The Conversion of Phytoene-14C to Acyclic, Monocyclic, and Dicyclic Carotenes and the Conversion of Lycopene-15,15'-3H to Mono- and Dicyclic Carotenes by Soluble Enzyme Systems Obtained from Plastids of Tomato Fruits*

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SUMMARY

Proof is presented in this paper for the formation of acyclic and cyclic carotenes from phytoene-14C by soluble extracts of plastids obtained from fruit of different genetic selections of the tomato. The carotenes biosynthesized from phytoene and the tomato selections used were the following: (a) cis- and trans-phytofluene, neurosporene, neolycopene, lycopene, and \( \alpha \)- and \( \beta \)-carotenes, red tomato fruit plastids; (b) cis- and trans-phytofluene, neurosporene, lycopene, and \( \gamma \), \( \delta \), \( \gamma \)-, \( \alpha \)-, and \( \beta \)-carotenes, "Hi-\( \beta \)" tomato fruit plastids; (c) cis- and trans-phytofluene, neurosporene, lycopene, \( \gamma \)- and \( \beta \)-carotenes, "Hi-\( \beta \)" tomato fruit plastids, and (d) cis- and trans-phytofluene, prolycopene, proneurosporene, lycopene, and \( \gamma \)- and \( \beta \)-carotenes, Golden Jubilee variety tomato fruit plastids. Experimental evidence for the conversion of lycopene-15,15'-3H to mono- and dicyclic carotenes by a soluble enzyme system obtained from red tomato fruit plastids is also presented in this paper.

Proof for the formation of the above carotenes from radioactive phytoene was obtained by cochromatography with authentic nonradioactive carotenes on an alumina chromatographic column. A close correspondence between radioactivity and absorbance for each carotene was observed. Further proof for the formation of acyclic and cyclic carotenes from radioactive phytoene was obtained by gas-liquid chromatography of the hydrogenated products. Coincidence between mass and radioactivity was also observed.

The conversion of phytoene to phytofluene by the tomato enzyme systems appears to be dependent upon the presence of NADP, whereas the conversion of the latter compound to lycopene appears to require FAD and Mn++. The formation of lycopene is also increased in the presence of Mg++ and dithiothreitol.

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The first detailed pathway of biosynthesis of carotenoids was proposed by Porter and Lincoln (1) in 1950. This pathway was proposed from the data available at that time on the structures of the acyclic carotenoids and on the occurrence of both cyclic and acyclic carotenoids in tomatoes. This biosynthetic pathway was modified in 1962 (2) and later in 1967 (3) when the structures of acyclic carotenoids were completely established through chemical synthesis and the results of tracer studies on the biosynthesis of terpenyl pyrophosphates, squalene, and the carotenoids became available.

The work of several investigators supported the proposed biosynthetic pathway (2, 3). Thus the isolation of mutants of Chlorella vulgaris (4), Rhodopseudomonas spheroides (5), and Neurospora crassa (6), which had lost the ability to form wild type carotenoids, and which contained one or more of the more saturated acyclic carotenoids, provided indirect evidence in support of the proposed pathway (2, 3). Further support for the proposed pathway was provided by Jensen et al. (7), who showed that a decrease in the concentration of the accumulated more saturated carotenoids, provided indirect evidence in support of the proposed pathway (2, 3). Further support for the proposed pathway was provided by Jensen et al. (7), who showed that a decrease in the concentration of the accumulated more saturated carotenoids of diphenylamine-inhibited Rhodospirillum rubrum cells was accompanied by a simultaneous increase in lycopene and spirilloxanthin when the inhibitor was removed from the culture.

More direct evidence for the proposed pathway (2, 3) was obtained from results of experiments from our laboratory and by Chichester and his associates. Early studies from our laboratory with ripening tomatoes and isolated tomato fruit plastids showed the incorporation of radioactivity of mevalonic acid (8, 9) and terpenyl pyrophosphates (10, 11) into each of the acyclic and cyclic carotenoids. The incorporation of radioactivity of isopentenyl pyrophosphate into lycopene by tomato fruit homogenates (13) and the synthesis of \( \beta \)-carotene from mevalonic acid-2,14C (14) by a cell-free extract of Phycomyces blakesleeanus were reported by Chichester and associates. Later Jungalwala and Porter (15) reported the solubilization, through preparation of a acetone powder of tomato fruit plastids, and the partial purification of the enzyme system for the synthesis of phytoene from isopentenyl and
farnesyl pyrophosphates. Shah et al. (16), using the same system, demonstrated the formation of phytoene from geranylgeranyl pyrophosphate-2,4C. Suzue and Porter (17) later reported the incorporation of radioactivity of isopentenyl pyrophosphate-4,4C into phytoene, phytofluene, neurosporene, and lycopene by the above soluble and partially purified tomato enzyme system. Lee and Chichester (18), using a cell-free enzyme system from a P. blakesleeanus R1 mutant, showed the conversion of geranylgeranyl pyrophosphate-2,4C to phytoene, phytofluene, δ-carotene, neurosporene, and lycopene.

