Substrate Specificity of Undecaprenyl Pyrophosphate Synthetase from *Lactobacillus plantarum*†

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ABSTRACT: Geranyl pyrophosphate (GPP), neryl pyrophosphate (NPP), *trans,trans*-farnesyl pyrophosphate (FPP), *c,t*-FPP, *all-trans*-geranylgeranyl pyrophosphate (GGPP), *c,t,t*-GGPP, and solanesyl pyrophosphate (SPP) were tested as substrates for undecaprenyl pyrophosphate synthetase from *Lactobacillus plantarum*. K_M and V_{max} were determined with each of these allylic substrates, as well as isopentenyl pyrophosphate (IPP). The reactivity of the allylic substrates increased with chain length, $C_{10} < C_{15} < C_{20}$, except for *all-trans*-SPP which was unreactive. Moreover, the enzyme was not only active with all-trans derivatives but was also active with those substrates having both cis and trans stereochemistry. The larger the number of *trans*-isoprene residues in the substrate, the more strongly the substrate bound to the enzyme. Farnesol, geraniol, geranyl monophosphate, and citronellyl pyrophosphate were found to have no stimulatory or inhibitory effect on the enzyme assayed with *t,t*-FPP as substrate. Geranylgeranyl monophosphate and farnesyl monophosphate, however, both stimulated enzyme activity. The enzyme activators, Triton X-100, cardiolipin, and myristic acid, all decreased the K_M for *t,t*-FPP. C_{55}-polyprenyl pyrophosphate was the only product synthesized from *t,t*-FPP or *c,t,t*-GGPP. The other allylic pyrophosphate substrates gave a mixture of products. The 50- and 55-carbon polyprenyl phosphates were the primary products from *c,t*-FPP, GPP, and NPP, whereas 55- and 60-carbon polyprenyl phosphates were the primary products from *t,t,t*-GGPP. It was also demonstrated with GPP, NPP, and *t,t*-FPP, utilizing (2*R*)-[2-^{3}H]IPP, that only *cis*-isoprene units are introduced by the enzyme. Large amounts of *c,c*-FPP were synthesized as a product when the cis derivative, NPP, was used as substrate, indicating an impaired ability of the enzyme to elongate the all-cis product.

The 55-carbon polyprenol, undecaprenol, obtained from the bacterium *Lactobacillus plantarum*, has two *trans*-isoprene residues adjacent to the ω-residue and eight *cis*-isoprene residues (Gough et al., 1970). Undecaprenyl pyrophosphate synthetase, which catalyzes the formation of long-chain polyprenyl pyrophosphates with up to 55 carbons from FPP[1] and IPP, has been extensively purified from *L. plantarum* in our laboratory (Allen et al., 1976). It was of interest to determine if the enzyme distinguishes between different geometric isomers of the allylic substrate, specifically about the double bond in the α residue. In the course of the earlier studies utilizing FPP of mixed stereochemistry, we occasionally found the formation of 50- and 45-carbon polyprenyl pyrophosphates in addition to the predominant 55-carbon product. It was not clear whether these shorter chain polyprenols were derived from the utilization of different isomeric substrates, the lack of specificity of the enzyme for synthesizing a specific chain length product, or both. Only the 55-carbon polyprenol is found naturally in this bacterium. Moreover, the enzyme was considerably active even with GPP, although the activity was one-fifth of that of *t,t*-FPP. Such findings prompted us to investigate the substrate specificity of the enzyme. For this purpose, we synthesized six geometrically pure substrates, GPP, NPP, *t,t*-FPP, *c,t*-FPP, *t,t,t*-GGPP, and *c,t,t*-GGPP. We report here the results of the utilization of these substrates, particularly in terms of the kinetic constants, product analysis, and the stereochemical course of the reaction.

Materials and Methods

Silica gel 60 for column and TLC were products of E. Merck. Triethyl phosphonoacetate was the product of Aldrich Chemical Co. Semicarbazide hydrochloride was obtained from Mallinckrodt Chemical Works. [^{3}H]$LiAlH_4$ (specific activity 171.45 mCi/mM) was purchased from New England Nuclear. (4*S*)- and (4*R*)-[4-^{3}H]mevalonic acid lactone were obtained from Amersham/Searle Corp. Technical grade farnesol (32% cis,trans and 68% trans,trans by GC) and nerol (>98%) were the products of Fluka, A. G. These were used for synthesis without further purification. Geraniol (geraniol 74% and citronellol 26% by GC) was obtained from ICN Corp. and purified by spinning-band column distillation (Corey and Achiwa, 1969). Fractions without citronellol were used for the synthesis of the farnesols. Geraniol and citronellol were thoroughly purified by column chromatography on 20% $AgNO_3$–silica gel 60 (Walter, 1967) with benzene–ethyl acetate in ratios of 5:1 and 10:1, respectively. Solanesol was a kind gift from Aromatics International Manufacturing Co., Inc., and was further purified by column chromatography on silica gel 60 with benzene–ethyl acetate (30:1).

