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Bile Acids in Bile of Cod, *Gadus callarias*

Hydroxylation of Deoxycholic Acid and Chenodeoxycholic Acid in Homogenates of Cod Liver. Bile Acids and Steroids 200 *

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Gall bladder bile of *Gadus callarias* was found to contain cholic acid, 3α,7β,12α-trihydroxy-5β-cholanoic acid, deoxycholic acid, chenodeoxycholic acid, and ursodeoxycholic acid. The ratio of trihydroxycholanoic acids to dihydroxycholanoic acids was about 5:1. Taurodeoxycholate as well as taurochenodeoxycholate were hydroxylated to taurocholic acid by cod liver homogenate, whereas no hydroxylation of the corresponding free acids took place. The free acids were partly conjugated with taurine *in vitro.*

The main bile acids of most teleosts are cholic acid and chenodeoxycholic acid which occur as taurine conjugates.[1] The bile salt pattern may, however, vary considerably throughout this large and heterogeneous group of animals.[2] Thus, sulfate esters of bile alcohols occur as bile salts in the Cyprinidae [3] and allocholic acid [4] is present in varying quantities in several species including "Gigi"-fish.[5] Recently, Sasaki [6] isolated a new bile acid, haemulcholic acid, 3α,7α,22β-trihydroxy-5β-cholanoic acid,[7] from *Papapristipoma trilineatum*, and Kallner [8] isolated an unsaturated bile acid, 3α,12α-dihydroxy-5β-chol-7-enoic acid, from *Cottus quadricornis*. Deoxycholic acid, which is a "secondary bile acid" among mammals, only seems to have been found previously in gall bladder of one teleostean species, the cod.[9] In a recent study [8] it was found that bile of *Cottus quadricornis* contained deoxycholic acid. The question was raised whether this acid is a primary bile acid

* The following systematic names are given to the compounds referred to by trivial names: cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; and ursodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid.

The following abbreviations are used: GLC, gas-liquid chromatography; TLC, thin layer chromatography; MS, mass spectrum; RRT, relative retention time; TFA, trifluoroacetate; TMSi, trimethylsilylether; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene; dpm, disintegrations per minute.

or a secondary in this species and if its presence in bile is due to the lack of hydroxylating enzyme systems in the liver of *Cottus quadricornis*.

In view of these findings it was considered of interest to analyze gall bladder bile of cod and, if deoxycholic acid should be present, also investigate the hydroxylating ability of cod liver homogenates.

EXPERIMENTAL

Gall bladder bile was obtained from fish caught off the Swedish coast in the neighbourhood of Lysekil. The gall bladders contained about 0.5 ml of bile each and the bile was collected as described previously.[8] Fish used in incubation experiments was caught in the Baltic, south of Stockholm, during September and October. The animals used weighed between 1 and 2 kg.

Analysis of bile. Isolation and purification of bile acids of gall bladder bile were performed as described previously.[8]

Analyses and characterization of the dihydroxycholanoic acid fraction were done by GLC of methyl esters, TFA of methyl esters and oxidation products. Methyl esters and oxidation products were run on a column with 3 % QF−1 as the stationary phase, whereas the TFA's were run on a column with 1 % SE−30 as the stationary phase. GLC−MS was performed with TFA of the methyl esters on a SE−30 column.

Analyses of the trihydroxycholanoic acid fraction were done by GLC of TFA and TMSi of the methyl esters and oxidation products. The TMSi's were prepared in pyridine which quantitatively yields the fully trimethyl-silylated compounds. All derivatives of trihydroxycholanoic acids were analyzed on a QF−1 column. GLC−MS was performed with TMSi of the methyl trihydroxycholanoates, using a QF−1 column.

The column temperature was 215−245° and argon pressure 2.2 kg/cm^2. GLC−MS were recorded with an LKB−9000 instrument.

Incubations. Liver homogenates, 30 % (liver wet weight per volume), were prepared at 0° in a buffer solution of pH 7.4, containing 10.1 g Na_2HPO_4, 1.4 g KH_2PO_4 and 43 g sucrose per liter[14] using a Potter-Elvehjem homogenizer with a tightly fitting Teflon pestle. Cell debris were removed by centrifugation at about 800 *g* for 15 min. During this procedure a large bulk of fat accumulated on top of the centrifugate (infranatant). The amount of fat varied between about 1/2 and 1/4 of the total volume of the centrifugate. No difference in the results of pilot incubations was achieved whether this fat was included or not in the experiments and therefore the fat was removed. Incubations were performed at 20° and 37° for 2 h. No qualitative difference was observed in pilot incubations and the higher temperature was used in the incubations.

