Mechanism of squalene biosynthesis: evidence against the involvement of free nerolidyl pyrophosphate

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ABSTRACT Several mechanisms that utilize farnesyl pyrophosphate and nerolidyl pyrophosphate as condensing substrates have been postulated for the asymmetric condensation reaction in squalene biosynthesis. Although there is ample evidence that farnesyl pyrophosphate is a substrate for this reaction, there has been no information concerning the role of nerolidyl pyrophosphate. We have made the following observations that demonstrate that nerolidyl pyrophosphate cannot be a free intermediate in squalene biosynthesis. (*a*) There is no significant interconversion of farnesyl pyrophosphate and nerolidyl pyrophosphate in a squalene-synthesizing system from yeast. (*b*) Nerolidyl-1-$^3H_2$ pyrophosphate is not converted to squalene in the presence or absence of farnesyl pyrophosphate. (*c*) The addition of unlabeled nerolidyl pyrophosphate to incubation mixtures does not alter the relative loss of α-hydrogens from farnesyl pyrophosphate during its conversion to squalene.

The synthesis of nerolidyl-1-$^3H_2$ pyrophosphate is described. Chromatographic methods for the separation of pyrophosphate esters of triprenols and terpenols are included.

SUPPLEMENTARY KEY WORDS yeast · farnesyl pyrophosphate · triprenol and terpenol pyrophosphates · chromatography · synthesis of nerolidyl pyrophosphate

Cornforth, Popják, and their collaborators (1–5) have shown that the synthesis of squalene, a symmetrical molecule, from two farnesyl pyrophosphate molecules is an asymmetric process. They considered that asymmetry might be introduced prior to the condensation reaction, and proposed several mechanisms for squalene biosynthesis in which asymmetry arose by the isomerization of one molecule of farnesyl pyrophosphate to nerolidyl pyrophosphate (4, 5). The condensation reaction was then hypothesized to be between farnesyl pyrophosphate and nerolidyl pyrophosphate. However, definite data on the role of nerolidyl pyrophosphate in squalene biosynthesis have been lacking.

In early studies of triprenyl pyrophosphate synthesis, Goodman and Popják (6) obtained evidence for the synthesis of both nerolidyl pyrophosphate and farnesyl pyrophosphate from mevalonic acid by an enzyme system from rat liver. More recently Krishna, Whitlock, Feldbruegge, and Porter (7) reported that nerolidyl pyrophosphate depressed the conversion of farnesyl pyrophosphate to squalene in an enzyme system from pig liver. This diminished conversion could have resulted from nerolidyl pyrophosphate inhibiting the utilization of farnesyl pyrophosphate or by its participating as a substrate with concomitant dilution of the conversion of farnesyl pyrophosphate to squalene. Thus, there has been no evidence concerning the participation of nerolidyl pyrophosphate in squalene biosynthesis. In this paper we will show that free nerolidyl pyrophosphate cannot be an intermediate in the biosynthesis of squalene.

METHODS

Trans-trans-farnesol was isolated from a *cis-trans*, *trans-trans* mixture (Aldrich Chemical Co., Inc., Milwaukee, Wis.) by crystallization of the diphenylurethane derivative (8). *Trans-trans*-methyl farnesoate was prepared by treating *trans-trans*-farnesoic acid, prepared via the *S*-benzylthiuronium salt (8), with diazomethane. Reduction of this ester with $LiAl^3H_4$ (New England Nuclear Corp., Boston, Mass.) yielded farnesol-1-$^3H_2$, specific activity 10 μc/μmole. Nerolidol-1-$^3H_2$ was obtained by the hydrolysis of farnesyl monophosphate-

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1-^3H_2 (a by-product of the synthesis of the pyrophosphate ester of farnesol) in 0.5 N HCl in methanol–water 1:1 for 45 min at 25°C. The products of acid hydrolysis, namely nerolidol (41%), farnesol (8%), and hydrocarbons, were extracted into hexane. Separation of the products was not attempted at this point. Redistilled *trans*-nerolidol (Aldrich) was then added to this mixture which, in turn, was phosphorylated. The nerolidyl pyrophosphate was purified by ion-exchange chromatography of the products of phosphorylation and by two crystallizations of the pyrophosphate ester from ammoniacal methanol. The specific activity, based on pyrophosphate content (9), was 0.48 μc/μmole.

