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Conjugation and Maximal Biliary Excretion of Bilirubin in the Rat During Pregnancy and Lactation and During Estroprogestogen Treatment

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Hepatic bilirubin conjugation and excretion were investigated during pregnancy and lactation in the rat. Bilirubin uridine diphosphate-glucuronyltransferase activity was decreased by 30% in pregnant rats, both when expressed per milligram of protein or as specific activity and per unit of liver weight. Liver size increased during pregnancy, and, as a consequence, total hepatic glucuronyltransferase activity was unchanged. The biliary bilirubin output was normal in pregnant rats, and when loaded with bilirubin, the maximal output in bile for the whole liver was also normal. In lactating rats, specific glucuronyltransferase activity returned to control values, but the activity per unit of liver weight was still lower, due to the decreased hepatic protein concentration. The liver remained enlarged during lactation, and total hepatic glucuronyltransferase activity was increased, together with the maximal output of bilirubin in bile. Two weeks after delivery, hepatic bilirubin conjugation and excretion in nonlactating mothers were comparable to those of virgin females. Parallel modifications of bilirubin glucuronyltransferase assayed *in vitro* and of maximal biliary output of the pigment *in vivo* were observed in all animals studied. The output of bilirubin diconjugates in bile was decreased during pregnancy but no changes of the proportion of the mono- to diconjugates in bile were observed 2 weeks after delivery both in lactating and in nonlactating rats. The modifications observed during pregnancy could not be reproduced by treatment with β-estradiol and progesterone. This suggests that different hormones or modifications of steroid metabolism are probably involved in the alterations of hepatic bilirubin metabolism in pregnant and lactating rats.

Bilirubin-IXα, the main breakdown product of heme turnover, is a model substance of endogenous hydrophobic compounds undergoing biotransformation in the liver prior to biliary excretion (1). Hepatic conjugation appears to be a critical step in determining the excretion of bilirubin in bile (2–5). It has been recently demonstrated that female rats exhibit both a higher hepatic conjugation rate and maximal excretory capacity (Tm) in bile than do males (5). The influence of pregnancy on bilirubin conjugation in female rat liver is however not clear. Previous investigations yielded contradictory results: the glucuronidation rate of bilirubin was found to be increased (6), unchanged (7) or decreased (8) in liver homogenates from pregnant rats, when compared to nonpregnant females. In one of these studies, bilirubin Tm seemed unaffected during pregnancy (6), despite the fact that a significantly increased bilirubin glucuronidation rate in liver homogenates was found. In the present work, bilirubin uridine diphosphate (UDP)-glucuronyltransferase activity was determined in virgin and pregnant female rats, both in untreated and in digitonin-activated liver homogenate, taking particular care in assessing the linearity of the reaction rate. Bilirubin Tm was established in rats having recovered from operation and anesthesia, which decreases bilirubin secretion in bile (5, 9). Because of the modifications which take place in biliary function during lactation (10), bilirubin conjugation and Tm were also determined in lactating rats. To check whether the changes observed during pregnancy are due to the increased production of estroprogestogens, similar investigations were performed in ovariectomized animals treated with estroprogestogens to yield plasma levels of estradiol and progesterone similar to those found in 20-day-old pregnant rats.

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MATERIALS AND METHODS

Bilirubin was obtained from Merck A.G. (Darmstadt, West Germany). UDP-glucuronic acid (ammonium salt), β-estradiol and progesterone were from Sigma Chemical Co. (St. Louis, Mo.).

Female inbred R/A Pfd rats of Wistar origin, 11 to 16 weeks old, were kept at 20° to 25°C, with humidity ranging from 45 to 80% and with 14 hr of light and 10 hr of darkness per day. The animals had free access to food and water, and were not starved before the experiment.

In the first study, four different groups of rats were investigated: virgin controls, 20- to 21-day-old pregnant animals, and two groups of mother rats studied 12 to 15 days after delivery. In one group, the animals were separated from their litters immediately after delivery, while in the other group they were allowed to lactate. Both control and pregnant rats were 2 weeks younger than lactating and nonlactating mothers.

