Bile acid metabolism in early life: studies of amniotic fluid

M. Nakagawa and K. D. R. Setchell

Clinical Mass Spectrometry Laboratories, Department of Pediatrics, Children's Hospital Medical Center, Cincinnati, OH 45229

Abstract Bile acid metabolism of the human fetus was examined in early gestation (weeks 13–19) and compared with the full-term fetus from the analysis of amniotic fluid collected from healthy pregnant women. Total individual bile acids were determined by gas–liquid chromatography–mass spectrometry after solvolysis and hydrolysis of bile acid conjugates. Additionally, bile acids were separated according to their mode of conjugation by lipophilic anion exchange chromatography. Qualitatively the bile acid profiles of amniotic fluid in early gestation were similar and markedly different from those of full-term fetuses. Chenodeoxycholic acid was the major bile acid identified in early gestation and concentrations exceeded those of cholic acid, but by full term this relationship was reversed. Over 50 bile acids were identified in the amniotic fluids, these included C-1, C-4, and C-6 hydroxylated species and reflected primary hepatic synthesis by the fetus. At full term, 7α,12α-dihydroxy-3-oxo-4-cholenoic acid was one of the major bile acids identified in amniotic fluid. The monohydroxy bile acids lithocholic and 3β-hydroxy-5-cholenoic acids were present in significant proportions during early gestation, but by full term these accounted for only a few percent of the total bile acids. Quantitatively the total bile acid concentration of amniotic fluid was <4 μmol/l. The majority of bile acids were found to be glyco-, tauro-, and sulfate-conjugates. The more hydrophobic bile acids tended to be preferentially sulfated. These data indicate that significant and major changes in bile acid metabolism take place between early and late gestation in the human fetus.—**Nakagawa, M., and K. D. R. Setchell.** Bile acid metabolism in early life: studies of amniotic fluid. *J. Lipid Res.* 1990. **31:** 1089–1098.

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Pathways of bile acid synthesis in the adult have been relatively well documented (1, 2). Commencing with cholesterol, the synthesis of the two primary bile acids in man, the amidated conjugates of cholic and chenodeoxycholic acids, involves at least nine principal reactions and each is catalyzed by distinct enzymes located in various subcellular fractions of the hepatocyte. In recent years a number of new inborn errors in bile acid synthesis have been described that are associated with conditions of familial idiopathic neonatal hepatitis (3–7). Furthermore, inborn errors in side-chain oxidation have been recognized for some time (reviewed in 2, 8). Since many of these metabolic defects have a relatively poor prognosis, their early detection has important implications, viz. specific therapy might be possible that would bypass the enzymatic defect or the administration of bile acids could facilitate bile flow and ameliorate the disease process (2, 6, 7).

In view of the fact that bile acid synthesis is relatively well developed in early life (9, 10) and the human fetus is capable of synthesizing chenodeoxycholic and cholic acids by the twelfth week of gestation, the detection of inborn errors in this pathway should potentially be possible in early pregnancy from the analysis of bile acids in amniotic fluid.

The objective of this study was to comprehensively examine the qualitative and quantitative composition of bile acids in amniotic fluid from healthy pregnant women obtained at different times during early gestation, including at full term, so that baseline information will be available for making future comparisons with the bile acid composition of amniotic fluid of high risk pregnancies and from pregnant mothers with a history of giving birth to infants with idiopathic neonatal hepatitis.

MATERIALS AND METHODS

Collection of amniotic fluids

Amniotic fluid specimens were obtained by amniocentesis from pregnant women (n = 28) between 13 and 19 weeks of gestation. Fetal age was assessed on the basis of clinical history and confirmed by ultrasound examination. In all cases, amniocentesis was indicated for determination of possible chromosome abnormalities and chromosome analysis showed normal results. Amniotic

Abbreviations: GLC–MS, gas–liquid chromatography–mass spectrometry; Me-TMS, methyl ester-trimethylsilyl.

fluids from full-term pregnancies (n = 5) were collected by needle centesis of the amniotic membrane just before delivery. None of the amniotic fluid samples were contaminated with blood or meconium. All the pregnancies were normal and none of the mothers had any obvious organic diseases or history of liver disease.

Analysis of amniotic fluids

Individual samples of amniotic fluids were analyzed for total individual bile acid composition. Gestational ages of these fetuses were 13–14 weeks (n = 5), 16 weeks (n = 5), 19 weeks (n = 5), and 38–41 weeks (n = 5).

Amniotic fluids obtained from a further 13 fetuses of 16 weeks gestation were collected, and after extraction of bile acids, the extracts were pooled to give two samples (A and B) representing the equivalent of 100 ml of amniotic fluid. These were used for more detailed analysis of bile acid composition; specifically, bile acids were fractionated into unconjugated, glycine, taurine, and sulfate conjugate groups by anion exchange chromatography (11).

Extraction of bile acids from amniotic fluid. Immediately after collection of amniotic fluids (15–20 ml), all samples were centrifuged at 10,000 rpm for 10 min. The supernatant was diluted with 4 vols of 0.04 M sodium hydroxide. After heating to 64°C in a water bath for 30 min, bile acids and their conjugates were quantiatively extracted by adsorption to small cartridges of octadecylsilane bonded silica (Bond-Elut C_{18}; Analytichem, Harbor City, CA) exactly as described by Setchell and Worthington (12).

