The effect of cholesterol feeding on gallbladder bile acids of the rabbit

Evidence that lithocholic acid is a primary bile acid in the rabbit

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The bile acids in gallbladder bile of rabbits fed a normal diet or one containing 2% (w/w) cholesterol have been determined by gas chromatography–mass spectrometry. The predominant bile acids in normally fed rabbits were 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid (cholic acid), 3 α ,12 α -dihydroxy-5 α -cholan-24-oic acid (allodeoxycholic acid) and 3 α ,12 α -dihydroxy-5 β -cholan-24-oic acid (deoxycholic acid) with very much smaller amounts of 3 α -hydroxy-5 β -cholan-24-oic acid (lithocholic acid) and 3 α ,12 β -dihydroxy-5 β -cholan-24-oic acid. In the cholesterol-fed animals the lithocholate became a predominant bile acid. Sulphated bile acids accounted for less than 1% of the total bile acids. It is proposed that lithocholic acid may be a primary bile acid in the cholesterol-fed rabbit, formed by an alternative pathway of biosynthesis involving hepatic mitochondria.

The bile acid composition of normal rabbit gallbladder bile is markedly different from that of many other mammalian species, including man. Deoxycholic acid (3 α ,12 α -dihydroxy-5 β -cholan-24-oic acid) accounts for over 80% of the total bile acids, of which only 10% are accounted for by cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid). Chenodeoxycholic acid (3 α ,7 α -dihydroxy-5 β -cholan-24-oic acid), a primary bile acid in many species, is present, if at all, in very small amounts in rabbit gallbladder bile (Fischer *et al.*, 1974; Taylor, 1977). Even more unusual is the presence of allodeoxycholic acid (3 α ,12 α -dihydroxy-5 α -cholan-24-oic acid) (Danielsson *et al.*, 1963), the amounts of which exceed those of cholic acid in some samples (Taylor, 1977). Lithocholic acid (3 α -hydroxy-5 β -cholan-24-oic acid), normally considered to be the secondary bile acid of chenodeoxycholic acid, is present in rabbit gallbladder bile only in trace amounts (Taylor, 1977) but can account for 0–20% of rabbit faecal bile acids (Hellström & Sjövall, 1962; Fischer *et al.*, 1974).

During the course of a study on the effects of feeding rabbits a cholesterol-enriched diet to induce changes in biliary lipid composition, changes in

individual bile acids have been investigated. The present paper describes the changes which were observed. In the cholesterol-fed rabbit lithocholic acid can account for as much as 20% of the total bile acids. The results raise questions about the biosynthesis of bile acids from cholesterol in the rabbit.

Materials and methods

Source of bile samples

Bile samples were obtained from the gallbladders of male New Zealand White rabbits fed a normal diet (six animals) and from eight animals fed the normal diet enriched with 2% (w/w) cholesterol. The animals weighed 2.5–3 kg, and were killed after 10 weeks of treatment. Bile from individual animals was stored at –20°C before being processed.

Determination of bile acids

Total bile acids in 0.10 ml of bile were determined by g.l.c. as described by Taylor (1977). Mono-, di- and tri-hydroxy bile acids were quantified by triangulation using lithocholic, deoxycholic and cholic acid standards, respectively. Full details of g.l.c. and g.l.c.–m.s. methods have been described by Taylor (1977).

Abbreviation used: g.l.c.–m.s., gas chromatography–mass spectrometry.

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Separation of sulphated and non-sulphated bile acids

Equal portions of individual bile samples were pooled to give 2.0 ml of bile from each group of animals. These were added to 20 ml of ethanol with ultrasonication, incubated for 17 h at 37°C and then centrifuged. The precipitates were resuspended in ethanol and incubated for 2 h at 37°C twice more. The combined supernatants were dried in a rotary evaporator at 50°C. The dry residues were transferred to columns (20 g) of Sephadex LH-20, equilibrated and eluted with chloroform/methanol (1:1, v/v) made 0.01 M with respect to NaCl. Non-sulphated bile acids were eluted with 350 ml of the mixed solvent. Sulphated bile acids were eluted with 200 ml of methanol. Full details of the method have been described by Taylor (1977). The bile acid sulphates were solvolysed by incubation with acidified ethyl acetate for 17 h at 37°C. The hydrolysed sulphate fractions and the non-sulphated fractions were then analysed by g.l.c.

