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On the Conversion of Cholestanol into Allocholic Acid in Rat Liver

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The early steps in the conversion of cholestanol into allocholic acid were studied in homogenates of rat liver. The $20000 \times g$ supernatant fluid was found to catalyze 7α-hydroxylation of cholestanol. Under the same conditions 7α-hydroxylation of 5α-cholestan-3α-ol and 5α-cholestan-3-one was insignificant. The microsomal fraction fortified with NAD catalyzed oxidation of 5α-cholestane-3β,7α-diol into 7α-hydroxy-5α-cholestan-3-one. The $100000 \times g$ supernatant fluid catalyzed reduction of 7α-hydroxy-5α-cholestan-3-one into 5α-cholestane-3α,7α-diol. The same reaction was effected by a partially purified 3α-hydroxysteroid dehydrogenase from rat liver in the presence of NADPH. The microsomal fraction fortified with NADPH catalyzed 12α-hydroxylation of 5α-cholestane-3α,7α-diol at a rate about six times faster than that with either 5α-cholestane-3β,7α-diol or 7α-hydroxy-5α-cholestan-3-one. In rats with a biliary fistula 5α-cholestane-3β,7α-diol and 5α-cholestane-3α,7α-diol were converted into allocholic acid. The results indicate that the main pathway for the conversion of cholestanol into allocholic acid involves the following steps: cholestanol → 5α-cholestane-3β,7α-diol → 7α-hydroxy-5α-cholestan-3-one → 5α-cholestane-3α,7α-diol → 5α-cholestane-3α,7α,12α-triol → allocholic acid.

5α-Bile acids are major bile acids in some lower species and may occur in small amounts in mammals [1,2]. 5β-Bile acids, cholesterol and cholestanol have been shown to be precursors of 5α-bile acids in mammals. The conversion of 5β-bile acids into 5α-bile acids is effected by intestinal microorganisms in reactions probably involving Δ^4-3-oxosteroids as intermediates [3]. The conversion of cholesterol into 5β-bile acids in the rat involves the intermediary formation of Δ^4-3-oxosteroids. These Δ^4-3-oxosteroids, *i.e.* 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one have been shown to be converted to some extent into the corresponding 3-oxo-5α-steroids in rat liver microsomes fortified with NADPH [4]. These 3-oxo-5α-steroids are converted into allocholic acid in rats with a biliary fistula [4]. A similar pathway from cholesterol to allocholic acid has been shown in the lizard, *Iguana iguana* [2], a species having allocholic acid as the main bile acid. Karavolas *et al.* [5] and Ziller, Doisy and Elliot [6] have shown that cholestanol is converted into allochenodeoxycholic acid and allocholic acid in rats with a biliary fistula. Hofmann and Mosbach [7] and Hofmann *et al.* [8] have found that also in the rabbit cholestanol is converted into allocholic acid. The sequence of reactions in the conversion of cholestanol into allocholic acid is largely unknown. Shefer, Hauser and Mosbach [9] have shown that cholestanol is 7α-hydroxylated by rat liver microsomes fortified with NADPH and have suggested that this reaction is the first step in the conversion of cholestanol into allocholic acid.

The present work deals with the sequence of nuclear changes in the conversion of cholestanol into allocholic acid in the rat. The specificity of the 7α- and 12α-hydroxylating systems has been tested with different 5α-C_{27}-steroids and the metabolism of 5α-cholestane-3β,7α-diol has been studied *in vitro* and *in vivo*.

Nomenclature. The following systematic names are given to compounds referred to by trivial names: cholestanol, 5α-cholestan-3β-ol; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; allocholic acid, 3α,7α,12α-trihydroxy-5α-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; allochenodeoxycholic acid, 3α,7α-dihydroxy-5α-cholanoic acid.

