Formation of Δ^{22}-bile acids in rats is not gender specific and occurs in the peroxisome

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Abstract We recently demonstrated that the formation of Δ^{22}-bile acids is a quantitatively major pathway for normal bile acid synthesis in the adult male Sprague-Dawley rat. This pathway is specific for 7β-hydroxy bile acids and, when ursodeoxycholic acid is administered, Δ^{22}-ursodeoxycholic acid appears as a major metabolite in the liver tissue, bile, intestinal contents, and plasma. The aims of this study were, therefore, to determine whether this metabolic pathway was gender specific, and to establish that the peroxisome is a site of formation of Δ^{22}-bile acids. Bile acids were determined by gas chromatography–mass spectrometry in liver tissue, jejunum, and plasma of adult female rats and in animals fed a diet containing 0.4% and 1% ursodeoxycholic acid. Bile acid metabolism in female rats was found to be similar to that of male rats, and Δ^{22}-β-muricholic acid, rather than β-muricholate, was likewise confirmed as the major muricholic acid synthesized. Ursodeoxycholic acid administration resulted in the appearance of Δ^{22}-ursodeoxycholic acid as a major metabolite. When adult male Sprague-Dawley rats were treated with clofibrate, a drug that induces peroxisomal proliferation, liver weight increased 40–60% and total bile acid synthesis decreased markedly, but the relative composition of individual bile acids was unchanged. When ursodeoxycholic acid was added to the diet, the proportion of Δ^{22}-bile acids relative to the corresponding saturated analogues increased significantly compared with untreated rats, indicating that clofibrate had "amplified" the pathway for formation of Δ^{22}-bile acids. When UDCA was incubated in vitro with a peroxisomal-enriched fraction from normal adult male rat liver, Δ^{22}-ursodeoxycholic acid was formed in proportions comparable to that observed in vivo when this bile acid was given orally. These studies establish that the pathway for the formation of Δ^{22}-bile acids is not gender specific and mainly occurs in hepatic peroxisomes.–**Rodrigues, C. M. P., B. T. Kren, C. J. Steer, and K. D. R. Setchell.** Formation of Δ^{22}-bile acids in rats is not gender specific and occurs in the peroxisome. *J. Lipid Res.* 1996. **37:** 540–550.

Supplementary key words Δ^{22}-bile acids • ursodeoxycholic acid • β-oxidation • clofibrate • peroxisomes • liver tissue • rats

We recently described the formation of Δ^{22}-bile acids as a quantitatively important pathway for bile acid synthesis in the adult male Sprague-Dawley rat (1). This pathway for bile acid synthesis was found to be selective toward bile acids that possessed a 7β-hydroxyl function, and consequently Δ^{22}-β-muricholic acid was shown to be a hitherto previously unidentified major muricholic acid isomer in normal rats, while the administration of ursodeoxycholic acid (UDCA) resulted in the formation of relatively large proportions of Δ^{22}-UDCA as a specific metabolite. The finding of high concentrations of these Δ^{22}-bile acids in the liver tissue and in the jejunum, with virtual absence in the colon, provided strong support for the liver as the site of formation. Separate studies by Thompson, Davis, and Morris (2) and Davis and Thompson (3) identified tauro-Δ^{22}-β-muricholic acid in the serum of rats treated with α-naphthylisothiocyananate, and showed in vitro that rat liver slices were capable of synthesizing these unique bile acids. In addition, our previous finding of Δ^{22}-bile acids in the bile collected from bile duct-canulated animals served to establish an hepatic origin for their synthesis. Earlier studies had hinted at the presence of several unknown unsaturated C_{24}-bile acids in rats (4–9); however, with the result of improvements in methodology it is now apparent that the formation of Δ^{22}-bile acids is a major biosynthetic pathway of normal bile acid metabolism in male Sprague-Dawley animals. At the time, it was not known whether this pathway was gender specific, as is

Abbreviations: chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholan-24-oic acid; GC–MS, gas chromatography–mass spectrometry; lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid; α-muricholic acid, 3α,6β,7α-trihydroxy-5β-cholan-24-oic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholan-24-oic acid; ω-muricholic acid, 3α,6α,7β-trihydroxy-5β-cholan-24-oic acid; Δ^{22}-β-muricholic acid, 3α,6β,7β-trihydroxy-5β-chol-22-en-24-oic acid; Δ^{22}-ω-muricholic acid; 3α,6α,7β-trihydroxy-5β-chol-22-en-24-oic acid; Δ^{22}-ursodeoxycholic acid (Δ^{22}-UDCA), 3α,7β-dihydroxy-5β-chol-22-en-24-oic acid; ursodeoxycholic acid (UDCA), 3α,7β-dihydroxy-5β-cholan-24-oic acid.

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the case for the biosynthesis of steroid hormones by Sprague-Dawley rats (10). The mechanism of formation was postulated to occur in the peroxisome, by a process involving partial β-oxidation of the side-chain (10). We have now extended these studies to show that Δ^{22}-bile acids are also present in biological samples from female animals, thereby demonstrating that this biosynthetic pathway is not gender specific. We also provide evidence that establishes the peroxisome as a specific organelle in which the synthesis of Δ^{22}-bile acids takes place.

EXPERIMENTAL PROCEDURES

Animal studies

To determine whether the formation of Δ^{22}-bile acids is unique to male rats and to examine possible gender differences in the metabolism of UDCA, an experiment was conducted in which female Sprague-Dawley rats (body weight 205–229 g) were fed diets of standard laboratory chow supplemented with either 0.4% (n = 2), 1% (n = 2) UDCA or no addition (control, n = 2) for 10 days. The animals were then killed by exsanguination under ether anesthesia. The plasma was collected and frozen at -20°C. The liver was immediately removed, rinsed in normal saline, and flash-frozen in liquid nitrogen. The intestine was removed, divided into four sections (jejunum, ileum, cecum, colon), and flash-frozen in liquid nitrogen. Data from this study were compared with previously published results (1) where male animals were fed the same diets and samples were collected and analyzed in an identical manner.

