CLEAVAGE OF CHOLESTEROL SIDE CHAIN BY ADRENAL CORTEX
III. IDENTIFICATION OF 20 α-22 ξ-DIHYDROXYCHOLESTEROL AS AN INTERMEDIATE*

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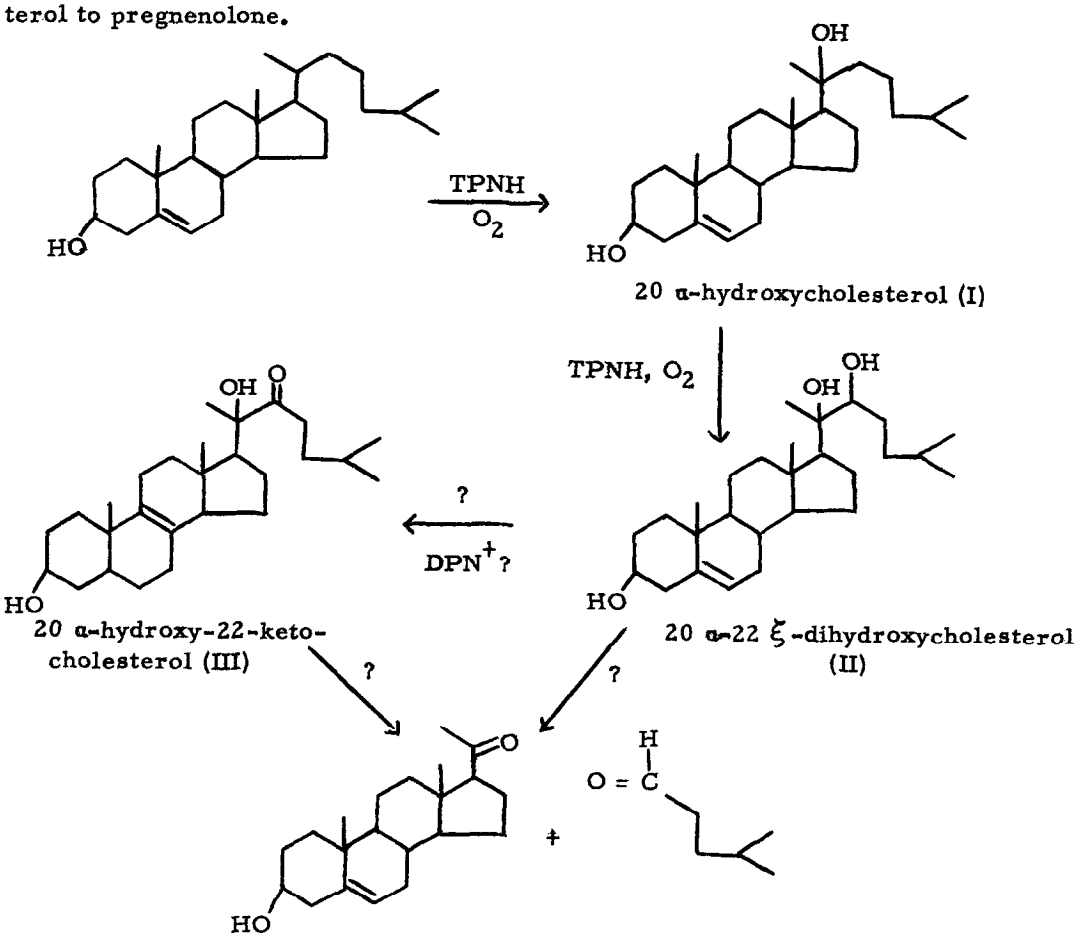
The results of several groups of investigators (Lynn et al., 1954; Solomon et al., 1956; Dorfmann, 1957; Shimizu et al., 1960 and 1961; Constantopoulos and Tchen, 1961 a and b) have led to the following scheme for the conversion of cholesterol to pregnenolone.



*Papers I and II of this series are the first two references cited in this paper.

While I has been clearly established as an intermediate, the occurrence and participation of II and III are largely hypothetical. The only direct evidence for the latter was the report by Shimizu et al. (1961) that, under proper conditions, a polar intermediate could be formed from I. This compound contains both the side chain and the ring of cholesterol and was tentatively identified as III. However, it was also reported (Halkerston and Hechter, 1961) that the conversion of this compound to pregnenolone still required TPNH, thus indicating another oxygenation reaction (see Mason, 1957). This was difficult to explain since III should be cleaved to pregnenolone and isocaproaldehyde by a hydrolytic (non-oxidative) reaction. Recently, using a soluble enzyme preparation (Constantopoulos and Tchen, 1961 b) prepared in a different manner from that used by the Worcester group, we have obtained a polar intermediate which appeared to be identical to that reported by Shimizu et al. (1961). We wish to report here the identification of this compound as 20 α-22 ξ-dihydroxycholesterol and present evidence that 20-α-22-ketocholestero is not an obligatory intermediate, if at all involved, in the biogenesis of pregnenolon

Materials and Methods

These were the same as reported previously (Constantopoulos and Tchen, 1961 a, b).

