Squalene Synthetase

III. MECHANISM OF THE REACTION*

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SUMMARY

Studies on the properties of the reaction and the mechanism of conversion of farnesyl pyrophosphate to squalene have been carried out with purified yeast squalene synthetase. The general properties of the yeast enzyme are similar to those of the enzyme obtained from mammalian liver. A divalent metal ion (Mg²⁺ or Mn⁺⁺) is essential for the formation of presqualene pyrophosphate, but not for the conversion of this compound to squalene. However, the rate of the latter reaction is increased in the presence of Mg⁺⁺. Yeast squalene synthetase activity is inhibited by concentrations above 50 mM of farnesyl pyrophosphate, by N-ethylmaleimide, and to a lesser extent by iodoacetamide. Hence it is evident that a sulfhydryl group or other nucleophile is required for enzyme activity.

Yeast squalene synthetase has a maximum enzyme activity at pH 7.3 to 7.5. However, great differences in enzyme activity were found, depending on the buffer used. These differences are due in part to ionic strength, and possibly to a direct effect of phosphate ion on enzymatic activity.

Initial velocity studies for the formation of squalene and presqualene pyrophosphate from farnesyl pyrophosphate have shown that the mechanism of the condensing reaction is ping-pong. Initial velocity kinetics and product inhibition studies for the reduction of presqualene pyrophosphate have shown that this reaction is sequential ordered. In the reduction reaction NADPH is the first substrate to bind to the enzyme, followed by presqualene pyrophosphate. The first product to be released is pyrophosphate, followed by squalene. The last product to leave the enzyme is NADP.

A chemical mechanism for squalene synthesis is presented. An isomerization analogous to that catalyzed by isopentenyl pyrophosphate isomerase (17, 18) follows. An attack of the π electrons of carbon 2 of one farnesyl pyrophosphate on carbon 1 of the other. This attack is helped by a nucleophilic group on the enzyme. The formation of the cyclopropane ring then results from a concerted attack on the carbon atom bound to the enzyme with the release of a proton from C-1 of the incoming farnesyl pyrophosphate molecule.

In the mechanism proposed by van Tamelen and Schwartz (10) the π system of carbon 2 of one farnesyl pyrophosphate attacks carbon 1 of the other farnesyl pyrophosphate with either intramolecular migration or external addition of pyrophosphate. An isomerization analogous to that catalyzed by isopentenyl pyrophosphate isomerase (17, 18) then follows. An attack of the newly formed double bond on the carbon atom binding pyrophosphate, with subsequent elimination of pyrophosphate, would yield the cyclopropane ring. Collapse of the carbonium ion by elimination of a proton from the tertiary carbon, would then result in the formation of presqualene pyrophosphate. Both of the above mechanisms result in the elimination of one of the hydrogen atoms from carbon 1 of one of the two condensing far-
The reduction of presqualene pyrophosphate to squalene is believed to involve as a first step the elimination of pyrophosphate (13, 14, 16). The carbonium ion formed would then undergo ring expansion to yield a cyclobutonium ion. The cyclobutonium ion would be opened and stabilized by the presence of allylic double bonds. Hydride transfer from NADPH would finally yield squalene as the product.

A second proposed mechanism (12) consists of a two-step reaction. In the first step, rearrangement of the presqualene pyrophosphate molecule results in ring expansion and migration of the pyrophosphate moiety. In the second step an attack by the hydride ion from NADPHII would result in ring opening and the elimination of the pyrophosphate anion.

In the present study we have attempted to work out the mechanism of squalene synthesis through kinetic analysis. Initial velocity kinetics of this reaction show that the first partial reaction is ping-pong. The second reaction, the reduction of presqualene pyrophosphate, is however sequential and ordered. From this information we have proposed a new mechanism for the synthesis of squalene.

**EXPERIMENTAL PROCEDURE**

**Materials**—The materials used in this study were purchased from the following sources: Tween 80 from E. H. Sargent; 1-butanol, benzene, and ethyl acetate from Fisher Scientific Laboratories; Silica Gel H and Silica Gel G from E. Merck; NADPH from Schwarz-Mann Laboratories; Good's buffers from Calbiochem; and Tris base from Sigma Chemical Co. All other reagents were of analytical grade.

The preparation of squalene synthetase and [3H]- and [14C]-farnesyl pyrophosphate were reported previously (1, 20).

