Hepatic biotransformation in rodents and physicochemical properties of 23(*R*)-hydroxychenodeoxycholic acid, a natural α-hydroxy bile acid[1]

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Abstract The hepatic biotransformation in the rat and hamster of 23(*R*)-hydroxychenodeoxycholic acid (23(*R*)OH-CDCA), the α-hydroxy derivative of CDCA, was defined; some physiological and physicochemical properties were also assessed. 23(*R*)OH-CDCA was isolated from duck bile; [24-^{14}C]23(*R*)OH-CDCA was synthesized. The compound was administered intravenously to anesthetized biliary fistula rats at doses of 1, 3, or 5 μmol/kg-min and to hamsters at 3 μmol/min-kg. Biliary bile acids and radioactivity were analyzed by thin-layer chromatography (TLC), high pressure liquid chromatography (HPLC), and gas chromatography–mass spectrometry (GC-MS). Recovery of radioactivity in bile was incomplete (50–70% of infused dose); some was also recovered as breath $^{14}CO_2$. Radioactivity in bile was present as unchanged compound (25–50%, dose-dependent) and its conjugates (with taurine, with glycine, or with glucuronate). Nor-CDCA (C_{23}) was present in bile (in both unconjugated and conjugated form), indicating that 23(*R*)OH-CDCA had undergone oxidative decarboxylation (α-oxidation) with loss of the C-24 carboxyl group. The α-oxidation was 20 ± 5% (mean ± SD) of administered dose in the rat and was not dose-dependent; in hamsters, α-oxidation was 35 ± 8%. In rats, the *S* isomer of 23OH-CDCA also underwent α-oxidation (10 ± 2%). Nor-CDCA also underwent 6β-hydroxylation to form nor-α-muricholic acid, as well as reduction of its C-23 carboxyl group to form the C_{23} alcohol. The taurine conjugate of 23(*R*)OH-CDCA [23(*R*)OH-CDC-tau] was prepared synthetically and characterized by ^{1}H-NMR. By surface tension measurements, it had a critical micellization concentration (CMC) of 3.5 mM (in 0.15 M Na^+), as compared to 1.8 mM for CDC-taurine. Aqueous solubility of 23(*R*)OH-CDCA increased markedly above pH 5, compared to pH 7 for CDCA. When incubated with cholylglycine hydrolase, 23(*R*)OH-CDC-tau was deconjugated at a rate one-fourth that of CDC-tau. It is concluded that the presence of a 23(*R*)-hydroxyl group in a 3α,7α-dihydroxy bile acid alters its metabolism in the rodent hepatocyte, as evidenced by inefficient conjugation with taurine or glycine, α-oxidation to nor (C_{23}) bile acid, and reduction of the nor bile acid to the primary alcohol. The taurine conjugate of 23(*R*)OH-CDCA, a major biliary bile acid of marine mammals and wading birds, is a biological detergent with properties superior to those of the taurine conjugate of CDCA. Natural C_{23} nor-bile acids may be formed by α-oxidation of α-hydroxy C_{24} bile acids.—**Merrill, J. R., C. D. Schteingart, L. R. Hagey, Y. Peng, H-T. Ton-Nu, E. Frick, M. Jirsa, and A. F. Hofmann.** Hepatic transformation in rodents and physicochemical properties of 23(*R*)-hydroxychenodeoxycholic acid, a natural α-hydroxy bile acid. *J. Lipid Res.* 1995. **36:** 98–112.

Supplementary key words α-oxidation • bile acid deconjugation • bile acid metabolism • critical micellization concentration • critical micellization pH • bile alcohols

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Abbreviations: 23(*R*)OH-CDCA, (23*R*)-3α,7α,23-trihydroxy-5β-cholan-24-oic acid; 23(*R*)OH-CDC-tau, its taurine conjugate (aminoacyl amidate); 23(*R*)OH-CDC-gly, its glycine conjugate; CA, cholic acid; DCA, deoxycholic acid; nor-CDCA, 3α,7α-dihydroxy-24-nor-5β-cholan-23-oic acid; α-MCA, α-muricholic acid, 3α,6β,7α-trihydroxy-5β-cholan-24-oic acid; β-MC-tau, its taurine conjugate; norα-MCA, 3α,6β,7α-trihydroxy-24-nor-5β-cholan-23-oic acid; β-MCA (β-muricholic acid, 3β,6β,7β-trihydroxy-5β-cholan-24-oic acid; MDCA, murideoxycholic acid (3α,6β-dihydroxy-5β-cholan-24-oic acid); β-HCA (β-hyocholic acid), 3α,6α,7β-trihydroxy-5β-cholan-24-oic acid; ODS, octadecylsilane; HPLC, high-pressure liquid chromatography; GC–MS, gas chromatography–mass spectrometry; NBS, N-bromosuccinimide; TFA, trifluoroacetic acid; ^{1}H-NMR, proton magnetic resonance; TMS, trimethylsilyl; TLC, thin-layer chromatography; RT, retention time; CMC, critical micellization concentration; and CMpH, critical micellization pH.

Dedicated to Professor Wolfgang Gerok, former Chairman of the Department of Medicine, Albert-Ludwigs University, Freiburg, Germany, on the occasion of his 70th birthday.

[1]Parts of this work were submitted for presentation at the annual meeting of the American Gastroenterological Association in 1991, and the American Association for the Study of Liver Disease in 1995, and published in abstract form (1, 2).

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The natural bile acid that has the structure of an α-hydroxy derivative of chenodeoxycholic acid (CDCA), has been known for several decades to be a major biliary bile acid in most marine mammals and in some snakes. The compound was originally isolated from the bile of a walrus by Hammarsten (3), who showed that it differed from cholic acid (CA) and that it was conjugated with taurine; he proposed the name phocaecholic acid (Gr. phocidae = seals). The basic chemical structure of phocaecholic acid was established as (23ξ)-3α,7α,23-trihydroxy-5β-cholan-24-oic acid by Windaus and Van Schoor in 1928 (4) and some years later was confirmed by Bergström, Krabish, and Lindeberg (5) (**Fig. 1, 4**). In 1982, Kutner and Jaworska (6) showed that the 23(*R*)-isomer of phocaecholic acid was present in marine mammal bile, and reported the chemical synthesis of both the 23(*R*)-isomer and the 23(*S*) isomer. Recently, Jirsa et al. (7) and Hagey et al. (8) found that phocaecholic acid is a major biliary bile acid in ducks and flamingos. As in marine mammals, the compound is present in the bile of these birds as the aminoacyl (N-acyl) amidate of taurine. [In the present paper, the trivial name phocaecholic acid has been replaced by the semi-systematic name 23(*R*)-hydroxychenodeoxycholic acid (abbreviated 23(*R*)OH-CDCA), in order to follow a recent recommendation regarding bile acid nomenclature (9).]

Using the individual 23(*R*)- and 23(*S*)-isomers prepared by Pellicciari et al. (10), Roda et al. (11) defined the physicochemical properties of these molecules. They found the aqueous solubility of the protonated form of 23(*R*)OH-CDCA to be 250 μM, a value nine times greater than that of CDCA (27 μM). As anticipated, 23(*R*)OH-CDCA was a stronger acid (pK_a = 3.8) than other C_{24} bile acids with an unsubstituted side chain (pK_a = 5.1) (11, 12). The CMC of 23(*R*)OH-CDCA was slightly higher than that of CDCA (11). Roda and his colleagues (11) also conducted hepatic biotransformation studies on 23(*R*)OH-CDCA in the rat. After intravenous infusion (2 μmol/min-kg), they reported that the bile acid was rapidly and efficiently secreted in bile, mostly in the form of the unchanged compound (11).

