Bile acid biosynthesis during development: hydroxylation of C_{27}-sterols in human fetal liver

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Abstract Several hydroxylase activities in bile acid biosynthesis were assayed in subcellular fractions of human fetal liver. The livers were obtained at legal abortions performed between gestational weeks 14 and 24. Microsomal 12α-hydroxylase and mitochondrial 12α- and 26-hydroxylase activities were detected from week 14. The microsomal fraction also had capacity for 25-hydroxylation, whereas 7α- and 26-hydroxylase activities were hardly detectable. The variation of the hydroxylase activities between different experiments can be explained by inactivation during the abortion or workup procedure. The results are discussed with respect to earlier studies of bile acid biosynthesis during development and adult life. — **Gustafsson, J.** Bile acid biosynthesis during development: hydroxylation of C_{27}-sterols in human fetal liver. *J. Lipid Res.* 1986. **27:** 801–806.

Supplementary key words hydroxylase activities • microsomes • mitochondria

The development of bile acid biosynthesis during fetal life is important for the process of lipid absorption in the newborn (1). Work by Watkins et al. (2) indicates a low capacity for bile acid biosynthesis in newborns, especially in prematures. This may be one explanation for the functional immaturity of neonatal lipid absorption (3). The presence of bile acids in the fetal gall bladder from 14 weeks of gestation (3) indicates the occurrence of fetal bile acid biosynthesis. However, the presence of secondary bile acids shows that at least part of the bile acids is derived from the mother (3).

There is little information available concerning individual reactions in fetal bile acid biosynthesis (3). Recently, the mitochondrial fraction of human fetal liver was shown to hydroxylate 5β-cholestane-3α,7α-diol in the sterol nucleus as well as in the side chain (4). In addition, fetal liver microsomes hydroxylated the same compound in the 12α-position.

The present work reports studies of hydroxylations of bile acid precursors in the mitochondrial and microsomal fractions of human fetal liver.

MATERIALS AND METHODS

Labeled compounds

[4-^{14}C]Cholesterol (sp act 61 Ci/mol) was obtained from the Radiochemical Centre (Amersham, England). Before use, the material was purified by chromatography on aluminium oxide, grade III (Woelm, Eschwege, West Germany) (5). 5-[7β-^{3}H]Cholestene-3β,7α-diol (sp act 5 Ci/mol), 7α-[6β-^{3}H]hydroxy-4-cholesten-3-one (sp act 7 Ci/mol), 5β-[7β-^{3}H]cholestane-3α,7α-diol (sp act 50 Ci/mol) and 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol (sp act 50 Ci/mol) were prepared as described previously (6).

Unlabeled compounds

5-Cholestene-3β,26-diol, 5-cholestene-3β,7α,26-triol, 7α,12α-dihydroxy-4-cholesten-3-one, 5β-cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α,26-triol, and 5β-cholestane-3α,7α,12α,26-tetrol were prepared as described previously (6).

Enzymes and cofactors

NADPH and isocitric acid were obtained from Sigma Chemical Co. (St. Louis, MO).

Experimental

Human fetuses were obtained at legal abortions performed in weeks 14–24 for socio-medical reasons. Fetal age was determined from data concerning the pregnancies and from height measurements. Consent was given by the ethical committee of the University of Uppsala. The abortions were performed by use of prostaglandins or via hysterotomy. The fetuses were taken to the laboratory and liver tissue was taken out and chilled in ice-cold buffer solution. Preparation of liver homogenates was started within 45–60 min after abortion. Liver homogenates (10%, w/v) were prepared in 0.25 M sucrose with a Potter-Elvehjem homogenizer equipped with a loosely fitting pestle (6). The microsomal fraction was obtained from the homogenate by centrifugations at 800 *g*, 20,000 *g*, and 100,000 *g* (6). The microsomal pellet was finally suspended and homogenized in 0.05 M Tris–acetate, pH 7.4, in a volume corresponding to 1/4 to 1/2 of the initial volume. The mitochondrial fraction was obtained by resuspension in 0.25 M sucrose of the pellet obtained after centrifugation at 20,000 *g* and recentrifugation twice at 6,400 *g* for 20 min (7). The final precipitate was sus-