Preparation of Presqualene Pyrophosphate—Two different preparations of presqualene pyrophosphate were used. Low specific activity presqualene pyrophosphate was synthesized enzymatically in the following incubation mixture: potassium phosphate buffer, pH 7.4, 100 mM; magnesium chloride, 10 mM; Tween 80, 2 mg; [4, 14C]farnesyl pyrophosphate (4 × 10^6 dpm), 1.37 mM; and DEAE-cellulose-purified yeast squalene synthetase, 16.5 mg of protein; in a total volume of 1 liter. After 4 hours of incubation under nitrogen, the reaction was stopped by the addition of 150 ml of concentrated ammonia and 150 ml of 0.25 M EDTA. The solution was incubated for an additional 10 min at 25°C. The [14C]presqualene pyrophosphate formed was extracted with 500-ml portions of 1-butanol until no radioactivity was found in the 1-butanol phase (generally six extractions). The 1-butanol was removed under vacuum by flash evaporation. The pale yellowish syrup was dissolved in benzene and the solvent was again evaporated. This process was repeated two more times to remove all traces of 1-butanol. The final syrup was dissolved in a minimum amount of benzene and applied to a series of glass plates, 20 × 20 cm, coated with Silica Gel II (0.5 mm thick). The thin layer plates were developed with ethyl acetate-isopropyl alcohol-ammonia (45:60:45); (RF values were the following: presqualene pyrophosphate, 0.40; presqualene monophosphate, 0.75; farnesyl pyrophosphate, 0.20; and farnesyl phosphate, 0.70). [3H]Presqualene pyrophosphate was located by assaying 1-cm strips of silica gel scraped from the plate for radioactivity with a Tri-Carb liquid scintillation spectrometer. The remainder of the desired region of the plate was scraped into a beaker and the [3H]presqualene pyrophosphate was eluted with 1% ammonia in methanol. Rechromatography of this material on thin layer plates (Silica Gel II) with isopropyl alcohol-concentrated ammonia-water (6:3:1) (4) showed a single spot of radioactivity (RF 0.40). Reverse phase thin layer chromatography (20) of the acid hydrolys products gave a single zone of radioactivity (RF 0.65), coincident with carrier acid-treated presqualene alcohol. Mass spectra of the purified [3H]presqualene pyrophosphate agreed with that obtained by Edmond et al. (12).

High specific activity [4, 14C]presqualene pyrophosphate, labeled in the same position as the material of low activity, was prepared under conditions similar to those reported above. The following components: potassium phosphate buffer, pH 7.5, 50 mM; magnesium chloride, 10 mM; Tween 80, 200 μg; [4, 14C]farnesyl pyrophosphate (2 × 10^6 dpm), 9.61 nmoles; and purified squalene synthetase, 1.37 mM of protein; in a total volume of 10 ml, at 25°C for 80 min. The reaction was stopped by the addition of 20 ml of 95% ethanol, and the reaction mixture was extracted three times with 5 ml of petroleum ether. Concentrated ammonia, 1.5 ml; EDTA, 0.25 m, 3 ml; and water, 50 ml, were added and the solution was incubated for 15 min at 25°C. The incubation mixture was extracted with three 100-ml aliquots of 1-butanol, and then with three 100 ml aliquots of benzene. The 1-butanol and benzene extracts were combined and the solvent each of the experiments reported in this paper the enzyme obtained after DEAE-cellulose column chromatography was used. Assays for the overall conversion of farnesyl pyrophosphate to squalene and the first partial reaction; i.e. the biosynthesis of presqualene pyrophosphate, were also reported previously (1, 20). Concentrations of the substrates used are shown in the legends to the figures.
was extracted under vacuum by flash evaporation. The residue was extracted first with 1-butanol-benzene (1:1), and then with 2% ammonium in methanol. The extracts were combined, the solvent removed by flash evaporation, and the residue applied to a thin layer plates of silica gel H. The plates were developed with ethyl acetate-isopropyl alcohol-ammonia (45:60:45). The $[^{14}C]$presqualene pyrophosphate region was scraped from the plate and eluted with 2% ammonium in methanol. Thin layer chromatography of the eluted material showed a single zone of radioactivity on chromatography on silica gel H in a system of isopropyl alcohol-ammonia-water (6:3:1). A single component was also observed on reverse phase chromatography of the acid hydrolysis products.

Assay for Reduction of Presqualene Pyrophosphate—The rate of reduction of presqualene pyrophosphate was determined in an incubation mixture that contained potassium phosphate buffer, pH 7.4, 25 μmoles; magnesium chloride, 5 μmoles; $[^{14}C]$presqualene pyrophosphate, 24 μmoles and 20,000 dpm; NADPH, 0.8 μmol; glycerol, 150 μl; and purified squalene synthetase, 5 μg of protein, in a total volume of 0.5 ml. After 2 to 10 min of incubation at 37° the reaction was stopped by the addition of 1 ml of 95% ethanol. The squalene formed was extracted with three 2-ml portions of petroleum ether. Carrier squalene, 0.5 mg, was added and the petroleum ether extract was evaporated to dryness under a stream of nitrogen. The residue was dissolved in petroleum ether and spotted on a thin layer plate of silica gel G, 0.3-mm thickness. The chromatogram was developed with petroleum ether-ethyl ether (98:2). After development, the plate was air-dried and sprayed with 1% lidoine in methanol. The squalene zone was scraped from the plate into a glass vial and radioactivity was measured by assaying in a Tri-Carb liquid scintillation spectrometer as described earlier (21).

Phosphate Determination—The concentration of phosphate was determined by measuring the concentration of phosphate by the method of Galliard et al. (22).

Protein Determination—Protein was determined by the method of Gornall et al. (23).