In the second study, female rats 11 to 12 weeks old were ovariectomized on the day of estrus, determined by microscopic examination of the vaginal smear. In one group, ovariectomized animals received 5 mg progesterone and 0.05 mg β-estradiol daily during 17 days, administered by subcutaneous injections twice a day. Separate hormone solutions were freshly made every day in 1 volume of absolute ethanol, to which 9 volumes of olive oil were then added, to give a final concentration of 2.5 mg per 0.1 ml for progesterone and 0.025 mg per 0.1 ml for β-estradiol. Injection volumes were 0.1 ml. A second group of ovariectomized animals received daily injections of the ethanol/olive oil vehicle. This protocol has been found to lead to plasma concentrations of estradiol and progesterone similar to those found in 20-day-old pregnant rats (11). Measurement of serum hormone levels by radioimmunoassay in three rats subjected to hormonal treatment gave values (mean ± S.D.) of 135 ± 28 ng per ml for plasma progesterone and 1,050 ± 482 pg per ml for plasma estradiol.

Determination of Maximal Biliary Output ("Tm") of Bilirubin

Body weight of the animals was recorded before each experiment. Under anesthesia with pentobarbitone (5 mg per 100 gm body weight, i.p.), a polyethylene catheter (no. 1, Biotrol Pharma, Paris, France) was inserted into a jugular vein, and a second one into the bile duct. In pregnant rats, the uterus was ligated and removed. The latter procedure was carried out to avoid possible unknown interferences during bilirubin infusion due to transplacental transport and to allow better comparison of the body weights with the other groups of rats. The animals were then put in a restraining cage and transferred into a premature infant incubator to keep body temperature constant (37° ± 0.3°C). A solution of NaCl (0.154 mole per liter) was infused through the jugular vein catheter at a rate of 3 ml per hr. Three hours after cannulation of the bile duct, when the animals had fully recovered from anesthesia, bile was collected at 10-min intervals on ice and in the dark, in preweighed tubes.

Bilirubin was dissolved in NaOH (0.05 mole per liter), adjusted to pH 9.0 with HCl (0.05 mole per liter) and diluted with NaCl (0.154 mole per liter) to the required volume. After three control collections of bile, bilirubin, 3.42 μmoles per 100 gm body weight, dissolved in a volume of 1 ml, were injected over 5 min, immediately followed by an infusion of 0.26 μmole × min^{-1} per 100 gm body weight administered over 1 hr in a total volume of 3 ml. At the end of the infusion, the rats were anesthetized with pentobarbitone and exsanguinated through the carotid artery. The liver was excised, weighed and stored at −20°C for the transferase assay. Bile samples were weighed and analyzed immediately. The concentration of conjugated bilirubins in bile was estimated by diazo-coupling with ethylanthranilate (12). Maximal bilirubin concentration and maximal bilirubin output in bile were calculated as the mean of the three values obtained during the last 30 min of bilirubin infusion, when a steady-state excretion was reached.

The relative proportions of bilirubin mono- and diconjugates in bile were determined by alkaline methanolysis (13).

Enzyme Assay

Homogenates (250 or 100 mg of wet liver per ml of suspension for assay of untreated or digitonin-activated homogenate, respectively) were prepared in 0.25 mole per liter sucrose. Bilirubin UDP-glucuronyltransferase activity was determined as described by Heirwegh et al. (14). The volume of the incubation mixture was increased five times, by proportional increases of all its components, to a total volume of 3.3 ml to allow kinetic analysis. The incubation was performed in a shaking water bath at 37°C. An aliquot (0.5 ml) of the mixture was removed at 3, 6, 10, 15, 20 and 25 min, and added to a test tube containing 2 ml of ice-cold 0.4 *M* glycine/HCl buffer (pH 2.7). The conjugated bilirubin formed was then measured by diazo-coupling with ethylanthranilate (12). The reaction was found to be linear up to 10 min when untreated homogenate was used, and up to 20 min with digitonin-activated liver homogenate.

Protein concentration in liver homogenates was measured according to Lowry et al. (15).

Statistics

Statistical analysis of differences between means was performed using the Student's test, with $p < 0.05$ considered as the lowest limit of significance.