The terpene alcohols were pyrophosphorylated by the method of Kandutsch, Paulus, Levin, and Bloch (10). After pyrophosphorylation, the reaction mixtures were taken to dryness on a rotary evaporator. The residue was suspended in 2% concentrated ammonium hydroxide in methanol, cleared by centrifugation, and applied to 1 × 14 cm columns of 200–400 mesh Bio-Rad AG 1-X8 (Bio-Rad Laboratories) in the formate form. The columns were eluted with a concentration gradient of ammonium formate in methanol. For the separation of nerolidyl and farnesyl pyrophosphates, a linear gradient of 0.053–0.43 M ammonium formate in a total volume of 300 ml of methanol was used (see Fig. 2). Farnesyl-^{14}C pyrophosphate (4μc/μmole) was enzymically prepared as previously described (11) except that it was purified by chromatography on Bio-Rad AG 1-X8.

A yeast particulate fraction (microsomes) was obtained by sonicating 5 g of baker's yeast (Fleishmann's) for 5 min in 7 ml of 0.05 M potassium phosphate buffer, pH 7.4, containing 0.001 M $MgCl_2$. A Branson LS-75 sonic oscillator was used at 80% of maximum output. After cell debris had been removed by centrifugation at 28,000 *g* for 15 min, the particulate fraction was obtained by centrifugation at 144,000 *g* for 2 hr. The particulate fraction was washed by centrifugation and suspended in 10 ml of the same buffer. Protein concentration was typically 14 mg/ml.

Typical incubation mixtures for the synthesis of squalene, unless otherwise specified, contained: glucose-6-phosphate, 0.2 μmole; a small crystal of glucose-6-phosphate dehydrogenase (Sigma Chemical Co.); potassium phosphate, pH 7.4, 2 μmole; $MgCl_2$, 1 μmole; KF, 1 μmole; 0.05 ml of the yeast particulate fraction; and 0.01–0.07 μmole of substrate in a total volume of 0.2 ml. Incubations were for 30 min at 30°C under nitrogen. For the isolation of squalene and the allylic pyrophosphates, the reaction mixtures were brought to 2 N NH_4OH and 1 M EDTA at the end of the incubation. Squalene and the allylic pyrophosphates were then extracted with hexane and *n*-butanol, respectively. In experiments where bacterial alkaline phosphatase (Sigma) was used to hydrolyze the allylic pyrophosphates, 2 μmoles of potassium oxalate, 300 μmoles of potassium phosphate (pH 8.0), and 0.5 mg of alkaline bacterial phosphatase were added to the incubation mixture after incubation. The volume was adjusted to 0.6 ml and the mixture was incubated at 37°C for 3–6 hr. The allylic alcohols released were extracted with hexane.

Thin-layer chromatography of the allylic pyrophosphates was performed on plates coated with 0.25 mm of a suspension of 20 g of Silica Gel H (E. Merck A. G.) in 60 ml of 0.1 M ammonium phosphate buffer solution (pH 6.5). The plates were developed with chloroform–methanol–water 60:40:9. The allylic alcohols were chromatographed on Silica Gel G (Merck) in ethyl acetate–hexane 30:70. Compounds were detected by exposure to iodine vapor. Allylic alcohols were further analyzed by gas–liquid chromatography on a 6 ft, $^1/_8$ inch column packed with 3% QF-1 (methyl fluoroalkyl silicone) on Gas-Chrom Q (Applied Science Laboratories). Retention times at 130°C were 3.1 min for *trans*-nerolidol and 7.8 min for *trans-trans*-farnesol.

A Packard liquid scintillation counter was used for radioactive measurements. The scintillation solution was 0.4% (w/v) 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]-thiophene in toluene. Aqueous samples were dissolved in the scintillation solution by the addition of absolute ethanol. Counting efficiencies were determined by internal standardization.