Studies on the enzymatic interconversion of carotenoids are relatively few. The conversion of phytoene to phytofluene by isolated tomato fruit plastids has been reported by Buehler and Porter (19) and the formation of β-carotene from phytol by chloroplasts of corn leaves has been reported by Costes (20). Decker and Uehleke (21) earlier reported the conversion of lycopene to β-carotene by spinach chloroplasts and the reverse conversion of β-carotene to lycopene by tomato slices. Recently, Kushwaha et al. (22) demonstrated the conversion of tritiated lycopene to cyclic carotenoids by soluble extracts of spinach chloroplasts and Hi-β and Hi-δ tomato fruit plastids, and Subbarayan et al. (23) demonstrated the enzymatic conversion of isopentenyl pyrophosphate and phytoene-14C to acyclic carotenoids by an ammonium sulfate-precipitated spinach enzyme system. From the above results it is generally assumed that the more unsaturated carotenoids are formed from phytoene through step-wise dehydration and that lycopene is then converted to mono- and dicyclic carotenoids.

Experimental evidence for the enzymatic conversion of phytoene to carotenoids other than phytol has not been reported, except for the data of Subbarayan et al. (23). Hence the present work was undertaken in an effort to show that phytoene-14C is converted to the more unsaturated acyclic and cyclic carotenoids by a soluble enzyme system obtained from tomato fruit plastids.

EXPERIMENTAL PROCEDURE

Materials—Red tomato fruits were purchased locally, whereas Hi-β, Hi-δ, and Golden Jubilee tomato fruits were generous gifts from Dr. Mark L. Tomes, Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana. Lycopene-15,15′-HI was a generous gift from Dr. O. Isler of Hoffmann-La Roche, Inc., Basel, Switzerland. γ-Carotene was isolated from a strain of Penicillium sclerotiorum, according to the method of Mase (24). δ-Carotene was isolated from fruit of Hi-δ tomato and proeneurosporene and prolycopene were isolated from Golden Jubilee tomatoes. cis- and trans-Phytofluene were isolated from red tomatoes. Other materials were obtained as follows: crystalline α- and β-carotenes, FAD, and NADP from Sigma; EDTA from Fisher; Tween 80 from E. H. Sargent (Chicago); alumina, chromatographic grade, from Merck; 2-mercaptoethanol and dithiothreitol (Cleland's reagent) from Calbiochem; Adam's catalyst from K and K Laboratories; and 2,4-C-mevalonic acid from New England Nuclear. Petroleum ether (30–60°, analytical reagent grade) was passed through silica gel, Grade 12 (Grace Division, Davison Chemical Company), and then distilled before use. All other reagents were of analytical grade.

Preparation of Substrates—Radioactive phytoene was prepared according to the method of Suzue and Porter (17). Incubations for the synthesis of radioactive phytoene were carried out as reported by the above authors, but a slightly different procedure was followed for the isolation of the product. Incubations were carried out for 6 hours and then the reaction was terminated by the addition of 4 ml of ethanol to the incubation mixture. Non-saponifiable compounds were extracted five times, each with 5-ml portions of petroleum ether. The combined petroleum ether extract was washed with water and dried over anhydrous sodium sulfate and then concentrated to a volume of 5.0 ml with a gentle stream of nitrogen. The extract was chromatographed on a column, 1.8 × 20 cm, of 1% (v/w) water-deactivated alumina (50 g). Carrier phytoene was not added to the column. The chromatographic column was developed with 0.5% and 1% peroxide-free diethyl ether in petroleum ether. Ten-milliliter eluate fractions were collected and an aliquot (100 μl) was assayed for radioactivity in a toluene-scintillator solution in a Packard Tri-Carb liquid scintillation spectrometer. Usually phytoene was eluted from the column with 1% diethyl ether in petroleum ether. The fractions containing radioactivity were combined, evaporated to dryness under oxygen-free nitrogen, and then redissolved in petroleum ether. The phytoene fraction was rechromatographed once more under the same conditions. An aliquot of rechromatographed material was mixed with authentic nonradioactive phytoene and the mixture was chromatographed on an alumina column. Absorbance at 280 mμ and radioactivity were determined on each eluate fraction. Good correspondence was obtained between the plots of radioactivity and absorbance. Aliquots of each of the fractions containing radioactivity were mixed, reduced to a small volume under oxygen-free nitrogen, hydrogenated overnight, and then subjected to gas-liquid chromatography to determine the purity of the product. A single component was found and almost all of the radioactivity was associated with the mass of the hydrogenated phytoene. This chromatographically purified phytoene was then used as a substrate in all incubations.