RESULTS

Liver and Body Weight

Liver weight increased in the rat during pregnancy and lactation. Liver weight relative to body weight did not change in pregnant rats when total body weight was considered (Table 1). When body weight was calculated by subtracting the content of the uterus (45 ± 13 gm), relative liver weight was increased in the pregnant animals (3.3 ± 0.3%; $p < 0.001$). In lactating mothers, relative liver weight was increased both when compared to controls and to nonlacting mothers (Table 1). Hepatic protein concentration did not change in pregnant rats, while it decreased in lactating animals (Table 1). Ovari-

TABLE 1. INFLUENCE OF PREGNANCY AND LACTATION ON BODY WEIGHT, LIVER WEIGHT AND LIVER PROTEIN CONCENTRATION IN THE RAT[a]

Group	No. of animals	Body weight (gm)	Liver weight (gm)	Relative liver weight (% of body weight)	Liver protein (mg/gm liver)
Virgin controls	6	192 ± 10	4.8 ± 0.2	2.6 ± 0.1	208 ± 7
Pregnant[b]	10	294 ± 8[c]	8.3 ± 0.8[c]	2.6 ± 0.2	205 ± 9
Lactating mothers	7	236 ± 22[d]	8.5 ± 1.1[c]	3.5 ± 0.3[c]	183 ± 11[d]
Nonlactating mothers	5	206 ± 23	5.5 ± 0.4[d]	2.7 ± 0.1	191 ± 15

[a] Results (mean ± S.D.) were obtained in 20 to 21-day-old pregnant rats and in mother rats 12 to 15 days after delivery.
[b] In pregnant rats, total body weight given included the uterus and its content (45 ± 13 gm); if subtracted, the relative liver weight increases to 3.3 ± 0.3%, comparable to values in the lactating mothers.
[c] $p < 0.001$.
[d] $p < 0.01$.

TABLE 2. INFLUENCE OF PREGNANCY AND LACTATION ON BILIRUBIN UDP-GLUCURONYLTRANSFERASE ACTIVITY IN RAT LIVER[a]

Group	No. of animals	Bilirubin UDP-glucuronyltransferase activity		
		nmoles/hr per mg of protein	nmoles/min per gm of liver	nmoles/min per whole liver
Virgin controls	6	29.9 ± 1.6	111 ± 7	538 ± 42
Pregnant	10	20.4 ± 2.2[b]	74 ± 5[b]	609 ± 72
Lactating mothers	7	30.8 ± 1.3	101 ± 4[c]	855 ± 122[b]
Nonlactating mothers	5	28.7 ± 2.9	103 ± 15	566 ± 56

[a] Bilirubin UDP-glucuronyltransferase activity was determined in digitonin-activated liver homogenates from 20- to 21-day-old pregnant rats and from mother rats 12 to 15 days after delivery. Values are means ± S.D.
[b] $p < 0.001$.
[c] $p < 0.01$.

TABLE 3. INFLUENCE OF PREGNANCY AND LACTATION ON BILE FLOW, BILIRUBIN CONCENTRATION AND OUTPUT AND PERCENTAGE OF BILIRUBIN DICONJUGATES IN BILE DURING BASAL CONDITIONS[a]

Group	No. of animals	Bilirubin concentration bile (μmoles/liter)	Bile flow (μl/min per gm of liver)	Bile flow (μl/min per whole liver)	Bilirubin output (nmoles/min per whole liver)	Diconjugates in bile (% of total conjugates)
Virgin controls	6	122 ± 14	2.4 ± 0.3	11.5 ± 1.1	1.4 ± 0.2	63 ± 12
Pregnant	10	99 ± 9[b]	1.9 ± 0.1[c]	16.0 ± 1.8[c]	1.6 ± 0.2	49 ± 4[c]
Lactating mothers	7	86 ± 9[c]	2.3 ± 0.2	19.9 ± 3.7[c]	1.7 ± 0.3	64 ± 5
Nonlactating mothers	5	115 ± 7	2.2 ± 0.2	12.3 ± 2.0	1.4 ± 0.2	62 ± 2

[a] Pregnant rats were studied 20 to 21 days after copulation plug, and lactating or nonlactating mothers 12 to 15 days after delivery. Values are means ± S.D.
[b] $p < 0.01$.
[c] $p < 0.001$.

ectomized rats treated with estroprogestogens (n = 6) had a lower body weight (165 ± 6 vs. 188 ± 14 gm; $p < 0.001$) and a higher liver weight (6.2 ± 0.4 ± 5.4 ± 0.4 gm; $p < 0.001$) than did the ovariectomized controls (n = 6; Table 5).

