Bile Salts of Germ-Free Domestic Fowl and Pigs

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(Received 18 January 1971)

1. The bile of germ-free domestic fowl contains taurine conjugates of 3 α ,7 α -dihydroxy-5 β -cholan-24-oic acid (chenodeoxycholic acid), 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid (cholic acid) and its 5 α -epimer (allocholic acid): that of germ-free pigs contains glycine and taurine conjugates of chenodeoxycholic acid, 3 α ,6 α -dihydroxy-5 β -cholan-24-oic acid (hyodeoxycholic acid), 3 α ,6 α ,7 α -trihydroxy-5 β -cholan-24-oic acid (hyocholic acid) and (probably) cholic acid. Keto acids were not found. 2. Allocholic acid and hyodeoxycholic acid are thus proved to be primary bile acids in intact animals. 3. The evolutionary and biochemical implications of these findings are briefly considered.

Germ-free (gnotobiotic) animals provide an opportunity of studying the intact fully physiologically functional organism, avoiding the criticism that incomplete animals or preparations of tissue imperfectly represent events *in vivo*. In the study of bile such animals are especially important, since making fistulae results in gross alterations of the rate of production of bile salts and may also produce qualitative effects. Bile of animals with normal gastrointestinal flora ('conventional' animals) contains a number of microbial artifacts of the enterohepatic circulation. The results of an examination of the bile salts of germ-free domestic chicks have been referred to previously (e.g. Haslewood, 1967) and the experimental basis for these is given below, together with an analysis of the bile salts of germ-free domestic pigs.

RESULTS

Bile from germ-free chicks was found to contain taurine conjugates of cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid), its 5 α -epimer, allocholic acid, and chenodeoxycholic acid (3 α ,7 α -dihydroxy-5 β -cholan-24-oic acid). Girard separation failed to detect keto acids. The amount of allocholic acid was at least as much as previously detected (e.g. by Yamasaki, 1951) in conventional fowls.

Samples of bile from germ-free pigs from two quite different sources contained both taurine and glycine conjugates; the proportion of taurine conjugates appeared to be markedly greater than in conventional pig bile. There was clear t.l.c. evidence for glycine- and taurine-conjugated hyodeoxycholic acid (3 α ,6 α -dihydroxy-5 β -cholan-24-oic acid). After

alkaline hydrolysis the bile acids obtained or detected were cholic acid, hyocholic acid (3 α ,6 α ,7 α -trihydroxy-5 β -cholan-24-oic acid), hyodeoxycholic acid and chenodeoxycholic acid. Keto acids were not detected.

EXPERIMENTAL

General

Details for preparation and purification of bile salts, hydrolysis and isolation of bile acids, their esterification, and determination of i.r. spectra were as given by Haslewood (1967). T.l.c. was carried out on silica gel G (E. Merck A-G., Darmstadt, Germany) on films 0.25 mm thick and the spraying reagent was 10% (w/v) phosphomolybdic acid in ethanol (Kritchevsky & Kirk, 1952) unless otherwise stated. G.l.c. of methylated bile acids was performed with a series 104 (model 24) dual flame ionization chromatograph (W. G. Pye and Co. Ltd., Cambridge, U.K.) with a coiled glass column (length 1.5 m, internal diam. 3.5 mm) packed with Gas Chrom Q (100-120 mesh) (Applied Science Laboratories, State College, Pa., U.S.A.) coated with Polysulfone (3%, w/w) (Applied Science Laboratories) and carrier gas (argon) flow of 50 ml/min at 315°C. Approximate retention times relative to methyl deoxycholate, which was used as standard, were: methyl chenodeoxycholate, 1.14; methyl hyodeoxycholate, 1.48; methyl cholate, 2.75; methyl hyocholate, 3.32. Methyl hyocholate was extensively decomposed on the column and the sharp peak with relative retention time about 3.32 appeared after broad shoulders, which we attribute to decomposition products.

Germ-free chicks

Bile salts. Bile (1.9 ml) from germ-free chicks, 4 weeks old, was preserved in ethanol (25 ml) by Dr N. Lev. Evaporation of the filtered solution left bile salts (0.385 g) as a light-green powder, which on paper chromatography

in the system 3-methylbutyl acetate–light petroleum (b.p. 80–100°C)–acetic acid–water (17:3:21:10, by vol.) gave a dense spot corresponding to taurochenodeoxycholate and a lighter spot with the chromatographic mobility of taurocholate; the same appearance was given by bile salts from conventional domestic hens.