Data Processing—Reciprocal velocities were plotted against the reciprocal of substrate concentration. Similar plots were made earlier by Dugan and Porter (24) for the over-all reaction catalyzed by pig liver squalene synthetase. The first partial reaction of squalene synthesis follows the general equation for a ping-pong mechanism (25) where

$$v = \frac{V_{A}^{2}}{(K_{A} + K_{A}')A + A'^{2}}$$

(1)

and $K_{A}$ and $K_{A}'$ are the Michaelis constants for the 1st and 2nd molecules of farnesyl pyrophosphate that bind to the enzyme, $A$ is the farnesyl pyrophosphate concentration; $V$ is the maximum velocity, and $n$ the rate of the reaction.

The data obtained from the initial velocities for the second partial reaction, the reduction of presqualene pyrophosphate to squalene, fit Equation 2. This equation fits a sequential Ordered Bi Ter reaction where

$$v = \frac{V_{A}B_{C}}{K_{A}K_{B} + K_{B}C + K_{B}B + BC}$$

(2)

and $B$ and $C$ represent the concentrations of NADPH and presqualene pyrophosphate, respectively; $K_{A}$ and $K_{B}$ the Michaelis constants for the respective substrates, and $K_{A}$ the dissociation constant for NADPH.

For product inhibition studies Equation 3, or parts of it, were used:

$$v = \frac{V_{A}V_{2}PO_{2}}{K_{eq} + V_{A}V_{2}PO_{2}}$$

$$v = \frac{V_{A}V_{2}BC}{K_{eq} + V_{A}V_{2}BC} + \frac{V_{A}K_{BP}}{K_{eq}K_{B}P}$$

$$v = \frac{V_{A}K_{BP}}{K_{eq}K_{B}P} + \frac{V_{A}K_{BP}}{K_{eq}K_{B}P} + \frac{V_{A}K_{BP}}{K_{eq}K_{B}P} + \frac{V_{A}K_{BP}}{K_{eq}K_{B}P} + \frac{V_{A}K_{BP}}{K_{eq}K_{B}P} + \frac{V_{A}K_{BP}}{K_{eq}K_{B}P} + \frac{V_{A}K_{BP}}{K_{eq}K_{B}P}$$

(3)

In this equation $P$, $Q$, and $R$ are the products of the reaction; namely, pyrophosphate, squalene, and NADP, respectively.

The other symbols follow Cleland's nomenclature (25, 26). The data were fitted to the above equations, assuming equal variance for the velocities (27).

RESULTS

General Properties of Reaction

Effect of Protein Concentration and Time of Incubation—Enzyme activity for the over-all conversion of farnesyl pyrophosphate to squalene and for the formation of presqualene pyrophosphate (Fig. 2, A and B) was proportional to the amount of protein up to 50 to 60 μg per ml. In the second partial reaction, the reduction of presqualene pyrophosphate to squalene, activity was linear with protein added to at least 30 μg per ml (Fig. 2C).

Enzyme activity for each of the above reactions was also linear with time, up to approximately 30 min (Fig. 3, A, B, and C).

pH Optimum and Effect of Ionic Strength—Yeast squalene synthetase showed maximum enzyme activity at pH 7.3 to 7.5 (Fig. 4). In addition enzyme activity was greater in phosphate buffer than in the buffers described by Good (28) and in Tris-HCl. To test whether activation by phosphate ions is a specific or non-specific ionic strength effect, experiments were performed with 50 mM Buffer A (N-tris(hydroxymethyl)methyl-2-aminoethane sulfonate), pH 7.5, and different concentrations of potassium phosphate or KCl. The results of these experiments (Fig. 5) show that the effect of phosphate ion is not completely specific. KCl replaces phosphate ions at high osmolarities. However, a specific effect of potassium phosphate cannot be ruled out since it was a better activator at low osmolarities. An increased enzyme activity in higher ionic strength buffer was observed for each of the three reactions studied (Fig. 5).

Metal Ion—Squalene synthetase requires a divalent metal ion for enzyme activity. Magnesium ion is essential for the over-all reaction (Fig. 6A) and for the formation of presqualene pyrophosphate (Fig. 6B). When 30 μmoles of EDTA were added instead of magnesium (the zero magnesium concentration, Fig. 6A), no reaction was observed. Magnesium ion is not essential for the reduction of presqualene pyrophosphate to squalene, but the reaction rate is enhanced nearly 50 times in the presence of 30 mM magnesium chloride (Fig. 6C).

Manganous ion can replace magnesium ion as an activator for the over-all reaction (Fig. 7). Maximum activity was reached at a concentration of approximately 1 mM. At higher concentrations manganous ion was inhibitory.
FIG. 2. The effect of the concentration of squalene synthetase on the rate of synthesis of presqualene pyrophosphate and squalene. A, the effect of protein concentration on the conversion of farnesyl pyrophosphate to squalene. The following were incubated in a total volume of 1 ml at 37° for 5 min: potassium phosphate, pH 7.5, 50 mM; MgCl₂, 10 mM; NADPH, 1.6 mM; glycerol, 0.3 ml; and [³⁵S]farnesyl pyrophosphate, 0.05 mM and 20,000 dpm. The amount of protein used in the incubation mixture is given on the figure. The conditions of assay were reported previously (1, 20). B, dependence of the rate of presqualene pyrophosphate formation on protein concentration. The incubation mixture contained the same components as reported for Fig. 2A, except for the omission of NADPH. The assay was carried out as described previously (1, 20). C, dependence of the rate of reduction of presqualene pyrophosphate to squalene on protein concentration. The following were incubated in a total volume of 0.5 ml for 5 min at 37°: potassium phosphate buffer, pH 7.4, 50 mM; MgCl₂, 10 mM; [³⁵S]presqualene pyrophosphate, 0.048 mM and 20,000 dpm; NADPH, 1.6 mM; and glycerol, 0.15 ml. The amount of protein used in each assay is shown on the figure. The conditions of assay are reported under "Experimental Procedure."  

