Δ^{22}-Ursodeoxycholic Acid, a Unique Metabolite of Administered Ursodeoxycholic Acid in Rats, Indicating Partial β-Oxidation as a Major Pathway for Bile Acid Metabolism†

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ABSTRACT: We describe for the first time the identification of 3α,7β-dihydroxy-5β-chol-22-en-24-oic acid (Δ^{22}-UDCA) in the plasma, bile, intestinal contents, and liver tissue of Sprague-Dawley rats after intravenous and oral administration of ursodeoxycholic acid (UDCA). Infusion of [2,2,4,4-$^2H_4$]UDCA confirmed Δ^{22}-UDCA to be a specific metabolite of UDCA. Definitive confirmation of this unique and major metabolite was established by liquid secondary ionization mass spectrometry and gas chromatography−mass spectrometry by comparison of the retention index and mass spectrum with an authentic standard of Δ^{22}-UDCA. When rats were fed a diet containing 1.0% UDCA, high concentrations of Δ^{22}-UDCA were found in the plasma (40.3 ± 11.8 μmol/L) and liver tissue (300.9 ± 64.2 nmol/g of tissue), and these represented 36% and 57%, respectively, of the UDCA concentration. In animals fed 0.4% and 1.0% UDCA, the mass of Δ^{22}-UDCA in the jejunum was high (7.5 ± 0.9 and 6.6 ± 0.6 mg, respectively), accounting for 50−60% of the total UDCA, but diminished markedly along the intestine, accounting for <3% of the total UDCA in the colon. Although Δ^{22}-UDCA was not found in biological samples from control rats, Δ^{22}-β-muricholic and Δ^{22}-ω-muricholic acids were normal constituents of plasma and intestinal contents and were major muricholate isomers in liver tissue and bile. Synthesis of Δ^{22}-bile acids appears to be highly specific toward bile acids possessing a functional 7β-hydroxyl group. We presume that, in common with pathways for endogenous bile acid synthesis, partial side-chain oxidation of UDCA occurs in the peroxisome with formation of α/β unsaturation; since UDCA has only a 5-carbon side chain, release of propionic or acetic acid is not possible, β-oxidation proceeds no further, and Δ^{22}-UDCA is formed. While the mechanism of formation and physiological significance of Δ^{22}-bile acids remain to be established, our data indicate that partial β-oxidation is a quantitatively important pathway for endogenous bile acid synthesis and for UDCA metabolism in this species.

Ursodeoxycholic acid (UDCA)[1] has been in clinical use for more than two decades, initially for the treatment of patients with cholelithiasis (Bachrach & Hofmann, 1982) and more recently for the treatment of a variety of liver diseases [for example, see Poupon et al. (1987), Hofmann and Popper (1987), Leuschner et al. (1989), O'Brien et al. (1991), Beuers et al. (1992), Colombo et al. (1990), Cotting et al. (1990), Crosignani et al. (1990), Palma et al. (1992), Erlinger et al. (1992), and Takano et al. (1994)]. Following the oral administration of unconjugated UDCA, extensive intestinal and hepatic biotransformation takes place, most of which results in the formation of glycine, taurine, sulfate, and glycosylated conjugates (Fedorowski et al., 1977; Hofmann, 1990; Crosignani et al., 1991; Setchell et al., 1992; Marschall et al., 1992; Batta et al., 1993). Other metabolites are formed, including ring and side-chain hydroxylated products, and the extent of biotransformation is species dependent (Yousef et al., 1972; Takikawa et al., 1991).

In early studies we observed that intravenous infusion of UDCA in rats led to the secretion of a unique and quantitatively important metabolite in bile. Subsequent studies showed that this metabolite appeared in plasma, liver tissue, and intestinal contents of Sprague-Dawley rats following oral administration of UDCA (Setchell et al., 1993). By the application of liquid−gel chromatography and gas chromatography−mass spectrometry we now describe the

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[1] Abbreviations: lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; ursodeoxycholic acid (UDCA), 3α,7β-dihydroxy-5β-cholan-24-oic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholan-24-oic acid; cholic acid, 3α,7α,12α-trihydroxy-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}-ursodeoxycholic acid (Δ^{22}-UDCA), 3α,7β-dihydroxy-5β-chol-22-en-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.

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definitive identification of 3α,7β-dihydroxy-5β-chol-22-en-24-oic acid (Δ^{22}-UDCA) and establish this as a specific and quantitatively important metabolite of intravenous and orally administered UDCA in the Sprague-Dawley rat. In addition to this metabolite, other Δ^{22}-metabolites of endogenous bile acids were identified in both control and UDCA-fed animals, indicating that formation of side-chain unsaturated bile acids is a normal and quantitatively important pathway for bile acid synthesis and metabolism in the rat. Our studies establish these unsaturated bile acids to be of hepatic origin, indicate that partial β-oxidation is a major pathway for bile acid metabolism by this species, and strongly suggest that this pathway is specific for 7β-hydroxylated bile acids.

EXPERIMENTAL PROCEDURES

Animal Studies

(i) Intravenous Infusion of UDCA and [$^2H_4$]UDCA. Male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN), weighing 200−230 g ($n = 6$), were anesthetized by an intraperitoneal injection of pentobarbital (Nembutal, 7.5 mg/100 g body weight) and maintained under sedation by additional doses. The right jugular vein and the common bile duct were cannulated using PE-50 polyethylene tubing (Clay-Adams, Parsippany, NJ). Body temperature was maintained throughout the experiment at 37 °C using a rectal probe and a thermostatically controlled heating pad (Harvard Apparatus Co., Inc., Millis, MA). Using a Harvard pump, saline was infused into the jugular vein at a rate of 1.0 mL/h for a control period of 2 h. After two 10-min bile samples were collected for baseline analysis, UDCA [2.0 μmol min^{-1} (100 g body weight)$^{-1}$ prepared in 3% human albumin in 0.45% saline] was infused for 30 min. Bile was collected into preweighed tubes and stored at −20 °C. In a separate experiment, a single adult male Sprague-Dawley rat was infused intravenously with 1.0 μmol min^{-1} (100 g body weight)$^{-1}$ of [2,2,4,4-$^2H_4$]UDCA (MSD Isotopes, Montreal, Canada; 98.2 atom % deuterium), and bile samples were collected in an identical fashion. This animal study protocol (no. 1B10044) was approved by the Bioethics Committee of the Children's Hospital Medical Center, Cincinnati, OH.

(ii) Oral Administration of Ursodeoxycholic Acid and Tauroursodeoxycholic Acid. Male Sprague-Dawley rats, weighing 160−175 g, were maintained on a 12-h light−dark cycle and fed standard laboratory chow *ad libitum* for 3 days. The animals were then transferred to metabolic cages and fed diets of standard laboratory chow (Bio-Serv, Frenchtown, NJ) supplemented with either 0.4% ($n = 6$), 1.0% ($n = 6$) UDCA, or no addition (control; $n = 6$) for 10 days. In a separate experiment, rats ($n = 3$) were fed a diet of standard laboratory chow supplemented with 250 mg/day tauroursodeoxycholic acid for 4 days. The animals were then sacrificed by exsanguination under ether anesthesia. The plasma was collected and frozen at −20 °C. The liver was 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. 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 (NIH Publication No. 86-23, revised 1985).