The purity and identification of the synthetic and commercial prenyl alcohols were confirmed by NMR spectroscopy. The stereochemical assignments by NMR were based on essentially the method used by Bates et al. (1963), who had drawn attention to small shift differences of methyl hydrogens in the substituted isoprenes. The following observed ppm and integrated ratios were obtained: nerol (1.75, 1.69, 1.61; 1:1:1), geraniol (1.68, 1.60, 2:1), citronellol (1.69, 1.60; 1:1), *cis,trans*-farnesol (1.75, 1.68, 1.60; 1:1:2.1), *trans,trans*-farnesol (1.68, 1.60; 1:1), *cis,trans,trans*-geranylgeraniol (1.75, 1.68, 1.60; 1:1:3), *all-trans*-geranylgeraniol (1.68, 1.60; 2:3.1), and

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[1] Abbreviations used are: IPP, $^3\Delta$-isopentenyl pyrophosphate; GPP, geranyl pyrophosphate; NPP, neryl pyrophosphate; CPP, citronellyl pyrophosphate; *t,t*-FPP, *trans,trans*-farnesyl pyrophosphate; *c,t*-FPP, *cis,trans*-farnesyl pyrophosphate; *t,t,t*-GGPP, all-*trans*-geranylgeranyl pyrophosphate; *c,t,t*-GGPP, *cis,trans,trans*-geranylgeranyl pyrophosphate; SPP, solanesyl pyrophosphate; C_{55}-PP, undecaprenyl pyrophosphate; GC, gas chromatography; $LiAlH_4$, lithium aluminum hydride; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

0006-2960/78/0417-5598$01.00/0

solanesol (1.66, 1.59; 1:3.2). NMR spectra were taken in $CDCl_3$ using tetramethylsilane as an internal standard.

Mass spectra and NMR spectra were taken on a single-focusing magnetic sector Dupont 490 F mass spectrometer (70 eV, EI) and a Varian 90 Mc NMR instrument, respectively. Analytical GC was carried out on a 4 ft by 0.25 in. column of 5% Carbowax 20M on Gas-Chrom CL.

Preparation of all-trans- and cis,trans,trans-Geranylgeraniols. Farnesyl bromide was prepared by an adaptation of the methods of Bates and Gale (1960) and Isler et al. (1958). A mixture of farnesol (8.64 g) and pyridine (1.47 g) in 30 mL of petroleum ether was cooled on a dry ice–acetone bath. Phosphorus tribromide (4.66 g) in 30 mL of petroleum ether was added dropwise under a stream of N_2, over 40 min, under vigorous stirring. Bromination was permitted to continue for another 2 h. The product was isolated by the methods described by Isler et al. (1958). The yield of crude farnesyl bromide was 8.3 g (80%).

Farnesylacetone was prepared from farnesyl bromide (8.23 g) and ethyl acetoacetate (6.37 g) by the method described by Isler et al. (1958). The yield of crude farnesylacetone was 7.3 g (90%).

Pure *trans,trans*-farnesylacetone was prepared from its semicarbazone. Crude farnesylacetone (7.3 g) was purified by column chromatography on silica gel 60 (100 g) with benzene–petroleum ether (1:1) (5.3 g, 72%). Semicarbazide hydrochloride (3.4 g) and sodium acetate (4 g) were suspended in 20 mL of CH_3OH and stirred for 1 h at room temperature. The suspension was filtered to remove salt, and the filtrate was mixed with purified farnesylacetone (5.3 g). Water was added to the mixture until the solution became turbid. The solution gave crystals of *trans,trans*-farnesylacetone semicarbazone on standing overnight. The fourth recrystallization from CH_3OH–H_2O gave 2.15 g (34% yield): mp 83–84 °C, lit. mp 81–82 °C (Isler et al., 1958).

trans,trans-Farnesylacetone was generated from the semicarbazone by acid treatment as described by Isler et al. (1958). Unreacted semicarbazone and other impurities were removed by column chromatography on silica gel 60 (130 g). Pure *trans,trans*-farnesylacetone (no *cis,trans*-farnesylacetone was detected by GC) was recovered from the column with benzene (1.5 g, 91%).

Ethyl geranylgeranate was prepared according to the method of Ogura et al. (1970). Triethyl phosphonoacetate (2.6 g) was added dropwise to a freshly prepared solution of sodium ethoxide (0.264 g of sodium metal and 5.7 mL of absolute ethanol) on a water bath which was maintained at 15 °C. This mixture was stirred at room temperature for 30 min. *trans,trans*-Farnesylacetone (1.5 g) was then added dropwise over 5 min at room temperature. The reaction was continued overnight with stirring under a N_2 stream. After dilution of the reaction mixture with water, the esters were extracted with ether. The ether layer was washed with water and saturated NaCl solution and was then dried over Na_2SO_4. Evaporation of the solvent gave a crude oil of ethyl geranylgeranate (99%). The *all-trans* isomer of ethyl geranylgeranate was separated from the cis,trans,trans isomer by column chromatography on silica gel 60 (130 g) with benzene–petroleum ether (1:2). The all-trans isomer was further purified by TLC (silica gel 60) in benzene–petroleum ether (10:1). Yields were *all-trans*-ethyl geranylgeranate (218 mg) and *cis,trans,trans*-ethyl geranylgeranate (119 mg). The purity of both esters was more than 96% by GC. Repeated column chromatography of partially purified esters was required to get more product.

all-trans-Ethyl geranylgeranate (218 mg) was reduced with $LiAl^3H_4$ (50 mg, 7.2 μCi/mM) at −30 °C according to the method of Popjak et al. (1962). The yield of crude *all-trans*-geranylgeraniol was 177 mg (93%). Crude *cis,trans,trans*-geranylgeraniol (150 mg) was obtained by the same method. These allylic alcohols were purified on a silica gel 60 column (30 g) eluting with benzene–ethyl acetate (20:1). The yield of pure *all-trans*-geranylgeraniol was 132 mg (75%) and of the pure *cis,trans,trans*-geranylgeraniol 101 mg (67%).