To examine the effect of the peroxisomal proliferating drug, clofibrate, on bile acid synthesis, male Sprague-Dawley rats (body weight 190–200 g) were maintained on a 12-h light–dark cycle and fed standard laboratory chow ad libitum for 3 days. The animals were then orally administered 500 mg/kg body weight/day of clofibrate (Fluka Chemical Corp., Ronkonkoma, NY) for 6 days. Starting on day 7, rats were fed diets supplemented with 0% (n = 5), 0.4% (n = 5), or 1% (n = 5) UDCA, and clofibrate gavage was continued for an additional 4 days. Biological samples were collected as described above. Adult male animals were fed the same diets, but no clofibrate was administered (control group), and data on the bile acid composition for control rats have been described elsewhere (1).

All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences (11).

In vitro studies

Peroxisome isolation. Peroxisomes were isolated from the rat liver of the normal male animals and the clofibrate-treated male animals as previously described (12, 13) with modifications. Unless otherwise indicated, all steps were performed at 4°C and buffers were stored on ice. Most of the chemicals were obtained from Fisher Scientific (Pittsburgh, PA). Liver tissue from normal rats and from those treated with clofibrate were weighed and ground to a fine powder in a mortar and pestle on dry ice. The powder was transferred to a chilled Douce homogenizer and dounced in 3 × (w/v) chilled homogenization buffer containing 0.25 M sucrose (ultrapure sucrose; Life Technologies, Gaithersburg, MD), 5 mM MOPS, pH 7.4 (United States Biochemical Corp., Cleveland, OH), and 1 mM EDTA. The homogenate was filtered through two layers of gauze into a 50 mL Oak Ridge centrifuge tube, and the volume was measured and adjusted with the homogenization buffer to a final volume of 5 mL per gram of liver tissue. The homogenate was centrifuged in a Sorvall SS34 rotor at 1,900 *g* (4,000 rpm) for 10 min at 4°C. The supernatant was removed and centrifuged a second time. The final supernatant was centrifuged in a Sorvall SS34 rotor at 25,300 *g* (14,550 rpm) for 20 min at 4°C and the resulting supernatant was discarded. The pellet was resuspended in 3 mL of homogenization buffer and the centrifugation was repeated. The resulting pellet was resuspended in 0.5 mL of homogenization buffer per 1.5 g of liver tissue. A volume of 0.5 mL of the resuspended pellet, which corresponded to the light mitochondrial fraction (12), was layered over a 4 mL 1.10–1.28 g/mL linear metrizamide gradient in 5 mM MOPS, pH 7.4, and 1 mM EDTA, and centrifuged in a Beckman SW 65 Ti rotor at 39,000 *g* (19,600 rpm) for 90 min at 4°C. The gradient was manually fractionated from the top, and aliquots were flash-frozen in liquid nitrogen. Catalase activity was assayed as the marker enzyme for peroxisomes (14) and proteins were determined using the Bio-Rad dye microassay (Bio-Rad, Chemical Laboratories, Richmond, CA). Fractions 5, 6, and 7 were pooled, and the catalase relative specific activity (i.e., the ratio of the catalase specific activity found in the fraction to that measured in the homogenate representing a crude preparation of peroxisomes) in these fractions was 19.2 ± 8.1%, 36.8 ± 1.7%, and 11.1 ± 2.3%. These lower gradient fractions were used in the incubation studies.

Peroxisome incubation. Incubations of the peroxisomal-enriched fraction with bile acids were performed in triplicate, according to Kase, Björkhem, and Pedersen (15) and modified as described below. The incubation mixture contained 7.5 mM ATP, 2 mM CoA, 10 mM $MgCl_2$, 7.5 μM FAD, 250–500 μg of peroxisomal proteins, and 0, 5, 10, or 20 μg UDCA or 5 μg chenodeoxycholic acid in 0.75 mL of 0.1 M Tris-HCl buffer, pH 8.0. Bile acid solutions were prepared in ethanol and less

than a 5 μL volume was added to the incubation mixture. After a 15-min preincubation at 37°C, the reaction was started by the addition of 2 mM NAD^+ and the incubation continued for 60 min. The reaction was terminated by the addition of 10 mL of methanol, and 5 μg of nordeoxycholic acid was added as an internal standard for bile acid quantification. Controls, in which peroxisomes and/or bile acids were not added to the reaction mixture, were also included.

Analytical techniques

Total and individual bile acid concentrations were determined in plasma, liver tissue, and jejunum from Sprague-Dawley rats and in the methanolic extracts of the peroxisomal incubation mixtures. Bile acids were measured by gas chromatography–mass spectrometry (GC–MS) after their extraction, hydrolysis, isolation by anion-exchange chromatography on Lipidex-DEAP, and conversion to volatile methyl ester-trimethylsilyl (Me-TMS) ether derivatives.

Extraction of bile acids

Plasma. After addition of an internal standard, nordeoxycholic acid (5 μg), plasma (1 mL) was diluted with 0.1 M sodium hydroxide (4 mL) and heated to 64°C for 20–30 min. Bile acids and their conjugates were quantitatively extracted by adsorption to small cartridges of octadecylsilane-bonded silica (Bond-Elut C_{18}; Analytichem, Harbor City, CA) and recovered with methanol (5 mL) as described by Setchell and Worthington (16). The methanolic extract was taken to dryness.

Intestinal contents and liver tissue. The intestinal contents of jejunum were weighed and dissected into small pieces using surgical scissors. Liver samples (200 mg) were ground to a fine paste in distilled water (20 mL) using a mortar and pestle and brought to a final concentration of 80% methanol (100 mL). All samples were sonicated for 30 min, refluxed for 2 h, and filtered (17). The residue was resuspended in 100 mL of chloroform–methanol 1:1 (v/v), refluxed for 1 h, and filtered. The combined extracts were taken to dryness on a rotary evaporator. The dried extract was resuspended in 80% methanol (20 mL) by sonication, 1/100 of the jejunum extract and 1/2 of the liver extract were taken and the internal standard, nordeoxycholic acid (10 and 5 μg, respectively), was added. The sample was diluted with 0.01 M acetic acid (19 mL) and passed through a column of Lipidex 1000 (bed size 4 × 1 cm; Packard Instrument Co., Groningen, The Netherlands). Aqueous acetic acid (20 mL) was passed through the gel bed, followed by distilled water (20 mL), and the combined effluent and washings were passed through a Bond-Elut C_{18} cartridge and discarded. Bile acids were recovered by elution of the Lipidex 1000 column and the Bond-Elut C_{18} cartridge with methanol, 20 mL and 5 mL, respectively, and the combined extracts were taken to dryness.

