Differential effects of 17α-ethinylestradiol on the neutral and acidic pathways of bile salt synthesis in the rat

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Abstract **Effects of 17α-ethinylestradiol (EE) on the neutral and acidic biosynthetic pathways of bile salt (BS) synthesis were evaluated in rats with an intact enterohepatic circulation and in rats with long-term bile diversion to induce BS synthesis. For this purpose, bile salt pool composition, synthesis of individual BS in vivo, hepatic activities, and expression levels of cholesterol 7α-hydroxylase (CYP7A), and sterol 27-hydroxylase (CYP27), as well as of other enzymes involved in BS synthesis, were analyzed in rats treated with EE (5 mg/kg, 3 days) or its vehicle. BS pool size was decreased by 27% but total BS synthesis was not affected by EE in intact rats. Synthesis of cholate was reduced by 68% in EE-treated rats, while that of chenodeoxycholate was increased by 60%. The recently identified Δ^{22}-isomer of β-muricholate contributed for 5.4% and 18.3 % ($P < 0.01$) to the pool in control and EE-treated rats, respectively, but could not be detected in bile after exhaustion of the pool. A clear reduction of BS synthesis was found in bile-diverted rats treated with EE, yet biliary BS composition was only minimally affected. Activity of CYP7A was decreased by EE in both intact and bile-diverted rats, whereas the activity of the CYP27 was not affected. Hepatic mRNA levels of CYP7A were significantly reduced by EE in bile-diverted rats only; CYP27 mRNA levels were not affected by EE. In addition, mRNA levels of sterol 12α-hydroxylase and lithocholate 6β-hydroxylase were increased by bile diversion and suppressed by EE. This study shows that 17α-ethinylestradiol (EE)-induced intrahepatic cholestasis in rats is associated with selective inhibition of the neutral pathway of bile salt (BS) synthesis. Simultaneous impairment of other enzymes in the BS biosynthetic pathways may contribute to overall effects of EE on BS synthesis.—**Koopen, N. R., S. M. Post, H. Wolters, R. Havinga, F. Stellaard, R. Boverhof, F. Kuipers, and H. M. G. Princen. **Differential effects of 17α-ethinylestradiol on the neutral and acidic pathways of bile salt synthesis in the rat.** *J. Lipid Res.* **1999.** 40: **100–108.**

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The synthetic estrogen 17α-ethinylestradiol (EE) induces cholestasis in rodents, mainly by reducing the bile salt-independent fraction of bile flow (BSIF) (1, 2). The mechanisms underlying the decrease in bile flow are not yet clear (3). In addition to reduced BSIF, several authors have reported that EE may also affect bile salt-dependent bile formation (BSDF) (2, 4, 5). This effect has been attributed to reduced hepatic BS synthesis (4–6) and to impaired activities of hepatic transport systems involved in vectorial transport from blood to bile (7–9). With respect to the first, reduced biliary BS secretion and altered biliary BS composition in EE-treated animals have been reported (4, 5, 10). In particular, the contribution of chenodeoxycholate and β-muricholate to the BS pool appears to increase at the expense of cholate. In a previous study (10), we found that conversion of endocytozed lipoprotein cholesterol to cholate was completely abolished in EE-treated rats. The metabolic basis for the changes in BS synthesis induced by EE are largely unknown. Earlier studies have shown that EE inhibits the activity of the cholesterol 7α-hydroxylase (CYP7A) (11, 12), the enzyme catalyzing the first step of the so-called neutral pathway of BS biosynthesis. Since then, however, it has become clear that an acidic pathway, initiated by 27-hydroxylation of cholesterol by the mitochondrial sterol 27-hydroxylase (CYP27), represents a quantitative important route for BS synthesis (13–15). Recently, data have been reported to indicate that in situations where the 'classical' neutral pathway is specifically suppressed, the acidic pathway becomes more important for maintenance of hepatic BS synthesis (16, 17). It is not known whether EE differentially affects both pathways. In addition, peroxisomal formation of a Δ^{22} isomer of muricholate has been suggested as a novel further downstream pathway in BS synthesis in rats (18, 19). Whether and to what extent formation of this species is affected under cholestatic conditions is not known. Finally,

Abbreviations: EE, 17α-ethinylestradiol; BS bile salt; Ch, cholate; CDC, chenodeoxycholate; UDC, ursodeoxycholate; LC, lithocholate; MC-β, muricholate; HC, hyocholate; BSDF, bile salt-dependent bile flow; BSIDF, bile salt-independent bile flow.

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EE has strong impact on hepatic cholesterol synthesis (e.g., 20–23). As the contribution of newly synthesized cholesterol to formation of individual BS may vary under different conditions (24–26), altered cholesterol synthesis may also affect BS synthesis.

To assess the quantitative contribution of the major pathways to hepatic BS synthesis in EE-treated rats, we related in vivo BS synthesis to the specific activities and expression levels of CYP7A and CYP27. In addition, mRNA levels of sterol 12α-hydroxylase and lithocholate 6β-hydroxylase, key enzymes in the formation of cholate and β-muricholate, respectively, were determined, as well as those of HMG-CoA synthase as a key enzyme in cholesterol synthesis. Experiments were performed in rats with an intact enterohepatic circulation and in rats with prolonged bile diversion. Bile diversion leads to pool depletion and to up-regulation of hepatic BS synthesis, thereby enabling us to directly assess the effects of EE on synthesis of the individual BS species and to relate these effects to hepatic enzyme activities.

MATERIALS AND METHODS

Materials

17α-Ethinylestradiol (EE) was purchased from Sigma Chemicals (St. Louis, MO). NADPH, isocitrate-dehydrogenase was obtained from Boehringer Mannheim (Mannheim, Germany). Cholesterol oxidase was obtained from Calbiochem (La Jolla, CA). All other chemicals were of reagent grade or the highest purity commercially available.