While this work was in progress, studies in our laboratory were also being conducted on the physicochemical and biological properties of 23(*R*)OH-CDCA. These studies were aimed at testing the hypothesis that 23(*R*) side chain hydroxylation improves the physiological properties of the corresponding bile acid with an unsubstituted side chain. The stimulus for this work was our observation (8) that the proportion of 23(*R*)OH-CDCA in biliary bile acids of ducks increased in parallel with the evolution of morphological features; this finding suggested that the presence of 23(*R*)OH-CDCA in bile conferred a survival advantage to the host, that is, that α-hydroxylation of CDCA improved the properties of this biological detergent.

In the present paper, we report a detailed study of the hepatic biotransformation of 23(*R*)OH-CDCA in the rat and hamster. Our results extend the data of Roda et al. (11) in showing that 23(*R*)OH-CDCA is not only incompletely amidated during hepatocyte transport, but in addition undergoes α-oxidation to form nor (C_{23}) bile acids. In addition, the CMpH of 23(*R*)OH-CDCA was determined as well as the CMC of its taurine conjugate which was synthesized as part of this study. To test the effect of an α-hydroxy group on bacterial deconjugation, the rate of hydrolysis by cholylglycine hydrolase of the taurine conjugate of 23(*R*)OH-CDCA was compared with that of the taurine conjugate of CDCA.

METHODS

Chemicals and radiochemicals

Bile acids. 23(*R*)OH-CDCA was isolated from 1 L of pooled duck bile (*Anas platyrhynchos v. domestica*) in Prague, Czechoslovakia, by one of us (MJ) using column adsorption chromatography as reported (13, 14); the compound was purified by repeated crystallization from ethyl acetate and sent to San Diego. As judged by GC–MS, it was about 98% pure, containing 2% CDCA. [24-^{14}C]CDCA was prepared by the method of Tserng and Klein (15). CDCA, pharmaceutical grade, was a gift from Diamalt, Pharmazell, Raubling, Germany (courtesy of Dr. Thilo Messerschmidt). Nor-CDCA was synthesized from CDCA as previously described (16).

Radiopure [24-^{14}C]23(*R*)OH-CDCA was prepared in four steps from [24-^{14}C]CDCA, **1,** following the sequence shown in Fig. 1.

[24-^{14}C]3α,7α-diformyloxy-5β-cholan-24-oic acid, **2.** A solution of [24-^{14}C]CDCA **1,** (sp act. 3.07 μCi/μmol, 20.0 mg, 160 μCi) in 150 μl formic acid containing 1.5 μl 60% perchloric acid was heated at 46–50°C for 1.5 h. Acetic anhydride (120 μl) was added and the solution was stirred at room temperature for 20 min. The reaction mixture was poured into 7 ml water and extracted with 7 ml ethyl acetate. The organic layer was washed with water (5 × 6 ml) and evaporated under nitrogen. Radiochemical yield: 158 μCi (99%), one spot identical to the reference compound (16) by TLC.

[24-^{14}C](23ξ)-23-bromo-3α,7α-diformyloxy-5β-cholan-24-oic acid, **3.** One hundred fifty-eight μCi **2** was treated with 4.5 mg N-bromosuccinimide in a mixture of 136 μl TFA and 181 μl trifluoroacetic anhydride for 20 h at room temperature. The reaction mixture was poured into 6 ml 1 M citrate buffer, pH 4.6, containing 100 mg sodium sulfite and extracted with 6 ml of ethyl acetate. The organic layer was washed with 6 ml citrate buffer, twice

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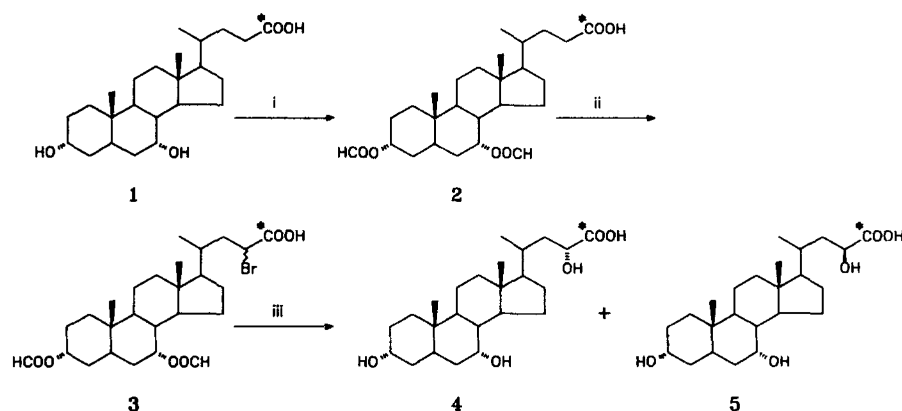

Fig. 1. Synthesis of a mixture of [24-^{14}C]23(*R*)OH-CDCA and [24-^{14}C]23(*S*)OH-CDCA. i: HCOOH, $HClO_4$; ii: NBS, TFA, TFA anhydride; iii: 1% NaOH, 80°C.

with 20% NaCl, evaporated, and dried. Radiochemical yield: 150 μCi (95%). TLC showed two spots (approximately 1:1 ratio) migrating with the same R_f as the standard mixture of (23*R*)- and (23*S*)-(23)-bromo-3α,7α-diformyloxy-5β-cholan-24-oic acids prepared according to (17). ^{1}H-NMR (360 MHz, Cl_3CD): 0.649 and 0.707 (s, 3H, Me-18), 0.94–1.0 (m, 6H, Me-19 and Me-21), 4.3–4.4 (m, 1H, H-23), 4.732 (m, 1H, H-3), 5.039 (bs, 1H, H-7), 8.033 (s, 1H, HCOO-), 8.084 (s, 1H, HCOO-).

[24-^{14}C](23R)-3α,7α,23-trihydroxy-5β-cholan-24-oic acid, **4**, *and [24-^{14}C](23S)-3α,7α,23-trihydroxy-5β-cholan-24-oic acid,* **5.** One hundred fifty μCi **3** was hydrolyzed in 5 ml 1% aqueous NaOH at 80°C for 3 h. The solution was acidified to pH 3 by addition of solid citric acid and the bile acids were extracted with 6 ml ethyl acetate. The organic layer was washed with 20% NaCl (× 3), dried, and evaporated under nitrogen to give a 1:1 (by radio-HPLC) mixture of **4** and **5.** Combined radiochemical yield: 140 μCi (93%). Yield based on TLC recovery was 87% of **1.** TLC of the methyl esters of the crude mixture showed only two spots corresponding to the methyl esters of 23(*R*)OH-CDCA and the unnatural 23(*S*)-isomer. The standards were prepared by a similar method and their structures were verified by ^{1}H-NMR (13).

