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THE BILE ACID COMPOSITION OF RABBIT AND CAT GALL-BLADDER BILE

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SUMMARY

Nonsulphated and sulphated bile acids from gall-bladder bile of rabbits and cats have been analysed by gas chromatography–mass spectrometry. In the nonsulphated fraction of rabbit bile deoxycholic acid was the predominant bile acid. The sulphate fraction (B.A.S.) contained lithocholic acid and small amounts of deoxycholic acid and allodeoxycholic acid and their 3- and 12-epimers. No sex difference in the composition of rabbit bile was found. The nonsulphated fraction of cat bile contained the normal primary and secondary bile acids, with smaller amounts of other bile acids. The B.A.S. fraction contained isomers of cholic and deoxycholic acids; allodeoxycholic and lithocholic acids predominated. Male cats produced about twice as much B.A.S. as did female cats, but the female animals produced more nonsulphated bile acids than the males.

INTRODUCTION

The structure, quantity and the extent of conjugation with taurine and glycine of the major bile acids present in mammalian bile vary from species to species[1, 2]. The presence in the bile of human subjects of the sulphate of conjugated lithocholic acid[3, 4] has aroused a great deal of interest in the sulphates of other bile acids in man and other species. Methods in general use for the extraction of nonsulphated bile acids after alkaline hydrolysis cause degradation of bile acid sulphates (B.A.S.)[5], and therefore the presence of B.A.S. in other biological media was not suspected. In recent years methods have been developed for the separation of nonsulphated bile acids from B.A.S., and this has resulted in the examination of biological fluids for the presence of these forms of bile acids. Such investigations were stimulated by the finding of elevated B.A.S. concentrations in the blood and urine of human subjects with various forms of hepatobiliary disease[6–8].

We have investigated the B.A.S. fraction of bile obtained from male and female rats by cannulation of the bile duct. There was a marked sex difference in the types and quantities of the bile acids in the B.A.S. fraction. Rather surprisingly, allochenodeoxycholic accounted for about 80% of bile acids in the B.A.S. fraction of female rat bile, whereas this bile acid could not be found in either the B.A.S. or nonsulphated fractions of male rat bile[9].

However, the rat does not have a gall bladder, and diversion of bile by cannulation of the bile duct may cause major changes in the types and amounts of bile acids synthesized in the liver due to removal of feedback control mechanisms, and secondary bile acids do not occur in bile because of interruption of the enterohepatic circulation. Therefore I have investigated the bile acid composition of gall-bladder bile obtained from intact male and female rabbits and cats. The cholesterol content of the bile samples was also determined. A preliminary account of part of this work has already been presented[10].

MATERIALS AND METHODS

Solvents. All were of analytical grade, and were twice distilled through a fractionating column (500 cm long, 7 cm diam.) packed with Raschig rings, in an all glass apparatus with exclusion of light. They were stored in the dark, and used within 2 weeks of distillation. Diethyl ether and hexane were stored in ground-glass stoppered bottles containing anhydrous sodium sulphate. Purity of solvents was checked by evaporating 10 ml of solvent to dryness in a stream of nitrogen at 60°C, methylating and silylating the residue as described below, and after removal of the reagents dissolving the residue in 50 μl of hexane and injecting 4 μl onto the gas chromatograph at very low attenuation. No peaks were observed in the bile acid region,

* The terminology used throughout this paper follows "*Recommendations for Bile Salt Nomenclature*" drawn up by an *ad hoc* Committee in August, 1975, and published in "*The Hepatobiliary System*", W. Taylor, editor. Plenum Press, New York (1976) 639–641.

† The permitted trivial names used in the text are: lithocholic acid, 3α-hydroxy-5β-cholan-24-oate; deoxycholic acid, 3α,12α-dihydroxy-5β-cholan-24-oate and its 5α-epimer, allodeoxycholic acid, 3α, 12α-dihydroxy-5α-cholan-24-oate; chenodeoxycholic acid, 3α, 7α-dihydroxy-5β-cholan-24-oate and its 5α-epimer, allochenodeoxycholic acid, 3α,7α-dihydroxy-5α-cholan-24-oate; cholic acid, 3α, 7α, 12α-trihydroxy-5β-cholan-24-oate and its 5α-epimer, allocholic acid, 3α, 7α, 12α-trihydroxy-5α-cholan-24-oate. "Iso" or "epi" as prefixes are not permitted: e.g. the 3β-epimer of deoxycholic acid should not be referred to as 3β-isodeoxycholic acid (see Table 1).

and only minor impurities occurred near the injection peak.