EXPERIMENTAL PROCEDURE

MATERIALS

[4-^{14}C]Cholestanol, 5α-[4-^{14}C]Cholestan-3α-ol and 5α-[4-^{14}C]Cholestan-3-one

[4-^{14}C]Cholestanol was prepared from [4-^{14}C]-cholesterol (Radiochemical Centre, Amersham, England) by reduction with hydrogen gas in acetic acid using platinum dioxide as catalyst. The [4-^{14}C]-cholestanol obtained was purified by means of thin-layer chromatography with benzene—ethyl acetate (1:1, v/v), as solvent, and was diluted with unlabeled cholestanol and crystallized twice. The specific radioactivity was 2.0×10^6 counts $\times$ min^{-1} $\times$ mg^{-1}. 5α-[4-^{14}C]Cholestan-3-one was prepared from

[4-^{14}C]cholestanol by oxidation with chromic acid under the conditions described in a previous communication [10] and the product was purified by thin-layer chromatography with benzene—ethyl acetate (9:1, v/v), as solvent. The specific radioactivity was 2.0×10^6 counts $\times$ min^{-1} $\times$ mg^{-1}. 5α-[4-^{14}C]Cholestan-3α-ol was prepared from 5α-[4-^{14}C]-cholestan-3-one by reduction with iridium(IV)-chloride [11]. 5α-[4-^{14}C]Cholestan-3-one, 5 mg, was refluxed with 1 mg of iridium(IV)chloride and 0.25 ml of trimethylphosphite in 2.5 ml of isopropanol and 0.25 ml of water for 48 h. The product was extracted with ether from the acidified solution and was purified by thin-layer chromatography with benzene—ethyl acetate (9:1, v/v), as solvent. The yield was 3.2 mg and the specific radioactivity was 2.0×10^6 counts $\times$ min^{-1} $\times$ mg^{-1}.

The [4-^{14}C]cholestanol, 5α-[4-^{14}C]cholestan-3-one and 5α-[4-^{14}C]cholestan-3α-ol prepared were homogeneous in thin-layer chromatography and in radiogas chromatography and had properties identical with the corresponding authentic compounds.

Unlabeled and 6β-^{3}H-Labeled 5α-Cholestane-3β,7α-diol, 7α-Hydroxy-5α-cholestan-3-one and 5α-Cholestane-3α,7α-diol

Unlabeled and 6β-^{3}H-labeled 5α-cholestane-3β,-7α-diol and 7α-hydroxy-5α-cholestan-3-one were prepared from unlabeled and 6β-^{3}H-labeled 7α-hydroxy-4-cholesten-3-one [10] by reduction with Li in liquid ammonia [4]. The specific radioactivity of 5α-[6β-^{3}H]cholestane-3β,7α-diol was 6.0×10^6 counts $\times$ min^{-1} $\times$ mg^{-1} and that of 7α-hydroxy-5α-[6β-^{3}H]-cholestan-3-one 3.6×10^6 counts $\times$ min^{-1} $\times$ mg^{-1}.

5α-Cholestane-3α,7α-diol was prepared from 7α-hydroxy-5α-cholestan-3-one by reduction with iridium(IV) chloride as described above with the exception that the reaction mixture was refluxed for 24 h. The 5α-cholestane-3α,7α-diol obtained was purified by chromatography on a column of aluminium oxide, grade III (Woelm, Eschwege, West-Germany). Benzene—ethyl acetate (2:3, v/v), eluted pure material which after crystallization twice from an acetone—water mixture had m.p. 159—160°. The trimethylsilyl ether of the compound was analyzed by combined gas chromatography-mass spectrometry using the LKB 9000 instrument equipped with a 1% SE-30 column. The mass spectrum was almost identical with those of the trimethylsilyl ethers of 5α-cholestane-3β,7α-diol and 5β-cholestane-3α,7α-diol. Prominent peaks were seen at M-90, M-(90 + 15), M-180, and M-(180 + 15). The fragment corresponding to M-15 was less prominent in the mass spectrum of the trimethylsilyl ether of 5β-cholestane-3α,7α-diol than in that of the 3β-epimer. Such a difference between 3-hydroxy epimers has been observed for some other 5α-steroids [12]. The retention time on an SE-30 column of the trimethylsilyl ether of 5α-cholestane-3α,7α-diol relative to the corresponding 3β-epimer was 0.93. A similar difference in retention time has been found for 3-hydroxy epimers of other 5α-steroids [12]. These properties as well as the method of synthesis [11] establish fully the stereochemistry at C-3 of the 5α-cholestane-3α,7α-diol prepared.

