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On the Bile Acid Metabolism in the Pig

Bile Acids and Steroids 81

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Pig bile, collected from bile fistulas at different times after fistulation, has been analyzed. The main bile acids found in samples collected during the first day after fistulation were chenodeoxycholic, hyocholic and hyodeoxycholic acid, conjugated with glycine and to a small extent with taurine. Bile samples collected later than 24 h after fistulation did not contain hyodeoxycholic acid, indicating that this acid is formed by the action of intestinal microorganisms during the enterohepatic circulation of the bile acids.

This was confirmed by the results of studies on the metabolism of labelled chenodeoxycholic, hyodeoxycholic and hyocholic acid in intact and bile-fistulated pigs.

24-^{14}C-chenodeoxycholic acid was 6α-hydroxylated to hyocholic acid in the fistulated pig, while 24-^{14}C-hyodeoxycholic acid was not metabolized except for conjugation. After circulating enterohepatically in intact pigs for 3 days, 50 % of tritium-labelled hyocholic acid had been transformed into hyodeoxycholic acid.

The metabolism of the main bile acids in the pig could then be summarized as follows:

$$\text{Chenodeoxycholic acid} \xrightarrow{\text{liver}} \text{hyocholic acid}$$

$$\text{Chenodeoxycholic acid} \xrightarrow[\text{microorganisms}]{\text{intestinal}} \text{hyodeoxycholic acid}$$

The main bile acids found in gall-bladder bile of pigs are hyocholic, hyodeoxycholic and chenodeoxycholic acid. They are present as conjugates with glycine and to a small extent with taurine [1]. Hyodeoxycholic (3α, 6α-dihydroxycholanic) acid was identified in 1923 by Windaus and Bohne [2]; the presence of chenodeoxycholic (3α, 7α-dihydroxycholanic) acid was observed in 1939 by Ido and Sakurai [3], but it was not until 1954 that Haslewood and Sjövall [4] demonstrated the presence of considerable amounts of a trihydroxy bile acid. Its structure was shortly thereafter shown to be 3α, 6α, 7α-trihydroxycholanic acid [5,6].

The present investigation is concerned with the metabolism of these three bile acids in the pig. We have prepared labelled hyocholic, hyodeoxycholic and chenodeoxycholic acid and injected them intraperitoneally into pigs. The bile acids were isolated from the bile by chromatography and the identity of the labelled compounds was established by repeated chromatography and cocrystallization with authentic material.

MATERIAL AND METHODS

Labelled compounds. 24-^{14}C-labelled chenodeoxycholic and hyodeoxycholic acid were prepared according to Bergström *et al.*[7] Hyocholic acid was labelled with tritium gas using the method of Wilzbach [8] in the apparatus described by Bergström and Lindstedt [9]. The labelled bile acids were purified by reversed phase partition chromatography in suitable systems (see below) and crystallized to constant specific activity with unlabelled material.

Chromatographic procedures. Reversed phase partition chromatography as described by Sjövall [10] and Norman [11] was used for the separations of the bile acids. The following solvent systems were employed:

	Moving phase	Stationary phase
C.	150 ml methanol	15 ml chloroform
	150 ml water	15 ml *iso*-octanol
D.	300 ml water	100 ml *n*-butanol
F.	165 ml methanol	45 ml chloroform
	135 ml water	5 ml heptane

Hyflo Supercel (Johns Manville and Co., USA) or Hostalene (Farbwerke Hoechst, G.m.b.H., West-Germany) [12] was used as supporting material. The procedure for making Hyflo Supercel hydrophobic has been described [10]. Prior to use, Hostalene was extracted with 95 % ethanol for 48 h in a Soxhlet extractor and then dried at 75°. Columns were prepared as described by Norman [11]. When Hostalene was used as supporting material only 3 ml of stationary phase were used per 4.5 g of this material.

Radioactivity assay. ^{14}C was determined with a Tracerlab end-window counter, tritium with a Tracerlab gas-flow counter.

