Formation of Bile Acids in Man: Conversion of Cholesterol into 5β-Cholestane-3α,7α,12α-triol in Liver Homogenates*

INGEMAR BJÖRKHEM, HENRY DANIELSSON, KURT EINARSSON, and GUNNAR JOHANSSON

From the Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

ABSTRACT The mechanisms of the conversion of cholesterol into bile acids in man were studied by examining the metabolism of cholesterol-1,2-^{3}H, cholest-5-ene-3β,7α-diol-7β-^{3}H, tritium-labeled 7α-hydroxycholest-4-en-3-one, 7α,12α-dihydroxycholest-4-en-3-one, and cholest-5-ene-3β,7α,12α-triol in fractions of liver homogenates. The 20,000 *g* supernatant fluid catalyzed the conversion of cholesterol into cholest-5-ene-3β,7α-diol, 7α-hydroxycholest-4-en-3-one, 7α-12α-dihydroxycholest-4-en-3-one, and 5β-cholestane-3α,7α,12α-triol. In the presence of microsomal fraction fortified with NAD$^+$, cholest-5-ene-3β,7α-diol was converted into 7α-hydroxycholest-4-en-3-one, and when this fraction was fortified with NADPH small amounts of cholest-5-ene-3β-7α,12α-triol were formed. 7α-Hydroxycholest-4-en-3-one was metabolized into 7α-12α-dihydroxycholest-4-en-3-one in the presence of microsomal fraction fortified with NADPH and into 5β-cholestane-3α,7α-diol in the presence of 100,000 *g* supernatant fluid. Cholest-5-ene-3β,7α,12α-triol was converted into 7α,12α-dihydroxycholest-4-en-3-one in the presence of microsomal fraction fortified with NAD$^+$. The 100,000 *g* supernatant fluid catalyzed the conversion of 7α,12α-dihydroxycholest-4-en-3-one into 5β-cholestane-3α,7α,12α-triol. The sequence of reactions in the conversion of cholesterol into 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol, the subcellular localization of the enzymes, and the cofactor requirements were found to be the same as those described for rat liver.

INTRODUCTION

Bile acids are major end products of cholesterol metabolism in vertebrates. (For a review, see reference 1.) The conversion of cholesterol into bile acids takes place in the liver, and the main bile acids formed are, in most mammalian species, cholic acid [1] and chenodeoxycholic acid (1). In bile, bile acids are present as conjugates with taurine and glycine. Bile acids are excreted with bile into the intestine, reabsorbed in the ileum, and again excreted with bile. A small amount of bile acids escapes reabsorption in each cycle of enterohepatic circulation, and these bile acids are excreted with the feces. During their enterohepatic circulation bile acids are subjected to the action of intestinal microorganisms. Major reactions involve the hydrolysis of the conjugates and the removal of the 7α-hydroxyl group yielding deoxycholic acid from cholic acid and lithocholic acid from chenodeoxycholic acid (1). Further struc-

* Bile acids and steroids 197.

Received for publication 5 February 1968 and in revised form 7 March 1968.

[1] The following systematic names are given to bile acids referred to by trivial names: cholic acid, 3α,7α, 12α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,-7α-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; lithocholic acid, 3α-hydroxy-5β-cholanoic acid.

tural modifications of the bile acids in the intestine lead to the complex mixture of bile acids excreted with the feces. The bile acids formed by microbial action on cholic acid and chenodeoxycholic acid can be reabsorbed, but of all the different metabolites formed only deoxycholic acid appears to be efficiently reabsorbed, and deoxycholic acid is a major bile acid in many species. Thus, the main bile acids in human bile are cholic acid, chenodeoxycholic acid, and deoxycholic acid.

The mechanisms of the conversion of cholesterol into cholic acid and chenodeoxycholic acid have been extensively studied in recent years (1). The results of investigations on the metabolism of cholesterol and other oxygenated C_{27} steroids in bile fistula rats and in homogenates of rat liver have led to the elucidation of the major pathways for the formation of bile acids in the rat (1). The conversion of cholesterol into bile acids in man has not been studied in any detail. Staple and Rabinowitz (2) and Carey (3) have isolated labeled 3α,7α,12α-trihydroxy-5β-cholestanoic acid from bile of patients treated with injection of labeled cholesterol. Herman, Weinstein, Staple, and Rabinowitz (4) have recently reported the isolation from human bile of small amounts of 5β-cholestane-3α,7α-diol, another probable intermediate in the conversion of cholesterol into bile acids. These results indicate that the pathways for the formation of bile acids in man are analogous to those in rat. However, it appeared of interest to attempt to define more closely the sequence of reactions in the conversion of cholesterol into bile acids in man by studying the metabolism of cholesterol and cholesterol metabolites in homogenates of human liver.

METHODS

Subjects. The patients in this investigation were: A.R., female, aged 60; I.-L.J., female, aged 41; and H.S., male, aged 57. One patient (I.-L.J.) was moderately jaundiced (serum bilirubin, 6.0 mg/100 ml; alkaline phosphatase, 17 Buch U; thymol turbidity test, 1 Maclagan U; glutamic oxaloacetic transaminase (GOT), 180 U; glutamic pyruvic transaminase (GPT), 480 U). The jaundice disappeared after operation. The results obtained with liver tissue from this patient were not different from those obtained with liver tissue from the other two patients. The patients suffered from cholelithiasis. Except for patient I.-L.J., there was no clinical or laboratory evidence for any complication or any other disease, and the recovery after operation was uneventful. 1 hr before cholecystectomy the patients were premedicated with 0.5 mg of atropine and 1 ml of Ketogin.[2] The general anaesthesia consisted of sodium hexobarbital, nitrous oxide, succinyl choline, and fluothane and was initiated 15 min or less before operation.