Animals

Male Wistar rats (Harlan Laboratories, Zeist, The Netherlands) weighing 290–330 g were used for these studies. Animals were kept in a light- and temperature-controlled environment and had free access to lab chow and tap water throughout the experiments. The animals received humane care and experimental protocols complied with the local guidelines for use of experimental animals.

To study the effects of EE on bile formation and composition under conditions with an intact enterohepatic circulation, rats were equipped with permanent catheters in bile duct and duodenum as described in detail elsewhere (27). Both catheters were immediately connected to each other to maintain an intact enterohepatic circulation. Subcutaneous EE (5 mg/kg) or solvent (1,2-propanediol) injections were given for 3 days, starting 4 days after surgery, i.e., after animals had regained their preoperative body weights. After 3 days of treatment, the connection between both catheters was interrupted and bile was collected for 6 h in 30-min intervals by means of a fraction collector. Bile volume was determined gravimetrically and samples were immediately stored at −20°C for later analysis. Separate groups of rats were used for the isolation of hepatic microsomes, mitochondria, total RNA, and for collection of blood.

In order to study the effects of EE on bile formation and composition after long-term bile diversion, when BS synthesis is maximally up-regulated, rats where equipped with a permanent catheter in the bile duct only. Bile was diverted for 5 days, prior to administration of EE or the solvent for 3 days. These animals were allowed to drink 0.9% NaCl to compensate for loss of electrolytes via bile. At day 8, bile samples were continuously collected by means of a fraction collector for 24 h in 90-min intervals. After the bile sampling, the animals were anesthetized with halothane. Blood was sampled by means of a cardiac puncture and the liver was removed for isolation of total RNA, microsomes and mitochondria.

Analyses

BS in plasma and bile were determined by an enzymatic fluorimetric assay (28). Plasma triglycerides, plasma and hepatic cholesterol were measured enzymatically using commercially available kits (Boehringer Mannheim, Mannheim, Germany). Aspartate transaminase (AST), alanine transaminase (ALT), and bilirubin in plasma were assessed by standard laboratory techniques.

Bile salt composition was studied by gas chromatography and gas chromatography–mass spectrometric techniques as described earlier for human bile (29). Briefly 5–50 μl bile was subjected to enzymatic hydrolysis with cholylglycine hydrolase. The free bile acids formed were extracted with C18 solid phase extraction, methylated, and silylated. The methyl-TMS derivatives were separated on a 25 m × 0.25 mm OV-1701 column (CP Sil19 CB, Chrompack Int., Middelburg, The Netherlands). As a modification, coprostanol was used as internal standard for the purpose of quantitation applying GC only. Identification of bile acids was performed by GC/MS (SSQ7000, Finnigan MAT, San Jose, CA) using the same GC separation system. Full scan data were recorded from m/z 50–850 and mass spectra were compared with reference spectra for definitive identification. In the absence of reference spectra, a tentative identification was done based on spectral information (19).

Preparation of microsomes and mitochondria

For the isolation of microsomes and mitochondria, livers were perfused with cold saline, removed, and 5 g of liver tissue was stored in 250 mm sucrose, 10 mm Tris, 1 mm EDTA, pH 7.4. All procedures were carried out at 4°C. Livers were cut into small pieces with scissors and homogenized in the same buffer using a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 10 min at 800 *g* and the supernatant was then centrifuged 12 min at 8500 *g*. The supernatant thus obtained was used for isolation of microsomes and the pellet was used for the isolation of mitochondria.

For the isolation of microsomes, the supernatant was centrifuged for 70 min at 100000 *g*. The pellet was resuspended by means of a Potter-Elvehjem homogenizer in 100 mm sucrose, 100 mm potassium phosphate, 2 mm EDTA, and 5 mm DTT, pH 7.4, and centrifuged for 1 h at 100000 *g*. Microsomes were resuspended in the same buffer and frozen quickly in fluid N_2 in small aliquots and stored at −80°C.

The mitochondrion-enriched pellet was resuspended by homogenization in 250 mm sucrose, 10 mm Tris, pH 7.4, and centrifuged for 12 min at 8500 *g*. This procedure was repeated 3 times. The final mitochondrial pellet was resuspended in this buffer and stored at −80°C. Protein concentration was measured according to Lowry et al. (30).

Assay of cholesterol 7α-hydroxylase and sterol 27-hydroxylase enzyme activity

Enzyme activities of CYP7A and CYP27 in isolated liver microsomes and mitochondria were determined essentially according to Chiang (31) measuring conversion of cholesterol into 7α- and 27-hydroxycholesterol, respectively. In short, 750 μg of protein of either microsomal or mitochondrial protein was incubated in 1 ml of buffer containing 0.1 m potassium phosphate, pH 7.2, 50 mm NaF, 5 mm DTT, 1 mm EDTA, 20% glycerol (w/v), and 0.015% (w/w) CHAPS. Twenty μl of 1 mg cholesterol in 45% (w/v) hydroxypropyl-β-cyclodextrin was added and the mixture was incubated with agitation for 10 min at 37°C. Then 200 μl of a

TABLE 1. Body and liver weights in control and EE-treated intact and bile-diverted rats

	Body Weight	Liver Weight	Liver (% Body Weight)
	g	*g*	*%*
Intact, control	325.0 ± 49.4	14.2 ± 2.6	4.3 ± 0.2
Intact, EE	332.6 ± 39.0	17.5 ± 2.5	5.3 ± 0.2[a]
Bile-diverted, control	322.0 ± 23.9	13.4 ± 0.9	4.2 ± 0.3
Bile-diverted, EE	298.3 ± 24.3	14.3 ± 0.7	4.8 ± 0.4[a]

Intact and bile-diverted rats were treated with 17α-ethinylestradiol (EE) (5 mg/kg) for 3 consecutive days. At 24 h after the last injection, animals were weighed, anesthetized with halothane, and a large blood sample was collected by cardiac puncture. Subsequently the liver was removed and weighed. Data are given as means ± SD; n = 3–5 per group.
[a]Significantly different ($P < 0.05$) from respective control.