Peroxisomal incubation mixtures. The peroxisomal incubation mixtures diluted with methanol and containing the internal standard nordeoxycholic acid were refluxed for 1 h at 65°C. After cooling and centrifugation, the methanolic supernatant was evaporated under nitrogen. The dried residue was redissolved in 2.5 mL of 0.2 M phosphate buffer, pH 5.6, and bile acid conjugates were enzymically hydrolyzed (18).

Hydrolysis of conjugated bile acids

Solvolysis was carried out in a mixture of methanol (1 mL), distilled tetrahydrofuran (9 mL), and 1 M trifluoracetic acid in dioxane (0.1 mL) heated to 45°C for 2 h (19). After the reagents were evaporated, the residue was subjected to enzymic hydrolysis (18). Hydrolysis was achieved by overnight incubation with 50 units choloylglycine hydrolase (Sigma Chemical Co., St. Louis, MO) in 2.5 mL of 0.2 M phosphate buffer, pH 5.6, at 37°C. After hydrolysis, bile acids were extracted by passage of the sample through a Bond-Elut C_{18} cartridge and recovered by elution with methanol (5 mL).

Isolation of bile acids following hydrolysis

The unconjugated bile acids, after hydrolysis, were isolated and separated from neutral sterols by lipophilic anion exchange chromatography on diethylaminohydroxypropyl Sephadex LH-20 (20) (Lipidex-DEAP; Packard Instrument Co.). The extract was diluted to 72% methanol and passed through a column of Lipidex-DEAP (bed size 13 × 0.4 cm, prepared in the acetate form in 72% ethanol). Neutral compounds pass directly through this anion exchange gel, while bile acids are retained. Recovery of unconjugated bile acids was achieved by elution with 0.1 M acetic acid in 72% ethanol (7 mL) and this fraction was evaporated to dryness.

Gas chromatography–mass spectrometry analysis

Me-TMS ether derivatives were prepared (21) and purified (22) prior to separation by gas chromatography on a 30 m × 0.32 mm DB-1 (0.25 μm film) fused silica capillary column (J & W Scientific, Folson, CA) using a temperature program from 225°C–295°C at a rate of 2°C/min, with initial and final isothermal periods of 2 min and 30 min, respectively. Helium was used as carrier gas with a flow rate of 1.8 mL/min. GC–MS analysis was carried out using a Finnigan 4635 quadrupole instrument housing an identical column and using the same chromatographic conditions. Electron ionization (70 eV) mass spectra were recorded over the mass range 50 to 800 Da by repetitive scanning of the eluting components. Quantification of bile acids was achieved using GC, by comparing the peak height response of the

TABLE 1. Principal bile acids present in liver tissue, jejunum, and plasma from normal adult male[a] and female[b] Sprague-Dawley rats fed standard laboratory rat chow, determined by gas chromatography–mass spectrometry

	Liver (nmol/g)			Jejunum (mg)			Plasma (μmol/L)		
	Male	Female 1	Female 2	Male	Female 1	Female 2	Male	Female 1	Female 2
Lithocholic acid	4.6 ± 0.3	6.1	9.1	0.02 ± 0.01	0.04	0.03	nd	0.3	0.1
Deoxycholic acid	29.0 ± 2.1	12.4	30.0	0.50 ± 0.03	0.39	0.32	3.3 ± 0.4	1.6	2.1
Chenodeoxycholic acid	17.2 ± 1.4	14.0	16.6	0.25 ± 0.05	1.15	0.72	5.2 ± 0.9	2.1	7.8
α-Muricholic acid	39.7 ± 4.7	18.3	30.0	1.48 ± 0.24	0.05	0.10	nd	nd	nd
Cholic acid	157.5 ± 11.7	83.2	136.5	6.82 ± 1.10	8.83	6.74	35.9 ± 4.0	17.5	22.6
UDCA	12.0 ± 2.5	10.8	14.1	0.03 ± 0.01	0.02	0.10	1.4 ± 0.3	0.2	0.6
Δ^{22}-UDCA	nd[c]	nd	nd	nd	nd	nd	nd	nd	nd
β-Muricholic acid	45.8 ± 3.0	24.7	60.3	0.64 ± 0.18	1.46	0.55	3.4 ± 0.6	0.9	1.8
Δ^{22}-β-Muricholic acid	129.1 ± 10.7	81.8	131.5	3.44 ± 0.57	0.57	1.40	4.3 ± 1.2	2.6	4.4
ω-Muricholic acid	nd	nd	nd	0.10 ± 0.01	0.10	0.10	1.9 ± 0.3	1.8	1.2
Δ^{22}-ω-Muricholic acid	nd	nd	nd	0.56 ± 0.08	0.05	0.05	0.7 ± 0.2	0.7	0.5
Total	434.9 ± 27.3	251.3	428.1	14.04 ± 2.00	12.84	10.11	56.0 ± 6.6	27.7	41.1

[a]Results are expressed as mean ± SEM for 6 animals. Values are taken from previously reported studies (1, 25).
[b]Results are expressed as individual values for each animal.
[c]nd, not detected.

individual bile acids with the peak height response obtained from the internal standard. Identification of a bile acid was determined by the GC retention index relative to a homologous series of *n*-alkanes, referred to as the methylene unit (MU) value, and the mass spectrum was compared with authentic standards. A comprehensive list of retention indices and mass spectra of bile acid Me-TMS ethers is published elsewhere (23).

RESULTS

Bile acid metabolism by adult female Sprague-Dawley rats

Normal bile acid metabolism. **Table 1** shows the liver tissue and plasma bile acid concentrations and the mass of bile acids found in the jejunum of normal adult female rats studied, and compares these values with previously published values for adult male animals (1). Bile acid metabolism was similar between male and female animals, with few exceptions. Lithocholic acid concentration was slightly higher in the female rats than in males for all biological samples examined. In the female animals, although there was a tendency toward lower concentrations for cholic acid and the muricholic acid isomers including the Δ^{22}-metabolites, these differences were not significant.

