AN ENZYME-BOUND INTERMEDIATE IN THE CONVERSION OF FARNESYL PYROPHOSPHATE TO SQUALENE*

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Previous reports demonstrated that farnesyl pyrophosphate is converted to squalene by isolated enzyme systems obtained from yeast (Lynen, et al., 1958), rat liver (Goodman and Popjak, 1960; Porter, 1960; Popjak, et al., 1962) and tomato fruit and carrot roots (Beeler, Anderson and Porter, 1963). Other studies by Popjak, et al., (1961) established the incorporation of one atom of tritium from $NADPH^3$ per molecule of squalene formed by rat liver microsomes. Negligible tritium was incorporated into squalene from H_2^3O when farnesyl pyrophosphate was the substrate. These results were confirmed by Childs and Bloch, (1962) in a reinvestigation of earlier work from the same laboratory.

Popjak, et al., (1961) proposed several mechanisms for the formation of squalene. Each of these proposals involved the formation of one or more stable intermediates between farnesyl pyrophosphate and squalene. A later report by Gosselin, (1962) presented some evidence for the formation of a

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hydrocarbon intermediate bound to a microsomal protein. However, the evidence was not very convincing, for direct proof was not given that the compound was a hydrocarbon and that it was converted to squalene. In addition the substrate used for these studies was mevalonic acid and not farnesyl pyrophosphate.

In all of the above studies the system used was particulate and, therefore, not completely amenable to a study of enzyme-bound intermediates in the conversion of farnesyl pyrophosphate to squalene. In an earlier study from our laboratory (Anderson, Rice and Porter, 1960) we reported the isolation, in low yield, of a soluble squalene synthetase from rat and beef liver microsomes. Studies with this preparation established requirements for Mg^{++} and TPNH for the formation of squalene. They also demonstrated the inhibition of squalene synthesis by S-H inhibitors.

In the present communication we report the formation of a " C_{30}" enzyme-bound ** compound from farnesyl pyrophosphate and the conversion of this compound to squalene on incubation with NADPH. A soluble enzyme, prepared through modification and extension of the method reported by Anderson, et al., (1960), was used. Pig liver squalene synthetase was extracted from microsomes with phosphate buffer, precipitated with ammonium sulfate and then subjected to chromatography on DEAE cellulose and carboxy methyl cellulose. The details of this procedure will be published elsewhere.

The incubation medium for the formation of enzyme-bound intermediates contained $MgCl_2$, 80 μmoles; KF, 120 μmoles; C^{14}-farnesyl pyrophosphate, 0.1 μmole (5.2×10^6 c.p.m./μmole); enzyme, 280 mgs. of protein; K phos-

** Absolute proof that the compound contains 30 carbon atoms is not available. However, it seems highly probable, based upon the fact that this compound is converted to squalene, and upon its behavior on gas chromatography, that it does contain this number of carbon atoms.

phate buffer, pH 7.4, 500 μmoles; 2 mercaptoethanol, 50 μmoles; in a total volume of 10 ml. The mixture was incubated for 15 minutes at 38°C under an atmosphere of nitrogen. At the end of the incubation period 2 μmoles of all-trans nonradioactive farnesyl pyrophosphate were added and incubation was continued for 5 more minutes. The protein was then precipitated by saturation of the solution to 40% with ammonium sulfate. The precipitated protein was dissolved in 0.05 M phosphate buffer, pH 7.4, and passed through Sephadex G 25, previously equilibrated with the same buffer plus 0.005 M mercaptoethanol. Eluted protein fractions of nearly equivalent specific radioactivities were combined and an aliquot was incubated with NADPH and Mg^{++}. Another aliquot was assayed for the presence of C^{14}-labeled free terpenols, farnesyl pyrophosphate, and C_{15}- and "C_{30}"-bound to enzyme, Table I. Assays were also made for these components and squalene after suitable time intervals of incubation, Table II.

TABLE I

DISTRIBUTION OF RADIOACTIVITY ASSOCIATED WITH SQUALENE SYNTHETASE

	Radioactivity in c.p.m.	Per cent of Total
Free terpenols	1625	8.3
Farnesyl pyrophosphate	1500	7.6
C_{15}-enzyme	2500	12.7
"C_{30}"-enzyme	14000	71.4

The conditions of incubation and assay for individual components are reported in the text.

TABLE II

Experiment No. 1		Reduction in enzyme-bound	
Incubation time, in minutes	Squalene formed c. p. m.	"C_{30}" compound c. p. m.	C_{15} compound c. p. m.
10	4910	4700	1520
20	6287	7315	1900
30	7540	8350	1940
Experiment No. 2			
15	5260	7800	4330
30	8660	10755	3800
60	8960	12330	3670
90	10512	11130	4154

The incubation mixture of experiment 1 contained: $MgCl_2$, 10 μmoles; KF, 15 μmoles; NADPH, 1.0 μmole; K phosphate buffer, pH 7.2, 50 μmoles; 2 mercaptoethanol, 5 μmoles; and enzyme-bound intermediate, 13 mg of protein and 9930 c. p. m.; in a total volume of 1.25 ml. In experiment 2 the same components were present at the same molar concentration, but in a volume of 2.5 ml. Enzyme-bound intermediate was present as 27.8 mg of protein and 22000 c. p. m. Each incubation was made at 38° under an atmosphere of N_2.