Inhibitors—Fluoride ion has been used by other investigators, and by us, to inhibit a very active phosphatase present in the microsomes and in crude preparations of squalene synthetase. Fig. 8 shows the effect of fluoride ion on purified bakers’ yeast squalene synthetase. Fluoride ion is a potent inhibitor of the three reactions studied. Fifty per cent inhibition was observed at fluoride concentrations between 5 to 8 mM. 

Krishna et al. (5) reported that pig liver squalene synthetase is strongly inhibited by p-hydroxymercurobenzoate and N-ethylmaleimide. We obtained similar results with the purified bakers yeast squalene synthetase. The inhibition of squalene synthetase by reagents that combine with sulfhydryl groups is shown in Fig. 9, A, B, and C. The condensation of farnesyl pyrophosphate to form presqualene pyrophosphate and the reduction of the latter to squalene are inhibited to the same extent, indicating that each of the partial reactions of squalene synthesis requires either a sulfhydryl or a nucleophilic group. Iodoacetamide, another reagent that combines with sulfhydryl groups, was not as effec-
The effect of pH on the rate of squalene synthesis. The assay mixture contained: MgCl$_2$, 10 mM; glycerol, 0.3 ml; [14C]farnesyl pyrophosphate, 0.05 mM and 20,000 dpm; and purified squalene synthetase, 40 µg of protein, in a total volume of 1 ml. The incubation time was 5 min. Squalene was extracted and purified as reported previously (1, 20). The following buffers were used at a concentration of 50 mM: O—O, Tris-HCl; O—O, potassium phosphate; and △—△, Gold's buffers (pH 5.55, 6.31, and 6.60); 2-(A-morpholino)ethane sulfonate (pH 6.70, 7.10, and 7.31); Buffer A (pH 7.75); and Tricine, pH 8.50.

Farnesyl pyrophosphate at high concentrations is an inhibitor of yeast squalene synthetase. Maximal activity was obtained with a farnesyl pyrophosphate concentration of 25 µM. At 600 µM, the enzymatic activity was 32% of the maximal activity (Fig. 10).

Reduced Nucleotide Specificity of Squalene Synthetase—Previously Shechter and Bloch (30) analyzed yeast squalene synthetase for its requirement for pyridine nucleotides. A similar analysis of our enzyme preparation for pyridine nucleotide specificity is shown in Table I. NADH can replace NADPH; however, the reaction proceeds at a much lower rate.

Formation of Lycopersene—Yeast squalene synthetase condenses 2 molecules of geranylgeranyl pyrophosphate to form the C$_{40}$ compound lycopersene, Table I. However, the rate of this reaction is much slower than that for the formation of squalene.

Initial Velocity Kinetics

Over-all Reaction—A series of kinetic experiments was carried out to obtain data on the mechanism of action of yeast squalene synthetase. When NADPH was the variable substrate, and four fixed farnesyl pyrophosphate concentrations were used, a parallel pattern of initial velocities was obtained for the reciprocal plots (Fig. 11). From replots of the intercepts of the above lines the Michaelis constants were determined for farnesyl pyrophosphate (4.4 × 10$^{-7}$ M) and for NADPH (6.1 × 10$^{-5}$ M). The parallel pattern of initial velocities obtained (Fig. 11), indicates that the Michaelis constants for farnesyl pyrophosphate and NADPH to the enzyme are irreversibly connected. When the reciprocal of the concentration of farnesyl pyrophosphate was plotted against the reciprocal of the rate of reaction, with NADPH held at several fixed substrate concentrations, a family of parallel straight lines was obtained, thus indicating that the binding of the 2 molecules of farnesyl pyrophosphate are irreversibly connected. Hence, a ping-pong-type mechanism is deduced for the first partial reaction of squalene synthesis.

First Partial Reaction—To further test this possibility, initial velocity studies of the first partial reaction were conducted. If the mechanism for the formation of presqualene pyrophosphate is ping-pong, the reaction rate would follow Equation 4.

\[
\frac{1}{v} = \frac{K_a + K_{a'}}{V} \cdot \frac{1}{A} + \frac{1}{V}
\]  

This equation predicts a straight line when a reciprocal plot of the data is made. If the reaction is sequential, the data would follow Equation 5, which predicts a parabolic line upon plotting the reciprocals.

\[
\frac{1}{v} = \frac{K_b \cdot K_{a'}}{V} \cdot \frac{1}{A^3} + \frac{K_a + K_a'}{V} \cdot \frac{1}{A} + \frac{1}{V}
\]  

