ACTA CHEMICA SCANDINAVICA 22 (1968) 1595–1605

Formation and Metabolism of 3β-Hydroxycholest-5-en-7-one and Cholest-5-ene-3β, 7β-diol

Bile Acids and Steroids 192 *

INGEMAR BJÖRKHEM, KURT EINARSSON and GUNNAR JOHANSSON

Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

The enzymatic formation of 3β-hydroxycholest-5-en-7-one and cholest-5-ene-3β,7β-diol was demonstrated in rat liver homogenates. The microsomal fraction fortified with NADPH catalyzed the conversion of cholesterol into 3β-hydroxycholest-5-en-7-one and the same subcellular fraction, when fortified with NADP, catalyzed the oxidation of cholest-5-ene-3β,7β-diol to 3β-hydroxycholest-5-en-7-one. The reverse reaction required the 20 000 *g* supernatant fluid. It was not possible to demonstrate any significant interconversion between cholest-5-ene-3β,7α-diol and 3β-hydroxycholest-5-en-7-one.

After administration of tritium-labeled 3β-hydroxycholest-5-en-7-one and cholest-5-ene-3β,7β-diol to bile fistula rats, 10–25 % of administered isotope was excreted in the first 48 h portion of bile. With the possible exception of a small fraction, the labeled products in bile were not identical with any of the bile acids that are normally present in rat bile. After administration of tritium-labeled 3β-hydroxycholest-5-en-7-one to bile fistula guinea pigs, 5–10 % of administered isotope was excreted in the first 24 h portion of bile. None of the labeled products excreted in bile was identical with 3α-hydroxy-7-keto-5β-cholanoic acid.

The main primary bile acids in all species have a 7α-hydroxyl group (*cf.* Ref. 1). The introduction of this group is the first reaction in the biosynthesis of cholic acid and in a major pathway for the biosynthesis of chenodeoxycholic acid. Bile acids with a 7-keto group or with a 7β-hydroxyl group occur in small amounts in bile of several species.[1] The main pathway for the formation of these bile acids requires the participation of enzymes in the intes-

* 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; lithocholic acid, 3α-hydroxy-5β-cholanoic acid.

tinal microorganisms, which act on the corresponding 7α-hydroxy bile acids during the enterohepatic circulation of bile.[1] Peric-Golia and Jones [2] have shown, however, that 3α-hydroxy-7-keto-5β-cholanoic acid can be a primary bile acid in the guinea pig. This finding has been confirmed by Danielsson and Einarsson.[3] It is conceivable that also 3α,7β-dihydroxy-5β-cholanoic acid (ursodeoxycholic acid) could be, in part, a primary bile acid. Possible precursors of 3α-hydroxy-7-keto-5β-cholanoic acid and 3α,7β-dihydroxy-5β-cholanoic acid under these circumstances appear to be 3β-hydroxycholest-5-en-7-one and cholest-5-ene-3β,7β-diol.

The enzymatic formation of cholest-5-ene-3β,7β-diol from cholesterol in the presence of 20 000 *g* supernatant fluid of rat liver homogenate was reported by Mendelsohn, Mendelsohn, and Staple [4] and Mitton and Boyd [5] described in a recent preliminary communication the enzymatic conversion of cholesterol into 3β-hydroxycholest-5-en-7-one in the presence of microsomal fraction of rat liver homogenate. Similar findings were made in this laboratory in connection with studies of the 7α-hydroxylation of cholesterol in fractions of rat liver homogenate. It appeared of interest to examine the formation and metabolism of 3β-hydroxycholest-5-en-7-one and cholest-5-ene-3β,7β-diol in more detail in an attempt to obtain information on the role of these steroids in the biosynthesis of bile acids.

EXPERIMENTAL

Materials. Cholesterol-4-^{14}C (specific activity, 60 μC/mg) was obtained from Radiochemical Centre, Amersham, England. Cholesterol-1,2-^{3}H (specific activity, 25 mC/mg) was obtained from New England Nuclear Corp., Boston, Mass. 3β-Hydroxycholest-5-en-7-one-1,2-^{3}H was prepared from cholesterol-1,2-^{3}H as described by Danielsson [6] and had a specific activity of 8.3 μC/mg. Cholest-5-ene-3β,7β-diol-1,2-^{3}H was prepared by reduction of 3β-acetoxycholest-5-en-7-one-1,2-^{3}H with sodium borohydride and had a specific activity of 8.3 μC/mg. Cholest-5-ene-3β,7α-diol-1,2-^{3}H was prepared biosynthetically by incubation of cholesterol-1,2-^{3}H with the 20 000 *g* supernatant fluid of homogenate of liver from bile fistula rats.[7] The cholest-5-ene-3β,7α-diol isolated was diluted with unlabeled material and was crystallized from an acetone-water mixture yielding material with a specific activity of 1.7 μC/mg. The cofactors were obtained from Sigma Chemical Co., St. Louis, Mo.