pended in 0.1 M Tris-Cl buffer, pH 7.4, in a volume corresponding to 1/6 to 1/4 of the initial volume of the homogenate. The protein content of the microsomal fraction was between 0.5 and 3.0 mg/ml and that of the mitochondrial fraction between 0.4 and 2.5 mg/ml when determined according to Lowry et al. (8). The lack of detectable 25-hydroxylase activity towards 5β-cholestane-3α,7α,12α-triol in the mitochondrial fraction as well as lack of 26-hydroxylase activity towards the same substrate in the microsomal fraction (c.f. Results) speak against occurrence of a significant contamination with microsomal protein in the mitochondrial fraction and vice versa. Both the microsomal and mitochondrial fractions were contaminated to some extent with peroxisomal protein as judged from measurements of the activity of urate oxidase, a peroxisomal marker enzyme (9). In an experiment with a liver from a 15-week-old fetus, about 75% of the total urate oxidase activity sedimented with the 800-*g* pellet, about 15% with the mitochondrial fraction, and about 10% with the microsomal fraction.

In one experiment the effect on microsomal and mitochondrial C_{27}-sterol hydroxylation of preincubation of whole liver at 37°C was studied. Thus, a liver from an 18-week-old fetus was divided into three parts. One of these was immediately used for preparation of subcellular fractions, whereas the other two were incubated at 37°C for 45 and 90 min, respectively, before preparations of subcellular fractions were started.

In another experiment the hepatic concentrations of cholic and chenodeoxycholic acid were determined by isotope dilution-mass spectrometry after addition of deuterium-labeled internal standards (10), alkaline hydrolysis, acidification, and ether extraction of liver tissue from two 18-week-old fetuses. The concentrations of cholic acid were 3.2 and 4.2 nmol per g of liver, respectively, and those of chenodeoxycholic acid were 1.7 and 4.1 nmol per g of liver, respectively. The hepatic concentration of deoxycholic acid was less than 1 nmol per g of liver.

Incubation procedures and analysis of incubation mixtures

In incubations with the microsomal fraction, 0.25 μmol of substrate dissolved in 50 μl of acetone was incubated with 2 ml of microsomal fraction in a total volume of 3 ml of homogenizing medium. The incubation mixtures were supplemented with 3 μmol of NADPH. Microsomal incubations with cholesterol were performed as described earlier (11). In incubations with the mitochondrial fraction, 0.25 μmol of substrate dissolved in 50 μl of acetone was incubated with 2 ml of mitochondrial fraction in a total volume of 3 ml of the homogenizing medium. The incubation mixtures were supplemented with 3 μmol of NADPH and 30 μmol of $MgCl_2$ (5). In some experiments NADPH was replaced by 4.6 μmol of isocitric acid (5). All incubations were performed for 20 min at 37°C and were terminated by the addition of 5 ml of 96% (v/v) ethanol. After acidification and ether extraction, the incubations were subjected to thin-layer chromatography using appropriate external standards (6). Radioactivity in the different chromatographic zones was assayed with a thin-layer scanner (Berthold, Karlsruhe, Germany) (12). The chromatographic zones corresponding to the products were extracted with methanol, converted into trimethyl silyl ethers (12), and analyzed by radio-gas-liquid chromatography using a Barber-Colman 5000 instrument equipped with a 3% QF-1 column (12). Knowing the specific radioactivity of the compounds used, the conversion into different products was calculated from the amount of radioactivity found in the thin-layer chromatographic zone and the amount of radioactivity found with the appropriate retention time in the radio-gas chromatogram. As judged from gas-liquid chromatography of extracts of microsomal and mitochondrial fractions, there was no endogenous dilution with any of the incubated C_{27}-sterols except for cholesterol.