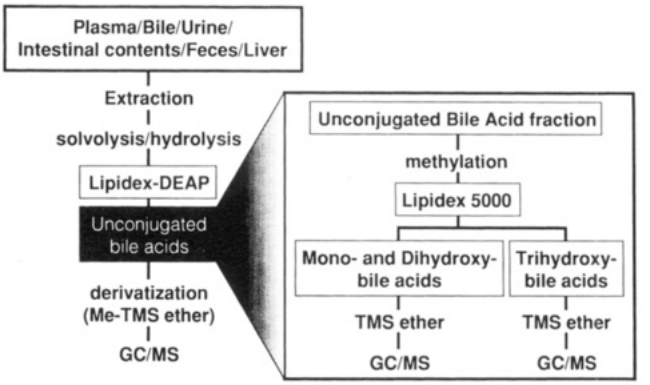

FIGURE 1: General scheme adopted for the analysis of bile acids in biological samples.

Analytical Techniques

The general scheme for the analysis of bile acids in all biological samples collected from these experiments is illustrated schematically in Figure 1. Total and individual bile acid concentrations were determined in plasma, bile, liver tissue, and intestinal contents by gas chromatography−mass spectrometry (GC−MS) following 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

(i) Plasma and Bile. After addition of an internal standard, nordeoxycholic acid (1−10 μg), plasma (0.5 mL), and bile (50 μL) were diluted to 1 mL with distilled water, and 0.1 M sodium hydroxide (4 mL) was added. After being heated to 64 °C, 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 (1982). The methanolic extract was taken to dryness.

(ii) Intestinal Contents and Liver Tissue. The intestinal contents were weighed and dissected into small pieces using surgical scissors. Liver samples 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 (Setchell et al., 1983). The residue was resuspended in 100 mL of chloroform−methanol (1:1 by volume), 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, $^1/_{20}$th of the extract was taken, and the internal standard, nordeoxycholic acid (10 μg), 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 cartridge with methanol, 20 and 5 mL, respectively, and the combined extracts were taken to dryness.

Hydrolysis of Conjugated Bile Acids. Solvolysis was carried out in a mixture of methanol (1 mL), distilled

tetrahydrofuran (9 mL), and 1 M trifluoroacetic acid in dioxane (0.1 mL) heated to 45 °C for 2 h (Hirano et al., 1987). After the reagents were evaporated, the residue was subjected to enzymic hydrolysis (Nair & Garcia, 1969). Hydrolysis was achieved by overnight incubation with 50 units of 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, following hydrolysis, were isolated and separated from neutral sterols by lipophilic anion-exchange chromatography on diethylaminohydroxypropyl-Sephadex LH-20 (Almé et al., 1977) (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.

Group Separation of Bile Acids According to Their Mode of Conjugation Using Lipophilic Anion-Exchange Chromatography. In a separate analysis of the intestinal contents, the extent of bile acid conjugation was examined. This was achieved by pooling the individual segments of intestine from each animal ($n = 6$) after extraction by reflux as described above. Bile acids were separated into groups on the basis of their state of conjugation using Lipidex-DEAP. The extracts were first diluted to 72% methanol and passed through a column of Lipidex-DEAP. Bile acids and their conjugates were retained and then recovered by stepwise elution of the gel bed with the following buffers: 0.1 M acetic acid in 72% ethanol (unconjugated bile acids), 0.3 M acetic acid in 72% ethanol, pH 5.0 (glycine conjugates), 0.15 M acetic acid in 72% ethanol, pH 6.5 (taurine conjugates), and 0.3 M acetic acid in 72% ethanol, pH 9.6 (sulfate conjugates). After evaporation of the buffers, bile acid conjugates were hydrolyzed as described above.

Preparation of Methyl Ester−Trimethylsilyl (Me−TMS) Ethers. The methyl ester derivative was prepared (Blau & King, 1978) by dissolving the sample in methanol (0.3 mL) and reacting with freshly distilled ethereal diazomethane (2.7 mL). After evaporation of the reagents, the methyl esters were converted to trimethylsilyl ethers by addition of 50 μL of Tri-Sil reagent (Pierce Chemicals, Rockford, IL). The derivatizing reagents were removed, and the sample was purified by passage through a small column of Lipidex 5000 (Packard Instrument Co.) in hexane (Axelson & Sjövall, 1974).

Separation of Bile Acids According to Number of Hydroxyl Groups. The unconjugated bile acid fraction was first methylated, and the methyl esters were separated by straight-phase chromatography on Lipidex 5000 into two groups. A combined monohydroxy- and dihydroxy-bile acid fraction was collected separately from a trihydroxy-bile acid fraction, exactly as described by Bremmelgaard and Sjövall (1980). The fractions were evaporated to dryness, and the TMS ether was prepared and analyzed by GC−MS.

Gas Chromatography−Mass Spectrometry (GC−MS) Analysis. The Me−TMS ether derivatives were separated by 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 to 295 °C at a rate of 2 °C/min, with initial and final isothermal periods of 2 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 either a Finnigan 4635 quadrupole instrument or a VG Autospec Q magnetic sector instrument housing identical columns and using the same chromatographic conditions. Electron ionization (70 eV) mass spectra were recorded over the mass range 50−800 Da by repetitive scanning of the eluting components.

Quantification and Identification of Bile Acids. Quantification of bile acids was achieved using GC, by comparing the peak height response of the individual bile acids with the peak height response obtained from the internal standard. Identification of a bile acid was made on the basis of 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 (Lawson & Setchell, 1988).

Liquid Secondary Ionization Mass Spectrometry (LSIMS) of Bile Acid Conjugates in Bile. Negative ion LSIMS spectra of bile samples were obtained after placing the equivalent of approximately 1 μL of the original bile extract onto a small drop of a glycerol matrix spotted on a probe. This probe was introduced directly into the ion source of a VG Autospec Q mass spectrometer, and bile acids were ionized in a beam of cesium ions (35 keV). Negative ion mass spectra were recorded over the mass range 50−800 Da.

RESULTS

Identification and Quantification of 3α,7β-Dihydroxy-5β-chol-22-en-24-oic Acid (Δ^{22}-UDCA) and Other Side-Chain Unsaturated Bile Acids

(i) LSIMS Analysis of Bile Following UDCA and [$^2H_4$]-UDCA Infusion. Biliary bile acid analysis by LSIMS prior to and following intravenous infusion of UDCA demonstrated that exogenous UDCA is taken up by the liver and extensively biotransformed. In bile from normal rats, the negative ion mass spectrum (Figure 2) is typically characterized by a prominent pseudomolecular ion corresponding to taurine-conjugated trihydroxycholanoic acids (*m/z* 514). This ion is accompanied by ions characteristic of glycotrihydroxy- and taurodihydroxycholanoic acids (*m/z* 464 and 498, respectively) and an ion, consistent with taurine-conjugated unsaturated trihydroxy-bile acids (*m/z* 512), shown by GC−MS to be Δ^{22}-muricholate isomers.