Separation and purification of **4** *and* **5.** The two isomers, **4** and **5,** were separated and purified by reversed phase HPLC on a 250 × 10 mm Altex Ultrasphere-ODS, 5 μm RP-C_{18} semipreparative column (flow 4.5 ml/min) as discussed in (8). Twenty μCi (approximately 2.7 mg) portions of the mixture were injected at a time and the radioactivity profile was determined by scintillation

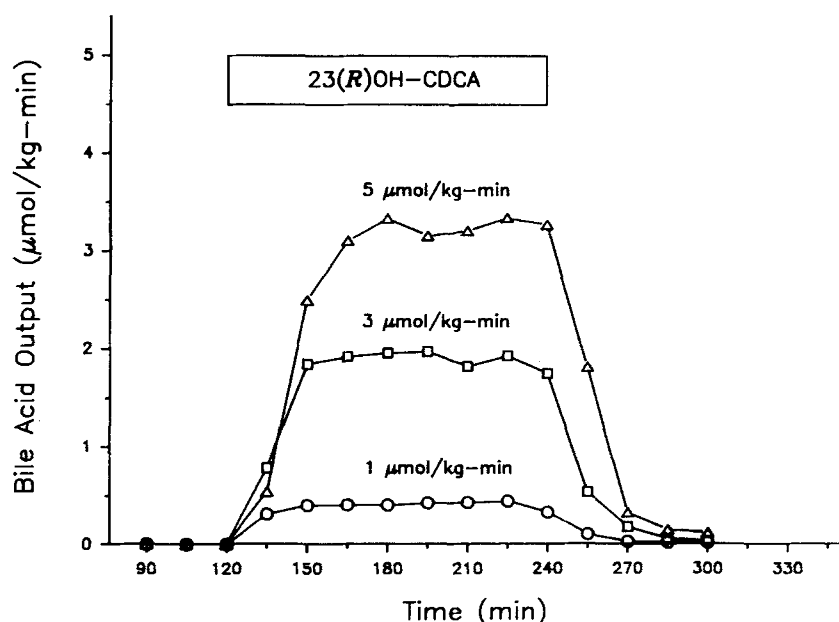

Fig. 2. Time course of biliary output of ^{14}C-bile acid radioactivity after infusion of [24-^{14}C]23(*R*)OH-CDCA. Steady state recovery of label was about 60–70% for all three doses.

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TABLE 1. Chemical form of biliary radioactivity and rate of bile acid conjugation after intravenous administration of [24-^{14}C] (23*R*)OH-CDCA to the anesthetized biliary fistula rat

Parameter	Dose (μmol/min-kg)[a]		
	1	3	5
Distribution of radioactivity in bile acid classes[b,c]			
23(*R*)OH-CDCA (unchanged)	26	44	55
Conjugates of 23(*R*)OH-CDCA			
C-24 aminoacylamidates (tau and gly)[c]	74	51	34
C-3 glucuronide (ethereal), non-amidated	1	6	11
Rate of C_{24} bile acid conjugation (μmol/min-kg)			
Amidation[c]	0.4	1.0	1.1
Glucuronidation	0.01	0.12	0.35

[a]Results are means of 5 animals for the dose of 1 μmol/min-kg; of 2 animals for 3 μmol/min-kg. Only a single animal was studied at 5 μmol/min-kg because the compound was poorly tolerated at this dose, including hemolysis and hemobilia. The rate of bile acid secretion in the healthy rat is 1–4 μmol/min-kg (66, 67).

[b]Recovery of radioactivity during steady state excretion was only 60–70% because of oxidative decarboxylation (see text); percent of biliary radioactivity.

[c]Conjugation with taurine always predominated; remainder was with glycine.

counting of small aliquots of the recovered fractions. The fractions containing **4** (RT 16.9 min) and **5** (RT 20.4 min) were evaporated under nitrogen, reconstituted with water, and adsorbed on 500 mg C_{18} reversed phase cartridges (BondElut C18, Analytichem International, Harbor City, CA). These were washed with water and the radioactive compounds were eluted with methanol to give **4** and **5** 99%+ pure by radio-HPLC.

Synthesis of reference metabolites

The taurine and glycine conjugates of 23(*R*)OH-CDCA were prepared by a modification of the method of Tserng and Klein (18) and used as chromatography standards. 23(*R*)OH-CDC-tau was further characterized by ^{1}H-NMR because it was used for the enzymatic hydrolysis and CMC experiments.

Sodium 2-[[(23R)-3a,7a,23-trihydroxy-5b-cholan-24-yl]amino] ethanesulfonate. ^{1}H-NMR (360 MHz, d_4-methanol): 0.715 (s, 3H, Me–18), 0.926 (s, 3H, Me–19), 1.007 (d, 6.5 Hz, 3H, Me–21), 2.263 (q, 12 Hz, 1H, H–4α), 2.969 (t, 6.5 Hz, 2H, $-CH_2SO_3^-$), 3.631 (m, 2H, $-CH_2CH_2SO_3^-$), 3.790 (bs, 1H, H–7), 4.052 (bd, 11.2 Hz, 1H, H-23). HPLC RT 0.61, relative to C-gly.

24-nor-5β-cholane-3α,7α,23-triol was prepared by reduction of nor-CDCA with borane in tetrahydrofuran (1 M) at 23°C for 30 min. The mass spectrum of the TMS derivative is shown in Fig. 4.

Animal pharmacology: hepatic biotransformation studies

Bile collection. Studies were performed in anesthetized biliary fistula rats and hamsters, prepared with a biliary fistula and a jugular vein catheter as previously described (cf. 19–23). Male Sprague-Dawley rats weighing 250–310 g (Charles River Labs, Wilmington, MA) were studied. Anesthesia was induced using intraperitoneal pentobarbital (70 mg/kg) and maintained by intravenously administered pentobarbital (10 mg every 3 h). Male golden Syrian hamsters weighing 120–130 g (Charles River, Wilmington, MA) were anesthetized intraperitoneally with ketamine HCl, 200 mg/kg, (Fort Dodge Laboratories Inc., Fort Dodge, IA) and Xylazine 10 mg/kg (Lloyd Laboratories, Shenendoah, IA). After 10 min, 100 mg/kg ketamine HCL (Fort Dodge Laboratories Inc., Fort Dodge, IA) was added intraperitoneally. After 90 min, 50 mg/kg pentobarbital (Abbott Laboratories, North Chicago, IL) was given intraperitoneally to maintain the anesthesia. During the study, animals were maintained at 37.5°C using a temperature controlling device (Yellow Springs Instruments Co., Yellow Springs, OH). The protocol was approved by the Animal Subjects Committee of UCSD.

After a control period of 2–3 h during which saline was infused at 2.2 ml/h, 23(*R*)OH-CDCA (together with [24-^{14}C]23(*R*)OH-CDCA, 0.5–1.0 μCi) was given intravenously at doses (expressed as μmol/kg-min) of 1.0 (5 rats), 3.0 (3 rats), and 5.0 (3 rats) for 2 h, after which saline was again infused at 2.2 ml/h. Bile was collected in 15-min intervals and its volume was determined by weighing, assuming a specific gravity of 1.00. At the completion of the study, blood and urine samples were also collected.

Decarboxylation: analysis of $^{14}CO_2$. A CO_2 collection train was constructed essentially as described by Mundlos, Rhodes, and Hofmann (24). CO_2 was collected in ethanolamine–2-methoxyethanol 1:1 (v:v). Breath was

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collected in 30-min intervals during the 2-h bile acid infusion period and for 30 min afterward. Radioactivity was determined by liquid scintillation counting.

Identification of biotransformation products present in bile

Thin-layer adsorption chromatography (TLC) of the whole bile was performed on silica gel G plates (DC-Fertigplatten, Kieselgel 60, Merck, Darmstadt, Germany) using two solvent systems. The first was isoamyl acetate–propionic acid–1-propanol–water 4:3:2:1 (v/v) (25), a system that resolves free and conjugated bile acids. The second was a double development system in which plates are developed first in chloroform–acetone–methanol–propionic acid–water 10:4:2:2:1 (v/v) and after drying overnight, developed in 1-butanol–propionic acid–water 10:1:1 (v/v) (26); (this system separates glucuronide conjugates from taurine conjugates). Selected bands were scraped from the TLC plate and the bile acids were eluted with $CHCl_3$–MeOH 2:1 (v/v). Bile acids were also obtained by collecting selected fractions after separation by HPLC (see below). For determination of the chemical form of radioactivity in bile, TLC-zonal scanning was used (c.f. 26). Zones of adsorbent, 1 mm in width, were scraped into scintillation vials; water, 0.25 ml, and ethanol, 2.0 ml, were added to desorb the labelled compound, and scintillant was then added.