RESULTS AND DISCUSSION

Some Properties of Nerolidyl Pyrophosphate

Since this paper deals with the role of nerolidyl pyrophosphate in sterol metabolism and since the characteristics of this compound that are important to biochemical studies have not been reported, some information regarding its properties is given here. The pyrophosphate ester of nerolidol is easily prepared (12) and isolated by ion-exchange chromatography. Hydrolysis by bacterial alkaline phosphatase of tritiated nerolidyl pyrophosphate, prepared as described in Methods, gave 99% nerolidol with a 1% farnesol contamination, as judged by gas chromatography. This indicated that rearrangements had not occurred during phosphorylation. Allylic phosphate esters are notorious for their susceptibility to acid hydrolysis (6, 13). Nerolidyl pyrophosphate, a tertiary allylic ester, is even more labile. The relative rates of acid hydrolysis of nerolidyl pyrophosphate and farnesyl pyrophosphate are given in Fig. 1. Even at pH 7.4, nerolidyl pyrophosphate decomposes at a rate of about 1% per minute at 95°C, a temperature and pH under which farnesyl pyrophosphate is stable. Because of this lability to acid, it is essential that nerolidyl pyro-

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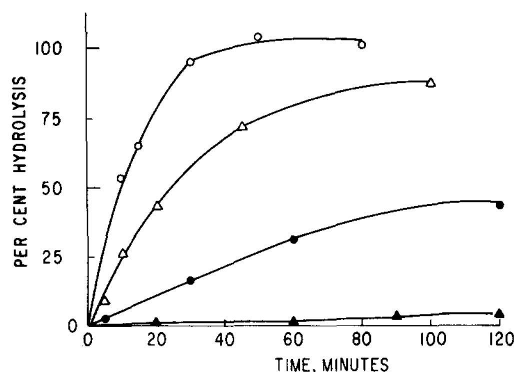

FIG. 1. Rates of hydrolysis of farnesyl and nerolidyl pyrophosphate in 0.67 M citrate buffers at 25°C. Quantifications were made by gas-liquid chromatography of hexane extracts of the indicated mixtures. The symbols used are: ○, nerolidyl pyrophosphate at pH 2.9; △, nerolidyl pyrophosphate at pH 3.9; ●, farnesyl pyrophosphate at pH 2.9; ▲, farnesyl pyrophosphate at pH 3.9.

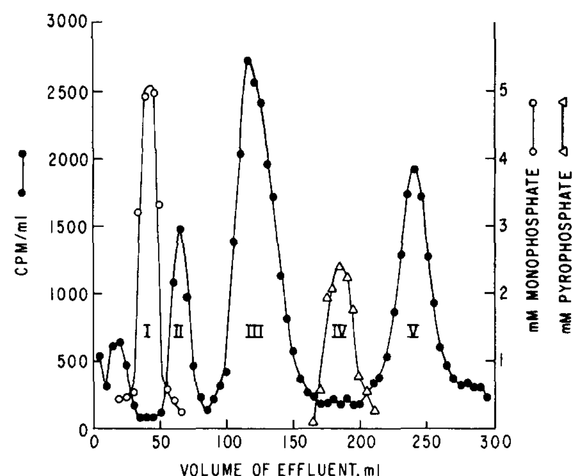

FIG. 2. Separation of nerolidyl and farnesyl pyrophosphates, showing the lack of incorporation of tritium into nerolidyl pyrophosphate. 20 µmoles of potassium phosphate (pH 7.4), 10 µmoles of $MgCl_2$, 10 µmoles of KF, 0.5 ml of the yeast particulate fraction, 0.2 µmole of nerolidyl pyrophosphate, 0.2 µmole of farnesyl pyrophosphate, and 3.26×10^6 dpm of farnesyl-1-$^3H_2$ pyrophosphate in a final volume of 2 ml were incubated under nitrogen at 30°C for 5 min. The butanol extracts of the incubation mixtures were then diluted with 80 ml of methanol, 50 µmoles each of nerolidyl monophosphate and nerolidyl pyrophosphate were added, and the mixture was chromatographed on Bio-Rad AG 1X-8. Nerolidyl monophosphate and nerolidyl pyrophosphate were located by phosphate determination (9). The positions of the other compounds were determined by radioisotopic measurements. The peaks are numbered as follows: nerolidyl monophosphate, I; farnesyl monophosphate, II; C_{30} phosphorylated intermediate in squalene synthesis, III; nerolidyl pyrophosphate, IV; farnesyl pyrophosphate, V.

phosphate be stored either in alkaline solution or dry, after lyophilization from ammoniacal solutions.