During UDCA infusion, the mass spectrum (Figure 2) revealed prominent ions at *m/z* 498 and 448 which correspond to the pseudomolecular ions $[M - H]^-$ for dihydroxycholanoates conjugated with taurine and glycine, respectively. Ions at *m/z* 391 and 407 indicate unconjugated dihydroxy- and trihydroxycholanoates, respectively, and reflect the presence of the unchanged bile acid and hydroxylated metabolites, while the ion at *m/z* 567 (paired with *m/z* 589, the sodium adduct) represents glucuronide conjugates of UDCA. A relatively intense ion at *m/z* 496 is present,

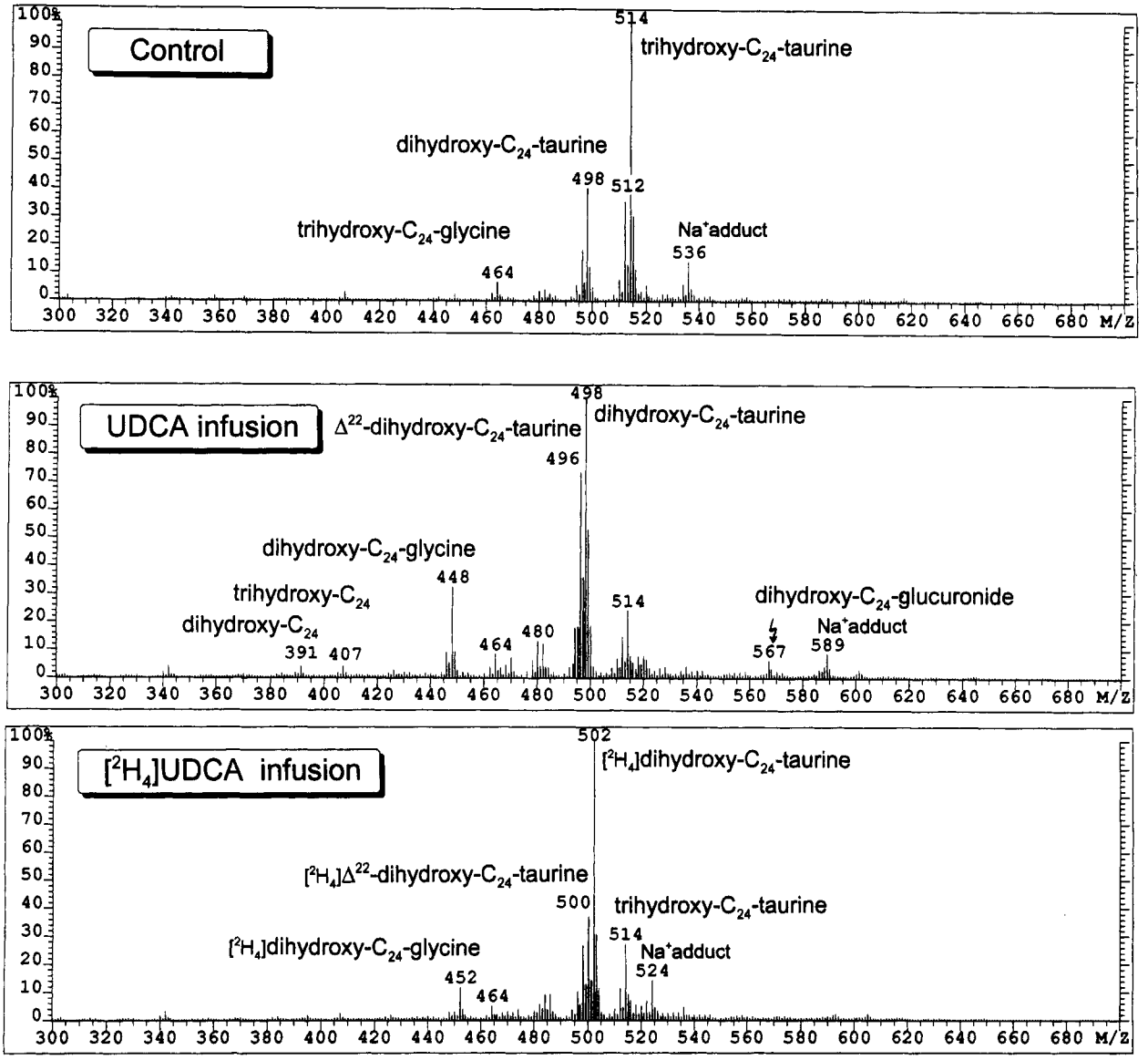

FIGURE 2: Negative ion LSIMS spectra of bile from a Sprague-Dawley rat (top panel) in the control period, (middle panel) during intravenous infusion of UDCA, and (bottom panel) during intravenous infusion of [2,2,4,4-$^2H_4$]UDCA. The major bile acids are abbreviated by trivial names, where C_{24} refers to a cholanoic acid nucleus.

which is consistent with the molecular weight of a taurine-conjugated dihydroxycholenoate (*unsaturated*) structure, subsequently confirmed to be Δ^{22}-UDCA.

When [$^2H_4$]UDCA was infused, the negative ion LSIMS spectrum of the bile (Figure 2) was essentially identical, except that many of the pseudomolecular ions were shifted by 4 atomic mass units (amu), confirming that they were derived from the infused [$^2H_4$]UDCA. This was the case for the ion at *m/z* 496 in the bile from the animals infused with UDCA, which subsequently appeared at *m/z* 500 when [$^2H_4$]UDCA was infused.

(ii) GC−MS Analysis of Bile Following UDCA and [$^2H_4$]-UDCA Infusion. GC−MS profiles of the Me−TMS ether derivatives of the bile acids isolated from the bile before and during infusion of UDCA are shown in Figure 3. Prior to UDCA infusion, the biliary bile acid composition revealed the major bile acids to be cholic acid, deoxycholic acid, and a series of muricholic acids, including α-, β-, and ω-isomers. However, β-muricholic acid was not the principal muricholate isomer. Rather, intense peaks with retention indices of 33.52 and 34.62 MU were found to have electron ionization spectra with fragmentation patterns similar to those of muricholic acids, but differing by 2 amu in molecular weight and indicating an unsaturated structure. The presence of an ion at *m/z* 253 confirmed that the ABCD-ring nucleus contained three derivatized hydroxyl groups that were lost on fragmentation, indicating that the unsaturation must reside in the side chain. The difference between the retention indices of these two compounds and the corresponding saturated analogues was consistent with published data for retention shifts between other Δ^{22}-bile acids and their corresponding saturated analogues (Kihira & Hoshita, 1985). Despite the lack of available authentic standards, it was concluded that these metabolites were Δ^{22}-β-muricholic and Δ^{22}-ω-muricholic acids and they were the major muricholic isomers of normal rat bile.

