Conversion of 7α,12α-dihydroxycholest-4-en-3-one to 5α-cholestane-3α,7α,12α-triol by iguana liver microsomes

T. HOSHITA,* S. SHEFER, and E. H. MOSBACH

Department of Laboratory Diagnosis, Public Health Research Institute of the City of New York, Inc., and the Bureau of Laboratories, New York City Department of Health, New York 10009

ABSTRACT The role of 7α,12α-dihydroxycholest-4-en-3-one as an intermediate in the formation of 5α-bile acids from cholesterol was investigated with liver preparations of *Iguana iguana* in vitro. The microsomal fraction of iguana liver catalyzed the transformation of 7α,12α-dihydroxycholest-4-en-3-one to 5α-cholestane-3α,7α,12α-triol in good yield. 7α,12α-dihydroxy-5α-cholestan-3-one served as an intermediate. Under the conditions employed, formation of the corresponding 5β-isomers could not be detected. High speed supernatant solution and mitochondrial fraction of iguana liver did not reduce 7α,12α-dihydroxycholest-4-en-3-one to a measurable extent. The microsomal enzyme system required NADPH as hydrogen donor and was inactive in the presence of NADH.

It is suggested that 7α,12α-dihydroxycholest-4-en-3-one may serve as a common intermediate in the formation of 5α- and 5β-bile acids from cholesterol.

KEY WORDS *Iguana iguana* · allo bile acids · biosynthesis · liver microsomes · 7α,12α-dihydroxycholest-4-en-3-one · 7α,12α-dihydroxy-5α-cholestan-3-one · 5α-cholestane-3α,7α,12α-triol · intermediary metabolism · bile acids

IN THE BIOSYNTHESIS OF BILE ACIDS the 5,6-double bond of cholesterol is reduced stereospecifically: the cholanoic acids formed in most mammals have the 5β-configuration (A/B *cis* juncture) (1). However, it has recently become apparent that bile acids with the 5α-configuration (A/B *trans*) do occur in the bile of some mammals, usually in small concentrations (1). In certain species of fish, reptiles, and birds 5α-bile acids predominate and 5β-bile acids may be absent altogether or may be present in trace amounts only (2). Since the available evidence suggests that cholesterol is the precursor of bile acids in all species we considered the possibility that the biosynthetic pathway leading from cholesterol to the bile acids involves an intermediate which can be transformed into either 5α- or 5β-cholanoic acids. An early step in the biogenesis of bile acids which could serve as a branching point is the reduction of the double bond of 7α,12α-dihydroxycholest-4-en-3-one, leading to the formation of either 7α,12α-dihydroxy-5α-cholestan-3-one or 7α,12α-dihydroxy-5β-cholestan-3-one (2). These saturated ketones could then be transformed into the corresponding triols, namely, 5α-cholestane-3α,7α,12α-triol or 5β-cholestane-3α,7α,12α-triol. Further conversion of the C_{27} compounds to 5α- or 5β-cholanoic acids would then proceed by a sequence of reactions which is currently being elucidated (2).

In testing the hypothesis that 7α,12α-dihydroxycholest-4-en-3-one is the intermediate common to both 5α- and 5β-bile acids it was necessary only to show that this unsaturated ketone can be converted to 5α-cholestane-3α,7α,12α-triol, since it is known that soluble enzymes of rat liver can catalyze the transformation of this substance to 5β-cholestane-3α,7α,12α-triol (3). The bile of the green iguana (*Iguana iguana*) contains the taurine conjugate of allocholic acid (3α,7α,12α-trihydroxy-5α-cholanoic acid) as the major bile acid constituent (K. Okuda, personal communication), which suggests that liver preparations of this lizard would be suitable for experiments on the reduction of 7α,12α-dihydroxy-cholest-4-en-3-one in vitro. Tritium-labeled 7α,12α-dihydroxycholest-4-en-3-one was incubated with ultracentrifugal fractions of iguana liver,

GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

*Fellow of the Public Health Research Institute, 1966–67. Present address: Department of Biochemistry, University of Hiroshima School of Medicine, Hiroshima, Japan.

and it was found that the microsomal fraction (but not the mitochondria or high speed supernatant solution) converted the unsaturated ketone to 5α-cholestane-3α,7α,12α-triol in excellent yield. The corresponding 5β-epimer was not produced by any of these tissue fractions in detectable quantities. These results indicate that a C_{27} compound, 7α,12α-dihydroxycholest-4-en-3-one, probably represents a branching point of the biosynthetic pathway leading from cholesterol to either 5α- or 5β-bile acids.