In case of microsomal and mitochondrial hydroxylations of cholesterol, 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol as well as mitochondrial 25-hydroxylation of 7α-hydroxy-4-cholest-3-one, the identities of the products were confirmed by gas-liquid chromatography-mass spectrometry (6) or by crystallization to constant specific radioactivity after dilution with unlabeled authentic material (cf. ref. 4).

RESULTS

Incubations with the microsomal fraction

Out of 15 tested livers, microsomal hydroxylase activities were detected in 11. In the active preparations the relative conversions of the compounds varied between 0.1 and 4%. The variation between duplicate incubations from the same liver was less than 15%. **Table 1** summarizes the hydroxylase activities and the number of metabolic products in incubations of different substrates with the microsomal fraction of livers from fetuses of different gestational ages. Due to the small amounts of liver tissue available, only a limited number of experiments could be performed with each preparation.

Microsomal hydroxylase activity was detected in gestational week 14. Regardless of fetal age, very low or no conversion was noted in the case of microsomal 7α-hydroxylation of cholesterol. With microsomes from one out of four livers, there was some 12α- and 26-hydroxylation of 5-cholestene-3β,7α-diol. In experiments with 7α-hydroxy-4-cholesten-3-one, some degree of 12α-hydroxylation was obtained. With one liver from week 24 there also was some 26-hydroxylation. 5β-Cholestane-3α,7α-diol was also hydroxylated predomi-

TABLE 1. Hydroxylation of C_{27}-sterols by the microsomal fraction of fetal liver

	Gestational Week										
	14	15	16	16	16	17	18	18	22	24	24
	pmol × mg prot^{-1} × min^{-1}										
7α-Hydroxylation											
Cholesterol	< 4	–[a]	–	< 4	–	–	–	–	–	< 4	< 4
12α-Hydroxylation											
5-Cholestene-3β,7α-diol	–	–	< 4	–	–	–	< 4	–	–	6	< 4
7α-Hydroxy-4-cholesten-3-one	–	–	–	21	4	–	–	–	4	–	23
5β-Cholestane-3α,7α-diol	6	26	< 4	–	–	< 4	7	30	4	4	12
25-Hydroxylation											
5β-Cholestane-3α,7α-diol	< 4	< 4	< 4	–	–	< 4	< 4	< 4	< 4	< 4	< 4
5β-Cholestane-3α,7α,12α-triol	–	–	9	4	7	19	< 4	–	22	4	< 4
26-Hydroxylation											
5-Cholestene-3β,7α-diol	–	–	< 4	–	–	–	< 4	–	–	5	< 4
7α-Hydroxy-4-cholesten-3-one	–	–	–	–	–	–	–	–	–	–	20
5β-Cholestane-3α,7α-diol	< 4	< 4	< 4	–	–	< 4	< 4	< 4	< 4	< 4	< 4
5β-Cholestane-3α,7α,12α-triol	–	–	< 4	< 4	< 4	< 4	< 4	–	< 4	< 4	< 4

[a] Not determined.

nantly in the 12α-position. Almost no 26-hydroxylation was obtained with this substrate. The product in incubations with 5β-cholestane-3α,7α,12α-triol was identified as 5β-cholestane-3α,7α,12α,25-tetrol. There was almost no 26-hydroxylation of this substrate.

Incubations with the mitochondrial fraction

Out of 15 tested livers, it was possible to detect one or several mitochondrial hydroxylase activities in 11. In the active preparations the relative conversions of the compounds varied between 0.1 and 6%. The variation when duplicate incubations from the same liver were performed was less than 15%.

Table 2 summarizes the hydroxylase activities and the number of metabolic products in incubations of different substrates with the mitochondrial fraction. Very low 26-hydroxylase activity towards cholesterol was found. Only 2 out of 11 preparations were active towards this substrate. In one experiment with a liver from a fetus in week 24, cholesterol was hydroxylated in the C-25 position to a small extent. Mitochondrial hydroxylation of 5-cholestene-3β,7α-diol was tested with two preparations from livers of fetuses in week 24. Both 12α- and 26-hydroxylations were obtained. Mitochondrial hydroxylation of 7α-hydroxy-4-cholesten-3-one was studied with two preparations. One of these, a preparation from the liver of a fetus in week 16, yielded only the 25-hydroxylated product, whereas the other preparation from a fetus in week 24 had 12α- as well as 26-hydroxylase activity. Mitochondrial 12α- and 26-hydroxylation of 5β-cholestane-