Reference compounds. Authentic bile acids were provided by Professor J. Sjövall. The cholic acid used as the quantitation standard, obtained from Maybridge Chemicals, Tintagel, U.K., was at least 99% pure when assayed by gas chromatography. A standard solution of 0.45 mg/ml in ethanol was stored at 4°C.

Sephadex LH-20. (Pharmacia Fine Chemicals); 100 g were gently stirred with 500 ml 5% acetic acid in ethanol (v/v) and allowed to settle for 2 h. Fines were removed by decantation of the supernatant. After two further washings, the resin was filtered with gentle suction in a sintered-glass funnel. The resin was washed in the funnel twice more with ethanolic acetic acid and, with gentle stirring with a plastic rod, with 2l each of water, ethanol, methanol and acetone. The resin was placed in a shallow box made from Whatman 3MM paper sheets and gently agitated. Any lumps were broken up by mild pressure with a plastic rod until and resin flowed freely. Most of the acetone was removed by evaporation at room temperature. The resin was then heated in an oven at 55°C until no odour of acetone remained, and was stored at room temperature in plastic screw-top bottles.

Separation of nonsulphated and sulphated bile acids by chromatography on Sephadex LH-20. The resin (20 g) was equilibrated overnight at room temperature in a 500 ml ground-glass stoppered conical flask with 200 ml chloroform–methanol (1:1, v/v) made 0.01 M with respect to sodium chloride ($CHCl_3$–MeOH–NaCl).

Glass columns, 2 cm i.d. and 1 m long were fitted with a 2 cm coarse sintered-glass disc fused into the base, and had a Teflon–glass tap to control flow rate and a spherical reservoir. The column was filled with $CHCl_3$–MeOH–NaCl and the equilibrated Sephadex LH-20 was slowly poured into the column to ensure even settling of the resin and that no air-bubbles became trapped. During the filling of the column solvent was allowed to flow at 1 drop/s. When all the resin has been transferred the column was allowed to settle for 2 h with a solvent flow-rate of 1 drop/s. The bile sample (see below) dissolved in 5 ml $CHCl_3$–MeOH–NaCl was applied to the column without disturbance of the resin surface, and the sample container rinsed three times with 5 ml of the same solvent, the washings being added to the column when the solvent was about 2 mm above the surface of the resin. The column was eluted with 350 ml of the mixed solvent at 1 drop/s, and the eluate discarded. The sulphate fraction was eluted with 250 ml methanol.

The methanol was removed on a rotary evaporator at 50°C, and the residue was applied to a second column of Sephadex LH-20 which was eluted with 150 ml and 200 ml $CHCl_3$–MeOH–NaCl and then with 250 ml methanol. The 150 ml and 200 ml mixed solvent fractions were dried on the rotary evaporator and hydrolysed and analysed for bile acids (see below). Small amounts of bile acids were detected in the 150 ml fraction, but no peaks corresponding to bile acids were observed in the 200 ml fraction, and the sulphate fraction was then considered to contain no nonsulphated bile acids.

After solvolysis of the sulphates (see under *Analysis of bile*), this fraction was chromatographed on a 20 g column, and the fraction eluted by 350 ml $CHCl_3$–MeOH–NaCl was collected, evaporated to dryness and hydrolysed in ethanolic sodium hydroxide to yield the free bile acids, originally present as conjugated sulphates. These were analysed by quantitative g.l.c. and GC–MS as described below.

Gall-bladder bile was obtained from male and female rabbits and cats by withdrawing the bile from the gall-bladders of the animals with a hypodermic syringe immediately after the animals had been killed or anesthetized and the abdomen opened. The animals were not fasted for any specific periods before bile collection. The male rabbit bile pool was obtained from 12 animals, and the female bile from 7 animals. Two pools of cat bile were obtained; pool 1, male from 12 animals, female from 10 animals; pool 2, male from 9 animals, female from 12 animals. The bile was stored at −30°C before being analysed.

Thin-layer chromatography was carried out on plates precoated with Silica gel 60 (Merck, Darmstadt) without prior activation of the adsorbent[11]. Spots were visualized in iodine vapour, or by spraying with 10% phosphomolybdic acid in ethanol and warming the plates in an oven at 80°C.

Cholesterol was determined colorimetrically[12], but the freezing, storage and thawing of the bile prevented the determination of phospholipids because of inconsistent and non-reproducible results.