Preparation of homogenates and analysis of incubations. Liver biopsies (3–5 g) were taken within 15 min after the abdomen had been opened and were immediately put in cold homogenizing medium. Homogenates were prepared within 30 min after the biopsy. The homogenates, 20% (liver wet weight per volume), were prepared in a modified Bucher medium (5), pH 7.4, using a Potter-Elvehjem homogenizer equipped with a loosely fitting pestle. The homogenate was centrifuged at 800 *g* for 10 min and then at 20,000 *g* for 10 min. The microsomal and soluble fractions were obtained by centrifuging the 20,000 *g* supernatant fluid at 100,000 *g* for 2 hr. The microsomal fraction was suspended in the same volume of homogenizing medium as the volume of the 20,000 *g* supernatant fluid from which it had been isolated. The labeled compounds were added to the incubation mixtures dissolved in 50 μl of acetone, and incubations were run at 37°C. Incubations with labeled cholesterol were run for 60 min, and those with the other substrates for 10 or 20 min. (See legends to figures.) Incubations were terminated by the addition of 20 volumes of chloroform-methanol (2:1, v/v). The precipitate was filtered off, and the chloroform-methanol solution was washed with 0.2 volumes of a 0.9% (w/v) solution of sodium chloride. The chloroform phase was collected, and the solvent was evaporated under reduced pressure. The methanol-saline phase, which in all experiments contained less than 10% of the total radioactivity, was discarded. The residue of the chloroform extract together with appropriate reference compounds was subjected to thin-layer chromatography with Kieselgel G [3] as adsorbent. The solvent systems used to develop the chromatoplates are given in the legends to the figures. The internal standards were visualized by exposing the chromatoplates to iodine vapor. The appropriate zones were scraped into test tubes and were extracted with methanol by vigorous stirring. The silica gel was allowed to settle by gravity, and an aliquot of the methanol solution was assayed for radioactivity. Further identification of the metabolites formed was done by crystallizing representative samples together with authentic material to constant specific radioactivity. The reference compounds were material synthesized in this laboratory and used in earlier studies in this series of investigations.

Labeled compounds. Cholesterol-1,2-^{3}H (specific radioactivity, 33 mc/mg) was obtained commercially.[4] Before use, the labeled cholesterol was purified by chromatography on a column of aluminum oxide,[5] grade III activity. Cholest-5-ene-3β,7α-diol-7β-^{3}H (specific radioactivity, 10 μc/mg), tritium-labeled 7α-hydroxycholest-4-en-3-one (specific radioactivity, 10 μc/mg), tritium-labeled 7α,12α-dihydroxycholest-4-en-3-one (specific radioactivity,

[2] Ketobemidon chloride, 5 mg/ml, and *N,N*-dimethyl-3,3-diphenyl-1-methylallylamine chloride, 25 mg/ml.

[3] Merck, Darmstadt, Germany.

[4] New England Nuclear Corp. Boston, Mass.

[5] Woelm, Eschwege, Germany.

6 μc/mg), and tritium-labeled cholest-5-ene-3β,7α,12α-triol (specific radioactivity, 7 μc/mg) were prepared as described previously (6–8).

Radioactivity assay. Radioactivity was assayed with a methane gas flow counter. Aliquots of the extracts were plated on aluminum planchettes. Under the conditions of the assay 1 μc of ^3H corresponded to 6×10^5 cpm.

RESULTS

Metabolism of cholesterol-1,2-^3H. Incubation of cholesterol-1,2-^3H with the 20,000 *g* supernatant fluid and with 800 *g* supernatant fluid resulted in the conversion of about 2% of added substrate into more polar products. Experiments were conducted with liver tissue from each of the three patients, and the extent of conversion of added cholesterol as well as the pattern of products formed were about the same in all the experiments. Fig. 1 A shows a thin-layer chromatogram of the extract of an incubation of cholesterol-1,2-^3H with the 20,000 *g* supernatant fluid. Rechromatography of the material in the most polar zone (1 and 2, Fig. 1 A) suggested that the main part of this labeled material (70%) was identical with cholestane-3β,5α,6β-triol (2, Fig. 1 B). About 10 % of the material had chromatographic properties of cholest-5-ene-3β,7α,12α-triol (1, Fig. 1 B). The identity of the main part of the labeled material with cholestane-3β,5α,6β-triol was established by crystallization together with authentic material to con-

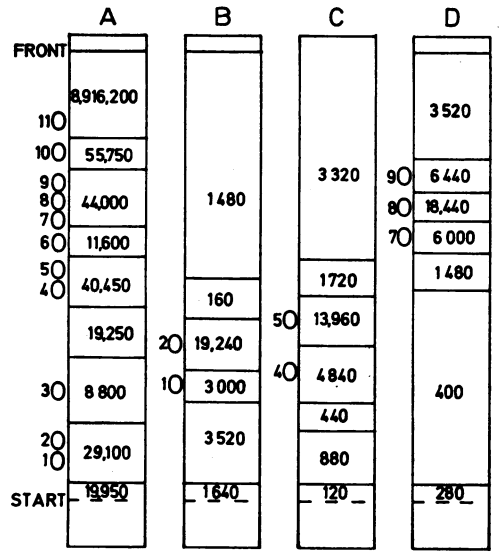

FIGURE 1 A, thin-layer chromatogram of chloroform extract of incubation of cholesterol-1,2-^3H with 20,000 *g* supernatant fluid. 4 ml of 20,000 *g* supernatant fluid, diluted with 2 ml of homogenizing medium, were incubated for 1 hr with 1μg of cholesterol-1,2-^3H. The numbers on the chromatogram represent counts per minute. Reference compounds were: 1, cholest-5-ene-3β,7α,12α-triol; 2, cholestane-3β,5α,6β-triol; 3, 5β-cholestane-3α,7α,12α-triol; 4, 7α,12α-dihydroxycholest-4-en-3-one; 5, cholest-5-ene-3β,-7α-diol; 6, cholest-5-ene-3β,7β-diol; 7, 5β-cholestane-3α,-7α-diol; 8, 3β-hydroxycholest-5-en-7-one; 9, cholest-5-ene-3β,26-diol; 10, 7α-hydroxycholest-4-en-3-one; 11, cholesterol. Solvent, benzene–ethyl acetate (3:7, v/v). B, rechromatography of material in the zone corresponding to reference compounds 1 and 2 of the chromatogram shown in A. Reference compounds were the same as in A. Solvent, ethyl acetate. C, rechromatography of material in the zone corresponding to reference compounds 4 and 5 of the chromatogram shown in A. Reference compounds were the same as in A. Solvent, benzene–ethyl acetate–trimethylpentane (3:7:3, v/v/v). The chromatogram was run twice in the same solvent with drying of the plate between the runs. D, rechromatography of material in the zone corresponding to reference compounds 7, 8, and 9 of the chromatogram shown in A. Reference compounds and solvent were the same as in A.