Preparation of Tomato Enzyme System—Tomato fruit plastids and an acetone powder of these plastids were prepared as reported by Jungalwala and Porter (15). The acetone powder was extracted with phosphate buffer as described previously (16) and then fractionated with ammonium sulfate under nitrogen. The protein fractions precipitating between 20 and 40% and between 40 and 60% of saturation were dissolved in 0.1 M phosphate buffer, pH 7.0, and the protein fraction was found to be stable for at least 4 weeks when stored under nitrogen at -20°. The stored enzyme fractions were kept at 4°C for 24 hours prior to use. The slurry was then centrifuged at 105,000 × g for 2 hours in a Spinco model L ultracentrifuge. The protein fraction of the supernatant solution precipitating between 0 and 80% of saturation was dissolved in 0.1 M phosphate buffer, pH 7.0, containing 0.001 M DTT. Each enzyme fraction was found to be stable for at least 4 weeks when stored under nitrogen at -20°. The stored enzyme fractions were dialyzed against 0.05 M phosphate buffer, pH 7.0, containing 0.001 M 2-mercaptoethanol for 3 hours, with a change of buffer at the end of 1.5 hours, just before use.

Soluble enzyme systems were prepared from Hi-β, Hi-δ, and Golden Jubilee tomato fruit plastids as follows. Plastids were added to an approximately equal quantity of 120 mesh glass beads and then ground with a pestle and mortar for 2 min to make a thick slurry. The glass beads and the pestle and mortar were kept at 4°C for 24 hours prior to use. The slurry was then extracted twice with 0.1 M phosphate buffer, pH 7.0, and the extract was centrifuged at 105,000 × g for 2 hours in a Spinco model L ultracentrifuge. The protein fraction of the supernatant solution precipitating between 0 and 80% of saturation with ammonium sulfate was dissolved in 0.1 M phosphate buffer, pH 7.0, containing 0.001 M DTT and stored as described above. The stored enzyme was dialyzed as described in the previous paragraph just before use.

1 The abbreviation used is: DTT, dithiothreitol.
Enzymatic Synthesis of Carotenes

Incubation System for Synthesis of Acyclic, Monocyclic, and Dicyclic Carotenes—A typical incubation system for the enzymatic synthesis of carotenes was prepared as follows. A petroleum ether (distilled twice) solution of phytoene-14C was placed in a 20-ml incubation tube and the solvent was removed by a gentle stream of oxygen-free nitrogen. Tween 80, 2 mg, in 200 µl of acetone (purified) was added and the acetone was removed with oxygen-free nitrogen. Borate buffer (pH 7.5, 250 µmoles), DTT (19.4 µmoles), MgCl₂ (3 µmoles), NADP (3.66 µmoles), FAD (3.8 mµmoles), and enzyme protein (ammonium sulfate precipitate, 20 to 40% saturation, 10 mg; 40 to 60% fraction, 10 mg) were added. The final volume of the incubation mixture was 3 ml. Incubations were carried out for 5 hours at 25° under nitrogen with gentle shaking on a Fisher clinical rotator.

<table>
<thead>
<tr>
<th>Carotene</th>
<th>Total radioactivity (dpm)</th>
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</thead>
<tbody>
<tr>
<td>cis-Phytofluene</td>
<td>9943</td>
</tr>
<tr>
<td>trans-Phytofluene</td>
<td>990</td>
</tr>
<tr>
<td>x-Carotene</td>
<td>2866</td>
</tr>
<tr>
<td>y-Carotene</td>
<td>1900</td>
</tr>
<tr>
<td>Neusorenone</td>
<td>2550</td>
</tr>
<tr>
<td>Neolycopenone</td>
<td>2788</td>
</tr>
<tr>
<td>Lycopene</td>
<td>3883</td>
</tr>
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</table>

**Table I**
Enzymatic conversion of phytoene-14C to more unsaturated carotenes by soluble enzyme system obtained from red tomato fruit plastids

The complete incubation system contained phytoene-14C, 2,393 mµmoles (250,000 dpm); Tween 80, 2 mg; borate buffer, pH 7.5, 250 µmoles; NADP, 3.66 µmoles; FAD, 3.81 µmoles; MgCl₂, 30 µmoles; MnCl₂, 3 µmoles; DTT, 19.4 µmoles; and enzyme protein (ammonium sulfate precipitate, 20 to 40% saturation, 10 mg; 40 to 60% saturation, 10 mg) in a final volume of 3 ml. Incubation was carried out for 5 hours in the dark at 25° under nitrogen.