Gas chromatography (g.l.c.) and gas chromatography–mass spectrometry (GC–MS). The bile acids were analyzed as the trimethylsilyl ethers of their methyl esters (TMS ethers). Methylation was carried out with freshly prepared diazomethane added to samples dissolved in dry diethyl ether–methanol (9:1, v/v) at 0°C for 30 min. The solvent was removed in a stream of nitrogen at 45°C; the dry residue was stored at 4°C in toluene or immediately silylated with pyridine–trimethylchlorosilane–hexamethyldisilazane (3:1:2, by vol.) overnight at room temperature. The samples were dried in a stream of nitrogen at 55°C and dissolved at once in dry hexane. The precipitate was dispersed by ultrasonication, and the tubes centrifuged. The hexane was decanted into a clean tube, and the precipitate dispersed and extracted three more times with hexane. The combined hexane extracts were evaporated to dryness as before, and the residues immediately dissolved in dry hexane. In quantitative analyses, triplicate solutions of standard cholic acid were treated with each batch of bile samples.

Quantitative analyses were carried out on 2.1 m × 4 mm columns of 1% Hi-Eff 8 BP on 80–100 mesh, Gas-chrom Q: Applied Science Labs., State

College, PA, in a Pye 104 gas chromatograph: oven temperature, 225°C; injection port and detector temperature, 250°C; carrier gas, nitrogen at 30 ml/min; flame ionisation detection. Bile acids were quantitated by triangulation and the weights calculated on the basis of the standard cholic acid derivative. No allowance was made for the different responses of individual bile acids in the flame ionisation detector, nor for procedural losses.

GC–MS was carried out on 2.5 m × 3.4 mm columns of 1.5% Hi-Eff 8 BP on the bile acid TMS-ethers, with repetitive magnetic scanning as previously described[13].

Analysis of bile

Nonsulphated bile acids. Duplicate 100 μl samples of whole bile were hydrolysed in 6 ml 15% sodium hydroxide in aqueous ethanol (1:1) in screw-topped Teflon-lined steel vessels at 110°C for 10 h. Bile acids were extracted from the acidified hydrolysis mixture with ethyl acetate, and the ethyl acetate extracts were washed with water until neutral. The ethyl acetate was evaporated in a rotary evaporator at 50°C. The dry residues were methylated as described above, and the solution divided into two equal portions, one for quantitative g.l.c., the other for GC–MS at the Department of Chemistry, Karolinska Institute, Stockholm.

Bile acid sulphates. 5.0 ml Samples of pooled rabbit bile, and pools 1 and 2 of cat bile were added drop by drop to 50 ml acetone–ethanol (1:1, v/v) in a conical flask held in an ultrasonic bath. The mixture was incubated at 37°C for 17 h, and the precipitate removed by centrifugation. The precipitate was resuspended in 30 ml acetone–ethanol by ultrasonication and incubated at 37°C for 2 h. The resultant precipitate was extracted twice more in the same way, and the pooled extracts taken to dryness in a rotary evaporator at 50°C. The sulphates were obtained by chromatography of the residues on Sephadex LH-20 as described above.

The sulphates were hydrolysed in acetone–ethanol–HCl for 48 h at room temperature[4]. The now nonsulphated conjugated bile acids were hydrolysed and methylated as described above; one half of this fraction was used for quantitative g.l.c., and the other half for GC–MS.

A compound was considered to be fully characterized when the retention time (t_R) relative to methyl cholate-TMS and the fragment ion patterns were identical with those of known compounds. Because of the small amounts of some bile acids found, or lack of reference compounds, some bile acids were only partially identified. The t_R values and the *m/e* values of the ions used for identification are shown in Table 1.

RESULTS

Thin-layer chromatography of whole bile showed that the bile acids of rabbit bile were conjugated mainly with glycine, and those of cat bile with taurine.

Tables 2–5 show the results of the quantitative GC analysis of the nonsulphated and sulphated fractions of male and female rabbit bile. The sulphates were determined in only one pool of cat bile (Table 5). The cholesterol concentrations in whole bile are also included in the Tables.

Fragment ion chromatograms of the sulphate fractions of male and female rabbit and cat bile are shown in Fig. 1.

