# Biosynthesis of presqualene pyrophosphate by liver microsomes

H. C. RILLING

Department of Biochemistry, University of Utah
College of Medicine, Salt Lake City, Utah 84112

ABSTRACT Microsomes from rat liver have been shown to synthesize a squalene precursor from farnesyl pyrophosphate. This intermediate is identical with presqualene pyrophosphate, a 30-carbon cyclopropane containing pyrophosphate ester that had previously been isolated from yeast. The squalene precursor was found to be tightly, but not covalently, bound to microsomes.

SUPPLEMENTARY KEY WORDS squalene biosynthesis · farnesyl pyrophosphate

THE CONVERSION of farnesyl pyrophosphate to squalene is a reductive process requiring TPNH. By omitting TPNH from yeast enzyme systems, some investigators have found free intermediates in this reaction (1, 2). These intermediates were characterized as $C_{30}$-pyrophosphate esters.[1] Although the squalene precursors from yeast could be converted to squalene by liver microsomes (evidence that the pathways in yeast and mammals are the same), attempts to demonstrate the synthesis of free intermediates by liver enzymes were unsuccessful (1, 2).

In contrast, Krishna, Whitlock, Feldbruegge, and Porter (5) proposed that a 30-carbon enzyme-bound intermediate was a squalene precursor in pig liver. This intermediate, which was postulated to be devoid of a phosphate or pyrophosphate residue, could be converted to squalene by the addition of TPNH.

In this paper the synthesis of a squalene precursor from farnesyl pyrophosphate by liver microsomes is reported. This intermediate is shown to be the same as presqualene pyrophosphate,[2] an intermediate synthesized by yeast enzymes (1,4). Presqualene pyrophosphate is also shown to be tightly, but not covalently, bound to microsomes.

## MATERIALS AND METHODS

The preparation of farnesyl-1-$^3$H pyrophosphate and farnesyl-$^{14}$C pyrophosphate has been described previously (1, 6). Yeast microsomes were prepared as before (6). All steps used in the preparation of microsomes from rat liver were done at 0°–4°C. 5g of fresh rat liver were minced and homogenized in 25 ml of 0.3 M sucrose by five strokes of a Potter–Elvehjem homogenizer. After the addition of 20 ml more of 0.3 M sucrose, the crude homogenate was centrifuged at 12,000 *g* for 20 min. The resulting supernatant fluid was then centrifuged for 1 hr at 144,000 *g*. The pellet thus obtained was suspended in 40 ml of 0.3 M sucrose by gentle homogenization with a Dounce homogenizer. The suspension was centrifuged again at 12,000 *g*, and the microsomes were sedimented at 144,000 *g* as before. The microsomes were then suspended in 10 ml of 0.3 M sucrose.

The conditions for the synthesis of squalene by yeast microsomes have been described (6). These conditions were also used for incubations with microsomes from rat liver. For the conversion of farnesyl pyrophosphate to presqualene pyrophosphate, TPN, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were omitted from the incubation mixtures. The protein concentration of these incubations with liver and yeast microsomes was typically 2.5 mg/ml as determined by the method of Lowry, Rosebrough, Farr, and Randall (7).

The products of farnesyl pyrophosphate metabolism were analyzed by chromatography on Whatman filter

[1] Agreement on the structure of the intermediate(s) has not been reached. However, one of the proposed structures (2) must now be discarded on chemical grounds (3, 4).

[2] This intermediate was originally named presqualene. For reasons of clarity, the name has been changed to presqualene pyrophosphate.

paper No. 1 impregnated with potassium oxalate at pH 5.5. To 0.2 ml of incubated mixtures, 0.02 ml of 0.1 M potassium oxalate, pH 7.4, was added, and the mixtures were extracted with 0.2 ml of *n*-butanol. Half of each butanol extract was spotted on the chromatography paper. The papers were equilibrated in the vapor phase of a saturated solution of $CaCl_2$ (30% humidity) for 20 min and then transferred to a chromatography jar for a 30-min equilibration prior to chromatography with chloroform–methanol–water 65:30:5. The $R_F$'s for farnesyl pyrophosphate, farnesyl monophosphate, and presqualene pyrophosphate were about 0.08, 0.5, and 0.25, respectively. Since these $R_F$ values are sensitive to variation in humidity, it was necessary to run farnesyl monophosphate and farnesyl pyrophosphate as standards with each group of chromatographs. A Packard radiochromatogram scanner was used for the measurement of radioactivity on the paper chromatograms.