Results and Discussion

The polar intermediate was obtained by incubating for 45 minutes 10^6 counts per minute (20 μg) of either 4-C^{14} or 26-C^{14}-cholesterol, 500 μg each of pregnenolone and progesterone, 100 mg of albumin (Baker, purified) and 1 μ mole of TPNH with soluble extract of adrenal mitochondria (from 2 gm of tissue) in a total volume of 10 ml. In both cases, chromatography (Zaffaroni and Burton, 1956) of the sterols after reaction revealed two bands of polar material. One band remained at the origin and was formed in larger amount from 4-C^{14}-cholesterol than from 26-C^{14}-cholesterol. This material was resistant to both

lead tetraacetate oxidation and enzymatic transformation and will therefore not be discussed here. The other polar material migrated at about half of the rate of migration of pregnenolone. It was formed in the same amount (approximately 3000 counts per minute) from 4-C^{14}- or 26-C^{14}-cholesterol. When re-incubated with the soluble enzymes and TPNH or DPNH, this polar material gave rise to labeled pregnenolone or isocaproic acid, depending upon the original position of C^{14} in the labeled cholesterol. If the incubation was carried out in the presence of unlabeled isocaproaldehyde (Constantopoulos and Tchen, 1961 b), labeled isocaproaldehyde was recovered instead of the acid. Both enzyme fractions A and B (Constantopoulos and Tchen, 1961 a, b) were still required and TPNH and DPNH could not be replaced by TPN^+ or DPN^+ (Table I).

When 3000 counts per minute of 4-C^{14}- or 26-C^{14}-labeled polar intermediates was treated overnight at room temperature with 20 mg of lead etraacetate in 2 ml of methanol in the presence of unlabeled isocaproaldehyde, labeled pregnenolone and isocaproaldehyde were obtained in 50 percent yield. Significantly, no labeled isocaproic acid was obtained. These results clearly established the polar intermediate as 20,22-dihydroxycholesterol, presumably with the 20-OH group still in the α-configuration.

Previously, both the Worcester group (Shimizu et al., 1961) and ourselves (Constantopoulos and Tchen, 1961 a) have postulated the oxidation of II to III prior to the cleavage reaction. Such an oxidation, the dehydrogenation of an alcohol, would probably use DPN^+ or TPN^+ as oxidant. Since the subsequent cleavage of the α-ketol to pregnenolone and isocaproaldehyde does not involve any oxidation or reduction, the overall conversion of II to pregnenolone and isocaproaldehyde should require DPN^+ or TPN^+ as cofactor, and not DPNH or TPNH. The current experimental finding is in disagreement with the above hypothesis. In fact, the requirement for TPNH or DPNH for this oxidation conversion suggests that another

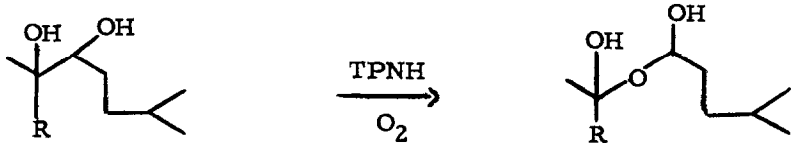
TABLE I

Enzymatic Conversion of a Polar Sterol to Pregnenolone and Isocaproic Acid

Enzyme	Cofactors	Pregnenolone*	Isocaproic Acid*
A+B	TPNH	1,200	218
A+B	DPNH	1,400	320
A+B	TPN^+	-	-
A+B	DPN^+	-	-
A	TPNH	-	-
B	TPNH	-	-

* Counts per minute of labeled pregnenolone and isocaproic acid obtained from 2,500 counts per minute of labeled intermediate derived from 4-C^{14}- and 26-C^{14}-cholesterol respectively. Under the same conditions 2,500 c.p.m. of 4-C^{14} and 26-C^{14}-cholesterol gave rise to 475 c.p.m. of pregnenolone and 226 c.p.m. of isocaproic acid respectively. The conversion of 26-C^{14}-labeled polar sterol in this series of experiments was poor due to poor emulsification of the substrate. In other experiments with both enzyme fraction and TPNH, the yield was 800 counts per minute.

oxygenation reaction ("mixed function oxygen transferase" in Mason's terminology, 1957) takes place. In view of these considerations, we wish to propose that the conversion of II to pregnenolone involves the insertion of an atom of oxygen between carbons 20 and 22:



The latter, containing isocaproaldehyde and pregnenolone masked in semiacetal and semiketal form, would spontaneously decompose to give pregnenolone and isocaproaldehyde.

This scheme does not involve a 22-keto derivative as intermediate and found support in experiments with various analogs of II. These were synthesized

in similar manner as II, (Petrow and Stuart-Webb, 1956), namely, by condensation of labeled pregnenolone with the Grignard of isoamyl bromide, ethyl bromide, isopropyl bromide and sec-butyl bromide. The latter two gave analogs of II where the C_{22} contained only one hydrogen and which therefore cannot form a 22-keto group. It was found that all these analogs can be cleaved to give pregnenolone. Thus, in a typical series of experiments where II was converted to the extent of 40, 16, 6, and 12 percent respectively. Since two of these analogs could not form 22-keto group, it is clear that the formation of 22-keto group is not an essential step in the cleavage of side chain of cholesterol. These results, by the exclusion of an obligatory intermediate with 22-keto group, indirectly lend support to the oxygenation reaction proposed in this paper.

Acknowledgment

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Addendum: Since the manuscript was submitted, Shimizu, Gut, and Dorfman (J. Biol. Chem., 237, 699, 1961) have reported the identification of this polar intermediate as one of the isomers of 20 α-22 ξ -dihydroxycholesterol. Also, rate studies suggested that it is converted to pregnenolone without the intermediary formation of 20 α-hydroxy-22-ketocholesterol, although the latter can also be converted to pregnenolone.