When the initial velocity experiment for the first partial reaction was carried out (Fig. 12), a straight line was obtained. A value of 3.9 × 10$^{-7}$ was obtained for ($K_a + K_{a'}$), from the extrapola-
Fig. 6. Effect of MgCl₂ concentration on the rates of the reactions catalyzed by squalene synthetase. A, dependence of the rate of the over-all conversion of farnesyl pyrophosphate to squalene on the concentration of magnesium ion. The following were incubated in a total volume of 1 ml for 5 min at 37°C: potassium phosphate buffer, pH 7.5, 50 mM; NADPH, 1.6 mM; [³⁵⁹]farnesyl pyrophosphate, 0.05 mM and 20,000 dpm; glycerol, 0.3 ml; and purified squalene synthetase, 10 µg of protein. The concentrations of MgCl₂ are reported on the figure. The assay for enzyme activity was carried out as reported previously (1, 20). B, dependence of rate of synthesis of presqualene pyrophosphate on the concentration of MgCl₂. The incubation mixture was the same as in Fig 6A, except for the omission of NADPH. The assay for enzyme activity was carried out as described previously (1, 20). C, dependence of the rate of reduction of presqualene pyrophosphate to squalene on the concentration of MgCl₂. The following were incubated in a total volume of 0.5 ml at 37°C for 5 min: potassium phosphate buffer, pH 7.4, 50 mM; [³⁵⁹]presqualene pyrophosphate, 0.048 mM and 20,000 dpm; NADPH, 1.6 mM; glycerol, 0.3 ml; and purified squalene synthetase, 5 µg of protein. The conditions of the assay are reported under "Experimental Procedure."

Fig. 7. Effect of concentration of manganous ion on the rate of synthesis of squalene from farnesyl pyrophosphate. The assay mixture was the same as reported in Fig. 6A, except for the replacement of MgCl₂ by MnCl₂.

Second Partial Reaction—The next series of experiments was designed to study the second partial reaction. Initial velocity studies (Fig. 13) showed an intersecting pattern when the reciprocal of NADPH concentration was plotted against the reciprocal of the rate of the reaction. From replots of the intercept against the reciprocal of the concentration of the changing fixed substrate, the Michaelis constants for NADPH and presqualene pyrophosphate, were calculated. Values of 7.0 x 10⁻⁵ and 7.7 x 10⁻⁷ M were obtained for NADPH and presqualene pyrophosphate, respectively.

The intersecting pattern obtained for the reduction of presqualene pyrophosphate indicates that the second partial reaction is sequential, i.e. that both substrates must be bound to the enzyme before the release of product.

Product Inhibition Studies—In order to determine whether this reaction is ordered or random, and also to know the order, if any, of the addition of substrate and release of products, experiments were carried out on the inhibition of the enzyme reaction by its products. The effect of inorganic pyrophosphate on the second partial reaction catalyzed by squalene synthetase is shown in Fig. 14. When the reciprocal of the reaction rate was plotted against the reciprocal of the concentration of NADPH, a constant non-saturating concentration of presqualene pyrophosphate, an S-linear, I-linear non-competitive inhibition was observed. When NADP was used as product inhibitor, and presqualene pyrophosphate the variable substrate, an S-linear, I-linear non-competitive inhibition was obtained (Fig. 15). When NADPH was the variable substrate and NADP was the inhibitor, double reciprocal plots showed a pattern of S-linear competitive inhibition (Fig. 16).
The effect of potassium fluoride on the over-all conversion of farnesyl pyrophosphate to squalene. The incubation mixture contained: potassium phosphate buffer, pH 7.5, 50 mM; MgCl₂, 10 mM; NADPH, 1.6 mM; glycerol, 0.3 ml; [³⁵⁸⁷]farnesyl pyrophosphate, 0.05 mM and 20,000 dpm; and enzyme protein, 20 μg. The total volume was 1 ml and incubations were carried out for 5 min at 37°C. The concentration of potassium fluoride used is shown on the figure. Inhibition of the synthesis of presqualene pyrophosphate by potassium fluoride (A). The incubation mixture was the same as for (A) except for omission of NADPH. Assays were carried out as described previously (1, 20). Inhibition of the reduction of presqualene pyrophosphate to squalene by potassium fluoride (B). The following were incubated in a total volume of 0.5 ml for 5 min at 37°C: potassium phosphate buffer, pH 7.4, 50 mM; MgCl₂, 10 mM; [³⁵C]presqualene pyrophosphate, 0.048 mM and 20,000 dpm; NADPH, 1.6 mM; glycerol, 0.15 ml; and purified squalene synthetase, 5 μg of protein. The conditions of assay are reported under "Experimental Procedure."

**Discussion**

Purified squalene synthetase catalyzes an over-all reaction in which three substrates and four products are involved. The over-all reaction is in turn composed of two partial reactions, namely, the condensation of 2 molecules of farnesyl pyrophosphate to presqualene pyrophosphate, and the reduction of this intermediate to form squalene.

The reaction for the formation of squalene is linear with respect to the concentration of protein, up to 60 μg per ml and with time, to 30 min. A marked deviation from linearity was observed, however, when more than 4% of the farnesyl pyrophosphate was used.