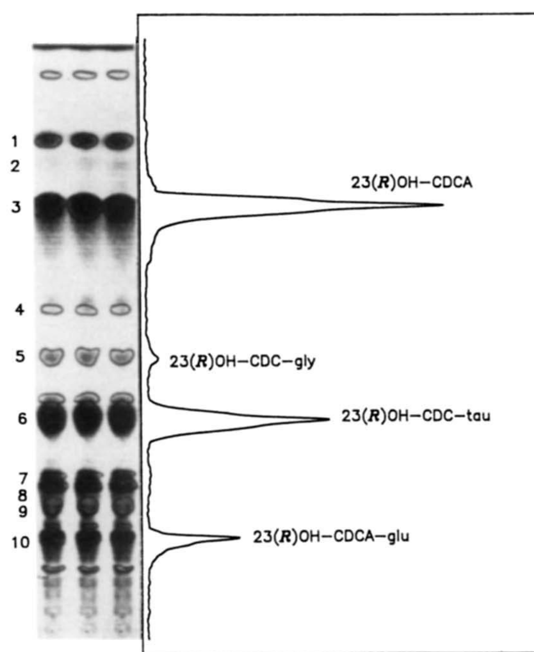

Fig. 3. TLC zonal scan of biliary metabolites recovered during 23(*R*)OH-CDCA infusion. For conditions, see text. Peak (spot) identification and [R_f] are as follows: 1. a mixture of unconjugated CDCA [0.84] and unconjugated nor-CDCA [0.84]; 2. unconjugated β-MCA [0.80]; 3. unconjugated 23(*R*)OH-CDCA [0.73]; 4. a mixture of CDC-gly [0.55] and DC-gly [0.55]; 5. 23(*R*)OH-CDC-gly [0.47]; 6. 23(*R*)OH-CDC-tau [0.37]; 7. a mixture of nor-CDCA-3-glucuronide [0.27] (ethereal) and nor-CDC-23-glucuronide [0.27] (ester); 8. β-MC-tau [0.25]; 9. C-tau [0.22]; and 10. (23*R*)OH-CDCA-3-glucuronide (or glucoside) [0.16] (ethereal).

Conjugated bile acids were analyzed by HPLC of whole bile essentially as previously described (27). The method used an octadecylsilane column (RP C-18) with elution at 0.75 ml/min using an isocratic buffer, apparent pH 5.4, composed of a mixture of methanol (67.4%) and 0.01 M KH_2PO_4; the effluent was monitored at 205 nm (amide bond of conjugated bile acids).

To determine biotransformation of the steroid moiety (fraction of C_{24} bile acids undergoing oxidative decarboxylation to C_{23} bile acids), bile samples from entire experiments were pooled, and bile acids were deconjugated chemically (1.0 N NaOH, 130°C, 4 h). The liberated unconjugated bile acids were esterified with methanol (diazomethane) and then converted to either per-acetate esters (using acetic anhydride in acetic acid with perchloric acid catalyst) or per-trimethylsilyl ethers (using Tri-Sil™, Pierce Chemicals, Rockford, IL). Bile acids were analyzed by GC–MS using a Hewlett-Packard 5890 Gas Chromatograph-5970 MSD, controlled by a HP/UX Chem Station program. The column was a Supelco 30m 0.25 mm ID SPB-35 operated at 277°C (isothermal). A splitless injection was used with an injection temperature of 290°C and interface temperature of 290°C. Helium was used as the carrier gas with a 6 psi column head pressure. Hydrolysis by β-glucuronidase (*Helix pomatia* β-glucuronidase, type H-2 (Sigma, St. Louis)) was performed by the addition of enzyme (1000 units/sample) at pH 5.0 (sodium acetate buffer) at 37°C. After overnight incubation, ethanol was added, the mixture was centrifuged, and the supernatant fluid was removed. The solution was dried, reconstituted in methanol, and analyzed by TLC using the double-development TLC method.

^{1}H-NMR spectra were recorded on a 360 MHz instrument equipped with a modified Varian MR-220 console, Oxford magnet and Nicolet 1180-E computer system. Chemical shifts are in ppm relative to tetramethylsilane.

Physicochemical properties

Determination of pH–solubility relationships. The effect of pH on the aqueous solubility of 23(*R*)OH-CDCA and of CDCA was determined by measuring the aqueous solubility of the two bile acids over the pH range from 2 to 10, as described previously (28). The aqueous solubility of any weak acid increases exponentially with pH. When the aqueous solubility reaches the CMC, the solubility (monomers and micelles) increases markedly over a narrow pH range, which can be considered to define a "critical micellization pH" (CMpH) (29).

Determination of CMC of 23(R)OH-CDC-tau. The CMC of 23(*R*)OH-CDC-tau was determined by the maximum

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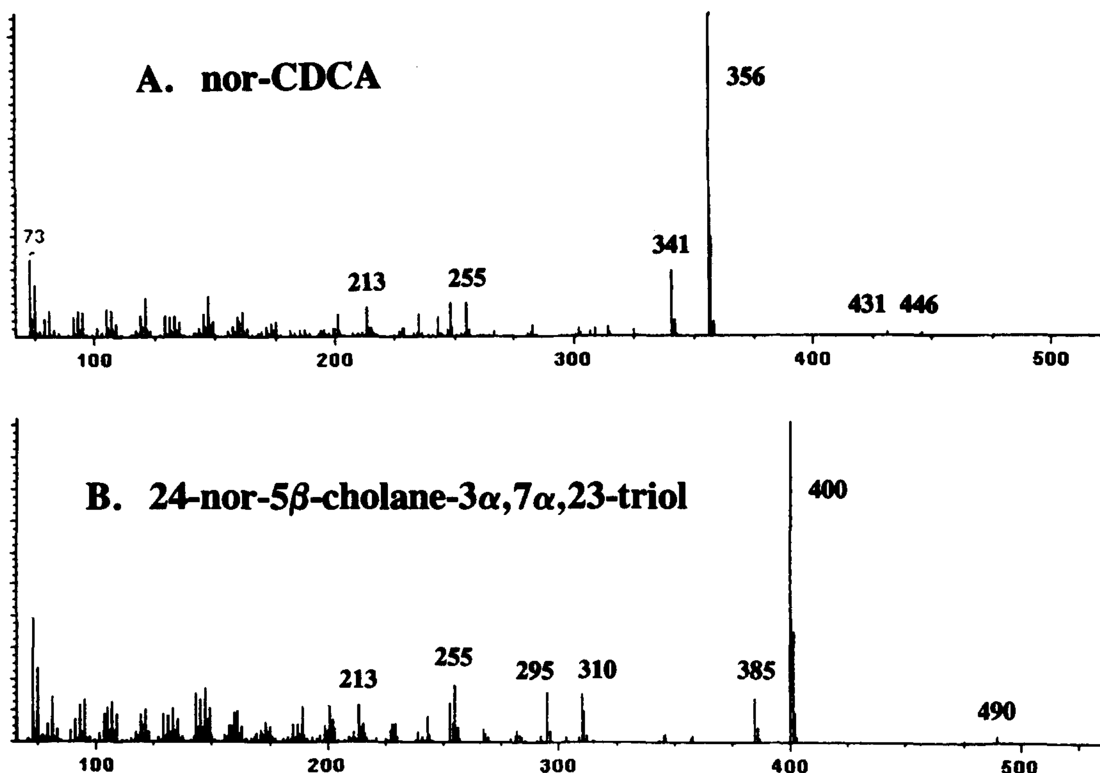

Fig. 4. A: Electron impact mass spectrum of the methyl ester trimethylsilyl derivative of nor-CDCA (24-nor-3α,7α-dihydroxy-5β-cholan-23-oic acid). The molecular ion (*m/z* 536) was not observed. The principal ions were found at *m/z* 446 (M-90 [TMSi]), 431 (M−15−90), 356 (M−90−90), 341 (M−90−90−15), 255 (M−90−90−101 [side chain]), and 213 (M−90−90−101−C_{15}−C_{16}−C_{17}). B: Electron impact mass spectrum of the trimethylsilyl derivative of 24-nor-5β-cholane-3α,7α,23-triol. The molecular ion (*m/z* 580) was not observed. Principal ions are found at *m/z* 490 (M−90), 400 (M−90−90), 385 (M−90−90−15), 310 (M−90−90−90), 295 (M−90−90−90−15), 255 (M−90−90−145 [side chain]), and 213 (M−90−90−145−C_{15}−C_{16}−C_{17}).

bubble pressure method using an apparatus constructed by Karol J. Mysels. This method determines changes in surface tension under dynamic conditions, and its principles (30) and application to bile acids (31) have been reported previously. Bubble pressure was measured over a concentration range of 0–50 mM bile acid at 25°C; the pH was adjusted to 8.6. The bubble pressure was plotted against the logarithm of the aqueous bile acid concentration. The intercept of the two lines defined by the method of least squares was defined as the CMC.