TABLE I

Identification of Products Formed from Cholesterol-1, 2-^3H

	No. of crystallizations	Solvent	Weight	Specific activity
			mg	*cpm/mg* $\times 10^{-3}$
Cholestane-3β, 5α, 6β-triol	0		18.3	0.58
	1	Methanol-water	11.2	0.72
	2	Acetone-water	7.0	0.63
	3	Methanol-water	3.7	0.57
	4	Acetone-water	3.0	0.58
5β-Cholestane-3α, 7α, 12α-triol	0		7.4	0.72
	1	Methanol-water	7.3	0.62
	2	Acetone-water	6.3	0.20
	3	Methanol-water	4.9	0.13
	4	Acetone-water	4.1	0.12
7α, 12α-Dihydroxy-cholest-4-en-3-one	0		7.7	0.36
	1	Methanol-water	7.5	0.35
	2	Acetone-water	6.8	0.30
	3	Methanol-water	4.6	0.32
Cholest-5-ene-3β, 7α-diol	0		10.3	1.30
	1	Methanol-water	9.7	1.33
	2	Acetone-water	9.4	1.27
	3	Methanol-water	3.9	1.25
3β-Hydroxycholest-5-en-7-one	0		15.2	0.58
	1	Acetone-water	10.5	0.50
	2	Methanol-water	8.2	0.42
	3	Acetone-water	6.2	0.43
7α-Hydroxycholest-4-en-3-one	0		7.6	2.77
	1	Methanol-water	7.3	2.48
	2	Acetone-water	6.9	1.63
	3	Methanol-water	4.6	1.66

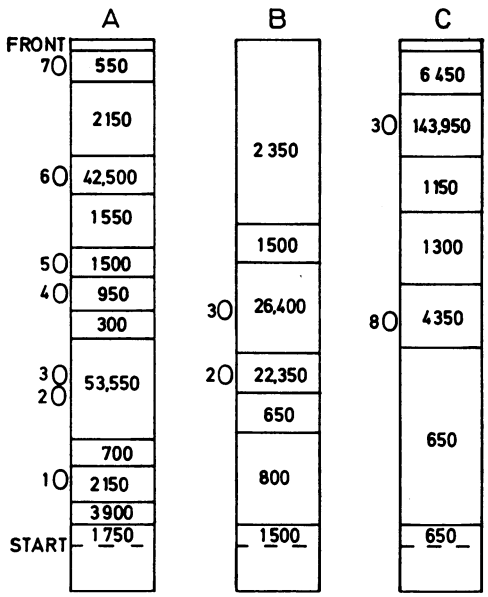

FIGURE 2 A, thin-layer chromatogram of chloroform extract of incubation of cholest-5-ene-3β,7α-diol-7β-^{3}H with 20,000 *g* supernatant fluid. 3 ml of 20,000 *g* supernatant fluid was incubated for 20 min with 40 μg of cholest-5-ene-3β,7α-diol-7β-^{3}H. The numbers on the chromatogram represent counts per minute. Reference compounds were: 1, 5β-cholestane-3α,7α,12α-triol; 2, 7α 12α-dihydroxycholest-4-en-3-one; 3, cholest-5-ene-3β,7α-diol; 4, 5β-cholestane-3α,7α-diol; 5, 7α,12α-dihydroxy-5β-cholestan-3-one; 6, 7α-hydroxycholest-4-en-3-one; 7, 7α-hydroxy-5β-cholestan-3-one. Solvent, benzene–ethyl acetate (1:1, v/v). B, rechromatography of material in the zone corresponding to reference compounds 2 and 3 of the chromatogram shown in A. Reference compounds were the same as in A. Solvent, benzene–ethyl acetate–trimethylpentane (3:7:3, v/v/v). The chromatogram was run twice in the same solvent with drying of the plate between the runs. C, thin-layer chromatogram of chloroform extract of incubation of cholest-5-ene-3β,7α-diol-7β-^{3}H with microsomal fraction fortified with NADPH. 1 ml of microsomal fraction was diluted with 2 ml of homogenizing medium, fortified with 3 μmoles of NADPH, and was incubated for 10 min with 40 μg of cholest-5-ene-3β,7α-diol-7β-^{3}H. The numbers on the chromatogram represent counts per minute. Reference compounds were: 3, cholest-5-ene-3β,7α-diol; 8, cholest-5-ene-3β,7α,12α-triol. Solvent, ethyl acetate.