EXPERIMENTAL PROCEDURES

Analytical Methods

GLC. The concentration of C_{27} steroids obtained during adsorption or partition column chromatography was determined with a Barber-Colman model 5000 gas chromatograph equipped with flame ionization detectors and disc integrators. The glass columns used were 6 ft in length and 4 mm in diameter and were packed with 3% QF-1 (methyl fluoroalkyl silicone) on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, Pa.). Column temperature was 265°C.

TLC. Thin-layer chromatography of steroids was performed as described previously (4).

Organic Synthesis

Tritium-Labeled 7α,12α-Dihydroxycholest-4-en-3-one. 7α,12α-dihydroxy-5β-cholestan-3-one was prepared as described by Berséus, Danielsson, and Kallner (3). The ketone was labeled with tritium by exposure to 3 curies of tritium gas for 2 wk (5). The labeled compound (2.1 mg, 20 mc) was diluted with 87 mg of unlabeled material and purified by preparative TLC (Silica Gel G, 2 mm layer, ethyl acetate–benzene 1:1). The purified sample (81 mg) was dissolved in 20 ml of 95% ethanol and treated with 65 mg of selenium dioxide at 60°C for 8 hr. The reaction mixture was filtered, and the solvent was evaporated under reduced pressure. The residue was chromatographed on a 10 g column of alumina, Woelm grade II, and 7α,12α-dihydroxycholest-4-en-3-one was eluted with 40% ethyl acetate in benzene. After recrystallization from methanol–water, the product melted at 228°C, λ_{max} (ethanol) 234 mμ, ϵ = 17,000 [recorded mp 228–229°C, λ_{max} (ethanol) 234 mμ, ϵ = 16,800 (3)] and had a specific radioactivity of 4×10^7 cpm/mg. As judged by TLC (Silica Gel G, chloroform–ethanol 9:1) and by GLC, the labeled dihydroxycholestenone contained no detectable contaminants. The specific radioactivity remained constant during three recrystallizations from different solvents: methanol, ethanol–water, and methanol–ethyl acetate (4.2×10^7, 4.3×10^7, and 4.3×10^7 cpm/mg).

5α-Cholestane-3α,7α,12α-triol. Deoxy-5α-cyprinol (5α-cholestane-3α,7α,12α,26-tetrol), 530 mg, prepared by $LiAlH_4$ reduction of anhydro-5α-cyprinol (6), was dissolved in 10 ml of dry pyridine; 450 mg of *p*-toluenesulfonyl chloride was added, and the mixture was allowed to stand at room temperature overnight. The solution was poured into an excess of ice-water and the resulting precipitate was collected and dried. TLC of the crude product (Silica Gel G, ethyl acetate–acetone 9:1) showed that all of the tetrol had been transformed into a less polar substance, namely, the 26-tosylate of the tetrol. This material was dissolved in 60 ml of dry benzene and the solution was concentrated to approximately 30 ml, added to a suspension of 300 mg of $LiAlH_4$ in 30 ml of anhydrous ether, and refluxed for 6 hr. The reaction mixture was treated with crushed ice and dilute sulfuric acid, and the product obtained was crystallized from methanol–water, yielding 430 mg of 5α-cholestane-3α,7α,12α-triol, mp 251°C. The infrared spectrum of this substance exhibited bands characteristic of the allocholic acid nucleus (7). GLC of the triol gave a single peak with a retention time of 1.10 relative to that of 5β-cholestane-3α,7α,12α-triol. The retention time of methyl allocholate relative to methyl cholate is likewise 1.10 under these conditions.

5α-Cholestane-3α,7α,12α-triol was prepared in addition from 5α-cholest-25-ene-3α,7α,12α-triol which had been synthesized as described by Hoshita, Hirofuji, Nakagawa, and Kazuno (8). The 5α-cholestanetriol synthesized by catalytic hydrogenation of the 25-ene with 5% palladium on carbon catalyst in ethanol was identical in all respects to the sample prepared by $LiAlH_4$ reduction of the 26-tosylate as described above.