regenerating system was added containing 10 mm sodium isocitrate, 10 mm $MgCl_2$, 1 mm NADPH, and 0.15 U isocitrate-dehydrogenase at 37°C. After 20 min of incubation, 60 μl of a stop solution containing 20% sodium cholate and 1 μg 20α-hydroxycholesterol, which served as an internal standard, was added. Steroid products were oxidized for 45 min with 100 μl buffer containing: 0.1% cholesterol oxidase, 10 mm K_2HPO_4, 1 mm DTT, and 20% glycerin at 37°C, and the reaction was stopped by addition of 2 ml ethanol. Cholesterol metabolites from this reaction mixture were extracted in petroleum ether and the ether layer was evaporated under a stream of nitrogen. Residues resuspended in a mixture of 60% acetonitril, 30% methanol and 10% chloroform (v/v) were analyzed by using C-18 reverse phase HPLC on a Tosohaas TSK gel-ODS 80TM column equilibrated with 70% acetonitrile and 30% methanol at a flow rate of 0.8 ml/min. The amount of products formed was determined by monitoring the absorbance at 240 nm. Peaks were integrated using Data Control software (Cecil Instruments, UK).

Determination of mRNA levels

Total RNA was isolated according to Chomczynski and Sacchi (32). Determination of steady state mRNA levels for CYP7A, CYP27, 12α-hydroxylase, lithocholic acid 6β-hydroxylase, HMG-CoA synthase, and LDL receptor by Northern blot and dot blot and hybridization conditions were performed as described previously (33–35). 18S ribosomal RNA was used as an internal standard to correct for differences in amounts of total RNA applied to the gel. mRNA levels were quantified by phospho-imager analysis (Fuij Fujix Bas 1000) by using the program TINA version 2.08c.

Calculations and statistics

Output rates of BS were determined by multiplying bile flow by BS concentrations, after correction for the dead space of the tubing system. Values are expressed as mean ± SD. Significance of difference between two groups was assessed by means of Mann-Whitney nonparametric test at $P < 0.05$ level of significance.

RESULTS

Animal characteristics

There were no significant differences in body weight between EE- or solvent-injected intact or bile-diverted rats (**Table 1**). The liver-to-body weight ratio increased significantly upon EE treatment in both conditions. **Table 2** shows the effects of EE treatment for 3 days on plasma markers of liver function. Aspartate transaminase, alanine transaminase, and bilirubin levels in plasma were not significantly affected by EE. The plasma BS concentration was significantly increased in intact rats upon EE treatment. As expected, BS levels were at the lower limits of detection in the untreated bile-diverted rats and did not increase after EE administration. Treatment with EE led to significant reductions in plasma cholesterol and triglyceride levels in both experimental models.

Bile formation

Figure 1A shows bile flow over a period of 6 h after interruption of the enterohepatic circulation of intact rats (left panel) and during a 24-h period after 8 days of bile diversion (right panel). EE treatment significantly decreased bile flow in intact rats, which, as shown previously (3), is mainly caused by reduction of the BSIF. EE also decreased bile flow in rats with long-term bile diversion; In this condition, the well-established diurnal variation in bile flow was absent. BS output was decreased upon EE treatment in both intact and bile-diverted rats (Fig. 1B). BS poolsizes, as calculated from the wash-out curves in the intact rats, were 53.0 ± 17.2 μmol/100 g in the controls and 37.0 ± 12.9 μmol/100 g in the EE-treated rats, respectively ($P < 0.05$). The BS synthesis rate, determined at the nadir of the wash-out curve, was not affected by EE treatment, i.e., 1.54 ± 0.16 μmol/h per 100 g and 1.57 ± 0.16 μmol/h per 100 g in control and EE-treated rats, respectively. After 8 days of bile diversion, BS synthesis rate increased to 3.37 ± 0.52 μmol/h per 100 g in control rats at day time, whereas in the EE-treated bile-diverted animals synthesis was significantly lower, i.e., 1.23 ± 0.04

TABLE 2. Effect of 17α-ethinylestradiol (EE) treatment for 3 days on plasma parameters in intact and bile-diverted rats

	AST	ALT	Bilirubin	Bile Salt	Cholesterol	Triglyceride
	IU/L	*IU/L*	*μM*	*μM*	*mM*	*mM*
Intact, control	89 ± 16	41 ± 2	5.5 ± 1.7	36 ± 12	1.33 ± 0.66	1.34 ± 0.66
Intact, EE	115 ± 50	40 ± 11	5.2 ± 1.1	77 ± 22[a]	0.32 ± 0.05[a]	0.21 ± 0.11[a]
Bile-diverted, control	222 ± 81	52 ± 14	8.3 ± 5.9	4 ± 0	1.39 ± 0.35	1.42 ± 0.65
Bile-diverted, EE	112 ± 74	30 ± 6	4.8 ± 0.5	2 ± 1	0.39 ± 0.14[a]	0.33 ± 0.27[a]

See legend to Table 1 for experimental details. Data are given as means ± SD, n = 3–5 per group; AST, aspartate transaminase; ALT, alanine transaminase.
[a]Significantly different from respective control, $P < 0.05$.