Bakers' yeast squalene synthetase has an optimum pH of 7.3 to 7.5. The same optimum pH was found for the rat (29) and pig liver (5) enzymes. Yeast squalene synthetase showed large differences in activity in the presence of different buffers even though they were used at identical molar concentrations. The optimum pH was, however, the same for all the buffers tested.

The increased activity of squalene synthetase observed with phosphate ion could be due to (a) a specific effect of this ion, (b) a nonspecific effect of ionic strength, or (c) to both effects. The results reported show that at high osmolarities, KCl can replace phosphate ion. However, the phosphate ion is a better activator for squalene synthesis at low ionic strength, thereby indicating that both high ionic strength and phosphate ion are important for maximum activity of squalene synthetase. Ionic strength and phosphate ion are also important for both partial reactions of squalene synthesis.

A divalent metal ion is an absolute requirement for activity of yeast squalene synthetase. Either magnesium or manganese will meet this requirement. At low concentrations manganese ion is a better activator than magnesium ion, but at higher concentrations manganese ion is inhibitory. This phenomenon has been observed with other enzymes as well, especially those which have phosphorylated intermediates (31-33). Activation of squalene synthetase by magnesium ion showed a biphasic curve for the three reactions studied; first, activity rapidly increased with an increase in magnesium ion concentration, up to 2.5 to 5 mM, and then it increased more slowly up to 30 mM (the highest concentration tested). This secondary increase in enzyme activity at high concentrations of metal ion could be due to an increase in ionic strength in the incubation mixture (compare with Fig. 4).

Magnesium is an absolute requirement for enzyme activity for the first partial reaction, but it does not appear to be essential for the reduction of presqualene pyrophosphate. The second partial reaction occurs at a significant rate, even in the presence of 30 mM EDTA and zero magnesium concentration (Fig. 6). However enzyme activity for this partial reaction is increased greatly in the presence of magnesium ion.

Fluoride ion has been widely used to inhibit a microsomal phosphatase in crude preparations of squalene synthetase (3, 6, 10, 31). We have found, however, that fluoride ion is a potent inhibitor of squalene synthetase and its inhibitory action has been demonstrated for both partial reactions of squalene synthesis.

The difference in inhibition of squalene synthetase obtained with N-ethylmaleimide and monooiodoacetamide cannot be explained at present. However, the same phenomenon has been observed by others (29, 34, 35). It appears very probable that squalene synthetase requires a sulphydryl group for full activity. This fact is supported not only by the inhibition studies but also by the requirement of 2-mercaptoethanol or dithiothreitol for stabilization of the enzyme. Krishna et al. (5) and Goodman et al. (29) previously reported inhibition of the over-all reaction of squalene synthesis by reagents that combine with sulphydryl groups when pig and rat liver squalene synthetases were used.

Both inhibitors, N-ethylmaleimide and monooiodoacetamide, inhibited squalene synthetase to nearly the same extent in each partial reaction (Fig. 9), thus indicating that a sulphydryl or a nucleophile group is important in each reaction.

Krishna et al. (5) found that high concentrations of farnesyl pyrophosphate are inhibitory for pig liver squalene synthetase. Our experiments with yeast squalene synthetase also showed that this enzyme is inhibited by farnesyl pyrophosphate. However, the inhibitory concentrations of farnesyl pyrophosphate that we found were higher than those required for the critical micellar concentration for farnesyl pyrophosphate. Possibly the inhibition by farnesyl pyrophosphate that we observed could be due to a nonspecific detergent effect. Schechter and Bloch (30) also reported inhibition of squalene synthetase by other detergents such as deoxycholate.

Truger and Porter (24) reached maximum velocity for squalene synthesis at 0.2 to 0.4 μM farnesyl pyrophosphate. They explained their results on the basis of micelle formation. We did not observe maximum velocity at this farnesyl pyrophosphate concentration with our assay procedure. Possibly this differ-
Mechanism of Reaction—The results of initial velocity experiments for the over-all reaction of squalene synthesis indicate that the binding of the 1st molecule of farnesyl pyrophosphate to the enzyme is irreversibly connected to the binding of NADPH (parallel pattern) and to the binding of the 2nd molecule of farnesyl pyrophosphate (straight lines in the plot of Fig. 11). From this information we can conclude that the first partial reaction has a ping-pong-type mechanism (25, 36) (see Scheme I). Confirmatory evidence for a ping-pong mechanism was obtained from an initial velocity study of the first partial reaction. The straight line obtained for the plot of the reciprocal of the initial velocity against the reciprocal of farnesyl pyrophosphate concentration in the first partial reaction (Fig. 12) agrees with our result on the initial velocity for the over-all reaction (Fig. 11).

