STEREOCHEMISTRY OF THE HYDROGEN TRANSFER TO SQUALENE DURING ITS BIOSYNTHESIS FROM FARNESYL PYROPHOSPHATE.*

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Recent studies have demonstrated that the biosynthesis of squalene from its immediate precursor, farnesyl pyrophosphate, is accompanied by the exchange of one hydrogen atom at C1, in one of the two precursor molecules, for a hydride ion derived from TPNH (Popjak et al., 1961 and 1962). In addition, there is evidence that this exchange of one hydrogen atom is a stereospecific process. This was suggested by the absence of isotope discrimination during squalene formation from 1-H^3_2-2-C^{14}-trans-trans-farnesyl pyrophosphate (Popjak et al., 1962). Subsequently it was shown that the hydrogen transferred to the center of squalene is entirely transferred from the "β" position of TPNH (Popjak, Schroepfer, and Cornforth, 1962).

In order to study further the stereochemistry of this hydrogen transfer, squalene was synthesized from C^{14}-farnesyl pyrophosphate, using rat liver microsomes and TPNH, and then directly converted to cholesterol. The two central carbon atoms of squalene, which become labeled with H^3 from $TPNH^3$, appear in cholesterol as carbon atoms 11 and 12. The distribution and stereochemistry of the tritium label in cholesterol was then determined by biological and chemical means.

Biosynthesis of C^{14}-H^3-squalene and conversion to C^{14}-H^3-cholesterol was achieved in a single, continuous two-stage incubation. In the first stage, the

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anaerobic microsomal squalene synthesizing system described by Goodman and Popjak (1960) was employed, together with $TPNH^3$ as cofactor. The substrate C^{14}-farnesyl pyrophosphate had been enzymically synthesized from 2-C^{14}-mevalonate (Goodman and Popjak, 1960), and had a specific radioactivity of 0.015 μc per μmole. After 60 minutes incubation under nitrogen at 37°, soluble supernatant fraction of the liver homogenate was added, and the incubation continued under nitrogen for 5 minutes more. It was expected that much of the tritium of the $TPNH^3$ would thus be lost from the pyridine nucleotide by equilibration with the hydrogens of water, since such an equilibration has been shown to occur in the presence of soluble supernatant plus microsomes (Popjak et al., 1961). Immediately thereafter a small aliquot was removed for analysis, unlabeled TPNH and glucose-6-phosphate were added, and the gas phase changed from nitrogen to oxygen. Incubation under oxygen was continued for 3 hours, to convert the squalene formed during the anaerobic first stage to cholesterol.

At the end of the incubation the mixture was saponified. The nonsaponifiable fraction was extracted with light petroleum ether, and was separated into squalene and sterol fractions by chromatography on alumina (Goodman, 1961). Portions of each fraction were simultaneously assayed for C^{14} and H^3 in a Packard liquid scintillation spectrometer. These assays indicated that the yield of squalene from C^{14}-farnesyl pyrophosphate had been 90%, and that of cholesterol from squalene 95%. No significant loss or gain of H^3 had occurred during the conversion of the C^{14}-H^3-squalene to cholesterol.

The isolated C^{14}-H^3-cholesterol was suspended in 0.5% bovine serum albumin and injected intraperitoneally into a 300 gm bile fistula rat. Bile was collected for eight days, after which the proteins were precipitated with ethanol and the ethanolic extracts evaporated to dryness with nitrogen. The conjugated bile acids were hydrolyzed in 2N NaOH at 115° for 6 hours and the free bile acids extracted with ether after acidification with HCl. Cholic and chenodeoxycholic acids were then isolated by reversed phase partition chromatography, using solvent systems C and F of Norman (1953) and Bergström and Sjövall (1951). These crystalline

bile acids were converted into the corresponding methyl esters by treatment with diazomethane.

A portion of the labeled methyl cholate was converted to methyl 3α, 7α-diacetoxy 12α-hydroxycholanate by treatment with pyridine and acetic anhydride (Fieser and Rajagopalan, 1950). Some of this was assayed for H^3 and C^{14}, and the remainder converted to the corresponding 12-keto derivative by oxidation with chromium trioxide-pyridine complex (Poos et al., 1959). This method of oxidation was chosen in order to avoid exchange of the hydrogens at C11 with the medium through enolization.

Radioassay of the various compounds indicated that methyl chenodeoxycholate and methyl cholate both had virtually the same isotope ratio as the injected cholesterol. Thus, the observed ratio of H^3/C^{14} counts per minute was 4.56 for cholesterol (corrected for the loss of one of the five C^{14}-labeled carbon atoms during conversion of cholesterol to bile acids), 4.22 for methyl chenodeoxycholate, and 4.47 for methyl cholate (for its 3α, 7α-diacetoxy derivative). This means that only a negligible amount of tritium label might have been present in the 12α-position of the biosynthesized cholesterol. Oxidation of the methyl 3α, 7α-diacetoxy, 12α-hydroxycholanate to the 12-keto derivative under non-enolizing conditions resulted in loss of approximately half the H^3 content (H^3/C^{14} ratio 2.06). Exchange of the hydrogens at C11 by enolization by treatment of the 12-keto compound with alkali resulted in almost complete loss of the tritium label (H^3/C^{14} ratio 0.132). These data therefore indicate that the tritium label introduced into squalene from $TPNH^3$, during its biosynthesis from farnesyl pyrophosphate, appeared in cholesterol in approximately equal amounts at C11 and at C12. The tritium present in the latter position was exclusively confined to the β position, whereas the steric position of the tritium at C11 has not been determined.

The presence of the tritium label at C12 exclusively in the β position unequivocally demonstrates that the introduction of the hydride ion to the squalene precursor is a stereospecific reaction, and further demonstrates that the asymmetric carbon atom created in squalene by introduction of the hydrogen isotope has

the absolute configuration of R (Cahn et al., 1956). This assignment of absolute configuration rests on the assumption that the introduction of the 12α hydroxyl group in cholic acid involves a direct replacement of hydrogen from the position hydroxylated -a mechanism which has been found to occur in all steroid hydroxylation reactions previously examined, and involving a variety of compounds and species (see Samuelsson, 1959).

Although the steric position of the tritium at C11 has not been determined, tritium labeled squalene with the R configuration at the central carbon containing the tritium will yield cholesterol containing tritium in 12β or 11α position, depending upon from which end of the molecule cyclization is initiated. It can thus be assumed with reasonable confidence that the H^3 not in the 12β position was present entirely in the 11α position.

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