MECHANISM OF SQUALENE BIOSYNTHESIS FROM MEVALONATE AND FARNESYL PYROPHOSPHATE

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It has been shown recently that the immediate precursor of squalene is farnesyl pyrophosphate (FPP) both in yeast and liver enzyme systems (Lynen et al., 1958; Popják, 1959; Goodman and Popják 1960), but the mechanism of the condensation of two molecules of FPP to the symmetrical molecule of squalene is still not solved.

In order to study this question we have determined the deuterium labeling of squalene biosynthesized from mevalonate-5-D_2-2-C^{14} and measured the incorporation of T from either the water of the incubation medium, or from T-labeled TPNH (TPNT), into squalene during its synthesis from C^{14}-farnesyl pyrophosphate, or from mevalonate, with liver enzyme preparations. The full experimental details - too extensive to be produced here - are being submitted for publication elsewhere.

Briefly, squalene was biosynthesized from mevalonate-5-D_2-2-C^{14} under N_2 in 10,000 g supernatants of rat liver homogenates fortified with ATP and TPNH according to a schedule described previously (Cornforth et al. 1959). The squalene obtained from 16 incubations of 50 ml. each was purified three times without carrier through the thiourea adduct. The final yield was 14.8 mg. of

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a hydrocarbon containing 18.5 μmoles of newly synthesized squalene. A 2 mg. sample of this was diluted with unlabeled squalene and purified again through the thiourea adduct (dilution factor 79.4). Samples of the diluted squalene were burnt and the D-content of the water of combustion determined in a mass-spectrometer. The bulk of the undiluted squalene specimen was degraded by ozonolysis (Cornforth and Popják, 1954) and the succinic acid, derived mostly from the four central carbons of squalene, analyzed as succinic anhydride and dimethyl succinate in the mass-spectrometer of the Karolinska Institutet.

The D-content of the mevalonic acid lactone-5-D_2-2-C^{14} was 18.58 ± 0.38 atom-% excess (5 determinations) and that of the diluted squalene specimen 0.2654 ± 0.0080 atom-% excess (6 determinations). Allowing for the 79.4-fold dilution, the D-content of the newly synthesized squalene was calculated to be 21.07 ± 0.63 atom-% excess. This value agrees best with the assumption that 11 out of a possible 12 atoms of labeled H-atoms attached to C-5 of mevalonate were incorporated into squalene. The mass-spectrometric analysis of the succinic acid showed that about 80% of the labeled molecules in the specimen contained 3 atoms of D. Hence the labeling of squalene biosynthesized from mevalonate-5-D_2-2-C^{14} must be as is shown in Fig. 1 (cf. Cornforth et al. 1958), the positions marked with full circles representing carbon atoms derived from C-2 of mevalonate.

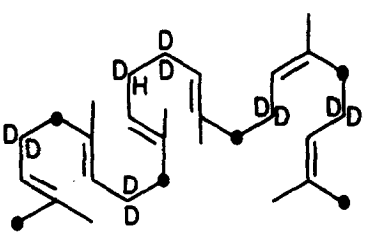

Fig. 1

Since C-5 of mevalonate, which furnishes the two central carbons of squalene, becomes C-1 of farnesyl pyrophosphate (cf. Popják and Cornforth, 1960), the results indicate that one of the H-atoms attached to C-1 of one of the two FPP molecules condensing to squalene is exchanged during the synthetic process. The origin of this exchanged H could be either the water of the incubation medium (H^+) or TPNH (H^-), a known coenzyme of the microsomal squalene synthetase system using

FPP as substrate (Goodman and Popják, 1960). When squalene was synthesized from C^{14}-FPP in an anaerobic system with washed microsomes alone (Goodman and Popják, 1960) in the presence of THO only traces of T were found in the squalene. However, when TPNT was used in the incubations the squalene contained 0.6 to 0.8 μg.-atom labeled H/μmole. One of several experiments of this kind is reproduced in Table I. By chemical degradation of the squalene we have shown that all the T incorporated into squalene from TPNT was confined to the central carbons, no doubt representing the one exchanged hydrogen atom from C-1 of one of the FPP molecules.