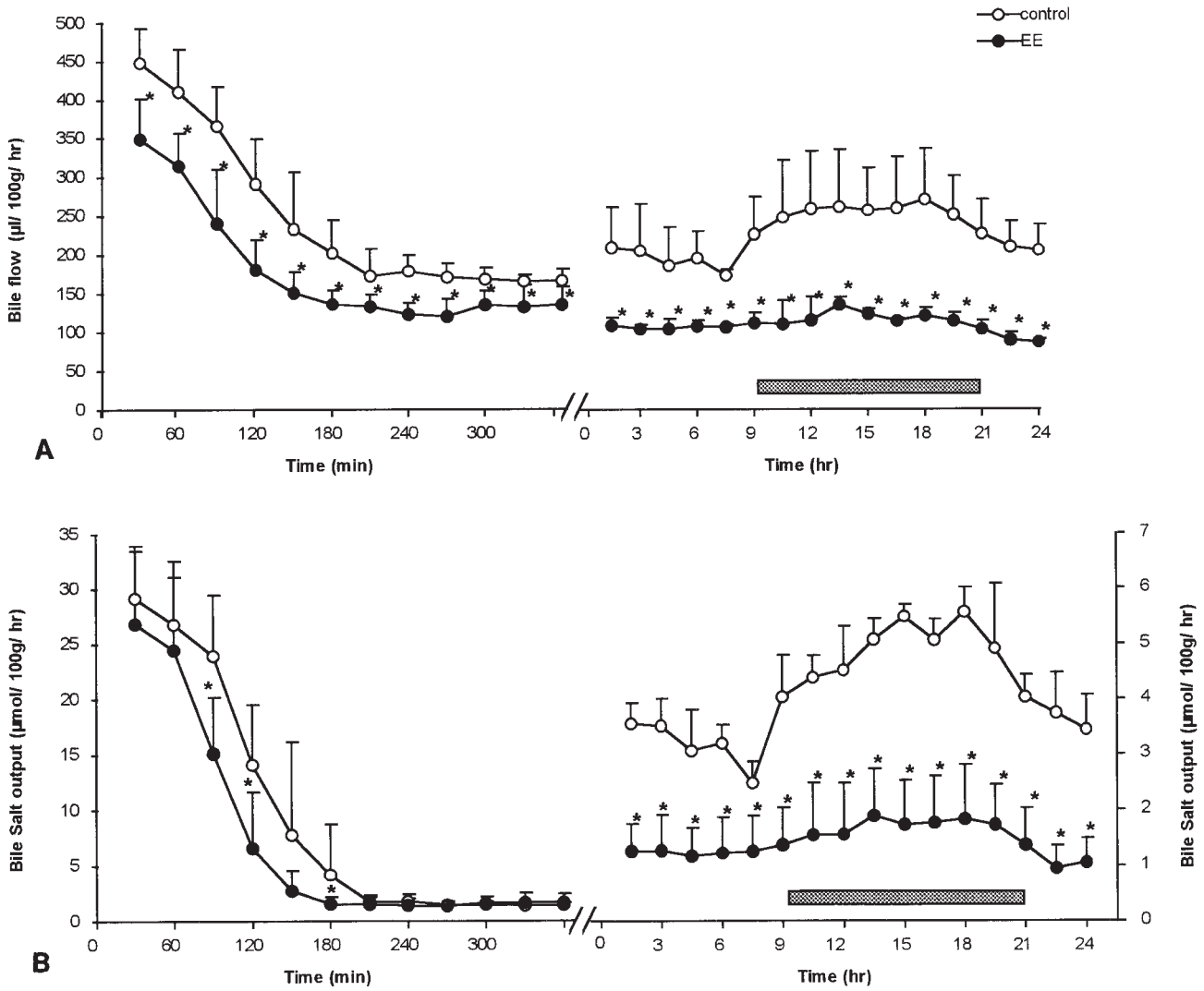

Fig. 1. The effect of EE on bile flow (A) and BS output (B) in intact rats over a period of 6 h after interruption of the enterohepatic circulation (left panel) and the effect of EE over a period of 24 h after 8 days of bile diversion (right panel). In intact rats bile was collected in 30-min fractions for 6 h after interruption of the enterohepatic circulation. In 8-day bile-diverted rats, bile was collected continuously during 24 h in 90-min fractions. Horizontal bars indicate the dark period. Values are means ± SD of 3–6 rats per group. *Significantly different from respective control ($P < 0.05$).

µmol/h per 100 g. This figure also shows that the characteristic increase in BS output during the dark period in bile-diverted control rats (36) was absent in the EE-treated rats. These results imply that, after long-term bile diversion, the estrogen also impairs the magnitude of the BSDF.

The relative contribution of the individual BS present in the pool was assessed by GC and GC–MS analysis (**Table 3**). In the BS pool, the contribution of cholate was decreased from 67.0 ± 3.7 to 50.8 ± 12.2%, probably caused by a decreased cholate synthesis, as determined after pool depletion. On the other hand, synthesis of CDC was relatively increased by EE. Secondary BS, i.e., DC and LC, were present in bile in low concentrations after 6 h pool depletion, probably reflecting their slow entry into the bloodstream from the colon. Their contribution was not affected by EE. The recently identified (18, 19) Δ^{22} isomer of β-muricholate comprised 5.4% of the BS pool in control rats and 18.3% in EE-treated rats. The identity of this unique rodent BS was confirmed by GC–MS, based upon the unique combination of fragment ions *m*/*z* 195, 285 (typical for muricholic acids) and 367, 456 and 546 (**Fig. 2**) indicating a combination of a 3α,6α- or 6β,7β-trihydroxy bile acid and a single double bond. According to Setchell et al. (19) the double bond is located at the C22 position. A similar Δ^{22} β-muricholic acid could be identified based on the similar fragmentation pattern and the retention time shift relative to ω-muricholic acid which is comparable with the retention time shift of Δ^{22} β-muricholic acid relative to β-muricholic acid. After exhaustion of the pool, only traces of Δ^{22}-β-muricholate could be detected in bile of both control and EE-treated rats. After long-term bile diversion, the isomer was not detectable at all.