Following infusion with UDCA there was a significant decrease in the proportion of cholic and muricholic acid isomers, and these bile acids were displaced by UDCA and a previously unidentified metabolite with a retention index of 32.95 MU. This metabolite comprised between 10% and 30% of the total biliary bile acids.

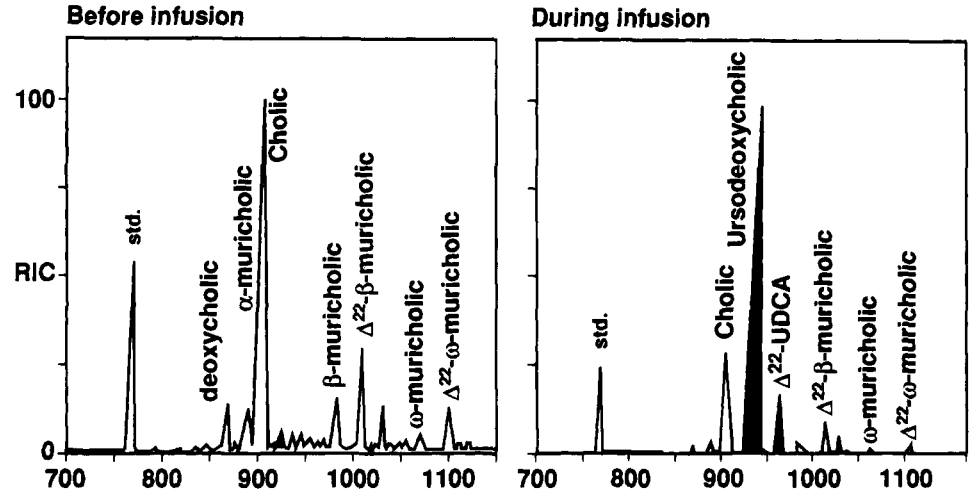

FIGURE 3: Typical GC–MS analyses showing the total ion current chromatograms of the methyl ester–trimethylsilyl ethers of the biliary bile acids of the Sprague-Dawley rat in the control period and during intravenous infusion of UDCA.

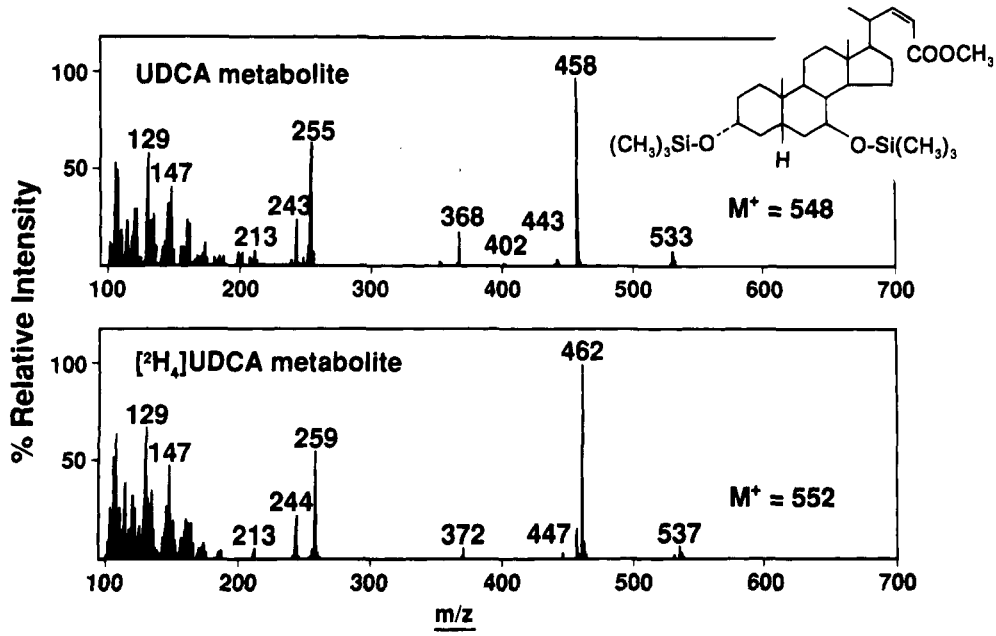

FIGURE 4: Electron ionization (70 eV) mass spectra of the methyl ester–trimethylsilyl ether of the metabolite of UDCA found in the bile of Sprague-Dawley rats having a retention time 32.95 MU and the corresponding metabolite formed during intravenous infusion of [2,2,4,4-$^2H_4$]UDCA.

The electron ionization mass spectrum of the Me–TMS ether is shown in Figure 4. The base peak in the spectrum, *m/z* 458, is derived from the ion of *m/z* 548 by loss of one trimethylsilanol group. The ion at *m/z* 368 reflects a further loss of trimethylsilanol (*m/z* 458 − 90) due to cleavage of a derivatized hydroxyl group. The fragmentation pattern thus confirms at least two hydroxyl groups in the molecule. Due to the facile fragmentation of this derivative, the ion of highest mass appeared at *m/z* 533, which is derived from *m/z* 548 by loss of a methyl group. It was not clear whether the molecular ion $[M]^+$ was at *m/z* 548, which would represent an unsaturated dihydroxy-C_{24}-bile acid, or at higher mass. The ion at *m/z* 255 is an ABCD-ring fragment, formed by the loss of the side chain and two nuclear hydroxyl groups, while *m/z* 243 is accounted for by an AB-ring fragment containing a C-3 and C-7 hydroxyl. On the basis of this spectrum, it was concluded that this metabolite was either a trihydroxycholanoate with one of the hydroxyl groups in the side chain, or a dihydroxycholenoate with a double bond in the side chain. The latter seemed more probable since there was a side-chain loss of 113 amu (*m/z* 368 − 255) in the mass spectrum, whereas the normal bile acid C-5 side-chain loss would be 115 amu.

Our failure to find this bile acid in the bile collected before infusion of UDCA indicated it to be a metabolite of UDCA. This was confirmed following administration of [$^2H_4$]UDCA. The electron ionization spectrum of the Me–TMS ether of the deuterium-labeled metabolite (Figure 4) revealed an identical fragmentation pattern; however, the ions were shifted by 4 amu, corresponding to the incorporation of four deuterium atoms in the infused [$^2H_4$]UDCA.