DISCUSSION

The rabbit and the cat provide two useful contrasting examples for investigating sex and species differences in bile acid metabolism. Both species do not have spontaneous estrus cycles, ovulation is induced by coitus, and environmental factors have only a minor effect on the state of estrus[14]. Therefore the effect of female sex hormones on bile acid metabolism

Table 1. Bile acids completely or partially identified in the nonsulphated and sulphated fractions of gall-bladder bile from male and female rabbits and cats (all acids identified were 5α- or 5β-cholanoates as trimethylsilyl ethers of their methyl esters).

Bile acid (-cholanoates)	t_R* Cholate	Ions used for qualitative† GC–MS analysis, *m/e*
3α,7α,12α-Trihydroxy-5α	0.82	623, 458, 368, 343, 261, 253
3α,7α,12α-Trihydroxy-5β	1.00	623, 458, 368, 343, 253
3α,12α-Dihydroxy-5α	1.21	535, 345, 255
Dihydroxy-5β (3, 12)?	1,31‡	255
	1.42‡	535, 345, 255
3α,12α-Dihydroxy-5β	1.63	535, 370, 345, 255
3α,12β-Dihydroxy-5β	1.71	535, 370, 345, 255, 208
3α,12α-Dihydroxy-5β	1.74	370, 262, 255, 249, 243
3α-Hydroxy-5α	1.89	462, 372, 357, 230, 215
3β,12α-Dihydroxy-5α	2.12	535, 460, 345, 255
3α-Hydroxy-5β	2.47	372, 357, 257, 230, 215

* Relative retention time (t_R) to the trimethylsilyl ether of methyl cholate. † The isotope peaks of the major fragment ions were sometimes used. ‡ Tentative; see text and Tables 2–5

Table 2. Nonsulphated bile acids and cholesterol in gall-bladder bile of rabbits

Bile acid (-cholanoates)	Male mg/ml	Male % of total	Female mg/ml	Female % of total
3α,7α,12α-Trihydroxy-5α	<0.1	—	<0.1	—
3α,7α,12α-Trihydroxy-5β	3.3	10.0	1.0	3.1
3α,12α-Dihydroxy-5α	2.6	7.9	2.6	8.0
Dihydroxy-5β (3, 12)?	<0.1*	—	<0.1*	—
3β,12α-Dihydroxy-5β	<0.1*	—	<0.1*	—
3α,12α-Dihydroxy-5β	27	82.1	29	89.0
3α,12β-Dihydroxy-5β	<0.1†	—	<0.1†	—
3α-Hydroxy-5β	<0.1	—	<0.1	—
TOTALS	32.9		32.6	
Cholesterol	5.8		6.4	

* Possible structure from mass spectrum and t_R relative to the derivative of cholic acid.
† Probable structure from mass spectrum and t_R.

Table 3. Sulphated bile acids in gall-bladder bile of rabbits

Bile acid (-cholanoates)	Male μg/ml	Female μg/ml
3α,7α,12α-Trihydroxy-5β	trace	trace
3α,12α-Dihydroxy-5α	27	31
3β,12α-Dihydroxy-5β	5	7
3α,12α-Dihydroxy-5β	92	88
3α,12β-Dihydroxy-5β	22†	17†
3α-Hydroxy-5α	2	5
3β,12α-Dihydroxy-5α	2*	2*
3α-Hydroxy-5β	10	21
TOTALS	160	171
% of nonsulphated bile acids	0.5	0.5

* Possible structure from mass spectrum and t_R relative to the derivative of cholic acid. † Probable structure from mass spectrum and t_R.

in these species should be minimal under normal laboratory conditions.

Bile acid metabolism in the rabbit has been the subject of many previous investigations[15–24]. The rabbit is herbivorous and produces bile acids conjugated mainly with glycine[24]; it has a very large bile acid pool compared to that of man and the rat[18], and has very high serum bile acid and cholesterol levels and can excrete up to 24% of its total bile acid and output as urinary metabolites[19]. Rabbit serum and liver contain high concentrations of 5α-cholestan-3β-ol (cholestanol)[21], a major precursor of allo bile acids[25, 26]. The rabbit produces a copious flow of bile under experimental conditions[27], although bile acid composition and concentration do not appear to be affected by normal rates of bile flow[24]. The bile acid composition of rabbit gall-bladder bile is less complex than that of other species for reasons discussed below. Studies on bile acid metabolism in the rabbit are complicated by the habit of this animal to indulge in coprophagy.