<table>
<thead>
<tr>
<th>Carotene</th>
<th>Total radioactivity (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-Phytofluene</td>
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<tr>
<td>trans-Phytofluene</td>
<td>890</td>
</tr>
<tr>
<td>α-Carotene</td>
<td>2,580</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>3,900</td>
</tr>
<tr>
<td>γ-Carotene</td>
<td>2,100</td>
</tr>
<tr>
<td>δ-Carotene</td>
<td>12,100</td>
</tr>
<tr>
<td>y-Carotene</td>
<td>5,300</td>
</tr>
<tr>
<td>Neurosporene</td>
<td>2,823</td>
</tr>
<tr>
<td>Lycopene</td>
<td>4,107</td>
</tr>
</tbody>
</table>

**Table II**
Enzymatic conversion of phytoene-14C to more unsaturated carotenes by soluble enzyme system prepared from plastids of Hi-6 tomato fruits

The incubation system contained phytoene-14C, 3,329 mµmoles (400,000 dpm); Tween 80, 2 mg; borate buffer, pH 7.5, 250 µmoles; NADP, 3.66 µmoles; FAD, 3.81 µmoles; MgCl₂, 30 µmoles; MnCl₂, 3 µmoles; DTT, 19.4 µmoles; and enzyme protein (ammonium sulfate precipitate, 0 to 80% saturation, 25 mg) in a final volume of 3 ml. Incubations were carried out for 5 hours in the dark at 25° under nitrogen.

**Extraction and Separation of Carotenes—**Enzyme activity in the incubation mixture was terminated by the addition of 4 volumes of ethanol. The nonsaponifiable compounds were then extracted with five 5-ml portions of petroleum ether. Known amounts of carrier carotenes, freshly prepared from tomatoes, were added to the petroleum ether extract to minimize the destruction of the labeled carotenes by light and oxygen in subsequent operations. The petroleum ether extract was washed three times with water, dried over anhydrous sodium sulfate, and then chromatographed on a column, 1.8 x 20 cm, of 1% (v/w) water-deactivated alumina, 50 g. The upper surface of the alumina column was protected by a small amount of anhydrous sodium sulfate. The chromatogram was developed with increasing amounts of peroxide-free diethyl ether in petroleum ether (see legend to Fig. 1).

**Assay of Mass and Radioactivity of Carotenes—**A 1-ml aliquot of each eluate fraction, 10 ml, was assayed for radioactivity with a Packard Tri-Carb liquid scintillation spectrometer. The light absorption spectrum of each fraction eluted from the column was then determined with a Beckman DU spectrophotometer. The light absorption maxima and minima for each pigment were also determined with a Beckman DU spectrophotometer. Absorbance for each carotene, at its principal absorption maximum, was measured with a Beckman DU spectrophotometer. Coincidence between the absorbance at the principal absorption maximum for each carotene and radioactivity was obtained.

**Hydrogenation of the Separated Carotenes (9)—**Radioactive and nonradioactive cis- and trans-phytofluene, proeneoprosepine, prolycopenone, neurosporene, lycopene, and β-carotenes, separated by column chromatography as indicated above, were hydrogenated. Hydrogenation was carried out overnight with Adam’s catalyst at a pressure of 40 p.s.i. of hydrogen in petroleum ether-isopropyl alcohol in a Parr hydrogenator. After hydrogenation the perhydrocarotenes were extracted with petroleum ether. The extract was washed with water, dried over anhydrous sodium sulfate, and then chromatographed on a column, 1.8 x 7 cm, of alumina. The hydrogenated carotenes were eluted with petroleum ether. Of the radioactivity originally present in a carotene, 90 to 95% was routinely recovered in the perhydrocarotene on chromatography. The petroleum ether solution of the perhydrocarotene eluted from the alumina column was evaporated to dryness with a gentle stream of air in a graduated centrifuge tube. The hydrogenated carotene was then dissolved in a minimum of petroleum ether and subjected to gas-liquid chromatography.

**Gas-Liquid Chromatography of Perhydrocarotenes and Detection of Radioactivity (9)—**Perhydrocarotenes were chromatographed in a Barber-Colman model 10 gas chromatograph. Separation between acyclic, monocyclic, and dicyclic perhydrocarotenes was achieved on a column, 8 feet x 6 mm (internal diameter), of 2% SE-30 on Gas-Chrom-Q at 25° with an Argon flow of 100 ml per min. Fractions emerging from the gas-liquid chromatographic column were collected on glass wool with a Packard fraction collector. Each effluent fraction, trapped on leaving the gas-liquid chromatographic column, was eluted with toluene-scintillator solution and then assayed for radioactivity. All toluene-soluble samples were assayed for radioactivity in a diphenyloxazolodimethyl-p-bis-2-(5'-phenyloxazoyl) benzene-toluene solution in a Packard liquid scintillation spectrometer. Corrections for quenching were made with an automatic external
TABLE III
Enzymatic conversion of phytoene-14C to more unsaturated carotenes by soluble enzyme system obtained from plastids of Hi-β tomato fruits

The incubation mixture contained radioactive phytoene, 1.914 mmoles (200,000 dpm); Tween 80, 2 mg; borate buffer, pH 7.5, 250 μmoles; NADP, 3.66 μmoles; FAD, 3.81 μmoles; MgCl₂, 30 μmoles; MnCl₂, 3 μmoles; DTT, 19.4 μmoles; and enzyme protein (ammonium sulfate precipitate, 0 to 80% saturation, 21 mg) in a final volume of 3 ml. Incubations were carried out for 5 hours at 25° under nitrogen.