Hydrolysis and esterification. Bile salts (351 mg) in 1.25 M-NaOH (7 ml) were hydrolysed at 112°C and the bile acids collected and converted into ethyl esters (265 mg). The acid liquors from the hydrolysed bile salts showed, with BaCl₂, a trace of SO₄²⁻. The esters, in benzene, were put on to a column of neutralized alumina (2.7 g) and eluted as follows (fraction no., ml of solvent as eluent, mg eluted): I, 120 benzene, 110; II, 50 ether, 46; III, 50 acetone, 95; IV, 50 ethanol, 13. The total weight eluted was 264 mg. T.l.c. of fractions I and II [solvent system, dry ether; spray reagent, 5% (w/v) uranyl nitrate in 10% (v/v) H₂SO₄–water followed by heating at 100°C] gave spots corresponding to ethyl chenodeoxycholate, but not to ethyl cholate or ethyl 3 α -hydroxy-7-oxo-5 β -cholan-24-oate. Fraction III, similarly treated, showed a faint spot corresponding to ethyl cholate and a dense spot with the mobility of ethyl chenodeoxycholate. Fraction I on Girard separation as previously described (Haslewood, 1956) gave no ketonic material. The non-ketonic material (100 mg) was separated on a column of Celite (10 g) in the system light petroleum (b.p. 80–100°C)–ethanol–water (7:5:2, by vol.) as described by Haslewood (1967). The moving phase (14–30 and 38–66 ml) eluted material (about 45 and 55 mg respectively) in two principal peaks, A and B. Material from peak A (45 mg) appeared to be chiefly fatty acid in type and that from peak B (55 mg) had the properties, including i.r. spectrum, of ethyl chenodeoxycholate. Fraction III, similarly treated on Celite, gave an ethyl chenodeoxycholate fraction (65 mg, eluted by 38–78 ml of moving phase) and an ethyl cholate fraction (8.5 mg, m.p. 154–157°C, eluted by 160–200 ml of moving phase); similar 'ethyl cholate' material (18.5 mg, m.p. 154–159°C) was stripped from the column by acetone (100 ml). Thus fraction III contained ethyl chenodeoxycholate (65 mg) and impure ethyl cholate (27 mg). Fraction II, which consisted apparently of ethyl chenodeoxycholate, was combined with the above corresponding Celite fractions and the total material (156 mg) was acetylated in the usual way to give diacetyl ethyl chenodeoxycholate, identical with crystalline authentic material, in a yield of 89% after crystallization from light petroleum (b.p. 40–60°C). Evaporation of the petroleum liquors left a residue which was hydrolysed with ethanolic 0.5 M-KOH to give, finally, an acid whose i.r. spectrum was almost identical with that of chenodeoxycholic acid. Fraction IV was a gel which with ether–acetone gave white crystals (about 7 mg), m.p. 203–207°C, which had the same i.r. spectrum as the ethyl ester of Ohta's acid [an approx. 2:1 (w/w) mixture of allocholic acid and cholic acid (Anderson & Haslewood, 1962)]. Thus, in summary, the bile acid esters (264 mg) contained approximately, by weight, 17% (46 mg) of oily material, probably largely fatty acid esters, 63% (166 mg) of ethyl chenodeoxycholate and 15% (40 mg) of ethyl cholate–allocholate, of which at least 5 mg (about 2% of the total esters) was ethyl allocholate. The total accounted for was about 95%, the remainder being lost in the Girard separation and in between-peak fractions from the Celite columns.

