Lycopersene and Prelycopersene Pyrophosphate
INTERMEDIATES IN CAROTENE BIOSYNTHESIS

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SUMMARY
This paper reports the enzymatic synthesis of lycopersene and prelycopersene pyrophosphate from $[4,8,12,16-\text{C}]$geranylgeranyl pyrophosphate by soluble enzyme systems obtained from yeast and tomato fruit plastids.

Incubation of a soluble extract of an acetone powder of tomato fruit plastids, or a purified squalene synthetase of yeast, with $[4,8,12,16-\text{C}]$geranylgeranyl pyrophosphate and NADPH yields a product which co-chromatographs with authentic lycopersene on thin layer, column, and gas-liquid chromatographic systems. These enzyme systems also form prelycopersene pyrophosphate from $[4,8,12,16-\text{C}]$geranylgeranyl pyrophosphate in the absence of NADPH. Both of the above compounds, lycopersene and prelycopersene pyrophosphate, are converted to phytoene and lycopene on incubation with the above soluble tomato enzyme system, NADP and FAD. Hence, it is concluded that these compounds are intermediates in the biosynthesis of acyclic and cyclic carotenes from geranylgeranyl pyrophosphate.

In 1961 Grob et al. (1) reported the formation of lycopersene from labeled geranylgeranyl pyrophosphate by an enzyme system obtained from Neurospora crassa. Further, they suggested that lycopersene is an intermediate in carotene biosynthesis. However, subsequent studies from other laboratories (2-4) failed to detect the formation of this compound in systems synthesizing phytoene and other carotenes. As a result of these studies, and the fact that lycopersene was characterized only by thin layer chromatography (1), most investigators concluded that lycopersene is not an intermediate in carotene biosynthesis.

In 1968 Shah et al. (5) demonstrated the conversion of $[4,8,12,16-\text{C}]$geranylgeranyl pyrophosphate to phytoene by a soluble plant enzyme system. Later this conversion was confirmed by two other laboratories (6, 7) with other enzyme systems. In none of these studies (5-7) was lycopersene detected as an intermediate in carotene biosynthesis.

Recently we reinvestigated the question of lycopersene involvement in the biosynthesis of phytoene with a partially purified tomato plastid enzyme system that converts $[4,16\text{C}]$isopentenyl pyrophosphate to acyclic and cyclic carotenes. The present preliminary report of these studies shows that lycopersene is indeed an intermediate in carotene biosynthesis.

The tomato enzyme system used for the synthesis of prelycopersene pyrophosphate, lycopersene, and other carotenes was prepared as follows: An acetone powder of tomato fruit plastids was prepared as reported previously (5, 8). Extraction of the acetone powder with phosphate buffer was then carried out by the procedure of Shah et al. (5). The extract was centrifuged at 105,000 x g for 45 min and the supernatant solution was fractionated under nitrogen between 0 and 65% saturation with solid ammonium sulfate. The precipitate was redissolved in 0.1 m potassium phosphate buffer, pH 7, containing 5 mM 2-mercaptoethanol and 5 mM EDTA. This solution was dialyzed for 2 hours against 3 liters of 0.05 m phosphate buffer, pH 7, containing 1.0 mM 2-mercaptoethanol, with a change of buffer at 1 hour. The dialyzed enzyme preparation was used immediately.

Incubations for the synthesis of prelycopersene pyrophosphate and lycopersene, and for the conversion of these compounds to phytoene and lycopene were carried out as reported in Tables I and II. All incubations were terminated by the addition of twice the volume of absolute alcohol. Lycopersene and other acyclic carotenes were extracted from incubation mixtures with three 4-ml aliquots of petroleum ether. The petroleum ether extracts were dried under anhydrous sodium sulfate and carrier lycopene, phytoene, and lycopene were added.

Lycopersene was separated from phytoene by chromatography on a column (1.5 x 8 cm) of 1% deactivated alumina, Fig. 1. Lycopersene was eluted from this column with 0.6% diethyl ether in petroleum ether.

FIG. 1. The separation of lycopersene and phytoene by chromatography on a column of 1% deactivated alumina. Lycopersene and phytoene were synthesized by the tomato enzyme system under the conditions reported in Table I. Three-fourths of the total petroleum ether extract of the incubation mixture was then subjected to thin layer chromatography (petroleum ether-benzene, 96:4; R of lycopersene = 0.65). The silica gel-lycopersene fraction was mixed with the remaining one-fourth of the petroleum ether extract of the incubation mixture and then applied to a 1% deactivated alumina chromographic column. The conditions of chromatographic separation are described in the text. Aliquots of 5.0 ml were collected and assayed for radioactivity (O—O) and absorbance at 285 nm (X-X-X).

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ether in petroleum ether. Phytoene was then eluted with approximately 0.9% diethyl ether in petroleum ether; lycopersene and phytoene were also separated by thin layer chromatography on Silica Gel G plates. A system of petroleum ether-benzene (96:4, v/v) was used. The \( R_F \) values for lycopersene and phytoene were 0.65 and 0.58, respectively. When yeast squalene synthetase was used (Table I), only lycopersene was formed, but with the tomato enzyme system both lycopersene and phytoene were synthesized.

Coincidence of radioactivity and mass of lycopersene, separated by thin layer chromatography, was demonstrated by gas-liquid chromatography on a Barber Colman model 10 instrument, Fig. 2. A column (6 mm x 8 feet) of 2% SE-30 on Chromosorb W was used and the column temperature was maintained at 275°C. An argon gas flow rate of 100 ml per min was used. Effluent fractions were trapped on glass wool plugs (soaked in dioxane-scintillator solution) in collection tubes cooled in a Dry Ice-acetone bath.

