The *in Vitro* Catabolism of Cholesterol: Formation of 5β-Cholestane-3α,7α-diol and 5β-Cholestane-3α,12α-diol from Cholesterol in Rat Liver*

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ABSTRACT: The catabolism of cholesterol to 3α,7α-dihydroxycoprostane and 3α,12α-dihydroxycoprostane has been studied using a rat liver preparation *in vitro*. The data demonstrate that rat liver is capable of forming 3α,7α-dihydroxycoprostane from cholesterol. Since 3α,7α-dihydroxycoprostane is converted to cholic acid and chenodeoxycholic acid in the bile-fistula rat, it is suggested that 3α,7α-dihydroxycoprostane is probably an intermediate in the formation of bile acids from cholesterol in the rat. The hypothesis has been put forward that cholic acid and chenodeoxycholic acid are formed via a common pathway until the stage of 3α,7α-dihydroxycoprostane.

Thereafter, regulatory mechanisms exist in the liver whereby hydroxylation at position 12 is controlled. Under similar circumstances virtually no transformation of cholesterol to 3α,12α-dihydroxycoprostane was observed, indicating that 3α,12α-dihydroxycoprostane is probably not an intermediate in bile acid metabolism in the rat.

Concerning the elucidation of the sequence and mechanisms of bile acid formation from cholesterol, *in vitro* studies on the degradation of cholesterol have so far yielded rather disappointing results. With the possible exception of 26-hydroxycholesterol no intermediary products have been isolated. In most instances the experiments have been performed by incubating labeled cholesterol with particulate fractions of rat liver followed by attempted isolation and identification of the radioactive compounds obtained. This method has not met with much success. In our studies we have employed a somewhat different approach to the problem. Various postulated intermediates in the sequence cholesterol to cholic acid have been synthesized and added as trapping agents in an *in vitro* system containing labeled cholesterol as substrate. The isolation and identification of each intermediate then becomes a relatively simple procedure. Using this technique we have recently described an enzyme system in rat liver capable of converting cholesterol to 5β-cholestane-3α,7α,12α-triol (THC)[1] (Mendelsohn and Staple, 1963) and to 7α-hydroxycholesterol (Mendelsohn *et al.*, 1965). These two compounds have both been shown to be converted to cholic acid in the bile-fistula rat, and are thus implicated as possible intermediates in the conversion of cholesterol to cholic acid (Bergstrom, 1955; Lindstedt, 1957). The enzyme system was also able to degrade cholesterol into a number of compounds apparently different from 7α-hydroxycholesterol or THC. Preliminary investigations into the nature of two of these compounds indicated that they might be 3α,7α-DHC and 3α,12α-DHC. As an extension of our previous studies this report describes the enzymatic conversion of cholesterol to 3α,7α-DHC and 3α,12α-DHC by a rat liver preparation *in vitro*.

Experimental

3α,7α-DHC and 3α,12α-DHC were prepared by anodic coupling of the appropriate bile acid and isovaleric acid using a slightly modified procedure of Kazuno and Mōri (1954). One g of chenodeoxycholic or deoxycholic acid was dissolved in 225 ml methanol containing 6.75 ml isovaleric acid and 145 mg sodium. The mixture was cooled in an ice bath and a current of 1.2 amp was allowed to pass through the solution for 4 hours. During this time the *p*H was maintained between 6 and 6.5. At the conclusion of the electrolysis the mixture was made alkaline with 0.1 N NaOH, when it became turbid. This was then poured into water and extracted several times with ether. The ether was washed with 0.01 N NaOH and then with water until the washings were neutral. After evaporation of the ether *in vacuo*, the residue was dissolved in 50 ml 5% alcoholic NaOH and refluxed for 2 hours. The cooled hydrolysate was poured into water and the solid which precipitated was collected. This was dissolved in 50 ml benzene, chromatographed on 30 g of activated alumina (for chromatographic absorption analysis

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[1] Abbreviations used in this work: 3α,7α-DHC, 5β-cholestane-3α,7α-diol (3α,7α-dihydroxycoprostane); 3α,12α-DHC, 5β-cholestane-3α,12α-diol (3α,12α-dihydroxycoprostane); THC, 5β-cholestane-3α,7α,12α-triol (3α,7α,12α-trihydroxycoprostane).

