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The Conversion of [3β-³H]5β-Cholestan-3α-ol into [3β-³H]Deoxycholic Acid in Man*

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ABSTRACT: Since 5β-cholestan-3α-ol possesses the structural features of bile acids at C-3 and at the A/B ring juncture, its conversion into bile acids was investigated. [3β-³H]5β-Cholestan-3α-ol was prepared and administered to a patient with a bile fistula. Over 15% of the radioactivity in the biliary steroids was associated with the dihydroxycholanic acids of which labeled deoxycholic acid comprised the major portion. Preparation of suitable derivatives showed that ³H was retained at C-3β through the transformation.

Two pathways have been proposed for the formation of C_{24} bile acids. The first (Danielsson, 1963) involves changes in the sterol nucleus prior to production of the carboxyl group. While the sequence is not fully established, it is probable that 7α hydroxylation of cholesterol is an early step (Bergstrom and Lindstedt, 1956; Bergstrom *et al.*, 1958) followed by 12α hydroxylation (Danielsson, 1962) which occurs either before or after the saturation of the double bond and inversion at C-3 (Danielsson, 1961a,b). Loss of three carbons leads to chenodeoxycholic[1] and cholic acid. In support of this pathway, Staple and coworkers have isolated radioactive 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid and 5β-cholestane-3α,7α-diol from human bile after the administration of [26-¹⁴C]cholesterol (Staple and Rabinowitz, 1962; Rabinowitz *et al.*, 1966). The second pathway, reported by Mitropoulos and Myant (1967) in rats and rat liver preparations, involves first the production of 3β-hydroxy-Δ⁵-cholenic acid followed by hydroxylation steps to afford bile acids oxygenated at C-3, -6, and -7. Whether such a pathway exists in man is not known. Bongiovanni (1965) was unable to find 3β-hydroxy-Δ⁵-cholenic acid in human gall bladder bile. Both of these pathways exclude 3,12-dihydroxy steroids from the biosynthetic sequence either as C_{27} intermediates or as primary bile acids. Deoxycholic acid (3α,12α-dihydroxy-5β-cholanic acid),

the principal bile acid of human feces and a major constituent of the bile of man and animals, is formed by bacterial reduction of cholic acid (Lindstedt, 1957; Ekdahl and Sjövall, 1957).

The metabolism of 5β-cholestan-3α-ol, structurally identical with naturally occurring bile acids at C-3 and at the A/B ring juncture, was studied in man. [3β-³H]5β-Cholestan-3α-ol was prepared and administered by vein to a subject with a bile fistula. The major portion of the radioactivity recovered from bile was found in neutral sterols. About 15% of the total, however, was associated with deoxycholic acid and the tritium was solely at the 3β position.
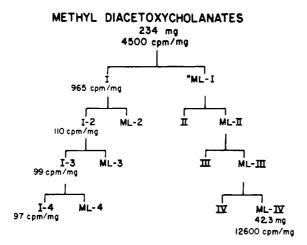
Materials and Methods

Radiochemical Purity of the Administered Sterol. [3β-³H]5β-Cholestan-3α-ol was prepared by the reduction of 5β-cholestan-3-one with [³H]lithium aluminum hydride (Rosenfeld *et al.*, 1967). To 100.4 mg of nonradioactive 5β-cholestan-3α-ol was added 1.0×10^7 cpm of the labeled compound (specific activity 99,600 cpm/mg). The material, in 2 ml of hot ethanol, was mixed with 5 ml of a solution of 1 g of digitonin in 80% ethanol. The nonprecipitable fraction was dissolved in 15 ml of dimethyl sulfoxide (Issidorides *et al.*, 1962) and extracted with petroleum ether (bp 30–60°). The petroleum ether was washed three times with 50% ethanol and afforded 99 mg of crystalline material. The digitonin separation was repeated to yield 97 mg in the nonprecipitable fraction. This was dissolved in 1 ml of pyridine and converted into the tosylate with 250 mg of *p*-toluenesulfonyl chloride in 1 ml of pyridine at room temperature overnight. After the usual extraction and washing procedures, 128 mg of 5β-cholestan-3α-yl tosylate was obtained and recrystallized twice from acetone. Radioactivity was measured at each

[1] Trivial names used: chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanic acid; allocholic acid, 3α,7α,12α-trihydroxy-5α-cholanic acid.

