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Bile Acid Metabolism during Development: Metabolism of Lithocholic Acid in Human Fetal Liver

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ABSTRACT. The metabolism of [24-^{14}C]lithocholic acid was studied in the microsomal fraction of fetal human liver homogenates. Fetal livers were obtained at legal abortions performed between wk 14 and 24. Product formation was analyzed by thin-layer chromatography, liquid chromatography, and gas chromatography-mass spectrometry. From wk 14 lithocholic acid was hydroxylated in several positions. Hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholanoic) and 1β,3α-dihydroxy-5β-cholanoic acid were identified among the products. The 3β-isomer of the former acid, 6α-hydroxy-3-oxo-5β-cholanoic acid and a 2-hydroxylithocholic acid were tentatively identified. Two other metabolites carried a hydroxyl group in an unknown position, one of them probably at an angular methyl group. The highest total conversions per mg of microsomal protein were obtained with preparations from 18 wk fetuses. The results are discussed with particular reference to the role of lithocholic acid in fetal hepatotoxicity. (*Pediatr Res* 21: 99–103, 1987)

Abbreviations

GLC, gas-liquid chromatography
GC/MS, gas chromatography-mass spectrometry

In humans the primary bile acids, cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid) and chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid) are excreted in bile as glycine and taurine conjugates (1). Deconjugation and dehydroxylation by intestinal microorgansims yield the secondary bile acids deoxycholic acid (3α,12α-di-hydroxy-5β-cholanoic acid) and lithocholic acid (3α-hydroxy-5β-cholanoic acid) (1). Deoxycholic acid is efficiently reabsorbed and returned to the liver where it is conjugated and to some extent hydroxylated (1). Lithocholic acid also is reabsorbed but less efficiently since it is poorly soluble in water and tightly bound to intestinal microorganisms (1).

Little information is available on the metabolism of primary and secondary bile acids during fetal life (2). There is evidence that human fetal liver can conjugate primary bile acids (2). Due to lack of microflora there is no formation of secondary bile acids (2). The occurence of deoxycholic and lithocholic acids in meconium has earlier been thought to reflect placental transfer of these compounds (2), but at least in case of lithocholic acid a primary fetal synthesis from cholesterol cannot be excluded (3). We have shown recently that fetal liver efficiently metabolizes taurodeoxycholic acid by hydroxylations at the 1β- and 7α-positions (4).

Lithocholic acid is a potent toxic compound causing cholestasis in many species (5). A role for lithocholic acid in the pathogenesis of neonatal cholestasis/extra-hepatic biliary atresia has been suggested (6). Presence of sulfated conjugates of lithocholic acid in meconium indicates that sulfation may represent one mechanism for elimination of this bile acid in the fetus (6, 7).

The present work reports on the metabolism of lithocholic acid by hydroxylation in the microsomal fraction of human fetal liver.

MATERIALS AND METHODS

Materials. [24-^{14}C]lithocholic acid (specific radioactivity, 55 Ci/mol) was obtained from Amersham, Buckinghamshire, England. Before use the material was purified by thin-layer chromatography with solvent system S 11 (8). Lithocholic, chenodeoxycholic, deoxycholic, and hyodeoxycholic (3α,6α-dihydroxy-5β-cholanoic) acids were obtained from Sigma Chemical Co., St. Louis, MO and NADPH was obtained from Sigma Chemical Co. Solvents were of reagent grade and redistilled. Diazomethane was prepared according to Schlenk and Gellerman (9). Pyridine, hexamethyldisilazane, and trimethylchlorosilane were redistilled. Unisil was from Clarkson Chem. Co (Williamsport, PA), SP-Sephadex from Pharmacia Fine Chemicals (Uppsala, Sweden), and Lipidex-DEAP from Packard Instr. Co. (Downers Grove, IL). The two ion exchangers were washed with aqueous ethanol and ethanol prior to use. GLC was carried out on 25 m × 0.32 mm (i.d.) fused silica capillary columns coated with OV-1 (0.20 μm film thickness, Orion Analytica Espoo, Finland) or cross-linked methyl silicone (0.25 μm film thickness, Quadrex Corp., New Haven, CT) in a Carlo Erba HRGC 4160 gas chromatograph. Helium was used as carrier gas at 50-100 kPa and column temperatures were 270–290°.