Deoxycholic acid and chenodeoxycholic acid were incubated as free acids and as taurine conjugates. The substrates were diluted with inactive material to a specific radioactivity of about 3×10^6 dpm/mg and 1 mg of substrate was added to 9 ml of the incubation mixture. For incubation of the free acids the incubation mixture was fortified with 2 mg NADPH, 6 mg ATP, and 1 mg each of Coenzyme A and taurine, whereas for incubations of the conjugates with 2 mg of NADPH only.[10]

Incubations were performed under oxygen for 2 h and terminated by the addition of 2 volumes of ethanol. The precipitate was removed by centrifugation, suspended in the same volume of chloroform-methanol (1:1) and again centrifuged. The extracts were combined, the solvent was evaporated and the residue dissolved in a suitable amount of methanol.

Aliquots of the incubates of the free acids were analyzed by TLC. The incubates of the conjugated acids were hydrolyzed in a sealed steel tube in 2 N ethanolic KOH at 120° for 6 h. The hydrolysate was acidified by the addition of HCl and extracted three times with ether. The combined extracts were washed neutral with water and the ether evaporated. The residue was dissolved in methanol and methylated by the addition of diazomethane. The methyl esters were analyzed by TLC, using phase system S6.[11] Methyl trihydroxycholanoates and methyl dihydroxycholanoates were separated on columns of aluminium oxide, activity grade IV.[8]

Incubates of free acids were analyzed with solvent system S6 [11] and butanol:water: acetic acid (10:1:1), BWA,[12] with cholic acid, deoxycholic acid, taurocholate, and taurodeoxycholate as reference compounds. Neither of the above-mentioned solvent systems separate deoxycholic acid and chenodeoxycholic acid or derivatives of these compounds. After development, the plates were sprayed with water to locate the reference compounds. The appropriate zones were scraped off into liquid scintillation vials containing PPO-dimethyl-POPOP (4 g and 50 mg, respectively, per liter toluene), 10 ml, and 0.5 ml of 1 % hydroxide of hyamine. The recovery with these procedures was 70—80 %, as estimated by the assay of the radioactivity.

Tritium-labeled taurochenodeoxycholate was prepared according to Norman [13] from randomly tritium-labeled chenodeoxycholate prepared by the method of Wilzbach.[14] Unlabeled taurochenodeoxycholate was added to the extract of the reaction mixture and the material purified by reversed phase partition chromatography on a column of Hyflo Super-Cel eluted with solvent system D.[15] The taurochenodeoxycholate had a specific radioactivity of about 18×10^6 dpm/mg.

RESULTS

Analysis of gall bladder bile. TLC of crude cod bile (solvent system BWA) showed the presence of material with the mobility of taurine conjugates of dihydroxycholanoic as well as trihydroxycholanoic acids. No material was present in the zones corresponding to glycine derivatives.

After alkaline hydrolysis and methylation of the product, the trihydroxycholanoic and dihydroxycholanoic acid fractions were separated by chromatography on a column of aluminium oxide, activity grade IV. From 40 ml of cod bile, a crude methyl dihydroxycholanoate fraction weighing 890 mg and a methyl trihydroxycholanoate fraction weighing 3550 mg were obtained. The methyl dihydroxycholanoate fraction was further purified by chromatography on a column of aluminium oxide, activity grade III. The column was eluted with increasing amounts of ethyl acetate in benzene. Ethyl acetate, 20 % in benzene, eluted 91 mg (fractions 9—11) of substance and 25 % ethyl acetate in benzene eluted an additional 597 mg (fractions 12—16) of substance with the TLC properties of bile acid derivatives. The remaining 190 mg was eluted before the bile acids and was not further analyzed. Three different spots were recognized on TLC of fractions 9—11 and fractions 12—16, using solvent system S12.[11] Two of the spots corresponded to those of reference methyl deoxycholate and methyl chenodeoxycholate, whereas the spot of the third compound showed the characteristic greenish color of a 3,7-dihydroxycholanoate and the same R_F value as simultaneously run reference samples of methyl ursodeoxycholate and methyl 3β,7α-dihydroxy-5α-cholanoate.[16] About equal amounts of this "third compound" and methyl deoxycholate were present in fractions 9—11, whereas the main part of fractions 12—16 consisted of methyl deoxycholate (Fig. 1). The identity of these compounds was further established by GLC of methyl esters and oxidation products on a QF—1 column and TFA's on an SE—30 column (Table 1) and by GLC—MS (Figs. 3, 4, and 5).