stant specific radioactivity (Table I). The labeled material in the zone corresponding to 5β-cholestane-3α,7α,12α-triol (3, Fig. 1 A) was crystallized together with authentic material (Table I). About 15 % of the labeled material could be accounted for as 5β-cholestane-3α,7α,12α-triol. The zone corresponding to 7α,12α-dihydroxycholest-4-en-3-one and cholest-5-ene-3β,7α-diol (4, 5, Fig. 1 A) contained about 0.5 % of the total radioactivity put on the chromatoplate. Rechromatography of this labeled material (Fig. 1 C) showed that about 20 % of the material had chromatographic properties of 7α,12α-dihydroxycholest-4-en-3-one (4, Fig. 1 C) and the remainder the properties of cholest-5-ene-3β,7α-diol (5, Fig. 1 C). Identification was established by crystallization to constant specific radioactivity with authentic material (Table I). The labeled material appearing in the zone corresponding to cholest-5-ene-3β,26-diol, 3β-hydroxycholest-5-en-7-one, and 5β-cholestane-3α,7α-diol (7, 8, and 9, Fig. 1 A) was rechromatographed (Fig. 1 D), and about 75 % of the labeled material could be identified as 3β-hydroxycholest-5-en-7-one (8, Fig. 1 D and Table I). The material in the zone just below that corresponding to cholesterol in the chromatogram shown in Fig. 1 A was rechromatographed with benzene–ethyl acetate (1:1, v/v) as solvent and with 7α-hydroxycholest-4-en-3-one and cholesterol as internal standards. Part of this labeled material could be identified as 7α-hydroxycholest-4-en-3-one (Table I). From these data is was calculated that about 0.1 % of added cholesterol had been converted into 7α-hydroxycholest-4-en-3-one.

Metabolism of cholest-5-ene-3β,7α-diol-7β-^{3}H. Cholest-5-ene-3β,7α-diol-7β-^{3}H was metabolized efficiently in the presence of 20,000 *g* supernatant fluid into compounds with chromatographic properties of 7α-hydroxycholest-4-en-3-one (6, Fig. 2 A) and 7α,12α-dihydroxycholest-4-en-3-one (2,

TABLE II

Identification of 7α-Hydroxycholest-4-en-3-one Formed from Cholest-5-ene-3β, 7α-diol-7β-^{3}H

Source of labeled compound	No. of crystallizations	Solvent	Weight	Specific activity
			mg	*cpm/mg* $\times 10^{-3}$
20,000 *g* supernatant	0		8.3	1.28
	1	Acetone-water	6.5	1.50
	2	Methanol-water	4.6	1.43
	3	Acetone-water	4.2	1.57
Microsomes+NAD$^+$	0		9.6	1.67
	1	Acetone-water	8.1	1.83
	2	Methanol-water	7.0	1.73
	3	Acetone-water	6.2	1.77

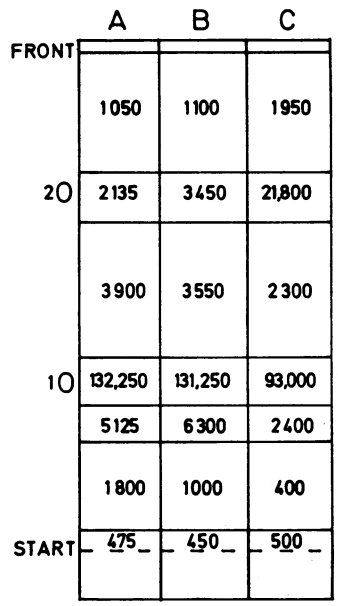

FIGURE 3 A, thin-layer chromatogram of chloroform extract of incubation of cholest-5-ene-3β,7α-diol-7β-^{3}H with microsomal fraction. 1 ml of microsomal fraction was diluted with 2 ml of homogenizing medium and was incubated with 40 μg of cholest-5-ene-3β,7α-diol-7β-^{3}H for 20 min. The numbers on the chromatogram represent counts per minute. Reference compounds were: 1, cholest-5-ene-3β,7α-diol; 2, 7α-hydroxycholest-4-en-3-one. Solvent, benzene–ethyl acetate (1:1, v/v). B, thin-layer chromatogram of chloroform extract of incubation of cholest-5-ene-3β,7α-diol-7β-^{3}H with microsomal fraction fortified with NADP$^+$. 1 ml of microsomal fraction was diluted with 2 ml of homogenizing medium, fortified with 0.3 μmoles of NADP$^+$ and was incubated for 20 min with 40 μg of cholest-5-ene-3β,7α-diol-7β-^{3}H. Reference compounds and chromatographic conditions were the same as in A. C, thin-layer chromatogram of chloroform extract of incubation of cholest-5-ene-3β,7α-diol-7β-^{3}H with microsomal fraction fortified with NAD$^+$. 1 ml of microsomal fraction was diluted with 2 ml of homogenizing medium, fortified with 0.3 μmoles of NAD$^+$ and was incubated for 20 min with 40 μg of cholest-5-ene-3β,7α-diol-7β-^{3}H. Reference compounds and chromatographic conditions were the same as in A.

Fig. 2B). The identity of the labeled 7α-hydroxycholest-4-en-3-one formed was established by crystallization to constant specific radioactivity with authentic material (Table II). In the presence of microsomal fraction, or microsomal fraction fortified by the addition of NADP$^+$, cholest-5-ene-3β,7α-diol-7β-^{3}H was metabolized only to a small extent (Fig. 3A and B). Addition of NAD$^+$ to the microsomal fraction resulted in a marked stimulation of the conversion of cholest-5-ene-3β,7α-diol into 7α-hydroxycholest-4-en-3-one (2, Fig. 3C and Table II). In one experiment, the microsomal fraction was fortified by the addition of NADPH resulting in the formation of small amounts of labeled material with the chromato-