For the assay of $^3H^+$ by distillation, 2 ml of methanol was added to 0.2- to 0.5-ml aqueous samples. The test tubes were connected to cooled receivers by a short glass tube and were heated in a sand bath. The first 0.5–1.0 ml of distilled methanol was collected, and a 0.5 ml portion was taken for liquid scintillation counting. For the calculation of total $^3H^+$ in the sample, the observed counts were multiplied by the ratio of the total exchangeable hydrogens in the sample plus methanol to the exchangeable hydrogens in the methanol that were counted.

To demonstrate the accuracy of this method for determining $^3H^+$, two identical mixtures (0.2 ml) for presqualene pyrophosphate synthesis were incubated to about 30% conversion. When the first sample was analyzed by lyophilization, a total of 2000 dpm $^3H$ was found in the $H_2O$. Analysis of the second sample by distillation of methanol showed 353 dpm $^3H$ in a 0.5 ml aliquot. Multiplying this number by a correction factor of 5.8 for the fraction of exchangeable hydrogens counted, gave a corrected value of 2040 dpm $^3H$, in excellent agreement with the 2000 dpm $^3H$ in $^3H_2O$ found by lyophilization of the first sample.

For the chromatography of presqualene alcohol and its acetate, 1 × 23 cm columns of Silica Gel G were used. Presqualene alcohol was eluted with benzene. The acetate was eluted with hexane–benzene 50:50 at 10 psi.

The methods for ion-exchange chromatography, thin-layer chromatography, and scintillation counting of both singly and doubly labeled samples have been described (6).

## RESULTS AND DISCUSSION

### *Biosynthesis of Presqualene Pyrophosphate by Liver Microsomes*

Microsomes prepared from rat liver as described in Methods catalyzed the formation of a radioactive compound from farnesyl-1-$^3H_2$ pyrophosphate that had the same mobility on paper (Fig. 1) and thin-layer chromatography (Fig. 2) as did presqualene pyrophosphate prepared in the yeast system. The yield of this compound was 30% or less of maximum, and attempts to improve the yield of product using other methods for the preparation of microsomes were unsuccessful. Other variables examined without significant improvement were pH, reaction time, temperature, concentration of protein, $Mg^{2+}$, $F^-$, and farnesyl pyrophosphate (Tables 1–3). The major reaction observed in all of these incubations was the hydrolysis of farnesyl pyrophosphate to farnesyl monophosphate and farnesol, and more often than not, the synthesis of the intermediate was precluded by the

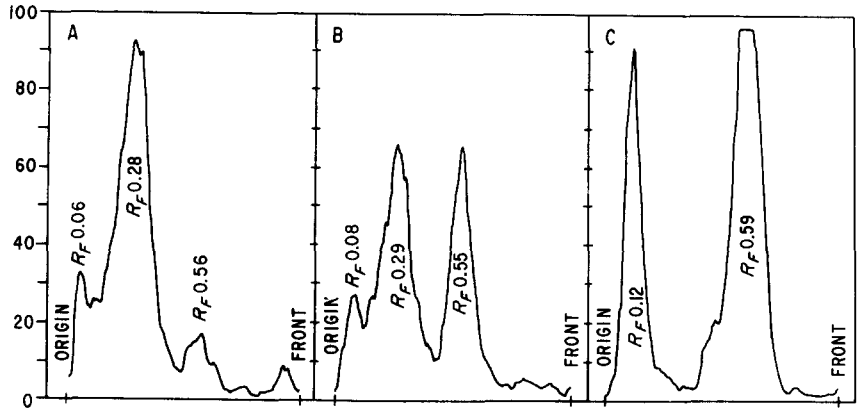


FIG. 1. Paper chromatography of the squalene precursor. (*A*) The chromatograph of a butanol extract of an incubation mixture with yeast enzymes and farnesyl-1-$^3H_2$ pyrophosphate as substrate. (*B*) The chromatograph of a butanol extract of an incubation mixture with rat liver microsomes and farnesyl-1-$^3H_2$ pyrophosphate. (*C*) The chromatograph of a mixture of farnesyl-1-$^3H_2$ monophosphate and farnesyl-1-$^3H_2$ pyrophosphate.