Analysis of amniotic fluids for total individual bile acid composition. After the addition of an internal standard, nordeoxycholic acid (2 μg), the methanolic extract from the Bond-Elut C_{18} cartridge was taken to dryness on a rotary evaporator and the residue was subjected to solvolysis. Solvolysis was achieved in a glass-stoppered tube by dissolving the dried extract in methanol (1 ml), adding 9 ml of a solution of freshly distilled tetrahydrofuran–0.1 M trifluoroacetic acid in dioxane 9:1 (v/v); the reaction was allowed to proceed for 2 h at 45°C (13). After the reagents were evaporated under a stream of nitrogen, the residue was hydrolyzed. Hydrolysis was achieved by incubation with 50 units of cholylglycine hydrolase (Sigma Chemical Co., St. Louis, MO) in 0.1 M phosphate buffer at 37°C for 16 h. After hydrolysis, unconjugated bile acids were extracted by passage of the sample through a Bond-Elut C_{18} cartridge (12) and recovered by elution with 5 ml of methanol. After addition of 1.25 ml of distilled water, the sample was applied directly to a column of diethylaminohydroxypropyl Sephadex LH-20 (DEAP Sephadex LH-20, commercially sold by the name Lipidex-DEAP: Packard Instruments, Groningen, Holland) prepared in the acetate form and packed in 72% ethanol (bed size 13 × 0.4 cm). Neutral compounds passed directly through this anion exchange gel and unconjugated bile acids were recovered with 7 ml of 0.1 M acetic acid in 72% ethanol (pH 4.0). After evaporation of the sample, bile acids were converted to the methyl ester-trimethylsilyl (Me-TMS) ether derivatives, purified by chromatography in Lipidex 5000, and analyzed by GLC and GLC-MS (14).

Analysis of the bile acid composition of unconjugated, glycine, taurine, and sulfate conjugate fractions. After extraction of bile acids (using Bond-Elut C_{18} cartridges) from each of the individual amniotic fluid samples, the methanolic extracts were pooled to give two separate pools (A and B), each representing the equivalent of a total volume of 100 ml of amniotic fluid. These pools came from a total of 13 individual pregnancies. The pooled samples were diluted with distilled water to give a final concentration of 70% methanol. This solution was passed through a glass column (30 × 1 cm diameter) containing 4 g of the cation exchange resin Amberlist A-15 in the [H^+] form and collected. The sample tube and resin were washed with a further 2 × 10 ml of 70% methanol. The sample and washings were pooled and applied directly to a column of Lipidex-DEAP (0.6 g gel; bed size 13 cm × 0.4 cm). Neutral compounds passed directly through this anion exchange gel and stepwise elution of *i)* unconjugated bile acids; *ii)* glycine conjugates; *iii)* taurine conjugates; and *iv)* sulfate conjugates was achieved with the following respective buffers: i) 7 ml of 0.1 M acetic acid in 72% ethanol, pH 4.5; ii) 5 ml of 0.3 M acetic acid in 72% ethanol, pH 5.0; iii) 4 ml of 0.15 M acetic acid in 72% ethanol, pH 6.6; and iv) 10 ml of 0.3 M acetic acid in 72% ethanol, pH 9.6. After the addition of the internal standard, nordeoxycholic acid (0.5 –5 μg), the fractions were taken to dryness.

Hydrolysis of the glycine and taurine conjugate fractions was carried out with cholylglycine hydrolase (14). Bile acid sulfates were first solvolyzed (13) and then hydrolyzed with cholylglycine hydrolase exactly as described above for the analysis of total bile acids. Final purification of these fractions was carried out by chromatography on Lipidex-DEAP and collection of the unconjugated bile acid fraction as described above. Bile acid fractions were converted to the Me-TMS ether derivatives and analyzed by GLC and GLC-MS.

Gas–liquid chromatography–mass spectrometry. The methyl ester-trimethylsilyl (Me-TMS) ether derivatives were chromatographed on a 30 meter × 4 mm DB-1 (0.25 μm film) fused silica capillary column (J and W Scientific Inc., Rancho Cordova, CA) using a temperature program between 225° and 295°C in increments of 2°C/min with initial and final isothermal periods of 5 min and 20 min respectively. Helium was used as carrier gas with a flow rate of 1.8 ml/min measured from the column end at ambient temperature.

GLC-MS analysis was carried out using a Finnigan 4635 quadrupole GLC-MS-DS instrument (Finnigan

Inc., San Jose, CA) housing an identical GLC column with the same chromatographic conditions. Electron impact ionization (70 eV) mass spectra were recorded over the mass range 50–800 daltons by repetitive scanning (2.0 secs/cycle) of the eluting components. Data were stored and processed using the Super Incos Data System (Finnigan Inc.).

Quantification and identification of bile acids. Quantification of bile acids was achieved using GLC, by comparing the peak height response of the individual bile acids with the peak height response obtained from the internal standard (15). The recovery of nordeoxycholic acid exceeded 90%, while quantitative recoveries (>95%) of radiolabeled cholic, chenodeoxycholic, glycocholic, and taurocholic acids added to amniotic fluids were obtained in separate studies. Identification of a bile acid was made on the basis of the GLC retention index relative to a homologous series of n-alkanes referred to as a Methylene Unit (MU) value, and the mass spectrum was compared with authentic standards (16–18). A compilation list of over 100 mass spectra of authentic bile acid standards and a list of retention indices was recently compiled as a reference source (16).