A dihydroxy-C_{24} structure was suggested following chromatographic separation on Lipidex 5000 of the unconjugated bile acid fraction that had been methylated (Figure 1). This straight-phase solvent system affords a separation of C_{24}-bile acid methyl esters into two distinct fractions: a combined mono- and dihydroxy fraction and a separate trihydroxy fraction. After the TMS ether derivative was prepared, GC and GC–MS analyses showed that the UDCA metabolite, together with UDCA, migrated to the combined mono- and

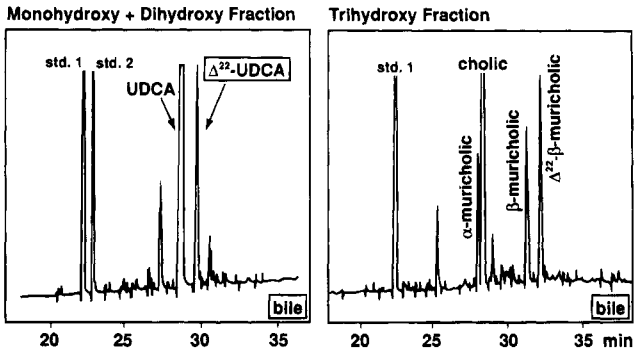

FIGURE 5: Typical GC profiles of the methyl ester–trimethylsilyl ether derivatives of biliary bile acids from Sprague-Dawley rats separated by lipophilic gel chromatography into distinct fractions containing (left panel) combined monohydroxy- and dihydroxy-bile acids and (right panel) trihydroxy-bile acids.

dihydroxy fraction (Figure 5). The trihydroxy fraction contained cholic and muricholic acid isomers, where, interestingly, Δ^{22}-β-muricholic acid was found to be present in larger proportions than β-muricholic acid. These chromatographic migration studies conclusively establish that this metabolite was a dihydroxycholenoate (unsaturated bile acid). The mass spectrum confirmed the unsaturation to be in the side chain, probably at the C-22 position. It was therefore concluded that the structure of this UDCA metabolite was 3α,7β-dihydroxy-5β-chol-22-en-24-oic acid (Δ^{22}-UDCA). Definitive confirmation was established by comparison of the GC retention index and electron ionization mass spectrum with an authentic standard of Δ^{22}-UDCA (kindly provided by Dr. K. Kihira, Hiroshima, Japan), which were identical to the biological metabolite. Figure 6 shows the electron ionization mass spectrum of the Me–TMS ether of the pure sample of Δ^{22}-UDCA. The chemical synthesis and characteristics, including the mass spectra and retention indices of Δ^{22}-UDCA and a number of other Δ^{22}-bile acids, are described in detail elsewhere (Kihira & Hoshita, 1985).

Distribution of Δ^{22}-Bile Acids in Normal and UDCA-Fed Animals

(i) Bile Acid Composition of Intestinal Contents during Oral Administration of UDCA. Bile acid profiles were obtained for the entire intestinal contents of the jejunum, ileum, cecum, and colon of male Sprague-Dawley rats after being fed a diet containing either 0%, 0.4%, or 1.0% UDCA. Figure 7 shows representative profiles of jejunum and colon for the animals fed 1.0% UDCA. UDCA was the predominant bile acid found in all segments of the intestinal tract of animals fed a diet containing UDCA, while in the control animals it was a relatively minor constituent. In the jejunum, Δ^{22}-UDCA was found in high proportions and concentrations. Levels of Δ^{22}-β-muricholic and Δ^{22}-ω-muricholic acids were consistently greater than β-muricholic and ω-muricholic acids, respectively, in accord with the biliary bile acid composition. In the control animals, Δ^{22}-UDCA was not found in the jejunum or in other segments of the intestine, yet Δ^{22}-β-muricholic and Δ^{22}-ω-muricholic acids were found

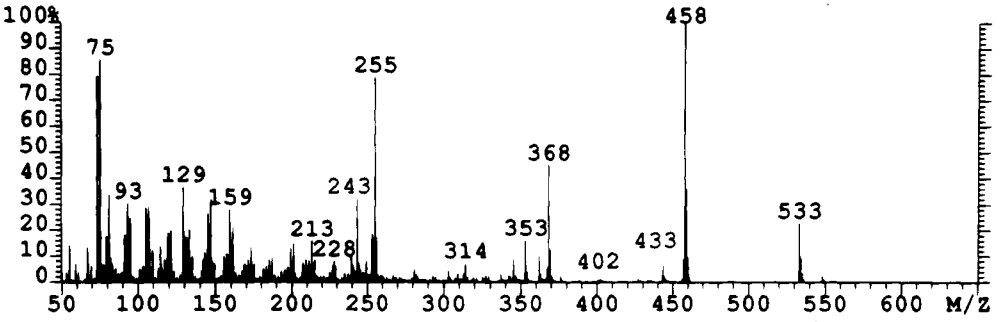

FIGURE 6: Electron ionization (70 eV) mass spectrum of the methyl ester–trimethylsilyl ether of an authentic sample of 3α,7β-dihydroxy-5β-chol-22-en-24-oic acid (Δ^{22}-UDCA).

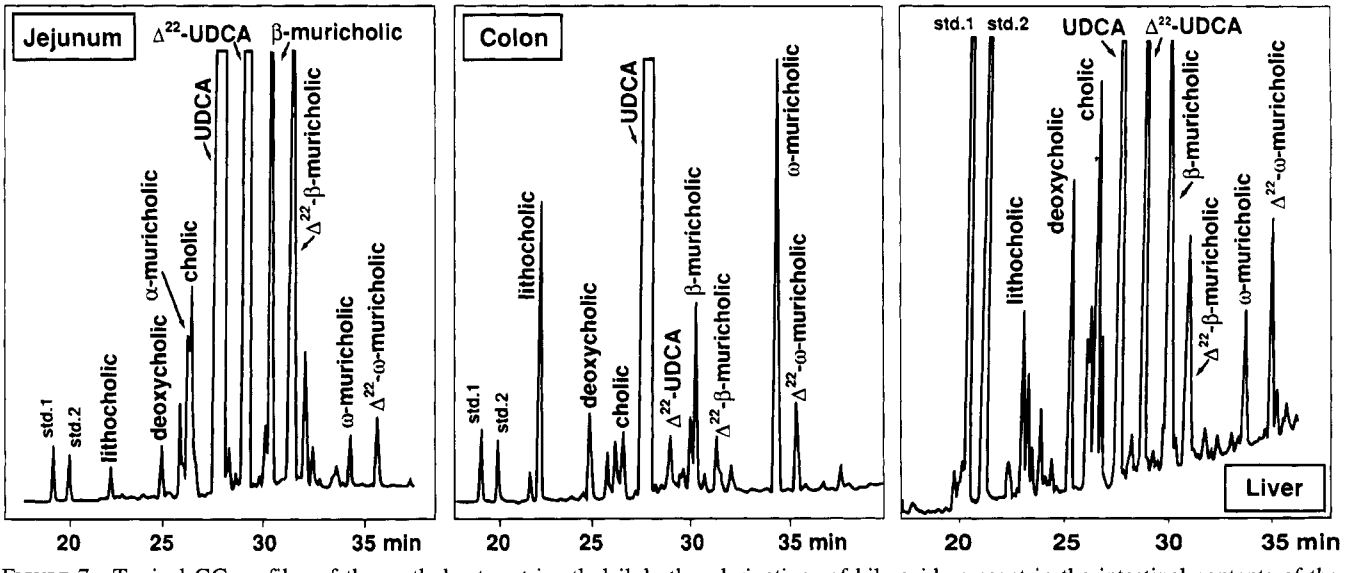

FIGURE 7: Typical GC profiles of the methyl ester–trimethylsilyl ether derivatives of bile acids present in the intestinal contents of the jejunum and colon and in the liver tissue of Sprague-Dawley rats fed a diet containing 1.0% UDCA.