HEPATIC BILIRUBIN UDP-GLUCURONYLTRANFERASE

In untreated liver homogenates, bilirubin UDP-glucuronyltransferase activity was similar in virgin controls (4.4 ± 1.1 nmoles per hr per mg of protein) and in pregnant rats (4.3 ± 1.0). In digitonin-activated homogenates, the activity was significantly decreased in pregnant compared to control rats, both when expressed as specific activity and per unit of liver weight (Table 2). However, because of the higher liver weight in pregnant rats, their total hepatic glucuronyltransferase activity was not different from controls (Table 2). When analyzed 12 to 15 days after delivery, the specific activity had returned to control levels both in lactating and in nonlactating mothers. The activity per unit of liver weight was however lower in lactating rats, in agreement with their lower hepatic protein concentration (Table 1). As a result of the higher liver weight in lactating mothers, their total hepatic glucuronyltransferase activity was higher than in the controls (Table 2). Ovariectomized rats treated with estroprogestogens had a higher UDP-glucuronyltransferase activity than did the ovariectomized controls (Table 5).

BILE FLOW AND BILIRUBIN OUTPUT IN BILE

Bile flow per unit of liver weight was reduced in pregnant rats, and returned to control values 2 weeks after delivery both in lactating and in nonlactating mothers. Total hepatic output of bile was, however, higher in pregnant and in lactating rats, compared to controls (Table 3). Bile flow was not affected by estroprogestogens administration (Table 5).

The maximal concentration of bilirubin in bile ob-

TABLE 4. INFLUENCE OF PREGNANCY AND LACTATION ON MAXIMAL BILIRUBIN OUTPUT IN BILE IN THE RAT[a]

Group	No. of animals	Maximum bilirubin concentration in bile (nmoles/liter)	nmoles/min per gm of liver	nmoles/min per whole liver
Virgin controls	6	28.6 ± 2.0	73 ± 5	368 ± 30
Pregnant	10	22.0 ± 1.8[b]	45 ± 3[b]	367 ± 35
Lactating mothers	7	23.8 ± 1.9[c]	53 ± 6[b]	452 ± 74[d]
Nonlactating mothers	5	28.4 ± 1.6	68 ± 6	382 ± 31

[a] A load of bilirubin was infused intravenously in 20- to 21-day-old pregnant rats and in mother rats 12 to 15 days after delivery. Results are expressed as means ± S.D.
[b] $p < 0.001$.
[c] $p < 0.01$.
[d] $p < 0.05$.

served during pigment loading *in vivo* was reduced both in pregnant and in lactating rats, while it returned to control values in nonlactating rats 2 weeks after delivery (Table 4).

Bilirubin Tm per unit of liver weight was reduced in pregnant and in lactating rats, while it was again comparable to control values in nonlactating mothers (Table 4). No difference between virgin and pregnant rats was found when total hepatic output of bilirubin was considered, while total output was increased in lactating animals (Table 4). When bilirubin UDP-glucuronyltransferase activity and bilirubin Tm, determined for each rat and expressed per gram of liver, were plotted against each other, a significant correlation was found ($r = 0.80$; $p < 0.001$) (Figure 1).