RESULTS AND DISCUSSION

Literature regarding the bile acid composition of amniotic fluid is sparse and confined exclusively to bile acid metabolism in the last trimester of pregnancy (19–24). Délèze, Sidiropoulos, and Paumgartner (19) first reported on the clinical utility of bile acid determinations in amniotic fluid and indicated that the prenatal diagnosis of intestinal obstruction could be made based upon markedly elevated total bile acid concentrations. In healthy pregnant women the total bile acid concentration of amniotic fluid (28–42 weeks gestation) was in the range 1.4–2.4 μmol/l (19), whereas in two patients who gave birth to infants with intestinal obstruction, total bile acid concentrations in the amniotic fluid increased to 30.3 and 83.1 μmol/l, respectively, possibly because of regurgitation of bile into the amniotic fluid (19). The specificity of such observations may be questionable, because it was later shown that the amniotic fluid bile acid concentrations of women with intrahepatic cholestasis of pregnancy were also significantly elevated (21).

After the recent discoveries of new inborn errors in bile acid synthesis associated with idiopathic neonatal hepatitis syndromes (3–7), we focused our attention on the possibility of detecting such metabolic defects in utero from the analysis of amniotic fluid obtained during amniocentesis. Therefore, while the absolute concentrations of bile acids may have some clinical utility, the qualitative profile may provide evidence of abnormal bile acid synthesis once normal profiles have been established.

Qualitative analysis of total bile acids

Typical capillary column GLC profiles of the total bile acids of amniotic fluid from early gestation and full term are compared in **Fig. 1**. Qualitatively the profiles obtained between weeks 13 and 19 were all similar and differed greatly from those of full term fetuses. In early gestation, chenodeoxycholic acid is the major bile acid of amniotic fluid and its concentration exceeds that of cholic acid; however, by full term this relationship is reversed. Table 1 lists the individual bile acids identified by GLC–MS in these samples. For many of these bile acids it was not possible to fully elucidate the structures because of the

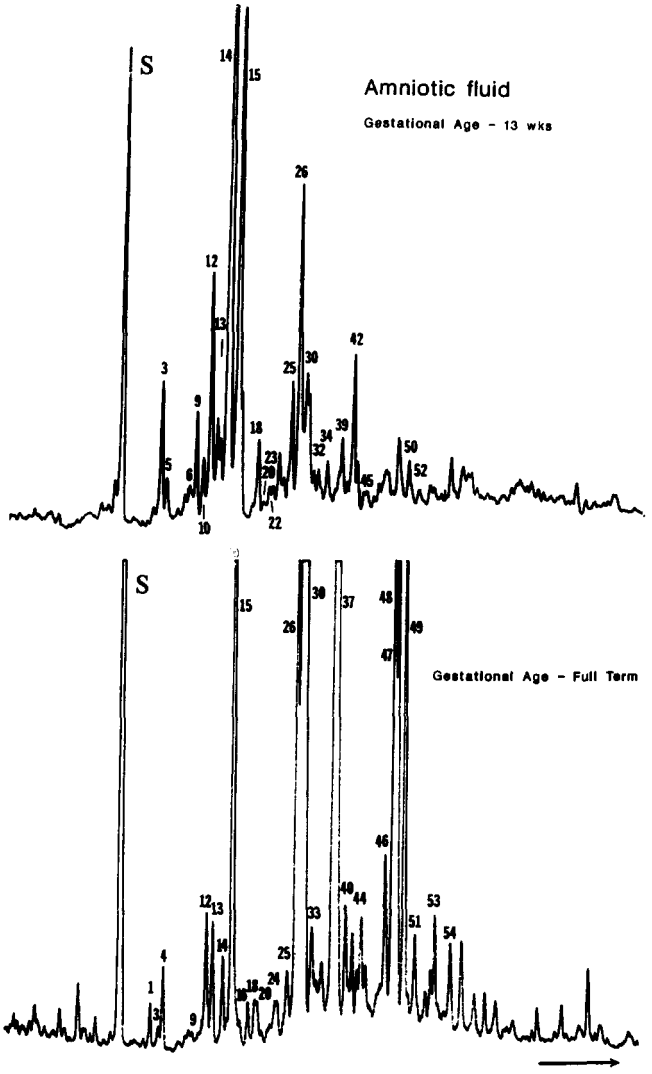

Fig. 1. Capillary column gas–liquid chromatograms comparing the total bile acid profiles of amniotic fluid from a 13-week gestational age fetus with that of a full-term fetus. Bile acids were analyzed as their methyl ester-trimethylsilyl ether derivatives and chromatographed on a 30 meter DB-1 capillary column using temperature programmed operation. Bile acids (eluting between 25 and 50 min) were identified by GLC–MS and the numbered peaks correspond to the bile acids listed in Table 1; S, internal standard, coprostanol.

lack of available authentic standards and in these instances the retention index (MU value) is given together with the general structure determined from the mass spectrometric fragmentation pattern (16–18). Interestingly, many of the bile acids identified in these amniotic fluids are also found in human fetal bile (9, 10) and are presumed to reflect primary hepatic synthesis by the fetus. These include several C-1, C-4, and C-6 hydroxylated species and these compounds are not typically found in appreciable amounts in biological fluids from healthy adults.