Identification of lithocholic acid

The occurrence during g.l.c. of a large peak corresponding to lithocholic acid in the non-sulphated fraction obtained from cholesterol-fed animals was surprising (see the Results section), and demanded fuller investigation. Two bile samples from cholesterol-fed rabbits (1.0 ml of each) containing 18% and 20% of apparent lithocholic acid respectively were pooled, diluted with 10 ml of ethanol/water (7:3, v/v) and extracted four times with *n*-hexane to remove cholesterol and other non-polar lipids. The aqueous ethanolic solution was taken to dryness in a rotary evaporator at 50°C. The dry residues were dissolved in 1.0 ml of water and 0.1 ml was hydrolysed as described above. The hydrolysed bile acids were combined and methylated twice with diazomethane. The methylated bile acids were separated into mono-, di- and tri-hydroxy fractions on Sephadex LH-20 by the method of Summerfield *et al.* (1976). G.l.c. of a portion of the monohydroxy fraction showed contamination of the lithocholic acid with small amounts of other bile acids. Most of these contaminants were removed by passing the monohydroxy fraction through a second column of Sephadex LH-20. G.l.c. of the fraction showed a major peak corresponding to lithocholic acid, estimated to be 95–97% pure.

Mass spectrometry of the isolated and of standard methyl lithocholate was carried out by direct inlet, with the probe temp. raised from 150–200°C.

Statistical analysis

The significance of differences (*P*) between bile acids in control and cholesterol-fed animals was assessed by the two-tailed Student's *t*-test; the level of significance was set at *P* < 0.05.

Results

Fig. 1 shows the g.l.c. analysis of samples of gallbladder bile typical of rabbits fed a normal diet or a diet containing 2% (w/w) cholesterol. In the former animals the predominant compounds were cholic, allodeoxycholic and deoxycholic acids, with only a small amount of lithocholic acid. In the cholesterol-fed rabbits the same major bile acids were present but a compound with the same retention time as lithocholic acid also became predominant. The amounts of the individual bile acids in the gallbladder biles of both groups of animals, expressed as $\mu\text{mol/ml}$ and as a percentage of the total bile acids, are shown in Table 1. There were no significant differences between the values for cholic and allodeoxycholic acids of the two groups. The concentration of deoxycholic acid was the same in the two groups of animals, but there was a significant decrease in the percentage of deoxycholic acid in the cholesterol-fed rabbits. Both 3 α ,12 β -dihydroxy-5 β -cholanoic and lithocholic acids increased significantly in the cholesterol-fed group in

Fig. 1. Gas-chromatographic separation of gallbladder bile acid methyl esters as trimethylsilyl ether derivatives from rabbits fed a normal diet (a), and those fed the same diet containing 2% (w/w) cholesterol (b).

The following compounds are indicated: 1, cholate; 2, allodeoxycholate; 3, deoxycholate; 4, 3 α ,12 β -dihydroxy-5 β -cholanoate; 5, lithocholate.

Table 1. *Bile acid composition of gallbladder bile of rabbits fed a normal diet (controls) or the same diet supplemented with 2% (w/w) cholesterol for 10 weeks*

Results are expressed as $\mu\text{mol/ml}$ of bile, and the amounts of individual bile acids as a percentage of the total (means and s.e.m.). The significance of difference was calculated by a two-tailed Student's *t*-test. *P* values are given in parentheses. Abbreviation: N.S., not significant.

	Composition in:			
	Controls (<i>n</i> = 6)		Cholesterol-fed (<i>n</i> = 8)	
	($\mu\text{mol/ml}$)	(% total)	($\mu\text{mol/ml}$)	(% total)
Cholic acid	19.7 $\pm$ 4.76	9.1 $\pm$ 1.94	19.1 $\pm$ 3.00 (N.S.)	7.2 $\pm$ 1.00 (N.S.)
Allodeoxycholic acid	14.9 $\pm$ 1.75	7.3 $\pm$ 0.93	14.0 $\pm$ 1.68 (N.S.)	5.4 $\pm$ 0.64 (N.S.)
Deoxycholic acid	169.1 $\pm$ 12.4	80.9 $\pm$ 1.34	171.4 $\pm$ 12.5 (N.S.)	65.2 $\pm$ 3.10 (0.001)
3 α ,12 β -Dihydroxy-5 β -cholan-24-oic acid	0 $\pm$ 0	0 $\pm$ 0	17.5 $\pm$ 1.00 (0.001)	6.9 $\pm$ 0.60 (0.001)
Lithocholic acid	5.9 $\pm$ 1.13	2.7 $\pm$ 0.42	41.7 $\pm$ 10.76 (0.013)	15.4 $\pm$ 3.53 (0.009)
Total bile acids	209.6 $\pm$ 15.8		263.7 $\pm$ 15.0 (0.03)	