The reduction of the esters with cold $LiAlH_4$ was also carried out. NMR spectra of the unlabeled alcohols showed that these allylic alcohols did not contain any other geranylgeraniol isomers. Mass spectral analysis gave parent ions and fragments characteristic of the desired products.

Preparation of cis,trans- and trans,trans-Farnesols. *trans,trans*-Ethyl and *cis,-trans*-ethyl farnesoates were synthesized by essentially the same method as described for the synthesis of ethyl geranylgeranate. From 8.5 g of spinning-band purified geraniol, 7.0 g (61% yield) of crude geranylacetone was obtained by the ethyl acetoacetate synthesis method. Crude geranylacetone was purified twice by column chromatography on silica gel 60 (100 g). Pure geranylacetone was eluted with benzene–petroleum ether (5:1) (2.14 g, 30.6%). Ethyl farnesoate was synthesized from geranylacetone (2.4 g), triethyl phosphonoacetate (4.5 g), and sodium ethoxide (0.45 g of sodium metal, 10 mL of absolute EtOH) in a yield of 2.8 g (99%). *trans,trans*-Ethyl farnesoate was separated by column chromatography on silica gel 60 (130 g). The esters were eluted with benzene–petroleum ether (1:1). The trans,trans isomer required further purification by TLC (silica gel 60) in benzene. The yields were 285 mg for *trans,trans*-ethyl farnesoate (purity >96% by GC) and 218 mg for *cis,trans*-ethyl farnesoate (purity >96% by GC). These esters were reduced with $LiAl^3H_4$, and the resulting alcohols were purified by silica gel chromatography using benzene–ethyl acetate (20:1). Yields were 136 mg for *trans,trans*-farnesol and 133 mg for *cis,-trans*-farnesol.

NMR spectra of unlabeled alcohols showed that these allylic alcohols did not contain any other isomeric farnesols. Mass spectral analyses were also satisfactory.

Phosphorylation of the Allylic Alcohols. *t,t,t*-GGPP, *c,t,t*-GGPP, *t,t*-FPP, *c,t*-FPP, GPP, NPP, CPP, GGP, GP, and FP were obtained by the phosphorylation of the corresponding alcohols by the method of Popjak et al. (1962). Purification of the mono- and pyrophosphate esters was accomplished by XAD-2 treatment and DEAE-cellulose chromatography (Holloway and Popjak, 1967). Solanesol was phosphorylated as described by Samuel et al. (1974). The reaction mixture was applied directly to a column of DEAE-Sephadex LH20 after removal of the solvent, and the column was washed with $CHCl_3$–CH_3OH (1:1). The mono- and pyrophosphate were eluted with 0.1 M ammonium formate in $CHCl_3$–CH_3OH (1:1). Salt in the fractions was removed by extraction with water. Complete separation of the pyrophosphate from monophosphate was attained by subsequent chromatography on DEAE-Sephadex LH20 using a linear gradient of 0 to 0.1 M ammonium formate in $CHCl_3$–CH_3OH (1:1). Phosphate was analyzed by the method of Chen et al. (1956).

The specific activities of tritiated *t,t*-FPP, *c,t*-FPP, *t,t,t*-GGPP, and *c,t,t*-GGPP were 2.20, 3.28, 4.60, and 4.68 μCi/μmol, respectively.

(2*R*)-[2-^{3}H]IPP (specific activity 6.1 μCi/μmol) and (2*S*)-[2-^{3}H]IPP (specific activity 3.05 μCi/μmol) were prepared as previously described (Allen et al., 1976) from (4*S*)-[4-^{3}H]mevalonic acid and (4*R*)-[4-^{3}H]mevalonic acid, respectively. (2*R*)- and (2*S*)-[2-^{3}H]IPP were purified by DEAE-cellulose column chromatography (Skilleter and Kekwick, 1967). (2*S*)-[2-^{3}H]IPP required further purification

TABLE I: Reactivities for the Various Terpenyl Pyrophosphates.[a]

	rate (units)[b]	rate rel to *t,t*-FPP (units)
citronellyl-PP	0	0
GPP	27	0.14
NPP	21	0.11
t,t-[^{3}H]FPP	188	1.0
c,t-[^{3}H]FPP	111	0.59
t,t,t-[^{3}H]GGPP	215	1.14
c,t,t-[^{3}H]GGPP	255	1.36
all-trans-SPP	0	0

[a] The reaction conditions were those described under Materials and Methods. The concentrations of the allylic pyrophosphate substrate and [^{14}C]IPP were 50 and 60 μM, respectively. [b] One unit is 1 nmol of [^{14}C]IPP incorporated into product min^{-1} (mg of protein)$^{-1}$.

by descending chromatography on Whatman 3MM paper in propanol–NH_4OH (conc)–H_2O (6:2:2). [^{14}C]IPP (specific activity 0.45 μCi/μmol) was the preparation previously described (Keenan and Allen, 1974).