Microbiology: rate of deconjugation by cholylglycine hydrolase or mixed enteric bacteria

The rate of deconjugation of 23(*R*)OH-CDC-tau was compared with that of CDC-tau using a commercial preparation of cholylglycine hydrolase from *Clostridium perfringens* (Sigma No. C-4018, Sigma Chemical Co., St. Louis, MO) using methodology previously reported from this laboratory (32). The substrate solution (2 ml total volume) contained bile acid, 1 mM, mercaptoethanol, 10 mM, and EDTA, 1.0 mM; it was buffered with sodium acetate buffer (5.0 mM) to pH 5.6. Enzyme was added to give a concentration of 7.5 U/ml. Incubation was performed at 37°C. Aliquots (100 μL) were removed from the substrate solution at 2 min, 5 min, 30 min, 60 min, and 1, 2, 3, 4, and 5 h later. The pH of the aliquots was adjusted to pH 7, and bile acids were isolated from the solution by adsorption to a hydrophobic column (Bond Elut C18). Bile acids were eluted from the column with methanol and the methanol was then evaporated. The residue was dissolved in HPLC mobile phase and the concentration of remaining taurine conjugate was determined by HPLC (27). The natural logarithm of the peak areas was plotted against time to determine the $t_{1/2}$. Additional experiments were performed with homogenates of small or large intestinal contents from the rat. As results with all of these experiments were identical, only results using cholylglycine hydrolase are reported.

RESULTS

Synthesis of [24-^{14}C]23(*R*)OH-CDCA

The most readily available precursor for the preparation of ^{14}C-labeled 23(*R*)OH-CDCA was [24-^{14}C]CDCA. Two procedures have been reported for the introduction

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TABLE 2. Metabolism of 23(*R*)OH-CDCA based on analysis of bile in anesthetized biliary fistula rats and hamsters

Dose	n	Chemical Form in Bile (mean ± SD)					
			C_{23} Acids				
		C_{24} Acids[a]	nor-CDCA[b]	nor-α-MCA[c]	nor-U[d]	C_{23} Alcohol[e]	α-Oxidation
		%	%	%	%	%	%
Rats							
1 μmol/min-kg	5	76.9 ± 9.3	20.4 ± 8.8	1.4 ± 0.6	0.7 ± 0.6	0.7 ± 0.4	23.1 ± 9.3
3 μmol/min-kg	3	85.2 ± 2.2	12.5 ± 2.6	1.2 ± 0.5	0.4 ± 0.3	0.7 ± 0.9	14.8 ± 2.2
5 μmol/min-kg	3	77.7 ± 4.6	18.3 ± 9.6	1.3 ± 0.2	0.3 ± 0.3	2.5 ± 1.8	22.3 ± 10.5
Mean ± SEM		79.4 ± 2.6	17.6 ± 2.4	1.3 ± 0.1	0.5 ± 0.1	1.2 ± 0.4	20.6 ± 2.6
		C_{24} Acids[a]	nor-CDCA[b]	nor-5β-OH-CDCA[f]	nor-U[d]	C_{23} Alcohol[e]	α-Oxidation
		%	%	%	%	%	%
Hamsters							
3 μmol/min-kg	3	65.1 ± 4.6	28.7 ± 5.3	6.2 ± 2.7	< 0.1	< 0.1	34.9 ± 4.6

[a]The chemical form of the C_{24} bile acids is summarized in Table 1.
[b]The nor-CDCA was present in bile as such, as a mixture of C-3 and C-7 C_{23} glucuronides, and as the taurine conjugate.
[c]The trihydroxy derivatives were present as such; a small fraction was present as glucuronides.
[d]Nor-U denotes an unidentified C_{23} trihydroxy bile acid, presumably nor-β-MCA or nor-β-hyocholic acid.
[e]The C_{23} bile alcohol is likely to be present as the sulfate conjugate in bile.
[f]The chemical structure of this metabolite was based on the MS of a synthetic standard of 3α,5β,7α-trihydroxy-5β-24-nor-cholan-23-oic acid (68).

of the 23-hydroxyl group on CDCA: lead tetraacetate oxidation of a trimethylsilyl ketene acetal derivative (10), or treatment of a protected (tetrahydropyranyl) bile acid ethyl ester with oxodiperoxymolybdenum(pyridine)-hexamethylphosphoramide complex (6). In both cases the lithium enolate of an appropriate bile acid derivative must be generated under anhydrous conditions in an inert atmosphere. Because these conditions are difficult to implement for very small amounts of material and very small reaction volumes, we elected to prepare [24-^{14}C]23(*R*)OH-CDCA using a more robust sequence of reactions (Fig. 1). The nuclear hydroxyl groups were protected as formates and the bile acid was quantitatively brominated at the α-position by means of N-bromosuccinimide in trifluoroacetic acid-trifluoroacetic anhydride (17). In order to avoid the handling of small amounts of radioactive precipitates, the workup procedures were carefully modified so that formic or trifluoroacetic acid could be eliminated by aqueous extractions without loss of the bile acid intermediates. The final alkaline treatment removed the protecting groups from the nuclear hydroxy groups and replaced the bromine atom on carbon 23 by a hydroxy group to give a 1:1 mixture of [24-^{14}C]23(*R*)OH-CDCA and [24-^{14}C]23(*S*)OH-CDCA in 87% yield (from [24-^{14}C]CDCA); these were then separated by HPLC to afford the desired compounds with high radiopurity (99%).

Hepatic biotransformation

C_{24} biotransformation products in rat and hamster. After administering [24-^{14}C]23(*R*)OH-CDCA, radioactivity appeared rapidly in bile (**Fig. 2**). Steady state rates of biliary secretion occurred within 30 min of starting the intravenous infusion. When the infusion was

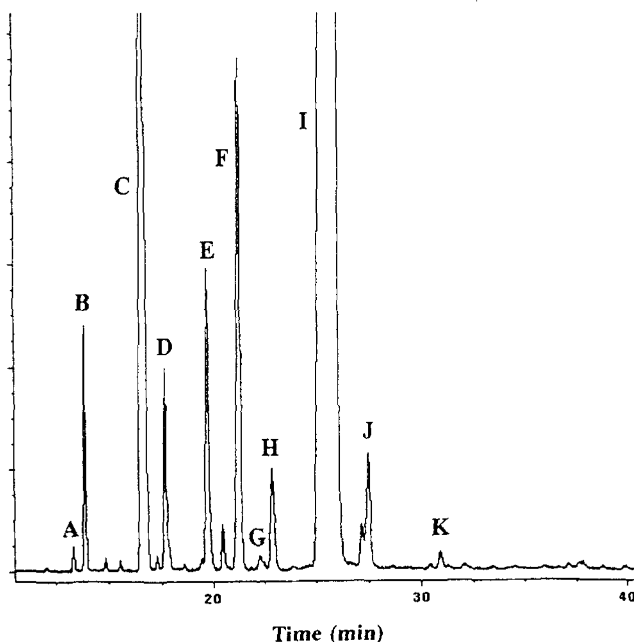

Fig. 5. GLC of TMS methyl esters of biliary bile acids from the rat infused with 23(*R*)OH-CDCA at 3 μmol/min-kg. For conditions, see text. Peak identifications and relative retention times (to CA) are as follows: A, 24-nor-5β-cholane-3α,7α,23-triol, 0.675; B, nor-αMCA, 0.703; C, nor-CDCA, 0.840; D, αMCA, 0.896; E, CA, 1.00; F, CDCA, 1.070; G, MDCA, 1.129; H, βMCA, 1.160; I, 23(*R*)OH-CDCA, 1.278; J, partial TMS ether of I, 1.398; and K, β-HCA, 1.571.

