ENZYMATIC SYNTHESIS OF CAROTENES AND RELATED COMPOUNDS

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ABSTRACT

Data are presented in this paper which establish many of the reactions involved in the biosynthesis of carotenes. Studies have shown that all of the enzymes required for the synthesis of acyclic and cyclic carotenes from mevalonic acid are present in plastids of tomato fruits. Thus, it has been demonstrated that a soluble extract of an acetone powder of tomato fruits converts mevalonic acid to geranylgeranyl pyrophosphate, and isopentenyl pyrophosphate to phytoene, phytofluene, neurosporene and lycopene. Finally, it has been demonstrated that lycopene is converted into mono- and dicyclic carotenes by soluble extracts of plastids of tomato fruits. Whether the enzymes for the conversion of acetyl-CoA to mevalonic acid are also present in tomato fruit plastids has not yet been determined.

INTRODUCTION

Studies on the enzymatic synthesis of carotenes were, until very recently, plagued by a number of problems. One of these was the fact that the enzymes for the synthesis of carotenes are located in a particulate body, namely chromoplasts or chloroplasts. Hence, a method of solubilization of the enzymes without appreciable loss of enzyme activity was needed. A second problem was concerned with the commercial unavailability of labelled substrate other than mevalonic acid. Thus it became necessary to synthesize other substrates either chemically or enzymatically and then to purify these compounds. Thirdly, the reactions in the synthesis of carotenes appear to proceed much more slowly than many other biochemical reactions. It is probable that this problem is not primarily one of activity of the enzyme, but instead is due to the presence of a relatively small amount of the enzyme in question in the cell. Finally, the hydrocarbon nature of the carotenes required the development of a system in which the hydrophobic substrate or intermediate could be kept in contact with the enzyme.

Fortunately, most of the difficulties mentioned above have been overcome. As a result, it is now possible to discuss carotene biosynthesis in terms of demonstrable reactions, cofactor requirements, and to some extent properties of enzymes. Thus, as will be shown in this paper, the main outlines of the pathway of carotene biosynthesis have been established. However, in spite of this progress, many questions concerning specific reactions, mechanisms

of reactions, and the properties of the proteins catalyzing the synthesis of carotenes remain to be answered in future symposia.

In this paper data are presented on the pathway of carotene biosynthesis and on some of the individual reactions of this pathway. Brief mention is also made of the characteristics of some of the enzymes. Most of the studies reported are concerned with enzymes obtained from tomato fruits. Other studies have utilized rat, pigeon and pig liver enzyme systems. Most of the information that is presented has been taken from studies performed in the author's laboratory.

FORMATION OF C_{20} INTERMEDIATE

Conversion of acetate to mevalonic acid

Acetate is considered as the starting compound in the biosynthesis of carotenes. This compound, and its coenzyme-A derivative, is converted through a series of reactions, to the various carotenes that are found in tomato fruits.

Relatively few investigations have been made on the conversion by plant enzymes of acetyl-CoA to intermediates in carotene biosynthesis. Hence, most of the information that will be considered on the earlier reactions in carotene biosynthesis will be drawn from experiments with

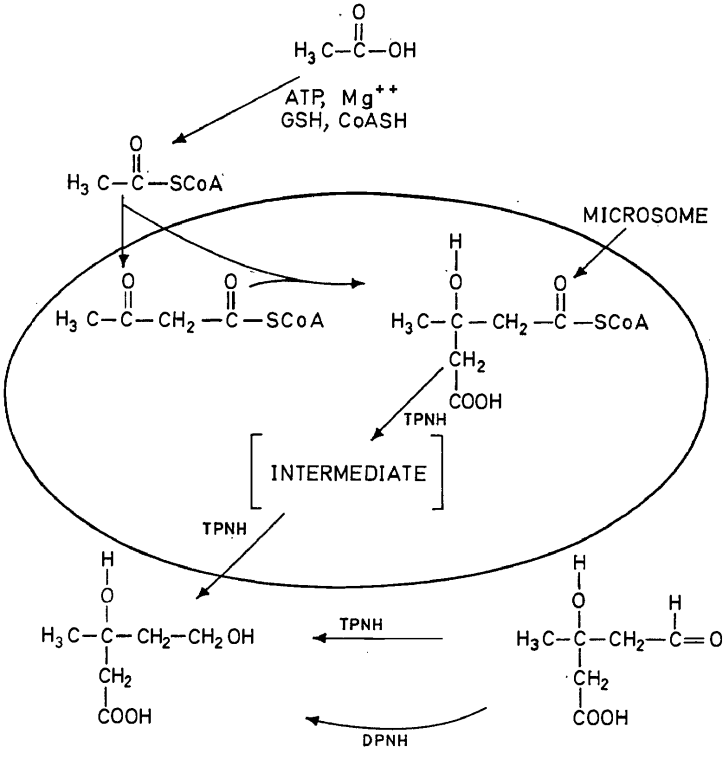

Figure 1. The pathway for the conversion of acetate to mevalonic acid by enzymes of mammalian liver.

animal enzyme systems. Early studies by Knauss, Porter and Wasson[1] demonstrated the synthesis of mevalonic acid from acetate by a combination of microsomes and soluble enzymes of rat liver. Both of these enzyme fractions were required for this conversion. A pool of nonradioactive mevalonic acid was used to trap radioactivity. Proof of the conversion of acetate to mevalonic acid was obtained by crystallization of derivatives of the latter to constant specific radioactivity. A proposal of the reactions that take place in the conversion of acetate to mevalonic acid and the site of these reactions in mammalian liver is presented in *Figure 1.*

Further studies by Brodie and Porter[2] demonstrated the conversion of ^{14}C-acetate to β-hydroxy, β-methyl glutaryl-CoA, mevaldic acid and mevalonic acid in the presence of a soluble enzyme system from pigeon liver. Prior reports by Rudney and associates[3, 4, 5] had presented evidence for the synthesis of β-hydroxy, β-methyl glutaryl-CoA from acetyl-CoA and acetoacetyl-CoA by beef liver microsomes and a yeast enzyme system. Further investigations in the laboratories of Lynen[6] and Rudney[7] resulted in the isolation of the yeast enzymes that catalyze the conversion of β-hydroxy, β-methyl glutaryl-CoA to mevalonic acid and the establishment of the characteristics of this reaction.

A second pathway for the synthesis of β-hydroxy, β-methyl glutaryl-CoA was discovered by Brodie *et al*[8, 9]. These workers found that a compound is formed by a partially purified pigeon liver fatty acid synthetase which chromatographs on paper with the same R_f as β-hydroxy, β-methyl glutaryl-CoA in an isobutyric acid-ammonia system (*Figure 2*). Further studies on

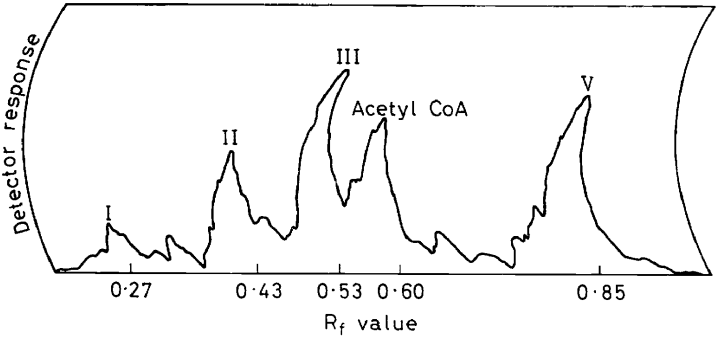

Figure 2. The enzymatic synthesis of β-hydroxy, β-methyl glutaryl-CoA by a partially purified pigeon liver fatty acid synthetase. The incubation mixture contained acetyl-CoA, 216 mμmoles and 82,500 cpm; malonyl-CoA, 216 mμmoles; enzyme protein, 3 mg; and potassium phosphate buffer, pH 7·0, 75 μmoles. After incubation for 1 hour at 38°, the solution was lyophilized and the residue was extracted with 99 per cent methanol. The entire methanol extract was applied to Whatman No. 3 MM paper and chromatographed in a descending system of 3 *N* NH_4OH, 0·1 M EDTA (pH 4·5), isobutyric acid-water (12:1:62:28). Peak III is β-hydroxy, β-methyl glutaryl-CoA.

this compound characterized it as β-hydroxy, β-methyl glutaryl-CoA. Both malonyl-CoA and acetyl-CoA are required for the formation of this compound (*Table 1*). Carbon atoms 1 and 2 of β-hydroxy, β-methyl glutaryl-CoA arise from malonyl-CoA and the remainder of the carbon atoms are supplied

Table 1. Participation of malonyl-CoA in the biosynthesis of HMG-CoA

Incubation No.	*Substrate*	HMG-CoA c.p.m.	HMG-CoA mμmoles
1	1-^{14}C-acetyl-CoA	1,150	0·6
2	1-^{14}C-acetyl-CoA + malonyl-CoA, 0·10 μmole	6,500	5·0
3	2-^{14}C-malonyl-CoA	7,000	2·0
4	2-^{14}C-malonyl-CoA + acetyl-CoA, 0·15 μmole	9,000	8·0
5	2-^{14}C-malonyl-CoA + acetyl-CoA, 1·05 μmoles	12,500	11·0
6	2-^{14}C-malonyl-CoA + 1-^{14}C-acetyl-CoA	20,900	11·0

The amount of 1-^{14}C-acetyl-CoA was 100 mμmoles and 65,000 c.p.m.; the amount of 2-^{14}C-malonyl-CoA was 55 mμmoles and 61,000 c.p.m. The incubation mixtures also contained 200 μmoles of phosphate buffer, pH 7·0, and 1 mg of 'DEAE enzyme' protein. The total volume was 2·0 ml and samples were incubated at 38° for 1 hour.

by acetyl-CoA. Further experiments proved that β-hydroxy, β-methyl glutaryl-CoA arises from acetoacetyl-enzyme and acetyl-CoA. Intermediates in the formation of β-hydroxy, β-methyl glutaryl-CoA are acetyl- and malonyl-enzyme, acetoacetyl-enzyme, and presumably β-hydroxy, β-methyl glutaryl-enzyme.

β-Hydroxy, β-methyl glutaryl-CoA is converted to mevalonic acid by an NADPH specific enzyme system[1, 2, 6, 7]. Free mevaldic acid is not an intermediate. The rate of this reaction is relatively slow, and in animals it is

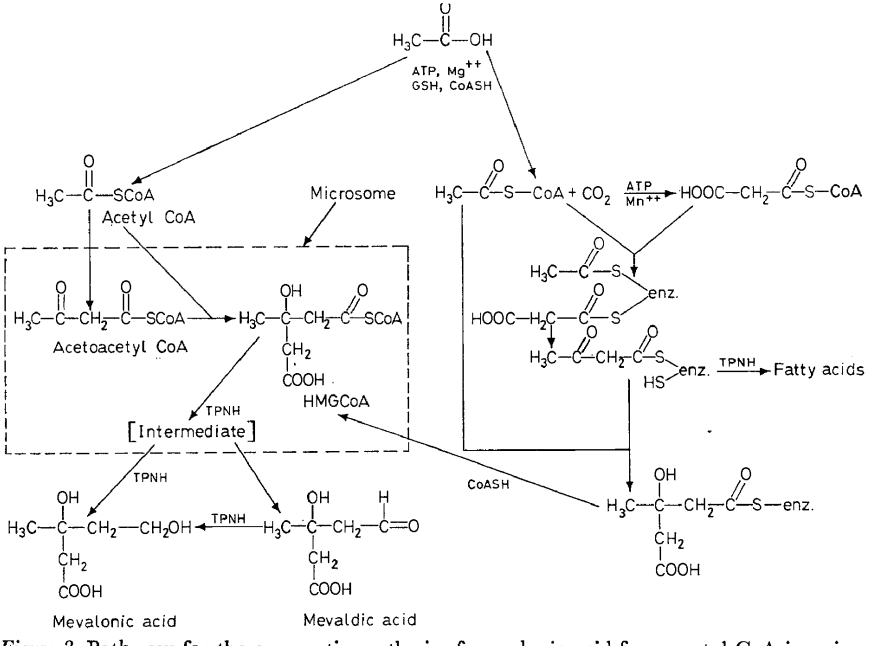

Figure 3. Pathways for the enzymatic synthesis of mevalonic acid from acetyl-CoA in avian and mammalian liver.

considered to be rate limiting in the biosynthesis of cholesterol. A summary of the reactions that take place in the conversion of acetate to mevalonic acid in avian and mammalian liver is presented in *Figure 3.*

Avian and mammalian liver also contain a very active enzyme for the

reduction of DL-mevaldic acid to mevalonic acid. This enzyme, surprisingly, utilizes both the D and the L isomers of mevaldic acid[10] (*Figure 4*). However, the rates of conversion differ for each of the isomers. Both NADH and NADPH specific mevaldic reductases have been reported[10, 11]. The significance of the mevaldic reductase in animal tissue is unknown, primarily because free mevaldic acid is not an intermediate in the formation of mevalonic acid, and secondly, to the writer's knowledge, no reactions have been reported that would yield free mevaldic acid in animal liver. This reaction has not been reported to occur in plants.