Enzyme Preparation and Assay. Undecaprenyl pyrophosphate synthetase was prepared through the hydroxylapatite or Sephadex G-100 purification step as previously described (Allen et al., 1976). The enzyme assay solutions, unless otherwise noted, contained in a final volume of 0.5 mL, 0.10 M Tris buffer (pH 7.5), 0.5% Triton X-100, 0.2 mM $MgCl_2$, and variable concentrations of allylic pyrophosphate and IPP as required. The reaction mixtures were incubated at 35 °C for 30 min, and the products were analyzed after acid hydrolysis as previously described (Allen et al., 1976). The radioactivity of all samples was determined using a toluene-based scintillation fluid containing 0.4% Omnifluor (New England Nuclear).

Hydrolysis and Analysis of Enzyme Products. Polyprenyl pyrophosphates were prepared in larger amounts by utilizing larger incubation mixtures containing 50–100 μM FPP, 60 μM IPP, and increased amounts of enzyme. The polyprenyl pyrophosphates were hydrolyzed to the free alcohols using a sequential treatment with *Micrococcus lysodeikticus* membrane and a crude yeast homogenate (see Figure 6, Keenan and Allen, 1974). The alcohols were extracted with either water-saturated butanol or petroleum ether. These extracts were evaporated to dryness, and the residues were redissolved in $CHCl_3$ and passed through a column of silicic acid previously equilibrated with $CHCl_3$. The free polyprenols were eluted with $CHCl_3$ and subsequently cochromatographed with ficaprenol and undecaprenol by reverse-phase TLC chromatography on Kieselguhr G plates in acetone–water (92:8, v/v) (Allen et al., 1976).

Results

Reactivities of Various Terpenyl Pyrophosphates. The substrate specificity of the enzyme was studied using a hydroxylapatite-purified enzyme preparation. Table I represents the relative reactivities of various allylic terpenyl pyrophosphates as substrates, each tested at a concentration of 50 μM. The reactivity of the substrate increased with chain length, $C_{10} < C_{15} < C_{20}$, except for the *all-trans*-solanesyl pyrophosphate, which was unreactive. Moreover, the enzyme was not only active with the trans derivatives but was also active with the substrates having both cis and trans stereochemistry. For example, *c,t,t*-GGPP was 1.2-fold more reactive than *t,t,t*-GGPP, although *c,t*-FPP was 60% as effective as *t,t*-FPP.

Comparisons of Kinetic Constants. The Michaelis constants obtained with each substrate were determined from Lineweaver–Burk plots and are summarized in Table II. The concentration of IPP used in the determination of K_M values with the allylic substrates was 60 μM, which was saturating when *t,t*-FPP was used as a substrate. The enzyme showed a threefold smaller K_M value with *t,t*-FPP than with *c,t*-FPP, whereas the V_{max} values were similar with both substrates. On the other hand, the Michaelis constant found with *c,t,t*-GGPP was similar to that observed with *t,t*-FPP, whereas the V_{max} with *c,t,t*-GGPP was the highest observed. In contrast, the enzyme had a K_M value with *t,t,t*-GGPP about one-third of that found for *t,t*-FPP, but the V_{max} values were similar with both substrates.

TABLE II: Kinetic Constants with Various Terpenyl Pyrophosphates.[a]

	K_M (μM)	V_{max} (units)
IPP	14	
GPP	3.2	42
NPP	32	27
t,t-FPP	108	364
t,t-[^{3}H]FPP	103	294
c,t-[^{3}H]FPP	305	333
t,t,t-[^{3}H]GGPP	37	236
c,t,t-[^{3}H]GGPP	134	421

[a] The reaction conditions were those described under Materials and Methods. The concentration of Triton X-100 was 0.5%. [^{14}C]IPP concentration was 60 μM when the constants for the allylic pyrophosphates were determined. *t,t*-FPP concentration was 50 μM when the constant for [^{14}C]IPP was determined.

TABLE III: Kinetic Constants for *t,t*-FPP with Various Enzyme Activators.[a]

expt	activator	K_M (μM)	V_{max} (units)
A	0.5% Triton X-100	103	294
	0.2% Triton X-100	163	286
	0.05% Triton X-100	434	388
	0.05% Triton X-100 + 0.25 μM myristic acid	60	260
B	0.5% Triton X-100	104	189
	1 μM cardiolipin	64	176
	0.1 μM cardiolipin	45	47

[a] The reaction conditions were those described under Materials and Methods. [^{14}C]IPP was used at a concentration of 60 μM and the *t,t*-[^{3}H]FPP concentration was varied. Triton X-100 was also varied as indicated. Different enzyme preparations were used for experiments A and B.

It is interesting that the enzyme had extremely small K_M values with GPP and NPP in comparison with that found with *t,t*-FPP, while the V_{max} values found with these substrates were about 10% of that seen with *t,t*-FPP.

Little change in the K_M found with FPP was observed on eightfold further purification of the hydroxylapatite enzyme (K_M = 108 μM) by a second DEAE-cellulose step (K_M = 129 μM) (Muth, unpublished observations).