Animal experiments and procedures for analysis of the bile. The bile acid was injected intraperitoneally in the form of its sodium salt, dissolved in about 10 ml of saline.

Young female pigs weighing about 20 kg were used. Bile fistulas were prepared using trichloroethylene anaesthesia by inserting a polyethylene cannula into the gall-bladder and tying off the ductus choledochus. During the collection of bile from the fistula the animals were restrained in a cage, to which they had previously been conditioned for approximately a week. The animals had free access to drinking water and were fed their normal diet. Bile was collected for 4 h periods into 95 % ethanol. After determination of their radioactivity, the bile samples were diluted with water, acidified with hydrochloric acid and extracted twice with water-saturated butanol. The combined butanol extracts were washed with small volumes of water until neutral and evaporated to dryness *in vacuo*. The residue was chromatographed in phase system C, the effluent was titrated with 0.02 N methanolic sodium hydroxide, and suitable aliquots were assayed for radioactivity. For purpose of further identification the conjugated bile acids were hydrolyzed with N NaOH in a closed steel tube for 6 h at 120°. The saponification mixtures were diluted with water, acidified with hydrochloric acid and extracted twice with ether. The combined ether-extracts, that contained the free bile acids, were washed until neutral with water, and evaporated to dryness. The residue was chromatographed on phase systems C or F, depending upon the acid being purified. The labelled bile acids were diluted with carrier to a weight of approximately 100 mg and crystallized four times from different solvents to a final weight of crystals of about one quarter the original weight. Hyocholic acid was crystallized from ethyl acetate / methanol / heptane, acetone/water, acetic acid/water and acetone/water; hyodeoxycholic acid from ethyl acetate / methanol / heptane, ethyl acetate, acetic acid / water and acetone / water; chenodeoxycholic acid from ethyl acetate / light petroleum, acetic acid / water and ethyl acetate / light petroleum twice.

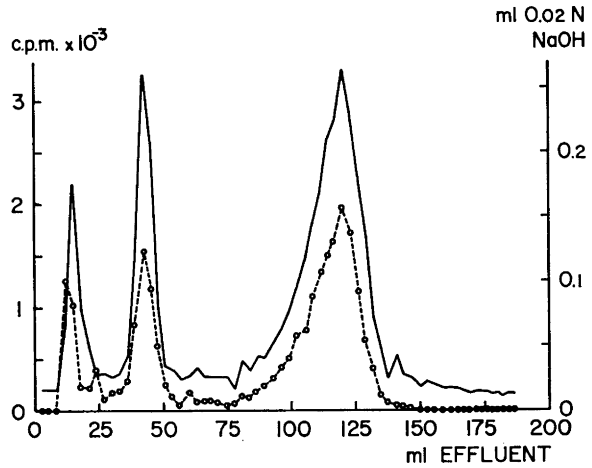


Fig. 1. Chromatogram of unhydrolyzed fistula bile from pig injected i. p. with 24-^{14}C-chenodeoxycholic acid. Fistulation 48 h before the injection. Phase system C. 4.5 g hydrophobic Hyflo Supercel. Solid line: titration. Broken line: c.p.m.

RESULTS

The composition of fistula bile. A sample of pig fistula bile, collected 48 h after the preparation of the fistula, was chromatographed on phase system C, and was found to contain three distinct titration peaks, as illustrated in Fig. 1. The bile acids constituting the first peak were identified as a mixture of taurochenodeoxycholic and taurohyocholic acid by rechromatography on phase system D and after saponification on C and F. The second titration peak was due to glycohyocholic acid, identified in the same manner as described above.