according to Brockman), and eluted with 250 ml each of ether, ether-acetone (1:1), and acetone. The ether and ether-acetone fractions were combined and taken to dryness. Attempted cyrstallization of the residue yielded only oily fractions. These were then combined and rechromatographed using the technique of preparative thin-layer chromatography with isooctane–isopropyl ether–acetic acid (50:25:25) as solvent. The samples were applied on the plates as bands; the reference compounds, which were synthesized according to the procedure described by Bergstrom and Krabisch (1957), were applied as single spots. After the plates were developed with solvent they were exposed to iodine vapor, and the area corresponding to the reference substance (3α,7α-DHC or 3α,12α-DHC) was eluted from the silica gel with acetone and recrystallized several times from aqueous acetone. 3α,7α-DHC, mp 78–80° (reported (Bergstrom and Krabisch, 1957) mp 84–86°), and 3α,12α-DHC, mp 104–106° (reported (Bergstrom and Krabisch, 1957) mp 108–110°) both gave a single spot when subjected to thin-layer chromatography in three different solvent systems.

Fractionation of the rat liver homogenates, incubation with [26-^{14}C]cholesterol, sterol extraction, and chromatographic procedures have been described previously (Mendelsohn and Staple, 1963). Immediately prior to use the radioactive cholesterol was purified by elution with benzene through a neutral alumina column (activity grade III).

Isolation of Labeled 3α,7α-DHC and 3α,12α-DHC. At the conclusion of the incubation period the contents of ten flasks were combined. Then 15 mg unlabeled carrier 3α,7α-DHC or 3α,12α-DHC was added followed by four volumes of 95% ethanol. The precipitate was removed by centrifugation and washed once with an equal volume of ethanol, and the combined supernatant solutions were evaporated to dryness *in vacuo* at 37°. After saponification of the residue by refluxing with 50 ml of 5% potassium hydroxide in methanol for 1 hour, the cooled digest was extracted four times with 60 ml of petroleum ether (bp 40–60°). The combined petroleum ether extracts were taken to dryness in a stream of nitrogen and the residue was subjected to reversed-phase partition-column chromatography (50%, v/v, aqueous 2-propanol, moving phase; 20%, v/v, chloroform in heptane, stationary phase; 4-ml stationary phase supported on 4.5 g hydrophobic Super-Cel) in order to separate the labeled compounds from unreacted cholesterol. The effluent fractions, which were thought to correspond to those containing 3α,7α-DHC or 3α,12α-DHC (by reference to elution volumes previously determined with authentic 3α,7α-DHC or 3α,12α-DHC under the same chromatographic conditions) were combined and evaporated to dryness under nitrogen. The material thus obtained was applied as bands onto thin-layer plates (0.5-mm-thick silica gel) and rechromatographed using isooctane–isopropyl ether–acetic acid (50:25:25) as solvent. Reference compounds (3α,7α-DHC or 3α,12α-DHC) were spotted on either side of the band. The plates were then exposed to iodine vapor for a few minutes to identify the position of the material on the plates. An area of the band corresponding to the position of the reference compound was scraped off the plate and the material eluted from the silica gel with acetone. The combined acetone eluates were taken to dryness and the residue was recrystallized to constant specific activity from aqueous acetone. After the second crystallization the chromatographic behavior on thin-layer chromatography of the isolated material was compared with that of authentic 3α,7α-DHC or 3α,12α-DHC in three different solvent systems: isooctane–isopropyl ether–acetic acid (50:25:25); petroleum ether (bp 60–80°)–ether–methanol–acetic acid (70:24:4:1); benzene–ethyl acetate (2:1). In all instances the material behaved exactly like pure 3α,7α-DHC or 3α,12α-DHC and, furthermore, radioactivity was detected only in those areas occupied by the material and nowhere else on the chromatogram. The identity of the appropriate isolated sterol was further characterized by mixed melting point determinations and comparison of the infrared spectrum with that of authentic material.

Radioactivity of all samples was assayed at "infinite thinness" in a windowless gas-flow counter. Approximately 1 mg of material was plated in each case and counting was continued until the error was less than ±3%. Each sample was investigated for a minimum of 10,000 counts.