Table 5. Sulphated bile acids in Pool 1 of gall-bladder bile of cats

Bile acid (-cholanoates)	Male μg/ml	Female μg/ml
3α,7α,12α-Trihydroxy-5α	17	7
3α,7α,12α-Trihydroxy-5β	3	7
3α,12α-Dihydroxy-5α	120	83
3β,12α-Dihydroxy-5β	22	15
3α,12α-Dihydroxy-5β	70	57
3α,12β-Dihydroxy-5β	<0.2*	<0.2*
3α,7α-Dihydroxy-5β	16	19
3α-Hydroxy-5α	11*	4*
3β,12α-Dihydroxy-5α	95	20
3α-Hydroxy-5β	105	41
TOTALS	459	253
% of nonsulphated bile acids	2.0	0.9

* Probable structure from mass spectrum and t_R

Table 4. Nonsulphated bile acids and cholesterol in two pools of gall-bladder bile of cats

Bile acid (-cholanoates)	Male Pool 1 mg/ml	Male Pool 1 % of total	Male Pool 2 mg/ml	Male Pool 2 % of total	Female Pool 1 mg/ml	Female Pool 1 % of total	Female Pool 2 mg/ml	Female Pool 2 % of total
3α,7α,12α-Trihydroxy-5α	0.5	2.1	1.0	4.3	0.7	2.4	1.5	4.4
3α,7α,12α-Trihydroxy-5β	14.0	60.9	17.1	73.0	22.0	76.7	22.0	65.0
3β,12α-Dihydroxy-5β	<0.1	—	<0.1	—	<0.1	—	<0.1	—
3α,12α-Dihydroxy-5β	2.5	10.9	4.1	17.5	3.3	11.5	6.2	18.3
3α,7α-Dihydroxy-5β	1.0	4.3	1.2	5.1	2.7	9.4	4.2	12.4
3α-Hydroxy-5β	5.0	21.7	<0.1	—	<0.1	—	<0.1	—
TOTALS	23.0		23.4		28.7		33.9	
Cholesterol	4.2		3.6		6.0		5.8	