7α,12α-dihydroxy-5α-cholestan-3-one. 5α-Cholestane-3α,7α,12α-triol, 200 mg, was dissolved in acetic anhydride and heated in the presence of anhydrous sodium acetate on a steam bath for 20 hr. The reaction mixture was poured into an excess of cold water and extracted with ether. The ether extract was washed with 5% $NaHCO_3$ solution and then with water. Evaporation of the solvent left a residue which on TLC (Silica Gel G, isooctane–ethyl acetate–acetic acid 10:10:0.1) (9) gave a single spot, presumably of the fully acetylated triol. This material was dissolved in 50 ml of methanol containing 1.5 ml of concentrated HCl and was allowed to stand at room temperature for 12 hr. The solution was diluted with water and extracted with ether. The ether extract was washed and dried and the ether was evaporated. The residue was analyzed by TLC in the solvent system just described (9); the results indicated that most of the triacetate had been partially hydrolyzed to give a diacetate, presumably 3α-hydroxy-7α,12α-diacetoxy-5α-cholestane. This material was dissolved in 5 ml of glacial acetic acid and treated with 50 mg of CrO_3 in 0.1 ml of water and 1

ml of acetic acid, and the solution was allowed to stand at room temperature for 2 hr. The reaction mixture was diluted with water and extracted with ether. The ether solution was washed with water and dried over sodium sulfate and the ether was evaporated. The residue was hydrolyzed in 5% methanolic KOH at 60°C for 2 hr. Dilution with water gave a precipitate which was collected by filtration. The dried precipitate was subjected to preparative TLC (2 mm layer of Silica Gel G, ethyl acetate–benzene 1:1), and the zone corresponding to the dihydroxymonoketone was extracted with ethanol. Recrystallization from methanol–water gave crystals of mp 190–192°C. On GLC this compound was homogeneous and its retention time was 1.10 relative to that of 7α,12α-dihydroxy-5β-cholestan-3-one.

Cholest-4-ene-3α,7α,12α-triol. This compound was prepared essentially as reported by Björkhem and Danielsson (10). 7α,12α-dihydroxycholest-4-en-3-one (40 mg in 10 ml of methanol) was treated with 30 mg of $NaBH_4$ at room temperature for 2 hr. The reaction mixture was extracted with ether after dilution with water and acidification with dilute HCl; the extract was washed with 5% Na_2CO_3 solution and then with water. The solvent was evaporated and the residue was subjected to preparative TLC (Silica Gel G, 2 mm layer, chloroform–ethanol 9:1). The zone corresponding to cholest-4-ene-3α,7α,12α-triol was extracted with ethanol. Crystallization from methanol–water gave crystals, mp 208–210°C [reported mp, 210-214°C (10)].

Preparation of Enzyme (11)

2-g portions of liver from *Iguana iguana* (the liver of the desert iguana *Dipsosaurus dorsalis* gave qualitatively similar results) were homogenized in a Potter-Elvehjem homogenizer with 18 ml of ice-cold 0.25 M sucrose solution containing 2.5 mM neutralized EDTA and 75 mM nicotinamide. Cellular debris and nuclei were removed by centrifugation at 500 *g* for 5 min. The supernatant solution was centrifuged at 20,000 *g* for 10 min to sediment the mitochondrial fraction. The microsomal fraction was obtained by centrifuging the 20,000 *g* supernatant solution at 100,000 *g* for 60 min. The mitochondrial and microsomal fractions were suspended in the homogenizing medium (same volume as original homogenate). A typical preparation of microsomes had a protein concentration of 1.6 mg/ml.

Assay of Enzyme Activity (12)

The enzymic conversion of 7α,12α-dihydroxycholest-4-en-3-one to 7α,12α-dihydroxy-5α-cholestan-3-one and 5α-cholestane-3α,7α,12α-triol was assayed as follows. A solution of 20 mμmoles of tritium-labeled 7α,12α-dihydroxycholest-4-en-3-one in 0.015 ml of acetone was added to an incubation mixture containing, in a volume of 1.75 ml: potassium phosphate buffer, pH 7.4, 0.167 mmole; $MgCl_2$, 11 μmoles; $NADP^+$, 1.5μmoles; glucose-6-phosphate, 2.7 μmoles; glucose-6-phosphate dehydrogenase (EC 1.1.1.49) 1 IU; and microsomes containing approximately 1.6 mg of protein. The conversion appeared to be optimal over the pH range 6.5–7.5.