<table>
<thead>
<tr>
<th>Carotene</th>
<th>Total radioactivity (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-Phytolene</td>
<td>3400</td>
</tr>
<tr>
<td>trans-Phytolene</td>
<td>756</td>
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<tr>
<td>β-Carotene</td>
<td>506</td>
</tr>
<tr>
<td>γ-Carotene</td>
<td>8003</td>
</tr>
<tr>
<td>Neoxyporphyene</td>
<td>1100</td>
</tr>
<tr>
<td>Lycopene</td>
<td>1723</td>
</tr>
</tbody>
</table>

FIG. 1. The separation of carotenes synthesized from phytoene-14C by a soluble enzyme system obtained from plastids of Hi-β tomato fruits on a 1% (v/w) water-deactivated alumina column. The chromatogram was developed with petroleum ether containing increasing amounts of peroxide-free diethyl ether. Radioactivity (●) and absorbance (○) were measured for each eluate fraction collected. Absorbance measurements were made at 348, 444, 345, 425, 455, 460, 440, and 470 nm, respectively for cis-phytfluene, α-carotene, trans-phytofluene, β-, δ-, 9-, and γ-carotenes, neurosporone, and lycopene. The agreement between radioactivity and absorbance was nearly the same, as shown when each carotene synthesized from either radioactive phytoene or lycopene-15,15'-3H by a soluble enzyme system obtained from plastids of red, Hi-β, or Golden Jubilee tomato fruits was chromatographed on alumina. The eluate fractions from this chromatogram were each 50 ml (Fractions 1 to 9) or 10 ml (Fractions 10 to 150). The per cent of ether and the fractions collected were the following: 0.5, 1 to 3; 1, 4 to 6; 2, 7 to 9; 3, 10 to 15; 4, 16 to 20; 5, 21 to 25; 6, 26 to 30; 7, 31 to 35; 8, 36 to 40; 9, 41 to 45; 10, 46 to 50; 11, 51 to 55; 12, 56 to 60; 13, 61 to 65; 14, 66 to 70; 15, 71 to 75; 16, 76 to 80; 17, 81 to 85; 18, 86 to 90; 19, 91 to 95; 20, 96 to 100; 21, 101 to 105; 22, 106 to 110; 23, 111 to 120; 24, 121 to 130; 25, 131 to 150. Unreacted phytoene was recovered in Fractions 1 to 6. The total radioactivity in phytoene was approximately 40% of that added to the incubation mixture. Presumably the major loss of phytoene on incubation resulted from oxidation of this very labile compound. It should be pointed out that each lot of alumina has somewhat different adsorptive characteristics. Hence, each new lot needs to be standardized as to the per cent of diethyl ether and the volume to use to effect the separation shown on this figure. The separation of neurosporone, prolycopene, and neolycopene is not shown on this figure. These compounds were each separated from only a single genetic selection of tomatoes. Each carotene was identified by its light absorption spectrum and by its position on chromatography as reported previously: neolycopene (25, 36), neurosporone (formerly called protetrahydrolycopene and unidentified I) (1, 27), and prolycopene (1, 25-27).
FIG. 2. The gas-liquid chromatographic separation of acyclic perhydrocarotenes. The carotenes were synthesized from radioactive phytoene by a soluble enzyme system obtained from plastids of Hi-6 tomato fruits. The carotenes were then purified, hydrogenated, and subjected to gas-liquid chromatography as reported under "Experimental Procedure." The effluents leaving the gas chromatograph were trapped on glass wool for 1 min as indicated. The trapped samples were eluted with toluene-scintillator solution and then assayed for radioactivity with a Tri-Carb liquid scintillation spectrometer. The shaded areas indicate radioactivity. Similar results were obtained when carotenes were synthesized by soluble enzyme systems obtained from plastids of Hi-β, red, or Golden Jubilee tomato fruits.
Conversion of radioactive phytoene to phytofluene, neurosporene, lycopene, 
\( \alpha \)-, \( \delta \)-, \( \gamma \)-, \( \chi \)-, and \( \beta \)-carotenes was shown by the coinocidence of radioactivity and mass of perhydrocarotene on gas-liquid chromatography.

**RESULTS**

**Conversion of Phytoene-\(^{14}C\) to Acyclic and Cyclic Carotenes by Soluble Enzyme Systems Prepared from Plastids of Tomato Fruit of Different Genetic Selections**

**Red Fruit**—The formation of cis- and trans-phytofluenes, neurosporene, neolycopene (25, 26), lycopene, and \( \alpha \)- and \( \gamma \)-carotenes from radioactive phytoene by a soluble enzyme system obtained from plastids of red tomato fruits is shown in Table I. The incorporation of radioactivity into cis-phytofluene was greater than that for any other carotene reported in this table. The amount of radioactivity in trans-phytofluene was approximately 10% of that in the cis-isomer.

