formation of lycopene from *trans*-phytoene would then involve four successive dehydrogenation reactions. Because of the difference in the initial step in the conversion of *cis*-phytoene to lycopene in *Phycomyces* and in higher plants, a comparative study of the organization of the enzymes in the two systems would be of interest.

A more extensive study on carotene biosynthesis has been carried out with a cell-free preparation from *Halobacterium cutirubrum* (Ref. 13). The enzymes involved in the conversion of

phytoene to other carotenes in this system are soluble, since they are located in the 270,000 x g supernatant fraction. With this enzyme system it was shown that radioactivity was readily incorporated into carotenes from *trans*-phytoene and *trans*-phytofluene, but there was little or no conversion when *cis*-phytoene or *cis*-phytofluene were used as substrate, Table 8. This is consistent with what is known of the pathway of carotene biosynthesis in this organism. Unlike the higher plant and *Phycomyces* systems discussed above, in *Halobacterium* the isomer of phytoene is the *trans*-compound (Ref. 25) and neither *cis*-phytoene nor *cis*-phytofluene is an intermediate in the formation of more unsaturated carotenes (Ref. 13). In agreement with other systems which have been examined, it was found that the formation of more unsaturated carotenes required the presence of $NADP^+$ and FAD (Ref. 13). It was also found that a high concentration of salt (4 *M* NaCl) was required for stability of the system from *Halobacterium*, an organism that grows in an extremely halophytic environment.

TABLE 8 Enzymatic conversion of *cis*-[^{14}C]phytoene, *trans*-[^{14}C]phytoene, *cis*-[^{14}C]phytofluene and *trans*-[^{14}C]phytofluene to more unsaturated carotenes by a soluble system from *H. cutirubrum*

Labeled products	Total radioactivity in carotenes			
	Precursor substrate			
	Trans-Phytoene	*Cis*-Phytoene	*Trans*-Phytofluene	*Cis*-Phytofluene
	dpm	dpm	dpm	dpm
Cis-Phytoene	1193	-	-	-
Trans-Phytoene	-	2500	-	-
Cis-Phytofluene	150	1800	1100	-
Trans-Phytofluene	6538	300	-	1500
ζ-Carotene	5565	150	10150	100
Neurosporene	4688	50	6010	-
Lycopene	3250	35	7543	-
γ-Carotene	2619	40	5800	-
β-Carotene	4225	70	8125	-

The complete incubation system contained either *cis*-[^{14}C]phytoene, 6.11 nmole (1.11 x 10^5 dpm), or *trans*-[^{14}C]phytoene, 5.3 nmol (1.0 x 10^5 dpm) or *cis*-[^{14}C]phytofluene, 4.59 nmol (8.5 x 10^4 dpm), or *trans*-[^{14}C]phytofluene, 5.61 nmol (1.05 x 10^5 dpm) as substrate. Incubations were carried out for 5 hours at 37°C under nitrogen in the dark (Ref. 13).

In 1975 Brown *et al.* reported the isolation of a cell-free preparation from a *Flavobacterium sp.* (Ref. 10), which was capable of synthesizing carotenes *in vitro*. A 100,000 x g supernatant efficiently incorporated radioactivity from 2-[^{14}C]mevalonate into phytoene. With the supernatant from a low-speed centrifugation (3000 - 5000 x g) 2-[^{14}C]mevalonate was also incorporated into phytofluene, ζ-carotene and its unsymmetrical isomer, neurosporene and lycopene. With the same preparation, the conversion of [^{14}C]phytoene (15-*cis*-isomer) into other carotenoids was also demonstrated. These results suggest that the enzymes involved in the conversion of phytoene to more unsaturated carotenes in this system are particulate rather than soluble. This appears to be the case in *Neurospora* as well (Ref. 12).