Table 2. *Sulphated bile acids in pooled gallbladder bile of rabbits fed a normal diet (controls) or the same diet supplemented with 2% (w/w) cholesterol for 10 weeks*

Results are expressed as nmol/ml of bile and the amounts of individual bile acids as a percentage of the total.

Bile acid	Amount of sulphated bile acids in:			
	Controls		Cholesterol-fed	
	(nmol/ml)	(% total)	(nmol/ml)	(% total)
Cholic	0	0	158	10.0
Allodeoxycholic	184	17.6	155	9.8
Deoxycholic	626	59.9	908	57.3
3 α ,12 β -Dihydroxy-5- β' -cholan-24-oic	149	14.3	201	12.7
Lithocholic*	71	6.8	90	5.7
3 β ,12 α -Dihydroxy-5- α -cholan-24-oic	15	1.4	35	2.2
3 β -Hydroxychol-5-en-24-oic	0	0	36	2.3
Total	1045		1583	
Percentage of total bile acids	0.5		0.6	

* Probable structure from mass spectrum and retention time.

terms of concentration and as a percentage of total bile acids. The total concentration of bile acids was slightly but significantly higher after cholesterol feeding. This increase is almost completely accounted for by the high lithocholic acid in cholesterol-fed animals.

Sulphated bile acids determined in a pool of bile from each group accounted for only 0.5–0.6% of the total bile acids (Table 2). Bile from both groups contained sulphated 3 β ,12 α -dihydroxy-5 α -cholan-24-oic acid, an allo compound not found in the non-sulphated bile acid fraction. The only qualitative difference between the normally fed and cholesterol-fed animals was the appearance in the bile from

the latter group of cholic and 3 β -hydroxychol-5-en-24-oic acids. Otherwise there were no major differences between the two groups of animals. It is particularly surprising that only a small amount of lithocholate was present in the sulphated form.

Because of the large amounts of lithocholic acid in the non-sulphated fraction of cholesterol-fed rabbit bile it was possible to obtain an almost pure sample of the methyl ester of this acid for analysis by direct probe mass spectrometry. The spectrum of the isolated compound was identical to that of methyl lithocholate. The molecular ion peak (*M* = 390) was very small because of the loss of the hydroxyl group. Major peaks were observed at *m/z*

372 ($M-18$) due to loss of water, and at m/z 215 ($M-175$) due to loss of the ABC ring plus water. Both spectra were virtually identical to that of methyl lithocholate described by Sjövall *et al.* (1971). Therefore the identity of the isolated compound was confirmed by mass spectrometry and retention time relative to that of cholic acid on g.l.c. The identity of the other bile acids using fragment-ion g.l.c.-m.s. has been described by Taylor (1977).

Discussion

There is convincing evidence that gallbladder bile of rabbits fed a normal diet contains mainly glycine conjugates of deoxycholic, cholic and allodeoxycholic acids with smaller amounts of $3\alpha,12\beta$ -dihydroxy-5 β -cholan-24-oic and lithocholic acids (Hofmann *et al.*, 1968; Fischer *et al.*, 1974; Taylor, 1977) (Table 1). Sulphated bile acids account for only about 0.5% of the total bile acids (Taylor, 1977) (Table 2). The absence of the primary bile acid, chenodeoxycholic acid, which occurs in gallbladder bile of most mammals, including man, is another peculiarity of rabbit gallbladder bile. However, rabbit liver is able to synthesize chenodeoxycholic acid. Gallbladder bile from two germ-free rabbits had the following percentage composition of bile acids: cholate, 93.4 and 94.2; allocholate, 4.4 and 5.1; chenodeoxycholate, 2.2 and 0.7 (Hofmann *et al.*, 1969). Those authors suggested that in germ-free rabbits allocholic acid must be a primary bile acid synthesized from cholesterol. Gregg & Foley (1966) obtained rabbit hepatic bile by cannulation of the hepatic bile duct. By the fifth day after cannulation, when the animals would have been almost completely cleared of secondary bile acids, the percentage composition of the conjugated bile acids was: cholate, 91; chenodeoxycholate, 5.8; deoxycholate, 2.2–5.8. Further evidence that cholic acid is the major primary bile acid in the rabbit was obtained by Hofmann *et al.* (1968). When rabbits were fed with the antibiotic neomycin the composition of the gallbladder bile was different from that of untreated animals. Consistent results were not obtained. In two of eight animals deoxycholate was almost completely replaced by cholate; in two animals deoxycholate was significantly decreased, but there was no change in the bile acid pattern of the other four rabbits. Neomycin affects only the coliform intestinal bacteria, and furthermore can sequester bile acids. Obligatory anaerobes able to dehydrate cholic acid at C-7 have been isolated from rabbit faeces by Mosbach *et al.* (1967). Of the above-mentioned authors only Taylor (1977) and Table 1 of the present paper showed the presence of lithocholate in normal rabbit gallbladder bile, probably because highly sensitive and specific g.l.c.-m.s. methods were used.