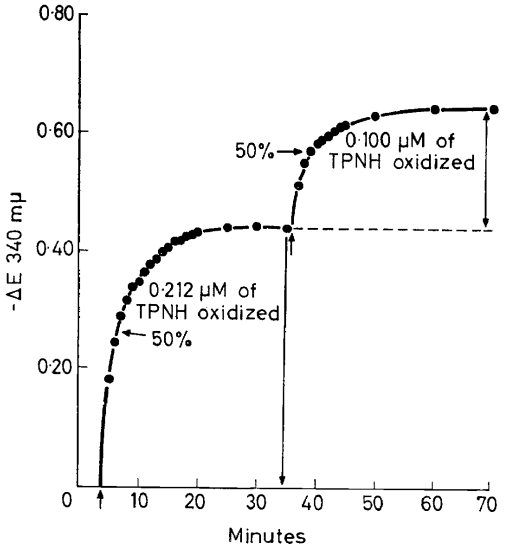

Figure 4. The enzymatic conversion of DL-mevaldic acid to mevalonic acid. The incubation mixture contained: phosphate buffer, pH 7·0, 100 μmoles; TPNH, 0·5 μmole; protein, 3 mg; and water to a final volume of 2·95 ml. At the first vertical arrow (4 minutes) 0·25 μmole of DL-mevaldic acid was added; at the second vertical arrow (36 minutes), 0·125 μmole was added. The horizontal arrows indicate the point of 50 per cent conversion of DL-mevaldic acid.

It is evident from the information that has been presented that two pathways exist in mammalian and avian liver for the formation of β-hydroxy, β-methyl glutaryl-CoA from acetyl-CoA (*Figure 3*). Neither of these pathways has been studied to any extent in plant materials. It has been shown, though, by Modi and Patwa[12] that mevalonic acid is present in carrot roots. It has also been shown by Oshima and Uritani[13, 14] that sweet potato tissue synthesizes β-hydroxy, β-methyl glutaryl-CoA from acetyl-CoA. This synthesis occurs in normal tissue in response to infection by *Ceratocystis fimbriata*. Preliminary investigations are in progress in our laboratory on the presence or absence of enzymes which catalyze the reactions, shown in *Figure 3*, in an extract of an acetone powder of tomato fruit plastids. However, these investigations have not proceeded far enough to warrant a report of the results.

Formation of isopentenyl pyrophosphate

Mevalonic acid is converted to isopentenyl pyrophosphate by the series of reactions shown in *Figure 5*[15]. In the conversion of one mole of mevalonic acid to isopentenyl pyrophosphate three moles of ATP are required. In

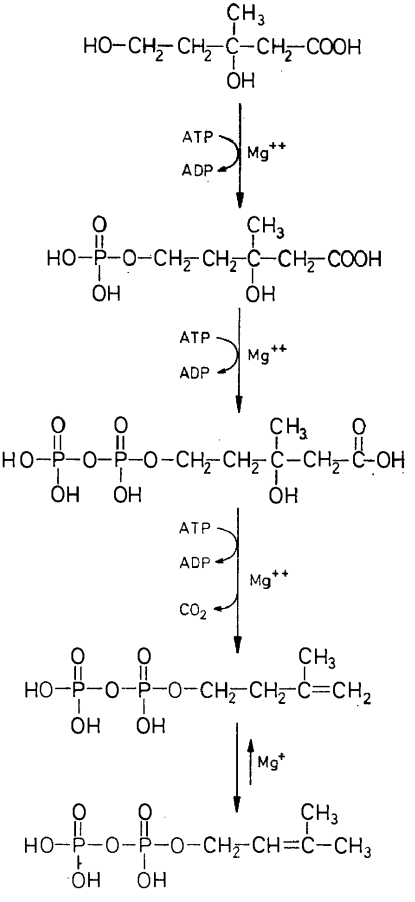

Figure 5. The reactions that occur in the conversion of mevalonic acid to isopentenyl pyrophosphate.

addition, magnesium is required as the metal ion. The enzymes of yeast and mammalian liver that convert mevalonic acid to isopentenyl pyrophosphate have been separated and either partially or highly purified. The initial reaction in this series of reactions is the conversion of mevalonic acid to mevalonic phosphate[16-18]. Kinetic studies on this reaction indicate that a sequential reaction takes place with mevalonic acid being bound to the enzyme first and then ATP[18] (*Figure 6*). Both substrates are bound to the enzyme before product is released. No evidence has been found to date that an enzyme bound intermediate is formed in this reaction.

In animal tissues mevalonic acid kinase is inhibited by geranyl

pyrophosphate and farnesyl pyrophosphate[19] (*Figure 7*). This inhibition is uncompetitive with respect to mevalonic acid and competitive with respect to ATP. The conversion of mevalonic acid to mevalonic phosphate has been demonstrated in plant tissue[20–25].

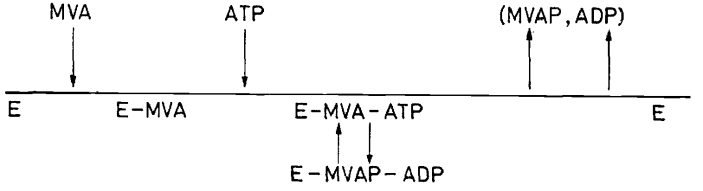

Figure 6. Mechanism of the enzymatic conversion of mevalonic acid to mevalonic phosphate.

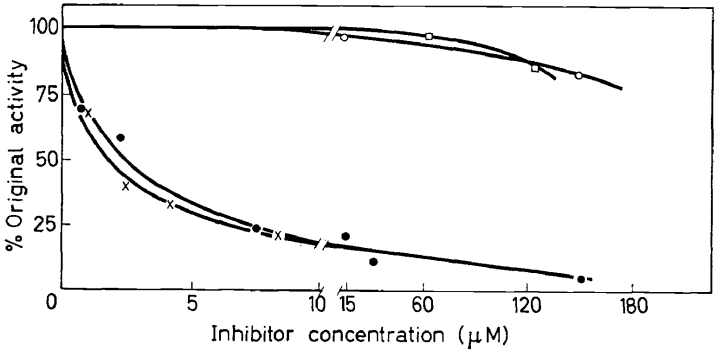

Figure 7. Inhibition of mevalonic kinase by terpenyl pyrophosphates. Enzyme activity was assayed as follows: phosphate buffer, pH 7·0, 100 μmoles; $MgCl_2$, 2·0 μmoles; DPNH, 0·165 μmole; phosphoenolpyruvate, 0·5 μmole; GSH, 10 μmoles; lactic dehydrogenase, 50 μg of protein and 31 units; pyruvic kinase, 50 μg of protein and 23 units; MgATP 0·44 μmole; mevalonic kinase, 7·1 μg of protein; and water, in a final volume of 1 ml were incubated for 3 minutes with the appropriate amount of inhibitor. 0·37 μmole of mevalonic acid was then added to start the reaction. Enzyme activity was followed spectrophotometrically at 340 mμ. The following compounds were added to give the final concentration indicated: isopentenyl pyrophosphate (□——□); dimethylallyl pyrophosphate (○——○); geranyl pyrophosphate (●——●); and farnesyl pyrophosphate ×——×).

The second reaction in the conversion of mevalonic acid to isopentenyl pyrophosphate is the phosphorylation of mevalonic phosphate to yield mevalonic pyrophosphate. This enzyme has been isolated from yeast[26, 27], pig liver[28], and Hevea latex[29] and partially purified. ATP and Mg^{++} are also required for this reaction. The mechanism of conversion of mevalonic phosphate to mevalonic pyrophosphate has not been established. It has been shown, however, that geranyl and farnesyl pyrophosphates do not affect the rate of the reaction catalyzed by this enzyme. It has also been demonstrated that this enzyme is present in plant tissue[20, 22, 24].

The last enzyme involved in the formation of isopentenyl pyrophosphate has also been isolated from pig liver and partially purified. However, most of the information on this enzyme and the reaction it catalyzes has been obtained from studies with a yeast enzyme[26, 30–32]. It has been shown through these studies that the enzyme catalyzes a phosphorylation (carbon atom 3)

and a decarboxylation of mevalonic pyrophosphate. Presumably a concerted reaction occurs. The presence of this enzyme in plant tissue has been implicated in studies with incubation systems that synthesize terpenyl pyrophosphates[22, 24], carotenes[33] or rubber[20].

Formation of farnesyl pyrophosphate and squalene

The reactions that occur in the conversion of isopentenyl pyrophosphate to squalene are shown in *Figure 8*. The initial reaction in the formation of

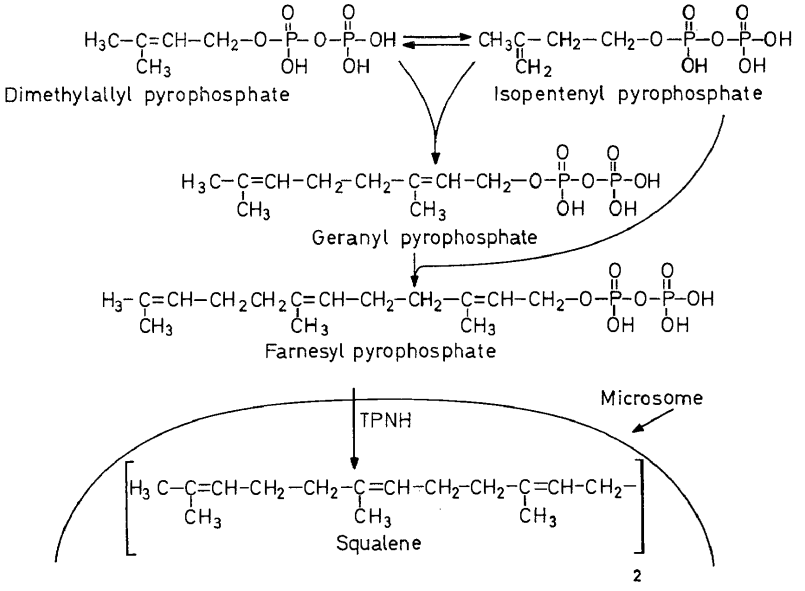

Figure 8. The reactions that occur in the enzymatic conversion of isopentenyl pyrophosphate to squalene in mammalian liver.

farnesyl pyrophosphate is the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate[34]. This reaction was first studied with a yeast enzyme system. Further studies on this reaction have been made with a partially purified enzyme obtained from pig liver[35]. The conversion of isopentenyl pyrophosphate to dimethylallyl pyrophosphate is an equilibrium reaction. At equilibrium approximately 87 per cent of dimethylallyl pyrophosphate and 13 per cent of isopentenyl pyrophosphate are present. This reaction is also stereospecific. The stereospecificity of the reaction has been established by two methods. One of these was reported from our laboratory[35]. In this study Shah *et al*[35]. determined the incorporation of tritium of ^3H_2O into isopentenyl and dimethylallyl pyrophosphates, and their degradation products, as a function of time of incubation after the reaction had achieved equilibrium. The second method utilized stereospecifically labelled tritiated mevalonic acid. Studies with this compound were reported by Cornforth *et al*[36]. The mechanism of isomerization deduced by Shah *et al*[35]. is shown in *Figure 9*.