5α-[6β-^{3}H]Cholestane-3α,7α-diol was prepared from 7α-hydroxy-5α-[6β-^{3}H]cholestan-3-one as described above and had a specific radioactivity of 6.0×10^6 counts $\times$ min^{-1} $\times$ mg^{-1}.

7α,12α-Dihydroxy-5α-cholestan-3-one, 5α-Cholestane-3β,7α,12α-triol and 5α-Cholestane-3α,7α,12α-triol

7α,12α-Dihydroxy-4-cholesten-3-one was reduced with Li in liquid ammonia [4]. The reduction mixture was chromatographed on a column of aluminium oxide, grade IV. Elution with benzene—ethyl acetate (3:2, v/v), yielded pure 7α,12α-dihydroxy-5α-cholestan-3-one. Crystallization twice from a methanol—water mixture yielded material with m.p. 192—193°, reported [2] m.p. 190—192° and [4] 194°. Further elution of the column with methanol yielded almost pure 5α-cholestane-3β,7α,12α-triol. Crystallization twice from a methanol—water mixture yielded material with m.p. 208—210°. The mass spectrum of the trimethylsilyl ether of this material was almost identical with those of the trimethylsilyl ethers of 5α-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α,12α-triol. Prominent peaks were seen at M-90, M-(90 + 15), M-180, M-(18 + 15), M-270, and M-(270 + 15). There was no significant difference in the intensity of the fragment corresponding to M-15 between the trimethylsilyl ethers of 5α-cholestane-3β,7α,12α-triol and 5α-cholestane-3α,7α,12α-triol. The expected difference in retention times on an SE-30 column between the two compounds was observed. This finding and the method of synthesis [11] provide sufficient evidence for the stereochemistry at C-3 of the 5α-cholestane-3β,7α,12α-triol prepared.

5α-Cholestane-3α,7α-12α-triol was prepared from 7α,12α-dihydroxy-5α-cholestan-3-one by reduction with iridium(IV)cholride as described above for synthesis of 5α-cholestane-3α,7α-diol. 5α-Cholestane-3α,7α,12α-triol was purified by means of thin-layer chromatography with benzene—ethyl acetate (4:1, v/v), as solvent. Crystallization from ethyl acetate yielded material with m.p. 249—251°, reported [2] m.p. 251°.

METHODS

Experiments with Rat Liver Homogenates

Male rats of the Sprague-Dawley strain weighing about 200 g were used. Homogenates of liver, 20% (w/v), were prepared in a modified Bucher medium