TABLE 2. Hydroxylation of C_{27}-sterols by the mitochondrial fraction of fetal liver

	Gestational Week										
	14	15	15	16	16	16	17	18	19	24	24
	pmol × mg protein^{-1} × min^{-1}										
12α-Hydroxylation											
5-Cholestene-3β,7α-diol	–[a]	–	–	–	–	–	–	–	–	119	6
7α-Hydroxy-4-cholesten-3-one	–	–	–	–	–	< 4	–	–	–	–	118
5β-Cholestane-3α,7α-diol	4	< 4	–	< 4	5	–	< 4	< 4	23	70	–
25-Hydroxylation											
Cholesterol	< 4	< 4	< 4	< 4	< 4	< 4	< 4	< 4	< 4	4	< 4
7α-Hydroxy-4-cholesten-3-one	–	–	–	–	–	9	–	–	–	–	< 4
26-Hydroxylation											
Cholesterol	< 4	15	< 4	< 4	< 4	< 4	< 4	< 4	< 4	< 4	4
5-Cholestene-3β,7α-diol	–	–	–	–	–	–	–	–	–	7	19
7α-Hydroxy-4-cholesten-3-one	–	–	–	–	–	< 4	–	–	–	–	48
5β-Cholestane-3α,7α-diol	23	< 4	–	< 4	29	–	< 4	< 4	21	40	–
5β-Cholestane-3α,7α,12α-triol	8	39	68	20	14	–	26	47	13	184	144

[a] Not determined.

3α,7α-diol occurred in four out of eight livers. The activities varied markedly between different liver preparations. Irrespective of fetal age, incubations of 5β-cholestane-3α,7α,12α-triol gave only 26-hydroxylated product. This was obtained in ten out of ten livers. In two experiments, incubation of 5β-cholestane-3α,7α,12α-triol with the mitochondrial fraction from fetuses in weeks 16 and 24, gave in addition to 5β-cholestane-3α,7α,12α,26-tetrol also small amounts of a product with thin-layer chromatographic properties of 3α,7α,12α-trihydroxy-5β-cholestanoic acid.

Effect on microsomal and mitochondrial hydroxylations of preincubation of whole liver at 37°C

When whole liver from an 18-week-old fetus was incubated at 37°C for 45 or 90 min, microsomal 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol was inhibited by about 40% and mitochondrial 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol by about 70 and 80%, respectively (**Table 3**).

Effect on microsomal and mitochondrial hydroxylations of bile acids

There was no inhibition of microsomal 25-hydroxylation or mitochondrial 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol when preparations from the liver of an 18-week-old fetus were incubated with the addition of taurocholic acid, 2.1 or 4.2 μM, and taurochenodeoxycholic acid, 2.1 or 4.2 μM, respectively (**Table 4**).

DISCUSSION

Cytochrome P-450-dependent, microsomal hydroxylations in human fetal liver have been shown with laurate (13), drugs (14), and several steroids (15). In addition to recent work on 12α-hydroxylation of 5β-cholestane-3α,7α-diol in fetal liver (4), the present work shows that the microsomal and mitochondrial fractions of fetal liver have capacity for several hydroxylations of C_{27}-sterols that are intermediates in bile acid synthesis in adult liver. There was no clear increase in hydroxylase activities with gestational age. In evaluating the efficiency of conversion, one has to consider the possibility of partial inactivation of the enzymes during abortion or during preparation of the subcellular fractions. Evidence for the possible occurrence of such inactivation was obtained in an experiment in which whole fetal liver was incubated at 37°C before preparation of subcellular fractions. However, in the overall investigation, the microsomal and/or the mitochondrial fractions of 13 out of 15 livers were active towards one or more of the substrates tested.