The RRT's of the various derivatives (Table 1) suggest that the dihydroxycholanoic acids present in cod bile are chenodeoxycholic acid, deoxycholic acid, and ursodeoxycholic acid and this was verified by MS. As seen in Fig. 5 the MS of TFA's of the isolated "third compound" and methyl 3β,7α-dihydroxy-

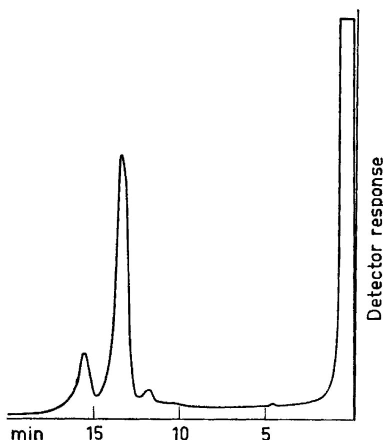

Fig. 1. GLC of TFA of fractions 12—16 (*cf.* text). Column: SE—30.

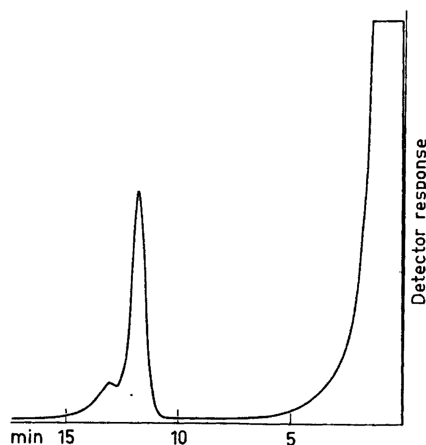

Fig. 2. GLC of TMSi of methyl trihydroxycholanoate fraction. Column: QF—1.

Table 1. RRT's of isolated bile acid fractions and suitable reference compounds. The retention time of methyl deoxycholate has arbitrarily been put to 1.000.

	QF—1 column				SE—30 column
	Methyl ester	TFA	Oxidation product	TMSi	TFA
Fractions 9—11	1.00		2.84		0.256
	1.23				0.354
Fractions 12—16	1.00				0.270
	1.16				0.311
Trihydroxycholanoic acid fraction		1.23	5.83	0.364	
		1.32		0.332	
Deoxycholic acid	1.000		2.82		0.262
Chenodeoxycholic acid	1.18		2.82		0.311
Ursodeoxycholic acid	1.22		2.82		0.362
3β,7α-Dihydroxy-5α-cholanoic acid	1.32				0.296
Cholic acid		1.33	5.90	0.332	
3α,7β,12α-Trihydroxy-5β-cholanoic acid		1.25	5.90	0.362	

5α-cholanoate are significantly different, the main difference being in the size of the peak corresponding to a fragment M—114 (*m*/*e* 484), equal to (M—CF_3COOH). On the other hand the MS of the isolated compound is identical with that of the TFA of methyl ursodeoxycholate.

Two compounds were present in the methyl trihydroxycholanoate fraction as shown by GLC of the TMSi derivatives (Fig. 2). The compounds also

