# THE CONVERSION OF FARNESYL PYROPHOSPHATE TO SQUALENE BY SOLUBLE EXTRACTS OF MICROSOMES*

David G. Anderson, Michael S. Rice and John W. Porter

The Radioisotope Unit, Veterans Administration Hospital
and the Department of Physiological Chemistry,
University of Wisconsin, Madison, Wisconsin

Received November 11, 1960

Previous studies have shown that farnesyl pyrophosphate is converted to squalene by microsomes of rat liver (Goodman and Popjak, 1960; Porter, 1960) and by particles of yeast (Lynen, et al., 1958). In the present report we show that a portion of the enzyme activity for the synthesis of squalene in beef and rat liver microsomes is solublized by extraction with $KHCO_3$-$KPO_4$ buffers. We also report on the cofactor requirements and on some of the properties of this soluble system.

Microsomes are prepared from rat liver homogenates, made according to Bucher and McGarrahan (1956), except that 0.1 M $KPO_4$, pH 7.4, is the homogenization medium. Young beef liver is chilled, diced and then homogenized in the same medium in a Waring Blendor for twenty seconds at a rheostat setting of 55, on a scale of 100. Following centrifugation at 500 x g and 8000 x g, the supernatant portion of each preparation is centrifuged at 100,000 x g for 40 min. The sedimented microsomal fractions are washed with at least 5 volumes of 0.1 M $KPO_4$, pH 7.0, then centrifuged again at 100,000 x g. Washed microsomes are resuspended in the

*This investigation was supported in part by research grants, A-1383 from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U. S. Public Health Service, G59-58 from the Life Insurance Medical Research Fund, and RF59:349 from the Wisconsin Alumni Research Foundation.

extracting buffer, 40 - 45 mg. protein/ml., and stirred in the cold for 6 hours. The buffers used (pH 7.0 or pH 8.0) are both 0.07 M in $KHCO_3$. The pH 8.0 buffer is 0.95 M in $KPO_4$; the pH 7.0 buffer is 0.11 M in $KPO_4$. The resultant mixture is centrifuged at 100,000 x g for 40 min., and the supernatant solution is carefully pipetted off.

Enzyme activity for the conversion of farnesyl pyrophosphate to squalene is determined with enzymatically prepared radioactive substrate which is purified by paper chromatography. Terpenoid pyrophosphates are formed in incubation mixtures which contain multiples of the following components: ATP, 10 μmoles; $MgCl_2$, 6.0 μmoles; BAL, 1 μmole; 2 $C^{14}$-mevalonic acid (MVA), 2.77 μmoles and 71,000 counts/min/μmole; $KPO_4$ buffer, pH 7.0, 50 μmoles; rat liver solubles, 10 mgs. of the 40 - 60% $(NH_4)_2SO_4$ fraction of Witting and Porter (1959), and water to 1.0 ml. Incubation is made at 37° for 90 min., and the reaction is stopped by the addition of absolute ethanol to 90%.

After centrifugation the supernatant solution is reduced in volume and lyophilized. The dry residue is taken up in 0.3% $NH_4OH$, streaked on specially washed Whatman No. 3 mm paper and chromatographed in a system of isopropanol, isobutanol, $NH_4OH$ and $H_2O$ (40:20:1:39). All radioactive acid-labile compounds move close to the front in this system, $R_f$ 0.90 - 0.95. They are eluted from the paper with 0.3% $NH_4OH$. Separation of these compounds from $PO_4$ and MVA is complete, and 70 - 100% of the radioactivity of the eluted fraction is present as acid-labile compounds. Some 85 - 90% of the acid-labile material is present as farnesyl pyrophosphate. The terpenoid moieties are identified by paper chromatography in a kerosene-acetic acid system and by gas chromatography. The pyrophosphate moiety is identified by paper chromatography in 3 systems and

by ionophoresis.

The reaction mixture for the conversion of terpenoid pyrophosphate to squalene contains: farnesyl pyrophosphate, 0.03 μmoles; $MgCl_2$, 0.12 μmoles; TPNH 0.8 - 1.6 μmoles; beef liver microsomal extract, 2.0 ml. and 3 - 5 mgs. of protein; in a final total volume of 2.5 ml. Samples are incubated for 90 min. at 37° in an atmosphere of nitrogen.