TABLE 3. Effect of preincubation of fetal liver at 37°C on microsomal and mitochondrial hydroxylations

	Percentage Relative Conversion at Time of Preincubation		
	0 min	45 min	90 min
Microsomal 25-hydroxylation 5β-cholestane-3α,7α,12α-triol	100	61	62
Mitochondrial 26-hydroxylation 5β-cholestane-3α,7α,12α-triol	100	30	17

The experiment was performed with liver tissue from an 18-week-old fetus. The incubations were performed as described in Methods. The conversion rate obtained with material that was not preincubated was set to 100%.

Microsomal hydroxylations

Regardless of fetal age, almost no microsomal 7α-hydroxylase activity towards cholesterol was detectable. Since microsomal 7α-hydroxylation of cholesterol is the rate-limiting step in bile acid synthesis in adult liver (1), the low degree of 7α-hydroxylation of cholesterol may support findings by Watkins et al. (2) of low bile acid pools in newborns, especially prematures, as compared to adults. 7α-Hydroxylation of taurodeoxycholic acid was recently shown to occur in fetal human liver microsomes (16). At least in rat liver, this reaction is catalyzed by a species of cytochrome P-450 different from that catalyzing cholesterol 7α-hydroxylation (17).

In similarity with in vitro preparations of adult human liver, major microsomal hydroxylations of fetal liver are 12α- and 25-hydroxylation (12).

The effect of addition of conjugated cholic and chenodeoxycholic acid, in concentrations occurring in liver, on mitochondrial and microsomal hydroxylation of 5β-cholestane-3α,7α,12α-triol was tested. Since taurine conjugates predominate in the fetus (3), taurocholic and taurochenodeoxycholic acid were used. Neither of these bile acids had any inhibitory effect on the hydroxylations tested. This is not consistent with an inhibitory effect of bile acids on hydroxylations of C_{27}-sterols in fetal liver. However, in view of the characteristics of the adult 7α-hydroxylase activity in vivo (1), it cannot be excluded that the low 7α-hydroxylase activity in the present study is due in part to an inhibition by intrahepatic bile acids.

Due to the sedimentation properties of fetal liver subcellular fractions, hydroxylase activities can be found already in the pellet obtained after centrifugation at 800 *g* (18). This was confirmed by assay of C_{27}-sterol hydroxylase activities in the 800 *g* pellet in some experiments. In relation to the amount of subcellular protein incubated, the microsomal hydroxylases were less active than the mitochondrial hydroxylases. This differs from what is found in adult liver preparations (12).

Mitochondrial hydroxylations

The low extent of 26-hydroxylation of cholesterol under the conditions used is surprising since 26-hydroxycholes-

TABLE 4. Effect of addition of taurocholic (TCA) and taurochenodeoxycholic (TCDA) acid on microsomal and mitochondrial hydroxylations of 5β-cholestane-3α,7α,12α-triol

	No Addition	TCA 2.1 μM + TCDA 2.1 μM	TCA 4.2 μM + TCDA 4.2 μM
		percentage relative conversion	
Microsomal 25-hydroxylation	100	90	90
Mitochondrial 26-hydroxylation	100	117	102

The experiment was performed with liver tissue from an 18-week-old fetus. The conversion rate in the incubations without any addition of bile acids was set to 100%.

terol has been found in meconium (19) and 3β-hydroxy-5-cholenoic acid has been found in both meconium and amniotic fluid (20, 21). 3β-Hydroxy-5-cholenoic acid has its origin in 26-hydroxycholesterol and is supposed to represent a "primitive" pathway for synthesis of monohydroxy bile acids in the fetus (21). On the other hand, Makino et al. (22) have shown that cholesterol was rather poorly converted into 3β-hydroxy-5-cholenoic acid in a child with biliary atresia. The authors suggested that another precursor than cholesterol itself might be involved in this particular transformation (22). In the present investigation, the low 26-hydroxylase activity towards cholesterol may well be due to partial inactivation of the 26-hydroxylase system. The lability of the system has been pointed out earlier both in work with adult human liver and rat liver (12, 23).