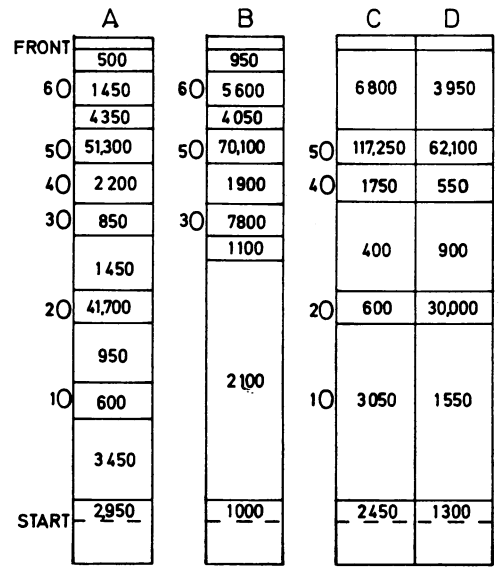

FIGURE 4 A, thin-layer chromatogram of chloroform extract of incubation of tritium-labeled 7α-hydroxycholest-4-en-3-one with 20,000 g supernatant fluid. 3 ml of 20,000 g supernatant fluid was incubated for 20 min with 40 μg of tritium-labeled 7α-hydroxycholest-4-en-3-one. The numbers on the chromatogram represent counts per minute. Reference compounds were: 1, 5β-cholestane-3α,7α,12α-triol; 2, 7α,12α-dihydroxycholest-4-en-3-one; 3, 5β-cholestane-3α,7α-diol; 4, 7α,12α-dihydroxy-5β-cholestan-3-one; 5, 7α-hydroxycholest-4-en-3-one; 6, 7α-hydroxy-5β-cholestane-3-one. Solvent, benzene–ethyl acetate (3:7, v/v). B, thin-layer chromatogram of chloroform extract of incubation of tritium-labeled 7α-hydroxycholest-4-en-3-one with 100,000 g supernatant fluid. 3 ml of 100,000 g supernatant fluid were incubated for 20 min with 40 μg of tritium-labeled 7α-hydroxycholest-4-en-3-one. Reference compounds and chromatographic conditions were the same as in A. C, thin-layer chromatogram of chloroform extract of incubation of tritium-labeled 7α-hydroxycholest-4-en-3-one with microsomal fraction. 3 ml of microsomal fraction was incubated for 10 min with 40 μg of tritium-labeled 7α-hydroxycholest-4-en-3-one. Reference compounds and chromatographic conditions were the same as in A. D, thin-layer chromatogram of chloroform extract of incubation of tritium-labeled 7α-hydroxycholest-4-en-3-one with microsomal fraction fortified with NADPH. 3 ml of microsomal fraction was fortified with 3 μmoles of NADPH and was incubated for 10 min with 40 μg of tritium-labeled 7α-hydroxycholest-4-en-3-one. Reference compounds and chromatographic conditions were the same as in A.

TABLE III

Identification of Products formed from Tritium-Labeled 7α-Hydroxycholest-4-en-3-one

Source of labeled compound	Compound	No. of crystallizations	Solvent	Weight	Specific activity
				mg	*cpm/mg* $\times 10^{-3}$
20,000 *g* supernatant	7α,12α-Dihydroxycholest-4-en-3-one	0		11.7	2.94
		1	Acetone-water	9.5	2.76
		2	Methanol-water	7.7	2.73
		3	Acetone-water	5.3	2.81
Microsomes + NADPH	7α,12α-Dihydroxycholest-4-en-3-one	0		11.2	1.22
		1	Acetone-water	10.0	1.06
		2	Methanol-water	7.0	1.07
		3	Acetone-water	3.3	1.05
100,000 *g* supernatant	7α-Hydroxy-5β-cholestan-3-one	0		9.9	0.21
		1	Acetone-water	8.5	0.21
		2	Methanol-water	6.9	0.24
		3	Acetone-water	5.4	0.21
100,000 *g* supernatant	5β-Cholestane-3α,7α-diol	0		12.6	0.41
		1	Acetone-water	11.8	0.39
		2	Acetone-water	4.7	0.36
		3	Acetone-water	3.2	0.35

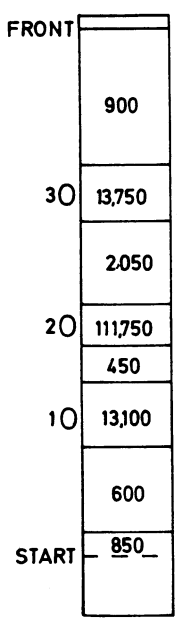

FIGURE 5 Thin-layer chromatogram of chloroform extract of incubation of tritium-labeled 7α,12α-dihydroxycholest-4-en-3-one with 100,000 *g* supernatant fluid. 3 ml of 100,000 *g* supernatant fluid were incubated for 20 min with 40 μg of tritium-labeled 7α,12α-dihydroxycholest-4-en-3-one. The numbers on the chromatogram represent counts per minute. Reference compounds were: 1, 5β-cholestane-3α,7α,12α-triol; 2, 7α,12α-dihydroxycholest-4-en-3-one; 3, 7α,12α-dihydroxy-5β-cholestan-3-one. Solvent, benzene–ethyl acetate (3:7, v/v).

graphic properties of cholest-5-ene-3β,7α,12α-triol (8, Fig. 2C).

Metabolism of tritium-labeled 7α-hydroxycholest-4-en-3-one. As shown in Fig. 4A, the main metabolite formed from tritium-labeled 7α-hydroxycholest-4-en-3-one in the presence of 20,000 *g* supernatant fluid was 7α-12α-dihydroxycholest-4-en-3-one (compound 2). The formation of this compound was also catalyzed efficiently by the microsomal fraction fortified by the addition of NADPH (2, Fig. 4C and D, and Table III). After incubation of tritium-labeled 7α-hydroxy-

TABLE IV

Identification of Products Formed from Tritium-Labeled 7α, 12α-Dihydroxycholest-4-en-3-one

	No. of crystallizations	Solvent	Weight	Specific activity
			mg	*cpm/mg* $\times 10^{-3}$
7α, 12α-Dihydroxy-5β-cholestan-3-one	0		11.5	0.53
	1	Acetone-water	8.7	0.43
	2	Methanol-water	7.6	0.43
	3	Acetone-water	6.0	0.45
5β-Cholestane-3α, 7α, 12α-triol	0		13.1	0.74
	1	Acetone-water	11.3	0.75
	2	Methanol-water	9.1	0.74
	3	Acetone-water	8.3	0.80