Table I

Synthesis of Squalene from C^{14}-farnesyl Pyrophosphate in the Presence of TPNT or of THO

Tube No.	Source of T	Radioactivity in sample counted c.p.m. C^{14}	T	Squalene formed μmole	μg.-atom H in squalene formed	μg.-atom H per μmole squalene
1	None	28,343	-	0.021	-	-
2	None	29,908	-	0.022	-	-
3	TPNT	21,945	3292	0.016	0.0134	0.82
4	TPNT	22,499	3139	0.017	0.0128	0.76
5	THO	27,562	240	0.0205	2.64×10^{-4}	0.013
6	THO	28,645	292	0.0213	3.22×10^{-4}	0.015

Each tube contained in a final volume of 1 ml.: microsome suspension, 0.1 ml.; ^{14}C-farnesyl pyrophosphate, 0.075 mM (0.075 μmole per ml.); $MgCl_2$, 5 mM; nicotineamide, 30 mM; NaF, 10 mM and K-PO_4 buffer, pH 7.4, 0.1 M. Tube Nos. 1, 2, 5 and 6 contained also 3.6 mM TPNH and Tube Nos. 3 and 4, 3.6 mM TPNT (3.5 μc. of T). Tube Nos. 5 and 6 contained 200 mc. of THO. Incubation under N_2 for 2 hours at 37°.

These results are contrary to the data of Rilling and Bloch (1959) who found only 10 atoms of D in squalene biosynthesized from mevalonate-5-D_2. They also reported that there were only two atoms of D attached to the two central carbons in the squalene thus synthesized, and that there was an uptake of two protons into the central carbons of squalene from the water of the incubation medium during biosynthesis of squalene from mevalonate. We believe

that the low values reported by Rilling and Bloch (1959) for the D-content of squalene biosynthesized from mevalonate-5-D_2 and the finding of the entry of two protons from water were probably due to almost unavoidable analytical errors under their experimental conditions.

We have further evidence showing that in the cruder enzyme system (which contains both soluble enzymes and microsomes) synthesizing squalene from mevalonate in the presence of THO one (not two) H-atom enters the central positions of squalene. However the true nature of this H-atom is not that of a proton as inferred by Rilling and Bloch (1959), but that of a hydride ion transferred from TPNH. The soluble enzymes catalyze a rapid exchange between TPNH and the H of water thus: $TPNH + H^+ + X \rightleftharpoons XH_2 + TPN$. When this reaction occurs in a medium containing THO the TPNH becomes labeled. Therefore a valid conclusion as to the origin of the one H-atom on one of the central carbons of squalene, and which does not originate from C-5 of mevalonate (or from C-1 of FPP), may be drawn only from a study of the process with FPP as substrate and the microsomal enzymes in which a H-exchange between TPNH and H_2O apparently does not occur.

From our results certain general features of the last step of squalene synthesis may be drawn. First, the condensation of two FPP molecules to

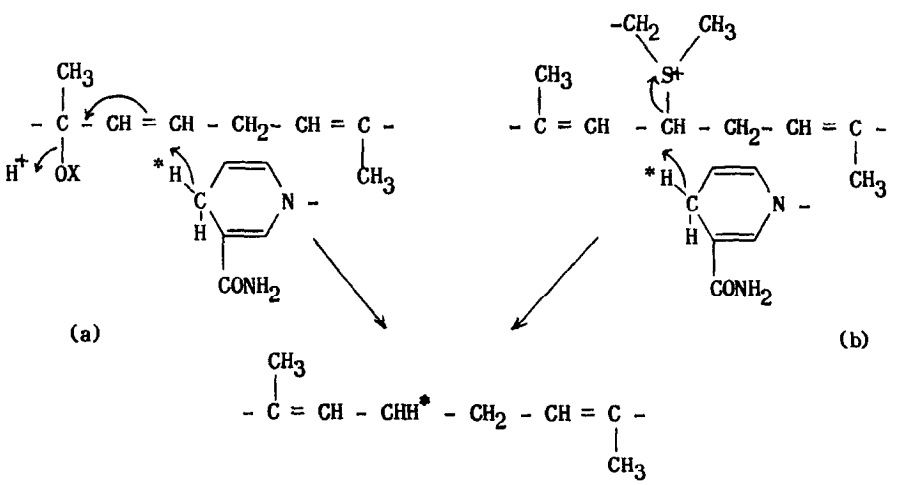

Fig. 2. Possible functions of TPNH in last step of squalene synthesis. Only the central section of the hypothetical intermediates and of squalene are represented.

squalene must be an asymmetric process in the sense that one of the FPP molecules condensing is subject to reactions different from those of the other. Second, the role of TPNH in the process cannot be the reduction of a double bond, for in that event not only a hydride ion but also a proton from the water should have entered the squalene molecule. The functions that we can ascribe to TPNH here is either the reduction of an allylic system, such as is shown in Fig. 2a, or the reductive cleavage of a bond linking one of the central carbons of a squalene intermediate to a functional group of an enzyme or coenzyme as, for example, in the sulfonium compound shown in Fig. 2b.

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