TABLE I
Activity of squalene synthetase with substrate analogs

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydrocarbons formed</th>
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<tbody>
<tr>
<td>NADPH, [14C]farnesyl pyrophosphate</td>
<td>100.0a</td>
</tr>
<tr>
<td>NADPH, [14C]geranylgeranyl pyrophosphate</td>
<td>6.3b</td>
</tr>
<tr>
<td>NADH, [14C]farnesyl pyrophosphate</td>
<td>37.4b</td>
</tr>
</tbody>
</table>

Squalene.

a The product isolated was identified as lycopersene.
FIG. 11. The effect of a fixed variable concentration of farnesyl pyrophosphate and a variable concentration of NADPH on the initial velocity of the squalene synthetase reaction. The following were incubated at 37°C in a volume of 1 ml: potassium phosphate buffer, pH 7.5, 50 mM; MgCl₂, 10 mM; glycerol, 0.3 ml; and protein, 20 μg. The concentrations of farnesyl pyrophosphate were: ▲—▲, 4.002 μM; △—△, 2.022 μM; □—□, 1.347 μM; ○—○, 0.991 μM. The concentrations of NADPH are indicated on the figure. The rate for each point was obtained from the slope of the line resulting from a plot of the squalene formed against incubation times of 0, 1, 2, and 5 min. The procedure of assay for squalene was described previously (1, 20). Inset, a replot of intercepts against the reciprocal of farnesyl pyrophosphate concentration.

FIG. 12. Double reciprocal plots of the initial velocity of presqualene pyrophosphate formation against substrate concentration. The following were incubated for 0, 1, 2, and 5 min at 37°C: potassium phosphate, pH 7.5, 50 mM; magnesium chloride, 10 mM; glycerol, 0.3 ml; and purified squalene synthetase, 20 μg of protein. The concentrations of farnesyl pyrophosphate are indicated on the figure. The procedure of assay for presqualene pyrophosphate was reported previously (1, 20).

If the condensation of 2 molecules of farnesyl pyrophosphate were sequential (both molecules of farnesyl pyrophosphate bound to the enzyme surface before product is released), the double reciprocal plot would generate a parabola (Equation 5). On the other hand, the equation for a ping-pong mechanism predicts straight lines (Equation 1). More direct evidence for this mechanism would involve the isolation of an “enzyme-farnesyl” intermediate which is postulated for this reaction. In earlier studies Krishna et al. (5) reported the isolation of a C₄₂ intermediate tightly bound to the enzyme. However, it was not shown that this group is covalently bound to squalene synthetase.

FIG. 13. A plot of the reciprocal of the initial velocity of the reduction of presqualene pyrophosphate catalyzed by squalene synthetase against the reciprocal of NADPH concentration. The following were incubated in a total volume of 0.5 ml at 37°C: potassium phosphate buffer, pH 7.4, 50 mM; MgCl₂, 10 mM; glycerol, 0.15 ml; and purified squalene synthetase, 5 μg of protein. The concentrations of presqualene pyrophosphate were: ○—○, 1.667 μM; □—□, 2.249 μM; △—△, 2.863 μM; and ●—●, 5.05 μM. The rate of reaction for each point was calculated from the slope of the line resulting from a plot of the squalene produced against incubations for 0, 1, 2, and 5 min. The assay procedure is reported under “Experimental Procedure.” Inset, a replot of intercepts and slopes against the reciprocal of presqualene pyrophosphate concentration.

FIG. 14. Effect of inorganic pyrophosphate on the rate of reduction of presqualene pyrophosphate to squalene. The following were incubated in a volume of 0.5 ml at 37°C: potassium phosphate buffer, pH 7.4, 50 mM; MgCl₂, 10 mM; glycerol, 0.15 ml; and squalene synthetase, 5 μg. The concentration of presqualene pyrophosphate was 1.02 μM. The concentrations of pyrophosphate were: ▲—▲, 0.08 mM; ○—○, 0.06 mM; △—△, 0.02 mM; and □—□, 0.00 mM. The rate for each point was obtained from the slope of the line resulting from a plot of squalene formed against incubation at 0, 2.5, and 5 min. The assay procedure is reported under “Experimental Procedure.” Inset, a replot of intercepts and slopes against pyrophosphate concentration.

Initial velocity studies for the second partial reaction showed that it is an Ordered Bi Ter type. The possibilities existing for this reaction are (a) a completely random addition and release of products, and (b) a sequential ordered mechanism. If the addition of substrates or the release of products were random, none of the products would give competitive inhibition with any
FIG. 15. The effect of fixed variable concentrations of NADP on the initial velocity of reduction of presqualene pyrophosphate to squalene. The following were incubated in a total volume of 0.5 ml at 37°C: potassium phosphate buffer, pH 7.4, 50 mM; MgCl₂, 10 mM; glycerol, 0.15 ml; and protein, 5 μg. The concentration of NADPH was 0.0174 mM. The concentration of presqualene pyrophosphate is indicated on the figure. The concentrations of NADP were: O---O, 0.186 mM; □---□, 0.069 mM; Δ---Δ, 0.035 mM; ●---●, 0.00 mM. The rate for each point was determined from the slope of the line resulting from a plot of squalene formed against incubation of 0, 1, and 2.5 min. The assay procedure is reported under “Experimental Procedure.” Inset, a replot of intercepts and slopes against NADP concentration.