**Hi-6 Fruit**—The conversion of phytoene-\(^{14}C\) to cis- and trans-phytofluenes, neurosporene, lycopene, and \( \alpha \)-, \( \delta \)-, \( \gamma \)-, \( \chi \)-, and \( \beta \)-carotenes by a soluble enzyme system (0 to 80% ammonium sulfate precipitate) obtained from plastids of Hi-6 tomato fruits is reported in Table II. The quantity of radioactivity found in \( \delta \)-carotene was greater than that found in any of the other carotenes, as expected from the pigment composition of these fruits.

**Hi-\( \beta \) Fruit**—The conversion of phytoene-\(^{14}C\) to cis- and trans-
phytoene-4C is converted to various acyclic and cyclic carotenes by soluble enzyme system (0 to 80% ammonium sulfate fraction) obtained from plastids of red tomato fruits. A good correspondence was obtained between radioactivity and absorbance for \( \alpha \)-, \( \beta \)-, \( \gamma \)-, and \( \delta \)-carotenes obtained from these fruits when they were chromatographed on a 1% (v/w) water-deactivated alumina column (Fig. 1). Similar results were obtained on rechromatography of each separated carotene.

The coincidence of radioactivity and mass of each perhydrocarotene on gas-liquid chromatography was similar to that shown in Figs. 3 and 4. Most of the radioactivity was recovered in the mass peak of each perhydrocarotene (Table VI). These results, along with our earlier findings on the conversion of lycopene to cyclic carotenes by soluble extracts of Hi-\( \beta \) and Hi-\( \delta \) tomato fruit plastids and spinach plastids (22), provide proof that tritiated lycopene is converted to \( \alpha \)-, \( \beta \)-, \( \gamma \)-, and \( \delta \)-carotenes by plant enzyme systems obtained from a diversity of sources.

**Enzymatic Conversion of Tritiated Lycopene to Mono- and Dicyclic Carotenes by Soluble Enzyme System Obtained from Plastids of Red Tomato Fruits**

Table V shows the conversion of lycopene-15,15'\(^3\)H to \( \alpha \)-, \( \beta \)-, \( \gamma \)-, and \( \delta \)-carotenes by a soluble enzyme system obtained from plastids of red tomato fruits (22). The incorporation of radioactivity into carotenes was maximal in prolycopene, as expected from the pigment composition of these fruits. A good correspondence was obtained between radioactivity and absorbance for \( \alpha \)-, \( \beta \)-, \( \gamma \)-, and \( \delta \)-carotenes obtained from these fruits when they were chromatographed on a 1% (v/w) water-deactivated alumina column (Fig. 1). Similar results were obtained on rechromatography of each separated carotene.

**Cofactor Requirements**

Data on the effect of various cofactors on the conversion of phytoene-\(^{14}\)C to phytofluene and lycopene by a soluble enzyme system obtained from plastids of red tomato fruits are presented in Table VIII. When the incubations were carried out without FAD, phytoene was converted only to phytofluene. No radioactivity was detected in other carotenes. When NADP was omitted the conversion of phytoene to phytofluene was decreased considerably. However, some lycopene was formed. The omission of Mn\(^{2+}\) had the same effect as the omission of FAD. Phytoene was not converted to lycopene but conversion to phytofluene was unaffected. These results suggest that NADP is required for the conversion of phytoene to phytofluene and that FAD and Mn\(^{2+}\) are absolute requirements for the conversion of phytoene to lycopene. Some reduction in the formation of lycopene was also observed when Mg\(^{2+}\) and DTT were omitted from the incubation mixture.

### Tables

**Table V**

<table>
<thead>
<tr>
<th>Carotene</th>
<th>Total radioactivity</th>
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<tr>
<td>( \alpha )-Carotene</td>
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</tr>
<tr>
<td>( \beta )-Carotene</td>
<td>49,154</td>
</tr>
<tr>
<td>( \delta )-Carotene</td>
<td>13,510</td>
</tr>
<tr>
<td>( \gamma )-Carotene</td>
<td>31,927</td>
</tr>
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**Table VI**

<table>
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<th>Hydrogenated carotene</th>
<th>Radioactivity</th>
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<tbody>
<tr>
<td></td>
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<td>Recovered*</td>
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<td>( \alpha )-Carotene</td>
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<td>( \delta )-Carotene</td>
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<td>( \gamma )-Carotene</td>
<td>28,550</td>
<td>26,873</td>
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</table>

* The values given are the radioactivities recovered with the perhydrocarotenes trapped on emergence from the gas-liquid chromatograph.
Furthermore, these conversions were effected by soluble enzyme systems obtained from the plastids of fruits of these selections. We have also demonstrated in the present paper that lycopene is converted to cyclic carotenes by spinach chloroplasts and by soluble preparations of these chloroplasts. We also showed that the same conversion could be effected with plastids (or soluble extracts thereof) of fruit of several genetic types of tomatoes. Previously, we had shown (22) that tritiated lycopene is converted to cyclic carotenes by spinach enzyme system obtained from plastids of red tomato fruits. Our results also raise a question, since red tomato fruits normally synthesize very small amounts of cyclic carotenes. If the latter is true, our procedure of solubilization of the enzyme system removes or inactivates the inhibitor.