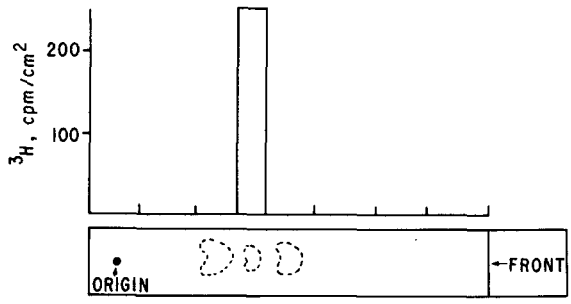


FIG. 2. Thin-layer chromatography of presqualene pyrophosphate. The upper portion shows the distribution of radioactivity of the squalene precursor synthesized by rat liver microsomes and purified by ion-exchange chromatography. The lower portion is a chromatogram of farnesyl pyrophosphate, farnesyl monophosphate, and presqualene pyrophosphate isolated from yeast. The central spot is presqualene pyrophosphate.

rapid hydrolysis of the substrate. The high ratio of phosphatase activity to presqualene pyrophosphate synthetase activity accounts for the previous difficulties in detecting the synthesis of presqualene pyrophosphate by liver microsomes. High levels of prenyl phosphatase activity were also found by Tsai and Gaylor in testicular microsomes (8).

The radioactive compound synthesized from farnesyl pyrophosphate by liver microsomes was shown to be identical with presqualene pyrophosphate from yeast by the following experiments. An incubation mixture (10 ml total volume) with liver microsomes as the source of enzyme and farnesyl-1-$^3H_2$ pyrophosphate as substrate was extracted five times with 5 ml of *n*-butanol. The butanol extracts were concentrated and then chromatographed on a 1 × 14 cm column of Dowex 1 X8 formate. The column was developed with a linear gradient of 0.053–0.43 M ammonium formate in a total volume of 300 ml of methanol, conditions which have been shown to resolve presqualene pyrophosphate from a mixture of farnesyl and nerolidyl monophosphates and pyrophosphates (6). In this instance the predominant fractions were nonpolar materials not retained by the column, farnesyl monophosphate, and presqualene pyrophosphate. The fractions containing the squalene precursor were combined with $^{14}C$-labeled presqualene pyrophosphate that had been synthesized by yeast microsomes and purified by ion-exchange chromatography. This doubly labeled mixture was then chromatographed as before on Dowex 1 X8 formate, and the fractions were evaporated to dryness, lyophilized, and analyzed for $^{14}C$ and $^3H$. A single radioactive peak with a nearly constant ratio of the two isotopes was observed (Fig. 3).

A doubly labeled mixture of presqualene pyrophosphate isolated from yeast and liver was treated with an ether solution of $LiAlH_4$, a reagent that cleaves the pyrophosphate ester to yield the parent alcohol and several hydrocarbons

TABLE 1 THE EFFECT OF VARIATION OF pH ON PRESQUALENE PYROPHOSPHATE SYNTHESIS*

| pH | % Conversion of Farnesyl Pyrophosphate to Presqualene Pyrophosphate |
|---|---|
| 5.0 | 8 |
| 6.0 | 21 |
| 6.8 | 27 |
| 7.4 | 30 |
| 8.0 | 12 |
| 9.0 | 16 |

* Each incubation mixture contained farnesyl-1-$^3H_2$ pyrophosphate, 4 nmole; potassium phosphate, 2 μmole; $MgCl_2$, 1μmole; KF, 1 μmole; and 0.5 mg of rat liver microsomes in a volume of 0.2 ml. Incubation was for 30 min at 30°C under nitrogen.

TABLE 2 THE EFFECT OF COFACTOR CONCENTRATION ON PRESQUALENE PYROPHOSPHATE SYNTHESIS*

| Concentration | | % Conversion of Farnesyl Pyrophosphate to Presqualene Pyrophosphate |
|---|---|---|
| $MgCl_2$ | KF | |
| 5 mM | 5 mM | 30 |
| — | 5 mM | 13 |
| 10 mM | 5 mM | 29 |
| 5 mM | — | 0 |
| 5 mM | 10 mM | 24 |

* The conditions were the same as in Table 1. Potassium phosphate, pH 7.4, was used as buffer.