Experimental. Nine human fetuses were obtained at legal abortions performed between wk 14 and 24 (c.f. Table 2). Consent was given by the ethical committee of the University of Uppsala. The abortions were performed with prostaglandins or via hysterotomy. Fetal age was determined from measurements of fetal height and from data concerning the pregnancies. After abortion, the fetuses were taken to the laboratory and liver tissue was taken out and chilled in ice-cold buffer solution. Liver homogenates (10%, w/v) were prepared within 45–60 min in 0.25 M sucrose using a Potter-Elvehjem homogenizer equipped with a loosely fitting pestle (10). The microsomal fraction was prepared by centrifugation of the homogenate at 800 × *g*, 20,000 and 100,000 × *g* (10). The final pellet was suspended in one-fourth to one-half of the initial volume of 0.05 M Tris-acetate

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buffer, pH 7.4, containing 0.1 mM EDTA, and 20% (v/v) glycerol. Microsomal protein was 0.9–3 mg/ml when determined according to Bradford (11). The lack of detectable 26-hydroxylase activity toward 5β-cholestane-3α,7α,12α-triol in the microsomal fraction indicates absence of significant contamination with mitochondrial protein in the microsomal fraction (*c.f.* Reference 12). With regard to peroxisomal protein some degree of contamination was found. Thus, the activity of urate oxidase, a peroxisomal marker enzyme (13), was about 15% of that in whole liver homogenate.

Incubation procedure and analysis of incubation mixtures. [24-^{14}C]lithocholic acid was diluted with nine parts unlabeled lithocholic acid and 0.25 μmol (specific radioactivity 5.5 Ci/mol, corresponding to about 8.5 atom percent excess of ^{14}C at C-24) was added in 40 μl of acetone. Of microsomal fraction 1.0 ml was used and total incubation volume was 2 ml. NADPH, 2 μmol, was added and incubations were performed for 20–40 min at 37° C and were terminated by addition of 95% (v/v) aqueous ethanol. After acidification, the mixtures were extracted twice with diethyl ether. The combined ether extracts were washed with water until neutral and taken to dryness. Less than 1% of the incubated radioactivity remained in the water phases. The residue was subjected to thin-layer chromatography using solvent system S 11 (8). Chenodeoxycholic, deoxycholic, and hyodeoxycholic acids were used as external standards. Product formation was determined by radioactivity scanning of the thin-layer chromatograms. For identification, zones containing radioactive products were scraped into a 0.8 cm id column on top of 2 cm of Unisil and eluted with 25 ml of water-saturated ethyl acetate. The extract was taken to dryness, dissolved in 5 ml 70% (v/v) aqueous ethanol and passed through a column of SP-Sephadex (40 × 4 mm in H^+-form) and a column of Lipidex-DEAP (20 × 4 mm in acetate form) (14). After washing with 10 ml 70% ethanol, the bile acids were eluted from the latter column with 8 ml 0.1 M acetic acid in 70% ethanol. After methylation with diazomethane, they were trimethylsilylated in pyridine/hexamethyldisilazane/trimethylchlorosilane, 3:2:1 (v/v/v), at 60° C, 30 min. The derivatives were analyzed by GLC. GC/MS was carried out on a VG 7070E instrument (VG Analytical Ltd, Manchester, England) with the capillary columns directly introduced to the ion source, The electron energy was 22.5 eV.