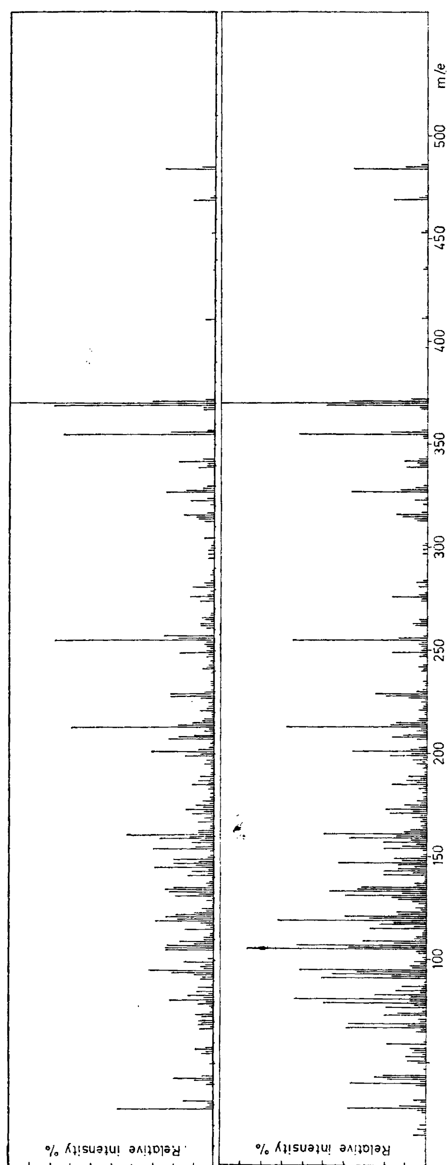

Fig. 3. MS of TFA of compound corresponding to the third peak in GLC of fractions 12–16 (left) and TFA of methyl chenodeoxycholate (right).

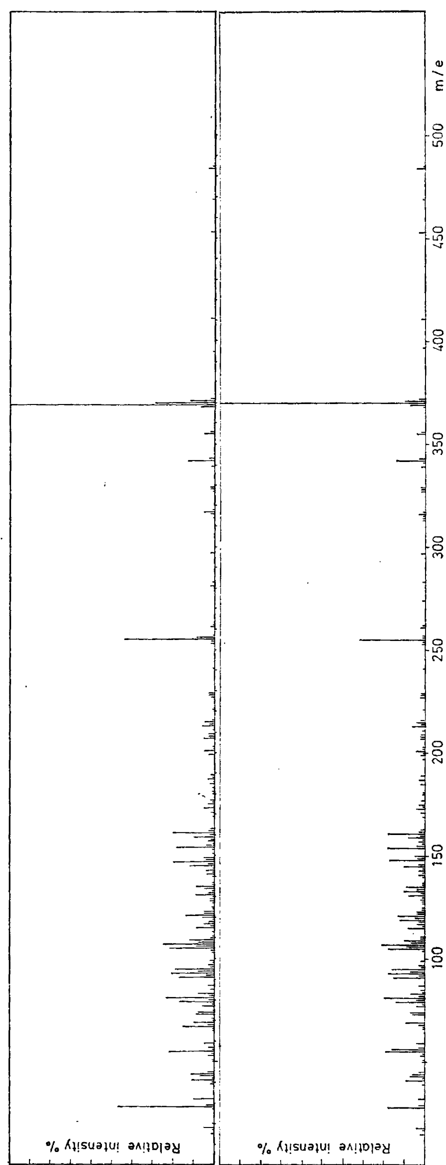

Fig. 4. MS of TFA of compound corresponding to the second peak in GLC of fractions 12–16 (left) and TFA of methyl deoxycholate (right).

separated as TFA's on the same column, although the elution order of the large peak and the small peak was reversed (Table 1). On oxidation of the methyl trihydroxycholanoate fraction only one peak was obtained and this peak had the same RRT as methyl 3,7,12-triketo-5β-cholanoate (Table 1). The GLC and MS (Figs. 6 and 7) verify that the two trihydroxycholanoic