Studies were also undertaken to evaluate the effect of enzyme activators such as Triton X-100, bovine cardiolipin, and myristic acid on the kinetic constants (Table III). The effect of varying Triton X-100 concentration on K_M values found with *t,t*-FPP is shown in experiment A. Increasing the concentration of Triton X-100 from 0.05 to 0.5% caused a fourfold decrease of the K_M value, whereas changes in V_{max} were relatively small. Similarly, myristic acid dramatically reduced

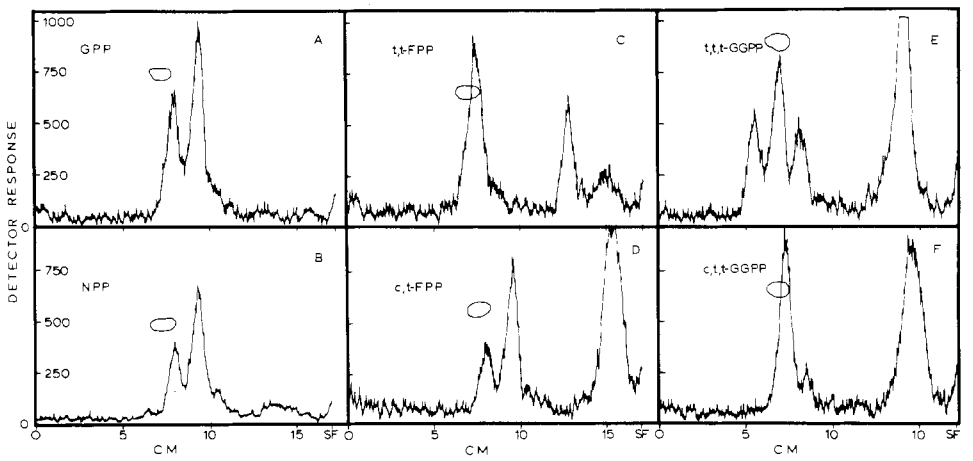


FIGURE 1: Reverse-phase TLC of the products derived from different isomeric allylic pyrophosphate substrates. Each allylic pyrophosphate substrate (100 μM) was incubated with [^{14}C]IPP (60 μM) under the usual incubation conditions for 5 h in a reaction volume of 1–2 mL. The products were hydrolyzed to the free polyprenols and chromatographed by reverse-phase TLC, as described under Materials and Methods. The tracings represent radioactive components detected by scanning the plate with a Packard radiochromatogram scanner with detector scale as indicated. The cochromatographing 55-carbon ficaprenol marker (circle) was detected by I_2 staining. Shorter chain-length polyprenols move faster than long-chain polyprenols in the reverse-phase system. The component chromatographing with an R_f of 0.75 in C was probably presqualene alcohol, since it was not present when substrates other than *t,t*-[^{3}H]FPP were used. The material chromatographing near the solvent front is hydrolyzed tritiated substrate.

TABLE IV: Effects of Prenyl Alcohols and Monophosphates on Synthetase Activity.[a]

effector	enhancement factor[b] effector concn 50 μM	100 μM	250 μM	500 μM
geraniol		0.97		1.22
t,t-farnesol		1.12		1.12
geranyl-P		0.91		1.09
t,t-farnesyl-P	1.90	2.01	1.88	1.31
t,t,t-geranylgeranyl-P	1.87	2.48	3.01	3.25
citronellyl-PP		0.93		1.13

[a] Incubation conditions were the same as described under Materials and Methods, except for FPP (20 μM), Triton X-100 (0.1%), and [^{14}C]IPP (60 μM). [b] Ratio of the extent of product formed in the presence of effector to the extent of product formed in the presence of 0.1% Triton X-100.

the K_M value even in the presence of 0.05% Triton X-100. The presence of cardiolipin affected both V_{max} and K_M values when compared to values obtained in 0.5% Triton X-100 (experiment B). The K_M values were similar to those obtained with the myristate–0.05% Triton X-100 mixture. The V_{max} value obtained in the presence of 0.1 μM cardiolipin was, however, also decreased.

Effects of Polyprenyl Alcohols and Monophosphates on Reaction Rates. The enzyme was studied with respect to the alteration of its activity by various polyprenol products (Table IV). The extent of the enzymatic reaction, using FPP as substrate, was not affected by farnesol, geraniol, GP, and CPP. *t,t,t*-GGP and *t,t*-FP, however, both stimulated enzymatic activity. This stimulatory effect was probably a function of the amphipatic properties of these compounds and was analogous to the observations seen with phospholipids (Allen and Muth, 1977).

Stereochemical Course of Polymerization. The stereochemical course of the polymerization reaction was studied using a mixture of [^{14}C]IPP and either (2*R*)- or (2*S*)-[2-^{3}H]IPP with different unlabeled allylic pyrophosphate substrates and the hydroxylapatite purified enzyme. In all cases studied (Table V), the normalized molar ratio of ^{3}H/^{14}C was 1 when (2*R*)-[2-^{3}H]IPP was used as the tritiated substrate and essentially zero when (2*S*)-[2-^{3}H]IPP was used as the tritiated substrate.

Product Analysis. The enzymatic products prepared using [^{3}H]allylic pyrophosphates, [^{14}C]IPP, and the hydroxylapatite-purified enzyme were hydrolyzed to the respective polyprenols and identified by comparison of their mobilities with authentic polyprenols on reverse-phase TLC. In addition, areas on the TLC plates corresponding to the radioactive products were extracted with $CHCl_3$, and the isotopic ratios in the double-labeled products were calculated. The chain lengths of the products determined from the ratios of ^{14}C/^{3}H obtained after 5 h of incubation were essentially coincident to that expected from direct comparison of the mobility of the products with authentic polyprenols on reverse-phase TLC (Table VI, Figure 1). The chain lengths determined from 10-min incubations were essentially the same but did not give quite as good a correlation. It was apparent that the 55-carbon polyprenol was the only enzymatic product synthesized from *t,t*-FPP or *c,t,t*-GGPP in both short- (10 min, not shown) and long-time incubations (5 h, Figure 1C,F). However, multiple products were formed with the other substrates. The 50- and 55-carbon polyprenols were the major and minor products, respectively, in reactions using *c,t*-FPP, GPP, and NPP (Figure 1D, 1A, and 1B). When *t,t,t*-GGPP was used as substrate, the 55-carbon polyprenol was the major product (Figure 1E) and was accompanied by lesser amounts of 50- and 60-carbon polyprenol. Little difference in product distribution was seen between short- (10 min) and long-time (5 h) incubation in all cases, except for NPP.