Table 4 shows BS composition in 8 day bile-diverted rats

TABLE 3. Effect of 17α-ethinylestradiol (EE) treatment on bile salt pool composition and on composition of newly synthesized bile salts immediately after exhaustion of the circulating pool

Bile Salt Species	Bile Salt Pool		Bile Salt Synthesis	
	Control	EE	Control	EE
	%		%	
LC	1.1 ± 0.2	1.5 ± 0.6		
DC	3.5 ± 1.3	5.2 ± 3.2		
Ch	67.0 ± 3.7	50.8 ± 12.2[a]	44.1 ± 5.5	14.9 ± 2.1[b]
CDC	9.6 ± 3.5	8.3 ± 4.4	25.2 ± 5.4	42.1 ± 10.5[b]
HDC	5.9 ± 1.9	8.8 ± 3.8	16.8 ± 4.4	22.7 ± 3.5
β-MC	5.5 ± 2.8	7.0 ± 1.9	14.6 ± 2.9	20.3 ± 6.1
UDC	2.2 ± 1.0	0.2 ± 0.5[b]		
Δ^{22}-β-MC	5.4 ± 1.7	18.3 ± 3.8[b]		

After interruption of the enterohepatic circulation, 30-min bile samples were collected. Pool composition was determined in the 0–30-min sample. Synthesis was measured at 300–330 min after the interruption, i.e., after exhaustion of the bile salt pool. Data are given as means ± SD, n = 4–5 per group; LC, lithocholate; DC, deoxycholate; Ch, cholate; CDC, chenodeoxycholate; HDC, hyodeoxycholate; β-MC, β-muricholate; UDC, ursodeoxycholate; Δ^{22}-β-MC, Δ^{22} isomer of β-muricholate.
[a] $P < 0.05$; [b] $P < 0.01$; significantly different from respective control.

TABLE 4. Effect of 7α-ethinylestradiol (EE) on bile salt composition in long-term bile-diverted rats

Bile Salt Species	Control	EE
	%	%
Ch	51.0 ± 7.2	46.3 ± 6.2
CDC	33.9 ± 3.2	28.4 ± 8.7
UDC	2.8 ± 0.9	4.0 ± 0.2
β-MC	13.0 ± 5.9	21.2 ± 4.8

Bile samples (90 min) were taken from long-term bile-diverted rats after 3 days of treatment with EE. A sample at the mid-light period (from 12–13:30 am) was used for determining bile salt composition. Data are given as means ± SD; n = 3–5 per group. Ch, cholate; CDC, chenodeoxycholate; UDC, ursodeoxycholate; β-MC, β-muricholate.

when BS synthesis is maximally up-regulated (27). As the intestinal BS pool of these rats has been depleted, only primary BS are present in bile. The relative decrease in cholate synthesis seen in EE-treated intact rats was not found after long-term bile diversion. Also, the other BS were not significantly affected by EE, although the relative contribution of β-MC tended to be increased by treatment with EE.

Activities and mRNA levels of cholesterol 7α-hydroxylase, sterol 27-hydroxylase, sterol 12α-hydroxylase and lithocholate 6β-hydroxylase

In order to gain insight into the metabolic background of the changes in BS composition induced by EE, the activities of the cholesterol 7α-hydroxylase and the sterol 27-hydroxylase were determined in isolated hepatic microsomes and mitochondria, respectively. **Figures 3a** and **b** show the activities of these enzymes, expressed as percentage of the solvent-treated intact controls. After 8 days of bile diversion, i.e., without administration of EE, the activity of CYP7A was increased 8-fold, as shown previously (37). Upon EE treatment, enzyme activity decreased from 2.87 ± 1.51 to 1.60 ± 0.34 nmol/mg per h ($P = 0.065$) in intact rats (−44%) and from 22.94 ± 4.32 to 7.07 ± 3.18 nmol/mg per h ($P < 0.05$) in bile-diverted animals (−70%). It should be noted that the activity of CYP7A in the EE-treated bile-diverted rats was still increased compared with the control situation. After 8 days of bile diversion, the activity of CYP27 was increased by about 100%; EE treatment did not change activity of this enzyme.

To investigate the level of EE interaction with BS synthesis, we determined steady state mRNA levels of *CYP7A* and *CYP27*. **Figure 4** shows that mRNA levels of *CYP7A* and *CYP27* as well as of the LDL receptor, measured as an in-