1β-Hydroxy bile acids have been found in the urine of cholestatic patients (25, 26), newborn infants (27), meconium (28–30), and more recently in arterial and venous umbilical cord blood (23). 1β-Hydroxylation has been demonstrated in vitro by human fetal microsomes (31) and the presence of several 1β-hydroxycholanoic acids in human fetal bile (10) indicates their hepatic origin. C-4 hydroxy bile acids were recently described for the first time in the bile of the human fetus (10, 32). In amniotic fluid from early gestation, 3α,4β,7α-trihydroxy-5β-cholanoic acid (i.e., 4β-hydroxy-chenodeoxycholic) was the major C-4 hydroxy bile acid and lesser proportions of 3α,4β-dihydroxy-(i.e., 4β-hydroxy-lithocholic) and 3α,4β,7α,12α-tetrahydroxy-5β-cholanoic (i.e., 4β-hydroxy-cholic) acids were present; however by full term the more polar tetrahydroxy isomer (3α,4β,7α,12α-tetrahydroxy-5β-cholanoic acid) became one of the major components. This change is in concert with differing proportions of chenodeoxycholic and cholic acids that are synthesized between early and late gestation.

Hyocholic (3α,6α,7α-trihydroxy-5β-cholanoic) and hyodeoxycholic (3α,6α-dihydroxy-5β-cholanoic) acid were the principal C-6 hydroxylated bile acids identified, and 2β,3α,7α,12α-tetrahydroxy-5β-cholanoic, which was first reported in the urine of newborns (27), was also present and in relatively high proportions in the amniotic fluid at full term.

The finding of the unsaturated bile acid, 7α,12α-dihydroxy-3-oxo-4-cholenoic acid is of particular interest. This compound, although not present in significant amounts during early gestation, was quantitatively one of the more important bile acids in amniotic fluid at full term. Indeed, several oxo-bile acids were apparent in the profiles. During the course of completing our studies, Shoda et al. (23) described the bile acid composition of amniotic fluid bile acids from full-term fetuses but there were no reports of the occurrence of oxo-bile acids. High levels of 3-oxo-Δ^4 bile acids in the absence of primary bile acids have been associated with a deficiency in the activity of the Δ^4-3-oxosteroid 5β-reductase enzyme (5, 6) that catalyzes the conversion of the Δ^4-3-oxo-C_{27} sterol intermediates into the 3-oxo-5β(H) products in the normal pathway for primary bile acid synthesis (1, 2). The finding of relatively high proportions of this bile acid, in the presence of low proportions of cholic acid at full term, might indicate that the enzyme may have limited capacity to cope with increased primary bile acid synthesis that presumably occurs in response to a demand for an expanding bile acid pool-size during early life. For example, the total bile acid pool-size of infants of 32 weeks gestation is approximately one-sixth that of adults when corrected for surface area differences (33) and the pool-sizes of cholic and chenodeoxycholic acids double with concurrent increases in intraluminal bile acid concentrations by 1 month postnatally (33). Similarly, term infants show a progressive enlargement of their pool-size in concert with increases in intraluminal bile acid concentrations postnatally (34).

Many of the early studies of amniotic fluid bile acid composition focused attention on the presence of 3β-hydroxy-5-cholenoic acid and it was suggested that measurements of this monohydroxy bile acid provide an indication of fetal maturity (35). In these studies 3β-hydroxy-5-cholenoic acid was present in amniotic fluid obtained during early gestation and at full term. The original reports of the quantitative importance of this bile acid, however, appear to be markedly over-estimated (33% of total bile acids). Our studies indicate that at full term, 3β-hydroxy-5-cholenoic acid accounts for 2 $\pm$ 1% (mean $\pm$ SD) of the total amniotic fluid bile acids, a value similar to that recently reported by Shoda et al. (23), while in early gestation greater proportions are to be found. Indeed, at full term, the concentrations of 3β-hydroxy-5-cholenoic acid are comparable to those of chenodeoxycholic acid, yet lithocholic acid concentrations are relatively low. Previous studies of human fetal gallbladder bile obtained during early gestation also found 3β-hydroxy-5-cholenoic acid mainly as the sulfate conjugate and average total concentrations were of the order of 1.3 μmol/l (9). These findings suggest that the "Yamasaki pathway" for bile acid synthesis involving side-chain hydroxylation and oxidation prior to nuclear changes (36–38) may be important for chenodeoxycholic synthesis in late gestation.

A number of short-chain monohydroxy bile acids (etianic acids) have been previously identified in meconium as glucuronide and sulfate conjugates (30, 39, 40); however, it should be pointed out that we made no attempt to identify this species of bile acids in amniotic fluid, even though the hydrolysis methods used would have cleaved the conjugate moiety.