3085

METHYL DIACETOXYCHOLANATES
234 mg
4500 cpm/mg

FIGURE 1: Fractional crystallization of methyl diacetoxy-cholanates.

TABLE II: Distribution of Biliary Radioactivity in Sterols and Bile Acid Derivatives.

Time (days)	Sterols[b]	Methyl Diacetoxy-cholanates[b]	Methyl Triacetoxy-cholanates[b]
0–0.5	226	140	12.3
0.5–1	764	141	
2–3	333	33	33.5[a]
4–9	615	74	
Total counts	1940	388	45.8
% of total	82	16	2

[a] Combined fraction: 0.5–9 days. [b] Values in cpm $\times$ 10^{-4}.

stage of the purification and the data are presented in Table I.

Subject and Administration of Sterol. The subject was a 70-year-old man (serum cholesterol, 143 mg/ 100 ml, 30.8 mg % free) with carcinoma of the gall bladder who, at the time of the study, had exterior drainage of the bile. [3β-³H]5β-Cholestan-3α-ol (14.4 mg, 5.0 $\times$ 10^8 cpm) was dissolved in 1 ml of ethanol, mixed with 30 ml of glucose solution, and immediately administered by vein over 3 min. Complete bile and stool collections were made for 9 days and blood samples were obtained at certain times during the period.

Isolation of Sterols and Bile Acids. Biliary sterols were obtained from the nonsaponifiable fraction of each bile collection. Crude bile acids were converted into methyl esters, acetylated, and chromatographed on silica gel columns according to a method reported earlier (Rosenfeld and Hellman, 1959) to afford a diacetoxy and a triacetoxy fraction. The diacetoxy fraction was crystalline, and infrared analysis, as well as behavior on thin-layer chromatography, showed no differences from authentic methyl 3α,7α-diacetoxy-cholanate; mp 124–127° after one recrystallization from ethanol. The triacetoxy fraction was oily and only after saponification could cholic acid be

TABLE I: Radiochemical Purity of Administered [3β-³H]5β-Cholestan-3α-ol.

Procedure	cpm/mg
[3β-³H]5β-Cholestan-3α-ol; carrier added	99,600
Digitonin treatment; α fraction (99 mg)	99,000
Repeat digitonin treatment; α fraction (97 mg)	94,000
5β-Cholestan-3α-yl tosylate; crystallized	92,100[a]
5β-Cholestan-3α-yl tosylate; recrystallized	95,000[a]

[a] Corrected for molecular weight difference.

obtained crystalline. The distribution of radioactivity in bile sterols and bile acids is shown in Table II. The methyl ester diacetates were pooled for further examination; specific activity of combined material, 4500 cpm/mg. A portion of this material (234 mg) was fractionally crystallized from ethanol according to Figure 1.

Both I-4 and ML-IV (Figure 1) were crystalline and had physical characteristics identical with methyl 3α,7α-diacetoxycholanate; however, the 100-fold difference in specific activity between the two fractions suggested that a major transformation product of radioactive 5β-cholestan-3α-ol had been concentrated in ML-IV.