In the case of NPP, a considerable amount of short-chain product was produced at short times (Figure 2A), which disappeared at long times (Figure 1B). This short-chain product after hydrolysis with phosphatases was identified as farnesol by both normal TLC (silica gel 60, benzene–ethyl acetate, 10:1, v/v, Figure 2B) and by reverse phase TLC (acetone–H_2O, 65:35, v/v, data not shown). Gas chromatographic analysis indicated that the farnesol was exclusively the cis,cis isomer (Figure 2C, peak I).

Small amounts of highly purified enzyme were extracted from polyacrylamide gels following electrophoresis in 0.5% Triton X-100 (Muth, unpublished observations). The product ratio of C_{55}/C_{50} obtained using GPP as a substrate, with this

TABLE V: Stereochemical Course of Polymerization.[a]

substrate	product	substrate incorporated from (2R)-[2-^{3}H]IPP + [^{14}C]IPP ^{3}H(nmol)[b]	^{14}C(nmol)[b]	normalized ^{3}H/^{14}C ratio	(2S)-[2-^{3}H]IPP + [^{14}C]IPP ^{3}H(nmol)	^{14}C(nmol)	normalized ^{3}H/^{14}C ratio
GPP	C_{50}	2.62	2.57	1.02	0.040	2.96	0.01
	C_{55}	2.23	2.16	1.03	0.013	1.88	0.01
NPP	C_{15}	1.05	1.02	1.03	0.058	0.87	0.07
	C_{50}	1.63	1.63	1.00	0.026	2.21	0.01
	C_{55}	0.64	0.63	1.02	0.003	0.46	0.01
t,t-FPP	C_{55}	7.26	6.41	1.13	0.069	4.80	0.01

[a] Incubations were carried out for 30 min under the standard conditions using 100 μM allylic pyrophosphate, a mixture of [^{14}C]IPP (6.45 nCi, sp act. 0.45 nCi/nmol), and either (2*R*)-[2-^{3}H]IPP (32.9 nCi, sp act. 3.05 nCi/nmol) or (2*S*)-[2-^{3}H]IPP (32.9 nCi, sp act. 6.1 nCi/nmol) and a hydroxylapatite-purified enzyme. The polyprenyl pyrophosphate products obtained using each allylic pyrophosphate substrate were hydrolyzed to the free polyprenols and separated by reverse-phase TLC in acetone–H_2O (85:15) as described under Materials and Methods. The areas corresponding to the polyprenols were scraped from the plate. The polyprenols were eluted from the gel with two 1-mL aliquots of $CHCl_3$ and three 1-mL aliquots of acetone. The pooled eluates were then analyzed for ^{3}H and ^{14}C. [b] The specific activities of the combined IPP substrates were 1.31 nCi/nmol for (2*R*)-[2-^{3}H]IPP and 0.257 nCi/nmol for [^{14}C]IPP when (2*R*)-[2-^{3}H]IPP was used, and 1.67 nCi/nmol for (2*S*)-[2-^{3}H]IPP, and 0.33 nCi/nmol for [^{14}C]IPP when (2*S*)-[2-^{3}H]IPP was used.

TABLE VI: Isotope Ratios in the Products Synthesized from Various Substrates.[a]

incubat time	substrate	polyprenol length[b]	expected isoprene units added[c]	obsd isoprene units added[d]
10 min	*t,t*-FPP	C_{55}	8	8
	c,t-FPP	C_{55}, **C_{50}**	8, 7	7.92, 7.35
	t,t,t-GGPP	C_{60}, **C_{55}**, C_{50}	8, 7, 6	8.88, 7.79, 6.03
	c,t,t-GGPP	C_{55}	7	7.19
5 h	*t,t*-FPP	C_{55}	8	8
	c,t-FPP	C_{55}, **C_{50}**	8, 7	8.04, 7.03
	t,t,t-GGPP	C_{60}, **C_{55}**, C_{50}	8, 7	8.21, 7.27
	c,t,t-GGPP	C_{55}	7	6.83

[a] Reactions were carried out in 1–8-mL incubation volumes, depending on the activity of the allylic substrate, for the times indicated under the conditions described under Materials and Methods. The concentrations of [^{14}C]IPP and each of the [^{3}H]allylic pyrophosphates were 60 and 50 μM, respectively. [b] Determined from mobilities on reverse-phase TLC, the predominant product is in boldface type. [c] Based on polyprenol length determined by TLC. [d] Determined from the observed ^{14}C/^{3}H ratios. The ratio of ^{14}C/^{3}H was defined as 8 using *t,t*-FPP as substrate, since its product contained only the 55-carbon polyprenol and, therefore, must have had 8 isoprene units added.

highly purified enzyme on long-time incubation (5 h), was essentially the same as that obtained using the hydroxylapatite enzyme (Figure 1A).