TABLE 3 THE EFFECT OF VARIATION OF ENZYME CONCENTRATION, TIME, AND TEMPERATURE ON PRESQUALENE PYROPHOSPHATE SYNTHESIS*

| Enzyme | Time | Temperature | % Conversion of Farnesyl Pyrophosphate to Presqualene Pyrophosphate |
|---|---|---|---|
| *mg* | *min* | °C | |
| 0.1 | 60 | 30 | 10 |
| 0.5 | 60 | 30 | 27 |
| 0.5 | 15 | 30 | 12 |
| 0.5 | 15 | 37 | 17 |
| 0.5 | 60 | 37 | 23 |

* The conditions are described in Table 2.

(9, 10). This mixture was applied to a 1 × 2 cm column of grade II Alumina, the hydrocarbons were eluted with hexane, and the alcohol was eluted with 1% methanol in benzene. Presqualene alcohol, derived from yeast and isolated in this manner, has been shown to be homogenous (9). When this alcohol was chromatographed on a column of Silica Gel G, a single peak of radioactivity was observed. However, there was a partial resolution of the two radioisotopes (Fig. 4). This separation could represent the fractionation of two different components

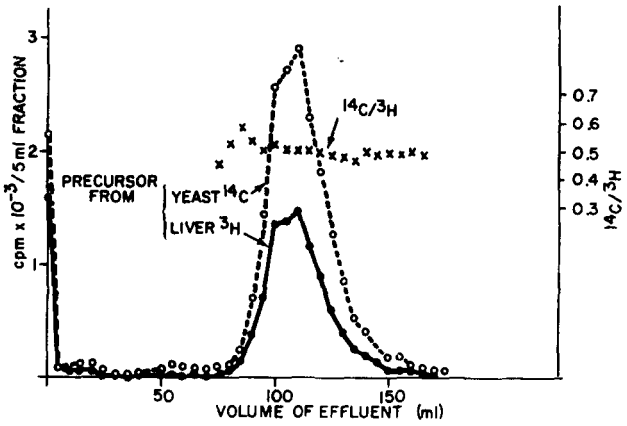


FIG. 3. Chromatography of squalene precursors from yeast and liver on Dowex 1. The radioactivity in fraction 1 is the total radioactivity found in a 100 ml fraction collected while loading the column and is a result of decomposition of presqualene pyrophosphate.

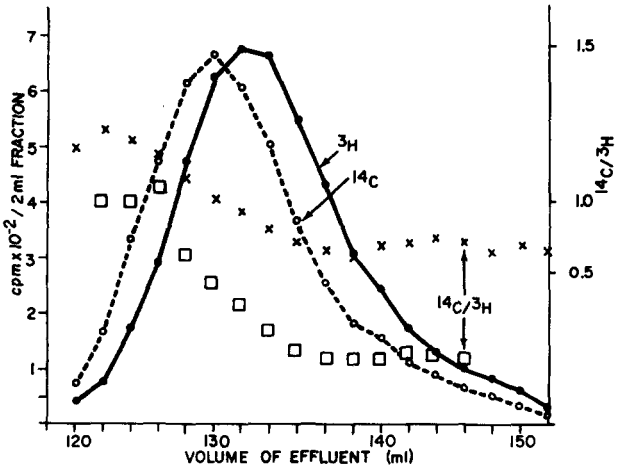


FIG. 4. Chromatography of the alcohol derived from doubly labeled squalene precursor. The alcohol-$^{14}$C was from a yeast preparation. The alcohol-$^{3}$H was from a liver preparation. The symbol × represents the $^{14}$C/$^{3}$H ratio for this chromatogram. The symbol □ represents the $^{14}$C/$^{3}$H ratio for doubly labeled presqualene alcohol derived solely from yeast.

or the separation of a doubly labeled compound by an isotope effect. To determine if an isotope effect was causing this separation, doubly labeled presqualene alcohol was prepared from presqualene-$^{3}$H,$^{14}$C pyrophosphate which had been synthesized from farnesyl pyrophosphate by yeast enzymes only. When this alcohol was chromatographed on Silica Gel G, very nearly the same resolution of radioisotopes was observed (Fig. 4, open squares). The location of two of three of the $^{3}$H on the same carbon as the hydroxyl group in this compound undoubtedly contributes to this isotope effect. Isotope fractionations of this magnitude have been observed in other instances (11).

