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EVIDENCE FOR *TRANS–TRANS* AND *CIS–CIS* FARNESYL PYROPHOSPHATE SYNTHESIS IN *GOSSIPIUM HIRSUTUM*

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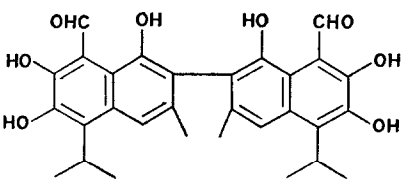
Key Word Index—*Gossipium hirsutum*; Malvaceae; gossypol biosynthesis; *cis–cis* farnesyl pyrophosphate synthesis; farnesyl pyrophosphate synthetase.

Abstract—A protein fraction capable of catalyzing the formation of all four geometrical isomers of farnesyl pyrophosphate has been isolated from cotton roots. Using neryl pyrophosphate and isopentenyl pyrophosphate as substrates the product was found to be *cis–cis* farnesyl pyrophosphate and possibly *trans–cis* farnesyl pyrophosphate. Geranyl pyrophosphate and isopentenyl pyrophosphate as substrates yielded *trans–trans* and possible *cis–trans* farnesyl pyrophosphate. During purification of the active protein fraction, the ratio of utilization of geranyl pyrophosphate and neryl pyrophosphate did not remain constant, indicating that two enzymes may be involved, one specific for *cis* C_{10}-substrate and the other for *trans* C_{10}-substrate.

INTRODUCTION†

GOSSYPOL (I), a phenolic sesquiterpene dimer found in the cotton plant, has been reported to be synthesized *via* the isoprenoid pathway[1] from acetate and MVA. However, further investigation[2] indicated that the enzymes responsible for gossypol biosynthesis preferentially utilized the *cis* C_{10} substrate (NPP) and the *cis–cis*-C_{15} substrate (FPP), rather than the *trans*, and *trans-trans* substrates as had been shown in cholesterol biosynthesis in mammalian systems. Similarly the *cis* C_{10} isomer, NPP, appeared to be the preferred substrate for pinene formation in *Pinus radiata* seedlings.[3] However, the proposed mechanisms of sequiterpene cyclization generally imply *trans–trans* or *trans–cis* FPP precursors.[4–6] Therefore, it appeared



(I)

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† Abbreviations used: MVA—mevalonic acid; IPP—isopentenyl pyrophosphate; GPP—geranyl pyrophosphate; NPP—neryl pyrophosphate; FPP—farnesyl pyrophosphate; EtSH—mercaptoethanol; DEDTC—diethyldithiocarbamate (disodium salt); DTT—dithiothreitol; ATP—adenosine-5′-triphosphate; EDTA—tetrasodium ethylene diamine tetraacetic acid.

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important to document the occurrence and therefore the formation of *cis–cis* FPP in plant material. Since *cis–cis* FPP is utilized by cotton root homogenates to synthesize gossypol, this cell-free system was used to isolate a protein fraction capable of forming *cis–cis* FPP from NPP and IPP.

RESULTS

Purification, Stabilization and General Properties of the Enzyme Activity

A typical purification profile with GPP as the substrate is shown in Table 1. Maximal purification was obtained after DEAE-cellulose chromatography. Further attempted purification through sucrose gradient centrifugation resulted in an apparent loss in enzyme activity. Therefore the DEAE-cellulose-purified protein fraction was used in subsequent experiments.

TABLE 1. PURIFICATION OF FPP SYNTHETASE FROM *Gossipium hirsutum* ROOT

Purification step	Specific activity (units/mg/min*)	Purification
Crude extract	0·03	—
105 000 *g* supernatant solution	0·08	2·7
$(NH_4)_2SO_4$precipitate		
Without glycerol	2·93	98
With glycerol	9·30	310
DEAE-cellulose	49·91	1663
Sucrose gradient	29·87	996

* One unit equals the incorporation of 1 nmol of 1-^{14}C-IPP into acid labile prenyl alcohols. Each incubation contained, in addition to enzyme solution, 2×10^{-4} M 1-^{14}C-IPP, 4×10^{-4} M GPP, $1{\cdot}6 \times 10^{-3}$ M ATP, and $3{\cdot}0 \times 10^{-3}$ M $MgCl_2$. Incubation was at 32° for 30 min.