Incubations were conducted at 37°C in air for 2 hr and terminated by addition of 20 ml of methylene chloride–ethanol 5:1 and 10 ml of water (11). The organic layer was pipetted into a small test tube and washed with 5 ml of water. Celite Filter-Aid (0.5 g) was added and the solution was filtered; the solvent was then evaporated to dryness in a current of air. Under these conditions 85–95% of the radioactivity was recovered from the incubation mixture.

The dried residue was dissolved in ethanol and an aliquot was applied as a spot to a thin-layer plate coated with 250 μ of Silica Gel G. Unlabeled reference compounds (5α-cholestane-3α,7α,12α-triol, 7α,12α-dihydroxycholest-4-en-3-one, and 7α,12α-dihydroxy-5α-cholestan-3-one) were applied at the same spot. The developing solvents were either benzene–ethyl acetate 1:3 or chloroform–ethanol 9:1. The position of the standard samples was established by exposure of the plates to iodine vapor. Pertinent spots were removed from the plates by suction, and the steroids were extracted from the silica gel with 5 ml of ethanol (heated at 60°C for 30 min). Half of each ethanol extract was transferred to a scintillation vial containing 12.5 ml of a 0.4% solution of 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]-thiophene in toluene in a Unilux counter (Nuclear-Chicago Corporation). Suitable corrections were made for background and quenching.

Identification of Reaction Products

5α-Cholestane-3α,7α,12α-triol. The identity and radioactive purity of this reaction product were established as follows. The labeled material obtained from the cholestanetriol spot of the thin-layer chromatogram was chromatographed together with the authentic, unlabeled reference compound on either an alumina (adsorption) column or a reversed-phase partition column, as shown in Figs. 1 and 2. In both cases the radioactivity was eluted together with the known 5α-cholestane-3α,7α,12α-triol, and the specific activity of the eluted fractions remained constant throughout the carrier band. Further evidence was obtained by cocrystallization of the biosynthetic triol with unlabeled, authentic carrier. The data summarized in Table 1 demonstrate that the specific radioactivity of the triol did not change during three recrystallizations from different solvents.

7α,12α-dihydroxy-5α-cholestan-3-one. The radioactive material recovered from the spot corresponding to this saturated ketone was mixed with unlabeled carrier and

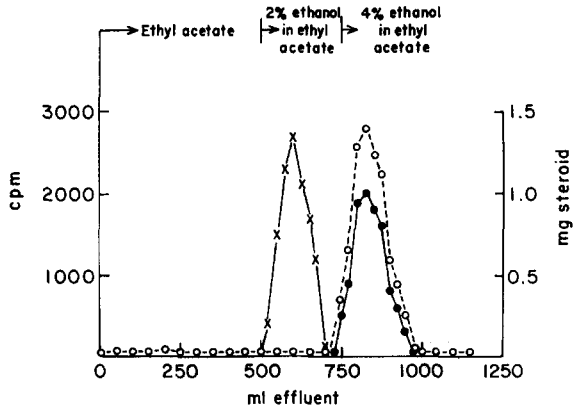

FIG. 1. Alumina column chromatography of the cholestane-3α,7α,12α-triol fraction obtained by TLC of the steroids formed when tritium-labeled 7α,12α-dihydroxycholest-4-en-3-one was incubated with iguana liver microsomes. Unlabeled 5α- and 5β-cholestanetriols were added as carriers. Column: 10 g of neutral alumina, Woelm activity grade IV. Weight of steroids in column fractions was determined by GLC. ×, 5β-cholestane-3α,7α,12α-triol; ●, 5α-cholestane-3α,7α,12α-triol; ○ cpm.

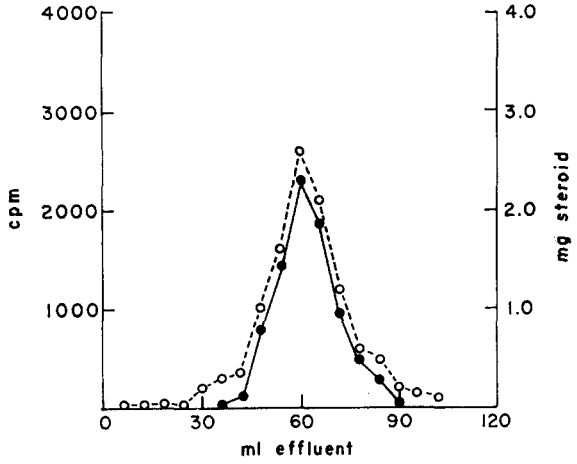

FIG. 2. Reversed-phase column partition chromatography of the cholestane-3α,7α,12α-triol fraction obtained by TLC of the steroids formed when tritium-labeled 7α,12α-dihydroxycholest-4-en-3-one was incubated with iguana liver microsomes. Unlabeled 5α-cholestanetriol was added as carrier. Column: 4.5 g of hydrophobic Hyflo Super-Cel with chloroform–n-heptane 1:4 as stationary phase and water–2-propanol 1:1 as mobile phase (18). ●, 5α-cholestane-3α,7α,12α-triol; ○, cpm.