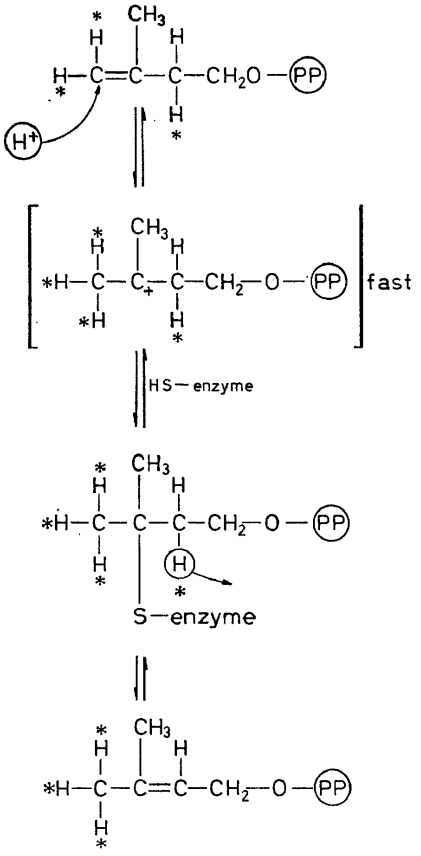

Figure 9. Mechanism of isomerization of isopentenyl pyrophosphate. The starred atoms indicate the exchangeable hydrogens of isopentenyl and dimethylallyl pyrophosphates.

The next reaction in the formation of farnesyl pyrophosphate involves the condensation of dimethylallyl and isopentenyl pyrophosphates. The enzyme effecting the formation of farnesyl pyrophosphate in pig liver has been isolated and highly purified by Dorsey, Dorsey and Porter[37]. This enzyme brings about the formation of both geranyl and farnesyl pyrophosphates, *Figure 10.* All evidence obtained to date indicates that both of these products are formed by a single enzyme, *Table 2.* The mechanism of this reaction has been studied by Holloway and Popjak through product inhibition kinetics[38]. Their results indicate that the reaction proceeds through the binding of first geranyl pyrophosphate and then isopentenyl pyrophosphate to the enzyme (*Figure 11*).

In animals two molecules of farnesyl pyrophosphate are condensed to form squalene. This reaction is effected by an enzyme system located in microsomes. Both NADPH and a metal ion (Mg^{++}) are required as cofactors. The study of this reaction in animals has proved difficult because of an inability to secure an appreciable yield of soluble enzyme from microsomes.

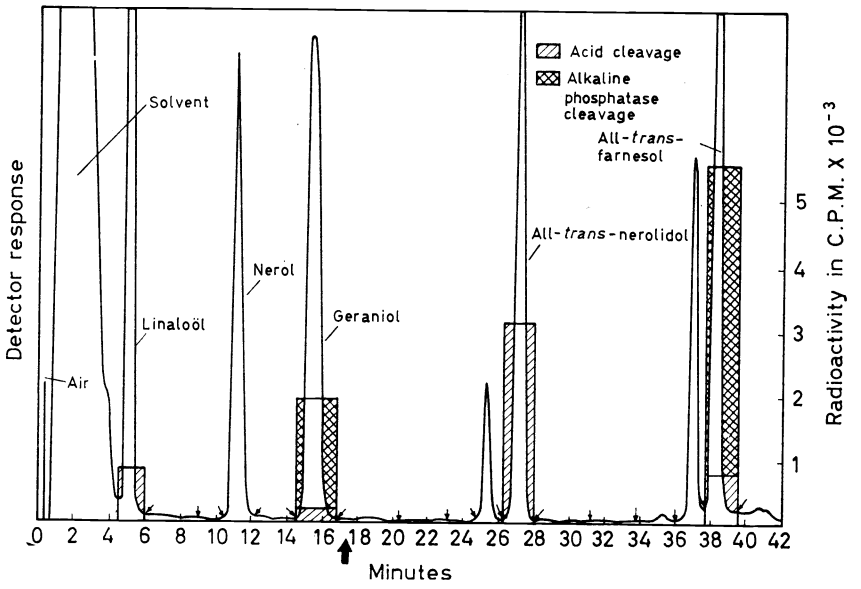

Figure 10. The gas chromatographic separation of terpenols liberated by either acid or alkaline phosphatase cleavage. The crosshatched areas indicate radioactivity. A temperature of 150° and an argon gas flow of 80 ml per minute were used. The heavy arrow at 17·5 minutes indicates the point at which the gas flow was increased to 100 ml per minute and the temperature was programmed to increase linearly at a rate of 2° per minute.

Table 2. Purification of geranyl pyrophosphate synthetase[a]

Purification step	*Total protein* (mg)	*Specific activity* (units/mg)	*Total activity* (units[c])	*Purification* (fold)	*Recovery* (%)	*Ratio of specific activities*[b]
144,000 × *g* supernatant	31,539	1·76	55,445			2·04
Ammonium sulphate I	13,164	3·20	42,085	1·82	76	1·87
Calcium phosphate gel supernatant	5,174	6·63	34,304	3·78	62	2·35
DEAE-cellulose	179	26·8	4,805	15·2	8·7	1·8
Sephadex G-200	66·7	45·3	3,019	25·7	5·5	1·62
Ammonium sulphate II	17·9	337·8	6,046	192	10·9	2·03

[a] The incubation mixture for the enzymatic synthesis of geranyl pyrophosphate contained Tris buffer, pH 7·2. 50 μmoles; $MgCl_2$, 1·0 μmole; dimethylallyl pyrophosphate, 20·6 mμmoles; 4-^{14}C-isopentenyl pyrophosphate 4·38 mμmoles and 20,900 cpm; and enzyme (1 to 50 μg of protein, depending on the stage of purification) in a final volume of 1·0 ml. The reaction mixture was incubated at 38; for 10 minutes. The assay system for the synthesis of farnesyl pyrophosphate contained phosphate buffer, pH 7·0, 50 μmoles; $MgCl_2$, 1·0 μmole; geranyl pyrophosphate, 50 mμmoles; 4-^{14}C-isopentenyl pyrophosphate, 4·38 mμmoles and 20,900 cpm; and enzyme protein as indicated above. This reaction mixture was incubated for 15 minutes at 38°.
[b] One unit = 1 mμmole of isopentenyl pyrophosphate converted to product per minute of incubation.
[c] The ratio of the specific activities for geranyl and farnesyl pyrophosphate formation.

This reaction has also been demonstrated in plants. The studies of Beeler, Anderson and Porter[39] showed that this reaction occurs in plastids of tomato fruits and carrots. Recently Graebe[40] has shown that squalene is synthesized by a cell-free system from pea fruits. The formation of squalene from farnesyl pyrophosphate by plant plastids also requires NADPH and Mg^{++} ions, and

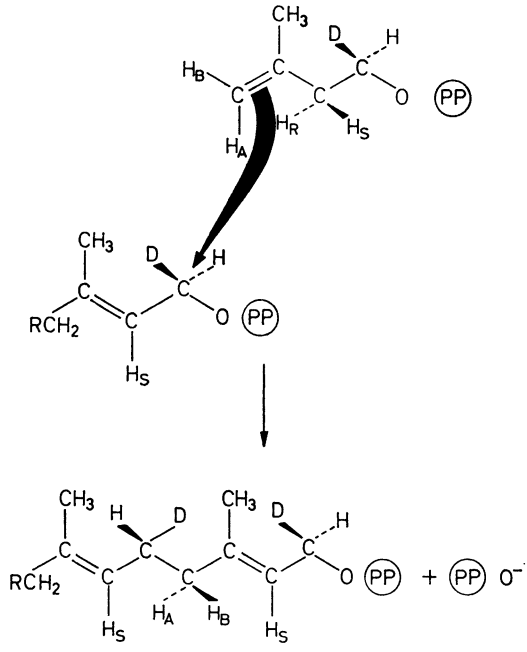

Figure 11. Summary of stereochemical changes that occur during C-C bond formation in the enzymatic synthesis of *trans-trans*-farnesyl pyrophosphate.

the substrate for the formation of squalene is all-*trans* farnesyl pyrophosphate[39] as it is in the animal system.

Formation of geranylgeranyl pyrophosphate

Most of the discussion thus far has been concerned with enzyme systems obtained from mammalian and avian liver and yeast. Some information has been obtained on the occurrence of these enzymes in plants and the reactions they catalyze. However, the amount of information available on plant systems is relatively sparse. As mentioned previously the formation of β-hydroxy, β-methyl glutaryl-CoA and the conversion of this compound to mevalonic acid has been demonstrated in sweet potato sections infected with *Ceratocystis fimbriata*[13, 14]. In addition a number of laboratories have demonstrated the existence of mevalonic kinase and the formation of mevalonic pyrophosphate and isopentenyl pyrophosphate in plant systems. Finally, Suzue *et al.*[41] recently demonstrated the formation of geranylgeranyl pyrophosphate from mevalonic acid by an extract of an acetone power of tomato fruit plastids.

Attention can now be turned to studies more directly concerned with the biosynthesis of carotenes. In doing this, I would like to review briefly a few of the early pertinent studies on the synthesis of carotenes. The first of these is illustrated by the data in *Table 3.* These data are taken from a publication by Anderson, Norgard and Porter[42] on the conversion of radioactive mevalonic acid to carotenes in ripening tomato fruits. In these

Table 3. Specific radioactivities of tomato carotenoids

Compound	*Sea Sorb chromatogram*	*Alumina or $Ca(OH)_2$ chromatogram Total radioactivity* (counts/min[a])	*After reduction*	*Specific radioactivity* (counts/min/mg)
Phytoene	2,060,000[b]	1295	1238	2870±200
Phytofluene	459,000[b]	1995[c]	1142	3080±200
ζ-Carotene	24,300[b]	440[b]	310	750±100
Neurosporene	20,500[b]	- - -[d]	45	780±150
γ-Carotene	37,000[b]	284[b]	224	3450±250
	Crystallization			
	Before	*After*		
Lycopene	162,000[b]	18,400[b,e]	- - -	3110± 50[b]
β-Carotene	242,000[b]	7,850[b,e]	- - -	6400±100[b]

[a] Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer.
[b] Radioactivity was measured with a thin end-window gas-flow Geiger-Muller tube, and values were corrected for comparison with those obtained with the liquid scintillation spectrometer.
[c] The radioactivity value is corrected for quenching.
[d] The radioactivity in neurosporene was too low for determination without serious loss of sample.
[e] Corrected for losses occurring during crystallizations.

experiments radioactive mevalonic acid was injected into the fruits and they were then allowed to ripen. The incorporation of radioactivity into several of the carotenes of tomato fruits is reported, *Table 3.* The data in this table also illustrate one of the early difficulties in such experimentation; namely, the very large amount of radioactive contaminants present with the small amount of radioactive carotenes.

The next study to consider is one on the conversion of terpenyl pyrophosphates to phytoene and other carotenes[43]. The results of such a study are shown in *Table 4.* In this study terpenyl pyrophosphates were generated

Table 4. Incorporation of ^{14}C of terpenyl pyrophosphates into carotenes by tomato plastids

	I	*Total radioactivity* II (counts/min)	III	IV
Phytoene	49,800	16,600	2940	5750
Phytofluene	870	1,125[a]	80[a]	200
ζ-Carotene	350	455[a]	62[a]	170
Neurosporene	- - - -	28	- - - -	- - - -
Lycopene	- - - -	340	120[a]	- - - -
γ-Carotene	- - - -	27	96[a]	- - - -
β-Carotene	- - - -	35[a]	45[a]	- - - -

[a] Corrected for losses of added carrier carotene.
Plastids derived from red tomatoes were used in Experiments I–III; plastids derived from the variety tangerine were used in Experiment IV.

from mevalonic acid by a rat liver enzyme system. The enzyme system was then heat-inactivated and plastids of tomato fruits were added. After an incubation of several hours, carotenes were isolated and assayed for the presence of radioactivity. The data of *Table 4* show the conversion of radio active terpenyl pyrophosphates to several carotenes by tomato fruit plastids.

The principal component in the terpenyl pyrophosphates was farnesyl pyrophosphate.

The last of the early studies that I wish to consider is one on the specific radioactivities of carotenes formed from terpenyl pyrophosphates. The results of such a study are shown in *Table 5*[44]. It will be noted that the specific radioactivities of tomato fruit carotenes declined with an increase in unsaturation of the carotene.