Experiments with rat liver homogenates. White male rats of the Sprague-Dawley strain weighing about 200 g were used. In the case of incubations with cholesterol-4-^{14}C livers were taken from bile fistula rats after 48—72 h of biliary drainage.[7] Homogenates, 20 % (liver wet weight/volume), were prepared in a modified Bucher medium, pH 7.4,[8] with a Potter-Elvehjem homogenizer equipped with a loosely fitting pestle. The homogenate was centrifuged at 800 *g* for 10 min. The 800 *g* supernatant fluid was then centrifuged at 20 000 *g* for 10 min. The 20 000 *g* supernatant fluid was centrifuged at 100 000 *g* for 2 h. The microsomal fraction was suspended in the homogenizing medium by homogenizing with a loosely fitting pestle. In some experiments, the suspended microsomal fraction was centrifuged once more at 100 000 *g* for 1 h and was then suspended as above. Boiled microsomes were prepared by heating the microsomal suspension for 5 min at 90° followed by centrifugation at 20 000 *g* for 10 min. Aliquots corresponding to 3 ml of 20 000 *g* supernatant fluid were used in each incubation. In incubations with cholesterol the incubation mixtures were diluted with 2 ml of Bucher medium. The labeled compounds were added dissolved in 50 μl of acetone. Incubations were conducted for 1 h and were terminated by addition of 20 volumes of chloroform-methanol, 2:1. Further analysis of the chloroform-methanol extract by thin layer chromatography was performed as described previously.[9] The chromatoplates were developed with benzene-ethyl acetate,

3:7, except in the case of incubations with cholest-5-ene-3β,7α-diol, when benzene-ethyl acetate, 1:1, was used as solvent.

Experiments with bile fistula rats and bile fistula guinea pigs. The same strain of rats as above was used. Guinea pigs weighing about 250 g were obtained from local suppliers. Bile fistulas were prepared in the usual manner. 3β-Hydroxycholest-5-en-7-one-1,2-^{3}H and cholest-5-ene-3β,7β-diol-1,2-^{3}H, 1.3—4.2 μC, were administered intraperitoneally in an emulsion stabilized with serum albumin 24 h after operation. Bile was collected for 24—48 h and was hydrolyzed at 110° for 12 h with 1 M sodium hydroxide in 50 % aqueous ethanol in closed steel tubes. The acidified hydrolysate was extracted with ether and the ether extract was washed with water until neutral. The residue of the ether extract was chromatographed on columns of hydrophobic Hyflo Super-Cel with phase systems F 1, F 2 and C 1.[10] The phase systems used for thin layer chromatography were S 11 and S 12 for bile acids [11] and benzene-ethyl acetate, 3:7, for steroids.

Radioactivity assay. Radioactivity was determined with a methane gas flow counter. Under the conditions of the assay 1 μC of ^{14}C corresponds to 1×10^{6} cpm and 1 μC of ^{3}H to 6×10^{5} cpm.

RESULTS

Conversion of cholesterol into 3β-hydroxycholest-5-en-7-one and cholest-5-ene-3β,7β-diol. Table 1 summarizes the results of incubations of cholesterol-4-^{14}C with different fractions of homogenate of liver from bile fistula rats. The main metabolites formed from cholesterol in the presence of 20 000 *g* supernatant fluid were 7α-hydroxylated products (*cf.* Fig. 1 A) as has been previously found.[7,9] In the presence of microsomal fraction fortified with

Table 1. Metabolism of cholesterol-4-^{14}C in fractions of homogenate of liver from bile fistula rat. The amounts of enzyme fraction used corresponded to 3 ml of 20 000 *g* supernatant fluid. 10 μg of substrate were added and incubations were run for 1 h. The percentages were calculated from the amounts of radioactivity in the different zones of the thin layer chromatograms.