In other experiments, the entire incubations were added to 50 ml of ethanol and heated at 60° C for 1 h. After filtration, the residue was extracted once again with 50 ml of ethanol. The combined extract was taken to dryness and the residue was partitioned between 0.1 M hydrochloric acid (20 ml) and ethyl acetate (50 ml). The water phase was extracted once more with ethyl acetate (50 ml), the combined extracts were washed with small portions of water and taken to dryness. The residue was dissolved in 20 ml 90% ethanol and a bile acid fraction was isolated, derivatized, and analyzed by GLC and GC/MS as described above. Samples in which lithocholic acid was added after termination of the incubations were analyzed as controls in the same way.

RESULTS

Incubation of [24-^{14}C]lithocholic acid with the microsomal fraction of fetal liver and NADPH gave three radioactive zones on thin-layer chromatography. In the active preparations the relative conversions were: 0.1–4.2% (zone I), 0.2–3.6% (zone II), and 0.1–3.6% (zone III). The products migrated as dihydroxycholanoic acids with mean R_f-values (migration of the product divided by migration of the solvent front) for the zones of 0.27 (zone I), 0.38 (zone II), and 0.53 (zone III), respectively. With the solvent system used the mobility of zone I corresponded to that of hyodeoxycholic acid (reference) and the mobility of zone II to that of chenodeoxycholic acid (reference). Except for the radioactivity found in zones I–III, all the remaining radioactivity migrated as lithocholic acid (reference). The gas chromatographic analyses of the incubation mixtures and the eluates of the thin-layer chromatographic zones indicated formation of several metabolites.

The GLC and GC/MS analyses of the crude incubation mixtures were difficult to interpret due to many overlapping peaks. However, besides lithocholic acid only cholic acid could be detected in the control experiments where lithocholic acid was added after termination of the incubation. The amount of cholic acid was 1–2% of that of the lithocholic acid. In contrast, the analyses of microsomal fractions incubated with lithocholic acid indicated formation of several dihydroxy bile acids which were studied after separation by thin-layer chromatography. The analyses of material from the thin-layer chromatograms also showed complex mixtures except in the case of zone I where hyodeoxycholic acid predominated. In all cases the origin of a compound from added lithocholic acid was definitively established by calculation of the percentage of ^{14}C-labeled molecules from the isotope pattern of suitable fragment ions in the mass spectra. About 8.5% of the incubated lithocholic acid contained ^{14}C. This abundance of heavy atoms was also seen in the spectra of the metabolites and could be determined with an accuracy of about ±20%. Cholic acid, also detected in the analyses, did not contain ^{14}C. Retention indices and mass spectral characteristics of the seven metabolites detected are given in Table 1.

The retention index and mass spectrum of the derivative of the major product in zone I were identical with those of the derivative of hyodeoxycholic acid (Fig. 1*A*). Prominent ions at *m/z* 405 (M-145, *i.e.* loss of C-1-C-4) and at *m/z* 323 (consisting of side chain, D-ring, C-8, C-7, and C-6 with its trimethylsiloxy group) are particularly characteristic for the 3,6-bis-trimethylsiloxy structure (*c.f.* Reference 15). A minor compound with a slightly longer retention time gave a very similar mass spectrum, showing that it was the derivative of an isomer of hyodeoxycholic acid. The 6β-isomer could be excluded since its derivative had a shorter retention time than that of hyodeoxycholic acid. This indicates a 3β-configuration of the metabolite. In the sample analyzed by GC/MS this compound constituted about 15% of the bile acids in zone I.

Zone II contained at least three metabolites occurring in similar amounts in the sample analyzed by GC/MS. The derivative of one of these (RI 3285) gave a mass spectrum with an intense base peak at *m/z* 217 typical of a 1,3-bis-trimethylsiloxy structure (Fig. 1*B*) (4, 14). Peaks at *m/z* 142 and 143 were also present (16). A peak of low intensity at *m/z* 535 (M-15) and peaks at *m/z* 460 (M-90) and 370 (M-2×90) showed the presence of two hydroxyl groups. The retention index (3285) compared with that of the derivative of 1β,3α,12α-trihydroxy-5β- cholanoic acid (RI 3293) indicated that the compound was the same as that previously obtained by hydroxylation of lithocholic acid with a 1β-hydroxylating Penicillium species (16). Thus, this metabolite is identified as 1β, 3α-dihydroxy-5β-cholanoic acid.