Results and Discussion

The data presented in Table I demonstrate the results of a typical experiment on the conversion of cholesterol to 3α,12α-DHC *in vitro*. The very low specific activity of the latter compound indicates that it is not an important product of cholesterol metabolism in rat liver. Earlier investigations by Bergstrom *et al.* (1960) have established the fact that, in the conversion of cholesterol to bile acids, modifications of the steroid nucleus occur prior to oxidation of the side chain. The order in which hydroxyl groups at C-7 and C-12 are introduced into the steroid nucleus in the rat is not known with certainty. While 7α-hydroxycholesterol is converted in good yield to cholic acid in the bile-fistula rat, 12α-hydroxycholesterol is not, indicating that hydroxylation at C-7 precedes hydroxylation at C-12 (Danielsson,

TABLE I: Melting Points and Specific Activities of 3α,12α-DHC Isolated from the Incubation Mixtures.[a]

Recrystallization	Melting Point	Specific Activity (cpm/mg)
1	103–105°	6
2	103–105°	8

[a] See text for details.

1963). Employing an *in vitro* system from rat liver we have shown that cholesterol can be converted into 7α-hydroxycholesterol (Mendelsohn *et al.*, 1965), 3α,7α-DHC (see Table II), and THC (Mendelsohn and Staple, 1963), whereas virtually no conversion into 3α,12α-DHC has been observed (Table I). Since rat liver must possess a 12α-hydroxylase, our findings also suggest that 7α-hydroxylation of the steroid nucleus is a prerequisite for 12α-hydroxylase activity.

A further point arises from the data presented in Table I. According to Norman and Sjövall (1958), the rat forms deoxycholic acid only by the action of intestinal microorganisms on cholic acid during the enterohepatic circulation of bile. Analogous to the postulate that cholic and chenodeoxycholic acids could be formed by side-chain oxidation of THC and 3α,7α-DHC, respectively (*vide infra*), deoxycholic acid could be formed from 3α,12α-DHC. Since we have not been able to demonstrate a significant conversion of cholesterol to 3α,12α-DHC (Table I), this seems to be confirmatory evidence that the liver is not the site of formation of deoxycholic acid in the rat.

Bergstrom and Lindstedt (1956) and Lindstedt (1957) investigated the biliary excretion of labeled products in the rat after administration of tritium-labeled 3α,7α-DHC. They isolated both labeled cholic acid and chenodeoxycholic acid in the bile, thus implicating 3α,7α-DHC as a possible intermediate in bile acid formation from cholesterol in the rat. The results presented in Table II demonstrate that rat liver is able

TABLE II: Melting Points and Specific Activities of 3α,7α-DHC Isolated from the Incubation Mixture.[a]

Recrystallization	Melting Point	Specific Activity (cpm/mg)
1	78–80°	68
2	78–80°	72

[a] See text for details.

to convert cholesterol to 3α,7α-DHC *in vitro*. This fact lends further considerable support to the contention that the latter is probably a normal and obligatory intermediate in the conversion of cholesterol to bile acids. We have also demonstrated the enzymatic conversion of cholesterol to 7α-hydroxycholesterol (Mendelsohn *et al.*, 1965) and to THC *in vitro* (Mendelsohn and Staple, 1963). While the exact sequence and nature of the transformations from cholesterol to THC is not definitely known, it is possible that the initial reaction is hydroxylation of cholesterol in the 7α position. Following this, there occurs saturation of the Δ^5 double bond and inversion of the 3β-hydroxyl group (not necessarily in this order) to give 3α,7α-DHC. These reactions might take place via a 3-keto compound, e.g., Δ^4-cholesten-7α-ol-3-one. The latter has been shown to give rise to cholic acid and chenodeoxycholic acid in the bile-fistula rat and has also been identified as a product of oxidation of 7α-hydroxycholesterol in rat and mouse liver mitochondria *in vitro* (Danielsson, 1961). After the formation of 3α,7α-DHC, 12α-hydroxylation would produce THC.

However, since the main bile acids in the rat are cholic acid (trihydroxylated) and chenodeoxycholic acid (dihydroxylated), the thought must be entertained that these two compounds could be formed via separate pathways in the liver. As a result of the present studies it might be postulated that once 3α,7α-DHC has been formed from cholesterol, regulatory mechanisms exist in the liver whereby hydroxylation at position 12 is controlled. Thus, 12α-hydroxylation of 3α,7α-DHC would produce THC, which could then be further metabolized to cholic acid, while degradation of 3α,7α-DHC alone would give rise to chenodeoxycholic acid.