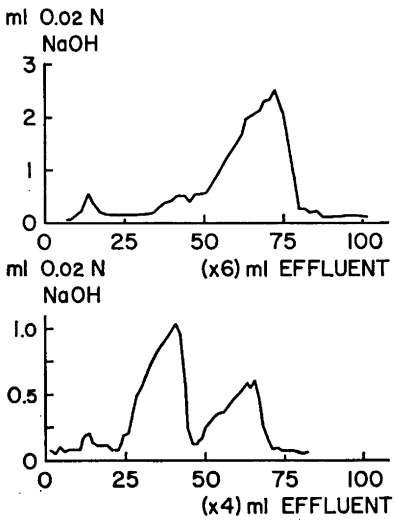


Fig. 2. Chromatogram of hydrolyzed dihydroxycholanic acid band from bile collected 48 h after fistulation (upper curve) and from bile collected immediately after fistulation (lower curve). Phase system F. 27 g hostalene (upper curve), 18 g hostalene (lower curve).

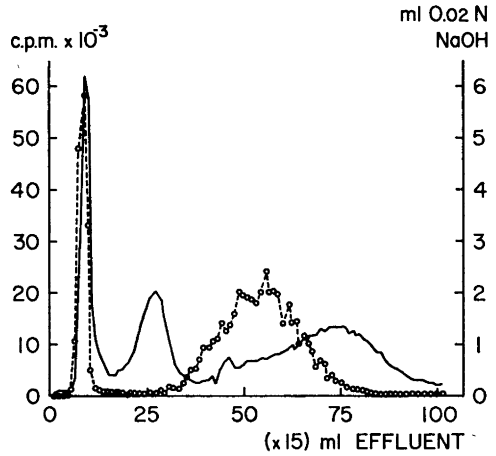


Fig. 3. Chromatogram of unhydrolyzed fistula bile from pig injected i.p. with 24-^{14}C-hyodeoxycholic acid. Fistulation 4 days before injection. Phase system C. 67 g hostalene. Solid line: titration. Broken line: c.p.m.

The third peak, appearing in the usual location for glycodihydroxycholanic acids was hydrolyzed and rechromatographed on phase system F and found to contain only chenodeoxycholic acid, *cf.* Fig. 2. For comparison, there is included in this figure a titration curve of the hydrolyzed glycodihydroxycholanic acids from fistula bile, collected immediately after fistulation, when the bile acids present in the entero-hepatic circulation were being excreted. The presence of a second titration peak, due to hyodeoxycholic acid, is clearly seen. This acid, however, is not present in the bile excreted through the fistula two days later.

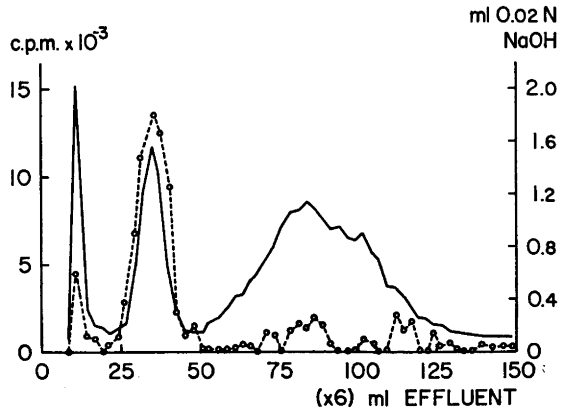


Fig. 4. Chromatogram of unhydrolyzed bile from pig injected i. p. with tritium-labelled hyocholic acid 24 h before sacrifice. Phase system C. 27 g hostalene. Solid line: titration. Broken line: c.p.m.

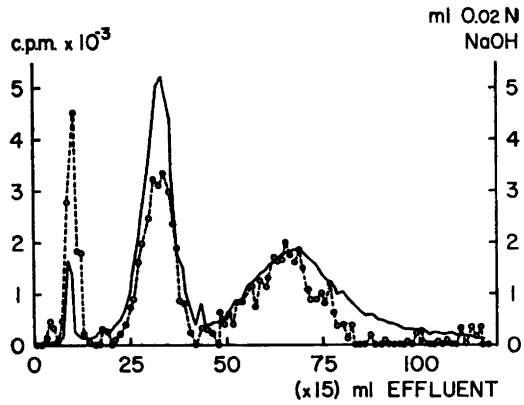


Fig. 5. Chromatogram of unhydrolyzed bile from pig injected i. p. with tritium-labelled hyocholic acid 72 h before collection of bile. Phase system C. 67 g hostalene. Solid line: titration. Broken line: c.p.m.