Quantitative analysis of bile acids in amniotic fluid

Table 1 summarizes the mean ($\pm$ SD) concentrations for the individual bile acids identified in the amniotic fluid at the different times of gestation. Total concentrations were low and comparable to those found in the urine of newborn infants (41) and adults (11). It is worth noting that the absolute concentrations for the individual and

TABLE 1. Identification and mean (± SD) concentrations (nmol/l) of individual bile acids identified in the amniotic fluids

Peak No.	Retention Index (MU)	Identification	Gestational Age 13–14 wk (n = 5)	16 wk (n = 5)	19 wk (n = 5)	38–41 wk (n = 5)
1	31.09	3α,7α,12α-trihydroxy-5β-norcholan-23-oic	N.D.	N.D.	N.D.	9 ± 4
2	31.12	3α-hydroxy-5α-cholanoic	N.D.	N.D.	23 ± 12	N.D.
3	31.25	3α-hydroxy-5β-cholanoic (lithocholic)	133 ± 23	134 ± 38	188 ± 102	2 ± 1
4	31.28	dihydroxy bile acid	N.D.	N.D.	N.D.	6 ± 2
5	31.32	cholesterol	—	—	—	—
6	31.57	3α,12α-dihydroxy-5α-cholanoic	23 ± 8	22 ± 8	19 ± 8	N.D.
7	31.62	dihydroxy bile acid	N.D.	N.D.	21 ± 10	N.D.
8	31.64	dihydroxy bile acid	N.D.	N.D.	N.D.	5 ± 2
9	31.72	3β,7α-dihydroxy-5β-cholanoic	79 ± 46	45 ± 15	21 ± 7	3 ± 1
10	31.81	3β,12α-dihydroxy-5β-cholanoic	42 ± 27	34 ± 13	49 ± 18	N.D.
11	31.83	dihydroxy bile acid (1β,3x-dihydroxy[a])	N.D.	N.D.	N.D.	4 ± 2
12	31.91	3α,12α-dihydroxy-5β-cholanoic (deoxycholic)	162 ± 38	133 ± 63	187 ± 75	40 ± 22
13	32.00	3β-hydroxy-5-cholenoic and trace of 3α,7α,12α-trihydroxy-5α-cholanoic	56 ± 26	98 ± 12	98 ± 33	32 ± 14
14	32.15	3α,7α-dihydroxy-5β-cholanoic (chenodeoxycholic)	706 ± 283	433 ± 136	221 ± 57	22 ± 7
15	32.28	3α,7α,12α-trihydroxy-5β-cholanoic (cholic) and trace of 3α,6α-dihydroxy-5β-cholanoic	489 ± 201	281 ± 114	184 ± 33	178 ± 39
16	32.46	3β,12α-dihydroxy-5-cholenoic	N.D.	N.D.	N.D.	10 ± 5
17	32.51	dihydroxy bile acid	N.D.	N.D.	7 ± 2	13 ± 4
18	32.57	3α,7β-dihydroxy-5β-cholanoic	56 ± 44	37 ± 22	31 ± 14	N.D.
19	32.59	trihydroxy cholenoic	N.D.	N.D.	N.D.	16 ± 10
20	32.65	3β,12α-dihydroxy-5α-cholanoic	21 ± 16	16 ± 10	38 ± 41	N.D.
21	32.73	3β,7β-dihydroxy-5β-cholanoic	N.D.	N.D.	12 ± 2	N.D.
22	32.73	7α-hydroxy-3-oxo-5β-cholanoic and trihydroxy bile acid	22 ± 13	11 ± 3	N.D.	N.D.
23	32.78	3α,7α-dihydroxy-5β-cholan-25-oic (homochenodeoxycholic)	30 ± 19	18 ± 10	N.D.	4 ± 1
24	32.85	3α,7α,12α-trihydroxy-5β-cholan-25-oic (homocholic)	N.D.	N.D.	N.D.	9 ± 3
25	33.05	1β,3α,12α-trihydroxy-5β-cholanoic	58 ± 27	42 ± 9	38 ± 17	20 ± 13
26	33.13	3α,6α,7α-trihydroxy-5β-cholanoic (hyocholic)	192 ± 57	90 ± 19	67 ± 18	—
27	33.13	3α,6α,7α,12α-tetrahydroxy-5β-cholanoic	—	—	—	55 ± 32
28	33.16	3α-hydroxy-7-oxo-5β-cholanoic	N.D.	N.D.	24 ± 11	N.D.
29	33.20	dihydroxy-oxo-cholenoic	41 ± 23	N.D.	N.D.	N.D.
30	33.24	1β,3α,7α,12α-tetrahydroxy-5β-cholanoic	58 ± 23	36 ± 7	29 ± 11	335 ± 243
31	33.30	dihydroxy-cholenoic	N.D.	N.D.	N.D.	158 ± 111
32	33.41	1β,3x,7x-trihydroxy-5β-cholanoic	21 ± 11	19 ± 5	22 ± 8	N.D.
33	33.42	trihydroxy-cholanoic	N.D.	N.D.	N.D.	19 ± 11
34	33.56	trihydroxy-cholanoic (3,7,22-trihydroxy)[a]	22 ± 8	8 ± 2	10 ± 3	N.D.
35	33.64	3,7,12-trihydroxy-cholanoic	N.D.	N.D.	19 ± 17	N.D.
36	33.67	dihydroxy bile acid	N.D.	N.D.	8 ± 4	N.D.
37	33.71	7α,12α-dihydroxy-3-oxo-4-cholenoic	18 ± 4	N.D.	N.D.	156 ± 81
38	33.75	1β,3x,7x-trihydroxy-5β-cholanoic	33 ± 11	17 ± 3	9 ± 7	N.D.
39	33.76	dihydroxy and trihydroxy bile acid	N.D.	N.D.	15 ± 7	N.D.
40	33.83	3α,12α-dihydroxy-7-oxo-5β-cholanoic and 1β-tetrahydroxy-cholanoic	N.D.	N.D.	N.D.	20 ± 6
41	33.83	monohydroxy-3-oxo-cholanoic	N.D.	12 ± 7	N.D.	N.D.
42	33.85	3α,4β,7α-trihydroxy-5β-cholanoic	79 ± 34	36 ± 20	8 ± 3	N.D.
43	34.00	1β-tetrahydroxy cholanoic and trihydroxy-oxo-cholanoic	N.D.	N.D.	N.D.	6 ± 3
44	34.06	tetrahydroxy-cholanoic	N.D.	N.D.	N.D.	16 ± 6
45	34.13	1β-tetrahydroxy-cholanoic	12 ± 5	N.D.	N.D.	N.D.
46	34.41	tetrahydroxy-cholanoic	N.D.	N.D.	N.D.	51 ± 40
47	34.51	2β,3α,7α,12α-tetrahydroxy-5β-cholanoic	N.D.	N.D.	N.D.	88 ± 48
48	34.58	tetrahydroxy-oxo-cholanoic	N.D.	N.D.	N.D.	100 ± 56
49	34.67	3α,4β,7α,12α-tetrahydroxy-5β-cholanoic	N.D.	N.D.	N.D.	68 ± 35
50	34.70	1β,3α-dihydroxy-7-oxo-5β-cholanoic[a]	34 ± 13	30 ± 7	29 ± 7	N.D.
51	34.81	1β-trihydroxy-oxo-5β-cholanoic[a]	N.D.	N.D.	N.D.	10 ± 7
52	34.89	1β-tetrahydroxy-cholanoic	12 ± 3	N.D.	N.D.	N.D.
53	34.96	1β-tetrahydroxy-cholanoic	N.D.	N.D.	N.D.	6 ± 3
54	35.33	tetrahydroxy-cholenoic	N.D.	N.D.	N.D.	10 ± 7
	Totals		2399 ± 963	1552 ± 523	1377 ± 527	1471 ± 819