The doubly labeled presqualene alcohol from yeast and liver preparations was acetylated by treatment for 12 hr with acetic anhydride and pyridine at 25°C. When the product was chromatographed on a column of Silica Gel G, again a single peak of radioactivity was found, but with a smaller shift in isotope ratio than was observed with the alcohol (Fig. 5). Since the same change in ratio was found on chromatography of the acetate of doubly labeled presqualene alcohol isolated from a single source, that is, yeast (Fig. 5, open squares), the partial separation of radioisotopes again can be attributed to an isotope effect.

When presqualene-$^{3}$H,$^{14}$C pyrophosphate from yeast and liver preparations was incubated with TPNH and microsomes from either yeast or liver, it was converted to squalene of very nearly the same isotope ratio (Table 4). Extensive hydrolysis of the substrate by liver microsomes accounts for the low yield with the liver system. This experiment and the demonstration that presqualene pyrophosphate and its derivatives isolated from either rat liver or yeast are chromatographically identical, provide good evidence that this compound is an intermediate common to both systems.

### *Enzyme-Bound Intermediates*

Krishna et al. found that after farnesyl pyrophosphate was incubated with a liver microsomes preparation, a substantial portion of the radioactivity could not be extracted with petroleum ether after treatment with

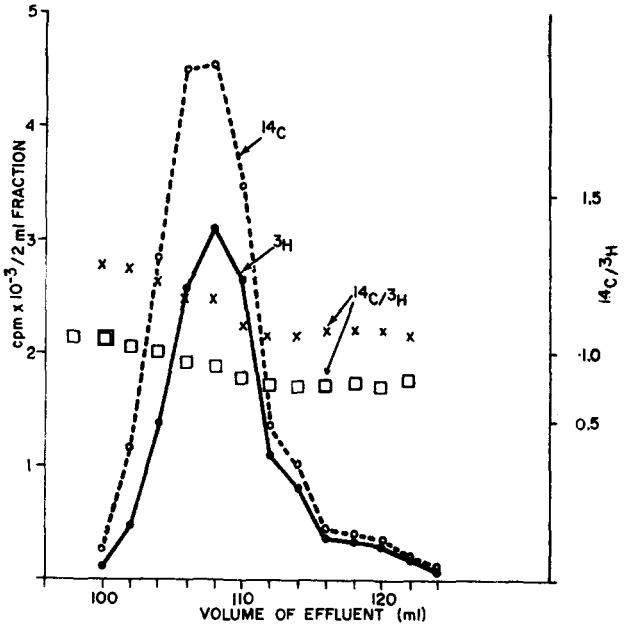


FIG. 5. Chromatography of the acetate of the alcohol derived from doubly labeled squalene precursor. The symbols are the same as for Fig. 4.

TABLE 4 THE ENZYMATIC CONVERSION OF PRESQUALENE PYROPHOSPHATE TO SQUALENE BY YEAST OR LIVER MICROSOMES*

| Compound | $^3H$ | $^{14}C/^3H$ |
|---|---|---|
| | *cpm* | |
| Presqualene pyrophosphate added | $1 \times 10^4$ | 0.83 |
| Squalene isolated from yeast | $5.5 \times 10^3$ | 0.80 |
| Squalene isolated from liver | $8 \times 10^2$ | 0.89 |

* The incubation mixtures contained the following: glucose-6-phosphate, 0.2 μmole; TPN, 0.1 μmole; a small crystal of glucose-6-phosphate dehydrogenase; potassium phosphate, pH 7.4, 2 μmole; $MgCl_2$, 1 μmole; KF, 1 μmole; and 0.13 mg of either yeast or rat liver microsomes. Incubation was for 30 min at 30°C.