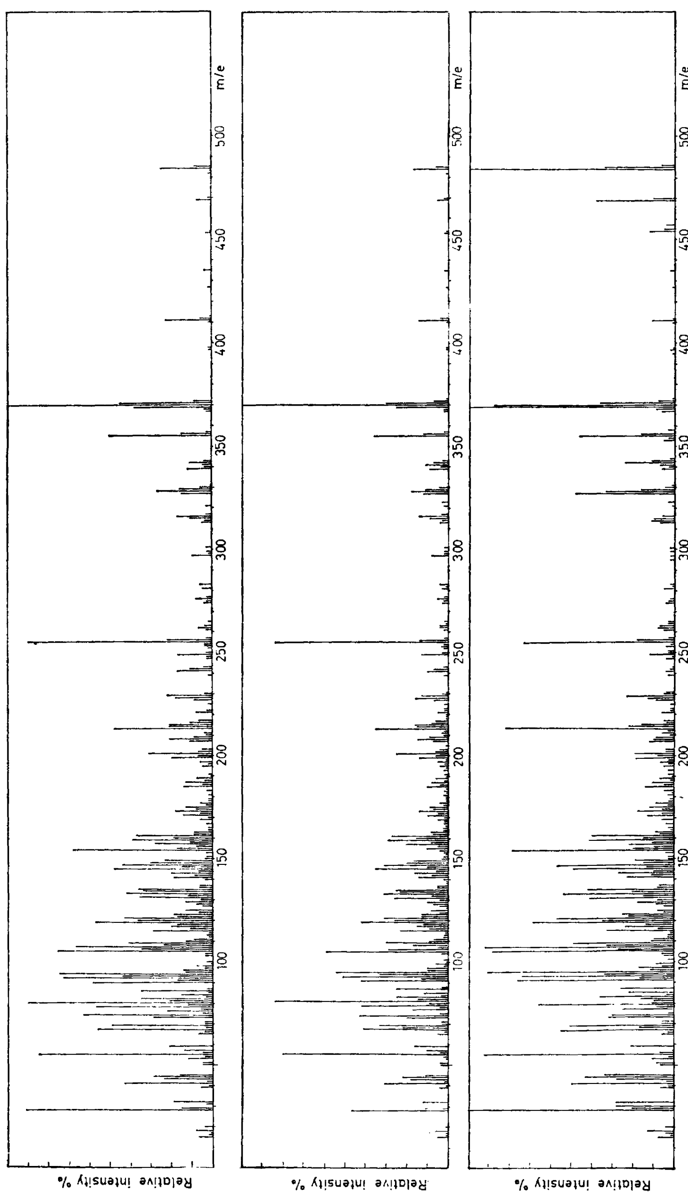

Fig. 5. MS of TFA of "third compound" (top), TFA of methyl ursodeoxycholate (middle) and TFA of methyl 3β,7α-dihydroxy-5α-cholanoate (bottom).

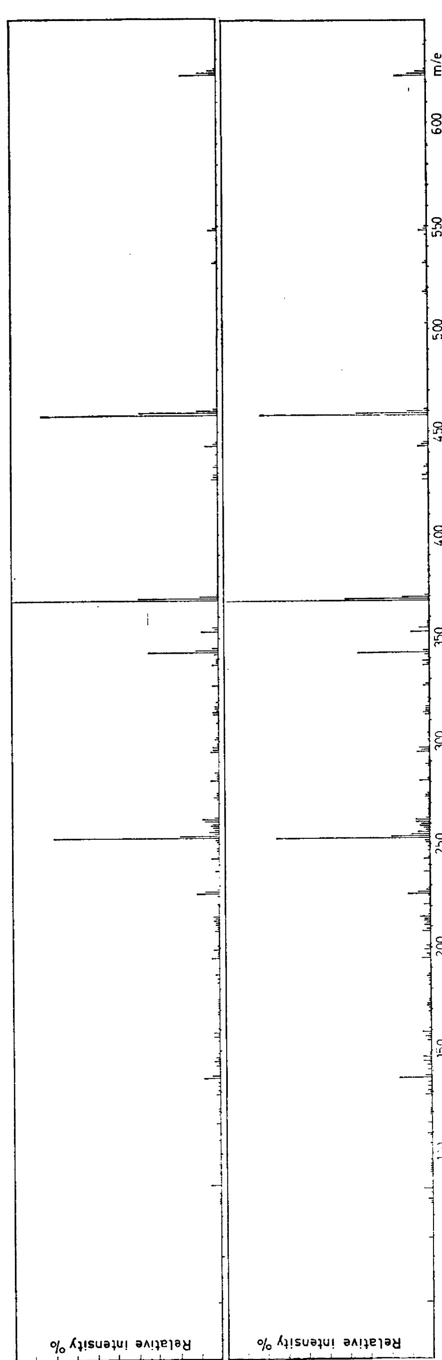

Fig. 6. **MS of TMSi of compound corresponding to the first peak in GLC shown in Fig. 2 (above) and TMSi of methyl cholate (below).**