Hydrolysis of allylic pyrophosphates with alkaline phosphatase has been used as a method for liberating the alcohol portion of the molecule for subsequent identification (6). We have found that farnesyl pyrophosphate yields only farnesol with this treatment. However, on enzymic hydrolysis of nerolidyl pyrophosphate, both the *trans-trans* and the *cis-trans* isomers of farnesol have been found in amounts totaling up to 15% of the alcohols formed. It is apparent that rearrangements can take place during the enzymic hydrolysis of nerolidyl pyrophosphate.

Goodman and Popják (6) used $LiAlH_4$ as a reagent for removal of the pyrophosphate moiety from allylic pyrophosphates for the purpose of identifying the alcohol fragment. We have found this method unsatisfactory since both farnesol and nerolidol, as well as hydrocarbons, are formed in various amounts during the treatment of either farnesyl or nerolidyl pyrophosphate with $LiAlH_4$, although the unrearranged alcohol was the predominant alcoholic product from either pyrophosphate ester. These results are in accord with those of Appelton, Fairlie, and McCrindle (14), who observed rearrangements during the $LiAlH_4$ reductions of tosylate esters.

Since Goodman and Popják (6) also used a hydrolysis method (alkaline phosphatase treatment) in which farnesyl pyrophosphate yields only farnesol and no nerolidol, their report of the synthesis of a nerolidyl derivative from mevalonic acid in a rat liver system must be accepted as valid. These experimenters did not establish the form of derivative that was present, but assumed that it was the pyrophosphate ester. In this laboratory, all attempts to demonstrate the formation of nerolidyl pyrophosphate from mevalonic acid with a yeast enzyme system have been unsuccessful.

Investigation of Interconversion of Farnesyl Pyrophosphate and Nerolidyl Pyrophosphate

If, in the yeast particulate fraction used for squalene biosynthesis, the rates of equilibration of farnesyl pyrophosphate and nerolidyl pyrophosphate were greater than the rate of squalene synthesis, it would be difficult to evaluate the individual roles of these compounds as substrates for squalene synthesis. Therefore, it was necessary to estimate the relative rates of interconversion of these two pyrophosphate esters.

Farnesyl-1-$^3H_2$ pyrophosphate was incubated with the yeast particulate fraction and unlabeled nerolidyl pyrophosphate for a short time. Ion-exchange chromatography of a butanol extract of the reaction mixture revealed radioactivity in the regions where farnesyl monophosphate, farnesyl pyrophosphate, and a C_{30} pyrophosphate intermediate[1] were eluted. No significant amounts of radioactivity were found in the nerolidyl pyrophosphate region (Fig. 2). These results indicate that, if there is a rapid interconversion of farnesyl pyrophosphate and

[1] Recent spectral data on this intermediate in squalene biosynthesis indicate that the tentatively proposed structure (11) will require modification. The essential features of this intermediate, a cyclopropane-containing C_{30} pyrophosphate ester, remain (16).

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nerolidyl pyrophosphate, the equilibrium position favors farnesyl pyrophosphate.