Effects of UDCA administration. Compared with control values, UDCA concentrations in the liver tissue of female animals increased markedly and in a dose-dependent fashion during the 0.4% UDCA (154.5 and 180.6 nmol/g) or 1.0% UDCA (519.3 and 368.7 nmol/g) feeding regimens. Proportionally, UDCA accounted for 52.8 and 55.5% or 63.0 and 69.9%, respectively, of the total bile acids identified in the liver of female animals fed the 0.4% or 1% UDCA diets, while in the controls this value was 3.7%. The increase in liver tissue UDCA with bile acid feeding was accompanied by substantial increases in the proportion of its major metabolite Δ^{22}-UDCA (**Fig. 1**). The liver tissue concentrations of Δ^{22}-UDCA were much lower in female animals fed UDCA than we previously reported for adult males (1). Similarly, Δ^{22}-β-muricholic acid concentrations were lower in female animals than in males (Fig. 1). Comparable observations were evident from the analysis of plasma and intestinal contents. The increased concentrations of UDCA and its Δ^{22}-metabolite compared with control animals were associated with substantial decreases in concentrations of cholic, deoxycholic and chenodeoxycholic acids (Table 1 and **Table 2**). Concomitant with these changes was a 2- to 3-fold elevation in the concentration of lithocholic acid in liver tissue and jejunum from rats fed the 1% UDCA diet.

Bile acid metabolism by clofibrate-treated male Sprague-Dawley rats

No change in body weight was observed between the control group of animals and those administered clofibrate alone or clofibrate combined with either 0.4% or 1% UDCA. However, compared with controls, liver weight increased 40–60% ($P < 0.001$) in all animals treated with clofibrate. Interestingly, the increased liver weight (expressed as a percentage of the body weight) due to clofibrate treatment was less pronounced ($P < 0.01$) in those animals administered UDCA, but did not differ depending upon the dose of bile acid administered. Electron microscopy of catalase-stained sections of the liver tissue confirmed peroxisomal proliferation by increased number of peroxisomes in the clofibrate-treated animals.

Effects of clofibrate on bile acid metabolism. Liver tissue concentrations of the principal bile acids of the male Sprague-Dawley rat published previously (1) were compared with values found in animals treated with clofi-

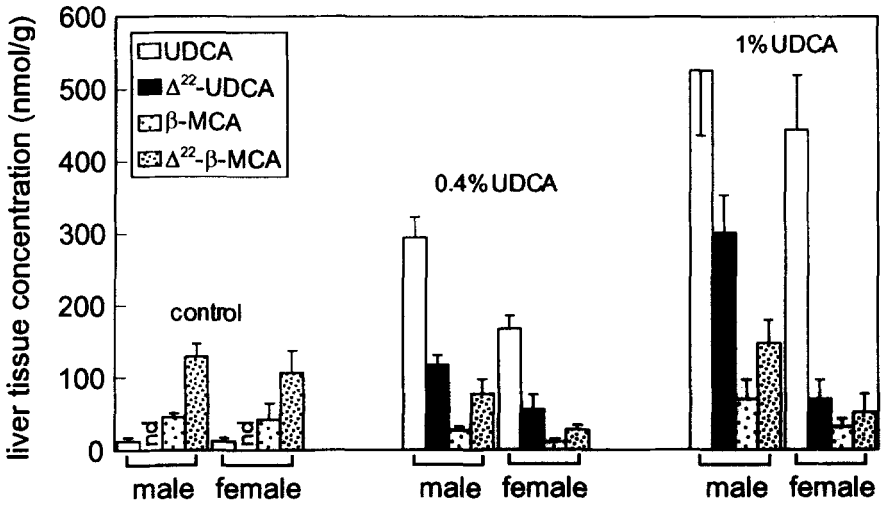

Fig. 1. Liver tissue concentrations of UDCA, β-muricholic acid (β-MCA), and the corresponding saturated analogues identified in male and female Sprague-Dawley rats fed a diet containing 0%, 0.4%, or 1% UDCA; nd, not detected. Values for male animals in the control group taken from previous data (1, 25).

brate alone (**Table 3**). Total bile acid concentration in the liver tissue of animals treated with clofibrate decreased markedly compared with untreated animals (158.5 ± 13.3 nmol/g versus 434.9 ± 27.3 nmol/g, $P < 0.001$). Similarly, there was a decrease in the plasma bile acid concentration and jejunal total bile acid mass with clofibrate treatment (Table 3). With regard to the principal individual liver tissue bile acids, all showed a consistent decrease in concentration with clofibrate treatment, while the proportion of cholic acid increased to account for 53.6 ± 5.6% of the total bile acids. However, in liver tissue, the Δ^{22}-β-muricholic acid/β-muricholic acid ratio in clofibrate treated rats (2.88 ± 0.45) did not increase compared with that of untreated animals (2.82 ± 0.28). Similarly, the Δ^{22}-ω-muricholate/ω-muricholate ratio did not change with clofibrate treatment. There were no detectable amounts of Δ^{22}-UDCA in any of the control animals treated with clofibrate alone (Table 3).

Formation of Δ^{22}-bile acids by clofibrate-treated animals administered UDCA. **Figure 2** shows the ratio of total Δ^{22}-bile acids to the total corresponding saturated analogues in the liver, jejunum, and plasma from untreated and clofibrate-treated rats fed diets containing 0%, 0.4%, and 1% UDCA. There was no significant change in the ratio of Δ^{22}-bile acids to the saturated analogues in the liver tissue, jejunum, and plasma with clofibrate treatment alone compared with normal rats. However, this ratio increased markedly with UDCA feeding, consistent with increased liver peroxisomes and substrate availability. With UDCA feeding, a significant increase in the Δ^{22}-UDCA/UDCA ratio was also found for liver tissue, jejunum, and plasma but the increase was less in the rats

TABLE 2. Concentrations of the principal bile acids in liver tissue from adult male[a] and female[b] Sprague-Dawley rats after consuming a diet of standard laboratory rat chow supplemented with 0.4% or 1% ursodeoxycholic acid (UDCA), determined by gas chromatography–mass spectrometry