It is clear from the above discussion that there are some significant variations in the enzyme systems effecting carotene biosynthesis in various organisms. The product formed in most instances is a *trans*-carotene, such as lycopene. There is a difference, though, as to whether *cis*- or *trans*-phytoene is formed. There is also a difference as to the step at which the *cis*-compound is converted to the *trans*-carotene (phytoene or phytofluene). There may also be a difference in the state of the enzyme; some are soluble, whereas others are particulate. Finally, it appears that there may be some differences in cofactors, particularly for the enzyme system from chloroplasts. These differences should prove to be interesting when they are concretely established through the isolation and purification of individual enzymes of carotene biosynthesis.

FORMATION OF CYCLIC CAROTENES

Cell-free extracts from tomato plastids that convert phytoene to lycopene also convert phytoene and lycopene to one or more of the four cyclic carotenes found in tomatoes. In red tom-

atoes small amounts of α-, β- and γ-carotenes are found, whereas large amounts of δ-carotene and β-carotene are found, respectively, in Hi δ and Hi β tomato selections. The reactions involved in the formation of these compounds from lycopene are shown in Fig. 13.

The incorporation of radioactivity from lycopene-15,15'-^{3}H into cyclic carotenes by soluble extracts of tomato fruit plastids was first demonstrated by Kushwaha *et al.* (Ref. 26). More

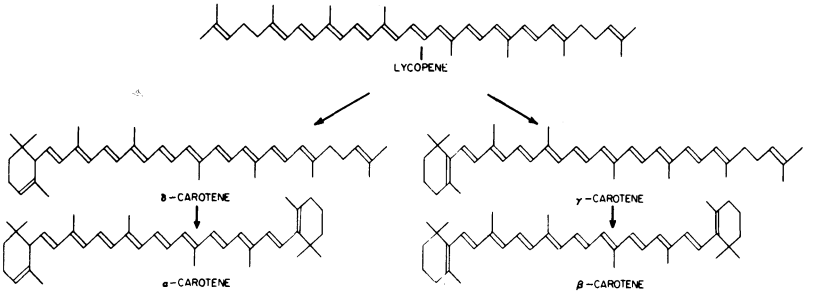

Fig. 13. The reactions that occur in the formation of cyclic carotenes from lycopene by a cell-free tomato enzyme system.

recently, Papastephanou *et al.* (Ref. 27) determined the biosynthesis of acyclic and cyclic carotenes from phytoene and lycopene by soluble extracts from plastids of fruit of several genetic selections of tomatoes. A summary of their results on the conversion of radioactive lycopene to cyclic carotenes is presented in Table 9. Extracts of red tomato fruit plastids

TABLE 9 Enzymatic conversion of radioactive lycopene to more unsaturated carotenes by soluble enzyme systems obtained from several genetic selections of tomatoes

Carotene	Genetic selection					
	Rutger's[a] normal red	Yellow[b] flesh (rr)	Tangerine[b] (tt)	Apricot[b] (at at)	Crimson[b] (og^{c} og^{c})	Verkerk's[b] 377-2αα (vo vo)
	dpm	dpm	dpm	dpm	dpm	dpm
γ-Carotene	31,427	200	18,146	1,500	4,206	2,135
β-Carotene	49,154	280	1,613	600	885	5,303
δ-Carotene	13,510	240	-	200	5,502	-
α-Carotene	5,069	200	-	360	726	-

[a] [^{3}H]Lycopene,, 1.64 nmoles (589,200 dpm) was used as substrate and the incubation of enzyme, substrate and cofactors was carried out for one hour in the dark at 25°C under nitrogen (Ref. 17).

[b] [^{14}C]Lycopene, 1.6 nmoles (60,000 dpm) was used as substrate and incubations were carried out for 5 hours under nitrogen in the dark (Ref. 27).

converted lycopene to all four cyclic carotenes. Similar results were obtained with extracts of plastids of yellow flesh (low total), apricot and crimson strains. The amounts of radioactivity incorporated into the cyclic carotenes were much lower, though, mainly because the specific activity of the substrate was much lower than that used for the red tomato plastid extracts. In extracts of the tangerine and Verkerk strains no conversion of radioactive lycopene to δ- or α-carotene was observed. Data have also been obtained on the cofactor requirements for the cyclization of lycopene to cyclic carotenes. A requirement for FAD for this reaction has been reported, Table 10.