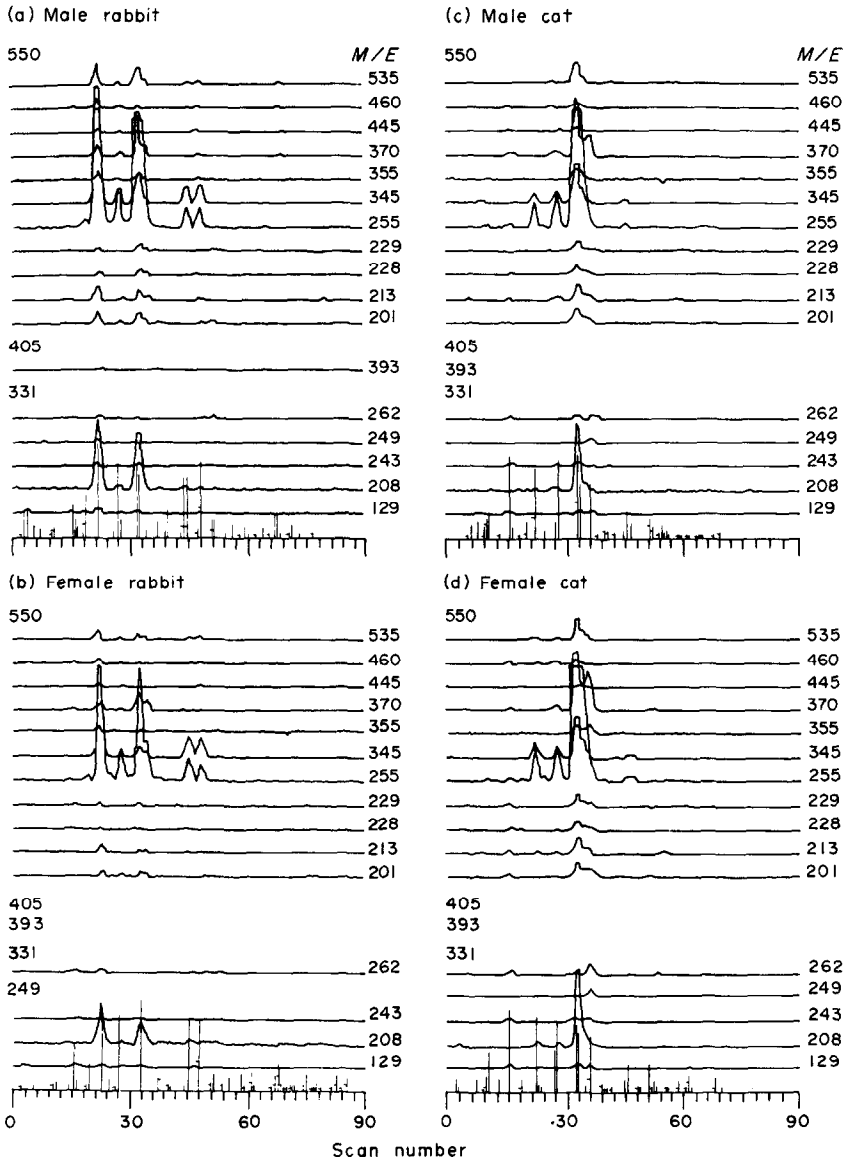

Fig. 1. Typical fragment ion current chromatograms constructed by the computer from the GC–MS analysis of the trimethylsilyl ethers of the methyl esters of bile acids of the sulphate fraction obtained from (a), male rabbit; (b), female rabbit; (c), male cat; (d), female cat. Column packing 1.5% Hi-Eff 8 BP. The first scan was started 7 min after injection of the sample. The interval between scans was 10.2 s and was increased by 0.6 s/min retention time. The scan numbers are not exactly the same for corresponding compounds.

This may be the reason why the rabbit has a profuse intestinal microflora, and it is probable that these microorganisms begin to metabolize bile salts higher up in the duodenum than in most other species[18].

However, in those earlier studies the methods used for bile acid analysis were less sensitive and specific than those used in the present study, and no previous investigation of sulphated bile acids (B.A.S.) has been reported.

Although the cat has been much used in the investigation of gastric and pancreatic function, nothing appears to be known about the nature or amounts of bile acids in cat urine, serum or feces. Analysis of cat bile by conventional chemical techniques has established that it contains cholic acid, and that its bile acids are conjugated almost entirely with taurine[1, 2].

Bile acid metabolism in rabbits

The data in Table 2 confirm and extend the results of previous investigations which showed that deoxycholic acid is quantitatively the major bile acid in gall-bladder bile of normal rabbits[16–19, 21, 24]. Allodeoxycholic acid has also been detected in rabbit bile[21], but it does not appear to have been realised that the quantities present are of the same order as those of cholic acid (Table 2). The allodeoxycholic acid could be derived from allocholic acid (see below)

or deoxycholic acid or from both. When labeled deoxycholic acid was administered to rabbits the specific activity of the allodeoxycholic acid and 3β,12α-dihydroxy-5β-cholanoic acid in the feces was about 70% of that of the deoxycholic acid in the same sample of feces[21]. Therefore considerable conversion of deoxycholic acid to its 5α-isomer can occur in the rabbit, and this may account for the large amount of allodeoxycholic acid found in gall-bladder bile (Table 2). Allodeoxycholic acid and its 3β-epimer, 3β,12α-dihydroxy-5α-cholanoic acid, are present in the B.A.S. fraction (Table 3). The functions of allo bile acids are still obscure[25, 26], but the presence of such large amounts of allodeoxycholic acid might be a positive disadvantage, since this acid is a major constituent of gall-stones induced in rabbits fed on a diet rich in cholestanol[22]. Another factor which would tend to favour gallstone formation in rabbits is that the principal bile acid in the mixed micelles of rabbit bile will be deoxycholic acid. These micelles would have a greater molecular weight and lower polarity than those containing trihydroxy bile acids, such as cholic acid, and rabbits should be more liable than other species to develop gallstones, but this is not so[24]. No gallstones were found in any of the 19 rabbits used in the present investigation.

Cholic and chenodeoxycholic acids are considered to be the major primary bile acids produced by most vertebrates[1, 2]. However, chenodeoxycholic acid could not be detected in rabbit gall-bladder bile in this and some earlier studies. An early report tentatively suggested that chenodeoxycholic acid is formed from cholesterol by rabbit liver[16]. Later, the secretion of this primary bile acid in rabbit bile was confirmed in two series of experiments. In the first, bile from two germ-free rabbits was found to have the following percentage composition: cholic acid, 93.4 and 94.2; allocholic acid 4.4 and 5.1; chenodeoxycholic acid 2.2 and 0.7. Of further interest was the relatively large amount of allocholic acid present in the bile, since the animals had been fed on a diet containing only about 0.1% by weight of cholestanol[23]. Therefore, under these circumstances allocholic acid must be a primary bile acid synthesized from cholesterol. In the present study only trace amounts of allocholic acid were found in gall-bladder bile, and 7α-dehydroxylation of this acid by intestinal microflora would add to the allodeoxycholic acid produced from deoxycholic acid as discussed above.