Enzyme fraction	Products %				
	Cholesterol	7α-Hydroxylated products [a]	3β-Hydroxy-cholest-5-en-7-one	Cholest-5-ene-3β,7β-diol	Unidentified products
20 000 *g* supernatant fluid	90.3	6.0	0.9	0.2	2.6
Microsomes	97.7	0.8	0.5	0.2	0.8
Microsomes + 5 μmoles of NADPH	91.5	1.6	3.7	0.8	2.4
Microsomes + 5 μmoles of NADH	97.0	0.8	0.9	0.4	0.9
Boiled microsomes + 5 μmoles of NADH	98.1	0.6	0.9	0.1	0.3

[a] The 7α-hydroxylated products included were cholest-5-ene-3β,7α-diol, 7α-hydroxycholest-4-en-3-one, and 7α,12α-dihydroxycholest-4-en-3-one.

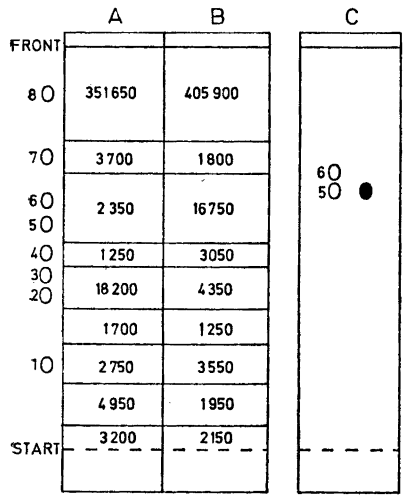

Fig. 1. A, thin layer chromatogram of chloroform extract of incubation of cholesterol-4-^{14}C (0.6×10^6 cpm, 10 μg) with the 20 000 *g* supernatant fluid of homogenate of liver from bile fistula rat. The *numbers* on the chromatogram represent counts per min. Reference compounds were: *1*, 5β-cholestane-3α,7α,-triol; *2*, 7α,12α-dihydroxycholest-4-en-3-one; *3*, cholest-5-ene-3β,7α-diol; *4*, cholest-5-ene-3β,7β-diol; *5*, 3β-hydroxycholest-5-en-7-one; *6*, cholest-5-ene-3β,26-diol; *7*, 7α-hydroxycholest-4-en-3-one; *8*, cholesterol. Solvent, benzene-ethyl acetate, 3:7. *B*, thin layer chromatogram of choloroform extract of incubation of cholesterol-4-^{14}C (0.6×10^6 cpm, 10 μg) with the microsomal fraction of homogenate of liver from bile fistula rat. The microsomal fraction was fortified with 5 μmoles of NADPH. Reference compounds and chromatographic conditions as in *A*. *C*, autoradiogram of thin layer chromatogram of the material in the zone corresponding to reference compounds *5* and *6* of the chromatogram shown in *B* (right-hand spot). Reference compounds as in *A* (left-hand spots). Solvent, benzene-ethyl acetate, 1:4.

NADPH the extent of conversion of cholesterol into 7α-hydroxylated products was low and the main metabolite was present in the chromatographic zone corresponding to 3β-hydroxycholest-5-en-7-one (*cf.* Fig. 1 B). Rechromatography (Fig. 1 C) of the material in this zone showed that the labeled material had the same chromatographic properties as 3β-hydroxycholest-5-en-7-one. The identity of the labeled material with 3β-hydroxycholest-5-en-7-one was

Table 2. Identification of 3β-acetoxycholest-5-en-7-one. 3β-Hydroxycholest-5-en-7-one was isolated by means of thin layer chromatography after incubation of cholesterol-4-^{14}C with the 20 000 *g* supernatant fluid (Incubation A) and of cholest-5-ene-3β,7β-diol,1,2-^{3}H with the microsomal fraction fortified with NADP (Incubation B). The materials were then treated with acetic anhydride in pyridine. All specific activities are given in thousands of counts per min per mg.