Table 5. Specific radioactivities of carotenes synthesized by isolated tomato plastids from ^{14}C-labelled terpenyl pyrophosphates

	Experiment 1		*Experiment 2*	
	(counts/min/mg)	(mg)	(counts/min/mg)	(mg)
Phytoene	489,600[a]	0·300	215,350[a]	0·764
Phytofluene	168,700[b]	0·034	12,100[a,b]	0·058
ζ-Carotene	149,800[b]	0·030	15,650[a,b]	0·094
Neurosporene	57,800[b]	0·006	4,500[d]	0·032
Lycopene	1,200[c]	1·020	40[c]	2·100
β-Carotene	2,480[c]	0·098	140[c]	0·340

[a] Determined by spectrophotometric assay after elution from an aluminum oxide chromatogram and by determination of the radiochemical purity of the reduced compound on gas-liquid chromatography.
[b] Determined by spectrophotometric assay after elution from an aluminum oxide chromatogram and/or a magnesium oxide-Super-Cel column and by a determination of the radioactivity coincident with lycopersane on reduction and gas-liquid chromatography.
[c] Determined through crystallization to constant specific radioactivity.
[d] Determined by spectrophotometric assay after elution from an aluminum oxide chromatogram and a $Ca(OH)_2$-Super-Cel column and by a determination of radioactivity coincident with lycopersane after reduction and chromatography on alumina. The absolute value for the specific radioactivity of this compound may be lower than the value given since the quantity of radioactivity present was insufficient for an analysis by gas-liquid chromatography.

The studies that have been mentioned thus far on the formation of carotenes utilized terpenyl pyrophosphates and isolated tomato fruit plastids. It became desirable at the time the previously mentioned studies were completed to use a single pure substrate, and if possible, a soluble enzyme system. Isopentenyl pyrophosphate was chosen as the pure substrate for subsequent studies. This compound was synthesized from mevalonic acid with a pig liver enzyme system, and then purified free of other radioactive compounds[35].

Further studies on the synthesis of carotenes were advanced greatly by the finding of Jungalwala and Porter[45] that an acetone powder of tomato fruit plastids retained activity for the synthesis of phytoene. Using this system, Jungalwala and Porter[45] demonstrated the conversion of isopentenyl and farnesyl pyrophosphates to phytoene, *Table 6*. The cofactor requirements for the conversion of farnesyl pyrophosphate to phytoene are isopentenyl pyrophosphate, Mn^{++}, Mg^{++}, and dithiothreitol. The requirement for isopentenyl pyrophosphate in the conversion of farnesyl pyrophosphate to phytoene suggests that geranylgeranyl pyrophosphate is an intermediate in this reaction; and the requirement for dithiothreitol suggests that an SH group of an enzyme is involved in the reactions that occur in the formation of phytoene, presumably in the condensation of geranylgeranyl pyrophosphate to phytoene.

Experimentation on the conversion of geranylgeranyl pyrophosphate to phytoene was plagued by a lack of a satisfactory system for the synthesis of

Table 6. Factors influencing the biosynthesis of phytoene from farnesyl pyrophosphate

Incubation system	*Total radioactivity in phytoene* (cpm)	*Acid labiles* *Nerolidol + farnesol* (%)	*Acid labiles* *Geranyllinalool + geranylgeraniol* (%)
Complete[a]	7,350	75·7	21·0
−Isopentenyl pyrophosphate	30	89·5	0·2
$-MnCl_2$	1,045	87·3	2·8
$-MgCl_2$	1,268	74·0	16·5
+NADP	6,758	77·1	22·8

[a] The complete incubation system contained farnesyl pyrophosphate-4,8,12-^{14}C, 9 mμmoles (97,700 cpm) nonradioactive isopentenyl pyrophosphate, 50 mμmoles; $MnCl_2$, 2 μmoles; $MgCl_2$, 15 μmoles; glycylglycine buffer, pH 8·0, 100 μmoles; dithiothreitol, 10 μmoles; and enzyme protein, 2·5 mg; in a final volume of 1 ml Incubations were carried out for 3 hours at 20° under an atmosphere of nitrogen. NADP, when added, was present in a concentration of 2 μmoles/ml. Acid labiles were assayed by gas-liquid chromatography.

appreciable quantities of the former compound, and secondly by the instability of this compound in the chromatographic systems used previously for the purification of other terpenyl pyrophosphates. Fortunately, Oster and West[46], working on the enzymatic synthesis of kaurene, had found an enzyme system that synthesized appreciable amounts of geranylgeranyl pyrophosphate. They had also developed a method of purification of this compound. A preparation of geranylgeranyl pyrophosphate secured from Oster and West[46] was incubated with an extract of an acetone powder of tomato fruit[47]. Phytoene was formed, *Figure 12.* It will be noted that a decrease in the quantity of geranylgeranyl pyrophosphate occurred with a simultaneous increase in the quantity of phytoene. The presence of radioactivity in the latter compound was proved through hydrogenation and gas-liquid chromatography.

Recently we have developed another procedure for the enzymatic synthesis of geranylgeranyl pyrophosphate[48]. In this procedure an extract of an acetone powder of tomato fruit plastids is used to convert radioactive mevalonic acid to geranylgeranyl pyrophosphate. Incubations are made at 38°. At this temperature the enzyme system for the conversion of geranylgeranyl pyrophosphate to phytoene is inactivated. It is interesting to note that it has been known for a long time that carotenes are formed in relatively small amounts when tomato fruits are ripened at temperatures above 30°. Apparently the failure of carotene formation at this temperature is largely or wholly attributable to the inactivation of the enzyme system that converts geranylgeranyl pyrophosphate to phytoene. The incubation mixture used by Suzue and Porter[48] is shown in *Table 7.* The procedure used for the isolation of geranylgeranyl pyrophosphate and the yield (radioactivity) obtained in each step of the procedure is given in *Table 8.*

Attention is now turned to studies on the separation and purification of isopentenyl pyrophosphate isomerase and geranylgeranyl pyrophosphate synthetase. A typical experiment in which an extract of an acetone powder of tomato fruit plastids was subjected to electrofocusing is shown in *Figure 13.* By this technique proteins are migrated in a pH gradient to their isoelectric points. Fortunately, both isopentenyl pyrophosphate isomerase and geranylgeranyl pyrophosphate synthetase are stable to this treatment. Also, these

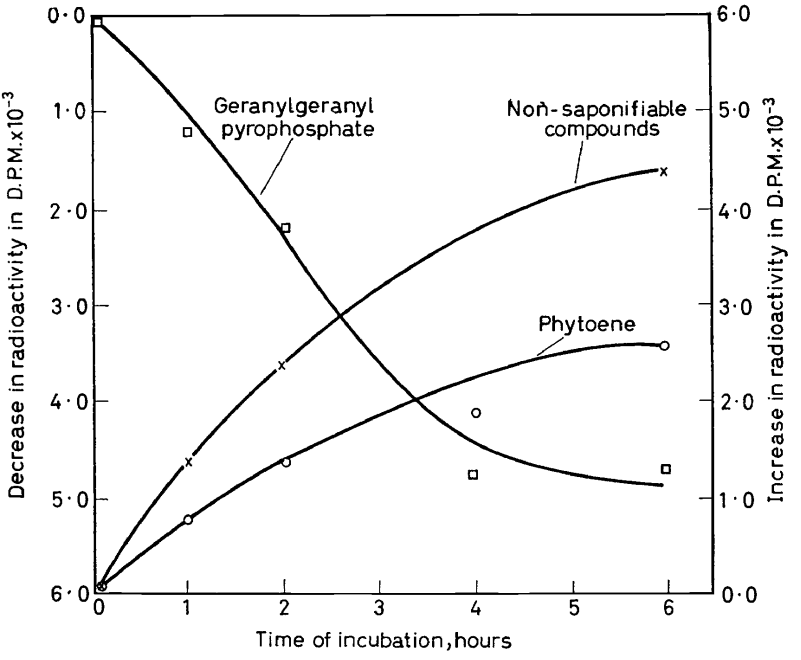

Figure 12. The synthesis of phytoene, as a function of time, from ^{14}C-labelled geranylgeranyl pyrophosphate by a tomato enzyme system. The incubation mixture contained ^{14}C-geranylgeranyl pyrophosphate, 0·5 mμmole and 12,200 dpm; $MnCl_2$, 2 μmoles; $MgCl_2$, 15 μmoles; borate buffer, pH 8·2, 100 μmoles; dithiothreitol, 10 μmoles; and enzyme protein, 3·5 mg [20–45 per cent $(NH_4)_2SO_4$ precipitate of an extract of an acetone powder of tomato fruit plastids] in a final volume of 1·0 ml. Incubations were carried out at 20°.

Table 7. Incubation system for the synthesis of geranylgeranyl pyrophosphate from mevalonic acid-2-^{14}C

Components	*Quantity*
Mevalonic acid-2-^{14}C ($1{\cdot}32 \times 10^7$ dpm)	0·001 mM
ATP	0·54 mM
$MgCl_2$	1·1 mM
$MnCl_2$	0·024 mM
Dithiothreitol	0·12 mM
Borate buffer (pH 7·5)	1·5 mM
KF	0·3 mM
H_2C	5·7 ml
Enzyme (protein conc. 8 mg/ml)	9·0 ml

Total volume: 22 ml
Incubation with shaking: 5 hours (at 38°)

Table 8. Purification and yield of geranylgeranyl pyrophosphate

	Radioactivity (dpm)	*Yield* (%)
Mevalonic acid-2-^{14}C-DL	$13{\cdot}2\times10^6$	
Collidine extract	$3{\cdot}7\times10^6$	56
$NaHCO_3$ extract	$2{\cdot}8\times10^6$	43
DEAE-Sephadex (A25) column chromatography	$1{\cdot}5\times10^6$	23
Lyophilized product	$1{\cdot}2\times10^6$	18

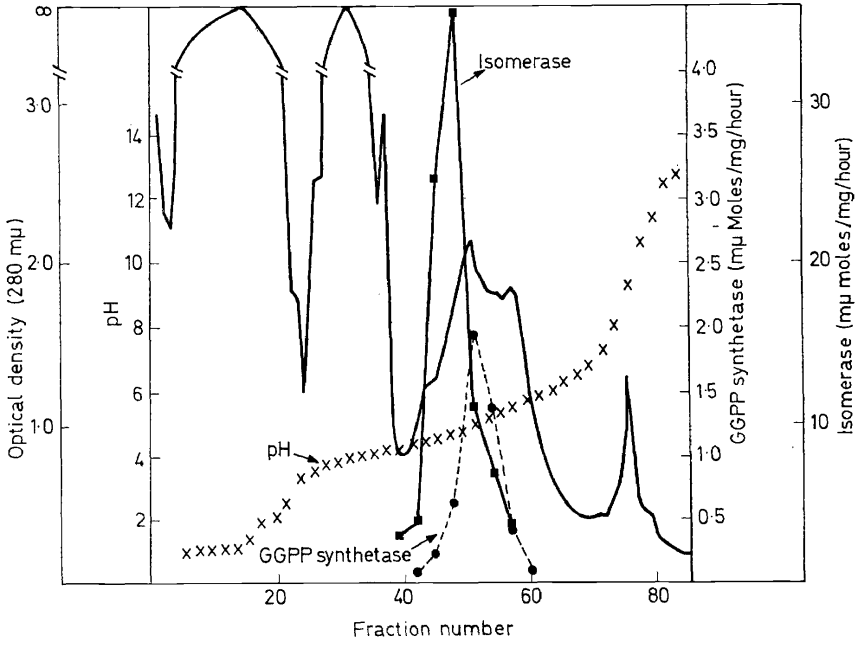

Figure 13. The partial purification of geranylgeranyl pyrophosphate synthetase by isoelectrofocusing. The protein used was an extract of an acetone powder of tomato fruit plastids. The column was the LKB 110 ml size and the ampholytes were those for the pH 4–6 range. Protein concentration was determined by measuring light absorption at 280 mμ and pH measurements were made with a radiometer Model TTT1c. The isoelectric point of geranylgeranyl pyrophosphate synthetase was 5.1. Geranylgeranyl pyrophosphate synthetase activity was measured by the standard assay procedure (see legend to *Figure 18*). Isomerase activity was measured by the same assay procedure except that dimethylallyl pyrophosphate was omitted from the incubation mixture. The total radioactivity of the acid labile terpenol fraction was taken as a measure of isomerase activity.