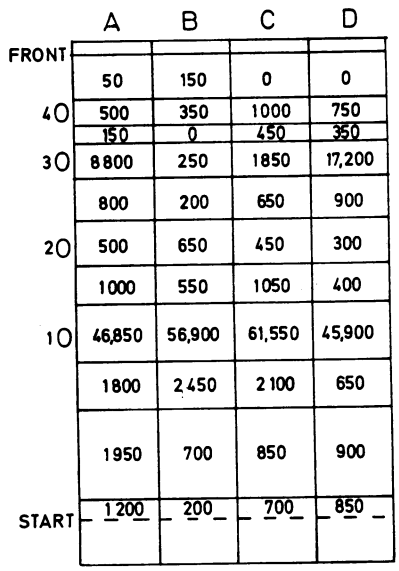

FIGURE 6 A, thin-layer chromatogram of chloroform extract of incubation of tritium-labeled cholest-5-ene-3β,-7α,12α-triol with 20,000 *g* supernatant fluid. 1 ml of 20,000 *g* supernatant fluid was diluted with 2 ml of homogenizing medium and was incubated for 20 min with 40 μg of tritium-labeled cholest-5-ene-3β,7α,12α-triol. The numbers on the chromatogram represent counts per minute. Reference compounds were: 1, cholest-5-ene-3β,7α,12α-triol; 2, 5β-cholestane-3α,7α,12α-triol; 3, 7α,12α-dihydroxycholest-4-en-3-one; 4, 7α,12α-dihydroxy-5β-cholestan-3-one. Solvent, ethyl acetate. B, thin-layer chromatogram of chloroform extract of incubation of tritium-labeled cholest-5-ene-3β,7α,12α-triol with microsomal fraction. 1 ml of microsomal fraction was diluted with 2 ml of homogenizing medium and was incubated for 20 min with 40 μg of tritium-labeled cholest-5-ene-3β,7α,12α-triol. Reference compounds and chromatographic conditions were the same as in A. C, thin-layer chromatogram of chloroform extract of incubation of tritium-labeled cholest-5-ene-3β,7α,12α-triol with microsomal fraction fortified with NADP$^+$. 1 ml of microsomal fraction was diluted with 2 ml of homogenizing medium, fortified with 0.3 μmoles of NADP$^+$ and was incubated for 20 min with 40 μg of tritium-labeled cholest-5-ene-3β,7α,12α-triol. Reference compounds and chromatographic conditions were the same as in A. D, thin-layer chromatogram of chloroform extract of incubation of tritium-labeled cholest-5-ene-3β,7α,12α-triol with microsomal fraction fortified with NAD$^+$. 1 ml of microsomal fraction was diluted with 2 ml of homogenizing medium, fortified with 0.3 μmoles of NAD$^+$ and incubated for 20 min with 40 μg of tritium-labeled cholest-5-ene-3β,7α,12α-triol. Reference compounds and chromatographic conditions were the same as in A.

cholest-4-en-3-one with the 100,000 *g* supernatant fluid the main products formed were 7α-hydroxy-5β-cholestan-3-one and 5β-cholestane-3α,7α-diol (6 and 3, Fig. 4 B and Table III).

TABLE V

Identification of 7α, 12α-Dihydroxycholest-4-en-3-one Formed from Tritium-Labeled Cholest-5-ene-3β, 7α, 12α-triol

Source of labeled compound	No. of crystallizations	Solvent	Weight	Specific activity
			mg	*cpm/mg* $\times 10^{-3}$
20,000 *g* supernatant	0		13.7	0.55
	1	Methanol-water	10.7	0.52
	2	Acetone-water	8.6	0.51
	3	Methanol-water	6.5	0.47
Microsomes+NAD$^+$	0		14.2	0.68
	1	Acetone-water	8.5	0.75
	2	Methanol-water	7.2	0.65
	3	Acetone-water	6.5	0.77

Metabolism of tritium-labeled 7α,12α-dihydroxycholest-4-en-3-one. Incubation of tritium-labeled 7α,12α-dihydroxycholest-4-en-3-one with the 100,000 *g* supernatant fluid yielded two main products, 7α,12α-dihydroxy-5β-cholestan-3-one and 5β-cholestane-3α,7α,12α-triol (3 and 1, Fig. 5 and Table IV).

Metabolism of tritium-labeled cholest-5-ene-3β, 7α,12α-triol. Upon incubation of tritium-labeled cholest-5-ene-3β,7α,12α-triol with the 20,000 *g* supernatant fluid, the added substrate was converted efficiently into 7α,12α-dihydroxycholest-4-en-3-one (3, Fig. 6 A and Table V). The substrate was also incubated with the microsomal fraction alone and with the microsomal fraction fortified by the addition of NADP$^+$ or NAD$^+$. Without any addition to the microsomal fraction, no conversion of the substrate was observed (Fig. 6 B), and the addition of NADP$^+$ had only a slight stimulatory effect (Fig. 6 C), whereas the addition of NAD$^+$ resulted in an efficient conversion of the substrate into 7α,12α-dihydroxycholest-4-en-3-one (3, Fig 6 D and Table V).

DISCUSSION

The major pathways for the conversion of cholesterol into cholic acid and chenodeoxycholic acid in the rat have now been elucidated (1). Fig. 7 summarizes the main sequences of reactions in the biosynthesis of cholic acid and chenodeoxycholic acid. Present information indicates the existence of several pathways for the formation of cholic acid as well as chenodeoxycholic acid (1), but the quantitative importance of the various pathways