Solvent	No. of crystallizations	3β-Acetoxycholest-5-en-7-one isolated from			
		Incubation A		Incubation B	
		Weight mg	Specific activity	Weight mg	Specific activity
None	0	41.0	1.28	43.6	0.22
Acetone-water	1	34.7	1.13	26.5	0.23
Acetone-water	2	32.1	1.06	23.4	0.25
Acetone-water	3	27.9	1.05	16.6	0.23

established by crystallization to constant specific radioactivity after addition of the authentic compound (Table 2). Substitution of NADPH with NADH in incubations with the microsomal fraction resulted in a much lower yield of 3β-hydroxycholest-5-en-7-one (Table 1). As seen in Table 1, small amounts of radioactivity appeared in the zone corresponding to cholest-5-ene-3β,7β-diol in all incubations. The amount of labeled material with chromatographic properties of cholest-5-ene-3β,7β-diol was significantly higher in incubations with the microsomal fraction fortified with NADPH than in any of the other incubations. No further identification of this labeled material was performed. When incubations were conducted with boiled microsomes fortified with NADPH, 3β-hydroxycholest-5-en-7-one and cholest-5-ene-3β,7β-diol were formed in only small amounts as compared with the amounts formed in incubations with fresh microsomes fortified with NADPH.

Conversion of cholest-5-ene-3β,7α-diol and cholest-5-ene-3β,7β-diol into 3β-hydroxycholest-5-en-7-one. Cholest-5-ene-3β,7α-diol-1,2-^{3}H was incubated with the 20 000 *g* supernatant fluid, the microsomal fraction, and the microsomal fraction fortified with 0.3 μmoles of NAD or NADP. In each case, 2—5 % of added labeled substrate was converted into material with chromatographic properties as those of 3β-hydroxycholest-5-en-7-one. Due to the small amounts of radioactivity, further identification was not attempted.

Table 3 summarizes the results of incubations of cholest-5-ene-3β,7β-diol-1,2-^{3}H with different fractions of rat liver homogenate. In the presence of microsomal fraction fortified with NADP the main metabolite was 3β-hydroxycholest-5-en-7-one (Fig. 2 and Table 2). Substitution of NADP with NAD resulted in a decrease in the extent of formation of 3β-hydroxycholest-5-en-7-one. The extent of conversion of cholest-5-ene-3β,7β-diol into 3β-hydroxycholest-5-en-7-one was low in incubations with the 20 000 *g* supernatant fluid even when NAD or NADP had been added.

Table 3. Metabolism of cholest-5-ene-3β,7β-diol-1,2-^{3}H in fractions of rat liver homogenate. The amounts of enzyme fraction used corresponded to 3 ml of 20 000 *g* supernatant fluid. 30 μg of substrate were added and incubations were run for 1 h. The percentages were calculated from the amounts of radioactivity in the different zones of the thin layer chromatograms.

Enzyme fraction	Cholest-5-ene-3β,7β-diol	3β-Hydroxy-cholest-5-en-7-one	7β-Hydroxy-cholest-4-en-3-one [a]	Unidentified products
20 000 *g* supernatant fluid	78.1	8.5	0.4	13.0
Microsomes	77.3	18.4	0.9	3.4
Microsomes + 0.3 μmoles of NADP	28.8	66.7	2.0	2.5
Microsomes + 0.3 μmoles of NAD	79.3	17.3	1.2	2.2
100 000 *g* supernatant fluid	90.6	5.6	1.0	2.8

[a] 7β-Hydroxycholest-4-en-3-one was prepared as described by Greenhalgh *et al.*[15]

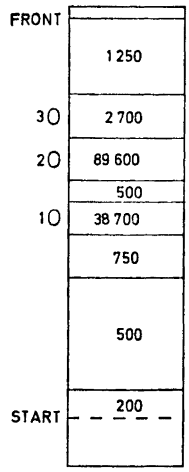

Fig. 2. Thin layer chromatogram of chloroform extract of incubation of cholest-5-ene-3β,7β-diol-1,2-^{3}H (0.15×10^6 cpm, 30 μg) with the microsomal fraction of rat liver homogenate fortified with 0.3 μmoles of NADP. The *numbers* on the chromatogram represent counts per min. Reference compounds were: *1*, cholest-5-ene-3β,7β-diol; *2*, 3β-hydroxycholest-5-en-7-one; *3*, 7β-hydroxycholest-4-en-3-one (prepared as described by Greenhalgh *et al.*[15]). Solvent, benzene-ethyl acetate, 3:7.