Germ-free pigs

Bile salts. Gall-bladder bile (preserved in excess of ethanol) from germ-free pigs (8–10 weeks old) was given by Professor C. K. Whitehair (Michigan State University, Mich., U.S.A.) and Dr P. Trexler (Royal Veterinary College, London N.W.1, U.K.). On t.l.c. in the system di-isopropyl ether–acetic acid–water (8:4:1, by vol.) bile salts from both sources showed dense spots corresponding to taurochenodeoxycholate (R_F 0.11) and glycochenodeoxycholate (R_F 0.69) and faint spots corresponding to taurocholate (R_F 0.07), glycocholate (R_F 0.45) and probably glycohyocholate (R_F 0.49) and glycohyodeoxycholate (R_F 0.64). Similar results were given by a Michigan sample from pigs mono-infected with *Escherichia coli*; this sample and the germ-free Michigan bile salts also gave an intense small spot (R_F about 0.22) between taurochenodeoxycholate and glycocholate, which was faintly visible in the chromatogram of the London sample and has not been identified. Three different samples of conventional pig bile salts run at the same time showed only faint spots corresponding to taurine conjugates and to glycocholate and glycohyocholate, but strong spots as glycochenodeoxycholate and probably glycohyodeoxycholate, with a spot running just ahead of glycochenodeoxycholate and showing up well after spraying with 0.5% (w/v) sodium borohydride in approx. 80% (v/v) methanol–water and development with Usui's (1963) reagent. This spot probably represents glyco-3 α -hydroxy-6-oxocholan-24-oate.

Bile acids. These (132 mg) were obtained as described above after hydrolysis of bile salts (200 mg) in 0.5 M-NaOH (4 ml) at 140°C for 17 h. With BaCl₂ the final acid liquors showed a trace of SO₄²⁻. On t.l.c. in system S₁₁ of Eneroth (1963) the bile acids showed borohydride corresponding to cholic acid, hyocholic acid, hyodeoxycholic acid and chenodeoxycholic acid, but not to lithocholic acid (3 α -hydroxy-5 β -cholan-24-oic acid) or to three of the (at least four) spots given by crude keto acids isolated from conventional pig bile by Girard separation. The fourth of these spots corresponded to chenodeoxycholic acid, perhaps carried over from the non-ketonic fraction. Girard separation of the methylated bile acids (134 mg) as described (Haslewood, 1956) gave 'ketones' (about 2 mg) that on g.l.c. showed none of the peaks (at least five) given by crude methylated conventional pig keto acids. The rest of the material was recovered in the non-ketonic fraction. Methylated bile acids (108 mg) were separated on Celite (11 g) as described above, in the system *n*-heptane–benzene–ethanol–water (5:2:5:2, by vol.) on a column (length 39 cm, diam. 1 cm; rate of flow about 3.5 ml/min). Some 83 fractions (2 ml) of moving phase were collected. The moving phase eluted material as follows (peak no, ml of moving phase, mg eluted, chief constituent): C, 14–24, 26, oil; D, 26–50, 28, methyl chenodeoxycholate; E, 52–92, 6, methyl hyodeoxycholate; F, 94–166, 47, methyl hyocholate. The esters mentioned in D and F were satisfactorily identified by their i.r. spectra, by t.l.c. and by g.l.c. by Dr I. G. Anderson. Small peaks or shoulders corresponding to methyl cholate were seen on g.l.c. of material eluted by 116–154 ml of moving phase; the small amount of methyl cholate was not separated on Celite from methyl hyocholate. There was some overlap between fractions E and F. Material from fraction E, showing only a spot

corresponding to methyl hyodeoxycholate on t.l.c. in system S₇ of Eneroth (1963) and eluted by 56–78 ml of moving phase, gave an i.r. spectrum identical with that of methyl hyodeoxycholate. This material (3 mg) was hydrolysed by warming with *m*-NaOH (0.1 ml) in methanol (0.2 ml) and the solvent removed by N₂. Acidification gave the acid, which was collected and washed. The product (2.2 mg) was dissolved in ethyl acetate and the solution was filtered; the filtrate was evaporated under N₂ and the residue crystallized with a drop of fresh ethyl acetate to give white globules (about 2 mg) of hyodeoxycholic acid, m.p. 195–196°C, not depressed by an authentic sample (m.p. 196–198°C). The material had the same i.r. spectrum and behaviour on t.l.c. as the reference compound and after methylation behaved on g.l.c. in a manner identical with that of the methyl ester of the reference compound. Dr L. Tökés reported that the mass spectra of corresponding samples (methylated) from both sources of bile were entirely compatible with their identity as methyl hyodeoxycholate.