The squalene formed during incubation is separated by chromatography on alumina according to the procedure of Anderson, Norgard and Porter (1960). Squalene synthesized with beef liver microsomes, and with solublized extracts of rat or beef liver microsomes has been converted to the hexahydrochloride and crystallized to constant specific radioactivity according to the method of Langdon and Bloch (1954).

Evidence that a major portion of the protein responsible for the formation of squalene from farnesyl pyrophosphate is actually in a soluble state is presented in Table I. A beef liver microsomal extract was centrifuged

TABLE I

Formation of Squalene by Various Protein Fractions

| Protein fraction | Protein concentration | Total squalene | Specific activity * |
|---|---|---|---|
| | mg./ml. | counts/min. | |
| Top | 1.2 | 1780 | 740 |
| Bottom | 1.6 | 3510 | 1090 |
| Original | 1.4 | 3000 | 1070 |

* Counts per min. per mg. of enzyme protein.
The components of the incubation mixture and the conditions of incubation are reported in the text.

a second time at 100; 000 x g for 1 hour. The minute pellet which formed was left undisturbed and aliquots of the top and bottom quarter sections of the supernatant solution were sampled and then tested in the standard incubation mixture.

The cofactor requirements for the incorporation of farnesyl pyrophosphate into squalene by the solublized beef liver enzyme are shown in Table II.

TABLE II

Cofactor Requirements for the Conversion of Farnesyl Pyrophosphate to Squalene

| System | Squalene |
|---|---|
| | counts/min. |
| Complete | 2760 |
| Minus TPNH | 20 |
| Minus $MgCl_2$ | 210 |
| Heat treated enzyme | 0 |
| Complete + BAL | 2840 |

The components and conditions of the complete incubation system are reported in the text.

DPNH can substitute for TPNH but it is not as effective a hydrogen donor at low concentrations. Plots of enzymic activity versus TPNH, DPNH and $MgCl_2$ concentrations are shown in Figure 1. The values plotted represent the squalene formed during 90 minute incubations, and they do not necessarily reflect initial reaction rates.

Further studies of the soluble enzyme system have demonstrated that enzymic activity for the conversion of farnesyl pyrophosphate to squalene is (1) a linear function of protein concentration, (2) a linear function of

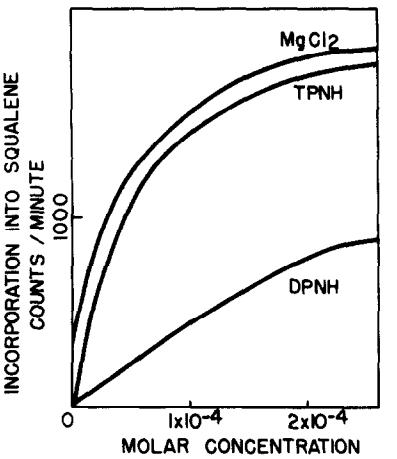


Figure 1. Incorporation of Farnesyl Pyrophosphate into Squalene as a Function of $MgCl_2$, TPNH or DPNH Concentration. Components and conditions of the incubation mixture are noted in the text.

time through 90 minutes, (3) stable to dialysis against the extraction medium, (4) inhibited by p-chlormercurisulfonic acid; 96% at 1 x $10^{-4}$ M, 52% at 6 x $10^{-5}$ M, and 8% at 2 x $10^{-5}$ M, and (5) weakly inhibited by iodoacetamide; 12% at 1 x $10^{-3}$ M.

We expect that solublization of this enzyme activity will greatly facilitate studies on the mechanism of conversion of farnesyl pyrophosphate to squalene.

The technical assistance of Mr. Jack Hipke is gratefully acknowledged.

## REFERENCES

Anderson, D. G., Norgard, D. W. and Porter, J. W.
Arch. Biochem. Biophys., 88, 68 (1960).
Bucher, N. L. R. and McGarrahan, K.
J. Biol. Chem., 222, 1 (1956).
Goodman, De W. S. and Popjak, G.
J. Lipid Research, 1, 286 (1960).
Langdon, R. G. and Bloch, K.
J. Biol. Chem., 200, 129 (1954).
Lynen, F., Eggerer, H., Henning, U. and Kessel, I.
Angew. Chem., 70, 739 (1958).
Porter, J. W.
International Symposium on "Drugs Affecting Lipid Metabolism," Milan, Italy, June (1960). In Press, Elsevier Publishing Co., Amsterdam.
Witting, L. and Porter, J. W.
J. Biol. Chem., 234, 2841 (1959).