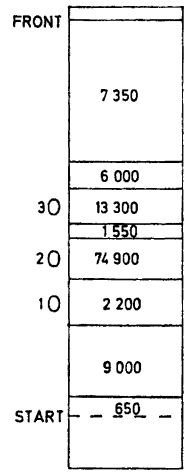

Fig. 3. Thin layer chromatogram of chloroform extract of incubation of 3β-hydroxycholest-5-en-7-one-1,2-^{3}H (0.15×10^6 cpm, 30 μg) with the 20 000 *g* supernatant fluid of rat liver homogenate. The *numbers* on the chromatogram represent counts per min. Reference compounds were: *1*, cholest-5-ene-3β,7α-diol; *2*, cholest-5-ene-3β,7β-diol; *3*, 3β-hydroxycholest-5-en-7-one. Solvent, benzene-ethyl acetate, 3:7.

Conversion of 3β-hydroxycholest-5-en-7-one into cholest-5-ene-3β,7β-diol. 3β-Hydroxycholest-5-en-7-one-1,2-^{3}H was incubated with different fractions of rat liver homogenate (Table 4). In the presence of 20 000 *g* supernatant fluid the main metabolite formed was cholest-5-ene-3β,7β-diol (Fig. 3 and Table 5). The conversion of 3β-hydroxycholest-5-en-7-one into cholest-5-ene-3β,7β-diol in the presence of microsomal fraction fortified with NADH or NADPH was about 20 % but after washing of the microsomes the extent of conversion decreased to 3—6 %. The yield of cholest-5-ene-3β,7β-diol was low in the presence of 100 000 *g* supernatant fluid. The addition of NADH, NADH together with FAD, NADPH or NADPH together with FAD to the microsomal fraction or to the 100 000 *g* supernatant fluid did not stimulate the formation of cholest-5-ene-3β,7β-diol.

Metabolism of 3β-hydroxycholest-5-en-7-one in bile fistula rat. After administration of 3β-hydroxycholest-5-en-7-one-1,2-^{3}H to bile fistula rats, 10—20 % of administered isotope was excreted in the first 48 h portion of bile. Fig. 4 shows a chromatogram of hydrolyzed bile with phase system F 2. The radioactivity was distributed in a number of peaks none of which coincided with the titration peaks of cholic acid and chenodeoxycholic acid. The nature of the labeled material in the different peaks was studied by chromatographying

Table 4. Metabolism of 3β-hydroxycholest-5-en-7-one-1,2-^{3}H in fractions of rat liver homogenate. The amounts of enzyme fraction used corresponded to 3 ml of 20 000 *g* supernatant fluid. The microsomal fraction was washed once (*cf.* Experimental). 30 μg of substrate were added and incubations were run for 1 h. The percentages were calculated from the amounts of radioactivity in the different zones of the thin layer chromatograms.

	Products %			
Enzyme fraction	3β-Hydroxy-cholest-5-en-7-one	Cholest-5-ene-3β,7β-diol	Cholest-5-ene-3β,7α-diol	Unidentified products
20 000 *g* super-natant fluid	13.3	63.8	2.3	20.6
Microsomes	83.6	6.0	0.2	10.2
Microsomes + 3 μmoles of NADPH	96.4	0.4	0.1	3.1
Microsomes + 3 μmoles of NADH	90.3	3.6	1.3	4.8
100 000 *g* super-natant fluid	91.7	1.2	0.7	6.4

Table 5. Identification of 3β,7β-dibenzoxycholest-5-ene. Cholest-5-ene-3β,7β-diol was isolated by means of thin layer chromatography after incubation of 3β-hydroxycholest-5-en-7-one-1,2-^{3}H with the 20 000 *g* supernatant fluid. The material was treated with benzoyl chloride in pyridine. All specific activities are given in thousands of counts per min per mg.

Solvent	No. of crystal-lizations	Weight mg	Specific activity
None	0	42.0	0.58
Acetone-water	1	17.3	0.42
Acetone-water	2	12.4	0.43
Acetone-water	3	7.9	0.41

the material in the individual peaks in suitable systems for reversed phase partition chromatography (C 1 for more polar material and F 1 and F 2 for less polar material) followed by thin layer chromatography in solvent systems S 11 and S 12. Special attention was paid to labeled material that could be identical with 3α,7β-dihydroxy-5β-cholanoic acid (20—32 ml of effluent, Fig. 4) and 3α-hydroxy-7-keto-5β-cholanoic acid (40—65 ml of effluent, Fig. 4). After the chromatographic analyses the labeled materials with chromatographic properties similar to those of 3α,7β-dihydroxy-5β-cholanoic acid and 3α-hydroxy-7-keto-5β-cholanoic acid, respectively, were crystallized together with the authentic bile acids. After four crystallizations, with the specific radioactivity of the crystals constantly falling, the crystals retained radioactivity that would correspond to the presence of less than 5 % of these two bile acids in the mixture of labeled compounds excreted in bile. With respect to the possible presence of labeled cholic acid, chenodeoxycholic acid, and