[13] and fractionated as described in previous communications [4,10]. In studies of 7α-hydroxylation, livers from rats with a biliary fistula were used since 7α-hydroxylation of cholestanol as well as of cholesterol is stimulated by biliary drainage [9,14]. In experiments concerning 7α-hydroxylation, 100 μg of labeled cholestanol, 5α-cholestan-3-one or 5α-cholestan-3α-ol, dissolved in 50 μl of acetone, were incubated for 1 h at 37° with 3 ml of 20000×*g* supernatant fluid fortified with 5 μmoles of NADPH. Incubations were terminated and extracted with chloroform—methanol (2:1, v/v), and subjected to thin-layer chromatography as described previously for incubations with cholesterol [14]. Cholestanol, 5α-cholestan-3-one, 5α-cholestan-3α-ol and the corresponding 7α-hydroxylated compounds were used as internal standards. The different chromatographic zones were detected and assayed for radioactivity as described in previous communications [4,10,14]. In experiments concerning oxidation of 5α-[6β-^{3}H]-cholestane-3β,7α-diol and reduction of 7α-hydroxy-5α-[6β-^{3}H]cholestan-3-one, 50 μg of the steroid dissolved in 50 μl of acetone were incubated for 20 min at 37° with 3 ml of microsomal fraction or 100000×*g* supernatant fluid or 3 ml of a solution containing 200-fold purified 3α-hydroxysteroid dehydrogenase [15]. When added, 1 μmole of NAD, NADP, NADH or NADPH was used. Incubations were terminated and extracted with chloroform—methanol (2:1, v/v). Extracts of incubations with 5α-[6β-^{3}H]cholestane-3β,7α-diol were analyzed by means of thin-layer chromatography with benzene—ethyl acetate (1:1, v/v), as solvent and 5α-cholestane-3β,7α-diol and 7α-hydroxy-5α-cholestan-3-one as internal standards. Extracts from incubations with 7α-hydroxy-5α-[6β-^{3}H]cholestan-3-one were analyzed by thin-layer chromatography with ethyl acetate—trimethylpentane—acetic acid (10:10:1, v/v/v), as solvent and 7α-hydroxy-5α-cholestan-3-one, 5α-cholestane-3β,7α-diol and 5α-cholestane-3α,7α-diol as internal standards. In experiments concerning 12α-hydroxylation, 50 μg of 7α-hydroxy-5α-[6β-^{3}H]-cholestan-3-one, 5α-[6β-^{3}H]cholestane-3β,7α-diol or 5α-[6β-^{3}H]cholestane-3α,7α-diol, dissolved in 50 μl of acetone, were incubated for 20 min at 37° with 3 ml of microsomal fraction fortified with 3 μmoles of NADPH. Incubations were terminated and extracted with chloroform—methanol (2:1, v/v). Thin-layer chromatography was performed with ethyl acetate as solvent in the case of incubations with 5α-[6β-^{3}H]cholestane-3β,7α-diol and 5α-[6β-^{3}H]-cholestane-3α,7α-diol and with ethyl acetate—benzene (4:1, v/v), in the case of incubations with 7α-hydroxy-5α-[6β-^{3}H]cholestan-3-one. Unlabeled 5α-cholestane-3β,7α-diol, 5α-cholestane-3α,7α-diol, 7α-hydroxy-5α-cholestan-3-one and the corresponding 12α-hydroxylated compounds were used as internal standards.

Experiments with Rats with a Biliary Fistula

Male rats of the same strain and size as above were used. The labeled compounds were administered intraperitoneally in an emulsion stabilized with serum albumin. Bile was collected in 24-h portions and hydrolyzed with 1 M sodium hydroxide in 50% (v/v) aqueous ethanol in sealed steel tubes at 110° for 12 h. The hydrolyzed mixture was acidified with hydrochloric acid and extracted with ether. The residue of the ether extract was chromatographed on a column of hydrophobic Hyflo Super-Cel with phase system C1 [16]. The trihydroxycholanoic acid fraction was methylated and the cholic- and allocholic-acid fractions were separated by means of thin-layer chromatography in system S6 [4,17]. The labeled methyl allocholate was further identified by means of radio-gas chromatography using a 1% QF-1 column.

Radioactivity Assay

Radioactivity was determined with a methane gas flow counter having an efficiency for ^{3}H of 26% and for ^{14}C of 43%.

RESULTS

Specificity of 7α-Hydroxylase

In incubations of [4-^{14}C]cholestanol with the 20000×*g* supernatant fluid of homogenate of liver from rat with a biliary fistula, 4—7% was converted into a product with properties of 5α-cholestane-3β,7α-diol in thin-layer chromatography and radio-gas chromatography. The identity was further established by crystallization to constant specific radioactivity after addition of the authentic compound (Table 1). In incubations of 5α-[4-^{14}C]cholestan-3-one or 5α-[4-^{14}C]cholestan-3α-ol, the conversion into products with the thin-layer chromatographic properties of the corresponding 7α-hydroxylated compound was less than 1%. From an analysis of pooled material by radio-gas chromatography it was calculated that less than 0.7% of 5α-cholestan-

Table 1. *Identification of 5α-cholestane-3β,7α-diol*
5α-Cholestane-3β,7α-diol was isolated by means of thin-layer chromatography from extracts of incubations of 5α-[4-^{14}C]cholestan-3β-ol with the 20000×*g* supernatant fluid