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TABLE 3. Comparison of hepatic metabolism of 23(R)- and 23(S)OH-CDCA in the rat

Treatment	C_{24} Compounds C_{24} Acids (Conjugated and unconjugated)	C_{23} Compounds C_{23} Acids (Conjugated and unconjugated)			C_{23} Alcohol	α-Oxidation
	(R)αOH-CDCA[b]	nor-CDCA	norαMCA	Other		
						%
I. 23(R)OH-CDCA[a]	86.4	11.6	1.4	0.3	0.3	13.6
	86.6	10.4	0.6	0.7	1.7	13.4
	82.7	15.4	1.6	0.2	0.1	17.3
	(S)αOH-CDCA					
II. 23(S)OH-CDCA[a]	88.2	8.7	2.4	0.0	0.7	11.8
	91.3	7.8	0.2	0.4	0.3	8.7

[a]Dose administered was 3 μmol/min-kg for 2 h, which is a physiological rate (65, 66). Data other than last column indicate % of biliary radioactivity.
[b]Data from Table 2.

stopped, biliary radioactivity declined rapidly, returning to near baseline levels within 30 min (Fig. 2). Steady state recovery of radioactive label in the rat was about 42–71% of the infused dose and did not appear to be dose dependent. Incomplete recovery was explained in part by the compound's undergoing oxidative decarboxylation (α-oxidation); see below. Urinary excretion of radioactivity was negligible.

By TLC-zonal scanning, radioactivity was present in four types of C_{24} bile acids, as summarized in **Table 1.** There were three classes of conjugates, taurine amidates, glycine amidates, and glucuronyl conjugates, as well as the unchanged compound. The proportion of the infused bile acid present in unconjugated form increased in a dose-dependent manner.

A thin-layer chromatogram showing the labeled and unlabeled rat biliary metabolites secreted during the period of 23(R)OH-CDCA infusion (5.0 μmol/min-kg) is shown in **Fig. 3.** In Fig. 3, spot 1: unconjugated dihydroxy bile acids (endogenous CDCA and nor-CDCA; spot 2: unconjugated trihydroxy bile acids (mostly endogenous β-MCA and nor-α-MCA); spot 4: CDC-gly and DC-gly; spot 8: β-MC-tau; and spot 9: cholyl-tau (C-tau) were identified by comparison with known standards.

Of the radioactively labeled peaks resolved by TLC zonal scan, peak 3 had a TLC R_f identical to that of unconjugated 23(R)OH-CDCA. It was isolated by TLC, derivatized, and shown by GC–MS to be authentic 23(R)OH-CDCA. Peaks 5 and 6 had TLC R_f values identical to that of 23(R)OH-CDC-gly and 23(R)OH-CDC-tau standards, respectively. The peaks were eluted from silica gel after separation by TLC. When subsequently examined by HPLC, they were found to have the same retention times, respectively, as those of standards of 23(R)OH-CDC-gly and 23(R)OH-CDC-tau. Also identified in peak 6 was a small amount of β-murideoxycholyltaurine (β-MDC-tau), a compound that is known to be an endogenous bile acid in the rat.

Peak 10 contained only an ethereal glycoside. The ^{1}H-NMR spectrum of peak 10 in d_4-methanol showed a doublet at 4.391 ppm (J = 7.6 Hz) corresponding to the anomeric proton of an ethereal glycoside with β-configuration where 2′-OH has an equatorial configuration (33).[5] It was concluded that peak 10 was a glycoside of 23(R)OH-CDCA at the 3 position (ethereal), most likely a glucuronide, although a glucoside could not be ruled out completely (because of the possibility that the β-glucuronidase preparation contained some glucosidase activity). R_f values for 23(R)OH-CDCA and its conjugates (adsorption TLC on silica gel layers) are summarized in the legend to Fig. 3.

TLC-zonal scanning was also performed in one hamster during infusion of [24-^{14}C]23(R)OH-CDCA at 3 μmol/min-kg. Radioactivity was mostly as glycine or taurine amidates (90%) with only 10% being recovered as the unchanged compound.

C23 Biotransformation products in the rat. TLC spot 1 was eluted, derivatized, and analyzed by GC–MS. It contained a significant proportion of nor-CDCA (**Fig. 4A**). Nor-CDCA had the same R_f as CDCA in the TLC solvent system used in Fig. 3.

After isolation by TLC, spot 7 and peak 10 were found to be susceptible to deconjugation by β-glucuronidase. The unconjugated bile acid present in spot 7 was shown by GC–MS to be nor-CDCA; that in peak 10 was 23(R)OH-CDCA. By 2-dimensional TLC (26), spot 7 was shown to be a mixture of ethereal (at C-3 or C-7) and ester (at C-23) glucuronides; such conjugates have been shown previously to be biotransformation products of nor-CDCA in the rat (21, 34). Peak 10 was a

[5]Details of the ^{1}H-NMR are as follows: (d_4-methanol): 0.716 (s, Me-18), 0.925 (s, Me-19), 1.025 (d, 6.1 Hz, Me-21), 3.4-3.6 (m, carbohydrate CHOH), 3.657 (m, H-3), 3.787 (bs, H-7), 3.942 (dd, 10.8, 2.1 Hz, H-23), 4.391 (d, 7.6 Hz, H-1′ of carbohydrate moiety).

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glucuronide, most likely the C-3 (ethereal) glucuronide of 23(R)OH-CDCA because of the relatively low pKa of α-hydroxy bile acids.

α-Oxidation: quantitation, recovery of $^{14}CO_2$ in breath, and stereospecificity

Quantitation. The proportion of 23(*R*)OH-CDCA converted into C_{23} biotransformation products could not be quantified by radiochromatography because the ^{14}C on the C_{24} carbon atom was lost from the bile acid side chain by α-oxidation. Because of this problem, bile collections were pooled, subjected to alkaline hydrolysis, and the results were analyzed by GC–MS (**Table 2**). These experiments indicated that 20.0 ± 4.6% [mean ± SE (n = 11)] of the compound had undergone α-oxidation in the rat and that the fraction of infused compound undergoing α-oxidation remained relatively constant over the range of infused doses. In the hamster, α-oxidation was somewhat greater, 34.9 ± 8.0% (n = 3).

In the rat, the 23(*R*)OH-CDCA was converted into four C_{23} metabolites, three of which were C_{23} bile acids, that is, with an isobutyric acid side chain: norCDCA, nor-α-MCA (the 6β-hydroxy-metabolite of norCDCA), and an unidentified C_{23} bile acid, probably nor-β-MCA or nor-HCA. The fourth metabolite was a C_{23} bile alcohol in which the C-23 carboxyl group had been reduced to a primary alcohol. The mass spectrum of this compound, 24-nor-5β-cholane-3α,7α,23-triol, is shown in Fig. 4. A capillary gas chromatogram of the bile acids present in the bile obtained from a biliary fistula rat during steady state excretion of 23(*R*)OH-CDCA and its metabolites (as well as persisting endogenous bile acids) is shown in **Fig. 5.**

In the hamster, the 23(*R*)OH-CDCA was converted to only two metabolites, both of which were C_{23} bile acids. The major metabolite was nor-CDCA; the minor metabolite was its 5β-hydroxy derivative. This compound has been shown previously to be a major metabolite of norCDCA in the hamster (34, 35).