DISCUSSION

Of the six bile acids listed (Haslewood, 1967) as occurring in domestic-fowl bile, 'isolithocholic acid' and the keto acids are apparently microbial artifacts or arise during hydrolysis of the bile salts. 3 α -Hydroxy-7-oxo-5 α -cholan-24-oic acid is apparently not formed, as for example in guinea pigs (Peric-Golia & Jones, 1961), without microbial intervention. The finding of allocholic acid in germ-free chicks was the first demonstration that 5 α bile salts could be primary products. Evidence has subsequently accumulated that confirms this conclusion, and Hoshita, Shefer & Mosbach (1968) have shown that liver microsomal preparations from the lizard *Iguana iguana*, having taurine-conjugated allocholic acid as its chief bile salt, reduce the presumed intermediate, 7 α ,12 α -dihydroxycholest-4-en-3-one, to a substituted 5 α -cholestane. It is curious that although chenodeoxycholic acid is the chief bile acid in both conventional and germ-free domestic fowls, no unequivocal evidence of its 5 α epimer (allochenodeoxycholic acid) could be adduced.

Bile salts from germ-free domestic pigs did not, apparently, contain conjugates of the 3 α ,6 β -dihydroxy-, 3 β ,6 α -dihydroxy- or 3 α -hydroxy-6-oxo-5 β -cholan-24-oic acids previously found in conventional pigs (Haslewood, 1967). However, they did contain about 4% (by wt.) of hyodeoxycholic acid and this is a most surprising result. Bergström, Danielsson & Göransson (1959) showed that chenodeoxycholic acid after injection into a pig with a bile fistula was converted into hyocholic acid, but hyodeoxycholic acid similarly treated was unchanged. Moreover, since hyocholic acid in a conventional intact pig could be converted partially into hyodeoxycholic acid and since this latter acid apparently vanished from the bile in the animal with a fistula, it has been assumed that hyodeoxy-

cholic acid was solely a microbial artifact, intestinal bacteria removing the 7 α -hydroxyl group from hyocholic acid as they do from cholic acid in certain other animals. The present work shows that this is only partially true and provides an illustration of the particular value of germ-free animals, for small amounts of hyodeoxycholic acid in fistula bile could never with certainty be disassociated from microbial origin in conventional pigs. It seems impossible that the hyodeoxycholic acid now isolated could have arisen during hydrolysis, a view supported by the t.l.c. evidence for conjugated hyodeoxycholic acid in the bile salts. Thus the pig can make hyodeoxycholic acid from cholesterol and normally adds to this by its intestinal microflora. The former process might be suppressed in the conventional pig, for Samuelsson (1959) failed to obtain labelled hyodeoxycholic acid after the intraperitoneal injection of [4-¹⁴C,6-³H]cholesterol to such animals having biliary fistulae. The situation in the pig is therefore physiologically different from that in the laboratory rabbit, for the conventional rabbit makes little chenodeoxycholic acid and uses deoxycholic acid (3 α ,12 α -dihydroxy-5 β -cholan-24-oic acid), which is made microbially from cholic acid and is not found at all in the germ-free animal (Hofmann, Mosbach & Sweeley, 1969). If it is true that the pig, like the rabbit, is an animal whose bile salts have become specialized for a vegetarian diet, its ability to make hyodeoxycholic acid must be taken into account in evaluating the selection pressures that led finally to its present condition.

The production of a primary bile acid with a molecule not containing oxygen at C-7 lends some support to the view of Mitropoulos & Myant (1967) that a route may exist for bile-acid biosynthesis from cholesterol that does not involve 7 α -hydroxycholesterol. Mitropoulos & Myant (1967) found that fortified rat liver mitochondrial preparations could convert cholesterol into, *inter alia*, lithocholic acid. On this evidence lithocholic acid would be a primary bile acid and could be 6 α -hydroxylated by pig liver to hyodeoxycholic acid. Lithocholic acid was not, however, detected in the present investigation.

I thank Dr N. Lev (National Institute for Research in Dairying, Shinfield, Berks., U.K.) for germ-free chick bile and Professor C. K. Whitehair (Michigan State University, Mich., U.S.A.) and especially Dr T. F. Kellogg (University of Notre Dame, Notre Dame, Ind., U.S.A.) and Dr P. Trexler and Mr A. Drummond (Royal Veterinary College, London N.W.1, U.K.) for germ-free pig bile. I also thank Dr I. G. Anderson for g.l.c. and Dr L. Tökés (Syntex Research, Palo Alto, Calif., U.S.A.) for mass spectra.

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