The derivative of another bile acid from zone II (RI 3254) gave a mass spectrum with a base peak at *m/z* 143 and an intense ion at *m/z* 142 (Fig. 1*C*). This is typical for steriods with a 2,3-bis-trimethylsiloxy structure which is supported by the presence of an ion at *m/z* 318 corresponding to loss of C-1-C-4 with two trimethylsiloxy groups (M-232). A molecular ion was not observed but the intensity of *m/z* 460 (corresponding to M-90) and the thin-layer chromatographic behavior indicate that the metabolite is a dihydroxy acid. It is tentatively identified as 2ξ,3α-dihydroxy-5β-cholanoic acid.

The derivative of a third metabolite in zone II gave an intense base peak at *m/z* 357, corresponding to M-90–103 (Fig. 1*D*). Loss of a fragment of mass 103 is seen in spectra of trimethylsilyl ethers of steroids with a derivatized primary hydroxyl group. The combined presence of ions at *m/z* 460, 370, and 255 indicates a side chain without a hydroxyl group. Thus, hydroxylation at C-18 or C-19 seems likely. The peak at *m/z* 249, if significant (*c.f.* Reference 15), favors C-19. There was no evidence of any formation of chenodeoxycholic acid from lithocholic acid.

Table 1. *Retention indices and mass spectral characteristics of methyl ester trimethylsilyl ethers of bile acids formed after incubation of lithocholic acid with preparations from human fetal liver*

RI*	TLC†	Masses, *m/z*, and relative intensities (%) of major ions	Bile acid identified
3225	I	550, 460, 405, 370, 331, 323, 315, 303, 263, 255, 249, 228, (7) (24) (21) (100) (12) (32) (18) (10) (14) (31) (11) (20) 213, 161, 143, 129 (31) (26) (10) (27)	Hyodeoxycholic acid
3238	I	550, 460, 405, 370, 331, 323, 315, 303, 263, 255, 249, 228, (7) (48) (49) (100) (15) (24) (9) (7) (7) (20) (14) (9) 213, 161, 143, 129 (16) (20) (34) (20)	3β,6α-dihydroxy-5β-cholanoic acid‡
3254	II	460, 431, 370, 339, 334, 331, 318, 262, 255, 249, 228, 213, (88) (10) (50) (12) (34) (34) (15) (7) (31) (18) (10) (24) 196, 171, 143, 142, 129 (16) (21) (100) (69) (39)	2ξ,3α-dihydroxy-5β-cholanoic acid‡
3262	II	535, 460, 370, 357, 339, 325, 255, 249, 241, 229, 213, 201, (2) (6) (30) (100) (3) (3) (7) (3) (3) (3) (4) (4) 161, 147, 131 (5) (9) (8)	19-hydroxylithocholic acid†
3285	II	535, 460, 370, 318, 255, 233, 217, 203, 161, 143, 142, 131, (1) (5) (2) (1) (1) (1) (100) (1) (1) (5) (4) (1) 117, 103 (1) (1)	1β,3α-dihydroxy-5β-cholanoic acid
3293	III	476, 461, 386, 371, 368, 323, 316, 313, 271, 253, 249, 244, (4) (33) (42) (15) (8) (54) (12) (12) (100) (13) (14) (15) 229, 129 (22) (22)	6ξ-hydroxy-3-oxo-5β-cholanoic acid
3326	II–III	550, 535, 460, 419, 371, 339, 331, 299, 275, 255, 249, 235, (2) (1) (7) (21) (28) (4) (100) (9) (3) (3) (4) (4) 209, 203, 195, 189, 175, 161, 142, 135, 129, 121, 115 (7) (7) (5) (6) (8) (9) (9) (8) (8) (9) (8)	Unknown dihydroxy bile acid