In the second series of experiments bile was collected from rabbits by cannulation of the bile duct. The composition of the bile changed with time, and by the fourth to fifth days after the cannulation the glycocholic acid increased to 91% and glycochenodeoxycholic acid to 5.8%, while the glycodeoxycholic acid decreased to 2.2–5.8% of the total bile acids[24].

Several bile acids have been identified in rabbit feces[21]. These are produced mainly by the action of the intestinal microflora on deoxycholic and lithocholic acids. The 3β-hydroxy-5β-cholanoic and lithocholic acids were probably derived from chenodeoxycholic acid, which partially explains the absence of the latter acid from the gall-bladder bile. Other acids, thought to be derived from cholic acid were: 3β-hydroxy-12-keto-5β-cholanoic, 3α-hydroxy-12-keto-5β-cholanoic, 3β, 12α-dihydroxy-5β-cholanoic and deoxycholic acids, as well as smaller amounts of allodeoxycholic acid. These acids are the same as, or closely related to, those found in bile (Tables 2 and 3). The 12-keto acids could be reduced in the liver to the corresponding 12α- or 12β-hydroxy acids. However, the possible presence of 12-keto acids in bile cannot be entirely ruled out, since these acids tend to be destroyed by the hydrolysis procedure used in the present work[5].

The presence of at least two isomers of deoxycholic acid (3β, 12α and 3α,12β and the partially identified isomer of t_R 1.31, Table 2) in both bile and feces is a reflection of the predominance of this acid in gall-bladder bile. These isomers are presumably produced by dehydrogenation of the 3α- and 12α-hydroxyl groups by the intestinal microflora to yield the corresponding 3- and 12-keto acids. Small amounts of these keto acids could be reduced in the intestine and enter the enterohepatic circulation, or the keto acids themselves could be reabsorbed reabsorbed from the gut and reduced to α- and β-hydroxy acids in the liver. Further work with labeled compounds is required to resolve the finer details of the formation of these isomers of deoxycholic acid.

Some bile acids were found in the B.A.S. fraction (Table 3) but not in the nonsulphated fraction (Table 2). Deoxycholic and allodeoxycholic acids were the predominant acids in both the nonsulphated and B.A.S. fractions, but it is unlikely that these acids in the B.A.S. fraction were contaminants from the nonsulphated fraction, because of the multiple Sephadex LH-20 separations carried out. In contrast, cholic acid, a major constituent of the nonsulphated fraction (Table 2) was only just detectable in trace amounts in the B.A.S. fraction (Table 3).

It is of interest that three of the eight acids in the B.A.S. fraction (Table 3) were allo bile acids.

A striking feature of the results obtained with rabbit bile is the very close similarity between the composition of both the nonsulphated and B.A.S. fractions from male and female bile, particularly since the bile samples represent the mean composition of twelve male and seven female rabbits. The similarity applies to both the qualitative and quantitative composition of the bile of the two sexes, in marked contrast to the rat[9] and the cat (see below). There was also no marked sex difference in the cholesterol content of the bile.

In conclusion, it may be said that the rabbit has an unusual gall-bladder bile composition which is the result of the low capacity of rabbit liver to synthesize chenodeoxycholic acid from cholesterol or recirculating deoxycholic acid, and a highly active intestinal microflora with the ability to convert deoxycholic

acid into allodeoxycholic acid. The absence of any sex difference in bile acid composition may be in part due to type of estrus cycle of this animal.

Bile acid metabolism in cats

The composition of gall-bladder bile of cats has not been reported previously, although the major nonsulphated bile acids have been isolated[1, 2]. Table 4 shows that cat bile has the normal nonsulphated bile acids typical of mammalian bile. The almost complete absence of the 3β- and 12β-isomers of deoxycholic acid may be a reflection of the different intestinal microflora of cats and rabbits and/or differences in the enterohepatic circulation of bile acids formed in the gut because of the different anatomy of the gastrointestinal tract of the cat and rabbit. Since no information about endogenous fecal bile acids or metabolism of exogenous bile acids in the cat is available, comparisons of biliary and fecal bile acids such as those made for the rabbit cannot be made for the cat.

In contrast to the rabbit, cat bile contains appreciable amounts of allocholic acid (cf. Tables 2 and 4), probably derived from dietary cholestanol.