Cholic acid is the predominant primary bile acid synthesized from cholesterol in rabbit liver. In the light of current concepts about the biosynthesis of bile acids (Danielsson, 1976), and the rate-limiting role of cholesterol 7 α -hydroxylase in bile acid synthesis (Myant & Mitropoulos, 1977) it is difficult to explain the relatively small amounts of chenodeoxycholic acid formed in rabbit liver except by supposing that this tissue contains a highly active 12 α -hydroxylase system which prevents the accumulation of chenodeoxycholate. The predominant secondary bile acid in rabbit bile is deoxycholate produced by the intestinal microflora, which shows that active dehydroxylating micro-organisms occur in the rabbit intestine and that rabbit liver has a low capacity for 7 α -hydroxylation of secondary bile acids. Therefore it is possible that chenodeoxycholate is not detectable in rabbit gallbladder bile because this bile acid is 7 α -dehydroxylated in the gut, without subsequent 7 α -hydroxylation of the re-absorbed lithocholate in the liver. Fischer *et al.* (1974) fed rabbits diets containing 0.05 and 0.5% of chenodeoxycholate or lithocholate. At both doses of chenodeoxycholate both lithocholate and chenodeoxycholate were found in gallbladder bile. After lithocholate feeding no chenodeoxycholate was found, indicating that of the large amounts of lithocholate presented to the liver none was 7 α -hydroxylated to chenodeoxycholate, results corresponding to those found in the present investigation in cholesterol-fed rabbits.

Mitropoulos & Myant (1967*a,b*) and Danielsson (1976) have suggested an alternative pathway of bile acid synthesis from cholesterol which does not depend upon initial 7 α -hydroxylation, and which may produce lithocholate as a primary bile acid. This pathway may have been followed by at least some of the cholesterol in the present investigation, but experiments with germ-free or bile-fistula animals would be required to provide direct evidence that lithocholate is a primary bile acid in cholesterol-fed rabbits.

Another feature of bile acid metabolism in rabbits may contribute to the amounts of lithocholate which accumulate in the bile after cholesterol feeding. In man and many other species any lithocholate which returns to the liver via the enterohepatic circulation and which is not hydroxylated to chenodeoxycholate is conjugated with taurine or glycine and then almost completely sulphated. These conjugated sulphates are not reabsorbed from the intestine to any appreciable extent and so are excreted in the faeces (Palmer, 1967, 1976). After feeding lithocholate or chenodeoxycholate to rabbits, Fischer *et al.* (1974) could not detect any sulphated bile acids in the bile. This inability of the rabbit to sulphate bile acids to any appreciable extent has been confirmed by Taylor (1977) and by the present work (Table 2).

Therefore the large amounts of lithocholate in the bile of cholesterol-fed rabbits may arise from reabsorption of the non-sulphated form of that bile acid.

In conclusion, the rabbit has a poor ability to secrete chenodeoxycholic acid and to sulphate lithocholic acid. The evidence presented strongly suggests that at least part of the bile acids synthesized in rabbit liver may arise from the alternative pathway involving C-26 hydroxylation of cholesterol and further metabolism in the mitochondria. However, the problem is complicated by the anatomy of the gastrointestinal tract of the rabbit, and the habit of that animal of indulging in coprophagy. Further work with germ-free or bile-fistula animals is required to clarify the situation.

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