* Retention index (RI) on a fused silica capillary column coated with a cross-linked methyl silicone (Quadrex, column temperature 280°). The derivative of cholic acid had an RI of 3216 under the same conditions. The retention index expresses retention time in relation to the retention times of a series of n-paraffins, *e.g.* 3200 corresponds to the retention time of dotriacontane.

† Thin-layer chromatography (TLC) zone containing the compound.

‡ Tentative structure, see text.

Two metabolites were found in zone III of the thin-layer chromatogram. The derivative of one of these (RI 3293) gave a mass spectrum with a molecular ion at *m/z* 476 corresponding to a methyl cholanoate with one trimethylsiloxy and one oxo group. The intense peak at *m/z* 323 (see above) and a peak at *m/z* 316 (386–70, *i.e.* loss of A-ring with oxo group) indicate that this metabolite was 6-hydroxy-3-oxo-5β-cholanoic acid.

The second metabolite in zone III had a lower mobility as indicated by its presence also in zone II. The derivative gave a mass spectrum with prominent ions corresponding to losses of fragments of mass 131 (*m/z* 419, M-131), 129 (*m/z* 331, M-90–129), and 89 (*m/z* 371, M-90–89). A molecular ion showed that two trimethylsiloxy groups were present and the peak at *m/z* 115 indicated an unsubstituted side chain. The structure remains unknown; it is likely that hydroxylation has occurred at a position close to C-3. In the sample analyzed by GC/MS this compound constituted about 75% of the metabolites identified in zone III.

Due to the complexity of the metabolite mixture and the low concentrations, the individual compounds could not be accurately quantified. However, most of the radioactivity in zone I was due to hyodeoxycholic acid and its isomer.

There was no continuous increase in conversion of lithocholic acid in relation to increasing gestational length (Table 2). Instead, the highest total conversions were obtained with three of four preparations from fetuses in wk 18.

DISCUSSION

Lithocholic acid, irrespective of its origin from the mother or the fetus, is potentially hepatotoxic to the fetus (7). Several pieces of evidence indicate that the fetus may detoxify lithocholic acid by sulfation or by excretion across the placenta (7). The present study shows that human fetal liver microsomes have the capacity for hydroxylation of lithocholic acid in 1β- and 6α-positions and most likely at C-2 and at an angular methyl group. Under the conditions used there was also oxidoreduction at C-3.

As mentioned above there was no correlation between gestational age and conversion. The marked variation between preparations from different fetal livers may in part be due to enzyme inactivation during the abortion and work-up procedure (*c.f.* Reference 12).

The results should be compared to those of studies on bile acid metabolism in adult human liver. In such studies Björkhem *et al.* (17) found no microsomal hydroxylation of free or conjugated lithocholic acid, whereas Trülsch *et al.* (18) reported the existence of microsomal 6α-hydroxylase activity toward taurolithocholic acid in liver biopsies. It should be pointed out that failure to detect certain 5β-cholanoic acid hydroxylase activities in the adult human liver *in vitro* does not necessarily mean a complete absence of such enzyme activities. In fact, the presence of small amounts of 1- and 6-hydroxylated bile acids in urine of normal (14) and cholestatic subjects (14, 19, 20) speaks in favor of the