Table 1: Mean (±SD) Mass of UDCA and Δ^{22}-UDCA along the Length of the Intestinal Tract[a] of Sprague-Dawley Rats Fed Diets Containing Different Amounts of UDCA

	intestinal UDCA (mg)			intestinal Δ^{22}-UDCA (mg)		
	control	0.4% UDCA diet	1.0% UDCA diet	control	0.4% UDCA diet	1.0% UDCA diet
jejunum	0.03 ± 0.02	13.43 ± 0.98	12.00 ± 1.71	nd[b]	7.50 ± 0.88	6.61 ± 0.55
ileum	0.01 ± 0.01	0.99 ± 0.29	1.64 ± 0.27	nd	0.41 ± 0.14	0.48 ± 0.08
cecum	0.02 ± 0.01	6.62 ± 1.68	14.23 ± 0.87	nd	0.32 ± 0.25	0.43 ± 0.08
colon	0.02 ± 0.01	5.27 ± 0.91	14.47 ± 2.11	nd	0.14 ± 0.07	0.36 ± 0.07

[a] Total weights (mean ± SD) of intestine and intestinal contents for the control animals were (i) jejunum 6.6 ± 1.2 mg, (ii) ileum 0.7 ± 0.2 mg, (iii) cecum 5.4 ± 1.2 mg, and (iv) colon 5.6 ± 0.4 mg, respectively, and these were similar in the animals fed 0.4% and 1.0% UDCA. [b] nd = not detectable.

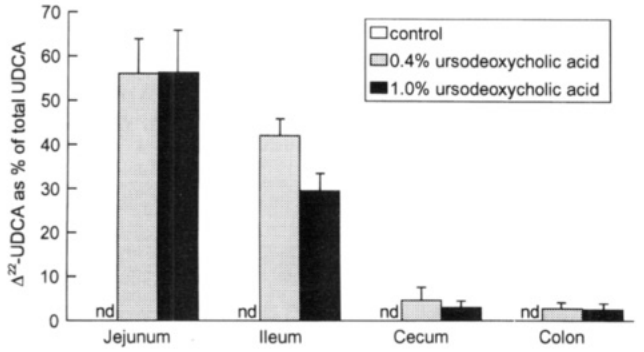

FIGURE 8: Distribution of Δ^{22}-UDCA along the length of the intestine of Sprague-Dawley rats, expressed relative to total UDCA; nd = not detectable.

in concentrations higher than the corresponding saturated muricholates, indicating that these bile acids are formed endogenously. By contrast, Δ^{22}-UDCA is a metabolite of exogenous UDCA administration, as demonstrated from stable-isotope studies.

Table 1 summarizes the masses of UDCA and Δ^{22}-UDCA in different segments of rat intestine from control animals and those fed a diet containing 0.4% and 1.0% UDCA. While a large mass of UDCA was consistently found along the entire length of the intestine (except for the ileum, because of its small size), the total mass of Δ^{22}-UDCA diminished markedly between jejunum and colon. In the jejunum, the mass of UDCA and Δ^{22}-UDCA was similar at both doses; however, the mass of these bile acids was in general 2.5-fold higher in the cecum and colon of the rats fed 1.0% UDCA compared with animals fed 0.4% UDCA. In the upper small intestine, Δ^{22}-UDCA was present at approximately 50−60% of the level of UDCA, while in the cecum and colon it represented <5%, indicating significant depletion of this metabolite along the length of the intestine, presumably due to either reabsorption or bacterial metabolism (Figure 8).

The distribution of Δ^{22}-UDCA and UDCA conjugates along the length of the intestine is shown in Table 2. In control animals, Δ^{22}-UDCA was not present. When 0.4% UDCA was added to the diet, all segments of small intestine contained predominantly taurine-conjugated Δ^{22}-UDCA, although significant quantities of the unconjugated species were present. With a 1.0% UDCA diet there was a proportional increase in the amount of unconjugated Δ^{22}-UDCA and a shift toward glycine conjugation.

(ii) Bile Acid Composition of Plasma during UDCA Administration. Δ^{22}-UDCA was not detected in the plasma of normal rats; however, Δ^{22}-β-muricholic and Δ^{22}-ω-muricholic acids were both present, indicating endogenous synthesis. Following UDCA administration, Δ^{22}-UDCA appeared in the plasma. The concentration of this metabolite was 1.4 ± 0.4 μmol/L (mean ± SEM) in animals fed 0.4% UDCA and 40.3 ± 11.8 μmol/L in animals fed 1.0% UDCA. In both instances, the values are lower than the corresponding plasma UDCA concentrations. In contrast to bile, the plasma concentration of Δ^{22}-β-muricholic and Δ^{22}-ω-muricholic acids after UDCA administration was lower than that of the corresponding saturated muricholates (Figure 9).

(iii) Bile Acid Composition of Liver Tissue during UDCA Administration. Δ^{22}-UDCA was not found in the liver tissue of the animals from the control group, while UDCA was detected at a concentration of 12.0 ± 2.5 nmol/g of tissue (4.8 ± 1.0 μg/g). A typical GC profile of the bile acids identified in the liver tissue from rats fed 1.0% UDCA is compared with profiles for jejunum and colon (Figure 7). Following UDCA feeding, there was a dose-dependent increase in the liver tissue concentration of UDCA, Δ^{22}-UDCA, and Δ^{22}-β-muricholic acid and a concomitant decrease in the concentration of cholic acid (Figure 9).

DISCUSSION

While oral UDCA therapy has been shown to have significant effects in lowering serum liver enzymes and improving clinical and histological markers of liver disease in a variety of clinical scenarios, it is apparent that the exact mechanism(s) by which these improvements occur is (are) still unclear. Analyses of biological fluids from patients undergoing UDCA therapy show that unconjugated UDCA is absorbed, transported to the liver, and efficiently biotransformed, predominantly by conjugation with glycine. In man, other conjugates are formed, and these include the C-3 sulfate (Setchell et al., 1991) and the 7β-glycosylated (Marschall et al., 1992) metabolites of UDCA, and our previous studies have indicated that little biotransformation of the steroid nucleus occurs (Nakagawa et al., 1990; Crosignani et al., 1991).