Relatively little is known about the number and characteristics of the enzymes that convert lycopene to cyclic carotenes in tomato fruit plastids. In studies on the conversion of radioactive lycopene to cyclic carotenes, extracts from plastids of fruits of two genetic strains catalyzed the formation of γ- and β-carotenes, but not δ- or α-carotenes, Table 9. This result is consistent with the presence in tomatoes of separate enzymes for the formation of γ- and β-carotenes which have β-ionone rings, and δ- and α-carotenes which have an α-

ionone ring. Another point of interest is whether the enzyme that forms the β-ionone ring in the conversion of lycopene to γ-carotene can also convert γ-carotene to β-carotene. Similarly, can this enzyme convert δ-carotene to α-carotene? Although it would probably be possible for one enzyme to effect each of these reactions, namely, lycopene → γ-carotene, γ-carotene → β-carotene and δ-carotene → α-carotene, it seems more likely that separate enzymes exist for each of these reactions.

TABLE 10 Enzymatic conversion of tritium-labeled lycopene to cyclic carotenes by soluble preparations of plastids from Hi β tomato fruits

Incubation system	Total radioactivity in carotenes			
	α-	β-	δ-	γ-
	dpm	dpm	dpm	dpm
Complete	624	1,730	146	15,515
− FAD	0	0	0	0
Complete, boiled	0	0	0	0

[^{3}H]Lycopene, 600,000 dpm and 1.67 nmoles, was used as substrate (Ref. 26).

The mechanism of cyclization of lycopene to cyclic carotenes is thought to involve a proton attack at C-2 of lycopene, followed by ring closure to yield a carbonium ion intermediate (Ref. 28). An α- or β-ionone ring is then formed, depending on which proton is lost from the intermediate. A scheme for the enzymatic synthesis of α- or β-carotenes is given in Fig. 14. In this scheme, separate enzymes are proposed for the formation of the α- and β-ionone rings, with the formation of an identical carbonium ion intermediate for each. The products formed by the first cyclization reaction are γ- and δ-carotenes. A second cyclization would lead to the formation of β- and α-carotenes. Whether this cyclization is brought about by one enzyme that recognizes either γ- or δ-carotenes or by enzymes that are specific for each of these compounds is unknown.

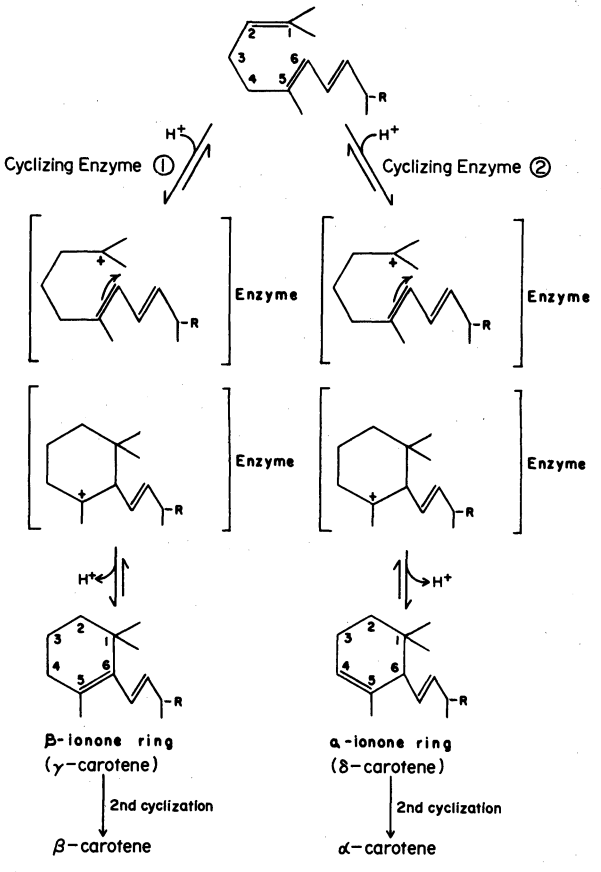

Fig. 14. Proposed mechanism for the enzymatic synthesis of cyclic carotenes from lycopene.