enzymes are separated from a large quantity of contaminating proteins by this procedure. They do not, however, separate appreciably from one another. The separation of these enzymes can be achieved by Sephadex gel filtration. A typical separation is shown in *Figure 14.*

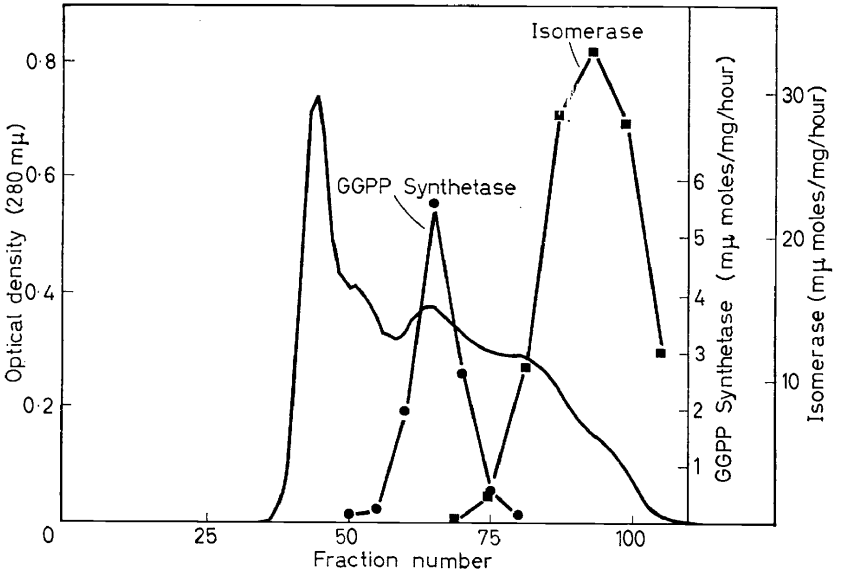

Figure 14. Sephadex G-100 gel filtration of geranylgeranyl pyrophosphate synthetase. The enzyme was partially purified by electrofocusing prior to gel filtration. A column of 1·4 × 90 cm was used. Protein concentration in the eluate fractions was determined by measurement of light absorption at 280 mμ. Geranylgeranyl pyrophosphate synthetase activity was measured by the standard assay procedure (see legend to *Figure 18*). Isomerase activity was measured with the same assay system, except that dimethylallyl pyrophosphate was omitted from the incubation mixture. The total radioactivity extractable with petroleum ether after the release of acid labile terpenols was measured.

Very few investigations have been made with isopentenyl pyrophosphate isomerase isolated from tomato fruit plastids. We have, however, demonstrated the occurrence of this enzyme and a few of its properties[49]. More time has been spent on the purification and properties of geranylgeranyl pyrophosphate synthetase. The next four figures (*Figures 15–18*) show some of the characteristics of the reaction catalyzed by this enzyme[49]. The conversion of isopentenyl pyrophosphate and dimethylallyl pyrophosphate to geranylgeranyl pyrophosphate is proportional to protein concentration, *Figure 15.* A metal ion is required for this reaction, and it has been found

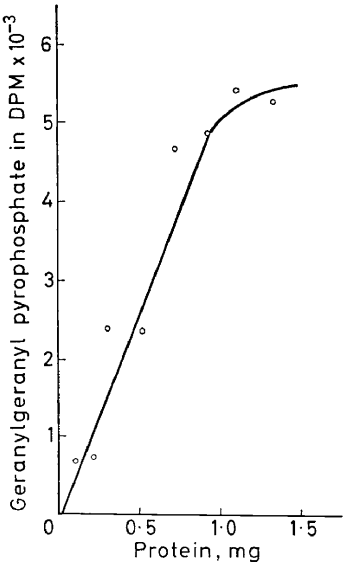

Figure 15. The dependence of geranylgeranyl pyrophosphate synthesis on protein concentration. The assay mixture contained TES buffer, pH 7·0, 50 μmoles; $MnCl_2$, 2 μmoles; isopentenyl pyrophosphate 4-^{14}C, 6·21 mμmoles and 69,010 dpm; and enzyme, in a total volume of 0·5 ml. The incubation time was 30 minutes. Acid labile terpenols were extracted with petroleum ether, identified by thin-layer chromatography and then assayed for radioactivity.

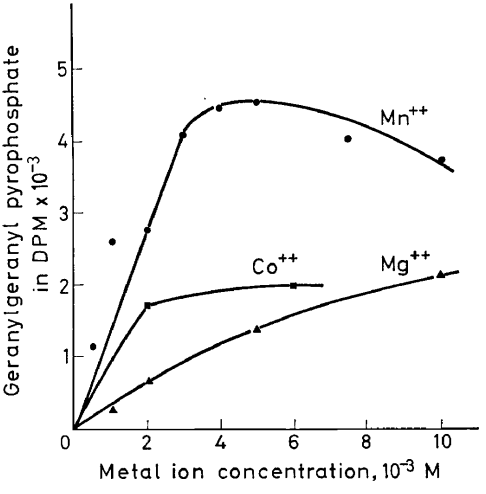

Figure 16. The effect of concentration of each of three divalent metal ions on the synthesis of geranylgeranyl pyrophosphate. Assay mixtures contained TES buffer, pH 7·0, 50 μmoles; isopentenyl pyrophosphate-4-^{14}C, 6·21 mμmoles and 69,010 dpm; dimethylallyl pyrophosphate, 7·05 mμmoles; enzyme, 40 μg of protein; and metal ions as indicated; in a final volume of 0·5 ml. The incubation was carried out for 1 hour at 37°. Acid labile terpenols were extracted with petroleum ether, identified by thin-layer chromatography and then assayed for radioactivity.

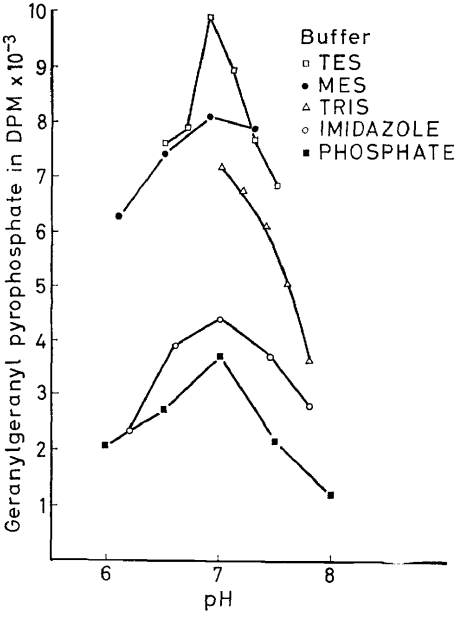

Figure 17. The effect of pH on the synthesis of geranylgeranyl pyrophosphate. The assay mixture contained buffer, 50 μmoles; $MnCl_2$, 2 μmoles; isopentenyl pyrophosphate-4-^{14}C, 6·21 mμmoles and 69,010 dpm; dimethylallyl pyrophosphate, 7·05 mμmoles and enzyme, 40 μg of protein; in a total volume of 0·5 ml. The incubation time was 1 hour. Acid labile terpenols were extracted with petroleum ether, identified by thin-layer chromatography and then assayed for radioactivity.

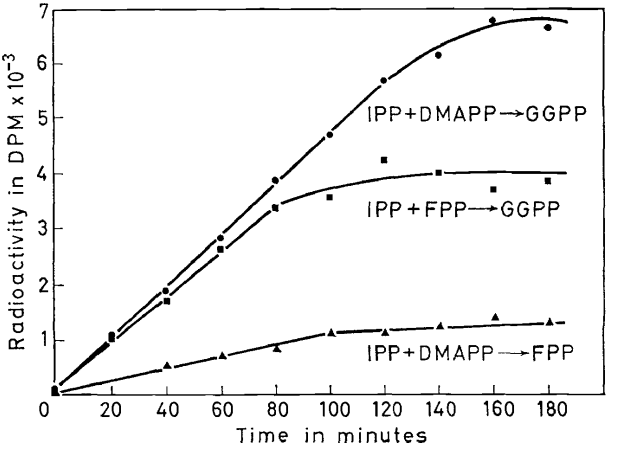

Figure 18. The dependence of geranylgeranyl pyrophosphate synthesis on time. Assay mixtures contained TES buffer, pH 7·0, 50 μmoles; $MnCl_2$, 2 μmoles; isopentenyl pyrophosphate-4-^{14}C, 6·21 mμmoles and 69,010 dpm; either dimethylallyl pyrophosphate, 7·05 mμmoles or farnesyl pyrophosphate, 8·78 mμmoles; and enzyme, 105 μg of protein; in a total volume of 0·5 ml. Acid labile terpenols were extracted with petroleum ether and then identified by thin-layer chromatography. Each terpenol fraction was scraped into a counting vial and assayed in a liquid-scintillation spectrometer for radioactivity.

that Mn^{++} is much more effective than Mg^{++}. Cobalt is intermediate between Mn^{++} and Mg^{++}, in its effect on this reaction, *Figure 16.* The pH optimum for the formation of geranylgeranyl pyrophosphate is approximately 7. However, buffers differ greatly in their effect on this reaction, *Figure 17.* Geranylgeranyl pyrophosphate synthetase condenses isopentenyl and farnesyl pyrophosphates as well as isopentenyl and dimethylallyl pyrophosphates. Both farnesyl and geranylgeranyl pyrophosphates are formed as products. However, much more geranylgeranyl pyrophosphate than farnesyl pyrophosphate is obtained, *Figure 18.*

The products formed by geranylgeranyl pyrophosphate synthetase have been identified by cleavage with alkaline phosphatase or acid, and subsequent thin-layer or gas-liquid chromatography. A typical separation of acid-released and alkaline phosphatase-released terpenols on thin-layer chromatography is shown in *Figure 19.*

Formation of phytoene and other acyclic carotenes

It was reported previously that phytoene is formed from isopentenyl pyrophosphate plus farnesyl pyrophosphate[45], and from geranylgeranyl pyrophosphate[47]. Only a metal ion (Mg^{++}) is required for the latter conversion. Dithiothreitol is also used in the incubation mixture, presumably to protect SH groups in phytoene synthetase. Studies with this enzyme have not progressed as far as those with geranylgeranyl pyrophosphate synthetase. One of the principal difficulties thus far has been the relative instability of this protein. It is partially or totally inactivated during electrofocusing. Hence, more standard methods of purification are currently being used. A partial purification of the enzyme[45] is shown in *Table 9.*

Table 9. Partial purification of phytoene synthetase

Purification step	*Total protein* (mg)	*Total activity* (cpm/hour[a])	*Specific activity* (cpm/mg/hour[a])	*Recovery* (%)	*Purification*
Plastids	484	198,924	411		
Acetone powder extract	230	159,160	692	80	1·6
Ammonium sulphate, 25–40% saturation	43·4	137,275	3,161	69	7·7

[a] Counts per minute incorporated into phytoene from isopentenyl pyrophosphate-4-^{14}C.

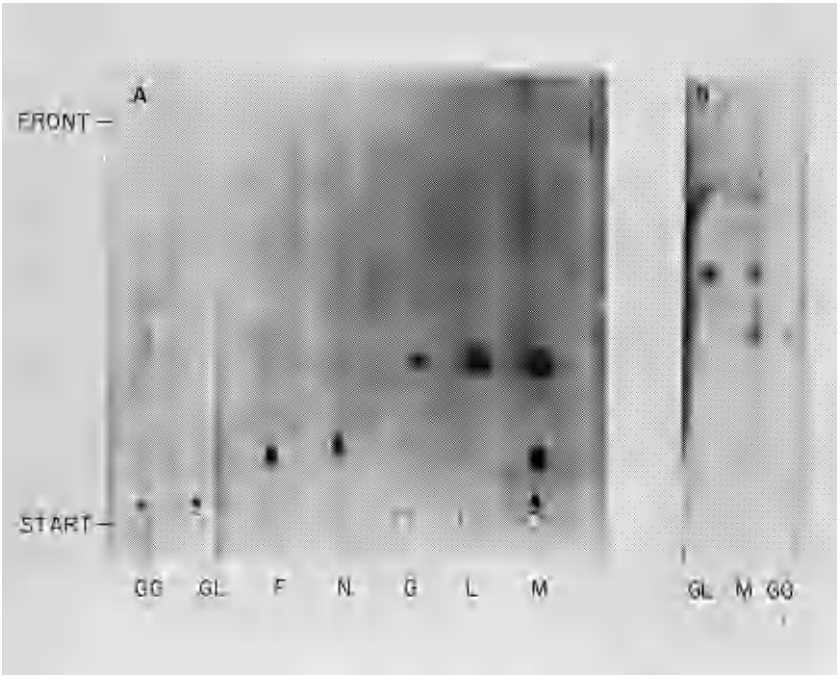

Figure 19A. The separation of terpenols on a thin layer of paraffin-impregnated Silica Gel G. The solvent was methanol:water (70:30) saturated with paraffin and the distance travelled was 15 cm (90 minutes). The plates were sprayed with a freshly prepared solution of phosphomolybdic acid (1·5 g) in 100 ml of ethanol. The plates were then heated to 120° for 5 minutes to effect the colour reaction. Geranylgeraniol (GG), geranyllinalool (GL), farnesol (F), nerolidol (N), geraniol (G), linalool (L) and mixture (M).