Discussion

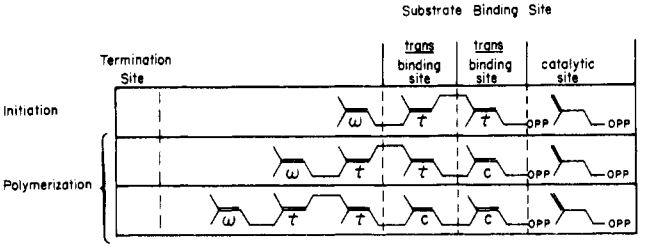
Studies on the mechanism of action of prenyltransferases, which form products with trans double bonds, have been directed toward elucidating the stereochemical course of the polymerization process (Hemming, 1974) and the specificity of the transferase for various natural (Popjak et al., 1969a) and synthetic isomers of the allylic pyrophosphate substrate (Popjak et al., 1969b; Ogura et al., 1970; Nishino et al., 1971). The study of undecaprenyl pyrophosphate synthetase offers the opportunity to examine a prenyltransferase which catalyzes the formation of a product having cis double bonds. The known stereochemical composition of undecaprenyl pyrophosphate and the preference of the synthetase for the 15-carbon allylic pyrophosphate, FPP, strongly suggest that *t,t*-FPP is the natural allylic substrate. The observation that geranyl pyrophosphate, a trans 10-carbon allylic substrate, could also serve as substrate, however, leads to several questions. What is the selectivity of the enzyme with respect to the chain length and stereochemistry of the allylic pyrophosphate substrate, the stereochemical course of the polymerization reaction, and the degree of polymerization with various substrates?

Kinetics. Comparison of kinetic parameters using a variety of substrates revealed that increasing the chain length of the allylic pyrophosphate substrate from 10 to 20 carbons results in an increased rate of product formation. The shortest possible substrate, dimethylallyl pyrophosphate (Allen et al., 1976), and longer *all-trans*-allylic pyrophosphate, solanesyl pyrophosphate, did not serve as substrates. It appears that a minimum chain length of three isoprene units is required for good substrate activity. This is particularly evident when one compares the V_{max} values found with substrates giving similar product distribution (333 and 42 for *c,t*-FPP and GPP, respectively).

Comparison of the Michaelis constants for substrates having the same number of carbon atoms revealed that the larger the number of *trans*-isoprene residues, the more strongly the substrate bound to the enzyme. This effect was more dramatic the shorter the chain. Binding of the allylic pyrophosphate substrate to the enzyme is also apparently dependent on the presence of an α-unsaturated isoprene unit and a pyrophosphate moiety, since no inhibition of activity was observed with the free polyprenols, their monophosphates, or citronellyl pyrophosphate.

Since detergents, phospholipids, and fatty acids have been described to have a marked stimulatory effect on enzymatic activity (Allen and Muth, 1977), it was of interest to see if their effects could be attributed to a change in the substrate-binding constant, V_{max}, or both. Triton X-100 and myristic acid–Triton

CHART I



X-100 mixtures both increased the binding of FPP to the enzyme with little effect on the V_{max} values. Cardiolipin also increased FPP binding.

Comparison of the kinetic constants alone does not permit one to determine if there is a unique allylic terpenyl pyrophosphate as the natural substrate of the enzyme. An evaluation of the stereochemical course of the reaction and product analysis is of equal importance.

Stereochemistry. The work of Cornforth et al. (1966) and Archer et al. (1966) first demonstrated that the presence of trans and cis double bonds in polyprenyl units can be determined with the use of stereospecifically labeled substrates. Our previous results showed that [^{14}C]IPP and (2*R*)-[2-^{3}H]IPP were incorporated into polyprenyl pyrophosphates in equal molar amounts when either FPP or GPP was used as substrate (Allen et al., 1976). This would indicate that all isoprene units were added with formation of cis double bonds. This is a particularly important point, since, conceivably, the C_{55} product formed from GPP could have had the naturally occurring ratio of 8 cis and 2 trans double bonds, meaning one new double bond would have been inserted with trans stereochemistry.

The incorporation of one isoprene unit in a trans configuration may not have been detected when using only the (2*R*)-[2-^{3}H] isomer, because of the large number of cis residues formed and the use of the ratios method of analysis. Use of the (2*S*)-[2-^{3}H] isomer provided a clear test of the formation of trans units, since only the (2*S*) proton is retained during the formation of the trans double bond (Archer et al., 1966). We have now conclusively shown that all isoprene units added by undecaprenyl pyrophosphate synthetase have a cis configuration, and geranyl pyrophosphate has been ruled out as a natural substrate.