Whether extracts of tomato plastids have enzymes which can cyclize neurosporene as well as lycopene cannot be determined until the individual cyclization enzymes are isolated and purified. Traces of α- and β-zeacarotene do occur in tomatoes but these compounds could arise if the specificity of the enzymes cyclizing lycopene is not absolute.

FORMATION OF CYCLIC CAROTENES IN OTHER SYSTEMS

Cell-free extracts of *Phycomyces blakesleeanus* have been prepared which incorporate radioactivity from lycopene, γ-carotene, and neurosporene into β-carotene (Ref. 29). The relative effects of carrier β-zeacarotene and lycopene in diluting out radioactivity incorporated from [^{14}C]neurosporene into β-carotene were also examined in this work. Both compounds reduced the amount of radioactivity incorporated into β-carotene. This result was interpreted as evidence for two cyclization pathways being operative, one involving cyclization of neurosporene to β-zeacarotene

and then to β-carotene, and a second involving cyclization of lycopene to γ-carotene and β-carotene. The possibility that these results could be due to low substrate specificity was also recognized, however.

Cell-free extracts from *Halobacterium cutirubrum* also incorporate radioactivity from [^{14}C]-neurosporene, [^{14}C]lycopene and [^{14}C]γ-carotene into β-carotene (Ref. 13). In this study [^{14}C]neurosporene was not as good a precursor as either [^{14}C]lycopene or [^{14}C]γ-carotene.

ADDENDUM

At these meetings it was reported by Dr. Moss that the structure of prolycopene is 7,9,7′,9′-tetra-*cis*-lycopene. The presence of the *trans* central double bond in prolycopene indicates that the isomerization reaction (the conversion of *cis*-phytofluene to *trans*-phytofluene) in the tangerine tomato is the same as that in the red tomato. *Cis*-ζ-carotene, whose *cis-trans*-structure is unknown might arise from *cis*-phytofluene (if the *cis*-bond is at the 15,15′ position) or it might arise from *trans*-ζ-carotene (if the *cis* bond is at the 11 position). It appears most likely, though, that *cis*-ζ-carotene has a central *cis* bond. Obviously, the characterization of the *cis-trans* structure of this compound is needed. If prolycopene is formed from *trans*-ζ-carotene, this would indicate the difference between red and tangerine tomatoes resides in the dehydrogenase that effects the terminal dehydrogenation reactions. If so, the dehydrogenase in the tangerine tomato would introduce 7 and 7′ *cis* double bonds into *trans*-ζ-carotene in the conversion of this compound to prolycopene. In the process the *trans*-double bonds at the 9 and 9′ positions would be converted to *cis* bonds. Proneurosporene would be an intermediate in this reaction. Obviously, a characterization of the *cis-trans* structure of this compound is also needed.

Relatively large amounts of radioactivity were also found in lycopene when neurosporene was used as substrate. These observations would suggest that cyclization proceeds from lycopene to β-carotene in this organism.

The incorporation of [^{3}H]lycopene into β-carotene and zeaxanthin by cell-free extracts of *Flavobacterium R*1519 has been reported in a short communication (Ref. 10). These same workers have also reported the hydroxylation of [^{14}C]β-carotene to zeaxanthin in similar cell-free preparations. This is the first report of the formation of a xanthophyll by a cell-free system. Hence, this may be a very useful system in studies on the enzymology of xanthophyll formation.

It is evident from data reported in this paper that a number of cell-free systems have been developed which will effect the biosynthesis of carotenes. It is also evident that a considerable number of new studies must be carried out with the enzymes of these systems if we are to establish the mechanisms of their actions. Hopefully, many of these studies will be carried out in the next three years.

Acknowledgment: This work was supported in part by the Research Service of the William S. Middleton Memorial VA Hospital and grants from the National Science Foundation (PCM76 06281) and the National Institutes of Arthritis, Metabolic and Digestive Diseases (AM 01383) of the National Institutes of Health, United States Public Health Service.