In one experiment some degree of mitochondrial 25-hydroxylation of cholesterol occurred and in another 25-hydroxylation of 7α-hydroxy-4-cholesten-3-one. 25-Hydroxylation of cholesterol has earlier been shown with rat (5) but not with human liver mitochondria (12). The importance of 25-hydroxylation of cholesterol in vivo is questionable since, at least in adults, 25-hydroxycholesterol is a poor precursor of bile acids (24). Liver mitochondrial 25-hydroxylation can be important in vitamin D activation (25), but it remains to be established whether fetal liver mitochondria has capacity for vitamin D 25-hydroxylation.

Pathways in fetal bile acid synthesis

The results of the present work are consistent with fetal bile acid synthesis occurring along pathways similar to those in adult human liver (1). In spite of the low degree of 7α-hydroxylation of cholesterol under the in vitro conditions used (11), it is still likely that this reaction initiates fetal bile acid synthesis in vivo. No other microsomal hydroxylation of cholesterol did in fact occur, making any other microsomal hydroxylation of cholesterol in vivo unlikely.

The present results do not rule out the possibility that mitochondrial 26-hydroxylation of cholesterol could initiate part of the fetal bile acid synthesis. Despite the low conversions obtained in the present investigation, the occurrence of fetal 26-hydroxycholesterol (19) and associated compounds (20, 21) makes it probable that some mitochondrial 26-hydroxylation of cholesterol occurs in vivo. To some extent, fetal 26-hydroxycholesterol could be derived from the mother by placental transfer, since the compound has been shown to occur in human serum (26).

As in adult liver, 12α-hydroxylation probably may occur with different substrates. In this work, 5-cholestene-3β,7α-diol, 7α-hydroxy-4-cholesten-3-one, and 5β-cholestane-3α,7α-diol were shown to be 12α-hydroxylated. The relative roles of the microsomal and mitochondrial 12α-hydroxylase activities cannot be assessed from the present work. The mitochondrial 12α-hydroxylase activity is probably lost during the further ontogeny (4), since it has not been found in vitro in preparations from livers of healthy adults (12, 27). As mentioned before, there was no evidence of any significant contamination with mitochondrial protein in the microsomal fraction or vice versa. The small amounts of fetal liver tissue available have prevented the preparation of a peroxisome-enriched fraction. Recent work by Thompson and Krisans (28) has provided evidence for the presence of peroxisomal side chain hydroxylase activity towards 5β-cholestane-3α,7α,12α-triol in rat liver. As judged from determinations of urate oxidase activity (9), the microsomal and mitochondrial fractions in the present investigation were contaminated with peroxisomal protein to about the same extent. This contamination was evidently not associated with any detectable peroxisomal side chain hydroxylase activity, neither in the microsomal nor in the mitochondrial fraction. If this had been the case, one would have expected the same stereospecific hydroxylations of 5β-cholestane-3α,7α,12α-triol to occur to some extent in both subcellular fractions.

Mitochondrial 26-hydroxylation in fetal bile acid biosynthesis may probably occur with several substrates. In cholic acid synthesis, 5β-cholestane-3α,7α,12α-triol may be a substrate in vivo, whereas in chenodeoxycholic acid synthesis 26-hydroxylation of several dioxygenated sterols is possible. 7α,26-Dihydroxy-4-cholesten-3-one was shown recently to be efficiently converted into chenodeoxycholic acid in vivo in the adult human (29). Probably the fetal liver also has capacity for further oxidation of the sterol

side chain, since 3α,7α,12α-trihydroxy-5β-cholestanoic acid was tentatively identified in two mitochondrial incubations with 5β-cholestane-3α,7α,12α-triol.

In cholic acid synthesis in the adult, a pathway involving 25-hydroxylation may exist (30). Such a pathway involves 24β-hydroxylation of 5β-cholestane-3α,7α,12α,25-tetrol. Although no microsomal 24β-hydroxylation of 5β-cholestane-3α,7α,12α-triol occurred under the present conditions, further studies are needed to evaluate the possibility of a 25-hydroxylation pathway in fetal liver.

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