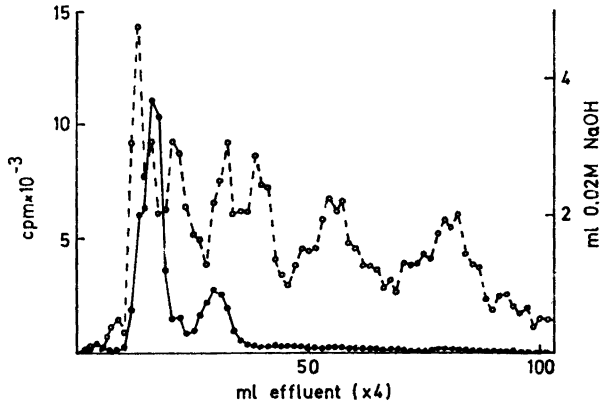

Fig. 4. Chromatogram of first 48 h portion of hydrolyzed bile from bile fistula rat treated with injection of 3β-hydroxycholest-5-en-7-one-1,2-^{3}H. Column, 18 g of hydrophobic Hyflo Super-Cel; phase system, F 2. ●, titration values; ○, radioactivity.

lithocholic acid the total amount of radioactivity that could be identical with these bile acids amounted altogether to less than 10 % of the total radioactivity present in bile.

Metabolism of 3β-hydroxycholest-5-en-7-one in bile fistula guinea pig. After administration of 3β-hydroxycholest-5-en-7-one-1,2-^{3}H to bile fistula guinea pigs, 5—10 % of administered isotope was excreted in the first 24 h portion of bile. The labeled products present in bile were analyzed as described above. No evidence for the presence of labeled 3α-hydroxy-7-keto-5β-cholanoic acid could be obtained.

Metabolism of cholest-5-ene-3β,7β-diol in bile fistula rat. After administration of cholest-5-ene-3β,7β-diol-1,2-^{3}H to bile fistula rats, 10—25 % of administered isotope was excreted in the first 48 h portion of bile. Fig. 5 shows a chromatogram of hydrolyzed bile with phase system F 2. The radioactivity was distributed in a number of peaks, the material in which was analyzed by repeated reversed phase partition chromatography and thin layer chromatography. As in the case of 3β-hydroxycholest-5-en-7-one, only very small amounts of the labeled products could possibly be identical with the normally occurring bile acids in rat bile including 3α,7β-dihydroxy-5β-cholanoic acid. It was estimated that less than 2 % of the labeled products excreted in bile could be identical with 3α,7β-dihydroxy-5β-cholanoic acid.

DISCUSSION

In the conversion of cholesterol to chenodeoxycholic acid and cholic acid a 7α-hydroxyl group is introduced. This reaction has been shown to be catalyzed by the 20 000 *g* supernatant fluid of rat liver homogenate.[1] Attempts have been made to characterize this enzyme system further. However, already

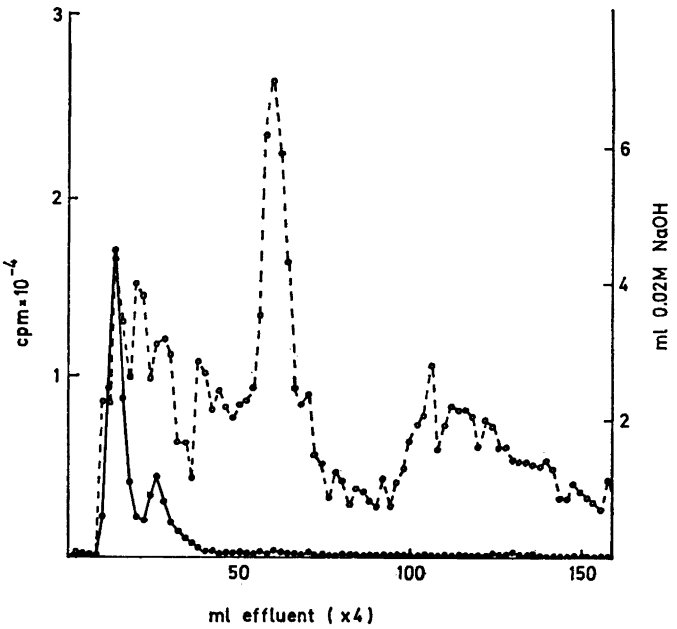

Fig. 5. Chromatogram of first 48 h portion of hydrolyzed bile from bile fistula rat treated with injection of cholest-5-ene-3β,7β-diol-1,2-^{3}H. Column, 18 g of hydrophobic Hyflo Super-Cel; phase system, F 2. ●, titration values; ○, radioactivity.