chromatographed on alumina (Fig. 3). The radioactivity was eluted together with the reference compound and the specific radioactivity of each column fraction remained constant throughout the carrier band.

Since the conversion of 7α,12α-dihydroxycholest-4-en-3-one to 7α,12α-dihydroxy-5α-cholestan-3-one and 5α-cholestane-3α,7α,12α-triol proceeded almost to completion, these reaction products could also be identified by their R_f values when analyzed by TLC in the absence of unlabeled carrier. After development of the thin-layer plate with benzene–ethyl acetate 1:3, the spots corresponding to the two reaction products could be made visible by means of phosphomolybdate spray reagent. The following R_f values were observed: 7α,12α-dihydroxycholest-4-en-3-one, 0.38; 7α,12α-dihydroxy-5α-cholestan-3-one, 0.52; 5α-cholestane-3α,7α,12α-triol, 0.15.

TABLE 1 IDENTIFICATION AND RADIOACTIVE PURITY OF BIOSYNTHETIC 5α-CHOLESTANE-3α,7α,12α-TRIOL

Solvent	Number of Crystallizations	Specific Activity
		cpm/mg
Methanol–water	1	1.14×10^4
Acetone–water	2	1.21×10^4
Ethyl acetate–n-hexane	3	1.18×10^4

RESULTS

Reduction of 7α,12α-Dihydroxycholest-4-en-3-one by Subcellular Fractions of Iguana Liver

The intracellular localization of the two enzyme activities involved in the conversion of 7α,12α-dihydroxycholest-4-en-3-one to 5α-cholestane-3α,7α,12α-triol via 7α,12α-dihydroxy-5α-cholestan-3-one was determined by ultracentrifugal separation of a 10% homogenate into mitochondria, microsomes, and supernatant solution. It was found, as summarized in Table 2, that the Δ^4-3-keto compound was efficiently reduced to the triol in the presence of the microsomal fraction and an NADPH-generating

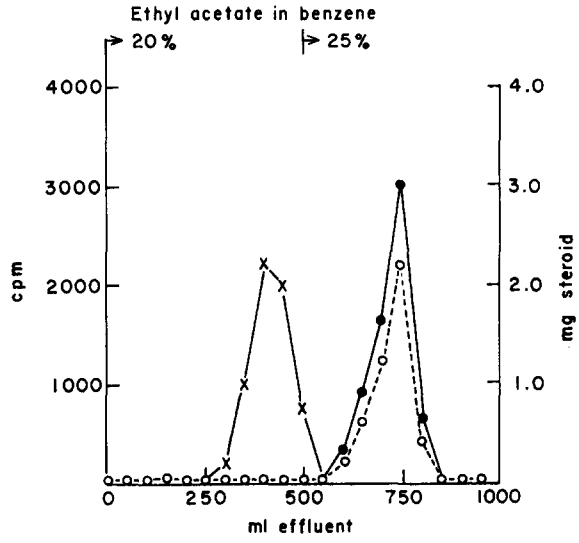

FIG. 3. Alumina column chromatography of the 7α,12α-dihydroxy-5α-cholestan-3-one fraction obtained by TLC of the steroids formed when tritium-labeled 7α,12α-dihydroxycholest-4-en-3-one was incubated with iguana liver microsomes. Unlabeled 5α- and 5β-7α,12α-dihydroxycholestan-3-one were added as carriers. Column: 10 g of neutral alumina, Woelm activity grade II. ×, 7α,12α-dihydroxy-5β-cholestan-3-one; ●, 7α,12α-dihydroxy-5α-cholestan-3-one; ○, cpm.

system. Small amounts of the saturated ketone were also present. No additional metabolites were detected under these conditions. When 7α,12α-dihydroxycholest-4-en-3-one was incubated with the mitochondrial or the soluble fractions of iguana liver all of the substrate radioactivity was recovered unchanged.