In this regard the findings of Erikssen (1957a,b) are of interest. In a study of the biliary excretion of cholic and chenodeoxycholic acid in eu-, hyper-, and hypothyroid bile-fistula rats this author found a decreased excretion of cholic acid in both hyper- and hypothyroidism confirming the earlier observations of Thompson and Vars (1953, 1954). In addition the amount of chenodeoxycholic acid excreted was increased in hyperthyroidism and decreased in hypothyroid rats as compared with normal animals. These results indicate that a possible regulatory effect on rat liver 12α-hydroxylase might be exerted by thyroxine.

Recently, Danielsson (1963) suggested an alternative scheme for the formation of cholic and chenodeoxycholic acid which does not include 3α,7α-DHC as an intermediate. Further research is obviously required to clarify this important metabolic problem.

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Studies on Steroid Conjugates. IV. Demonstration and Identification of Solvolyzable Corticosteroids in Human Urine and Plasma*

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ABSTRACT: The procedure of S. Burstein and S. Lieberman (*J. Biol. Chem. 233*, 331 (1958a)) for solvolytic cleavage of ketosteroid hydrogen sulfates has been modified so as to be applicable to the markedly more polar corticosteroid sulfates. The overall recoveries of the steroid moieties of synthetic corticosteroid mono- and disulfates subjected to this modified procedure were 85–98% with a reproducibility of ±2.5%. This procedure was then incorporated into a fractionated extraction, hydrolysis, and solvolysis method, the application of which to biological fluids resulted in the demonstration of a *consistent presence* of solvolyzable (presumably sulfate) conjugated corticosteroids in human blood and urine. These conjugates were shown to constitute a sizable fraction of urinary (15–33%) and plasma (12–21%) total 17-hydroxycorticosteroids. The steroidal moieties of these conjugates have been identified. It is of interest that along with cortisol, tetrahydrocortisol, and other ring A-reduced steroids, 6β-hydroxycortisol was also isolated from the solvolyzable fraction.

Our interest in conjugated corticosteroids stemming from the results of our studies on corticosteroid metabolism in hypertension (Kornel, 1958, 1960, 1964a; Kornel and Motohashi, 1963) stimulated us to undertake an investigation into the possible biological importance of some steroid conjugates (Kornel, 1963, 1964b; Kornel *et al.*, 1964; Kornel and Lee, 1964). The finding of a substantial fraction of urinary radioactive conjugated metabolites of tracer [4-^{14}C]cortisol, which could not be split by even most exhaustive β-glucuronidase hydrolysis (Kornel and Eik-Nes, 1961), indicated the presence of nonglucuronide conjugated corticosteroids and called for their isolation and identification.

In the first attempts to obtain some clues with regard to the nature of these conjugates, a method was sought which would specifically hydrolyze steroid sulfates. The procedure of solvolysis described by Burstein and Lieberman (1958a) for cleavage of C_{19} steroid sulfates seemed to be well suited for our purpose. However, the extraction methods for steroid conjugates, intrinsic to this solvolysis procedure, were found to be inadequate for work with more polar conjugates (Kornel, 1963). In an attempt to modify the method so as to make it applicable to work with conjugated corticosteroids, a method has been developed which gave 95–100% solvolytic yield of synthetic corticosteroid sulfates.

Application of this procedure to extracts from biological fluids resulted in the demonstration of a consistent presence of solvolyzable (presumably sulfate) conjugated corticosteroids in urine and plasma.[1] The steroidal moieties of these conjugates have been identified.

Experimental Procedure

Materials. All solvents used were J. T. Baker, analytical grade, and were redistilled without further purification, with the exception of diethyl ether which was redistilled over KOH before use, and ethyl acetate which was washed with a saturated aqueous solution of sodium bisulfite, then distilled twice. The latter procedures give peroxide-free solvents, as determined with acidified KI solution (Burstein and Kimball, 1963).

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[1] Preliminary reports of these findings appeared as abstracts (Kornel and Lee, 1962; Kornel and Hill, 1962).