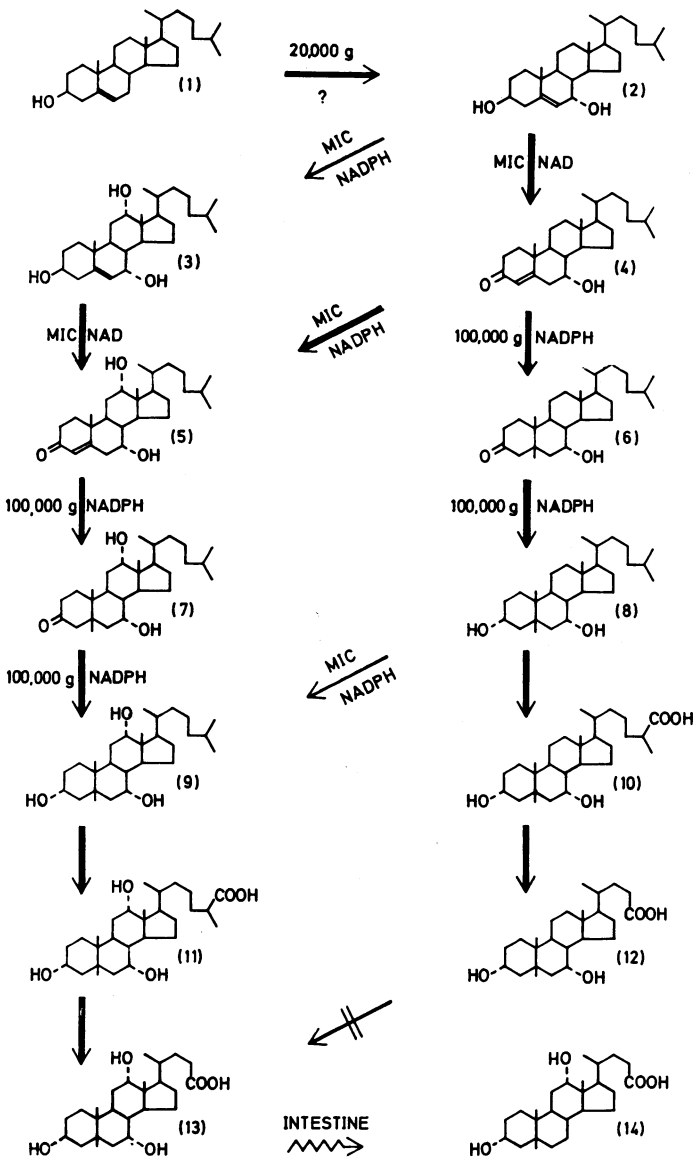

FIGURE 7 The biological formation of cholic acid, chenodeoxycholic acid, and deoxycholic acid from cholesterol. Abbreviations: 20,000 *g*, 20,000 *g* supernatant fluid; *mic*, microsomal fraction. Compounds: 1, cholesterol; 2, cholest-5-ene-3β,7α-diol; 3, cholest-5-ene-3β,7α,12α-triol; 4, 7α-hydroxycholest-4-en-3-one; 5, 7α,12α-dihydroxycholest-4-en-3-one; 6, 7α-hydroxy-5β-cholestan-3-one; 7, 7α,12α-dihydroxy-5β-cholestane-3-one; 8, 5β-cholestane-3α,7α-diol; 9, 5β-cholestane-3α,7α,-12α-triol; 10, 3α,7α-dihydroxy-5β-cholestanoic acid; 11, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; 12, chenodeoxycholic acid; 13, cholic acid; 14, deoxycholic acid.

has not been finally established. Many of the individual reactions have been studied in detail in the rat, and some of the enzymes catalyzing these reactions have been partially characterized. The aim of the present investigation was to study in detail the conversion of cholesterol into 5β-cholestane-3α,7α,12α-triol in man in order to provide a basis for further studies of factors influencing the formation of bile acids in man. It was thus considered of importance to identify as many of the intermediary products as possible and to obtain information on the subcellular localization of the enzymes catalyzing the reactions. The results obtained show that the sequence of reactions in the conversion of cholesterol into 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α-diol is the same in homogenates of human liver as in rat liver homogenates (6, 7, 9–14). The subcellular distribution as well as the cofactor requirements of the enzymes catalyzing these reactions were also found to be the same. (See Fig. 7.)

In the presence of 20,000 *g* supernatant fluid of homogenates of human liver, cholesterol (1, Fig. 7) was converted into cholest-5-ene-3β,7α-diol (2, Fig. 7), 7α-hydroxycholest-4-en-3-one (4, Fig. 7), 7α,12α-dihydroxycholest-4-en-3-one (5, Fig. 7),

and 5β-cholestane-3α,7α,12α-triol (9, Fig. 7). In addition, small amounts of cholestane-3β,5α,6β-triol and 3β-hydroxycholest-5-en-7-one were formed. These compounds are well known autoxidation products of cholesterol (15), but at least 3β-hydroxycholest-5-en-7-one can be formed also by enzymatic action on cholesterol (16). The experiments do not permit an evaluation of the extent of enzymatic formation of 3β-hydroxycholest-5-en-7-one. This compound is not an intermediate in the biosynthesis of cholic acid or chenodeoxycholic acid (16), and its metabolic role has not been established. Evidence has been obtained to indicate that the enzymatic formation of 3β-hydroxycholest-5-en-7-one increases concomitant with a decrease in the formation of cholest-5-ene-3β,7α-diol (16). The enzyme catalyzing the 7α-hydroxylation of cholesterol has been found to be easily inactivated (16), and it is possible that the anaesthesia of the patients as well as the time lag in the preparation of the homogenates might have had an influence on this enzyme system.