REFERENCES

1. Jungalwala, F. B. and Porter, J. W., *Arch. Biochem. Biophys.* *119*, 209-219 (1967)
2. Maudinas, B., Bucholtz, M. L., Papastephanou, C., Katiyar, S. S., Briedis, A. V. and Porter, J. W., *Arch. Biochem. Biophys.* *180*, 354-362 (1977)
3. Islam, M. A., Lyrene, S. A., Miller, E. M., Jr. and Porter, J. W., *J. Biol Chem.* *252*, 1523-1525 (1977).
4. Islam, M. A., Spurgeon, S. L. and Porter, J. W., *J. Biol. Chem.*, To be submitted (1978).
5. Graebe, J. W., *Phytochem.* *7*, 2003-2020 (1968).
6. Gregonis, D. E. and Rilling, H. C., *Biochem. Biophys. Res. Commun.* *54*, 449-454 (1973).
7. Yokoyama, H., Nakayama, T. O. M. and Chichester, C. O., *J. Biol. Chem.* *237*, 681-686 (1962).
8. Bramley, P. M. and Davies, B. H., *Phytochem.* *14*, 463-469 (1975).
9. Gregonis, D. E. and Rilling, H. C., *Biochemistry* *13*, 1538-1542 (1974).
10. Brown, D. J., Britton, G. and Goodwin, T. W., *Biochem. Soc. Trans.* *3*, 741-742 (1975).
11. Harding, R. W., Spurgeon, S. L. and Turner, R. V., *Fifth Int. Symp. on Carotenoids*, Abst. p. 19 (1978)
12. Mitzka-Schnabel, U. and Rau, W., *Fifth Int. Symp. on Carotenoids*, Abst. p. 40 (1978).
13. Kushwaha, S. C., Kates, M. and Porter, J. W., *Can. J. Biochem.* *54*, 816-823 (1976).
14. Than, A., Bramley, P. M., Davies, B. H. and Rees, A. V., *Phytochem.* *11*, 3187-3192 (1972).
15. Weeks, O. B. (1963) *in Aspects of Terpenoid Chemistry and Biochemistry*, Academic Press, New York, p. 301.

16. Weedon, B. C. L. (1971) *in Carotenoids*, Birkhäuser, Basel, p. 268.
17. Kushwaha, S. C., Suzue, G., Subbarayan, C. and Porter, J. W., *J. Biol. Chem.* **245**, 4708-4717 (1970).
18. Qureshi, A. A., Andrewes, A. G., Qureshi, N. and Porter, J. W., *Arch. Biochem. Biophys.* **162**, 93-107 (1974).
19. Qureshi, A. A., Kim, M., Qureshi, N. and Porter, J. W., *Arch. Biochem. Biophys.* **162**, 108-116 (1974).
20. Qureshi, A. A., Qureshi, N., Kim, M. and Porter, J. W., *Arch. Biochem. Biophys.* **162**, 117-125 (1974).
21. Subbarayan, C., Kushwaha, S. C., Suzue, G. and Porter, J. W., *Arch. Biochem. Biophys.* **137**, 547-557. (1970).
22. Bramley, P. M. and Davies, B. H., *Phytochem.* **14**, 463-469 (1975).
23. Davies, B. H., *Pure & Appl. Chem.* **35**, 1-28 (1972).
24. Aragón, C. M. G., Murillo, F. J., de la Guardia, M.D. and Cerdá-Olmedo, E., *Eur. J. Biochem.* **63**, 71-75 (1976).
25. Kushwaha, S. C., Pugh, E. L., Cramer, J. K. G. and Kates, M., *Biochim. Biophys. Acta* **260**, 492-506 (1972).
26. Kushwaha, S. C., Subbarayan, C., Beeler, D. A. and Porter, J. W., *J. Biol. Chem.* **244**, 3635-3642 (1969).
27. Papastephanou, C., Barnes, F. J., Briedis, A. V. and Porter, J. W., *Arch. Biochem. Biophys.* **157**, 415-425 (1973).
28. Britton, G., *Pure & Appl. Chem.* **47**, 223-226 (1976).
29. Bramley, P. M., Than, A. and Davies, B. H., *Phytochem.* **16**, 235-238 (1977).