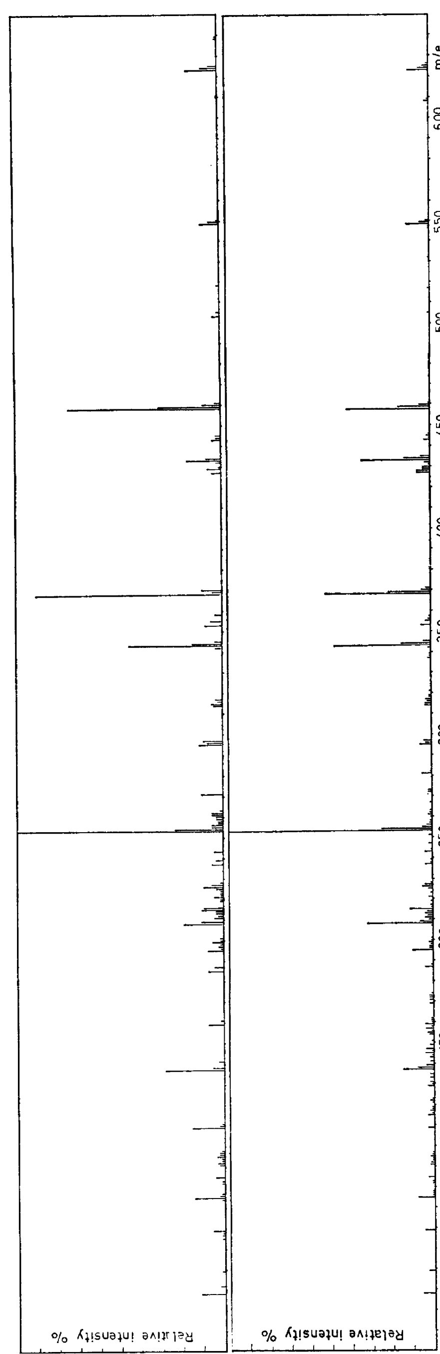

Fig. 7. **MS of TMSi of compound corresponding to the second peak in GLC shown in Fig. 2 (above) and TMSi of methyl 3α,7β,12α-trihydroxy-5β-cholanoate (below).**

acids present in cod bile are cholic acid and 3α,7β,12α-trihydroxy-5β-cholanoic acid, the main component being cholic acid. In the MS of methyl cholate TMSi the base peak at *m/e* 367 corresponds to a fragment (M−3TMSiOH), whereas the base peak of the TMSi of methyl 3α,7β,12α-trihydroxy-5β-cholanoate is found at *m/e* 253, equal to (M−(3TMSiOH+the elements of the side chain)).[17]

Incubation of deoxycholic acid and chenodeoxycholic acid. Aliquots of the centrifugate obtained after incubations with the free acids were analyzed by TLC which was developed with solvent system BWA. In this system free bile acids migrate with the solvent front, whereas taurine conjugates of dihydroxycholanoates and trihydroxycholanoates separate. TLC of the incubate of deoxycholic acid showed that 90−95 % of the radioactivity moved with the solvent front. The remainder of the radioactivity was isolated from the zone corresponding to taurocholate. In the same manner it was shown that after incubation of chenodeoxycholic acid an average of 30 % of the activity recovered from the plate was present in the zones corresponding to conjugates. About 80 % of this radioactivity was present in the trihydroxycholanoate fraction.

The incubation mixtures were also analyzed by TLC in solvent system S6. The only unconjugated radioactive material recovered from the plates occurred at the place of the incubated substrates. Thus, under the conditions used, the cod liver homogenate is capable of catalyzing the conjugation of chenodeoxycholic and deoxycholic acids with taurine. No direct hydroxylation of the substrates could be found.

Incubation of taurodeoxycholate and taurochenodeoxycholate. Preparative TLC of the methylated hydrolysates of the incubation mixtures of taurodeoxycholate and taurochenodeoxycholate showed that a conversion to trihydroxycholanoic acids had taken place to an extent of 80 % and 65 %, respectively. The methyl trihydroxycholanoates were isolated by chromatography on columns of aluminium oxide, activity grade IV, diluted with un-

Table 2. Recrystallization of methyl cholate isolated from incubations of taurochenodeoxycholate (A) and taurodeoxycholate (B). Unlabeled methyl cholate, 25 mg, has been added to each sample.