Product Chain Length. The enzyme was active with each of the 15- and 20-carbon terpenyl pyrophosphates examined. Product characterization provided the strongest support for *t,t*-FPP being the natural substrate. *t,t*-FPP and *c,t,t*-GGPP, the first expected product of *t,t*-FPP metabolism by this enzyme, were the only substrates that led to the formation of

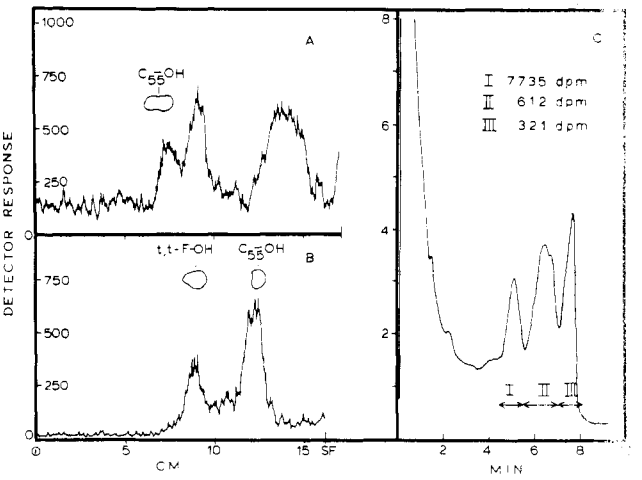


FIGURE 2: Characterization of short-chain product using NPP as a substrate. The polyprenyl pyrophosphate products synthesized in a 10-min incubation with NPP under conditions described in Figure 1 were hydrolyzed to the free alcohols by phosphatase treatment and then analyzed by reverse-phase TLC (A) as described under Materials and Methods and normal phase TLC on silica gel 60 in benzene–ethyl acetate (5:1, v/v) (B). The shorter chain products were separated by silica gel 60 column chromatography in benzene–ethyl acetate (20:1, v/v) and analyzed by GC (C). A mixture of isomeric farnesols was cochromatographed on GC with the radioactive components. Three peaks were collected with the use of a splitter fitted column and correlated with the retention times of farnesols of different stereochemistry. Peak I corresponds to *c,c*-FOH, peak II corresponds to a mixture of *c,t* and *t,c*-FOH, and peak III corresponds to *t,t*-FOH. The radioactivity found in each of the three peaks is indicated.

undecaprenol as the single product. The results obtained with the other stereochemical isomers could be accounted for if we propose three determinants to regulate product chain length: (1) the enzyme recognizes an optimal length of the product corresponding to a 55-carbon polyprenol, with no rigid requirement for its geometry; (2) the enzyme recognizes the total number of *cis*-isoprene residues (eight) in the product; (3) the enzyme adds eight *cis*-isoprene residues to the substrate no matter how many cis residues it contains.

The observation that *t,t*-FPP and *c,t,t*-GGPP gave the same product indicates that determinant 3 must be of little importance in these two cases, since there is no 60-carbon product formed from *c,t,t*-GGPP. It would appear in the case of the substrates GPP and *c,t*-FPP that the number of *cis*-isoprene units present in the product (determinant 2) is the predominant force determining the chain length distribution. On the other hand, in the metabolism of NPP, it is clear that determinant 3 predominates in establishing the product chain length. Poor

CHART II: Terminal Chain Arrangement Required to Give Major Products.

Substrate		Chain Termination Recognition Site: trans	trans	cis	Substrate Binding Site	Predominantly Observed and Predicted Product
t,t-FPP	ω	t	t	c	(c)$_7$-OPP	C_{55}
c,t,t-GGPP	ω	t	t	c	(c)$_7$-OPP	C_{55}
t,t,t-GGPP	ω	t	t	t	(c)$_7$-OPP	C_{55}
c,t-FPP		ω	t	c	(c)$_7$-OPP	C_{50}
GPP		ω	t	c	(c)$_7$-OPP	C_{50}
NPP		ω	c	c	(c)$_7$-OPP	C_{50}
No. of Isoprene Residue	11	10	9	8	7	

adaptability of *c,c*-FPP (an intermediate in NPP metabolism) to the enzyme active site, however, apparently resulted in a slow rate of its utilization to form *c,c,c*-GGPP, which ca ısed an accumulation of *c,c,c*-FPP. Since there was little accumulation of polyprenols other than 15-, 50-, and 55-carbon polyprenols, the elongation of a *c,c,c*-GGPP to product must be very rapid. Elongation of the substrate *t,t,t*-GGPP gave the 55-carbon polyprenol as the major product. In this case, determinant 1 is apparently predominating.

It would appear, therefore, that none of these determinants alone can predict the product chain length in all cases of the unnatural substrates examined here. However, each determinant may have varying influence, depending on the substrate used.

The enzyme lacks strict geometric or chain-length specificity for the allylic pyrophosphate substrate, but it polymerizes most effectively those having at least three isoprene residues containing two trans residues. The substrate binding site, therefore, must preferentially bind trans residues but accommodate cis residues during polymerization (Chart I). The lack of strict geometric specificity in binding the substrate may be caused by this requirement of the substrate binding site to accommodate both cis and trans residues during polymerization.

The enzyme showed, however, a considerable strictness in the chain length of the final products (50–60 carbons). Once polymerization starts, the reaction proceeds to the final products without accumulation of intermediates (except in the case where NPP was substrate). This might be explained by a model simply represented in Chart II. That portion of the product binding site located between the site of substrate binding and the site of chain termination would be relatively nonspecific with respect to the stereochemistry of the elongating chain. Hence, polymerization proceeds without the accumulation of interme diates. The chain-length distribution of the products could be explained if termi ion of elongation was signaled by recognition of the sequen of isoprene units trans,trans,cis. Recognition of this exact sec nce arrangement would have to be somewhat flexible to accommodate sequence arrangements not ordinarily encountered in vivo and would account for some variation in chain termination when using unnatural substrates.

Acknowledgments

We are grateful to Dr. Katherine Scott for recording and analyzing the NMR spectra of the prenyl alcohols and Dr. Brian Andresen for recording the mass spectra. We also thank Janine D. Muth for her excellent technical assistance in preparing the enzymes.

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