	0.4% UDCA Diet			1% UDCA Diet		
	Male	Female 1	Female 2	Male	Female 1	Female 2
Lithocholic acid	12.3 ± 3.3	12.8	11.8	17.3 ± 5.2	25.9	19.8
Deoxycholic acid	24.0 ± 3.7	0.5	7.8	13.4 ± 0.7	10.4	4.5
Chenodeoxycholic acid	25.9 ± 2.6	12.6	13.5	26.7 ± 8.8	17.7	11.2
α-Muricholic acid	13.3 ± 2.0	0.8	1.5	18.0 ± 3.5	3.5	0.5
Cholic acid	36.4 ± 2.7	13.0	15.2	28.8 ± 3.6	16.8	11.8
UDCA	294.3 ± 30.0	154.5	180.6	525.9 ± 83.1	519.3	368.7
Δ^{22}-UDCA	117.2 ± 12.2	39.5	70.6	301.0 ± 64.2	82.0	112.0
β-Muricholic acid	26.4 ± 4.2	11.0	10.4	69.6 ± 27.9	39.7	23.8
Δ^{22}-β-Muricholic acid	77.0 ± 21.8	29.5	26.3	147.4 ± 30.8	79.7	23.7
ω-Muricholic acid	8.2 ± 1.2	3.0	2.5	35.5 ± 8.9	23.6	6.1
Δ^{22}-ω-Muricholic acid	8.0 ± 1.2	1.0	0.8	12.8 ± 1.9	5.5	1.5
Total	642.8 ± 59.5	278.2	341.0	1196.2 ± 212.8	824.1	583.6

[a]Results are expressed as mean ± SEM for 6 animals.
[b]Results are expressed as individual values for each animal.

TABLE 3. Principal bile acids present in liver tissue, jejunum, and plasma from normal (control) adult male Sprague-Dawley rats and from rats orally administered clofibrate, determined by gas chromatography–mass spectrometry

	Liver (nmol/g)		Jejunum (mg)		Plasma (μmol/L)	
	Control	C lofibrate	Control	C lofibrate	Control	C lofibrate
Cholic acid	157.5 ± 11.7	85.0 ± 8.9	6.82 ± 1.10	6.91 ± 1.57	35.9 ± 4.0	33.5 ± 5.3
UDCA	12.0 ± 2.5	5.6 ± 0.8	0.03 ± 0.01	0.06 ± 0.04	1.4 ± 0.3	0.6 ± 0.2
Δ^{22}-UDCA	nd	nd	nd	nd	nd	nd
β-Muricholic acid	45.8 ± 3.0	11.3 ± 1.0	0.64 ± 0.18	0.42 ± 0.10	3.4 ± 0.6	1.7 ± 0.4
Δ^{22}-β-Muricholic acid	129.1 ± 10.7	32.2 ± 2.9	3.44 ± 0.57	2.24 ± 0.55	4.3 ± 1.2	1.9 ± 0.6
Total	434.9 ± 27.3	158.5 ± 13.3	14.04 ± 2.00	10.96 ± 2.46	56.0 ± 6.6	42.6 ± 7.0

Results are expressed as mean ± SEM; nd, not detected. Values for male animals are taken from previously published data (1, 25).

fed the 1% UDCA diet ($P < 0.01$) compared with those given a 0.4% UDCA diet ($P < 0.001$) (**Fig. 3**).

In vitro synthesis of Δ^{22}-UDCA from UDCA by peroxisomes

After incubation of the peroxisomal-enriched fraction isolated from normal rat liver with UDCA, in the presence of CoA, ATP, Mg^{2+}, FAD, and NAD^+, gas chromatographic analysis of the Me-TMS ether derivatives of the isolated bile acids revealed two major compounds having retention indices of 32.56 MU and 32.95 MU (**Fig. 4**). These were consistent with the retention indices of UDCA and Δ^{22}-UDCA, respectively, and their structures were confirmed by mass spectrometry, by comparing electron ionization mass spectra with the authentic compounds (1). These bile acids were not identified in peroxisomes isolated from rat liver unless UDCA was added to the incubation mixture (Fig. 4). The proportion of Δ^{22}-UDCA relative to UDCA after a 60-min incubation of peroxisomes with 5 and 10 μg of UDCA was 62.5 ± 5.4% and 56.5 ± 1.2%, respectively, and this was similar to the proportion found in liver tissue of intact animals (1). When a larger amount of UDCA (20 μg) was incubated, proportionally less Δ^{22}-UDCA was formed (24.2%). When peroxisomes isolated from clofibrate-treated rats were incubated with 10 μg UDCA, the proportion of Δ^{22}-UDCA formed relative to UDCA was 34.9 ± 8.6%.

Figure 4 also shows the gas chromatographic profile of the Me-TMS ether derivatives of bile acids isolated after incubation of the peroxisomal-enriched fraction from normal rat liver with chenodeoxycholic acid (5 μg). There was no evidence for formation of Δ^{22}-chenodeoxycho lic acid.

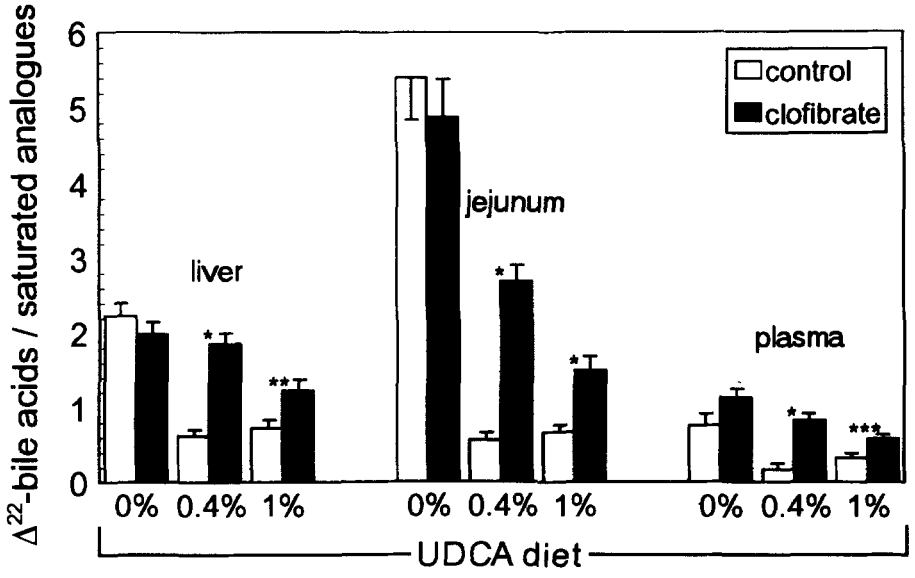

Fig. 2. Ratio of Δ^{22}-bile acids to the corresponding saturated analogues in liver tissue, jejunum, and plasma from control and clofibrate-treated adult male Sprague-Dawley rats fed a diet containing 0%, 0.4%, or 1% UDCA. Statistical comparisons were made between clofibrate and untreated groups: *, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$.