A rapid loss of enzyme activity was observed at all purification steps. This could be overcome by the addition of 10–15% glycerol and 0·1 mM DTT to the buffers used. Furthermore a protein concentration of 1–2 mg/ml was essential. Under these conditions the enzyme was stable at −18° for 3 weeks. The enzyme activity was markedly increased in the crude extract, 105 000 g supernatant solution and $(NH_4)_2SO_4$ precipitate, upon addition of ATP and $MgCl_2$ to the assay mixtures. However, the DEAE-cellulose-purified enzyme was fully active without ATP and $MgCl_2$.

The pH optimum was found to be 7·6–8·5. Although sulfhydryl agents stabilized the enzyme, iodoacetamide did not inhibit the enzymatic activity (Table 2). Furthermore, this insensitivity toward iodoacetamide indicated absence of IPP-DMAPP isomerase.

Products of the FPP Synthetase Reaction

The products formed by the enzyme system utilizing the different substrates and varying conditions are shown in Table 3. GLC analysis clearly indicated that when the enzyme system is supplied with the *cis*-C_{10} substrate the main product is *cis–cis* FPP (appearing as the alcohol after hydrolysis). No radioactivity was found in the *trans–trans* farnesol. Appreciable

activity in the combination peak of the *cis–trans* and *trans–cis* farnesols was probably due to the *trans–cis* isomer, since the Δ^6 double bond of FPP was fixed as *cis* by using the *cis*-C_{10} substrate. Furthermore, the absence of appreciable radioactivity in any of the C_{10} alcohols in the GLC analysis indicated the purified enzyme fraction was free of any IPP–DMAPP isomerase activity. Incubations using *trans*-C_{10} substrate resulted in incorporation of radioactivity into *trans–trans* FPP (Table 3). Again high radioactivity was found in the mixed (*cis–trans* and *trans–cis*) farnesol peak.

TABLE 2. EFFECT OF IODOACETAMIDE ON FPP SYNTHETASE ACTIVITY

Iodoacetamide	1-^{14}C-IPP incorporation
	(%)
−	1·56
+	1·31

0·1 ml of DEAE purified FPP synthetase incubated for 30 min at 32° with 0·2 μmol 1-^{14}C-IPP ($4{\cdot}32 \times 10^4$ dpm), 0·4 μmol GPP, 1·6 μmol ATP, 3·0 μmol $MgCl_2$, and 2·0 μmol iodoacetamide as indicated, total volume 1·1 ml.

Substrate competition between the *cis*-C_{10} (NPP) and the *trans*-C_{10} (GPP) isoprenyl pyrophosphates for the active site of the protein catalyst revealed an interesting product formation (Table 3). When assayed after elution from the DEAE cellulose column with

TABLE 3. DISTRIBUTION OF RADIOACTIVITY IN FPP SYNTHETASE PRODUCTS

	Products								
		Nerol	Geraniol	Nerolidol			Farnesol		
C_{10}-Substratres	Linalool (cpm)	*cis* (cpm)	*trans* (cpm)	*cis* (cpm)	*trans* (cpm)	*cis–cis* (cpm)	*cis–trans trans–cis* (cpm)	*trans–trans* (cpm)	Conversion of IPP (%)
Geranyl-PP*	9	3	1	1	56	10	219	50	5·3
Neryl-PP†	9	0	0	0	0	693	562	0	9·6
Geranyl-PP (70)‡ Neryl-PP (30)	10	18	9	3	2	19	90	190	5·2
Geranyl-PP (70)§ Neryl-PP (30)	27	2	0	0	0	76	179	0	4·3

* Acid hydrolysis; incubation after DEAE-elution, in phosphate buffer, pH 8·0.
† Alkaline phosphatase hydrolysis; incubation after buffer was changed to Tris-HCl, pH 8·6.
‡ Alkaline phosphatase hydrolysis; incubation after DEAE-elution, in phosphate buffer, pH 8·0.
§ Alkaline phosphatase hydrolysis; incubation after buffer was changed to Tris-HCl, pH 8·6.