Bile acid metabolism is highly species-specific (Haslewood, 1978), and this should be considered when interpreting biological and physiological effects of exogenously administered compounds. Compared with man, bile acid synthesis and metabolism are significantly different in the rat. Amidation with taurine is the major metabolic pathway for conjugation (Danielsson & Rutter, 1968), cholic acid and 6β-hydroxylated metabolites (muricholic acids) are the major bile acids of bile (Bergström & Sjövall, 1954), chenodeoxycholic acid is of negligible quantitative importance, and the rat is one of only a few species that can 7α-hydroxylate the secondary bile acid, deoxycholic acid (Bergström et al., 1953; Mahowald et al., 1957). Nevertheless, the Sprague-Dawley rat is a useful animal model for bile acid studies.

Table 2: Distribution of UDCA and Δ^{22}-UDCA Conjugates in Pooled Sections of the Intestinal Tract of Sprague-Dawley Rats Fed Diets Containing UDCA

		UDCA (mg)				Δ^{22}-UDCA (mg)			
		unconj	glycine	taurine	sulfate	unconj	glycine	taurine	sulfate
control	jejunum	0.01	0.03	0.05	nd[a]	nd	nd	nd	nd
	ileum	0.01	nd	nd	nd	nd	nd	nd	nd
	cecum	0.03	nd	0.01	nd	nd	nd	nd	nd
	colon	0.02	nd	nd	nd	nd	nd	nd	nd
0.4% UDCA	jejunum	3.70	13.49	4.75	0.03	0.57	2.92	4.30	0.01
	ileum	0.47	0.69	0.28	0.01	0.04	0.19	0.25	nd
	cecum	6.97	0.33	0.26	0.03	0.25	0.08	0.14	nd
	colon	4.60	0.14	0.09	0.02	0.10	0.03	0.04	nd
1.0% UDCA	jejunum	8.23	3.56	1.68	0.10	2.64	2.62	1.59	0.01
	ileum	1.31	0.30	0.23	0.02	0.43	0.13	0.08	nd
	cecum	16.11	0.58	0.15	0.06	0.25	0.20	0.06	nd
	colon	15.41	0.77	0.20	0.02	0.24	0.28	0.07	nd

[a] nd = not detectable.

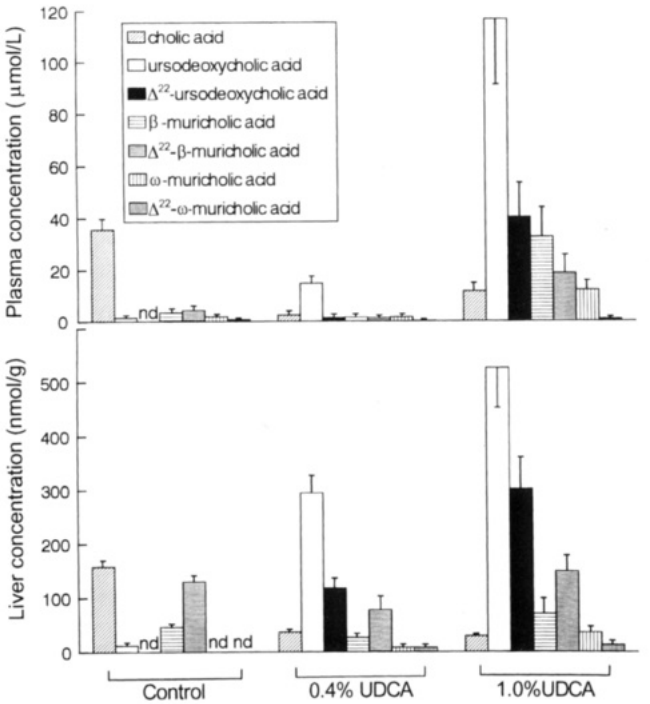

FIGURE 9: Plasma and liver tissue concentrations of cholic acid, Δ^{22}-bile acids, and the corresponding saturated analogues identified in Sprague-Dawley rats fed a diet containing 0%, 0.4%, or 1.0% UDCA; nd = not detectable.

UDCA has a potent hypercholeretic effect (Lake et al., 1987; Gurantz et al., 1991) in the rat, and during its enterohepatic recycling it is efficiently biotransformed, most notably by conjugation with taurine. In studies of the metabolism and physiological effects of a number of analogues of UDCA, we consistently observed a quantitatively important metabolite of UDCA, which we have now established to be Δ^{22}-UDCA (Setchell et al., 1993). This metabolite is not found in the plasma, bile, liver tissue, and intestinal contents of normal animals. Δ^{22}-UDCA accounted for between 10% and 30% of the total biliary bile acids following intravenous infusion of UDCA, and definitive confirmation that it was derived from UDCA was established following infusion of [$^2H_4$]UDCA and identification of [$^2H_4$]-Δ^{22}-UDCA as a specific metabolite. Differentiating dihydroxycholenoates (unsaturated) from trihydroxycholanoates (saturated) by mass spectrometry is often difficult because of the facile loss of the derivatized groups on electron ionization. In many instances, a molecular ion is not observed in the mass spectrum, and fragmentation patterns are consequently similar (Lawson & Setchell, 1988). Following methylation of a hydrolyzed bile sample and straight-phase chromatography on Lipidex 5000, the biliary metabolite was found to migrate to the dihydroxy fraction (Figure 5), and the side-chain loss of 113 amu in the mass spectrum provided evidence for the presence of an unsaturated side chain (Figure 4). The chemical synthesis of Δ^{22}-UDCA and several other Δ^{22}-bile acids has been described previously, and the structure of the UDCA metabolite was definitively established to be Δ^{22}-UDCA by comparison of the GC retention index and mass spectrum of the Me−TMS ether derivative with the authentic compound.

Other saturated bile acids were present in all of the biological samples examined, and these were shown to be Δ^{22}-β-muricholic and Δ^{22}-ω-muricholic acids on the basis of mass spectral fragmentation patterns and shifts in GC retention indices. However, in contrast to Δ^{22}-UDCA, the side-chain unsaturated muricholates are normal constituents of the bile, liver tissue, plasma, and intestinal contents of Sprague-Dawley rats, and concentrations of Δ^{22}-β-muricholic acid in the liver tissue and plasma of normal animals exceeds β-muricholic acid.

Despite extensive studies of bile acid synthesis and metabolism in the Sprague-Dawley rat, it is surprising that little attention has been given to Δ^{22}-bile acids. It is now evident that their formation represents a quantitatively significant pathway of bile acid synthesis in this species. Early studies alluded to the presence of unsaturated bile acids in the bile of rats. More than 20 years ago, Danielsson (1973) described the mass spectrum of the Me−TMS ether derivative of an unknown bile acid, suggested to be side-chain unsaturated, that accounted for 13−50% of the muricholates in rat bile. Later, in a more detailed study, Kern et al. (1977) reported the presence of several unsaturated muricholates in bile-fistula bile, accounting for 10−50% of the total muricholates. These metabolites were later found by several other groups (Kuriyama et al., 1979; Eyssen et al., 1983, 1985; Robben et al., 1986), and during the course of our studies, the definitive identification of tauro-Δ^{22}-β-muricholate was described and shown to be a major bile acid of the serum of rats treated with α-naphthylisothiocyanate (Thompson et al., 1993; Davis & Thompson, 1993). Our studies corroborate this latter finding and, furthermore,

demonstrate that the formation of Δ^{22}-bile acids is a quantitatively major pathway for normal bile acid synthesis by the rat. The concentration of Δ^{22}-β-muricholic acid exceeds β-muricholic acid in the plasma, liver, and bile of normal rats, and the higher proportions that we find, compared with earlier reports, can best be explained by improvements in methodology.