Injection of 24-^{14}C-chenodeoxycholic acid. Fig. 1. shows a chromatogram of a sample of unhydrolyzed fistula bile from an animal, where the fistula was prepared 2 days prior to the injection of 6×10^6 c.p.m. 24-^{14}C-chenodeoxycholic acid (approx. 40 μC, 2 mg). The radioactivity was found to be distributed in all three titration peaks. 5 % of the radioactivity was retained in the stationary phase and consisted of unchanged chenodeoxycholic acid. More than 90 % of the radioactivity of the first peak was present as taurochenodeoxycholic acid. After hydrolysis and rechromatography on phase system F, this material was diluted with carrier chenodeoxycholic acid, and the specific activity remained constant through four crystallizations. The second radioactive peak was shown to be glycohyocholic acid by rechromatography after saponification on phase system C and crystallization with unlabelled material. The radioactive compound appearing in the third titration peak was in a similar manner shown to be glycochenodeoxycholic acid.

Injection of 24-^{14}C-hyodeoxycholic acid. In Fig. 3 is shown a chromatogram of the unhydrolyzed bile acids from an animal, that had been injected with 9×10^6 c.p.m. 24-^{14}C-hyodeoxycholic acid (3 mg) four days after the preparation of the bile fistula. The radioactivity in the first peak was identified by rechromatography as taurohyodeoxycholic acid. After additional chromatography as the free acid, it was crystallized four times with unlabelled hyodeoxycholic acid, the specific activity remaining constant. The second radioactive elution band was found to be glycohyodeoxycholic acid, the identity of which was confirmed by cocrystallization with authentic hyodeoxycholic acid. 12 % of the radioactivity was retained in the column and consisted of unchanged free hyodeoxycholic acid.

Injection of tritium-labelled hyocholic acid. Two animals were injected each with 1×10^7 c.p.m. hyocholic acid (2 mg). One animal was sacrificed 24 h later and the bile was collected from the gall-bladder. In the other animal a bile fistula was prepared 72 h after the injection and bile was collected for a

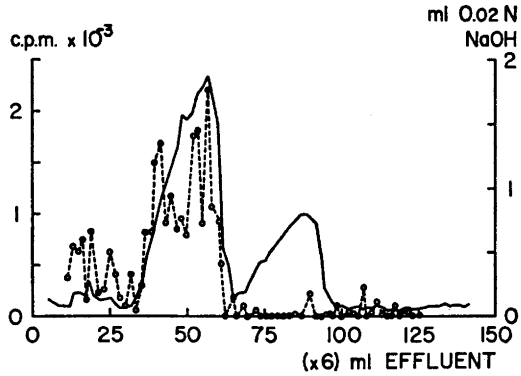


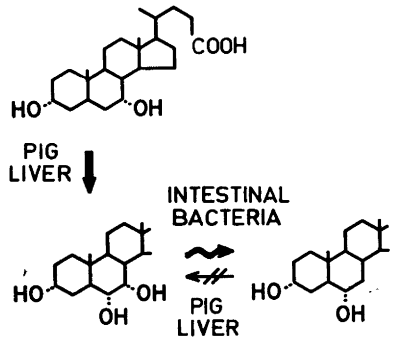
Fig. 6. Rechromatography of dihydroxycholanic acid band (*cf.* fig. 5) after hydrolysis. Phase system F. 27 g hydrophobic Hyflo Supercel. Solid line: titration. Broken line: c.p.m.

few hours. In Fig. 4 is shown a chromatogram of the non-saponified bile from the former animal, where the labelled hyocholic acid had been present in the entero-hepatic circulation of the bile for a maximum period of 24 h. The injected acid had been conjugated with glycine (second radioactive peak), and to a lesser degree with taurine (first peak). Small amounts of labelled material were found in the glycodihydroxycholanic acid band.