Prelycopersene pyrophosphate was extracted from incubation mixtures as follows. Concentrated ammonia, 0.1 ml, and 0.25 mM EDTA, pH 7.0, 0.2 ml, were added to the reaction mixture. Incubation was then carried out at 37°C for 10 min, at which time 2 ml of water were added. Extraction of prelycopersene pyrophosphate was effected with 1-butanol (two 2-ml aliquots) and benzene (three 3-ml aliquots). The extracts were combined and evaporated under vacuum at 50°C. The residue was extracted into benzene (five 3-ml aliquots), and evaporated under vacuum at 50°C. The residue was again dissolved in a small amount of benzene and then chromatographed on a plate of Silica Gel G. A system of ethyl acetate-concentrated ammonium isopropanol (3:3:4) was used. Prelycopersene pyrophosphate (\( R_F \) 0.52) was eluted from the plate with a mixture of 2% \( \text{NH}_4 \)OH in CH\(_2\)OH-ethyl acetate (1:1). This compound was either assayed directly or it was converted to the free alcohol by treatment with LiAlH\(_4\) in diethyl ether for 30 min at room temperature.

The incorporation of \([4,8,12,16-^{14}C]\)geranylgeranyl pyrophosphate into lycopersene was first observed in this laboratory with a purified yeast squalene synthetase (Table I). It was also demonstrated (Table I) that this enzyme system converts geranylgeranyl pyrophosphate to prelycopersene pyrophosphate. Proof that the tomato enzyme system that synthesizes carotenes from geranylgeranyl pyrophosphate also synthesizes lycopersene and prelycopersene pyrophosphate. Prelycopersene pyrophosphate was separated from geranylgeranyl pyrophosphate and other compounds by thin layer chromatography. The presence of the pyrophosphate moiety in this compound was established by the reductive color test of Rosenberg (9) which gave a positive purple color, and by mass spectral analysis, which showed a very small molecular ion peak at \( m/e \) 722 at low temperature (90°C). This peak readily loses 178 mass units, giving rise to an intense peak at \( m/e \) 544 (170). The fragmentation pattern observed for the \( m/e \) 544+ species (Fig. 3a) is analogous to that observed for presqualene pyrophosphate (10).

Chemical reduction of prelycopersene pyrophosphate by lithium aluminum hydride gave an alcohol whose mass spectrum
I Fig. 3. a, mass spectrum of prelycopersene pyrophosphate obtained at an injection temperature of 170°C. b, mass spectrum of prelycopersene alcohol obtained at an injection temperature of 160°C. All spectra were taken with an MS-9 high resolution instrument (A.E.I. Ltd., Manchester).

<table>
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<th>Conversion of lycopersene and prelycopersene pyrophosphate to phytoene and lycopene by a soluble tomato enzyme system†,b</th>
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† The incubation system contained: borate buffer, pH 7.5, 100 amoles; MgCl₂, 30 amoles; MnCl₂, 4 amoles; dithiothreitol, 3 mg; NADP⁺, 4.0 amoles; FAD⁺, 10 amoles; and [4,4',8,8',12,12',16,16'-14C]lycopersene, 0.17 amole and 8,500 dpm; and enzyme protein, 4.0 mg of a 0 to 60% (NH₄)₂SO₄ fraction, in a final volume of 1.1 ml. Incubations were carried out at 25°C under nitrogen for 234 hours.

b In this system [4,4',8,8',12,12',16,16'-14C]lycopersene was replaced by [3',7',11',15',19',5',9',13'-14C]prelycopersene pyrophosphate, 0.20 amole and 10,000 dpm, and NADPH, 1.6 amoles, was added.

† Phytoene and lycopene were separated on a column of 1% deactivated alumina by the procedure reported by Subbarayan et al. (11). Coincidence of mass and radioactivity was observed. The compounds were then hydrogenated and the products were subjected to gas-liquid chromatography (11). Again, coincidence of mass and radioactivity was obtained.

(Fig. 3b) showed a fragmentation pattern characteristic of a primary alcohol with a molecular ion at m/e 562⁺. This corresponds to a molecular formula of C₄₀H₆₀O. This formula has been further confirmed by the mass spectrum of its trimethylsilyl ether derivative which has a molecular ion peak at m/e 634⁺.

Both lycopersene and prelycopersene pyrophosphate are converted to phytoene and lycopene by incubation with a tomato enzyme system in the presence of NADP and FAD (Table II). It will be noted that good conversion of each of these compounds to phytoene and lycopene was effected by the tomato plastid enzyme system.

It is evident from the results reported in Tables I and II that prelycopersene pyrophosphate and lycopersene are intermediates in the biosynthesis of carotenes. It is also evident from these results that dehydrogenation of lycopersene to form phytoene is a required reaction of carotene formation. At present it appears that NADP is the hydrogen acceptor in this reaction. The pathway of carotene biosynthesis from geranylgeranyl pyrophosphate to phytoene and the cofactors involved would then be the following: Geranylgeranyl pyrophosphate \( \text{Mg}^{++} \rightarrow \text{prelycopersene pyrophosphate} \rightarrow \text{NADPH} \rightarrow \text{lycopersene} \rightarrow \text{NADP} \rightarrow \text{phytoene}.

REFERENCES

After this investigation was completed a preliminary communication appeared (12) which reported the chemical synthesis and the biosynthesis from geranylgeranyl pyrophosphate of a compound termed "prephytoene pyrophosphate." This compound was converted to a mixture of carotenes by an enzyme system obtained from a species of Mycobacteria. The properties of this compound and our prelycopersene pyrophosphate appear to be identical. We suggest, therefore, that both compounds should be properly designated as "prelycopersene pyrophosphate."
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