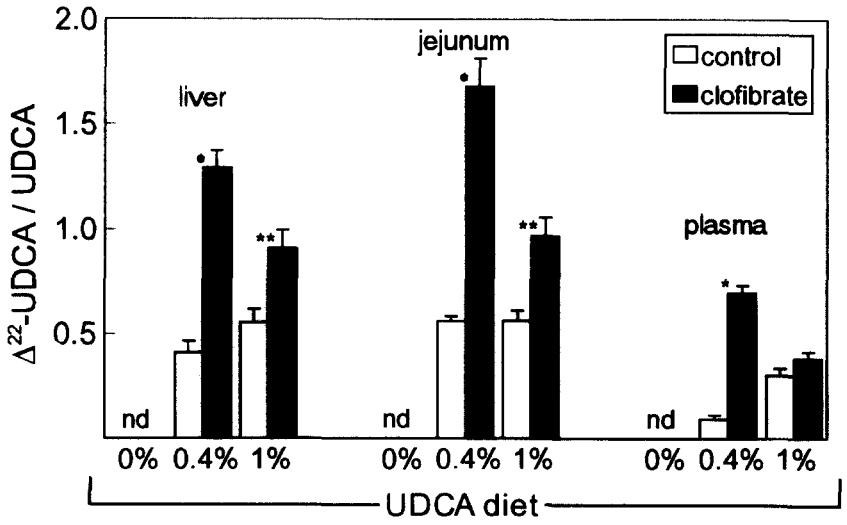

Fig. 3. Ratio of Δ^{22}-UDCA to UDCA in liver tissue, jejunum, and plasma from control and clofibrate-treated adult male Sprague-Dawley rats fed a diet containing 0%, 0.4%, or 1% UDCA. Statistical comparisons were made between clofibrate and untreated groups: *, $P < 0.001$; **, $P < 0.01$; nd, not detected.

DISCUSSION

We have previously shown that intravenous and oral administration of UDCA to adult male Sprague-Dawley rats was followed by the appearance in bile, liver tissue, jejunal contents, and plasma of relatively large proportions and concentrations of a unique metabolite, identified as Δ^{22}-UDCA (1, 24). Further analysis of these biological samples led to the recognition that the formation of Δ^{22}-bile acids was a normal pathway for endogenous bile acid synthesis by the adult male Sprague-Dawley rat, and bile acid feeding experiments indicated that this pathway was selective toward bile acids with a 7β-hydroxyl group (1). For example, in normal adult rats, Δ^{22}-β-muricholic acid (3α,6β,7β-trihydroxy-5β-chol-22-en-24-oic acid) and Δ^{22}-ω-muricholic acid (3α,6α,7β-trihydroxy-5β-chol-22-en-24-oic acid) were both identified, but there was no evidence for the presence of Δ^{22}-analogues of α-muricholic or hyocholic acid, two bile acids possessing a 7α-hydroxyl group. Additionally, while UDCA administration led to the appearance of large amounts of Δ^{22}-UDCA in biological samples, Δ^{22}-deoxycholic acid was not formed when deoxycholic acid was fed to rats (25). Detailed quantitative analysis of the bile, liver tissue, intraluminal contents along the entire intestinal tract, feces, and plasma established that, con-

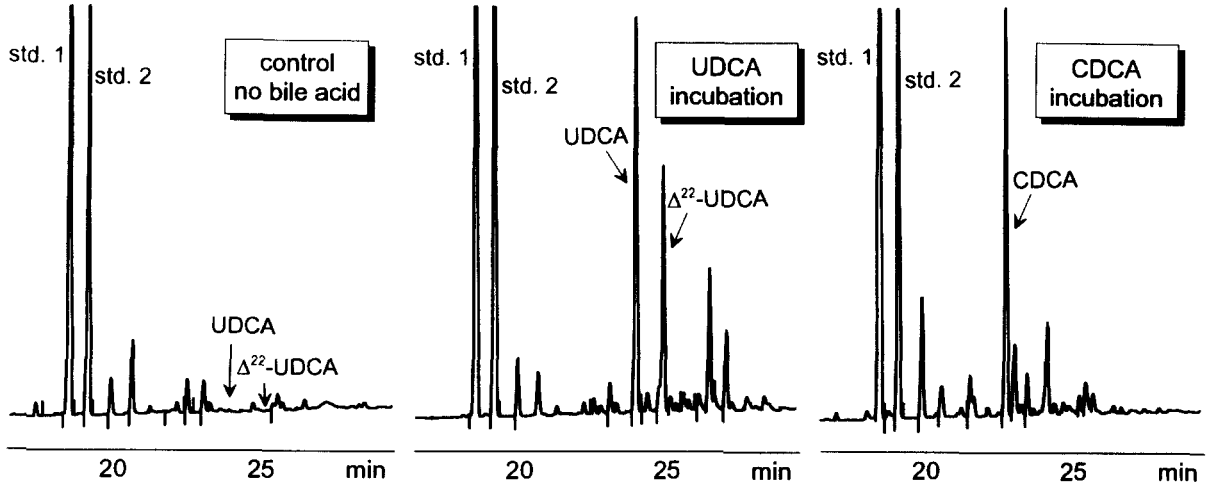

Fig. 4. Typical gas chromatographic profiles of the methyl ester-trimethylsilyl ether derivatives of bile acids isolated after incubation of a peroxisomal-enriched fraction of normal adult male rat liver with no bile acid, UDCA (5 μg), or chenodeoxycholic acid (CDCA) (5 μg).