Recovery of $^{14}CO_2$ from 24-^{14}C-labeled 23(R)OH-CDCA. When [24-^{14}C]23(*R*)OH-CDCA was infused, about 1% of the radioactivity was recovered as $^{14}CO_2$ in breath (data not shown). However, the fraction of infused radioactivity recovered as $^{14}CO_2$ is a gross underestimate of the amount of $^{14}CO_2$ formed because of the known entry of $^{14}CO_2$ into metabolic pools inside the hepatocyte (36).

Stereospecificity of α-oxidation. Two experiments were performed with the 23*S* isomer of 23OH-CDCA in the rat (**Table 3**). (Additional experiments could not be performed because of insufficient amount of material.) In addition to conjugation with taurine and excretion as such, the unnatural *S* isomer also underwent α-oxidation, and the nor-CDCA that was formed underwent 6β-hydroxylation on the nucleus. As with the *R* isomer, a small fraction of the *S* isomer underwent subsequent reduction of the C-23 carboxyl group to a C-23 primary alcohol.

Physiological properties: effect of infused 23(*R*)OH-CDCA on bile flow

Infusion of 23(*R*)OH-CDCA into rats caused a dose-dependent choleresis at the two lower doses of 1 and 3 μmol/min-kg. The increment in bile flow per increment in bile acid recovered in bile is termed the apparent choleretic activity (21), but could not be calculated due to the unknown rate of canalicular secretion of the very complex mixture of 23(*R*)OH-CDCA and its biotransformation products. In addition, at the doses of 1 and 3 μmol/min-kg, the majority of bile flow in the rat is bile acid-independent (20, 21). At the highest dose (5 μmol/min-kg), 23(*R*)OH-CDCA was hemolytic and induced hemobilia, suggesting damage to the sinusoidal endothelial cells.

Physicochemical measurements: pH–solubility relationships and micellar aggregation

pH–solubility relationships. The solubility of 23(*R*)OH-CDCA and CDCA in relation to pH is shown in **Fig. 6.** The solubility of 23(*R*)OH-CDCA began to increase at about pH 5 and showed a marked increase at pH 5.3, the pH value that is defined as the CMpH. In contrast, the aqueous solubility of CDCA began to increase at about 6.5 and the CMpH was about 6.8, in agreement with a previous study (37).

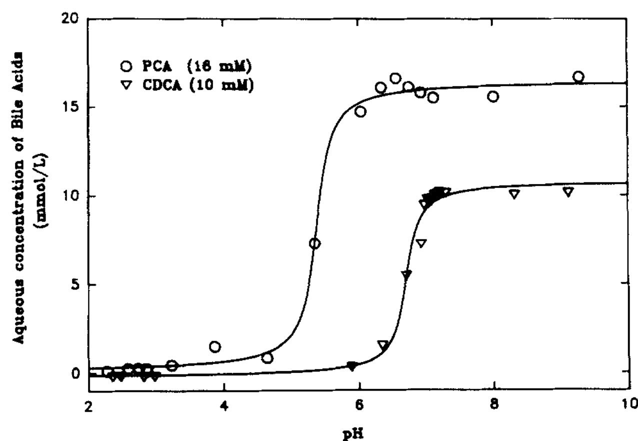

Fig. 6. Relationship between pH (abscissa) and aqueous solubility of 23(*R*)OH-CDCA and CDCA (ordinate). The pH at which the aqueous solubility reaches the CMC is defined as the CMpH (see text). The CMpH of 23OH-CDCA is lower than that of CDCA because the presence of an α-hydroxy group increases the solubility of the protonated acid considerably, lowers the pK_a, but has relatively little effect on the CMC. The apparent upper limit of solubility at alkaline pH is artifactual and merely indicates that all of the bile acid was solubilized at pH values above the CMpH.

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CMC of 23(R)OH-CDC-tau. The CMC of 23(*R*)OH-CDC-tau was found to be 4.6 mM at 25°C (Na^+ concentration 0.15 M), using the maximum bubble pressure method. Under these conditions, CDC-tau, differing only in the absence of the α-hydroxyl group, has a CMC of 1.8 mM (31).

Microbiology: deconjugation of 23(*R*)OH-CDC-tau by bacterial hydrolases

To define the influence of the α-hydroxy group on the rate of hydrolysis of taurine-conjugated bile acids, the relative rate of hydrolysis of 23(*R*)OH-CDC-tau by cholylglycine hydrolase was compared with that of CDC-tau. The hydrolysis of 23(*R*)OH-CDC-tau ($t_{1/2}$ = 23.9 min) was considerably slower than that of CDC-tau ($t_{1/2}$ = 6.7 min) as shown in **Fig. 7.**

DISCUSSION

Hepatic metabolism of 23(*R*)OH-CDCA

The first major finding of this paper was that in the biliary fistula rat and hamster 23(*R*)OH-CDCA, an α-hydroxy C_{24} bile acid, not only was incompletely conjugated with taurine or glycine, but in addition underwent oxidative decarboxylation (α-oxidation) to form nor-CDCA. Evidence for the biotransformation of 23(*R*)OH-CDCA into nor-CDCA was provided by the structural identification of nor-CDCA. Additional evidence for oxidative decarboxylation of 23(*R*)OH-CDCA was provided by recovery of $^{14}CO_2$ derived from the labeled C-24 atom in breath. The α-oxidation process had little steric specificity because nor-CDCA was formed from both the (*R*) and (*S*) isomers of 23OH-CDCA.

Oxidative decarboxylation of α-hydroxy bile acids as a mechanism for the formation of nor-bile acids has been proposed previously (38, 39); our data would appear to be the first to provide experimental evidence for the existence of this pathway. The present study suggests that α-hydroxy bile acids undergo oxidative decarboxylation when they enter the hepatocytes of a species that is unable to conjugate them efficiently with either taurine or glycine. Small amounts of nor-bile acids have been reported in human bile (40), urine (41), and feces (42), and this paper describes a possible biosynthetic pathway.

The process of α-oxidation has been well described for fatty acids, is known to occur in microsomes, and has been proposed to involve a peroxylactone intermediate (43, 44). The data suggest that incomplete esterification of 23(*R*)OH-CDCA with Coenzyme A (due to the presence of the 23(*R*)-hydroxyl group) allows the unesterified compound to enter the microsomal compartment, despite its relatively low pK_a. Another possibility is that the Coenzyme A ester of 23(*R*)OH-CDCA is a poor substrate for the CoA:amino acid N-acyltransferase as has been shown for norcholic acid (45).

In the present study, the rate of conjugation for 23(*R*)OH-CDCA with taurine or glycine was about 1 μmol/min-kg. Under similar conditions, ursodeoxycholic acid (UDCA) is conjugated at a rate of 10 μmol/min-kg (46). At the highest infusion dose, the proportion of 23(*R*)OH-CDCA conjugated with glucuronate increased relative to that conjugated with taurine or glycine, as has been shown to occur with UDCA in the rat (47) and in the dog (48). Our observation that 23(*R*)OH-CDCA undergoes incomplete conjugation in the rat is in agreement with the report of Roda et al. (11), although in our studies a greater proportion of 23(*R*)OH-CDCA underwent conjugation. Presumably, in vertebrates such as sea mammals and birds in which 23(*R*)OH-CDCA is efficiently conjugated with taurine, enzymatic adaptations have occurred that permit efficient CoA thioester formation and subsequent transfer of the bile acid moiety to the amino acid acceptor.