Figure 19B. The separation of terpenols on a thin layer of Silica Gel G, activated for 30 minutes at 110°. A solvent system of benzene: ethyl acetate (80:20) was used. The details of the development of coloured spots ae given in A. Geranylgeraniol (GG) and geranyl linalool (GL) were spotted as a mixture (M).

Structures of phytoene, phytofluene and ζ-carotene

One of our early studies on phytoene was concerned with the geometric configuration of this compound[50]. These studies were influenced appreciably by the syntheses of phytoene, phytofluene and ζ-carotene performed by Weedon and associates[51]. Some of their data, as well as that of Rabourn *et al.*[52, 53] suggested that phytoene and phytofluene are *cis*-carotenes. Jungalwala and Porter[50] achieved a complete separation of naturally occurring and chemically synthesized phytoene by chromatography on alumina. This separation is shown in *Figure 20*. Further studies with the naturally occurring compound and chemically synthesized *trans*-phytoene provided conclusive evidence that naturally occurring phytoene is a *cis*-

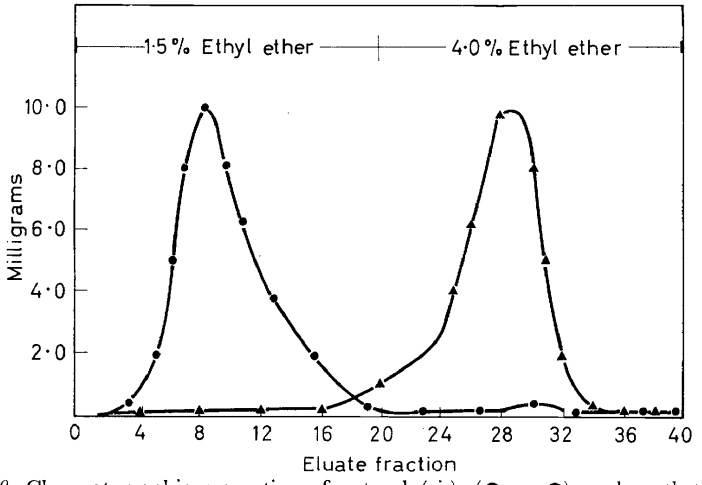

Figure 20. Chromatographic separation of natural (*cis*) (●——●) and synthetic (*trans*) (▲——▲) phytoene on a column of alumina.

isomer. The behaviour of naturally occurring phytoene on iodine isomerization is shown in *Figure 21*. It will be noted that a shift in light absorption towards longer wavelengths, with sharper maxima and minima, occurred on isomerization. Further studies included analyses of naturally occurring and *trans*-phytoene by infra-red light absorption and nuclear magnetic resonance. From these studies it was shown that phytoene and phytofluene are 15,15′-*cis*-carotenes, whereas ζ-carotene has the all-*trans* structure[50], *Figure 22*.

Formation of other acyclic carotenes

Early experiments by Beeler and Porter[54] provided evidence for the conversion of a small amount of phytoene to phytofluene by a tomato plastid enzyme system. The chromatographic separation of phytoene and phytofluene formed by this incubation mixture is shown in *Figure 23*. Proof of the incorporation of radioactivity into phytofluene was obtained through hydrogenation and gas-liquid chromatography of the product.

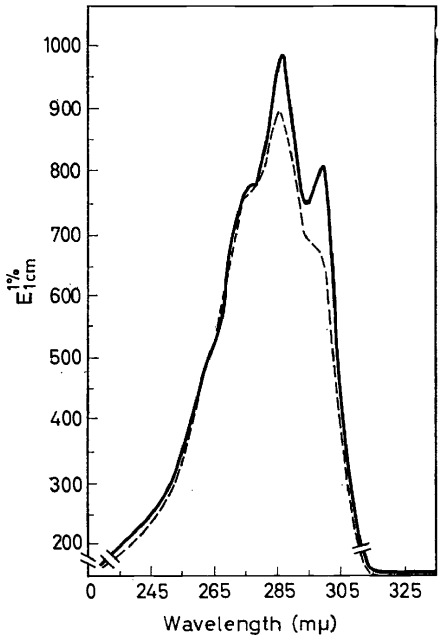

Figure 21. Ultraviolet light absorption curves of natural phytoene in petroleum ether: (— — — —) fresh solution; and (————) after isomerization by iodine.

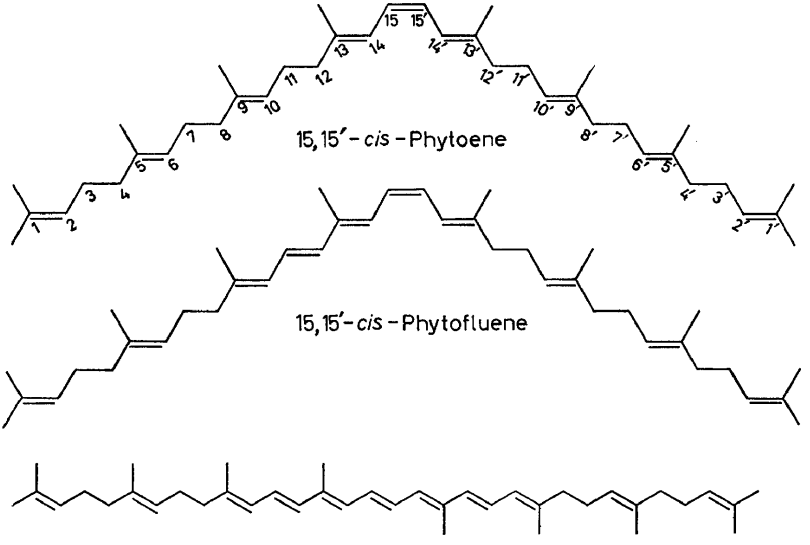

Figure 22. Structures of naturally occurring phytoene, phytofluene and ζ-carotene.

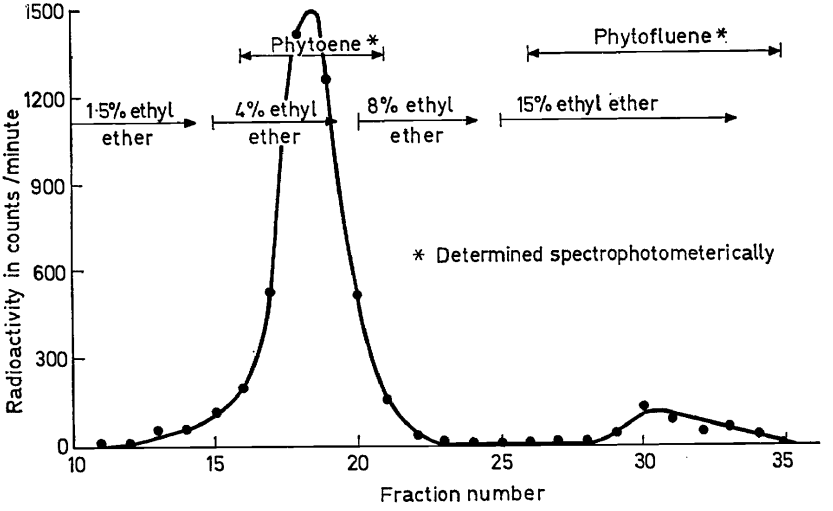

Figure 23. The chromatographic separation of phytoene and phytofluene.

Recently Suzue and Porter[55] reported that an extract of an acetone powder of tomato fruit plastids forms phytoene, phytofluene, neurosporene, and lycopene from isopentenyl pyrophosphate. The quantity of each of the products formed from isopentenyl pyrophosphate is shown in *Table 10.* Proof of the conversion of isopentenyl pyrophosphate to the above carotenes was obtained by hydrogenation and gas-liquid chromatography of the product. The coincidence of radioactivity and mass of lycopersane, obtained from the lycopene synthesized in this system, on gas-liquid chromatography, is shown in *Figure 24.* Since it was previously shown that isopentenyl pyrophosphate is converted to phytoene by this system[45], it is concluded that the system also converts phytoene to phytofluene, neurosporene and lycopene. The sequence of reactions proposed for these conversions is shown in *Figure 25.*

It is evident from the data that have been presented that a soluble extract of an acetone powder of tomato fruit plastids contains all the enzymes required for the conversion of mevalonic acid to lycopene. Whether such a preparation contains the enzymes for the formation of mevalonic acid from acetate or acetyl-CoA awaits the completion of experiments now in progress.

Table 10. Incorporation of isopentenyl pyrophosphate-4-^{14}C into carotenes

	Experiment 1 (d.p.m.)	*Experiment 2* (d.p.m.)
Phytoene	950,580	968,145
Phytofluene	14,080	17,090
Neurosporene	540	3,371
Lycopene	17,520	19,434

The incubation mixture for the synthesis of lycopene, neurosporene phytofluene and phytoene contained isopentenyl pyrophosphate-4-^{14}C, 124 mμmoles and $1{\cdot}38 \times 10^6$ d.p.m.; $MnCl_2$, 4 μmoles; $MgCl_2$, 30 μmoles; borate buffer, pH 8·2, 200 μmoles; dithiothreitol, 3·0 mg; and enzyme protein (25–40 per cent saturation, 10·9 mg; 40–65 per cent saturation, 1·1 mg) in a final volume of 1 ml. Incubations were carried out for 6 hours at 18°.

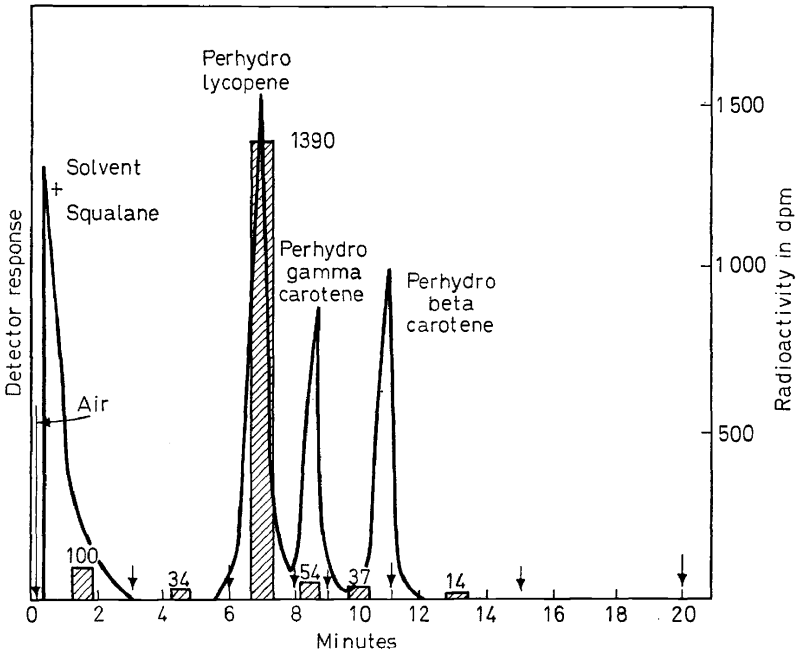

Figure 24. Gas-liquid chromatography of hydrogenated lycopene (lycopersane) synthesized from isopentenyl pyrophosphate -4-^{14}C. Separations were made on a 6 ft×6 mm column of 1 per cent SE-30 on Gaschrome Q, at a temperature of 250° and an argon flow rate of 100 ml/min. The amount of radioactivity in each effluent fraction trapped on leaving the chromatographic column is given on the figure in dpm. A total of 2055 dpm of hydrogenated lycopene was subjected to gas-liquid chromatography and 1390 dpm were recovered in lycopersane.