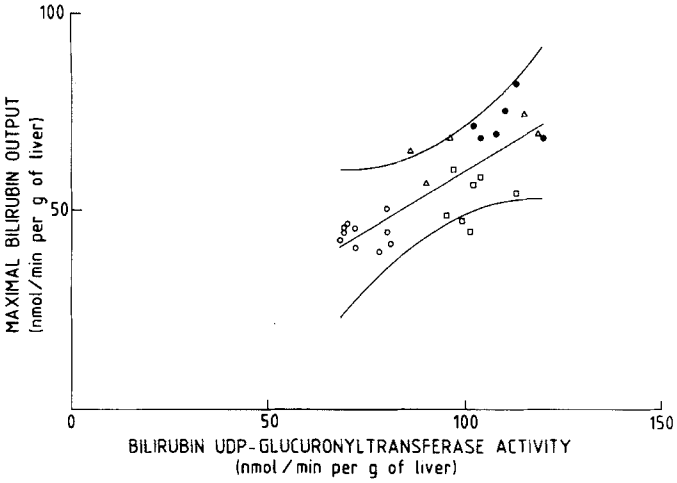

FIG. 1. Correlation between bilirubin UDP-glucuronyltransferase activity and maximal bilirubin excretion in bile (Tm) in virgin female (●), pregnant (○), nonlatacting mother (△) and lactating mother (□) rats. $r = 0.80$; $p < 0.001$. The regression line with 95% confidence limits is given; the slope is 0.6, the intercept on the y axis is 0.6.

PROPORTION OF BILIRUBIN DICONJUGATES RELATIVE TO TOTAL CONJUGATES IN BILE

The proportion of bilirubin diconjugates in bile was decreased in pregnant rats, while it was comparable to virgin controls in lactating and nonlactating mothers (Table 3). Administration of estroprogestogens to ovariectomized rats increased the percentage of diconjugates in bile (Table 5).

DISCUSSION

Pregnancy alters the hepatic metabolism of several endogenous and exogenous compounds. Reports include reduced specific activity of various drug-metabolizing enzymes (16–19) reduced glucuronidation of endogenous and exogenous substrates (8, 16, 20, 21), increased glutathione-*S*-transferase activity (22) and impaired biliary excretion of xenobiotics (23).

In the present work, bilirubin UDP-glucuronyltransferase activity was decreased by 30% in digitonin-activated liver homogenates from pregnant rats, both when expressed per unit of liver weight and of liver protein. However, because of the increase in liver size occurring during pregnancy, these modifications are not apparent when enzyme activities per whole liver are considered (Table 2; Ref. 21). Total transferase activity for the pregnant animals *in vivo* thus is comparable with that of the virgin controls. Our data agree with the previously reported 30% decrease of bilirubin and *p*-nitrophenol (8) and estrone and estradiol-glucuronyltransferase specific activity (21). *p*-Nitrophenol belongs to the so-called "late-fetal" group of substrates whereas bilirubin and estradiol are included in the "neonatal" group (24); a diverging behaviour for these groups of substrates has been described in several conditions (25). However, pregnancy in the rat leads to a similar reduction of transferase activities for several substrates as well as to a decrease of other microsomal enzymatic reactions (26), pointing to comparable, nonspecific alterations in the endoplasmic reticulum. This phenomenon has been related to changes in fatty acid and phospholipid composition which probably alter the structure of the microsomal membrane (27). The decreased bilirubin glucuronyltransferase activity was observed only in preparations treated with digitonin, presumably because of the relative insensitivity of the assay when carried out with nonactivated liver homogenates. Our finding that the linearity of the reaction was lost after 20 min in digitonin-activated and after 10 min in untreated liver homogenates stresses the importance of assessing the kinetics of the reaction. Loss of linearity due to longer incubation times, use of nonactivated liver preparations or of different methodology probably account for some of the different results reported previously (6, 7).

Bile flow per gram of liver was decreased in the pregnant rats but due to the higher liver weight, total bile flow was on the contrary increased (Table 2). *In vivo*,

TABLE 5. EFFECT OF ESTROPROGESTOGENS ON BILE FLOW, PERCENTAGE OF BILIRUBIN DICONJUGATES IN BILE AND HEPATIC BILIRUBIN UDP-GLUCURONYLTRANSFERASE ACTIVITY IN THE RAT[a]

	Bile flow (μl/min per whole liver)	Diconjugates in bile (% of total conjugates)	Bilirubin UDP-glucuronyltransferase activity		
			nmoles/hr per mg of protein	nmoles/hr per gm of liver	nmoles/hr per whole liver
Ovariectomized controls	11.8 ± 3.1	63 ± 2	21.7 ± 1.7	80 ± 6	432 ± 34
Ovariectomized + estroprogestogens	13.6 ± 0.8	70 ± 1[b]	32.6 ± 2.4[b]	120 ± 10[b]	743 ± 33[b]

[a] Female rats, 12 to 13 weeks old, were ovariectomized on the day of estrus and treated with 5 mg progesterone and 0.05 mg β-oestradiol by daily s.c. injections for 17 days. Ovariectomized controls received vehicle only. Results are expressed as means ± S.D. for groups of 6 (enzyme activities) or 4 animals (bile flow and diconjugates in bile).