FIG. 16. The effect of fixed variable concentrations of NADP and variable concentrations of NADPH on the initial velocity of reduction of presqualene pyrophosphate to squalene. The following were incubated in a total volume of 0.5 ml at 37°C: potassium phosphate buffer, pH 7.4, 50 mM; MgCl₂, 10 mM; glycerol, 0.15 ml; and purified squalene synthetase, 5 μg of protein. The concentration of presqualene pyrophosphate was 2.802 mM. The concentrations of NADPH are indicated on the figure. The concentrations of NADP were: O---O, 0.186 mM; □---□, 0.069 mM; Δ---Δ, 0.035 mM; ●---●, 0.00 mM. The rate for each point was determined from the slope of the line resulting from a plot of squalene formed against incubation of 0, 1, and 2.5 min. The assay procedure is reported under “Experimental Procedure.” Inset, a replot of intercepts and slopes against NADP concentration.

substrate. As we obtained an S-linear, competitive inhibition when NADP was added as an inhibitor and NADPH was the variable substrate, a random, or a partially random mechanism for the addition of substrates, the release of products, or both, must be discarded. Therefore, it is concluded that the second partial reaction is sequential ordered.

When F (the first product to be released from the enzyme) is present during the reaction, Equation 3 can be rearranged and simplified to Equation 6, which predicts an S-linear, I-linear noncompetitive inhibition when the first substrate is varied.

\[
\frac{1}{v} = \frac{1}{v} \left[ \frac{K_b}{C} + \frac{K_{P}K_{R}K_{PSQPP}}{K_{PSQPP}K_{FPP}K_{PP}} \right] \frac{1}{B} +
\]

\[
\frac{1}{v} \left[ C \frac{1 + \frac{K_{PSQPP}}{K_{FPP}K_{PP}}} {1 + \frac{K_{PSQPP}}{K_{FPP}K_{PP}}} \right] \frac{1}{B} +
\]

When R (the last product to leave the enzyme) is present in the incubation mixture, Equations 7 and 8 are obtained by rearrangement and simplification of Equation 3.

\[
\frac{1}{v} = \frac{1}{v} \left[ \frac{K_{PSQPP}b}{C} \left( 1 + \frac{K_{R}K_{PSQPP}}{K_{R}K_{PSQPP}} \right) + K_{c} \right] \frac{1}{C} +
\]

\[
\frac{1}{v} \left[ C \frac{1 + \frac{K_{PSQPP}}{K_{R}K_{PSQPP}}} {1 + \frac{K_{PSQPP}}{K_{R}K_{PSQPP}}} \right] \frac{1}{C} +
\]

Some of the possibilities that can be predicted by the use of Equations 6, 7, and 8, and the experimental results, are summarized in Table II. The results suggest a sequential ordered
Several mechanisms have been proposed for the formation of presqualene pyrophosphate (10-13). All of these mechanisms involve the sequential addition of farnesyl pyrophosphate. Our results, and those of Dugan and Porter (24) for the pig liver squalene synthetase, point to a ping-pong type of reaction with the formation of a farnesyl-enzyme intermediate. A mechanism for the reaction that fits the kinetic data is presented in Scheme II. In the first reaction an $S_N2$ displacement of the pyrophosphate moiety by a nucleophilic group of the enzyme yields a farnesyl-squalene synthetase intermediate. The $\pi$ system of C-2 of a 2nd molecule of farnesyl pyrophosphate then displaces the enzyme from C-1 of farnesyl-enzyme. This displacement is helped by a second nucleophilic group of the enzyme. The overall effect of this double displacement is retention of the configuration around C-1 of both of the original molecules of farnesyl pyrophosphate. One or both of the nucleophilic groups on the enzyme could be a sulphydryl group. This could explain the inhibitory action of N-ethylmaleimide and other $-SH$ reagents. The next step is isomerization of the double bond as postulated by van Tamelen and Schwartz (16) with the displacement of one of the hydrogens of the C-1 from the 1st farnesyl pyrophosphate molecule. The third step would then be an intramolecular $S_N2$ displacement of the enzyme by the newly formed $\pi$ electrons to form a cyclopropane ring. The last step would involve the formation of a trans double bond by elimination of a proton on the tertiary carbon with the establishment of an olefinic bond at the original site and the formation of presqualene pyrophosphate. Alternatively a direct removal of $H_3$ from III and an attack on the carbon atom would displace the $B$ group from the enzyme and form the cyclopropane ring (Scheme III). Both mechanisms agree with the results of Cornforth et al. (37) who showed that only 1 farnesyl residue loses a hydrogen from the C-1 position on conversion of 2 molecules of farnesyl pyrophosphate to squalene.

Our kinetic data for the second partial reaction of squalene synthesis are consistent with an ordered sequential mechanism as presented in Scheme IV. The chemistry of such a mechanism has been proposed by Edmond et al. (12). Their proposal involves the rearrangement of the cyclopropane ring with the migration of the pyrophosphate moiety to form a tautomeric cyclobutyl derivative. This reaction is then followed by a direct

Scheme II. Proposed mechanism for the first partial reaction, the conversion of farnesyl pyrophosphate to presqualene pyrophosphate.

Scheme III. Alternative mechanism for the first partial reaction, the conversion of farnesyl pyrophosphate to presqualene pyrophosphate.

Scheme IV. Proposed mechanism for the reduction of presqualene pyrophosphate. PSQPP, presqualene pyrophosphate; PP, inorganic pyrophosphate; SQ, squalene; and E, squalene synthetase.
transfer of the hydride from NADPH and elimination of the pyrophosphate anion to form squalene.

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