To evaluate the conversion of nerolidyl pyrophosphate to farnesyl pyrophosphate, we incubated both radioactive compounds with their nonradioactive counterparts in the presence of the yeast particulate fraction. The incubation mixtures were extracted with butanol and the extract was divided into two parts. One part was treated with alkaline phosphatase and the liberated alcohols were chromatographed in a thin-layer system that separated farnesol from nerolidol (Table 1). Since the alcohol from hydrolysis of a pyrophosphorylated intermediate between farnesyl pyrophosphate and squalene cochromatographs with nerolidol in this system, it was necessary to chromatograph the second portion of the butanol extract in a thin-layer system that separated this intermediate from farnesyl and nerolidyl pyrophosphates (Table 2). The data presented in Tables 1 and 2 show that the conversion of nerolidyl pyrophosphate to the C_{30} intermediate or farnesyl pyrophosphate by the yeast enzyme system is slight. In fact, the ratio of nerolidol to farnesol found after bacterial alkaline phosphatase treatment is the same for the incubated mixture and the unincubated control.

TABLE 1 Thin-Layer Chromatography of the Allylic Alcohols Liberated by Alkaline Phosphatase

R_f	Standard Compound	Hydrolysis Products			
		A	B	Farnesyl Pyro-phosphate	Nerolidyl Pyro-phosphate
		cpm/unit area			
0.89–1.0		55	0	0	1
0.80–0.89	Squalene	10,200	13	0	24
0.65–0.80		120	9	0	6
0.52–0.65	Nerolidol	220	561	1	1,230
0.46–0.52		310	3	22	11
0.35–0.46	Farnesol	11,600	52	4,190	100
0.08–0.35		200	9	120	4
Origin		1,780	226	9	0

A is from an incubation with 80,000 cpm of farnesyl-1-$^3H_2$ pyrophosphate and 0.06 μmole of nerolidyl pyrophosphate as substrates; B with 20,000 cpm of nerolidyl-1-$^3H_2$ pyrophosphate and 0.06 μmole of farnesyl pyrophosphate. The last two columns show products of enzymatic hydrolysis of the substrates used. The indicated zones on the thin-layer plates were removed and counted.

TABLE 2 Thin-Layer Chromatography of the Allylic Pyrophosphates

R_f	Standard Compound	A	B
		cpm/unit area	
0.91–1.0		3240	190
0.71–0.91		30	3
0.48–0.71		80	15
0.42–0.48	Phosphorylated C_{30} intermediate	780	2
0.37–0.42		140	2
0.23–0.37	Nerolidyl and farnesyl pyrophosphates	2840	800
0.08–0.23		50	6
Origin		16	2

A and B refer to the incubations cited in Table 1. The indicated zones were removed and counted.

Failure to Observe Incorporation of Nerolidyl-1-$^3H_2$ Pyrophosphate into Squalene

Incubation of nerolidyl-1-$^3H_2$ pyrophosphate in a squalene-synthesizing system in the presence or absence of farnesyl pyrophosphate failed to give significant incorporation of radioactivity into squalene. Small amounts of radioactive hydrocarbons were found, but this material did not cocrystallize with squalene as the thiourea adduct. Thin-layer chromatography of a butanol extract of a similar incubation mixture showed the presence of nerolidyl pyrophosphate, which indicated that this compound was present throughout incubation and that excessive hydrolysis of it had not occurred. This experiment provides strong evidence that nerolidyl pyrophosphate cannot be a free intermediate in squalene biosynthesis.

However, because of the difficulty in completely and unequivocally characterizing the small amount of tritiated nerolidyl pyrophosphate prepared, an alternative method for testing its role as an intermediate was devised.

Lack of Effect of Nerolidyl Pyrophosphate on the Relative Incorporation of α-Hydrogens of Farnesyl Pyrophosphate into Squalene

Cornforth, Popják, and their collaborators have shown (1–3) that the condensation of two triprenyl pyrophosphates to form squalene is an asymmetric process during which one of the four α-hydrogens of the two farnesyl pyrophosphate molecules is lost. If one of the farnesyl pyrophosphates were rearranged to nerolidyl pyrophosphate prior to coupling, it follows that this hydrogen would be lost from either the farnesyl or nerolidyl moiety.[2] If farnesyl pyrophosphate were converted to squalene in the presence of nerolidyl pyrophosphate and nerolidyl pyrophosphate were participating in squalene biosynthesis, there would be a change in the relative loss of the α-hydrogens from farnesyl pyrophosphate. Further, if the α-hydrogen loss were to occur from the nerolidyl moiety, there would be none from the farnesyl residue and vice versa. Only if the reactants were either both farnesyl pyrophosphate or nerolidyl pyrophosphate would the observed loss of α-hydrogens from farnesyl

[2] The exception to this would be if a symmetrical C_{30} intermediate were formed prior to proton loss. In view of available evidence concerning the mechanism of squalene biosynthesis, this possibility is extremely remote.