	Crystallizing solvent	Weight mg	Spec. activity cpm/mg
Methyl cholate from incubation A	Methanol-water	26.9	18 350
		23.8	18 250
		19.4	17 800
		17.7	19 900
		16.4	17 300
Methyl cholate from incubation B	Methanol-water	26.1	8 800
		23.6	7 350
		17.7	5 650
		16.2	5 850
		15.0	5 200

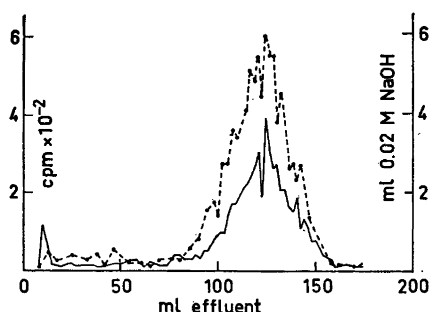

Fig. 8. Reversed phase partition chromatography of hydrolyzed mother liquors obtained after crystallization of metabolites from the incubation of taurochenodeoxycholate. 5 mg of unlabeled cholic acid had been added. Solid line, titration values; broken line, radioactivity.

labeled methyl cholate and crystallized from methanol/water (Table 2). In both cases a constant specific radioactivity of the crystals was obtained.

The mother liquors from the crystallization of the metabolite isolated from the incubation of taurochenodeoxycholate were combined and hydrolyzed. The free acids were analyzed after extraction, on reversed phase partition chromatography (phase system C)[16] after addition of 5 mg of unlabeled cholic acid (Fig. 8). This phase system separates cholic acid from other trihydroxycholanoic acids such as hyocholic acid[19] and pytocholic acid.[23] The chromatogram was assayed for weight by titration and for radioactivity. As shown in Fig. 8 the two curves coincide.

Thus, enzymes of the cod liver are capable of catalyzing the 7α-hydroxylation of taurodeoxycholate as well as the 12α-hydroxylation of taurochenodeoxycholate *in vitro.*

DISCUSSION

The main bile acid of gall bladder bile of cod is cholic acid and a small portion of the trihydroxycholanoic acid fraction consists of 3α,7β,12α-trihydroxy-5β-cholanoic acid. About 20 % of the bile acids are dihydroxycholanoic acids and the main constituent of this fraction is deoxycholic acid. Chenodeoxycholic acid and ursodeoxycholic acids occur in almost equal amounts as two minor components. The origin and metabolic interrelationship of these bile acids are not known. However, bile acids with a 7β-hydroxyl group have previously been isolated from bile of certain bear species, from the coypu and the rat as well as from man.[18,20,21] It has been shown that several strains of *E. coli* can catalyze the oxidation of a 7-hydroxyl group[22] and further that 3α-hydroxy-7-keto-5β-cholanoic acid is stereospecifically reduced to ursodeoxycholic acid by the rat liver *in vivo.*[23] It seems probable that a similar mechanism gives rise to ursodeoxycholic acid in the cod, although a direct formation of ursodeoxycholic acid from chenodeoxycholic acid similar to that found in mammals[20,24] is not excluded.

The cod liver was found to catalyze the hydroxylation of taurodeoxycholate as well as taurochenodeoxycholate in a buffer and cofactor system previously known to facilitate the *in vitro* 7α-hydroxylation of taurodeoxycholate in mammals.[10]

The ability of liver enzymes to hydroxylate deoxycholic acid is common to many mammals. However, the bile content of deoxycholic acid in such mammals, *e.g.* the rat, is low, whereas deoxycholic acid is one of the main bile acids in the bile of animals which cannot bring about 7α-hydroxylation, *e.g.* the rabbit. The hydroxylating capacity of cod liver *in vivo* cannot be estimated by the results of the present study, partly because the incubation temperature (37°) was far above what can be considered physiological for the cod.

12α-Hydroxylation of taurochenodeoxycholate has previously been observed *in vivo* in the python snake of the genus Boidae,[25] whereas this reaction has not been found to take place in higher animals.[2] It is not known if this reaction is limited to taurochenodeoxycholate in the cod or if tauroursodeoxycholate could also serve as a substrate, yielding the tauroconjugate of 3α,7β,12α-trihydroxy-5β-cholanoic acid. The biosynthesis of the latter acid could also occur by means of the intermediate formation of 3α,12α-dihydroxy-7-keto-5β-cholanoate.

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