N.D., not detected; x denotes unknown position of stereochemistry or substituent.
[a]Tentative.

total bile acids are, in general, much higher in amniotic fluid from early gestation compared with full term and this is explained by the greater dilution as the volume of amniotic fluid increases in utero towards term (42). Furthermore, it is probable that many bile acids are preferentially removed from amniotic fluid by fetal swallowing and subsequent sequestration in the fetal gut. In this regard, lithocholic acid, which is present in appreciable concentrations in amniotic fluid during early gestation, is virtually undetectable in amniotic fluid at full term, but is one of the major bile acids of meconium (28, 30). This recompartmentalization of the bile acid pool is more marked with the more hydrophobic and less soluble bile acids. Indeed, the very polar 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic acid constitutes the major bile acid of amniotic fluid at full term but represents only a small proportion of the bile acids in amniotic fluid during early gestation.

The quantitative increase in the extent of additional nuclear hydroxylation pathways for bile acid synthesis is a striking feature of the ontogeny of bile acid synthesis and metabolism during gestation. This is evident in **Fig. 2** which summarizes the percentage of the individual groups of bile acids during early and late gestation. These data indicate that the bile acid pool during development becomes progressively more hydrophilic. One can speculate as to possible reasons for these changes. For example, these multiple hydroxylation pathways may be activated in response to a sluggish enterohepatic circulation in the fetus due to an immaturity in bile acid uptake and transport (43, 44), and may serve to protect the fetal liver from the accumulation of potentially more cytotoxic/hepatotoxic hydrophobic bile acids (45). In early gestation, when it is evident from the analysis of human fetal bile that the total bile acid pool must be very small (9, 10), these additional hydroxylation pathways are quantitatively less important; however, as the bile acid pool expands and accumulation of primary bile acids occurs within the hepatocyte, the liver presumably responds by a switch-on of these hydroxylation pathways in a manner similar to the situation that occurs in adults with severe cholestatic syndromes (25). Cholestasis in adults is characterized by an accumulation of primary bile acids with a reduction in bile acid synthesis (46). Teleologically, these pathways may thus served to metabolize primary bile acids to limit their feedback inhibition on bile acid synthesis in a period where there is a demand to increase markedly the bile acid pool-size in preparation for neonatal life.

Conjugation of bile acids

Because of the relatively low concentrations of bile acids and the small volumes of amniotic fluid obtained from each pregnancy, it was not possible to obtain information about the state of bile acid conjugation in each individual sample of amniotic fluid. Therefore, two sepa-