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TABLE 3 Conversion of Farnesyl-1-$^{3}H_2$-^{14}C Pyrophosphate to Squalene in the Presence of Farnesyl Pyrophosphate or Nerolidyl Pyrophosphate

Incubation	Added Unlabeled Substrate	Squalene Isolated	
		^{14}C	$^{14}C/^{3}H$ Ratio
		dpm × 10^{-3}	
A_1	None	19	0.60
A_2	Farnesyl pyrophosphate (0.01 μmole)	18	0.63
A_3	Farnesyl pyrophosphate (0.06 μmole)	4.5	0.63
B_1	Nerolidyl pyrophosphate (0.01 μmole)	15	0.63
B_2	Nerolidyl pyrophosphate (0.06 μmole)	5*	0.62

Each incubation mixture contained farnesyl-1-$^{3}H_2$-^{14}C pyrophosphate, 3 × 10^4 dpm of ^{14}C. The (dpm) ratio $^{14}C/^{3}H$ was 0.46. In addition, incubations A_2 and A_3 contained farnesyl pyrophosphate while B_1 and B_2 contained nerolidyl pyrophosphate.

* The inhibition of squalene biosynthesis by nerolidyl pyrophosphate was also observed by Krishna et al. (7).

TABLE 4 Crystallization of the Thiourea Adduct of Squalene-^{3}H-^{14}C

Sample	A Series		B Series	
	$^{14}C/^{3}H$ Ratio	^{14}C	$^{14}C/^{3}H$ Ratio	^{14}C
		dpm/mg		*dpm/mg*
1st Supernate	1.05	246	0.66	135
1st Crystals	0.62	340	0.61	200
2nd Supernate	0.64	341	0.64	212
2nd Crystals	0.55	322	0.62	200
3rd Supernate	0.62	326	0.63	200
3rd Crystals	0.61	330	0.62	200

The squalene fractions isolated from incubations A_1, A_2, and A_3 were combined, as were those isolated from B_1 and B_2 (Table 3). To each sample, 100 mg of squalene was added and the thiourea adducts were prepared (15). The squalene isolated from the thiourea adduct was carried through two successive adduct formations.

pyrophosphate remain unchanged by the addition of nerolidyl pyrophosphate.

To test these possibilities, we prepared doubly-labeled farnesyl pyrophosphate by mixing farnesyl-1-$^{3}H_2$ pyrophosphate with farnesyl-^{14}C pyrophosphate and converted it to squalene by means of the yeast enzyme system in the presence of various levels of nerolidyl pyrophosphate or farnesyl pyrophosphate. In these experiments (Table 3), the $^{14}C/^{3}H$ ratio of the squalene isolated from the incubation mixtures containing nerolidyl pyrophosphate would be expected to approach either 0.92 or 0.46 if nerolidyl pyrophosphate were participating in squalene biosynthesis with the retention or loss of one of its α-hydrogens. However, in all instances the observed $^{14}C/^{3}H$ ratio was unchanged by the addition of nerolidyl pyrophosphate and was as predicted (0.61) for the loss of one of the four α-hydrogens from farnesyl pyrophosphate. These isotope ratios remained constant through several crystallizations of the squalene–thiourea adduct (Table 4). Thus, the condensation reaction cannot take place between farnesyl pyrophosphate and nerolidyl pyrophosphate. Since nerolidyl pyrophosphate is not formed in appreciable quantities from farnesyl pyrophosphate and since nerolidyl-^{3}H pyrophosphate is not converted to squalene, it follows that farnesyl pyrophosphate is the only substrate for the condensation reaction, although these data do not rule out the existence of a transient enzyme–nerolidol or enzyme–nerolidyl pyrophosphate complex.

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