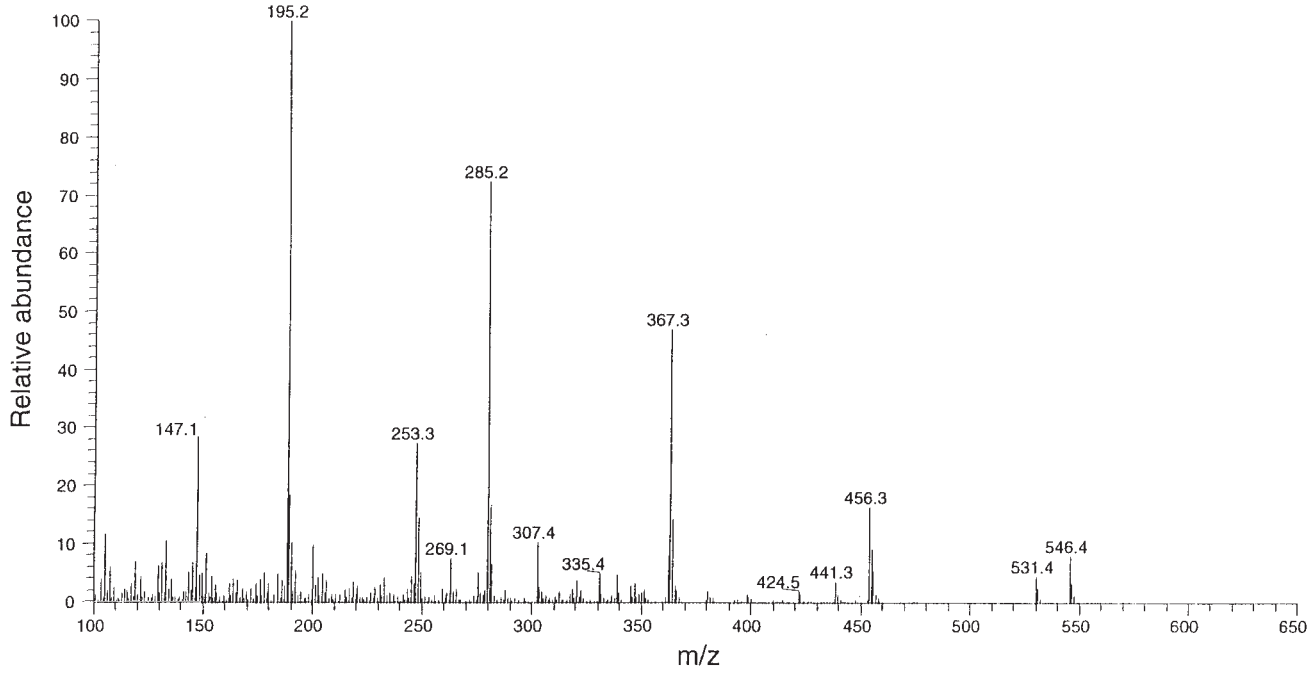

Fig. 2. Mass spectrum of a bile salt present in rat bile tentatively identified as Δ^{22}-β-muricholate.

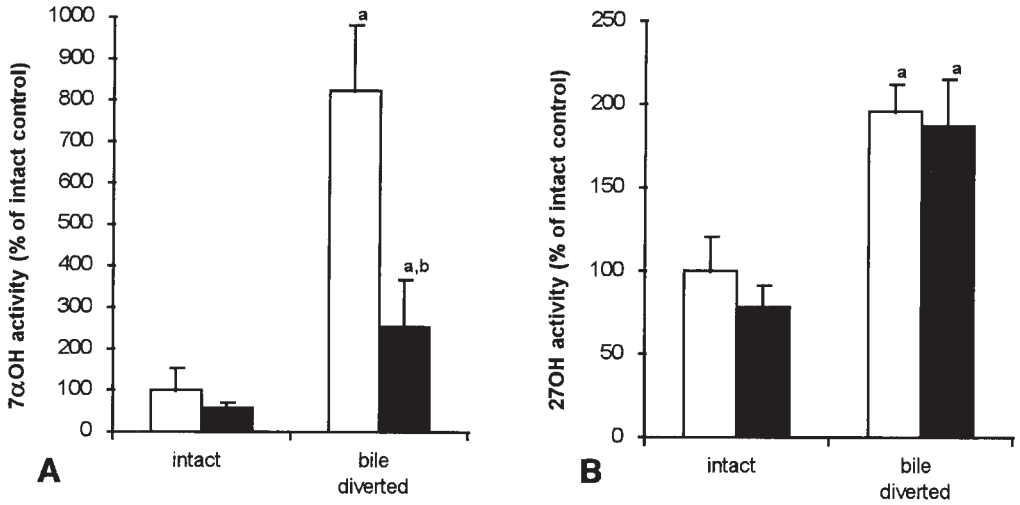

Fig. 3. Microsomal cholesterol 7α-hydroxylase (A) and mitochondrial sterol 27-hydroxylase (B) activities in intact and bile-diverted solvent-treated (control) and EE-treated rats. Microsomes and mitochondria were prepared from livers of the experimental groups harvested at 9 am and enzyme activities were measured as described in Materials and Methods. White bars indicate solvent-treated control groups and black bars indicate EE-treated groups. Values are means ± SD for 3–6 rats per group and expressed as percentage of the untreated, intact control group. The 100% value is 2.78 ± 1.51 nmol/mg per h for cholesterol 7α-hydroxylase and 1.28 ± 0.26 nmol/mg per h for sterol 27-hydroxylase; a, significantly different $P < 0.05$ from intact control; b, significantly different $P < 0.05$ from bile diverted control.

ternal control signal, increased upon bile diversion. The *CYP7A* mRNA levels were not significantly lowered by EE in intact rats but were clearly decreased in bile-diverted rats after treatment with the estrogen. *CYP27* mRNA levels, however, were not significantly affected by EE in either situation. The levels of LDL receptor mRNA were increased upon EE treatment both in intact and bile-diverted animals, as reported previously for intact rats (21, 22, 38, 39).

The mRNA levels of sterol 12α-hydroxylase, essential for cholate synthesis, and of lithocholate 6β-hydroxylase, involved in β-muricholate formation, were clearly increased in bile-diverted rats compared to intact animals and markedly down-regulated by EE treatment (**Fig. 5**). Furthermore, HMG-CoA synthase mRNA levels were increased after bile diversion, as expected, and clearly reduced by EE treatment, confirming earlier reports (20, 22) showing reduced cholesterol synthesis in EE-treated rats.

DISCUSSION

The effects of EE on BS synthesis and on expression and activity of key enzymes of the neutral and acidic pathways of BS synthesis were evaluated in rats under physiological conditions and in the situation with maximally