centrifugation of the 20 000 *g* supernatant fluid to separate the microsomal and soluble fractions results in partial inactivation of the 7α-hydroxylase system, since the recombined microsomal and soluble fractions, with or without NADPH added, show considerably less 7α-hydroxylase activity than the 20 000 *g* supernatant fluid. The microsomal fraction, when fortified with NADPH, shows 20—30 % of the 7α-hydroxylase activity exhibited by the 20 000 *g* supernatant fluid. In incubations with recombined microsomal and soluble fractions as well as in incubations with the microsomal fraction fortified with NADPH, material with chromatographic properties of 3β-hydroxycholest-5-en-7-one is formed. The amount of such material formed is about the same as the decrease in the formation of 7α-hydroxylated products. These observations, many of which have also been made by Mitton and Boyd [5,12] and have been reported in preliminary communications, prompted a more detailed study of the formation and metabolism of 3β-hydroxycholest-5-en-7-one.

The present investigation has established the enzymatic conversion of cholesterol into 3β-hydroxycholest-5-en-7-one. The reaction is catalyzed by the microsomal fraction of rat liver homogenate when fortified with NADPH. Substitution of NADPH with NADH results in a substantial reduction of the formation of 3β-hydroxycholest-5-en-7-one. This result and the low extent of formation of 3β-hydroxycholest-5-en-7-one in the presence of boiled micro-

somal fraction and NADPH constitute strong evidence that the conversion of cholesterol into 3β-hydroxycholest-5-en-7-one observed in the microsomal fraction fortified with NADPH is the result of enzymatic catalysis. The mechanism of the introduction of the keto group at position C-7 has not been established. The autoxidation of cholesterol leads to the formation of among other compounds 3β-hydroxycholest-5-en-7-one and the epimeric 7-hydroxycholesterols.[13] Bergström [13] has suggested that these compounds derive from intermediary hydroperoxides, which could be the initial products of the attack of oxygen on the cholesterol molecule. A similar mechanism of reaction for the enzymatic conversion of cholesterol into cholest-5-ene-3β,7α-diol and 3β-hydroxycholest-5-en-7-one might explain the requirement for NADPH in the formation of 3β-hydroxycholest-5-en-7-one and the finding that a decrease in the formation of cholest-5-ene-3β,7α-diol and its metabolites was almost parallelled by an increase in the formation of 3β-hydroxycholest-5-en-7-one. Assuming the intermediary formation of 7α-hydroperoxycholest-5-en-3β-ol, the direction of its conversion into cholest-5-ene-3β,7α-diol or 3β-hydroxycholest-5-en-7-one might be the critical event. In the presence of 20 000 *g* supernatant fluid the predominant products are cholest-5-ene-3β,7α-diol and its metabolites, whereas in the presence of microsomal fraction fortified with NADPH 3β-hydroxycholest-5-en-7-one is the major product. Thus, the 20 000 *g* supernatant fluid might contain a factor that could direct the conversion of an hypothetical, intermediary 7α-hydroperoxycholest-5-en-3β-ol into cholest-5-ene-3β,7α-diol. To test the possibility of the intermediary formation of a hydroperoxide, tritium-labeled 7α-hydroperoxycholest-5-en-7-one has been prepared and has been incubated with different fractions of rat liver homogenate.[14] However, due to the instability of this compound in an aqueous medium, no conclusion concerning the possible involvment of 7α-hydroperoxycholest-5-en-3β-ol in the enzymatic conversion of cholesterol into cholest-5-ene-3β,7α-diol and 3β-hydroxycholest-5-en-7-one can be drawn at present.