We have also demonstrated in the present paper that lycopene-15,15'-3H is converted to β-, γ-, α- and β-carotenes by a soluble enzyme system obtained from plastids of red tomato fruits. Previously, we had shown (22) that tritiated lycopene is converted to cyclic carotenes by spinach chloroplasts and by soluble preparations of these chloroplasts. We also showed that the same conversion could be effected with plastids (or soluble extracts thereof) of fruit of several genetic types of tomatoes. The results reported in this paper on the conversion of tritiated lycopene to more unsaturated carotenes has shown that the conversion of phytoene to phytofluene was greatly reduced when NADP was omitted from the incubation mixture. When FAD was omitted from the incubation mixture, no reports have been made on the conversion of phytoene to more unsaturated carotenes. In the present paper we have demonstrated that phytoene-1,15-3H is converted to β-, γ-, α- and β-carotenes by a soluble enzyme system obtained from plastids of red tomato fruits. Previously, we had shown (22) that tritiated lycopene is converted to cyclic carotenes by spinach chloroplasts and by soluble preparations of these chloroplasts. We also showed that the same conversion could be effected with plastids (or soluble extracts thereof) of fruit of several genetic types of tomatoes. The results reported in this paper on the conversion of tritiated lycopene to more unsaturated carotenes has shown that the conversion of phytoene to phytofluene was greatly reduced when NADP was omitted from the incubation mixture. When FAD was omitted from the incubation mixture, no reports have been made on the conversion of phytoene to more unsaturated carotenes.

### TABLE VII

Recovery of radioactivity of hydrogenated ^14C-labeled carotenes on gas-liquid chromatography

<table>
<thead>
<tr>
<th>Hydrogenated carotene</th>
<th>Red tomato</th>
<th>Hi-α</th>
<th>Hi-β</th>
<th>Golden Jubilee</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-Phytofluene</td>
<td>7,580</td>
<td>7,380</td>
<td>3,208</td>
<td>2,991</td>
</tr>
<tr>
<td>trans-Phytofluene</td>
<td>2,150</td>
<td>2,000</td>
<td>1,075</td>
<td>840</td>
</tr>
<tr>
<td>α-Carotene</td>
<td>1,891</td>
<td>1,821</td>
<td>3,196</td>
<td>3,039</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>3,650</td>
<td>2,442</td>
<td>2,000</td>
<td>1,918</td>
</tr>
<tr>
<td>γ Carotene</td>
<td>1,788</td>
<td>1,638</td>
<td>5,011</td>
<td>4,388</td>
</tr>
<tr>
<td>Prolycopene</td>
<td>2,500</td>
<td>2,350</td>
<td>2,335</td>
<td>2,218</td>
</tr>
<tr>
<td>Lycopene</td>
<td>2,714</td>
<td>2,501</td>
<td>3,039</td>
<td>2,190</td>
</tr>
</tbody>
</table>

* The values given are the radioactivities recovered with each perhydrocarotene trapped on emergence from the gas-liquid chromatograph.

### TABLE VIII

Effect of various factors on enzymatic conversion of phytoene-^14C to more unsaturated carotenes

<table>
<thead>
<tr>
<th>System</th>
<th>Phytofluene</th>
<th>β-Carotene</th>
<th>Lycopene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cis-</td>
<td>trans-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dpm</td>
<td>dpm</td>
<td>dpm</td>
</tr>
<tr>
<td>Complete*</td>
<td>2616</td>
<td>144</td>
<td>1100</td>
</tr>
<tr>
<td>- FAD</td>
<td>2321</td>
<td>198</td>
<td>0</td>
</tr>
<tr>
<td>- NADP</td>
<td>746</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>- MnCl₂</td>
<td>2901</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>- MgCl₂</td>
<td>3046</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>- DTT</td>
<td>1966</td>
<td>-</td>
<td>334</td>
</tr>
</tbody>
</table>

a The complete incubation system contained phytoene-^14C, 0.354 mmole (37,050 dpm): Tween 80, 2 mg; borate buffer, pH 7.5, 250 μMoles; NADP, 3.66 μMoles; FAD, 1.27 μMoles; MgCl₂, 30 μMoles; MnCl₂, 3 μMoles; DTT, 19.4 μMoles; and enzyme protein (ammonium sulfate precipitate, 20 to 40% saturation, 15 mg, and 40 to 60% saturation, 15 mg) in a final volume of 2 ml. Incubations were carried out for 6 hours in the dark at 25°C under nitrogen. b Determinations of radioactivity were not made.