70% GPP and 30% NPP the enzyme system incorporated the 1-^{14}C IPP most efficiently into the *trans–trans* FPP intermediate. But if the enzyme system was transferred to a Tris buffer (by elution on a Sephadex G25 column) and assayed, incorporation into the *trans–trans* isomer was lost while incorporation into *cis–cis* FPP was enhanced (Table 3).

Trans *FPP Synthetase vs* cis *FPP Synthetase*

Although maximum activity of *cis–cis* FPP synthetase appeared to be slightly behind the maximum of *trans–trans* FPP synthesis in the DEAE column profile (Fig. 1), attempts to separate physically the synthesis of *cis–cis* FPP from the formation of *trans–trans* FPP on DEAE-cellulose by different pH and salt gradients were unsuccessful.

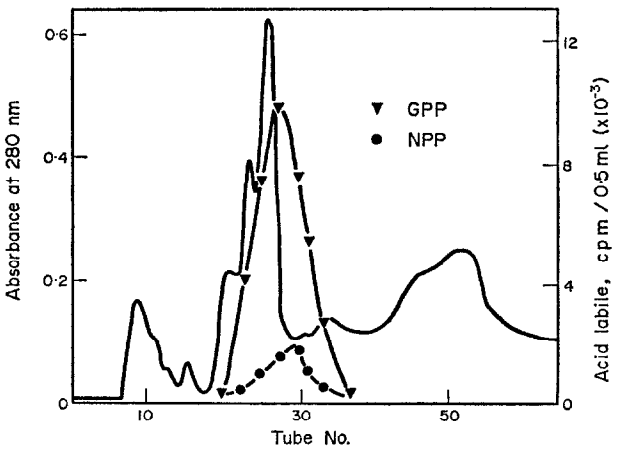

FIG. 1. ELUTION OF FPP SYNTHETASE ACTIVITY ON A DEAE-CELLULOSE COLUMN.
The solid line represents protein concentration (absorbancy at 280 nm). Each fraction was assayed with ^{14}C-IPP and with GPP or with NPP as substrates.

DISCUSSION

Isolation of an enzymatically active fraction of protein from cottonseed roots which catalyzed the formation of *cis–cis* FPP as the sequiterpene intermediate in gossypol[2] has been achieved. This fraction also catalyzed the formation of *trans–trans* FPP, although the *cis–cis* isomer was preferentially formed under certain conditions (Table 4). Since no *trans–trans* FPP was formed when the *cis*-C_{10} substrate (NPP) was utilized (Table 3), it was assumed that no *trans–trans* to *cis–cis* FPP isomerase was present in these preparations.

Incubation of the active protein fraction with NPP (which fixed the Δ^6 double bond of FPP as *cis*) and with IPP as the substrates yielded both *cis–cis* FPP and *trans–cis* FPP. Similarly when GPP (Δ^6 double bond of FPP must be *trans*) and IPP were the substrates the products were *trans–trans* FPP and *cis–trans* FPP. It appeared, therefore, that the active protein fraction could add the C_5 isoprenoid pyrophosphate (IPP) to the C_{10} substrate either *cis* or *trans* (Table 3). The radioactivity in the *trans* nerolidol fraction was assumed to originate from *trans–trans* or *cis–trans* FPP and *cis* nerolidol from *cis–cis* or *trans–cis* FPP.

Results presented herein did not conclusively answer the question as to whether one enzyme catalyzed both *cis–cis* and *trans–trans* FPP synthesis or whether two enzymes existed, specific for the *cis* C_{10} and *trans* C_{10} substrates respectively. Some of the experiments (Table 4) supported the existence of two enzymes. When the ratio of cpm of radioactivity incorporated with GPP to that with NPP was calculated at each purification step, the ratio drastically changed. Maximal activity for GPP was exhibited by the enzyme system in phosphate buffer after elution from the DEAE-cellulose column. On the other hand, maximum activity for NPP (as indicated by the very low ratio) was exhibited by the enzyme

system in a *tris* buffer. Furthermore, the DEAE-cellulose elution profile and enzyme activity (Fig. 1) revealed that the maximum activity peak for the utilization of the *trans* C_{10} substrate (GPP) did not exactly coincide with that for the *cis* C_{10} substrate (NPP). However it should be emphasized that at no time have the two enyzme activities been separated.