[b] $p < 0.001$.

total endogenous bilirubin output was normal as well as the maximal bilirubin output (Tm) calculated for the whole animal. The decreased glucuronyltransferase activity per gram of liver was parallelled by a 40% reduction in Tm expressed per gram of liver. The latter figure contrasts with a previous study, which reported no difference between control and pregnant female rats (6). The depressive effects of hypothermia and anaesthesia on the biliary secretion of bilirubin (4, 8) as well as differences in strains are possible explanations for the different results. In fact, the maximal output of bilirubin in bile reported for control females (6) was less than half the output observed in the present study.

The finding of a reduced proportion of bilirubin diconjugates in bile suggests that some alteration of bilirubin conjugation does exist in pregnant rats. The activity of β-glucuronidase was not different in pregnant and virgin rat bile (Muraca and Fevery, unpublished observations). It is not cear whether the reduced amount of diconjugates is due to a reduced availability of glucuronic acid or to a different enzyme activity in pregnant rats. The agents responsible for the reported modifications in pregnant rat liver are not known. Since the secretion of estrogens and progesterone is increased during pregnancy, these hormones are often regarded as a possible etiological factor for altered hepatic metabolism (28). Administration of estradiol and progesterone to ovariectomized rats to yield plasma concentrations comparable to those reached during pregnancy increased liver weight, hepatic UDP-glucuronyltransferase activity and the percentage of diconjugates in bile (Table 5). Total conjugating activity even exceeded values in pregnant rats. The latter however had a high liver weight but a low specific transferase activity and a reduced excretion of diconjugates in bile. Therefore, changes observed during pregnancy cannot be reproduced by treatment with estradiol and progesterone. Different steroids might be involved or other hormones might be required to allow an effect of estrogens and progestogens to become manifest, thus exerting a permissing effect. It has also been proposed that alterations of drug-metabolizing enzymes during pregnancy is a consequence of a shift of hepatic progesterone metabolism from the hydroxylation pathway, producing inducers of microsomal enzymes such as pregnenolone 16α-carbonitrile, to the reduction pathway, producing inhibitors of microsomal enzymes such as pregnanediol (29). This hypothesis could also explain the present results, since pregnenolone-16α-carbonitrile was found to induce hepatic bilirubin conjugation (29) while pregnanendiol was found to inhibit it (30).

Two weeks after delivery, all parameters determined in nonlactating mothers had returned to control values. In lactating mothers, specific bilirubin UDP-glucuronyltransferase activity was comparable to control values, but the activity per gram of liver was still lower. This discrepancy is explained by the lower protein concentration in lactating rat liver, reported also by other authors (31). Since liver weight in these animals was 40% higher than in controls, their total transferase activity was however increased as was their maximal bilirubin secretion. The relative amounts of bilirubin diconjugates in bile was similar to control values. Bile flow was normal when expressed per unit of liver weight, but total hepatic bile flow was 60% higher than in control animals. This presumably explains the faster excretion in lactating rats of dibromosulfophthalein and indocyanine green (10), two organic anions which are not metabolized by the liver and which are believed to share a common excretory pathway with bilirubin (1).

Bilirubin needs hepatic conjugation prior to biliary excretion (1, 2). When considering all animals investigated, maximal bilirubin output in bile correlated with glucuronyltransferase activity (Figure 1), suggesting that conjugation was the major determinant of bilirubin Tm, a conclusion reached also in different experimental conditions (3–5).

Pregnancy thus leads to a decreased specific activity of bilirubin UDP-glucuronyltransferase, but due to the increased liver size, total hepatic enzyme activity is normal. *In vivo*, endogenous and maximal biliary bilirubin output are normal, bile flow is increased and the diconjugate excretion decreased. Increased plasma concentrations of β-estradiol and progesterone do not seem to be the only mechanism by which the aforementioned alterations are induced.

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