Results of experiments dealing with the pyridine nucleotide specificity of the microsomal enzymes involved in the reduction of 7α,12α-dihydroxycholest-4-en-3-one are listed in Table 3. The enzymes utilized NADPH as electron donor; no activity was detected in the presence of NADH or an NADH-generating system.

The effect of changing the substrate concentration on the nature of the reaction products is illustrated in Fig. 4. At low substrate concentrations only 5α-cholestane-3α, 7α,12α-triol could be detected. When the amount of substrate was in excess of 20 mμmoles small amounts of the saturated ketone were also present.

Fig. 5 illustrates the relationship between enzyme concentration and the extent of conversion of 7α,12α-dihydroxycholest-4-en-3-one to the saturated ketone and the triol. At microsomal protein concentrations below 0.5 mg/1.75 ml the saturated ketone predominated; above this concentration the triol was the major reaction product.

TABLE 2 INTRACELLULAR LOCALIZATION OF ENZYMES CATALYZING THE REDUCTION OF 7α,12α-DIHYDROXYCHOLEST-4-EN-3-ONE*

Subcellular Fraction	5α-Cholestane-3α,7α,12α-triol Formed	7α,12α-Dihydroxy-5α-cholestan-3-one Formed
	mμmoles	
Mitochondria	0	0
Microsomes	17.5	2.0
100,000 *g* supernatant solution	0	0

* 20 mμmoles of tritium-labeled substrate were incubated for 2 hr as described in Experimental Procedures.

TABLE 3 PYRIDINE NUCLEOTIDE REQUIREMENT OF MICROSOMAL ENZYMES CATALYZING THE FORMATION OF 7α,12α-DIHYDROXY-5α-CHOLESTAN-3-ONE AND 5α-CHOLESTANE-3α,7α,12α-TRIOL FROM 7α,12α-DIHYDROXYCHOLEST-4-EN-3-ONE

Pyridine Nucleotide Added	5α-Cholestane-3α,7α,12α-triol Formed	7α,12α-Dihydroxy-5α-cholestan-3-one Formed
	mμmoles	
NADPH-generating system*	17.5	2.0
NADPH, 0.86 mM	16.8	1.8
NADH-generating system†	0	0
NADH, 0.86 mM	0	0

* Consisting of NADP, 0.86 mM; glucose-6-phosphate, 1.54 mM; glucose-6-phosphate dehydrogenase, 0.59 IU/ml.

† Consisting of NAD, 0.58 mM; 95% ethanol, 0.02 ml, 0.249 M; alcohol dehydrogenase (E.C. 1.1.1.1) 500 μg.

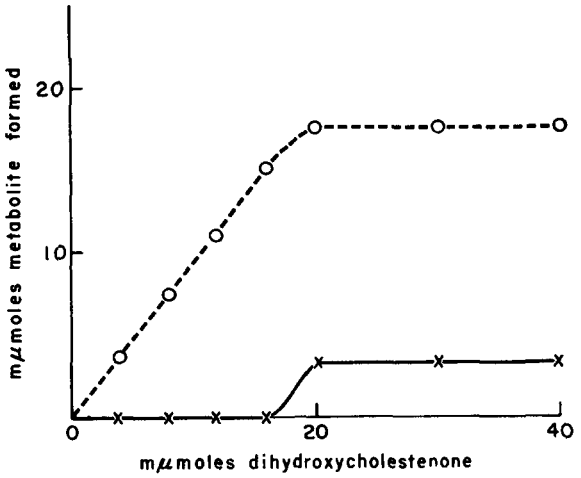

FIG. 4. Effect of substrate concentration on reduction of 7α,12α-dihydroxycholest-4-en-3-one to 7α,12α-dihydroxy-5α-cholestan-3-one and 5α-cholestane-3α,7α,12α-triol. ×, 7α,12α-dihydroxy-5α-cholestan-3-one formed; ○, 5α-cholestane-3α,7α,12α-triol formed.