TABLE 4. RATIOS OF *trans* FPP-SYNTHETASE ACTIVITY TO *cis* FPP-SYNTHETASE ACTIVITY

Purification step	Specific activity units/mg/min GPP	NPP	Ratio
Crude extract	0·04	0·0041	9·84
105 000 *g* supernatant solution	0·075	0·0103	7·25
37–50% $(NH_4)_2SO_4$ precipitate	8·2	1·72	4·78
DEAE-cellulose (phosphate buffer, pH 8·0)	54·2	4·6	11·76
DEAE-cellulose (Tris buffer, pH 8·0)	44·1	59·6	0·74
Sucrose gradient	27·2	9·9	2·75

Each incubation contained, in addition to enzyme solution, 2×10^{-4} M 1-^{14}C-IPP, 4×10^{-4} M GPP or NPP, $1{\cdot}6 \times 10^{-3}$ M ATP, and 3×10^{-3} M $MgCl_2$. Incubation was at 32° for 30 min.

On the other hand, some of the data could be interpreted to indicate the participation of only one enzyme. Although comparison of recovered sucrose gradient activity with that of the DEAE-cellulose stage of purification (Table 1) indicated that no purification was achieved the sucrose gradient fractionation profile indicated that significant amounts of inert protein were separated from the active fraction. This reduction in specific activity (Table 1) was possibly due to excessive time and/or speed of centrifugation, a phenomenon sometimes exhibited by enzymes with functional subunits. Furthermore, since the ratio GPP/NPP incorporation (Table 4) drastically decreased from the DEAE-cellulose-phosphate buffer step to the sucrose gradient step, it appears that *trans–trans* FPP synthetase activity is preferentially lost. If one assumes one enzyme with separate binding sites for NPP and GPP and therefore possibly separate subunits catalyzing *cis–cis* and *trans–trans* FPP formation, this loss could be due to a partial disintegration (loss of GPP binding subunit) of the enzyme. This variation in substrate utilization could represent a possible regulatory mechanism operating in the intact plant. Depending on the plant's requirement for triterpenoids (*trans*-isoprenoid precursors) and for gossypol (*cis*-isoprenoid precursors), a change in subunit activity could result in increased or decreased triterpenoid and/or gossypol biosynthesis.

Nevertheless, the results present for the first time, to our knowledge, evidence that all four geometrical isomers of FPP can be enzymatically synthesized in plant tissue and supports some of the postulated cyclization schemes from either of the four FPP isomers. Furthermore, the enzymatic synthesis of *cis–cis* FPP in the cotton plant supports the idea of biosynthesis of gossypol via a *cis–cis* isoprenoid pathway,[2] indicating the availability of *cis–cis* FPP in cotton root tissue for cyclization to gossypol.

EXPERIMENTAL

Preparation of substrates. The radioactive C_5-substrate, 1-^{14}C-IPP, was prepared from the Grignard reagent of 1-chloro-2-methyl-2-propene and $^{14}CO_2$ liberated from $Ba^{14}CO_3$. The resulting acid 1-^{14}C-3-methyl-3-butenoic acid was reduced with lithium aluminum hydride to 1-^{14}C-isopentenol, which was pyro-

phosphorylated.[7] The 1-^{14}C-IPP was purified from the mono-phosphate and possibly tri- and tetra-phosphates by chromatography on Whatman 3MM paper in a solvent system consisting of isopropanol–NH_4OH–H_2O (6:3:1). The radioactive band corresponding to authentic IPP was cut out, eluted with 5% NH_4OH, lyophylized and dissolved in 1% NH_4OH to a final concentration of 3 mM (*ca.* 2×10^5 dpm/μmol). NPP and GPP were prepared from authentic nerol or geraniol, respectively through pyrophosphorylation[7] and the pyrophosphates were purified on paper as described above for 1-^{14}C-IPP.