In the chromatogram (Fig. 5) of unhydrolyzed bile from the second animal, that had an intact entero-hepatic circulation for 72 h after the injection of the labelled hyocholic acid, it is seen, that almost half of the radioactivity is present in a band corresponding to a glycine conjugated dihydroxycholanic acid. This material was subsequently saponified and rerun with phase system F (Fig. 6). The radioactivity was found to coincide with the titration peak of hyodeoxycholic acid. The identity of the above-mentioned radioactive compounds was confirmed by cocrystallization with unlabelled material, where the specific activity remained constant through four recrystallizations.

DISCUSSION

The results obtained can be summarized in the following scheme:



The observation that hyodeoxycholic acid was present only in the first portions of the fistula bile but later disappeared, indicated that this acid might be a secondary acid not produced by the liver cells. This was further strengthened by the results with labelled bile acids, that showed that chenodeoxycholic acid was a precursor of hyocholic acid, whereas hyodeoxycholic acid remained unchanged except for its conjugation when injected intraperitoneally into pigs with a functioning bile fistula.

It was established that hyodeoxycholic acid indeed was a secondary product when labelled hyocholic acid was injected into intact pigs. After 24 h some labelled hyodeoxycholic acid had been formed, and after three days about half of the activity was present in the hyodeoxycholic acid formed by elimination of the 7α-hydroxyl group from hyocholic acid.

These results are thus in certain respects very similar to the bile acid metabolism in man [13] and rabbit [14]. In the latter cases intestinal microorganisms eliminate the 7α-hydroxyl from cholic acid resulting in the formation of deoxycholic acid, and in both instances deoxycholic acid — normally present in gallbladder bile of man and rabbit — disappears from the fistula bile, when the bile acids present in the enterohepatic circulation have been excreted.

In the rat the situation is also similar in that deoxycholic acid is formed from cholic acid in the intestine [15,16], but in this case the deoxycholic acid formed is rehydroxylated at position 7α to cholic acid in the liver [17].

In all these cases the intestinal microorganisms thus eliminate a 7α hydroxyl irrespective of whether the other hydroxyls are in position 3α and 12α or 3α and 6α.

A common feature in each of these cases, the rabbit excluded, is that chenodeoxycholic acid occurs in the primary bile, *i. e.* 3α and 7α hydroxyls are present. In most animals the third hydroxyl is then at position 12α, but in the pig it is at 6α, as is apparent from the reviews published by Haslewood [18,19]. There are some exceptions, for example the seals which are reported to have the third hydroxyl at C_{23} (β-phocaecholic acid, 3α, 7α, 23-trihydroxycholanic acid) [20]. Among the animals with trihydroxylated C_{24}-bile acids the only species known to have bile acids unsubstituted at C_7 are the Python and Constrictor snakes. They have pythocholic acid (3α, 12α, 16-trihydroxycholanic acid) as their main bile acid [21]. In these cases the primary bile acids are not known, but considering the long duration of the entero-hepatic circulation in these animals, there appears to be unusually favorable conditions for secondary changes by the intestinal microorganisms, possibly followed by further changes by the liver enzymes.

Earlier work has indicated, that at least some of the changes on the ring system of cholesterol take place before the side chain is degraded. Furthermore, Lindstedt [22] has found that 7α-hydroxycholesterol is transformed rapidly into cholic acid in the rat. It therefore appears possible, that the 7α-hydroxylation of cholesterol is a common and early step in the degradation of cholesterol to bile acids in most higher animals. Further work is needed to clarify the details of these reactions and their possible rôle as rate limiting steps in the degradation of cholesterol to bile acids.

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