Another possible pathway for the formation of 3β-hydroxycholest-5-en-7-one is through oxidation of cholest-5-ene-3β,7α-diol and/or cholest-5-ene-3β,7β-diol. No significant conversion of cholest-5-ene-3β,7α-diol into 3β-hydroxycholest-5-en-7-one could be shown. Cholest-5-ene-3β,7β-diol was, however, converted efficiently into 3β-hydroxycholest-5-en-7-one in the presence of microsomal fraction fortified with NADP. The experiments do not permit any definite conclusion concerning the importance of a pathway from cholesterol to 3β-hydroxycholest-5-en-7-one involving the intermediate formation of cholest-5-ene-3β,7β-diol. It might be added that the intermediate formation of cholest-5-ene-3β,7β-diol might explain the requirement for NADPH in the conversion of cholesterol into 3β-hydroxycholest-5-en-7-one provided that the 7β-hydroxylation requires NADPH.

It is worth mentioning that the reduction of 3β-hydroxycholest-5-en-7-one to cholest-5-ene-3β,7β-diol required the 20 000 *g* supernatant fluid. The extent of reduction observed in incubations with the microsomal fraction, with or without reduced pyridine nucleotides added, was one tenth or less of that observed in incubations with the 20 000 *g* supernatant fluid. In all cases, no significant amounts of cholest-5-ene-3β,7α-diol were formed from 3β-hydroxycholest-5-en-7-one.

The role of 3β-hydroxycholest-5-en-7-one and cholest-5-ene-3β,7β-diol in cholesterol catabolism remains to be established. It appeared possible that these compounds might be precursors of 3α-hydroxy-7-keto-5β-cholanoic acid and 3α,7β-dihydroxy-5β-cholanoic acid. These bile acids are present in small amounts in bile of a number of species.[1] In guinea pig 3α-hydroxy-7-keto-5β-cholanoic acid is in part a primary bile acid,[2,3] *i.e.* an acid formed from cholesterol in the liver, whereas no evidence has yet been presented to indicate that 3α,7β-dihydroxy-5β-cholanoic acid could be a primary bile acid. The results of the present investigation show that 3β-hydroxycholest-5-en-7-one and cholest-5-ene-3β,7β-diol are converted in the bile fistula guinea pig and the bile fistula rat predominantly into compounds that are not identical with the bile acids normally occurring in guinea pig and rat bile. The conversion of 3β-hydroxycholest-5-en-7-one and cholest-5-ene-3β,7β-diol into 3α-hydroxy-7-keto-5β-cholanoic acid and 3α,7β-dihydroxy-5β-cholanoic acid was insignificant.

Acknowledgements. The able technical assistance of Miss Madelaine Ekebrand is gratefully acknowledged.

This work is part of investigations supported by the *Swedish Medical Research Council* (Project 13X—218) and has also been supported by grants from *Karolinska Institutets Reservationsanslag.*

REFERENCES

1. Danielsson, H. and Tchen, T. T. In Greenberg, D. M. *Metabolic Pathways*, Academic, New York 1968, vol. II, p. 117.
2. Peric-Golia, L. and Jones, R. S. *Proc. Soc. Exptl. Biol. Med.* **106** (1961) 177.
3. Danielsson, H. and Einarsson, K. *Acta Chem. Scand.* **18** (1964) 732.
4. Mendelsohn, D., Mendelsohn, L. and Staple, E. *Biochim. Biophys. Acta* **97** (1965) 379.
5. Mitton, J. R. and Boyd, G. S. *Biochem. J.* **96** (1965) 60 p.
6. Danielsson, H. *Acta Chem. Scand.* **14** (1960) 846.
7. Danielsson, H., Einarsson, K. and Johansson, G. *European J. Biochem.* **2** (1967) 44.
8. Bergström, S. and Gloor, U. *Acta Chem. Scand.* **9** (1955) 34.
9. Danielsson, H. and Einarsson, K. *J. Biol. Chem.* **241** (1966) 1449.
10. Norman, A. and Sjövall, J. *J. Biol. Chem.* **233** (1958) 872.
11. Eneroth, P. *J. Lipid Res.* **4** (1963) 11.
12. Mitton, J. R. and Boyd, G. S. *Biochem. J.* **103** (1967) 17 p.
13. Bergström, S. *Arkiv Kemi, Mineral Geol.* **A 16** (1943) No. 10.
14. Björkhem, I., Einarsson, K. and Johansson, G. *To be published.*
15. Greenhalgh, C. W., Henbest, H. B. and Jones, E. R. H. *J. Chem. Soc.* **1952** 2375.

Received January 11, 1968.