trary to the published literature, the major muricholic acid isomer of the Sprague-Dawley rat is not β-muricholate but rather Δ^{22}-β-muricholic acid. Our studies, therefore, cast doubt on the reliability of previously published quantitative data on the family of muricholic acid isomers in rats. Interestingly, the presence of a quantitatively important unsaturated bile acid, with characteristics similar to the muricholic acids, was recognized in early studies of bile acid metabolism but its structure was never identified (4–9). The similarity in chemical structure and physicochemical properties of Δ^{22}-β-muricholic acid and the saturated analogue, β-muricholic acid, would easily explain the difficulty in recognizing its presence, as these bile acids would not chromatographically resolve when analyzed by reverse phase high performance liquid chromatography or packed column GC. Furthermore, the rigorous sample workup procedures used in most of the early bile acid methods could account for losses of unsaturated bile acids.

As all of our studies were performed on adult male Sprague-Dawley rats, and because of the known gender differences in steroid hormone synthesis and metabolism by this species (10), we examined whether the formation of Δ^{22}-bile acids was gender specific. At the time of these studies, Thompson, Davis, and Morris (2) and Davis and Thompson (3) identified the taurine conjugate of Δ^{22}-β-muricholic acid in the plasma of Sprague-Dawley rats treated with α-naphthylisothiocyananate. The fact that these were female animals suggested that gender differences in the formation of Δ^{22}-bile acids was unlikely.

Surprisingly, few studies have examined in detail gender differences in bile acid metabolism by rats (26–28), and perhaps because of the different species studied, data are conflicting in their findings. A study of bile-fistula Wistar rats showed gender differences in biliary bile acids, with female animals secreting greater amounts of cholic and lithocholic acid and less β-muricholic, deoxycholic, and hyodeoxycholic acids than male animals (27). Different observations were reported by Beher, Casazza, and Lin (26) for Sprague-Dawley rats. Using radioactive isotopes, it was found that the bile acid pool of female animals contained high concentrations of cholic and chenodeoxycholic acids, while male rats totally lacked chenodeoxycholic acid. Puzzling, however, was the absence of gender differences with respect to the concentrations of α-muricholic, β-muricholic, and deoxycholic acids. Interestingly, in neither study was there a mention of Δ^{22}-bile acids, and the identification of several bile acids appears equivocal.

In the present study, bile acid concentrations in liver tissue and plasma (Table 1) were found to be similar to values we previously reported for adult male rats (1). These findings suggest that there are no major gender differences in normal bile acid metabolism. Total bile acid concentrations in the biological samples from the female animals were slightly lower than values we previously reported for males. With regard to Δ^{22}-bile acids, Δ^{22}-β-muricholic acid concentrations in the liver tissue of female animals greatly exceeded values for β-muricholic acid and this bile acid was the major muricholic acid isomer of both sexes. Δ^{22}-UDCA was not detected in any of the biological samples analyzed and is therefore not a usual endogenous bile acid in this species. However, the administration of UDCA to female rats resulted in an enrichment in UDCA in excess of 50% for all biological samples and in the appearance of relatively large proportions of Δ^{22}-UDCA (Table 2). These changes were accompanied by decreases in the concentrations of cholic, deoxycholic, and chenodeoxycholic acids, whereas liver tissue concentrations of lithocholic acid increased several fold (Table 2). This is consistent with our previous observations (24). Overall, there was a tendency for the proportions of Δ^{22}-bile acids formed by female rats during UDCA administration to be lower than we had previously found for male animals studied under identical conditions (Fig. 1). The proportion of Δ^{22}-UDCA formed from UDCA was not dose-dependent in female rats, suggesting that the synthesis of Δ^{22}-UDCA may be limited or is saturated when the 1.0% UDCA diet was fed. This may reflect a gender difference in bile acid synthesis. It was noteworthy that the total bile acid concentration and the concentration of UDCA in the liver tissue were lower than we reported previously for male animals fed the same diets, suggesting that there may also be gender differences in the absorption of UDCA, although the reason for this is unknown.

We had previously suggested that the formation of Δ^{22}-UDCA, (and endogenous Δ^{22}-bile acids) takes place within the peroxisome (1) by reactions analogous to those involved in the β-oxidation of cholestanoic acids (C_{27}-bile acids) (29). Because UDCA has only a five carbon atom side-chain, release of propionic or acetic acids is not possible, and therefore the reaction does not proceed beyond formation of the Δ^{22}-intermediate. In an attempt to confirm the role of peroxisomes in the formation of Δ^{22}-bile acids, we first examined the effects of clofibrate, a drug known to induce peroxisomal proliferation in rat liver (30–32), on the synthesis of Δ^{22}-bile acids and on the formation of Δ^{22}-UDCA during administration of UDCA. We then isolated peroxisomes from the liver of normal and clofibrate-treated male animals and examined in vitro the formation of Δ^{22}-UDCA from UDCA.

Clofibrate administration caused an increase in liver weight in all animals, consistent with its known effects (30–32). However, it was remarkable that the increase