There is an indication that cats exhibit a sex difference in the amounts of nonsulphated bile acids and of cholesterol in gall-bladder bile. Female cats have a higher concentration of bile acids, mainly cholic acid, and of cholesterol than do male cats (Table 4). The estrus state of the animals from which the bile was collected was not assessed, but none were pregnant. Therefore, the possibility that the sex difference is due to differences in sex hormones cannot be assessed at this stage.

The pattern of bile acids in the B.A.S. fraction of cat bile (Table 5) was much more complex than that of rabbit bile (Table 3). An important feature of the cat bile B.A.S. fraction is the very small amount of cholic acid present. This finding justifies the conclusion that the multiple Sephadex LH-20 fractionation method used completely separated the sulphated from the nonsulphated bile acids, and that the relatively large amounts of deoxycholic acid in the B.A.S. fraction of rabbit bile (Table 3) were not due to contamination of the sulphate fraction with nonsulphated bile acids. Although Sephadex LH-20 has been much used for the separation of sulphated and nonsulphated bile acids, it is now known that this resin is not entirely suitable for that purpose. Sephadex LH-20 columns tend to be easily overloaded, to suffer from 'memory' effects and to produce artifacts. The liphophilic anion exchange resin diethylaminohydroxypropyl Sephadex LH-20[28] used in the analysis of rat bile[9] was not available in this laboratory. However, the disadvantages of Sephadex LH-20 were minimized in the present study by the washing of the resin before use, excluding light by enclosing the columns in aluminium foil, using 20 g of resin for 5 ml bile and applying the B.A.S. fractions from the first column to a second 20 g column.

The major bile acids in the B.A.S. fraction (Table 5) are allodeoxycholic and lithocholic acids. The presence of so much of the latter acid in the sulphated form is in accord with the concept that this hepatotoxic bile acid is sulphated to protect the liver[3, 4]. It is probable that the 5α-bile acids in the B.A.S. fraction are formed, at least in part, by the mechanism involving conversion of the saturated A-ring to a 3-one-4-ene structure with subsequent reduction of this structure to 5- and 3-epimeric bile acids by the intestinal microflora and/or by the liver[29, 30].

In contrast to the rabbit, there are appreciable sex differences between the amounts of B.A.S. in the cat, and also in the amount of these sulphates expressed as a percentage of sulphates. This is the reverse of the situation in the rat[9].

CONCLUSIONS

Sulphation of hydroxylated compounds by the liver and other tissues is a well-established method of detoxication of a wide range of endogenous and xenobiotic compounds[31]. The sulphation of the hydroxyl groups of bile acids, therefore, is not a specific phenomenon: metabolites of steroid hormones, for example, also undergo sulphation and biliary excretion in many mammalian species[27]. It is also not surprising that some hydroxy bile acids may also be converted into highly polar glucuronides[8]. No search for bile acid glucuronides was made in the present investigation. The question arises as to what may be the advantage, if any, derived from converting bile acids into sulphates and glucuronides, apart from increasing their polarity and water-solubility. A reduction in hepatotoxicity is one obvious advantage; lithocholic acid sulphate is considerably less toxic than the nonsulphated bile acid[3, 4]. However, the B.A.S. fractions of rabbit and cat contain the sulphated forms of bile acids which, as far as we know, are not hepatotoxic; e.g. cholic and deoxycholic acids. Nevertheless, in the B.A.S. fractions of the rabbit and cat, and of the female rat[9], allo bile acids tend to predominate; e.g. allodeoxycholic acid is a major component of the B.A.S. fraction of cat bile (Table 5), although this acid is not found in the nonsulphated fraction (Table 4); allochenodeoxycholic acid is the major bile acid in the B.A.S. fraction of female rat bile[9]. Little appears to be known about the hepatotoxicity of these bile acids. One possible explanation of the sulphation (and glucuronidation) of certain bile acids is that these bile acids which are partly reabsorbed in the enterohepatic circulation, but mainly excreted in the feces, are preferentially sulphated in the liver by relatively specific enzyme systems which can distinguish between nontoxic and toxic bile acids. Such sulphates, and glucuronides, could be more readily excreted by the kidney although it is evident that some are also excreted in bile.

In cholestatic conditions, the normal, non-toxic bile acids accumulate in the liver and would tend to satu-

rate the sulphating enzyme systems so that these normal bile acids would be sulphated (and conjugated with glucuronic acid) and be excreted by the kidney. This would account for the great increase in bile acid sulphates (and glucuronides) found in the serum and urine of patients with cholestatic liver disease[6–8].

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