discussion

Except for the demonstrated conversion of phytoene to phytofluene by tomato fruit plastids (19), and the recent report by Subbarayan et al. (23) on the conversion of phytoene to phytofluene and lycopene by a spinach enzyme system, no reports have been made on the conversion of phytoene to more unsaturated carotenes. In the present paper we have demonstrated that phytoene-^14C is converted to each of the principal acyclic and cyclic carotenes found in four genetic types of tomato fruits. Furthermore, these conversions were effected by soluble enzyme systems obtained from the plastids of fruits of these selections. Proof for the conversion of phytoene to acyclic and cyclic carotenes was obtained through the demonstration of (a) the coincidence of radioactivity and absorbance for each caroten separated on chromatography on alumina, and (b) the coincidence of mass and radioactivity for each hydrogenated carotene subjected to gas-liquid chromatography.

An examination of the cofactor requirements for the conversion of radioactive phytoene to more unsaturated carotenes has shown that the conversion of phytoene to phytofluene was greatly reduced when NADP was omitted from the incubation mixture. When FAD was omitted from the incubation mixture, lycopene was not formed. However, the conversion of phytoene to phytofluene was unaffected. These results suggest that NADP participates in the conversion of phytoene to phytofluene, whereas FAD functions in the conversion of phytofluene to lycopene. The elimination of Mn²⁺ from the incubation mixture had the same effect as the removal of FAD on the formation of lycopene. Hence, Mn²⁺ appears to be an absolute requirement for the conversion of phytoene to lycopene. The removal of Mg²⁺ and DTT from the incubation mixture also reduced the conversion of phytoene to lycopene.

We have also demonstrated in the present paper that lycopene-15,15'-3H is converted to β-, γ-, α-, and β-carotenes by a soluble enzyme system obtained from plastids of red tomato fruits. Previously, we had shown (22) that tritiated lycopene is converted to cyclic carotenes by spinach chloroplasts and by soluble preparations of these chloroplasts. We also showed that the same conversion could be effected with plastids (or soluble extracts thereof) of fruit of several genetic types of tomatoes. The results reported in this paper on the conversion of tritiated lycopene to cyclic carotenes by a soluble enzyme system obtained from plastids of red tomato fruits confirm our previous findings (22). Our results also raise a question, since red tomato fruits normally synthesize very small amounts of cyclic carotenes. This result suggests that the failure of red tomato fruits to form cyclic carotenes is either due to the lack of an essential cofactor or to the presence of an inhibitor that prevents the cyclization of lycopene. If the latter is true, our procedure of solubilization of the enzyme system removes or inactivates the inhibitor.

The results reported in this paper are summarized in Fig. 5. The results, and those reported previously (22), provide direct experimental support for the enzymatic synthesis of carotenes by...
the pathway shown in this figure. Thus, it has been shown that isopentenyl pyrophosphate is converted to geranylgeranyl pyrophosphate (15) and several of the carotenes listed in this figure (17, 23). It has also been shown that geranylgeranyl pyrophosphate is converted to phytoene (16). The conversion of the latter compound to each of the carotenes shown on this figure is demonstrated in the present paper. Each of these conversions is effected by enzymes from tomato fruit and spinach leaves (23). In addition, it was shown in a previous publication (22) that lycopene is converted to cyclic carotenes by soluble enzymes obtained from tomato fruits and spinach leaves. The isolation of radioactive trans-phytofluene from the incubation mixture suggests that this compound may be an intermediate between cis-phytofluene and trans-\( \epsilon \)-carotene. Whether this is true will have to await the isolation of the individual enzymes involved in the conversion of phytoene to lycopene and studies on each individual reaction. We also show in Fig. 5 the conversion of \( \gamma \)-carotene to proneurosene and prolycopene. Whether this conversion occurs, or these compounds arise from neurosporene and lycopene, will have to await studies with more purified enzyme systems.

Prior to the present investigation a report appeared in the literature (28, 29) on the conversion of bacterial phytoene (dehydrosqualene) to \( \delta \)-carotene by enzymes extracted from S. aureus. However, the identification of \( \delta \)-carotene from this bacteria was dependent only upon its light absorption spectrum (30). Therefore, proof of the incorporation of radioactivity into \( \delta \)-carotene in this system is equivocal. However, the suc-
cessive dehydrogenation of dehydrosqualene (29) with N-bromo-
succinimide to C30 phytofluene-like and C30 \( \gamma \) -carotene-like sub-
stances, as identified by light absorption spectra, might suggest
the presence of a C30 carotene series in this microorganism. If
dehydrosqualene were the precursor of the C40 carotenes, the
Porter-Lincoln series of reactions (1) would need some modifica-
tion. However, it is very doubtful that a pathway for the for-
mation of carotenoids from dehydrosqualene exists in tomatoes,
inasmuch as it has been shown that geranylgeranyl pyrophos-
phate is converted to phytoene by soluble extracts of tomato
fruit plastids (16).

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