The further metabolism of cholest-5- ene-3β,7α-diol, 7α-hydroxycholest-4-en-3-one and 7α,12α-dihydroxycholest-4-en-3-one in homogenates of human liver was completely analogous to the metabolism of these compounds in rat liver homogenates (6, 7, 12–14). Cholest-5-ene-3β,7α-diol (2, Fig. 7) was readily converted into 7α-hydroxycholest-4-en-3-one (4, Fig. 7) in the presence of microsomal fraction fortified with NAD^+. In the presence of microsomal fraction fortified with NADPH cholest-5-ene-3β,7α-diol (2, Fig. 7) was converted only to a limited extent into cholest-5-ene-3β,7α,12α-triol (3, Fig. 7). This contrasts with the efficient 12α-hydroxylation of 7α-hydroxycholest-4-en-3-one (4, Fig. 7) in the same system indicating that this compound is the major substrate for the 12α-hydroxylase in cholic acid formation in man as well as in the rat (6). Soluble enzymes were found to catalyze the conversion of 7α-hydroxycholest-4-en-3-one (4, Fig. 7) into 5β-cholestane-3α,7α-diol (8, Fig. 7) and of 7α,12α-dihydroxycholest-4-en-3-one (5, Fig. 7) into 5β-cholestane-3α,7α,12α-triol (9, Fig. 7) by means of the intermediary formation of the corresponding 3-keto-5β-steroids. It might be added that these reactions occurred only to a limited extent in incubations with the 20,000 *g* supernatant fluid. Evidence has been obtained to indicate an inhibition by the microsomal fraction of the Δ^4-3-keto-steroid 5β-reductase(s) catalyzing the saturation of the Δ^4 double bond in 7α-hydroxycholest-4-en-3-one and 7α,12α-dihydroxycholest-4-en-3-one (17). It is possible that a similar inhibition occurs also in homogenates of human liver.

No studies of the further metabolism of 5β-cholestane-3α,7α-diol (8, Fig. 7) and 5β-cholestane-3α,7α,12α-triol (9, Fig. 7) were performed in the present investigation. As shown in Fig. 7, these compounds are in the rat the substrates for the enzyme(s) catalyzing the 26-hydroxylation, the initial reaction in the degradation of the C_{27} side chain in bile acid formation (1). It is probable that 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol are metabolized in the same way in man.

ACKNOWLEDGMENTS

The authors wish to express their sincere gratitude to Dr. Olof Johansson, Department of Surgery I, Södersjukhuset, Stockholm, for generous help in supplying the liver specimens. The skillful technical assistance of Miss Madelaine Ekebrand and Miss Britt-Marie Gravander is gratefully acknowledged.

This work is part of investigations supported by the Swedish Medical Research Council (Project 13X-218) and by the Loo and Hans Ostermans Foundation.

REFERENCES

1. Danielsson, H., and T. T. Tchen. 1968. Steroid metabolism. *In* Metabolic Pathways. D. M. Greenberg, editor. Academic Press Inc., New York, 3rd edition. **2**: 117.
2. Staple, E., and J. L. Rabinowitz. 1962. Formation of trihydroxycoprostanic acid from cholesterol in man. *Biochim. Biophys. Acta.* **59**: 735.
3. Carey, J. B., Jr. 1964. Conversion of cholesterol to trihydroxycoprostanic acid and cholic acid in man. *J. Clin. Invest.* **43**: 1443.
4. Rabinowitz, J. L., R. H. Herman, D. Weinstein, and E. Staple. 1966. Isolation of 3α,7α-dihydroxycoprostane derived from cholesterol in human bile. *Arch. Biochem. Biophys.* **114**: 233.
5. Bergström, S., and U. Gloor. 1955. Studies on the 7α-hydroxylation of taurodeoxycholic acid in rat liver homogenates. *Acta Chem. Scand.* **9**: 34.
6. Danielsson, H., and K. Einarsson. 1966. On the conversion of cholesterol to 7α,12α-dihydroxycholest-4-en-3-one. Bile acids and steroids 168. *J. Biol. Chem.* **241**: 1449.
7. Berséus, O., H. Danielsson, and A. Kallner. 1965. Synthesis and metabolism of cholest-4-ene-7α,12α-diol-3-one and 5β-cholestane-7α,12α-diol-3-one. *J. Biol. Chem.* **240**: 2396.

8. Björkhem, I., H. Danielsson, C. Issidorides, and A. Kallner. 1965. On the synthesis and metabolism of cholest-4-en-7α-ol-3-one. Bile acids and steroids 156. *Acta Chem. Scand.* **19**: 2151.
9. Mendelsohn, D., and E. Staple. 1963. The *in vitro* catabolism of cholesterol: formation of 3α,7α,12α-trihydroxycoprostane from cholesterol in rat liver. *Biochemistry.* **2**: 577.
10. Mendelsohn, D., L. Mendelsohn, and E. Staple. 1965. The *in vitro* catabolism of cholesterol: formation of 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,12α-diol from cholesterol in rat liver. *Biochemistry.* **4**: 441.
11. Mendelsohn, D., L. Mendelsohn, and E. Staple. 1966. The *in vitro* catabolism of cholesterol: a comparison of the formation of cholest-4-en-7α-ol-3-one and 5β-cholestan-7α-ol-3-one from cholesterol in rat liver. *Biochemistry.* **5**: 1286.
12. Hutton, H. R. B., and G. S. Boyd. 1966. The metabolism of cholest-5-en-3β,7α-diol by rat-liver cell fractions. *Biochim. Biophys. Acta.* **116**: 336.
13. Berséus, O., and K. Einarsson. 1967. On the conversion of cholest-5-ene-3β,7α-diol to 7α-hydroxycholest-4-en-3-one in rat liver homogenates. Bile acids and steroids 186. Acta Chem. Scand. **21**: 1105.
14. Berséus, O., H. Danielsson, and K. Einarsson. 1967. Synthesis and metabolism of cholest-5-ene-3β,7α,12α-triol. Bile acids and steroids 169. *J. Biol. Chem.* **242**: 1211.
15. Danielsson, H. 1960. On the oxidation of cholesterol in liver mitochondrial preparations. *Acta Chem. Scand.* **14**: 846.
16. Björkhem, I., K. Einarsson, and G. Johansson. Formation and metabolism of 3β-hydroxycholest-5-en-7-one and cholest-5-ene-3β-7β-diol. *Acta Chem. Scand.* In press.
17. Björkhem, I., H. Danielsson, and K. Einarsson. 1967. On the conversion of cholesterol to 5β-cholestane-3α,7α-diol in guinea pig liver homogenates. *European J. Biochem.* **2**: 294.