CONVERSION OF LYCOPENE TO CYCLIC CAROTENES

Recently we completed a study on the conversion of lycopene to cyclic carotenes[56]. In this study lycopene 15,15'-^{3}H was used as the substrate. This compound was a generous gift from Dr. Isler of Hoffman La Roche, Incorporated. The tritated lycopene was subjected to column chromatography prior to use. Also, an aliquot of the chromatographically purified lycopene was crystallized repeatedly with carrier nonradioactive lycopene. No change in the specific radioactivity of the chromatographically purified lycopene was noted through four crystallizations.

Since prior papers had suggested that neurosporene[15, 57, 58] and lycopene[15, 59, 60] are substrates for the formation of cyclized carotenes, *Figure 26*, the current investigations were undertaken in an effort to establish that lycopene is or is not an intermediate in the formation of α- and β-ionine rings.

Initially experiments were performed with chloroplasts obtained from spinach leaves. Chloroplasts and tritium labelled lycopene were incubated with the components shown in *Table 11*. Later, incubations were made with

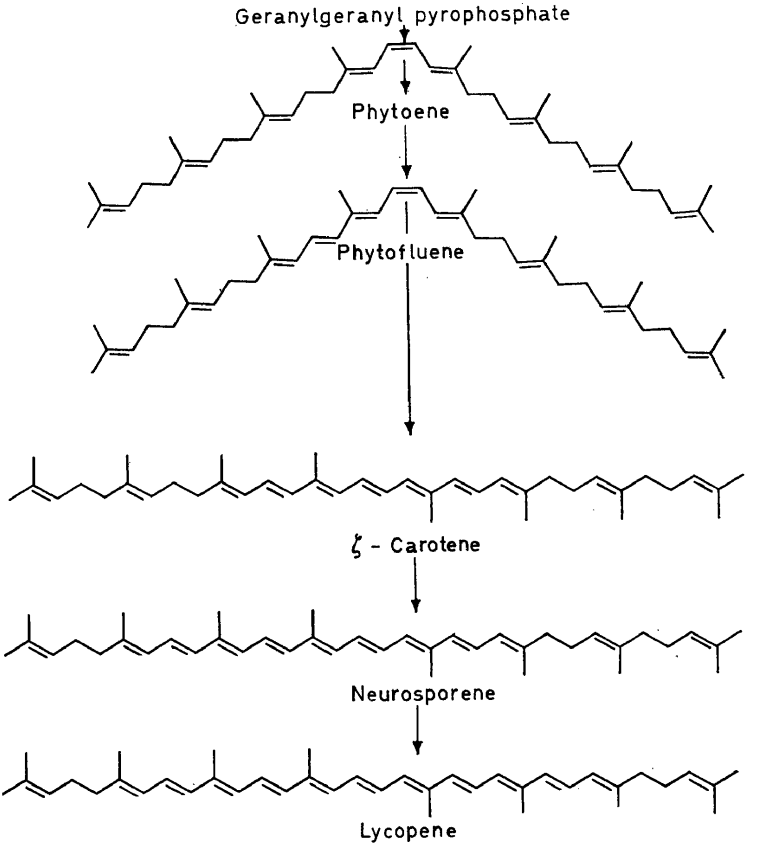

Figure 25. Proposed reactions for the conversion of geranylgeranyl pyrophosphate to lycopene.

soluble preparations obtained from spinach chloroplasts. The components for these incubations are also shown in *Table 11.* Initial experiments were concerned with proof of the conversion of lycopene to β-carotene, and the conditions under which maximal conversion could be effected. *Table 12* presents the results of a typical experiment. Lycopene was converted to β-carotene by spinach chloroplasts in the presence of light or darkness, in air or nitrogen at 25°. At 0°, or in the presence of boiled chloroplasts, no conversion occurred. Proof of the incorporation of lycopene into β-carotene was obtained through chromatography on two adsorbents and crystallization

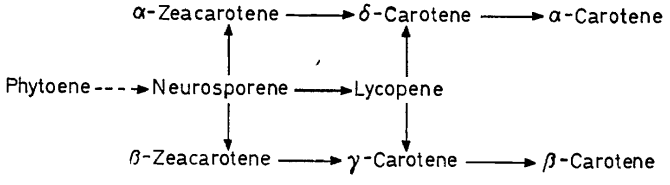

Figure 26. Proposed pathways for the formation of cyclic carotenes.

Table 11. Incubation mixture for the enzymatic conversion of 15,15′-^{3}H-lycopene to cyclic carotenes

Components	*Quantity*
	Plastids
Lycopene-^{3}H ($3{\cdot}3 \times 10^8$ dpm/μmole)	$3–6 \times 10^5$ dpm
Tween-80	2 mg
Phosphate buffer 0·1 M (pH 7·0)	50 μmoles
NADP	3 μmoles
Plastids	1 ml
	Soluble System
Above (except plastids) plus	
FAD	4 μmoles
$MgCl_2$	30 μmoles
$MnCl_2$	3 μmoles
Dithiothreitol	20 μmoles
Soluble preparation of enzyme	1 ml
Total volume	2·0 ml

Table 12. Conversion of tritium labelled lycopene to β-carotene by spinach plastids under various incubation conditions

Conditions[a]	*Total radioactivity in β-carotene* *Experiment 1* (dpm)	*Experiment 2* (dpm)
Darkness, 0°, and nitrogen[a]	0	0
Light, 0°, and nitrogen	0	0
Darkness, 25°, and nitrogen	35,635	33,925
Light, 25°, and nitrogen	44,000	43,209
Light, 25°, and nitrogen, minus NADP	27,682	29,897
Light, 25°, and air	31,098	32,906
Light, 25°, and nitrogen (boiled plastids)[b]	0	0

[a] Incubation mixtures contained lycopene-15,15′-^{3}H, 3×10^6 dpm and 8·4 mμmoles; Tween 80, 2 mg; phosphate buffer, pH 7·0, 50 μmoles; NADP, 2 mg; and plastids, 1 ml, in a final volume of 1·5 ml. The incubations were carried out for 1 hour.

[b] The incubation system contained 1 ml of boiled plastids instead of 1 ml of fresh plastids. All other components were the same as reported in[a].

of the β-carotene to constant specific radioactivity. No appreciable change occurred in the specific radioactivity of the chromatographically purified β-carotene on crystallization. Since a somewhat higher conversion of lycopene to β-carotene was obtained in the presence of light and nitrogen, subsequent incubations were made under these conditions at 25°.

Further studies were concerned with the conversion of lycopene to other carotenes by spinach chloroplasts, hi-*beta* and hi-*delta* tomato fruit plastids, and soluble extracts of each of these plastids. The carotenes formed from radioactive lycopene by these systems were separated from one another by chromatography on alumina and subsequent rechromatography on magnesium oxide Super Cel columns. A typical separation of the various carotenes found in an incubation mixture is shown in *Figure 27.* In these experiments, authentic carrier carotenes were added to incubation mixtures when these carotenes were not already present.

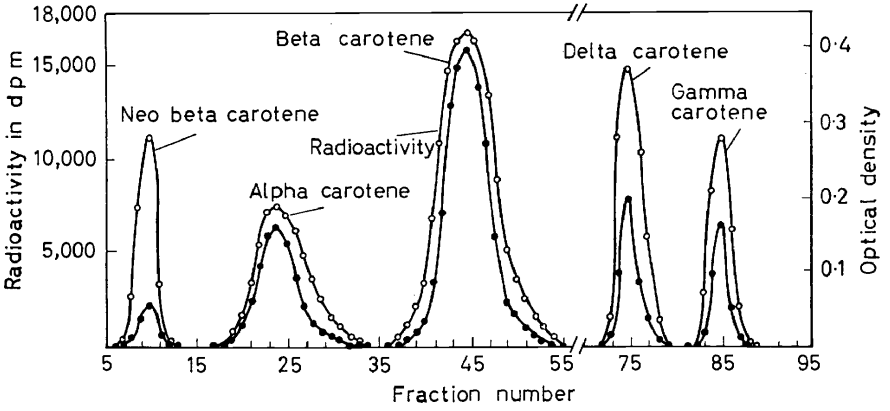

Figure 27. The separation of carotenes synthesized from lycopene-15-15′-^{3}H by spinach plastids on a column of 3 per cent (v/w) water deactivated alumina. The chromatogram was developed with petroleum ether containing increasing amounts of diethyl ether. Radioactivity (●——●) and optical density (○——○) measurements were made on each eluate collected. Optical density measurements were made at 445, 444, 450, 455 and 460 mμ, respectively, for neoβ, α-, β-, δ- and γ-carotenes. The coincidence of radioactivity and optical density were nearly the same, as shown in this figure, for each carotene synthesized from lycopene-15,15′-^{3}H by a soluble preparation of spinach plastids or by plastids or soluble preparations of plastids of 'hi-*beta*' and 'hi-*delta*' tomatoes.

The conversion of lycopene to cyclic carotenes by spinach chloroplasts and a soluble extract of chloroplasts is shown in *Table 13.* The spinach enzyme system converts lycopene to neo β-, α-, γ-, δ- and β-carotenes. Similarly, the soluble extract of chloroplasts converts lycopene to neo β- and β-carotene. Presumably the soluble extract would also effect the conversion of lycopene to the other cyclic carotenes. However, analyses were not made for the incorporation of radioactivity into these compounds in the experiment reported in *Table 13.*

Table 13. Enzymatic conversion of tritium labelled lycopene to cyclic carotenes by plastids and a soluble preparation of plastids from spinach

System	*Total radioactivity in various carotenes*[e]				
	Neo β-	*α-*	*β-* (dpm)	*δ-*	*γ-*
Plastids					
1[a]	4,589	35,000	93,386	17,600	15,265
2[b]	1,012	—[f]	10,604	—	—
Soluble extract					
1[c]	2,688	—	29,670	—	—
2[d]	0	0	0	—	—

[a] The incubation mixture contained lycopene-^{3}H, 600,000 dpm and 1·67 mμmoles; Tween 80, 2 mg; phosphate buffer, pH 7·0, 50 μmoles; NADP, 2 mg; and plastids, 2 ml, in a final volume of 2·5 ml.
[b] The incubation mixture contained lycopene-^{3}H, 480,000 dpm and 1·34 mμmoles; Tween 80, 2 mg; phosphate buffer, pH 7·0, 50 μmoles; NADP, 2 mg; and plastids, 1 ml, in a final volume of 1·5 ml.
[c] The incubation mixture contained lycopene-^{3}H, 480,000 dpm and 1·34 mμmoles; Tween 80, 2 mg; phosphate buffer, pH 7·0, 50 μmoles; NADP, 2 mg; FAD, 30 μg; $MgCl_2$, 30 μmoles; $MnCl_2$, 3 μmoles; and soluble extract, 1·0 ml, in a final volume of 2·0 ml.
[d] All components were the same as reported in [c], except that the incubation mixture contained 1 ml of boiled soluble extract instead of 1 ml of soluble extract.
[e] All incubations were one hour in duration.
[f] Indicates that determinations of radioactivity were not made.