Solvent	Number of crystallizations	Weight	Specific radioactivity
		mg	counts × min^{-1} × mg^{-1}
None	0	9.1	1150
Methanol—water	1	6.2	960
Methanol—water	2	4.4	950
Methanol—water	3	3.0	880
Methanol—water	4	2.3	1000

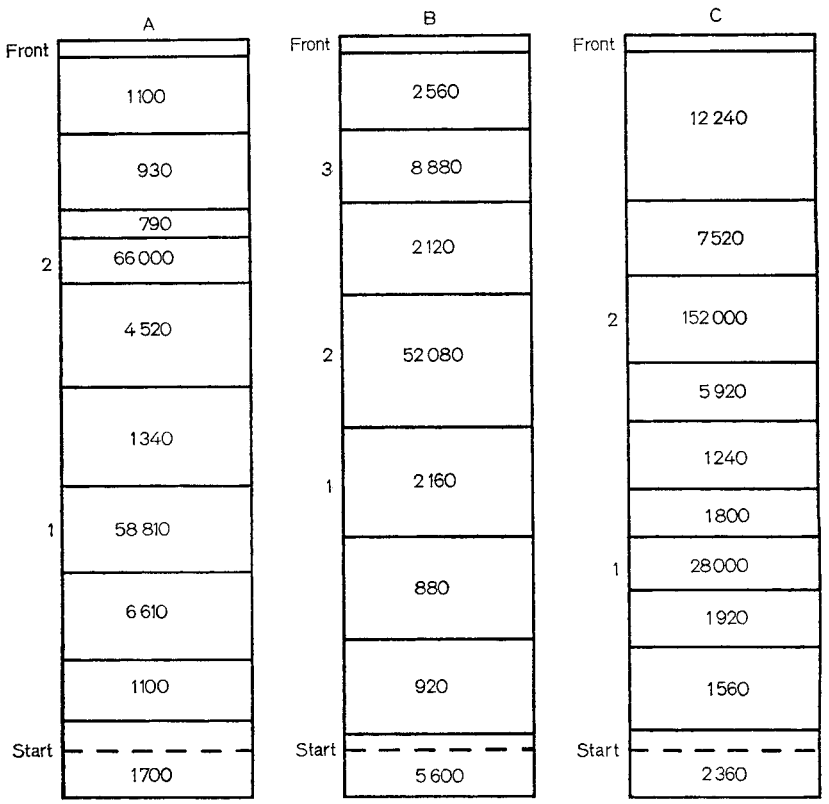

Fig. 1. *Thin-layer chromatogram of extract of incubation of (A) 5α-[6β-^{3}H]cholestane-3β,7α-diol with the microsomal fraction fortified with NAD, (B) 7α-hydroxy-5α-[6β-^{3}H]cholestan-3-one with the 100000×g supernatant fluid and (C) 5α-[6β-^{3}H]cholestane-3α,7α-diol with the microsomal fraction fortified with NADPH.* The numbers on the chromatograms represent counts/min. The reference compounds were in (A): (1) 5α-cholestane-3β,7α-diol; (2) 7α-hydroxy-5α-cholestan-3-one; in (B): (1) 5α-cholestane-3β,7α-diol; (2) 5α-cholestane-3α,7α-diol; (3) 7α-hydroxy-5α-cholestan-3-one; in (C): (1) 5α-cholestane-3α,7α,12α-triol; (2) 5α-cholestane-3α,7α-diol. The solvent systems were in (A) benzene—ethyl acetate (1:1, v/v), in (B) ethyl acetate—trimethylpentane—acetic acid (10:10:1, v/v/v) and in (C) ethyl acetate

3-one and less than 0.3% of 5α-cholestan-3α-ol had been 7α-hydroxylated. The extent of 7α-hydroxylation of cholestanol was somewhat lower in incubations with microsomal fraction according to Shefer, Hauser and Mosbach [9]. Also in this case, there was insignificant 7α-hydroxylation of 5α-cholestan-3-one and 5α-cholestan-3α-ol. No significant conversion into compounds with chromatographic properties of the corresponding 12α-hydroxylated compounds was detected in any of the incubations.