To our knowledge, we describe for the first time the identification of Δ^{22}-UDCA as a quantitatively important metabolite of exogenously administered UDCA in the rat. The distribution and site of formation of this specific metabolite were established by incorporating UDCA into the diet. Δ^{22}-UDCA formation was found to be dose-dependent, with the liver tissue concentration of Δ^{22}-UDCA increasing proportionally with the dose of UDCA administered (117.2 $\pm$ 12.2 nmol/g for 0.4% UDCA, 301.0 $\pm$ 64.2 nmol/g for 1.0% UDCA) and accounting for between 35% and 60% of the liver UDCA concentration. The total mass of Δ^{22}-UDCA residing in the upper small bowel was similar irrespective of the concentration of UDCA in the diet, and this metabolite accounted for 50−60% of the UDCA in the jejunum. In contrast to UDCA, a marked decline in the total mass of Δ^{22}-UDCA along the length of the intestine was found, so that, in the colon, Δ^{22}-UDCA accounted for only a small proportion (2−3%) of the total UDCA. This observation can be explained either by efficient reabsorption in the small intestine or by bacterial degradation. An early finding of Δ^{22}-β-muricholic acid in the feces of gnotobiotic rats inoculated with strains of Eubacteria and Clostridia led to the suggestion that this unsaturated bile acid was formed by intestinal metabolism of β-muricholic acid (Eysson et al., 1983, 1985; Robben et al., 1986). If this were the case, concentrations and proportions of Δ^{22}-unsaturated metabolites would far exceed the corresponding saturated metabolites in the cecum and colon where bacterial colonization is greatest; however, our findings do not support this suggestion. Furthermore, in bile-duct cannulated animals where the enterohepatic circulation is interrupted, Δ^{22}-UDCA appeared in bile following intravenous infusion of UDCA. Our findings clearly indicate a hepatic origin for the formation of Δ^{22}-bile acids, and this is confirmed by the observation that Δ^{22}-β-muricholate is synthesized *in vitro* by liver slices (Thompson et al., 1993).

Interestingly, the formation of Δ^{22}-bile acids appears to be substrate-specific. In separate studies, when rats were fed a diet containing deoxycholic acid under identical conditions, we found no evidence for the formation of Δ^{22}-deoxycholic acid (Kren et al., unpublished data). Furthermore, Δ^{22}-UDCA was not formed in rat liver when UDCA 7-sulfate and UDCA 3,7-disulfate were perfused (Setchell & Yamashita, 1994) or added to the diet (unpublished data); however, Δ^{22}-UDCA was formed from UDCA 3-sulfate (unpublished data). This would suggest that only bile acids with a functional (unblocked) 7β-hydroxyl group form unsaturated side-chain metabolites. In support of this contention is our failure to find the Δ^{22}-species of α-muricholic acid (3α,6β,7α-triol) or hyocholic acid (3α,6α,7α-triol) in any of the biological samples from the control animals, whereas Δ^{22}-β-muricholic acid (3α,6β,7β-triol) and Δ^{22}-ω-muricholic acid (3α,6α,7β-triol) are present as normal constituents. Selective metabolism of bile acids having specific functional groups is not unusual. For example, glucuronidation is a specific pathway of metabolism for bile acids with a 6α-hydroxyl group (Marschall et al., 1987; Radominska-Pyrek et al., 1987; Parquet et al., 1988), while the formation of *N*-acetylglucosaminides is specific to 7β-hydroxylated bile acids (Marschall et al., 1992).

The effect of amidation of the side chain was not examined in detail. Previously reported *in vitro* studies using liver slices demonstrated that Δ^{22}-β-muricholic acid could not be formed with incubation of tauro-β-muricholate (Thompson et al., 1993). When we administered tauroursodeoxycholic acid to rats, Δ^{22}-UDCA was found in the liver tissue, intestinal contents, feces, urine, and plasma (Rodrigues et al., 1995), and it is possible that this was formed from UDCA following bacterial deconjugation of tauroursodeoxycholic acid during enterohepatic recycling.

Whether the formation of Δ^{22}-UDCA is species-specific is uncertain. Detailed analyses of the bile, urine, and serum of patients with liver disease undergoing oral UDCA therapy have failed to reveal Δ^{22}-UDCA as a metabolite (Colombo et al., 1990; Crosignani et al., 1991); however, on a body weight basis the therapeutic clinical dose (10−15 mg/kg) is much lower than the dose administered to the animals in this study. Perhaps at a comparable weight-adjusted dosage, Δ^{22}-UDCA may be formed by the human liver, particularly if concentrations of unconjugated UDCA were to exceed the capacity for hepatic conjugation.

The exact mechanism by which formation of Δ^{22}-UDCA occurs remains to be established. This metabolite, and the endogenous Δ^{22}-muricholic acids, could be formed following side-chain hydroxylation at C-22 and dehydration with release of water; however, we have failed to find C-22 hydroxylated metabolites in any of the biological samples examined. Alternatively, it is possible that desaturation may take place in the endoplasmic reticulum by the action of a mixed function oxidase in a similar fashion to the formation of monoenoic fatty acids. However, the desaturase enzyme for this reaction requires that the substrate contain more than six carbon atoms. In the case of bile acids, the side chain is only five carbon atoms in length. Consequently, we presume that Δ^{22}-bile acids are formed in the peroxisome, in common with the normal pathway for bile acid synthesis, which involves the β-oxidation of cholestanoic acid intermediates (Russell & Setchell, 1992). We speculate that, because of the hydrophilic nature of 7β-hydroxylated bile acids, preferential uptake by the peroxisome occurs followed by partial β-oxidation. The first step in this reaction would involve formation of α,β unsaturation (Russell & Setchell, 1992). Since UDCA has only a five carbon atom side chain, release of propionic or acetic acid is not possible, oxidation proceeds no further, and Δ^{22}-UDCA is consequently formed. This bile acid is then conjugated and secreted in bile.

The physiologic significance of a pathway involving partial β-oxidation of 7β-hydroxylated bile acids is unclear. Incorporation of a double bond in the side chain imparts a small increase in hydrophobicity of the molecule which may facilitate canalicular secretion. Irrespective of the function of this metabolic pathway, our data clearly indicate that partial β-oxidation is a quantitatively major and normal pathway for bile acid metabolism in the rat that is relatively specific for 7β-hydroxylated bile acids of both endogenous and exogenous origin.

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