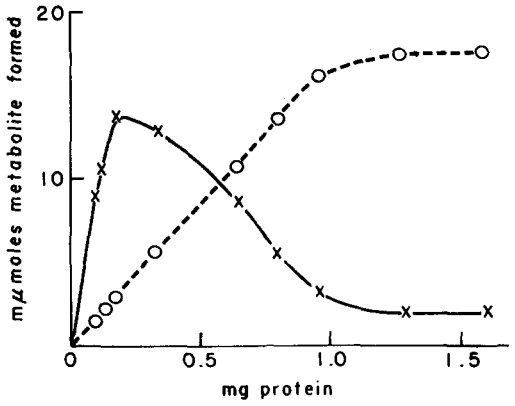

FIG. 5. Effect of increasing amounts of microsomes on the quantity and nature of reaction products during incubation of iguana liver microsomes with tritium-labeled 7α,12α-dihydroxycholest-4-en-3-one. ×, 7α,12α-dihydroxy-5α-cholestan-3-one formed; ○, 5α-cholestane-3α,7α,12α-triol formed.

The inhibition studies summarized in Table 4 were carried out to obtain further information on the nature of the intermediate in the reduction of 7α,12α-dihydroxycholest-4-en-3-one to 5α-cholestane-3α,7α,12α-triol. The data show that 7α,12α-dihydroxy-5α-cholestan-3-one served as a "trapping agent," thereby partially inhibiting the formation of 5α-cholestane-3α,7α,12α-triol. The other possible intermediate in the conversion of 7α,12α-dihydroxycholest-4-en-3-one to 5α-cholestane-3α,7α, 12α-triol, namely cholest-4-ene-3α,7α,12α-triol did not "trap" substrate radioactivity and had little effect on the nature and relative amounts of products formed. The major reaction product, 5α-cholestane-3α,7α,12α-triol, did not inhibit the conversion of 7α,12α-dihydroxycholest-4-en-3-one to this triol but reduced the amount of saturated ketone in the reaction mixture.

TABLE 4 INHIBITION OF MICROSOMAL ENZYME SYSTEM BY C_{27} STEROIDS

Inhibitor	Concentration of Inhibitor	% Conversion of 7α,12α-Dihydroxycholest-4-en-3-one to: 7α,12α-Dihydroxy-5α-cholestan-3-one	5α-Cholestane-3α,7α,12α-triol	Cholest-4-ene-3α,7α,-12α-triol
	mM			
None	—	17.5	32.9	0
7α,12α-Dihydroxy-5α-cholestan-3-one	0.228	21.5	7.0	0
	0.343	18.6	4.3	0
5α-Cholestane-3α,7α,12α-triol	0.114	4.7	31.6	0
Cholest-4-ene-3α,7α,12α-triol	0.228	13.0	34.0	0
	0.343	23.4	35.8	0

Assay procedure as described in Experimental Procedures, except that substrate concentration was 44 mμmoles/1.75 ml.

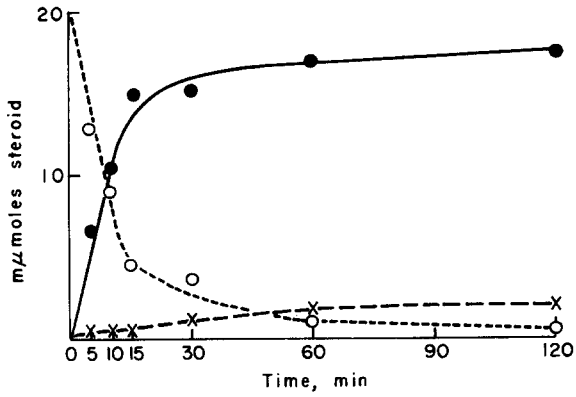

FIG. 6. Time study of the conversion of 7α,12α-dihydroxycholest-4-en-3-one to 7α,12α-dihydroxy-5α-cholestan-3-one and 5α-cholestane-3α,7α,12α-triol. ○, 7α,12α-dihydroxycholest-4-en-3-one; ×, 7α,12α-dihydroxy-5α-cholestan-3-one; ●, 5α-cholestane-3α,7α,12α-triol.

Fig. 6 illustrates the conversion of 7α,12α-dihydroxycholest-4-en-3-one to 7α,12α-dihydroxy-5α-cholestan-3-one and 5α-cholestane-3α,7α,12α-triol as a function of time. Since the enzyme concentration was relatively high, the intermediate, 7α,12α-dihydroxy-5α-cholestan-3-one, did not accumulate in the reaction mixture.