in liver weight in the rats fed the diets containing UDCA was not as great as in those animals given clofibrate alone, suggesting that UDCA may either have a direct hepatoprotective effect against this drug or may inhibit the intestinal absorption of clofibrate, thereby limiting the drug's overall effectiveness. Clofibrate administration to adult male rats led to a marked decrease in the liver tissue concentrations of the total and principal individual bile acids, and these changes were paralleled by similar reductions in bile acid mass within the jejunum and in plasma bile acid concentrations (Table 3). These changes indicate a reduction in bile acid synthesis with clofibrate and are supported by clinical studies that show primary bile acid synthesis, as measured from fecal bile acid excretion, to be reduced in patients with hyperlipidemias treated with clofibrate (33–35). The effects on hepatic bile acid synthesis could be secondary to the hypocholesterolemic effect of the drug, as clofibrate has been shown to directly inhibit cholesterol biosynthesis in rats (36), and in humans (37) by inhibiting mevalonate synthesis from acetate, an effect that would reduce the available supply of cholesterol for bile acid synthesis. However, more recently, Ståhlberg et al. (38) reported a 60% reduction in cholesterol 7α-hydroxylase activity after administration of bezafibrate, a second-generation fibrate analogue, in patients with gallstones, which seems the more possible explanation for the reduced bile acid synthesis by clofibrate. Although bile acid concentrations were reduced after clofibrate treatment, there was a significant increase in the proportion of cholic acid in liver tissue, consistent with previous studies of hypolipidemic drugs shown to stimulate cholesterol 12α-hydroxylase activity (39–41). With regard to Δ^{22}-bile acids, there was no difference between the proportion of Δ^{22}-bile acids relative to the corresponding saturated analogues in all biological samples from clofibrate-treated versus untreated control rats (Fig. 2). Furthermore, Δ^{22}-UDCA remained undetectable in the animals not fed UDCA, even after clofibrate treatment. However, when 0.4% UDCA was added to the diet of clofibrate-treated animals, there was a large increase in the ratio of Δ^{22}-bile acids to saturated analogues compared with rats fed standard laboratory chow with no bile acid added (Fig. 2). This is explained by the greater substrate availability of UDCA and its metabolite, β-muricholic acid, when UDCA was fed; in the absence of bile acid feeding, clofibrate causes a decrease in substrate because of repression of bile acid synthesis, leading to reduced β-muricholic acid levels. The increase in the proportion of Δ^{22}-bile acids after UDCA feeding was indicative of an "amplification" of the pathway for formation of Δ^{22}-bile acids and consistent with increased numbers of peroxisomes. When UDCA was added to the diet, the proportion of Δ^{22}-UDCA relative to UDCA was significantly greater in the clofibrate-treated animals compared with the untreated controls (Fig. 3). The increase, however, was not directly proportional to the amount of UDCA in the diet, because the Δ^{22}-UDCA/UDCA ratio and the total Δ^{22}-bile acids/saturated bile acids ratio were lower for the 1.0% UDCA diet than for the 0.4% UDCA. This suggests that the formation of Δ^{22}-bile acids is saturable, even though clofibrate induced peroxisomal proliferation. Nevertheless, these animal studies provide further evidence that the pathway for the formation of Δ^{22}-bile acids occurs in the peroxisome.

Finally, confirmation that Δ^{22}-UDCA is formed in the peroxisome was established from in vitro studies by incubations of a peroxisomal fraction from rat liver of normal adult male Sprague-Dawley rats with UDCA. These studies were essentially identical to those reported by Kase, Björkhem, and Pedersen (15) which demonstrated that formation of cholic acid from 3α,7α,12α-trihydroxy-5β-cholestanoic acid occurs by β-oxidation in the peroxisome. When UDCA was incubated for 60 min with the peroxisome-enriched fraction from rat liver, Δ^{22}-UDCA was isolated and identified by GC–MS as a major metabolite (Fig. 4). The proportion of Δ^{22}-UDCA relative to UDCA formed in vitro was similar to that found in rat liver tissue when UDCA was added to the diet. Furthermore, there was less Δ^{22}-UDCA formed in vitro from UDCA after incubation of a peroxisomal-enriched fraction obtained from the liver of clofibrate-treated rats. This is consistent with the well-characterized decline in activity of peroxisomal oxidases associated with clofibrate treatment (30, 32, 42), even though this drug induces peroxisomal proliferation (30–32).

Previous infusion studies and bile acid feeding experiments had indicated that the formation of Δ^{22}-bile acids was specific for bile acids having an unblocked 7β-hydroxyl group (1). Δ^{22}-Bile acid metabolites were not formed when rats were fed deoxycholic acid (25) or when UDCA was sulfated at position C-7 (43). Incubation of the peroxisomal-enriched fraction with chenodeoxycholic acid did not result in formation of Δ^{22}-chenodeoxycholic acid (Fig. 4). Furthermore, we have failed to find any detectable amounts of Δ^{22}-isomers of hyocholic, hyodeoxycholic, lithocholic, cholic, or α-muricholic acids in any biological samples from adult rats thus far examined. It is not known whether this pathway is specific to the rat but, interestingly, a Δ^{22}-metabolite of 7-oxo-lithocholic acid was recently reported to be a major biliary bile acid of other rodents (44). The synthesis of Δ^{22}-bile acids would require the formation of a CoA ester, and it is possible that this reaction may be rate-limiting, as was demonstrated for cholic acid formation from 3α,7α,12α-trihydroxy-5β-cholestanoic

acid (15). This would explain the reduced proportion of Δ^{22}-UDCA formed when peroxisomes were incubated with relatively large amounts (20 μg) of UDCA. The next step leads to α/β unsaturation, a reaction catalyzed by a bile acid-CoA oxidase, which must be assumed by analogy to the steps in the β-oxidation of bile acids (29) to be distinct from the fatty acid CoA oxidase responsible for the peroxisomal β-oxidation of very long chain fatty acids. As the substrate is a C_{24} bile acid, a Δ^{22}-bond is formed and the reaction can proceed no further. In the rat liver, the Δ^{22}-bile acid is then efficiently conjugated, mainly with taurine, and secreted in bile (1). Why this synthetic pathway is so specific for 7β-hydroxy bile acids is unclear, but selective metabolic pathways for other bile acids have been described, including glucuronidation and *N*-acetylglucosaminidation of the 6α-hydroxyl (45–47) and 7β-hydroxyl groups (48), respectively.

In summary, these studies demonstrate that the formation of Δ^{22}-bile acids is not a gender specific metabolic pathway in the Sprague-Dawley rat. In addition, Δ^{22}-β-muricholic acid and not β-muricholic acid, as is the general belief, is the major muricholic acid isomer synthesized by the liver of both adult male and female Sprague-Dawley rats. The results of these studies clearly indicate that the conversion of UDCA to Δ^{22}-UDCA takes place mainly in the peroxisomal fraction of rat liver. Furthermore, we report, for the first time, the effect of clofibrate treatment on bile acid metabolism in the rat, confirming that this peroxisomal proliferating drug causes a marked reduction in total bile acid levels and a proportional increase in the synthesis of Δ^{22}-bile acids.

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