Epimerization of 3β-Hydroxyl Group in 5α-Cholestane-3β,7α-diol

Incubation of [6β-^{3}H]cholestane-3β,7α-diol with the microsomal fraction fortified with NAD resulted in efficient conversion into a product with the thin-layer chromatographic properties of 7α-hydroxy-5α-cholestan-3-one (Fig. 1 A). The identity of the material was established by crystallization to constant specific radioactivity (Table 2). The conversion was reduced to about one tenth when NAD was substituted with NADP.

Table 2. *Identification of 7α-hydroxy-5α-cholestan-3-one*
7α-Hydroxy-5α-cholestan-3-one was isolated by means of thin-layer chromatography (*cf.* Fig. 1) from extracts of incubations of 5α-[6β-^{3}H]cholestane-3β,7α-diol with the microsomal fraction fortified with NAD

Solvent	Number of crystallizations	Weight	Specific radioactivity
		mg	counts × min^{-1} × mg^{-1}
None	0	10.2	6500
Acetone—water	1	9.8	6600
Acetone—water	2	6.4	6100
Acetone—water	3	5.9	5500
Acetone—water	4	4.2	6800

Incubation of 7α-hydroxy-5α-[6β-^{3}H]cholestan-3-one with the 100000×*g* supernatant fluid resulted in efficient conversion into 5α-cholestane-3α,7α-diol (Fig. 1 B and Table 3). The same reaction was

Table 3. *Identification of 5α-cholestane-3α,7α-diol*
5α-Cholestane-3α,7α-diol was isolated by means of thin-layer chromatography (*cf.* Fig. 2) from extracts of incubations of 7α-hydroxy-5α-[6β-^{3}H]cholestan-3-one with the 100000×*g* supernatant fluid

Solvent	Number of crystallizations	Weight	Specific radioactivity
		mg	counts×min^{-1}×mg^{-1}
None	0	5.3	9400
Acetone—water	1	4.3	10700
Acetone—water	2	3.6	8900
Acetone—water	3	2.7	9500

Table 4. *Identification of 5α-cholestane-3α,7α,12α-triol*
5α-Cholestane-3α,7α,12α-triol was isolated by means of thin-layer chromatography (*cf.* Fig. 3) from extracts of incubations of 5α-[6β-^{3}H]cholestane-3α,7α-diol with the microsomal fraction fortified with NADPH

Solvent	Number of crystallizations	Weight	Specific radioactivity
		mg	counts×min^{-1}×mg^{-1}
None	0	6.9	3800
Ethyl acetate	1	5.0	3900
Ethyl acetate	2	2.9	3300
Ethyl acetate	3	1.9	3600

catalyzed by a partially purified 3α-hydroxysteroid dehydrogenase [15] fortified with NADPH. NADH could not substitute for NADPH in this reaction.

Specificity of 12α-Hydroxylase

After incubation of 5α-[6β-^{3}H]cholestane-3α,7α-diol with the microsomal fraction fortified with NADPH, about 13% of the material was converted into 5α-cholestane-3α,7α,12α-triol (Fig. 1C, Table 4). NADH could not substitute for NADPH in the reaction. After incubation of 7α-hydroxy-5α-[6β-^{3}H]-cholestan-3-one with the microsomal fraction fortified with NADPH, about 7% was converted into material with thin-layer chromatographic properties similar to those of 7α,12α-dihydroxy-5α-cholestan-3-one. However, with the solvent system used, 7α,12α-dihydroxy-5α-cholestan-3-one does not separate from 5α-cholestane-3β,7α-diol which is also formed from 7α-hydroxy-5α-cholestan-3-one under these conditions [4]. After crystallization of the material with authentic 7α,12α-dihydroxy-5α-cholestan-3-one, the specific radioactivity decreased to about one fourth of the initial value after four crystallizations. It was calculated that less than 2% of 7α-hydroxy-5α-[6β-^{3}H]cholestan-3-one had been 12α-hydroxylated. After incubation of 5α-[6β-^{3}H]-cholestane-3β,7α-diol with the microsomal fraction fortified with NADPH, about 5% was converted into a product with the thin-layer chromatographic properties of 5α-cholestane-3β,7α,12α-triol. After crystallization of the material with authentic 5α-cholestane-3β,7α,12α-triol, the specific radioactivity decreased to about one sixth of the initial value. It was calculated that less than 1% of 5α-cholestane-3β,7α-diol had been converted into the 12α-hydroxy derivative.