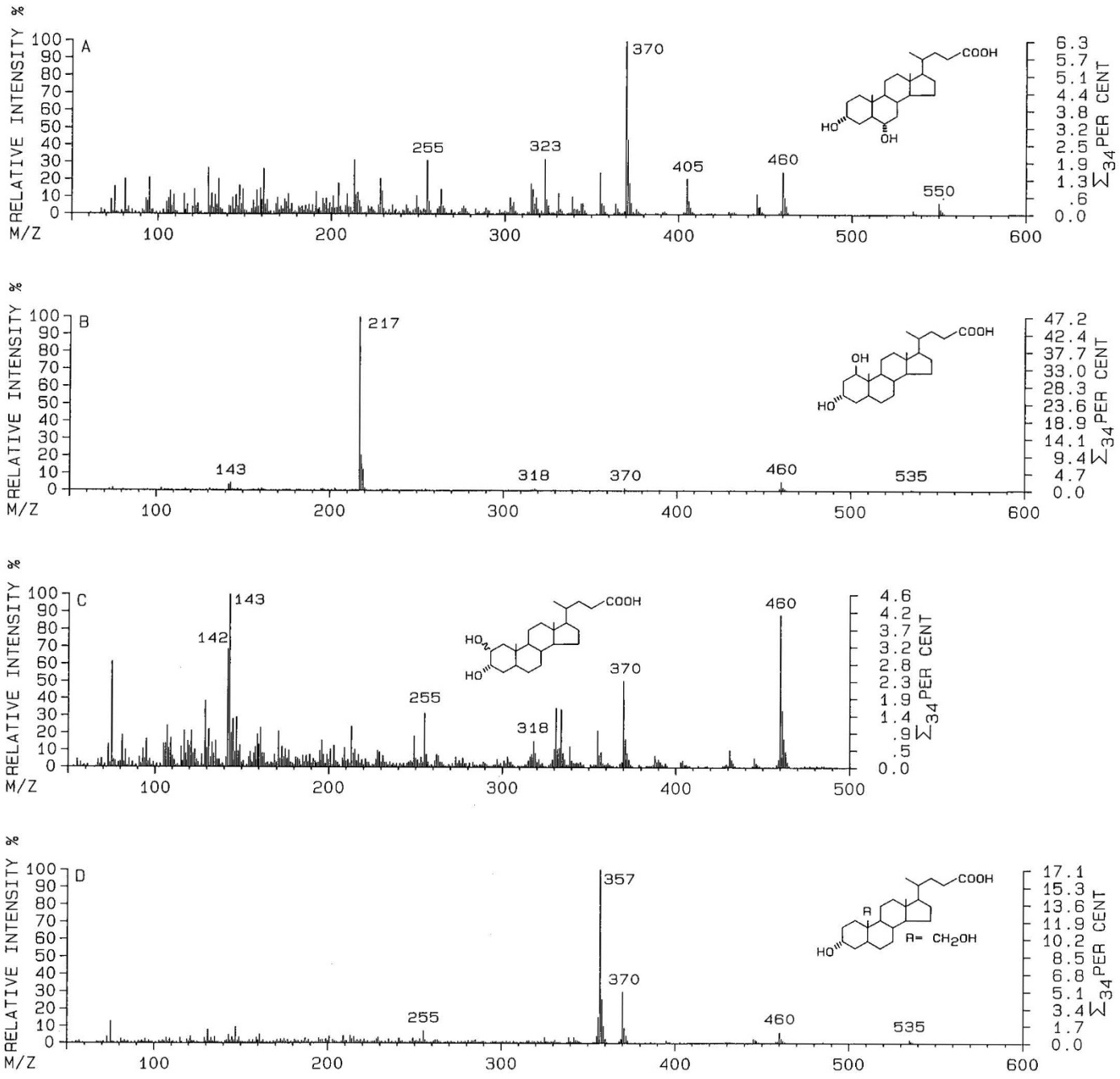

Fig. 1. Mass spectra of the methyl ester trimethylsilyl ethers of four hydroxylated metabolites of lithocholic acid. *A*, derivative of 3α,6α-dihydroxy-5β-cholanoic acid; *B*, derivative of 1β,3α-dihydroxy-5β-cholanoic acid; *C*, derivative of tentatively identified 2ξ,3α-dihydroxy-5β-cholanoic acid; *D*, derivative of dihydroxy-cholanoic acid possibly hydroxylated at C-19 (see text).