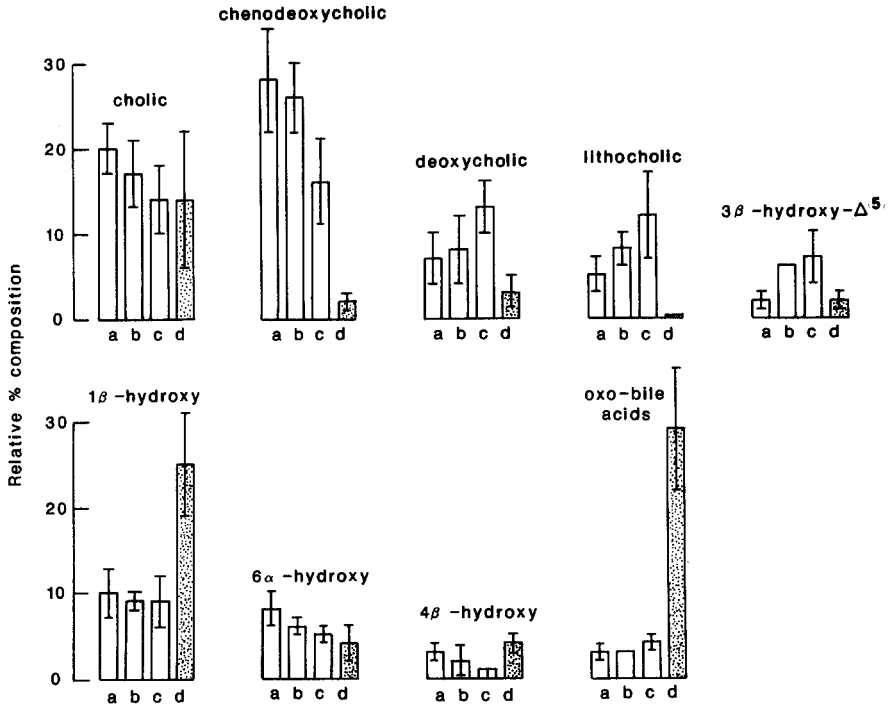

Fig. 2. Histograms showing the relative proportions of the principal bile acids found in the amniotic fluid at different times of gestation; a, 13 wk; b, 16 wk; c, 19 wk; d, full term.

TABLE 2. Concentrations (nmol/l) of unconjugated, glycine, taurine, and sulfate-conjugated bile acids in two separate pooled samples of amniotic fluids from pregnancies during early (16 weeks) gestation

Retention Index (MU)	Identification	Unconjugated		Conjugate Fractions: Glycine		Taurine		Sulfate	
		A	B	A	B	A	B	A	B
31.18	3α-hydroxy-5α-cholanoic	N.D.	N.D.	1	1	N.D.	N.D.	N.D.	N.D.
31.23	3α-hydroxy-5β-cholanoic	N.D.	N.D.	1	5	2	2	165	180
31.32	cholesterol	—	—	—	—	—	—	—	—
31.50	dihydroxy bile acid	N.D.	N.D.	1	1	N.D.	N.D.	N.D.	N.D.
31.60	3α,12α-dihydroxy-5α-cholanoic	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	8	11
31.69	3β,7α-dihydroxy-5β-cholanoic	N.D.	N.D.	17	13	16	19	22	16
31.80	3β,7α,12α-trihydroxy-5β-cholanoic	N.D.	N.D.	2	4	N.D.	N.D.	N.D.	N.D.
31.80	3β,12α-dihydroxy-5β-cholanoic	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	28	40
31.88	3α,12α-dihydroxy-5β-cholanoic	N.D.	N.D.	22	37	14	15	102	138
32.00	3β-hydroxy-5-cholenoic and trace of 3α,7α,12α-trihydroxy-5α-cholanoic	N.D.	N.D.	5	5	47	39	97	118
32.16	3α,7α-dihydroxy-5β-cholanoic	16	9	70	59	265	250	195	104
32.29	3α,7α,12α-trihydroxy-5β-cholanoic	29	28	77	92	120	143	9	16
32.49	3β,12α-dihydroxy-5-cholenoic	N.D.	N.D.	N.D.	N.D.	3	4	8	11
32.56	3α,7β-dihydroxy-5β-cholanoic	5	16	52	17	6	4	39	18
32.64	3β,12α-dihydroxy-5α-cholanoic	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	10	20
32.66	trihydroxy bile acid	1	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
32.70	dihydroxy bile acid	N.D.	N.D.	N.D.	N.D.	7	9	13	10
32.77	3α,7α-dihydroxy-5β-cholan-25-oic	N.D.	N.D.	N.D.	N.D.	7	9	N.D.	N.D.
32.85	3α,7α,12α-trihydroxy-5β-cholan-25-oic	N.D.	N.D.	4	2	5	8	N.D.	N.D.
33.02	1β,3α,12α-trihydroxy-5β-cholanoic	N.D.	N.D.	10	14	26	35	8	9
33.13	3α-hydroxy-7-oxo-5β-cholanoic	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	20	14
33.14	3α,6α,7α-trihydroxy-5β-cholanoic	N.D.	N.D.	15	16	74	81	N.D.	N.D.
33.24	7α,12α-dihydroxy-3-oxo-5β-cholanoic and 1β-hydroxy bile acid	N.D.	N.D.	3	5	33	30	N.D.	N.D.
33.29	7β-hydroxy-3-oxo-5β-cholanoic[a]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	12	7
33.34	1β-trihydroxy-bile acid	N.D.	N.D.	2	4	N.D.	N.D.	N.D.	N.D.
33.40	3α,4β-dihydroxy-5β-cholanoic	N.D.	N.D.	N.D.	N.D.	11	12	8	9
33.53	trihydroxy cholanoic (3,7,22-trihydroxy)[a]	N.D.	N.D.	1	2	9	8	N.D.	N.D.
33.53	dihydroxy bile	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	8	8
33.62	3β,7β-dihydroxy-5α-cholanoic	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	8	4
33.68	7α,12α-dihydroxy-3-oxo-4-cholenoic	7	14	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
33.70	dihydroxy bile acid	N.D.	N.D.	5	2	N.D.	N.D.	N.D.	N.D.
33.74	1β,3x,7x-trihydroxy-5β-cholanoic	N.D.	N.D.	1	1	12	13	3	2
33.86	trihydroxy and dihydroxy bile acid	N.D.	N.D.	4	<1	N.D.	N.D.	N.D.	N.D.
33.91	3α,4β,7α-trihydroxy-5β-cholanoic	N.D.	N.D.	3	16	17	30	7	6
34.05	dihydroxy-oxo-cholenoic	N.D.	N.D.	3	5	4	5	7	11
34.15	3α,7α-dihydroxy-12-oxo-5β-cholanoic	N.D.	N.D.	1	2	N.D.	N.D.	N.D.	N.D.
34.19	1β-trihydroxy bile acid	N.D.	N.D.	1	<1	N.D.	N.D.	N.D.	N.D.
34.35	tetrahydroxy bile acid	3	3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
34.53	3α,4β,7α,12α-tetrahydroxy-5β-cholanoic	N.D.	N.D.	N.D.	N.D.	4	7	N.D.	N.D.
34.69	1β,3α-dihydroxy-7-oxo-5β-cholanoic	N.D.	N.D.	3	4	10	14	20	17
34.88	1β-trihydroxy bile acid	N.D.	N.D.	N.D.	N.D.	3	4	N.D.	N.D.
	Totals	61	71	304	307	695	741	797	769