DISCUSSION

Recent methodological improvements in the analysis and synthesis of bile acids led to the finding that allo bile acids (5α-cholanoic acids) are more prevalent in mammalian bile than heretofore supposed. Allocholic acid (3α,7α,12α-trihydroxy-5α-cholanoic acid) has been detected in the bile of man and of other mammals, although usually only in trace amounts (1, 2). The presence of 5α-bile acid in the bile of these species could possibly be ascribed to the action of the bacterial intestinal flora on the primary 5β-bile acids (13, 14) and need not necessarily indicate hepatic biosynthesis of the allo compound. However, Hofmann and Mosbach (unpublished observations) have recently detected up to 5% allocholic acid in rabbit bile under conditions in which the participation of the intestinal flora could be ruled out (germ-free rabbits and rabbits with chronic bile fistulas). It seems likely, therefore, that certain mammalian species have retained some capacity to synthesize allo bile acids. In lower vertebrates, such as fish, birds, and reptiles, bile salts that have the 5α-configuration are much more common, which suggests that 5α-bile salts represent a more primitive form that has been replaced in higher vertebrates by the more modern 5β-compounds (14).

While it is now generally accepted that cholesterol is an obligatory precursor of the 5β-cholanoic acids of mammalian bile, it had to be considered that 5α-bile salts might arise instead from cholestanol (5α-cholestan-3β-ol), the ubiquitous companion of cholesterol. This possibility was suggested by previous studies that proved that dietary cholestanol is transformed into glycoallodeoxycholate in the rabbit (15) and by the observation that the 7α-hydroxylation of cholestanol by rat liver microsomes proceeds as readily as the hydroxylation of cholesterol (16). However, evidence arguing against a direct precursor role for cholestanol in the biosynthesis of 5α-bile salts derives from the finding that even in those species that produce predominantly 5α-bile salts, such as carp (7) and iguana, cholesterol (and not cholestanol) is the major hepatic sterol constituent. (Unpublished experiments from this laboratory showed that the liver sterol fraction of *Iguana iguana* contained approximately 97% cholesterol and 3% cholestanol.) In addition, in the carp 7α-hydroxycholesterol, and therefore presumably cholesterol, is transformed more readily into 5α-bile salts than cholestanol (17).

The results of the present study are in accord with the suggestion of Haslewood (2) that 7α,12α-dihydroxycholest-4-en-3-one is the biological precursor common to both the 5α- and 5β-series of bile salts. The transformation of the Δ^4-3-keto intermediate to 5α-cholestane-3α,7α,12α-triol by iguana liver microsomes demonstrated here is closely analogous to its conversion to 5β-cholestanetriol in the presence of high speed supernatant solution of rat liver, demonstrated by Berséus et al. (3). These authors showed that the reduction of 7α,12α-dihydroxycholest-4-en-3-one by soluble enzymes of rat liver required NADPH as hydrogen donor and involved 7α,12α-dihydroxy-5β-cholestan-3-one as an intermediate. In the present study the possibility was considered that cholest-4-ene-3α,7α,12α-triol rather than 7α,12α-dihydroxy-5α-cholestan-3-one was the initial reaction product. However, in the trapping experiments (Table 4) the forma-

tion of labeled cholest-4-ene-3α,7α,12α-triol could not be detected. Moreover, in the present study, as in the experiments of Berséus et al. (3) just cited, the saturated ketone predominated in the incubation mixture when the concentration of microsomal protein was low (Fig. 5). Studies on the metabolism of 7α,12α-dihydroxy-5α-cholestan-3-one in iguana liver should further clarify this point.

At present there exists no direct evidence that iguana liver contains enzymes capable of catalyzing the formation of 7α,12α-dihydroxycholest-4-en-3-one from cholesterol, although this transformation has been demonstrated in the rat (19). However, it is known that in the carp, during the biosynthesis of 5α-cyprinol (a C_{27} 5α-bile alcohol) from cholesterol, the 4β-hydrogen of the latter is largely lost, which suggests that a Δ^4-intermediate is involved (20). Obviously, the pathway for the formation of 5α-bile acids in the iguana suggested in the present paper must be considered tentative until the conversion of cholesterol to 7α,12α-dihydroxycholest-4-en-3-one, and the further transformation of 5α-cholestane-3α,7α,12α-triol to allocholic acid, has been demonstrated in this species.

This work was supported by Research Grants AM-05222 and HE-10894 from the United States Public Health Service.

Manuscript received 18 October 1967; accepted 4 December 1967.

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