Conversion of 5α-Cholestane-3β-7α-diol and 5α-Cholestane-3α,7α-diol into Allocholic Acid

After administration of 5α-[6β-^{3}H]cholestane-3β,7α-diol, 0.3 mg, to a rat with a biliary fistula, 11% of the administered dose was excreted in the first 24-hour portion of bile. The hydrolyzed bile was chromatographed with phase system C1 and about 80% of the total radioactivity was found in the trihydroxycholanoic acid fraction. The main part of this radioactivity had the thin-layer chromatographic properties of allocholic acid. The identity of the material was further established by radio-gas chromatography.

After administration of 0.2 mg 5α-[6β-^{3}H]cholestane-3α,7α-diol to a rat with a biliary fistula, about 70% of the radioactivity was excreted in the first 24-h portion of bile. About 70% of this radioactivity was found in the trihydroxycholanoic acid fraction. The main part of this radioactivity was shown to be identical with allocholic acid in thin-layer chromatography and radio-gas chromatography.

DISCUSSION

The conversion of cholestanol into allocholic acid involves introduction of hydroxyl groups in the 7α- and 12α-positions, epimerization of the 3β-hydroxyl group and oxidation of the side chain to yield a C_{24}-acid. From the results of the present investigation it may be concluded that 7α-hydroxylation precedes epimerization of the 3β-hydroxyl group. This conclusion is based on the finding that there was insignificant 7α-hydroxylation of 5α-cholestan-3-one and 5α-cholestan-3α-ol under conditions of efficient 7α-hydroxylation of cholestanol. The possibility that 12α-hydroxylation might precede 7α-hydroxylation appears excluded since there was no significant conversion of cholestanol, 5α-cholestan-3-one or 5α-cholestan-3α-ol into compounds with chromatographic properties expected for the corresponding 12α-hydroxylated compounds in incubations with the 20000×*g* supernatant fluid or with the microsomal fraction fortified with NADPH. The specificity of the microsomal, NADPH-dependent 12α-hydroxylase [18] was tested with 5α-cholestane-3β,7α-diol, 7α-hydroxy-5α-cholestan-3-one and 5α-cholestane-3α,7α-diol. The most efficient 12α-hydroxylation was observed with 5α-cholestane-3α,7α-diol indicating that epimerization of the 3β-hydroxy