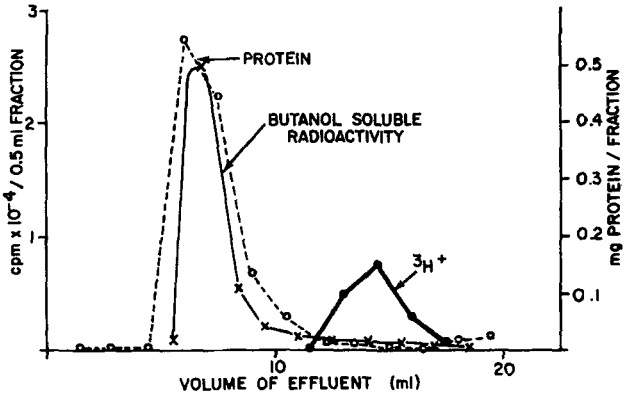


FIG. 6. Chromatography of an incubation mixture on Sephadex G-25. Farnesyl-1-$^3H_2$ pyrophosphate ($2.4 \times 10^5$ cpm) was incubated for 15 min at 30°C with 5 mg of yeast microsomes and 0.5 μmole $MgCl_2$ in 0.5 ml of $H_2O$. The incubation mixture was then applied to a 1 × 10 cm column of Sephadex G-25, previously equilibrated with 0.05 M potassium phosphate, pH 7.4, and 0.001 M $MgCl_2$. Alternate fractions were analyzed for protein, butanol-soluble radioactivity, and $^3H^+$ as described in Methods.

alkaline phosphatase, and they concluded that an enzyme-bound intermediate was involved in squalene synthesis (5). The following experiments are similar to those that Krishna et al. used to demonstrate the enzyme-bound intermediate, except that *n*-butanol was used as a solvent for extraction, and yeast microsomes were used for enzymes since liver microsomal preparations had high levels of phosphatase activity. In the first experiment yeast microsomes (3 mg) were incubated with 8 nmoles of farnesyl-1-$^3H_2$ pyrophosphate for 15 min at 30°C. The microsomes were reisolated by ultracentrifugation or by ammonium sulfate precipitation. In both instances two-thirds of the radioactivity remained with the microsomes. However, this radioactive material could be extracted quantitatively into *n*-butanol, and it cochromatographed with presqualene pyrophosphate on either paper or thin-layer chromatography. In another experiment the incubation mixture was applied to a column of Sephadex G-25. On elution with buffer, two peaks of radioactivity and one of protein was found (Fig. 6). The second radioactive peak coincided with the total volume of the column and was identified as $^3H^+$ by distillation as $CH_3O^3H$.[3] The radioactivity associated with the protein band could be extracted into butanol or into hexane after acid hydrolysis. The material extracted into butanol cochromatographed in thin-layer chromatography with presqualene pyrophosphate. This experiment was repeated with a column of the lipophilic Sephadex LH-20, in 4 M urea used for chromatography. Again two peaks of radioactivity were found, but the amount associated with the protein band was about 10% of that previously noted. The column material was then extruded, dried, and counted. Most of the radioactivity that had been applied to the column was found on the Sephadex LH-20.

In a final experiment, $6.5 \times 10^4$ cpm of farnesyl-1-$^3H_2$ pyrophosphate and 5 mg of yeast microsomes were incubated at 30°C for 15 min. After incubation, the mixtures were repeatedly extracted with butanol and then ether until no more radioactivity could be extracted. About 3% ($2.2 \times 10^3$ cpm) of the added tritium remained with the microsomes. When the concentration of radioactive farnesyl pyrophosphate was changed, a constant fraction of radioactivity remained with the microsomes. The radioactivity remaining bound to the microsomes could not be converted to ether-soluble products by treatment with 5 N KOH or 4 N HCl for 18 hr at 25°C, or by trituration with an ether solution of $LiAlH_4$. The finding that enzyme-bound radioactivity was also found in comparable amounts if the microsomes were boiled for 5 min prior to incubation, makes these observations difficult to interpret.[4] It is clear, however, that the intermediate presqualene pyrophosphate is tightly associated with the microsomes, but not covalently, linked to them.

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[3] A $^3H^+$ is a product of the following reaction: 2 farnesyl-1-$^3H_2$ pyrophosphate = presqualene pyrophosphate + PPi + $^3H^+$.

[4] It is possible that contaminants in the farnesyl-1-$^3H_2$ pyrophosphate such as the 2,3-dihydroanalogue (12) are responsible for this apparent binding.

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