Assay. Throughout the procedures, FPP synthetase was assayed by incubation with 1-^{14}C-IPP and either GPP and/or NPP and cofactors in the following concentrations: 1-^{14}C-IPP, 2×10^{-4} M (3×10^4 to 2×10^5 dpm/μmole); GPP and/or NPP, 4×10^{-4} M; ATP, $1{\cdot}6 \times 10^{-3}$ M; $MgCl_2$, $3{\cdot}0 \times 10^{-3}$ M; EtSH, 0·2 M; potassium phosphate or Tris-HCl buffer, 0·01 M, pH 8·0. ATP and $MgCl_2$ were omitted from assays of DEAE-cellulose purified preparations. Incubations were placed in a shaking water bath at 32° for 30 min. After stopping the reaction with 2 N HCl in 80% EtOH, the acid labile isoprene alcohols were extracted 3× with equal volumes of light petrol. (30–60°) as previously described.[8] An aliquot of the petrol extract was then taken and its radioactivity determined in a Beckman LS100 liquid scintillation counter.

Purification of FPP synthetase. Delinted cottonseed (*Gossypium hirsutum* var. Carolina Queen) were germinated in moist vermiculate at 32° for 48 hr. The roots were harvested, chilled in buffer, and homogenized in a Sorvall Omnimixer at full speed for 1 min with sufficient addititional buffer to make a final ratio of 2:1, buffer to roots. The buffer used was 0·05 M K_2HPO_4–KH_2PO_4, pH 8·0, 0·02 M EtSH, 0·02 M thioglycolate, and 0·02 M DEDTC. The resulting slurry was strained through cheesecloth to give the crude extract. This was centrifuged in a Beckman L2 65B Preparative Ultracentrifuge at 105 000 *g* for 1 hr. The FPP synthetase activity was contained in the supernatant solution. An $(NH_4)_2SO_4$ precipitation was next performed on the supernatant solution, maintained at pH 8·0. The protein precipitating between 37% saturation and 50% saturation was collected and redissolved in a minimal amount of 0·01 M K_2HPO_4–KH_2PO_4 buffer, pH 8·0, 0·02 M EtSH. This was then dialyzed against 80–100 vols. of 0·01 M K_2HPO_4–KH_2PO_4, pH 8·0, 0·02 M EtSH, 10^{-4} M EDTA for 4 hr, with a buffer change after 2 hr. The dialyzed solution was applied to a DEAE-cellulose (Whatman DE 23) column (1·9 × 25 cm) and eluted with a linear gradient of 0–0·7 M potassium chloride in the buffer used to dissolve the $(NH_4)_2SO_4$ precipitate. The elution of protein was monitored with a LKB Uvicord at 280 nm (Fig. 1) and the tubes containing FPP synthetase activity (as determined by assay) were combined and concentrated in an Amicon Ultrafiltration cell. The final step in purification was a 5–20% sucrose gradient fractionation.[9] An aliquot of the concentrated DEAE-cellulose-purified protein fraction was placed on the sucrose gradient which was centrifuged for 36 hr at 200 000 *g* in a Beckman Ultracentrifuge using a SW40 rotor. The sucrose gradient fractions were collected[9] and assayed to locate enzymatic activity.

Product determination. Bulk incubations were prepared using the same substrates and conditions described above except the incubations were stopped in some cases with alkaline phosphatase instead of with acid. Alkaline phosphatase hydrolyzed the phosphate esters of the synthesized isoprene alcohols without the primary to tertiary alcohol rearrangement observed in acid hydrolysis.[8] The radioactive products were extracted with petrol. (30–60°) mixed with carrier compounds (nerol, geraniol, linalool, *cis* and *trans* nerolidol, *cis–cis*, *cis–trans*, *trans–cis*, and *trans–trans* farnesol) and analyzed by GLC. The column (6·3 mm × 152 cm) used was packed with 10% Carbowax 20M on chromosorb W 60/80.[10] The temp. was programmed from 160° to 225°. From published retention times[10] of the individual alcohols and from NMR spectras[11] of previously collected standards, identification of the various *cis* and *trans* isomers obtained by GLC analysis was possible. The radioactivity associated with the individual peaks was determined by collection of the GLC effluents of each peak and counting in a Beckman liquid scintillation counter.

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