Table 14. Enzymatic conversion of tritium labelled lycopene to cyclic carotenes by plastids and a soluble preparation of plastids from 'hi-*beta*' tomato fruits

System	*Time of incubation* (hr)	*Total radioactivity in carotenes*				
		Neo β-	α-	β- (dpm)	δ-	γ-
Plastids						
1[a]	12	309	50	3,360	—[h]	—
2[b]	5	203	126	2,285	346	8,227
3[c]	5	0	0	0	0	0
Soluble extract						
1[d]	4	—	—	780	—	3,450
2[e]	4	—	624	1,730	146	17,515
3[f]	4	0	0	0	0	0
4[g]	4	0	0	0	0	0

[a] The incubation mixture contained lycopene 15,15′-^{3}H, 345,000 dpm and 0·96 mμmoles; Tween 80, 2 mg; NADP, 2 mg; phosphate buffer, pH 7·0, 50 μmoles; and plastids, 1 ml, in a final volume of 1·5 ml.
[b,c] The incubation mixtures contained 2 ml of plastids and 1 ml of boiled plastids, respectively, instead of 1·0 ml of fresh plastids as described under [a]; all other components were the same.
[d] The incubation mixture contained lycopene-^{3}H, 600,000 dpm and 1·67 mμmoles; Tween 80, 2 mg; phosphate buffer, pH 7·0, 50 μmoles; $MgCl_2$, 30 μmoles; $MnCl_2$, 3 μmoles; NADP, 2 mg; FAD, 30 μg; DTT, 3 mg; and soluble extract, 1 ml, in a final volume of 2·0 ml.
[e] All components of the incubation mixture were the same as in [d], except that 2 ml instead of 1 ml of soluble extract was used.
[f] All components of the incubation mixture were the same as in [d], except that FAD was not added.
[g] All components of the incubation mixture were the same as in [d], except that 1 ml of soluble extract was replaced by 1 ml of boiled extract.
[h] Indicates that determinations of radioactivity were not made.

The results of experiments on the conversion of radioactive lycopene to cyclic carotenes by plastids and soluble extracts of plastids of hi-*beta* and hi-*delta* tomato fruits are shown in *Tables 14* and *15*. The hi-*beta* and hi-*delta* tomato fruits were a generous gift from Dr. Mark Tomes of Purdue University. The characteristics of these tomatoes have been reported previously[59, 61–63]. The plastids of hi-*beta* tomato fruits and soluble extracts of these plastids convert lycopene to δ-, γ-, α- and β-carotenes (*Table 14*). The

Table 15. Enzymatic conversion of tritium labelled lycopene to cyclic carotenes by plastids and a soluble preparation of plastids from 'hi-*delta*' tomato fruits

System	*Time of incubation* (h)	*Total radioactivity in carotenes*		
		α-	β- (dpm)	δ-
Plastids				
1[a]	12	986	526	8,328
2[b]	5	686	290	4,338
Soluble system				
1[c]	2	389	1,083	7,240
2[d]	4	1,669	960	7,895
3[e]	4	0	0	0

[a] The incubation mixture contained lycopene-15,15′-^{3}H, 345,000 dpm and 0·96 mμmoles; NADP, 2 mg; Tween 80, 2 mg; phosphate buffer, pH 7·0, 50 μmoles; and plastids, 1 ml; in a final volume of 1·5 ml.
[b] The incubation mixture contained 2 ml instead of 1 ml of plastids; all other components were the same as in [a].
[c,d] Incubation mixtures contained lycopene-^{3}H, 345,000 dpm and 0·96 mμmoles; Tween 80, 2 mg; phosphate buffer, pH 7·0, 50 μmoles; $MgCl_2$, 30 μmoles; $MnCl_2$, 3 μmoles; NADP, 2 mg; FAD, 30 μg; DTT, 3 mg; and soluble extract of plastids, 1 ml; in a final volume of 2·0 ml.
[e] The incubation mixture contained 1 ml boiled soluble extract instead of 1·0 ml of fresh extract; all other components were the same as described in [c] and [d].

principal radioactive compounds formed are γ- and β-carotene, as expected from the pigment composition of these tomato fruits.

The results of experiments on the conversion of lycopene to cyclic carotenes by plastids and a soluble extract of plastids of hi-*delta* tomato fruits are reported in *Table 15.* Lycopene is converted to α-, δ- and β-carotenes by an enzyme system present in the plastids and a soluble extract of plastids of these fruits. As expected, the major radioactive compounds were δ- and α-carotene.

Further proof for the conversion of lycopene to mono- and di-cyclic carotenes was obtained through hydrogenation of each chromatographically purified carotene and subsequent gas-liquid chromatography of the hydrogenated product. Almost all of the radioactivity subjected to gas-liquid chromatography was coincident with the mass of the respective perhydro carotene (*Figures 28* and *29*). Thus it is evident that plastids and soluble extracts of plastids from spinach leaves and hi-*beta* and hi-*delta* fruits effect the conversion of lycopene to mono- and dicyclic carotenes.

Studies have been made of the cofactor requirements for the formation of cyclic carotenes from lycopene. *Table 16* shows the effect of the removal of various components from a complete incubation system on the conversion

Table 16. Cofactor requirements for the conversion of lycopene to cyclic carotenes

System	*Total radioactivity in carotenes* β (dpm)	α (dpm)
Complete (light)	4894	876
Complete (dark)	4598	709
$-MgCl_2$	1659	0
$-MnCl_2$	0	0
−NADP	0	0
−FAD	0	0

of lycopene to carotenes containing α- and β-ionone rings. It is evident from this table that FAD, NADP, Mn^{++} and Mg^{++} are required for the formation of cyclic carotenes. The exact function of these components in the conversion of lycopene to cyclic carotenes is not known. However, it is evident that each of these components is required for the cyclization reaction.

A mechanism has been proposed[56] for the conversion of lycopene to cyclic carotenes. This mechanism is shown in *Figure 30.* In this proposal it is suggested that the reaction is initiated by the addition of a proton to carbon atom 2 of lycopene. A carbonium ion is formed at carbon atom 1. Condensation then takes place between carbon atom 1 and carbon atom 6 of the lycopene molecule. This reaction results in the formation of a second carbonium ion which is stabilized by the removal of a proton. Stereospecific removal of a proton from the molecule would result in the formation of an α- or β-ionone ring. Presumably separate and specific enzymes exist in tomato and spinach plastids for the formation of α- and β-ionone rings.

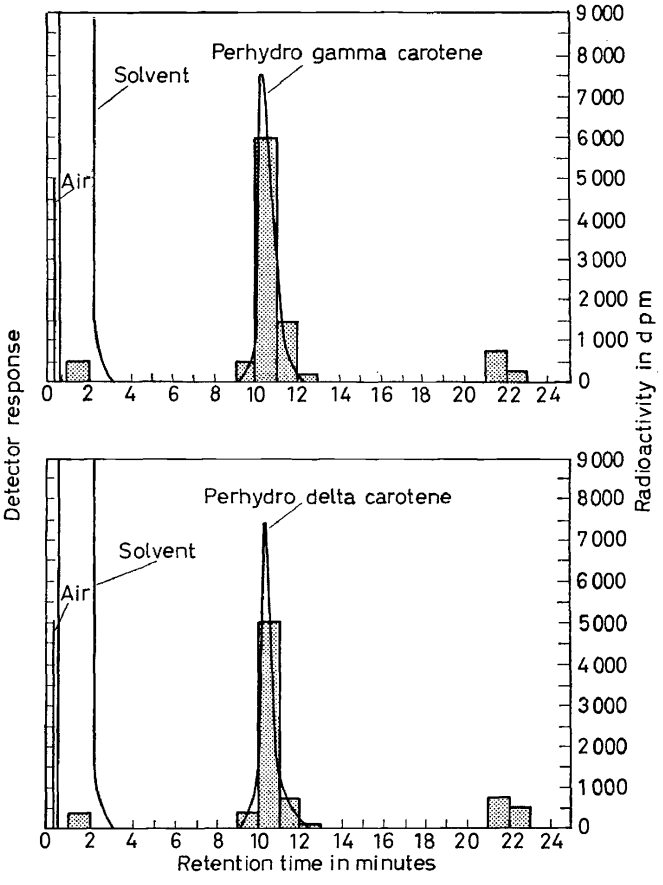

Figure 28. The gas-liquid chromatographic separation of monocyclic perhydro carotenes. These carotenes were synthesized from lycopene-15-15′-^{3}H by spinach plastids. Effluents were trapped on glass wool for one minute as indicated on the figure. The shaded areas indicate radioactivity. Similar results were obtained when the enzyme source was a soluble preparation of spinach plastids, 'hi-*beta*' or 'hi-*delta*' tomato fruit plastids, or soluble extracts of these plastids.

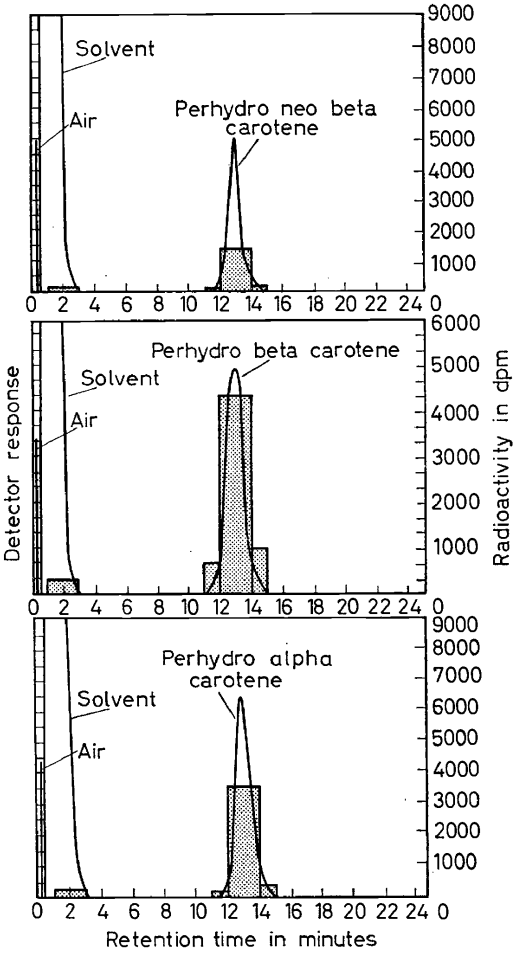

Figure 29. The gas-liquid chromatographic separation of dicyclic perhydro carotenes. These carotenes were synthesized from lycopene-15-15′-^{3}H by spinach plastids. Effluents were trapped on glass wool for one or two minutes as indicated on the figure. The shaded areas indicate radioactivity. Similar results were obtained when the enzyme source was a soluble preparation of spinach plastids, or plastids or soluble preparations of 'hi-*beta*' and 'hi-*delta*' tomato fruit plastids.

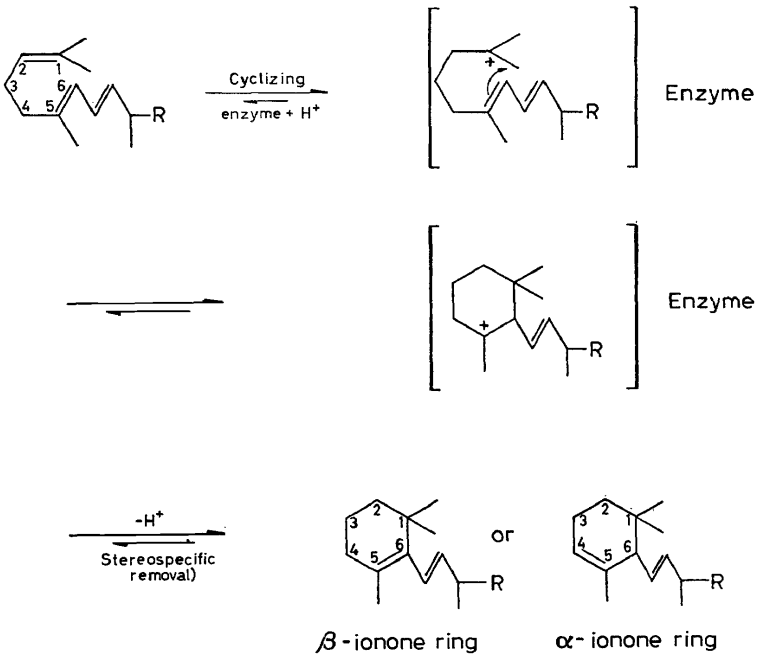

Figure 30. A possible mechanism for the conversion of lycopene to cyclic carotenes.

It is a pleasure to acknowledge the research contributions of those individuals who have investigated various aspects of carotene biosynthesis in the author's laboratory. Special mention should be made of Drs. Anderson, Beeler, Jungalwala, Shah, Suzue, Kushwaha and Subbarayan, and Mr. Feldbruegge. It should also be mentioned that investigations with animal enzymes performed in the author's laboratory have had a considerable influence on our studies on carotene biosynthesis. The names of the individuals involved in these studies are found in the list of references.

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