Radioactive C_{15}- and "C_{30}"-components bound to or associated with the enzyme were quantitated by the following methods: C^{14}-farnesyl pyrophosphate associated with squalene synthetase was cleaved with alkaline phosphatase (Beeler, Anderson and Porter, 1963) after heat denaturation of the synthetase and extraction of the associated free terpenols with petroleum ether. [Farnesyl pyrophosphate is quantitatively cleaved with alkaline phosphatase under these conditions while radioactive compounds bound to enzyme are unaffected.] C^{14}-farnesol, liberated from farnesyl pyrophosphate, was extracted with petroleum ether and an aliquot was subjected to gas-liquid chromatography. The quantity of C^{14} in association with farnesol was determined on elution from the column. Squalene and free terpenols were extracted from another aliquot of enzyme at zero time and after incubation

with NADPH for various time intervals. Extraction was made with petroleum ether after the incubation mixture was saponified with alcoholic KOH. Squalene was separated from other compounds by chromatography on a column of alumina and then by gas-liquid chromatography at 224° on a column of 5% SE-30 on Chromosorb W with an argon flow rate of 100 ml/minute. The amount of radioactivity in association with the squalene eluted on gas-liquid chromatography was determined. The aqueous layer remaining after saponification and extraction of the incubation mixture was acidified to pH 1.0 and then incubated at 38°C for one hour to liberate the radioactive components bound to protein. These radioactive compounds were extracted with petroleum ether. The C_{15}- and "C_{30}"-compounds liberated from protein were subjected to gas-liquid chromatography under the conditions used for squalene. C_{15}-compounds (mostly nerolidol) emerged from the column shortly after the solvent front whereas "C_{30}"-compounds emerged slightly before squalene, Fig. 1. All radioactive compounds were assayed for radioactivity with a Packard Tri-Carb liquid scintillation spectrometer.

A complete analysis of radioactivity bound to enzyme is given in Table I. It can be seen that less than 8% of the total radioactivity was present as farnesyl pyrophosphate whereas over 70% of the radioactivity was present as a "C_{30}"-compound. Table II presents data which show a decline in this "C_{30}"-compound with a concomittant increase in squalene during incubation. Since the increase in squalene can be accounted for only by the decline in "C_{30}"-compound, it is evident that this compound is an intermediate in the biosynthesis of squalene.

The exact structure of the "C_{30}"-compound is unknown. The compounds that are obtained from the enzyme after treatment with acid have a slightly shorter retention time than squalene on gas-liquid chromatography on SE-30,

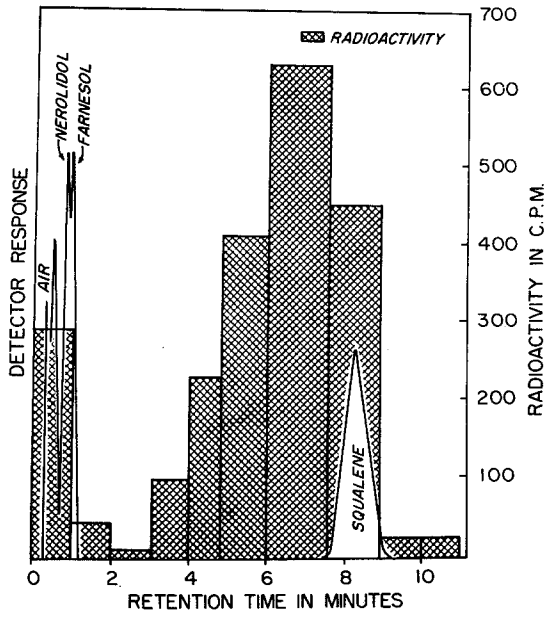

Fig. 1. Gas liquid chromatographic separation of acid-cleaved, enzyme-bound intermediates. Separation was achieved on a 6 ft x 6 mm i. d. column of 5% SE-30 on Chromosorb W at 224° with an argon flow rate of 100 ml/minute. Radioactivity was trapped on glass wool, eluted and counted in a Packard Tri Carb liquid scintillation spectrometer. The major radioactive component overlaps squalene in emergence from the column. However, no radioactivity was present in squalene on chromatography on an alumina column.

Fig. 1. These compounds also appear to be more polar than squalene, and less polar than farnesol, on reversed phase chromatography on vaseline impregnated paper when the chromatogram is developed with n-propanol-water (4:1). They are also more polar than squalene on chromatography on an alumina column.

The results reported in this note, plus the results obtained earlier by Popjak and associates and confirmed by Bloch, et al., permit a more definitive proposal of the pathway of squalene formation from farnesyl pyrophosphate. It may be postulated from current evidence that farnesyl pyrophosphate is converted to C_{15}-enzyme and that this compound is then condensed

with a molecule of farnesyl pyrophosphate to form "C_{30}"-enzyme. "C_{30}"-enzyme is then reduced to squalene in the presence of NADPH. The nature of the binding of the "C_{30}" compound to enzyme is not known, but attempts are being made to establish the identity of this compound and its binding to enzyme.

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