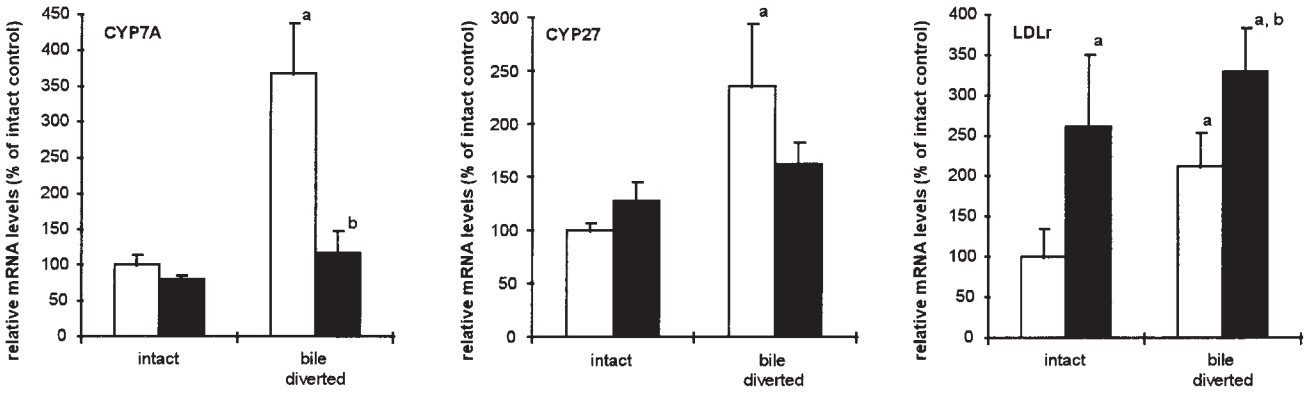

Fig. 4. Relative mRNA levels of cholesterol 7α-hydroxylase (CYP7), sterol 27-hydroxylase (CYP27), and LDL receptor (LDLr) in intact and bile-diverted solvent-treated (control) and EE-treated rats. Liver material used for RNA isolation was harvested at 9 am. White bars indicate solvent-treated control groups and black bars indicate EE-treated groups. mRNA levels were quantified relative to 18S RNA signal. Data are mean ± SD for 3–5 rats per group, expressed as percentage of the intact solvent-treated control group; a, significantly different $P < 0.05$ from intact control; b, significantly different $P < 0.05$ from bile diverted control.

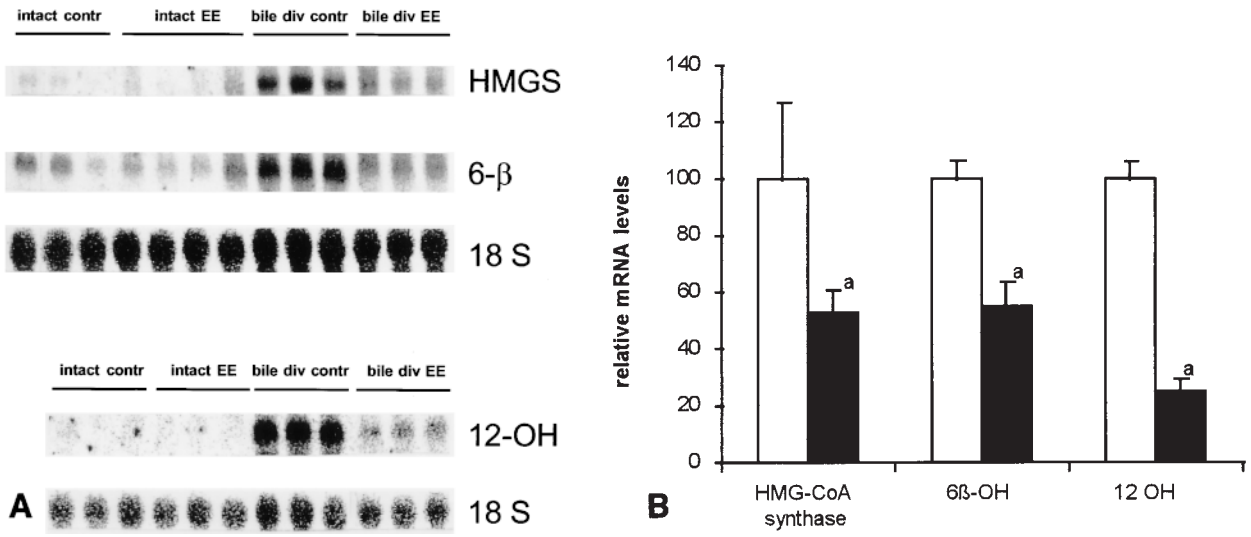

Fig. 5. Northern blot analysis of lithocholate 6β-hydroxylase, sterol 12α-hydroxylase, and HMG-CoA synthase in intact and bile-diverted rats treated with solvent or EE (A) and relative mRNA levels in 8-day bile-diverted rats with and without EE treatment (B). Liver material used for RNA isolation was harvested at 9 am. White bars indicate solvent-treated control groups and black bars indicate EE-treated groups. mRNA levels were quantified relative to 18S RNA signal. Data are mean ± SD for 3–5 rats per group, expressed as percentage of the intact solvent-treated control group; a, significantly different $P < 0.05$ from intact control.

up-regulated BS synthesis (27). Rats were treated with the estrogen for 3 days. Based on previous experiments (3), we anticipated that, with this treatment schedule, changes observed would be attributable to EE rather than to secondary effects of full-blown cholestasis. The presence of cholestasis per se, for instance in the bile duct-ligated rat (40, 41), strongly affects BS synthesis and CYP7A activity. Based on the minimal changes in plasma bilirubin and transaminases, it can be concluded that, although bile flow was markedly reduced in EE-treated animals, there was no accumulation of bile components in the plasma nor was liver damage induced in this experimental set-up. Slightly elevated plasma BS concentrations in EE-treated intact rats probably reflect their increased spill-over to the systemic circulation due to down-regulation of the Na^+-taurocholate cotransporting protein (Ntcp) (3, 8).

Results of the present study confirm earlier reports on the effects of long-term bile diversion on BS synthesis, CYP7A activity and *CYP7A* mRNA levels in rats (see 14, 15) and demonstrate that interruption of the enterohepatic circulation for 8 days also leads to a 2-fold increase in CYP27 activity and a 2.5-fold increase in *CYP27* mRNA levels. The latter results support previous findings in cholestyramine-fed rats and in cultured rat hepatocytes, demonstrating feed-back regulation of CYP27 by BS at a transcriptional level (25, 42, 43). In contrast to the situation in rats, CYP7A and CYP27 do not appear to be coordinately regulated by recirculating BS in the rabbit liver (17, 44), delineating the remarkable inter-species differences in regulation of BS metabolism. In addition to the anticipated effects on *CYP7A* and *CYP27* as well as on *HMGS* expression, it is shown for the first time that mRNA levels of sterol 12α-hydroxylase and lithocholate 6β-hydroxylase are markedly increased in the bile-diverted rat, indicating that BS exert regulatory actions at multiple sites of their biosynthetic pathways. Alternatively, it may be that hepatic accumulation of BS precursors, due to increased activities of the rate-limiting enzymes, increases gene transcription and/or mRNA stability of enzymes catalyzing conversions further down-stream in the biosynthetic cascade. It is also interesting to note that the well-established diurnal variation of BS synthesis in bile-depleted rats (27) is completely abrogated by EE. As this diurnal rhythm is thought to be mediated by glucocorticoids (45), it is tempting to speculate that EE renders the BS synthetic cascade insensitive to stimulatory actions of glucocorticoids.