Identification of Methyl 3α,12α-Diacetoxycholanate as the Principal Radioactive Substance in ML-IV. Nonradioactive methyl 3α,12α-diacetoxycholanate (53.8 mg) was mixed with 0.85 mg of ML-IV (12,600 cpm/ mg); specific activity of mixture, 196 cpm/mg. Recrystallization from ethanol yielded 30.4 mg; specific activity, 150 cpm/mg. Two additional recrystallizations from petroleum ether afforded material with no further change in specific activity. This reverse isotope dilution procedure was repeated twice, but with ten times the amount of ML-IV and carrier. The sequence of solvents in the crystallization steps was varied, but the final specific activities were essentially unchanged (148 and 158 cpm per mg, respectively). The samples of methyl 3α,12α-diacetoxycholanate, crystallized three times, were combined and 345 mg (specific activity 158 cpm/mg) was used for further demonstration of constant specific activity and to locate the position of tritium. By conventional saponification, acidification, and ether extraction, deoxycholic acid was obtained which was esterified with diazomethane to afford methyl deoxycholate. Acetylation by the procedure of Gallagher and Long (1946) yielded two products after chromatography on alumina: 78 mg of methyl 3α,12α-diacetoxycholanate (154 cpm/mg) and 154 mg of methyl 3α-acetoxy-12α-hydroxycholanate (154 cpm/mg). Oxidation of 139 mg of the monoacetate in

2% chromic acid in 95% acetic acid yielded 130 mg of methyl 3α-acetoxy-12-ketocholanate; 146 cpm/mg after crystallization from ethyl acetate. Alkaline hydrolysis of 128 mg of the acetoxy ketone followed by esterification and chromatography of the product on alumina afforded 99 mg of material (152 cpm/mg) which was crystallized from acetone–water to give 58 mg of methyl 3α-hydroxy-12-ketocholanate (155 cpm/mg). The hydroxy ketone was oxidized with the chromic oxide reagent and the product was chromatographed on alumina to yield methyl 3,12-diketocholanate which was devoid of radioactivity. All compounds in the above sequence had physical constants and infrared spectra identical with authentic materials. The specific activities have been adjusted for changes in molecular weight of the substances.

Radioactivity Measurements. The samples were counted in a Packard liquid scintillation counter. The choice of scintillants depended upon the solubility of the substances in water or organic solvents; the procedure has been recently described (Bradlow *et al.*, 1966).

Results

Table I shows that the administered [3β-³H]5β-cholestan-3α-ol was over 95% pure. The principal contaminant was [3α-³H]5β-cholestan-3β-ol (Rosenfeld *et al.*, 1967) which is formed as a minor product in the reduction of 5β-cholestan-3-one with metal hydrides.

From the distribution of radioactivity in bile steroids shown in Table II, it can be calculated that sterols, dihydroxy, and trihydroxy bile acids contained approximately 82, 16, and 2% of the counts, respectively. It can be safely assumed that radioactivity in the sterols was due to the presence of [³H]5β-cholestan-3α-ol since plasma and the bile sterols interchange rapidly (Rosenfeld and Hellman, 1959) and it has been shown that plasma sterol radioactivity in this subject was over 95% associated with the administered substance (R. S. Rosenfeld and L. Hellman, unpublished data). Both bile acid fractions lost considerable amounts of radioactivity when attempts were made to purify the principal constituents, methyl 3α,7α-diacetoxycholanate and methyl 3α,7α,12α-triacetoxycholanate. When constant specific activity of methyl 3α,7α-diacetoxycholanate was achieved after four recrystallizations (I-4, Figure 1), only 2.3% of the radioactivity in the fraction was associated with it. Since the triacetoxycholanate fraction contained so little tritium at the outset, no further examination was undertaken.

Because a significant amount of radioactivity was found in the methyl diacetoxycholanate fraction and since deoxycholic acid is notoriously difficult to separate from chenodeoxycholic acid, it seemed reasonable to suspect that the tritium might be associated with the former substance; this proved to be the case. Dilution of highly radioactive mother liquors (ML-IV, Figure 1), remaining after removal of four crops of methyl 3α,7α-diacetoxycholanate by repeated crystallization, with more than a 60-fold excess of methyl 3α,12α-diacetoxy-

cholanate gave a mixture which attained constant specific activity after one recrystallization. Thus it was demonstrated (see Experimental Section) that 74–80% of the ³H was present in methyl 3α,12α-diacetoxycholanate. Further, when this deoxycholic acid derivative was carried through six additional purification steps, including three derivatives, there was no loss in radioactivity. Complete loss of ³H was achieved only after conversion of methyl 3α-hydroxy-12-ketocholanate into methyl-3,12-diketocholanate which demonstrates that tritium must have been β at C-3; that is, there had been no alteration in the A ring or the A/B ring junction during transformation of a portion of [3β-³H]5β-cholestan-3α-ol to deoxycholic acid.