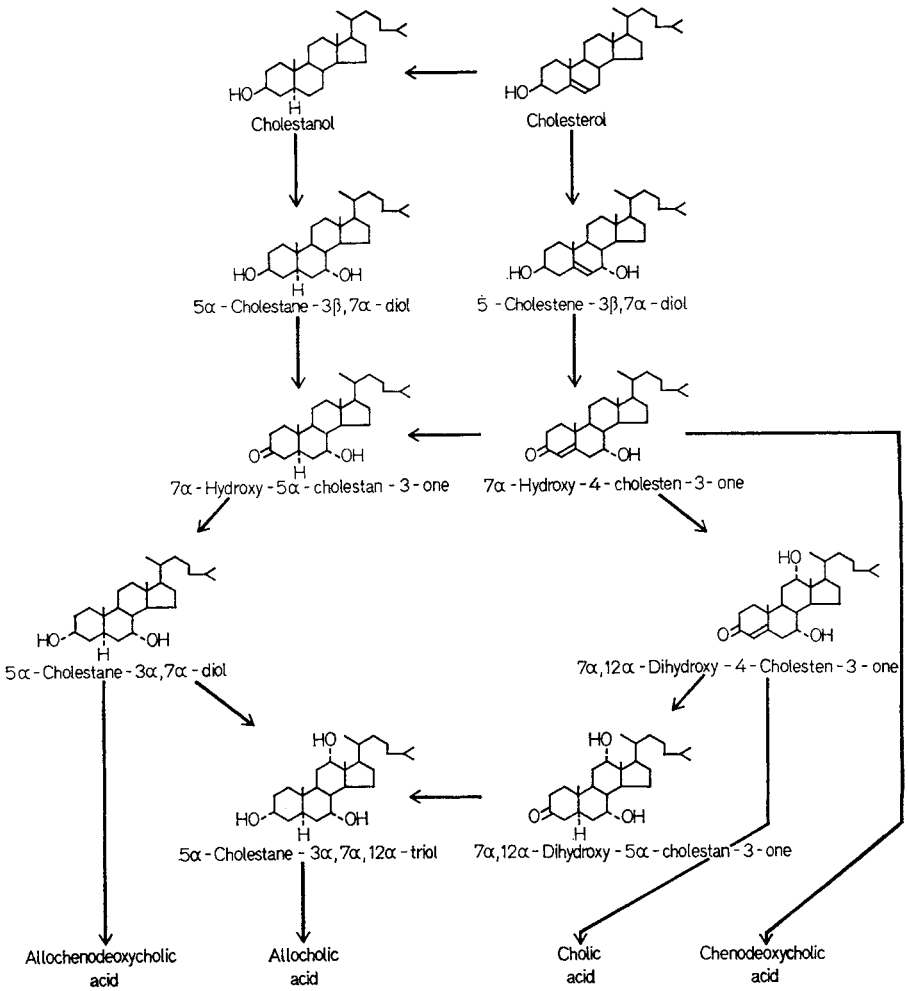

Fig. 2. *Sequences of transformations of the steroid nucleus in the biosynthesis of bile acids*

group precedes 12α-hydroxylation. Epimerization of the 3β-hydroxyl group in 5α-cholestane-3β,7α-diol was effected by a microsomal NAD-dependent 3β-hydroxysteroid dehydrogenase and a soluble NADP-dependent 3α-hydroxysteroid dehydrogenase. Since 5α-cholestane-3β,7α-diol and 5α-cholestane-3α,7α-diol as well as 7α-hydroxy-5α-cholestan-3-one [4] were converted into allocholic acid in rats with a biliary fistula, the following sequence of reactions in the conversion of cholestanol into allocholic acid in the rat is proposed: cholestanol $\rightarrow$ 5α-cholestane-3β,7α-diol $\rightarrow$ 7α-hydroxy-5α-cholestan-3-one $\rightarrow$ 5α-cholestane-3α,7α-diol $\rightarrow$ 5α-cholestane-3α,7α,12α-triol $\rightarrow$ allocholic acid (Fig. 2).

In a previous communication [4] evidence was presented to indicate the presence of a pathway for the conversion of cholesterol into allocholic acid involving 7α-hydroxy-4-cholesten-3-one and 7α-hydroxy-5α-cholestan-3-one as intermediates (*cf.* Fig. 2). The importance of the different pathways for allocholic acid formation is not known.

The similarity between the sequences of the early steps in the conversion of cholestanol into allocholic acid and of cholesterol into cholic acid is striking. Whether or not the same enzymes are involved is an interesting question also from the aspect of the evolution of bile salt [1,3]. Some information relevant to this question is available. Shefer, Hauser and Mosbach [9] have shown that biliary drainage, cholestyramine feeding and phenobarbital treatment influence 7α-hydroxylation of cholestanol and of cholesterol in the same way and to about the same extent. These findings indicate that the same enzyme system may catalyze 7α-hydroxylation of cholestanol and of cholesterol. As shown in the present investigation, reduction of the 3-oxo group in 7α-hydroxy-5α-cholestan-3-one is catalyzed by a 200-fold purified 3α-hydroxysteroid

dehydrogenase, which also catalyzes reduction of the 3-oxo group in 3-oxo-5β-steroids [15].

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