	Total Amniotic Fluid Bile Acid Concentrations	
	A = 1857 nmol/l	B = 1888 nmol/l
Relative % unconjugated bile acids	3.3	3.8
Relative % glycine conjugates	16.4	16.3
Relative % taurine conjugates	37.4	39.2
Relative % sulfate conjugates	42.9	40.7

N.D., not detected.
[a]Tentative.

rate pools of amniotic fluid, made from 13 different pregnancies of 16 weeks gestation were analyzed after separation of the bile acids into conjugate groups using a lipophilic anion exchange gel. No attempt was made to examine whether glucuronides were present, although in the analysis of total bile acids (Table 1) the hydrolytic steps would cleave glucuronic acid and these conjugates would be included.

Almost all of the bile acids identified in the two pools of amniotic fluid (**Table 2**) were conjugated; the unconjugated bile acids accounted for ca. 3% of the total. The major fraction of bile acids were taurine (37.4–39.2%) and sulfate (40.7–42.9%) conjugates. The sulfate fraction would also comprise amidated sulfated bile acids. Glycine conjugates accounted for 16–20% of the total bile acids. These findings are in contrast to those of human fetal bile where taurine conjugates make up >85% of the biliary bile acids of the fetus at the same gestational age and bile acid sulfates are barely detectable (9). The origin of these sulfates is difficult to ascertain. It is possible that sulfation takes place in the fetal liver and bile acid sulfates are preferentially excreted by renal clearance rather than by canalicular secretion into bile. Alternatively, sulfation by the kidney or maternal–fetal transfer could explain these findings. The finding of deoxycholic and lithocholic acids as sulfate conjugates might support a degree of maternal–fetal transfer. Certainly, the transplacental passage of bile acids in animals has been demonstrated (47, 48) while this group has also confirmed the transport of bile acids by the human placenta (49).

Examination of the extent of conjugation of some of the individual bile acids indicates a trend whereby the more hydrophobic compounds are preferentially sulfated (**Fig. 3**), i.e., lithocholic and 3β-hydroxy-5-cholenoic acids are found almost exclusively as sulfate conjugates, whereas the more hydrophilic bile acids, such as ursodeoxycholic and cholic acids, are found in lesser proportions as sulfate conjugates.

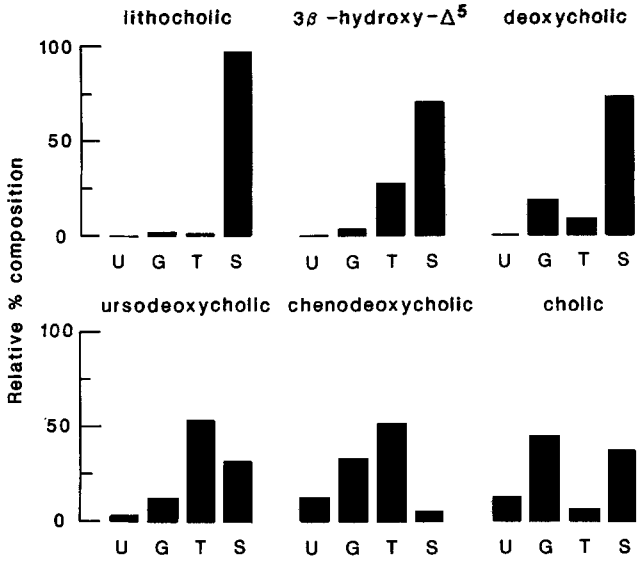

Fig. 3. The relative % distribution within the various conjugate fractions for a range of the bile acids with differing hydrophobicities found in amniotic fluid; U, unconjugated fraction; G, glycine conjugate; T, taurine conjugate; S, sulfate conjugate.

In summary, these data provide comprehensive information regarding the extent of bile acid metabolism during early gestation and indicate that very significant and major changes occur between the 20th week of gestation and full term. What triggers these events is uncertain, but comparable studies of amniotic fluid during the final trimester of pregnancy or examination of biological fluids from premature infants may provide some insight into the timing of these qualitative and quantitative changes. It is clear however, that the profile of amniotic fluid at full term, unlike early gestation, is almost indistinguishable from the urinary profile of patients with chronic cholestasis and this would be consistent with the period of physiological cholestasis (50–52) that is recognized in the early days postnatally.

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