Discussion

The available evidence suggests that one of the initial transformations of the steroid nucleus in the biosynthesis of bile acids from cholesterol is introduction of oxygen in ring B. Thus Δ⁵-cholestene-3β,7α-diol appears to be an early intermediate in the route where nuclear alterations precede loss of the three-carbon fragment (Mendelsohn *et al.*, 1965). In the alternate sequence, where 3β-hydroxy-Δ⁵-cholenic acid may occupy an intermediate position, further changes in the nucleus include hydroxylations at C-7 and C-6 (Mitropoulos and Myant, 1967); in the latter case, the investigators found no evidence of C-12 hydroxylation. With regard to the role of saturated 3-monohydroxy sterols in the biosynthesis of bile acids from cholesterol, both 5α-cholestan-3β-ol and 5β-cholestan-3β-ol are unlikely intermediates since the former, with the A/B *trans* juncture, is converted into allocholic acid (Karavolas *et al.*, 1965) and the latter, although transformed to normal bile acids (Bell *et al.*, 1965) is of bacterial origin. Because 5β-cholestan-3α-ol possesses structural features in the A ring and at the A/B ring juncture identical with the principal, primary bile acids, chenodeoxycholic and cholic acid, it seemed possible that this sterol might be an intermediate in an alternate route to bile acids at a point preceding 7 hydroxylation. However, 7 hydroxylation of [3β-³H]5β-cholestan-3α-ol proceeded poorly; 12 hydroxylation was over 17 times more efficient as evidenced by the identification of deoxycholic acid as the principal radioactive bile acid, but it cannot be stated with certainty whether the hydroxylation occurred before or after side-chain shortening.

It is unlikely that the [3β-³H]deoxycholic acid arose from dehydroxylation of a trihydroxycholanic acid, since fractions containing the latter material, even from bile collected during the first few days of the study, possessed no more than 11% of the bile acid radioactivity and pure cholic acid was not radioactive. It is also improbable that the labeled deoxycholic acid was derived from the radioactive impurity, [3α-³H]5β-cholestan-3β-ol (about 5% of the administered dose); for this to happen, the 3β-hydroxy must undergo inversion to the 3α-hydroxy without loss of tritium at C-3, a process which is not supported by earlier findings (Rosenfeld and Hellman, 1961; Green and Samuelson, 1964).

3087

The possibility that deoxycholic acid might have been formed from 5β-cholestan-3α-ol by microorganisms must be considered since there was uncertainty as to the completeness of the bile fistula. In this event, a portion of the bile acids could be involved in an enterohepatic circulation with resulting exposure to intestinal bacteria. Four factors make the participation by microorganisms unlikely. (1) Although [3β-³H]-deoxycholic acid was derived from [3β-³H]5β-cholestan-3α-ol, there was no measurable mass of deoxycholic acid in the bile. (2) Hydroxylation at 12α in the cholanic acids by microorganisms has not been reported. (3) There was no measurable bile acid in the stool by a gas–liquid partition chromatographic technique (Danielsson *et al.*, 1963) capable of detecting about 10 mg/day. (4) Over 93% of the radioactivity in stool was present in the sterol fraction.

It can be concluded from this study that deoxycholic acid was formed from 5β-cholestan-3α-ol as a primary bile acid with retention of configuration at C-3. This demonstrates a heretofore unknown capacity of the bile acid synthesizing system in man.

Acknowledgments

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