Both in intact rats and in bile-diverted animals, EE reduced CYP7A activity whereas *CYP7A* mRNA levels were reduced in the bile-diverted animals only. The fact that the decrease in enzyme activity just failed to reach statistical significance in intact rats in our hands is probably due to the relatively short treatment period: 5 days of treatment has repeatedly been shown to inhibit CYP7A activity in rat liver (11, 12). The divergent findings for *CYP7A* mRNA levels in intact and bile-diverted rats may point towards a differential effect of EE in both situations. In fact, Davis et al. (11) have provided evidence that EE may act directly on microsomal membranes and thereby inhibit CYP7A activity in livers of intact rats. On the other hand, in the derepressed state of BS synthesis in bile-diverted animals, the estrogen also acts at a pretranslational level. In marked contrast, EE had no effect on CYP27 activity or *CYP27* mRNA levels in either situation, but mRNA levels of HMG-CoA synthase, sterol 12α-hydroxylase, and lithocholate 6β-hydroxylase were also clearly suppressed by EE in the bile-diverted rats. Taken together, this suggests that EE affects expression of endoplasmic reticulum-localized enzymes but is without effect on the mitochondrial system. At first sight, these findings seem to indicate that EE rather selectively suppresses the contribution of the neu-

tral pathway of BS synthesis. In the intact rats only, this is apparently compensated for by increased flux via the acidic pathway. In spite of the unchanged BS synthesis rate in intact rats, however, BS pool size was significantly reduced, as previously reported by Davis and Kern (4). This may indicate that EE-treated rats are unable to up-regulate hepatic BS synthesis adequately to compensate for fecal BS loss; it may be that EE treatment down-regulates the recently identified intestinal Na^+-dependent BS transporter (ibst) similar to its reported effects on expression of the hepatic Na^+-dependent BS transporter (Ntcp) (3, 8).

Our data also indicate that, in particular in EE-treated bile-diverted animals, steps prior to or beyond the initial hydroxylations may become rate-limiting, leading to a reduced BS synthesis in a situation when both CYP7A and CYP27 are up-regulated in comparison with the control situation. This suggestion is supported by the absence of significant changes in biliary BS composition under these conditions. A factor that may become rate-limiting in bile-diverted animals is the supply of substrate, i.e., of (newly synthesized) cholesterol. We (26) and others (24) have shown that the contribution of the novo synthesized cholesterol to BS synthesis amounts up to 12% in the intact rat with low BS synthesis and up to 40–50% in bile-diverted rats. This shift in relative contribution of newly synthesized cholesterol to BS synthesis is most likely due to the fact that both synthetic processes are physically separated under normal conditions, i.e., are localized in different hepatocyte populations (25). This zonal distribution is largely lost when BS synthesis and cholesterol synthesis are derepressed by interruption of the enterohepatic circulation (25). As EE inhibits hepatic cholesterol synthesis (20–22), as confirmed in our study by decreased levels of *HMGS* mRNA in livers of EE-treated rats, it is conceivable that reduced substrate availability may contribute to reduced BS synthesis.

Another interesting finding of this study concerns the effects of EE on pool composition. A decreased contribution of cholate to the pool was found, as also previously reported by Kern et al. (5). Yet, synthesis of cholate was still appreciable in EE-treated intact rats. This is apparently in contrast to a previous study from our laboratory (10), in which we showed that the conversion of LDL-cholesterol to cholate is completely blocked in EE-treated rats. The combination of data implies that LDL-cholesterol is processed differently than cholesterol from other sources after EE administration. This may be a result of selective induction of LDL-receptors by EE in cell populations different from those expressing *CYP7A*, i.e., the periportal hepatocytes. Alternatively, it may be that in EE-treated rats LDL-cholesterol is preferably delivered to mitochondria for 27-hydroxylation rather than to the endoplasmic reticulum, where CYP7A resides.

The contribution of Δ^{22} β-muricholate to the pool was significantly increased in EE-treated rats. This newly identified rodent BS species is thought to represent a product of partial peroxisomal β-oxidation of the β-muricholate side chain (18, 19, 46). It is likely that the increased amounts of 6-hydroxylated BS species tentatively identified in bile of EE-treated rats by Kern et al. (5) actually represent Δ^{22} β-muricholate. Its increased contribution to the pool is probably the result of increased β-muricholate formation via the alternative pathway induced by EE; the fact that Δ^{22} β-muricholate disappears from the bile after interruption of the enterohepatic circulation is most likely explained by the assumption that β-muricholate can only be metabolized to Δ^{22} β-muricholate during entrohepatic cycling, i.e., after uptake from the intestine and transport back to the liver. In this scenario, therefore, Δ^{22} β-muricholate cannot be considered a primary BS but represents a tertiary species.

In conclusion, our studies show that EE treatment selectively suppresses the initial step in the neutral pathway of BS synthesis controlled by CYP7A in rats, at least in part at transcriptional level. EE does not affect CYP27 activity and mRNA levels, probably leading to preferential BS synthesis via the alternative route. Yet, EE effects on substrate availability and on enzymes further down the synthetic cascade may determine the ratios between the various end products of both synthetic pathways that are secreted into bile.

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