Table 2. *Hydroxylation of lithocholic acid by microsomal preparations from livers of fetuses of different gestational ages**

	Gestational wk								
	14	15	17	18	18	18	18	24	24
	(Product formation pmol/mg protein x min)								
Products in zone I	21	15	<10	69	80	<10	39	39	34
Products in zone II	21	15	<10	138	48	<10	21	11	10
Products in zone III	15	10	<10	<10	69	<10	47	36	23

* Incubations were performed as described in "Materials and methods" using 0.25 μmol of lithocholic acid in each incubation. Every incubation corresponds to one fetal liver microsomal preparation.

existence of corresponding hydroxylase activities in liver. However, the quantitative importance of such hydroxylations cannot be estimated from the rate of excretion of the corresponding bile acids in urine, since renal clearance for such bile acids apparently is high (20). The formation of 1-, 2-, and 6-hydroxylated bile acids in fetal liver is also in agreement with work by Strandvik and Wikström (21) on tetrahydroxylated bile acids in urine of newborn infants.

Back and Walter (22) have described a pattern of "immature" bile acids in meconium. The excretion of immature bile acids in urine of cholestatic subjects has led to the suggestion that cholestasis induces a return to a fetal stage of metabolism (22). To establish whether a fetal mode of bile acid metabolism exists under such conditions, it would be necessary to study hydroxylase activities in liver preparations from cholestatic subjects.

Lithocholic acid appears to be most toxic in species unable to convert it into more polar compounds (17). Conjugation with glycine or taurine does little to decrease the toxic potential of lithocholic acid (23), but sulfation (6, 7) and hydroxylation may represent ways for detoxification and elimination of this bile acid. Such metabolic transformations make this nonpolar compound more water soluble and promote its excretion (7).

The formation of 6-hydroxy-3-oxo-5β-cholanoic and 3β-hydroxy-5β-cholanoic acids from lithocholic acid in fetal liver is interesting in view of earlier work on lithocholic acid metabolism (17). Thus, Björkhem *et al.* (17) reported that an adult liver homogenate was capable of converting lithocholic acid into 3β-hydroxy-5β-cholanoic acid (isolithocholic acid) with the intermediate formation of 3-oxo-5β-cholanoic acid. Since the aim was to identify hydroxylated products, a possible isomerization of lithocholic acid was not investigated. Isolithocholic acid may well have been formed in the incubations, but under the conditions used for thin-layer chromatography lithocholic acid will not separate from 3β-hydroxy-5β-cholanoic acid. The lithocholic acid zone was not analyzed by GC/MS. Epimerization of the 3α-hydroxyl group may have physiological implications since it has been reported that 3β-hydroxysteroids are less toxic regarding hemolytic activity than 3α-hydroxysteriods (24). Further, isolithocholic acid is less active than lithocholic acid with respect to pyrogenic activity (25).

The present results suggest that the fetus is more capable than the adult of eliminating lithocholic acid by way of hydroxylations at several positions of the C_{24}-molecule. Such metabolism could be particularly important in recurrent cholestasis of pregnancy, a condition known to be associated with elevated fetal bile acid levels (26).

The absence or low amounts of secondary bile acids during the first years of life have been explained by the fact that there is a slow colonization of intestinal microorganisms in the infant (1). However, metabolism of lithocholic acid by hydroxylation may also explain the small amounts of lithocholic acid in this period of life as has been previously suggested in the case of deoxycholic acid (4).

If lithocholic acid plays a role in the initiation of neonatal liver disease (6, 27), such conditions could be associated with impaired fetal metabolism of lithocholic acid. It would therefore be of interest to compare lithocholic acid metabolism in microsomal preparations from infants with extrahepatic biliary atresia to that in preparations from infants without liver disease.

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