Previous studies from this and other laboratories have shown that nor-bile acids are poor substrates for the enzyme bile acid Coenzyme A ligase (49), as well as for the cholyl CoA amino acid transferase (45). As a consequence, when infused into the biliary fistula rodent, nor-CDCA is secreted, in part, into bile in unchanged form, with the majority being secreted after undergoing conjugation with glucuronate, and to a lesser extent with sulfate or glucose (21). An additional fraction is hydroxylated at the 6β-position in rats (21, 35) and at the 5β-position in hamsters and secreted in unconjugated form (34, 35). In this study, the nor-CDCA formed from

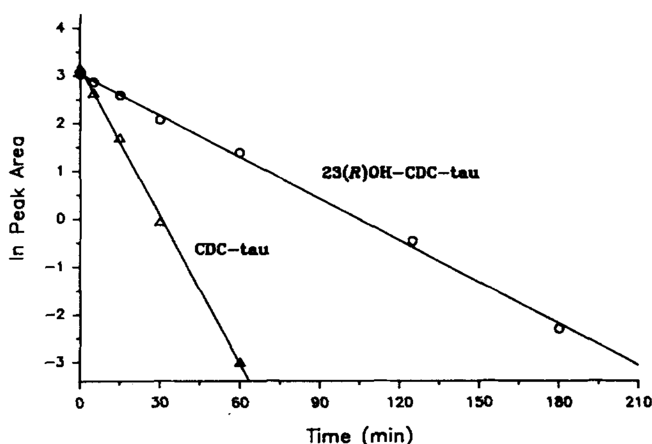

Fig. 7. Time course of deconjugation of 23(*R*)OH-CDC-tau by cholylglycine hydrolase from *C. perfringens* compared with that of CDC-tau. The natural logarithm of the concentration of remaining taurine conjugate (ln of HPLC peak area) is plotted against time. Hydrolysis of 23(*R*)OH-CDC-tau ($t_{1/2}$ = 23.9 min) was considerably slower than that of CDC-tau ($t_{1/2}$ = 6.7 min).

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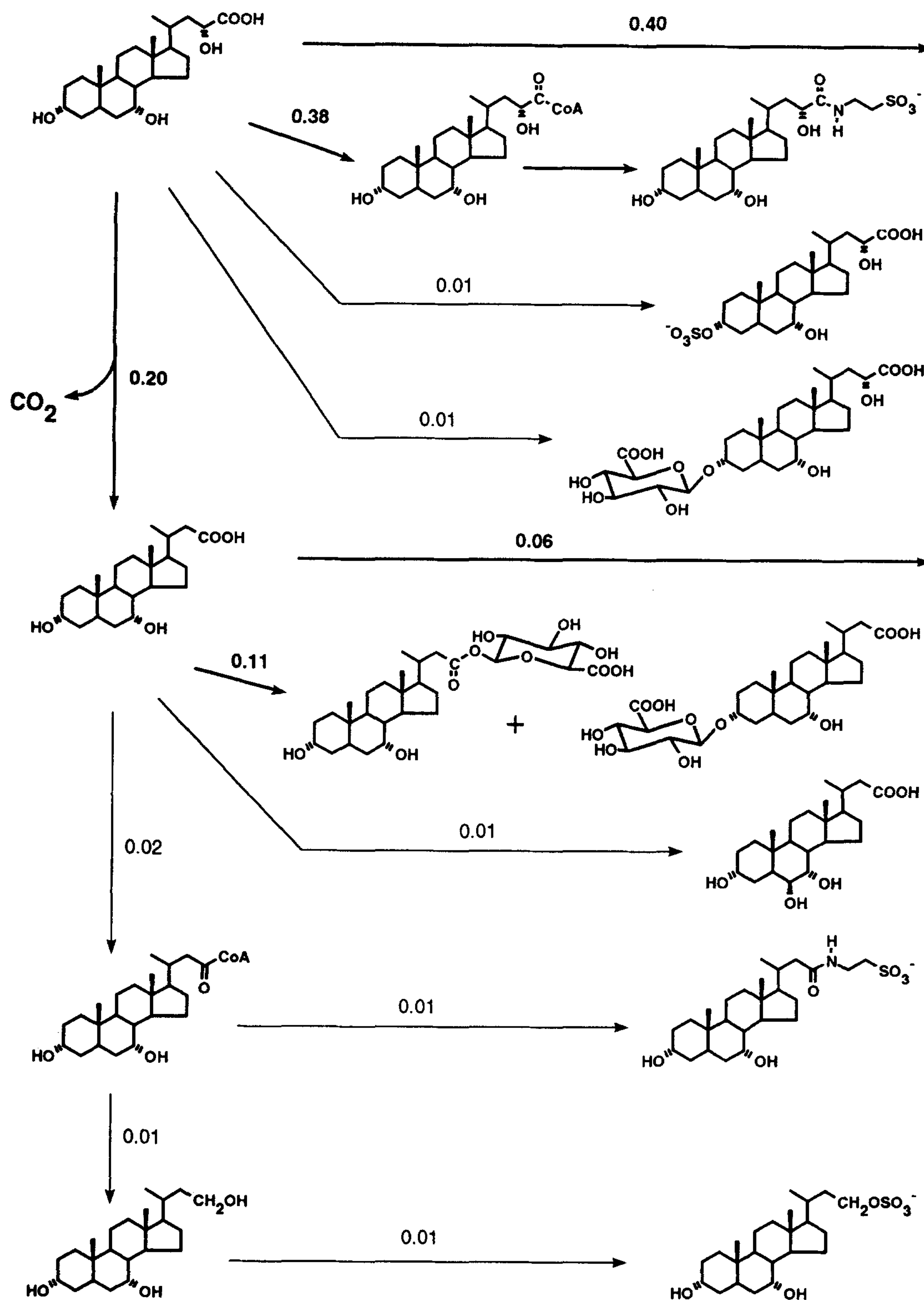

Fig. 8. Metabolism of 23(*R*)OH-CDCA in the rat based on the studies reported in this paper. The numbers indicate the fraction of the administered dose. Not shown are the glucuronides of α-MCA or the possible formation of β-HCA. CDCA is metabolized predominantly to its taurine amidate (> 95%), but about 1% is converted to the 3-sulfate or β-MCA (21).

23(*R*)OH-CDCA was metabolized in a manner similar to that of infused nor-CDCA (21, 34, 35).

An additional finding was that a small fraction of the metabolite nor-CDCA underwent reduction of the C-23 carboxyl group to a primary bile alcohol. C_{24} bile alcohols have been identified previously as trace constituents of biliary bile acids in many species (50), and are the major cholanoid in lungfish (51). Reduction of a

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carboxyl group to a primary alcohol is known to occur with long chain fatty acids in ether lipid synthesis (52), and is mediated by peroxisomal enzymes (53). Presumably, such a pathway is present for bile acids, also. The metabolism in the rat of 23(*R*)OH-CDCA is shown in **Fig. 8.**

Metabolism and physical state of 23(*R*)OH-CDCA during enterohepatic cycling

The second major finding in this paper was that the synthetically prepared taurine conjugate of 23(*R*)OH-CDCA was considerably more resistant to bacterial deconjugation than the taurine conjugate of CDCA. Thus, the presence of a 23(*R*)-hydroxy group decreased the rate of deconjugation for this conjugated bile acid, as was also observed by Roda et al. (11). Substituents around the peptide bond of conjugates are known to inhibit the rate of deconjugation by bacterial enzymes, presumably because of steric hindrance. For example, bile acids conjugated with N-methyltaurine or N-methylglycine are hydrolyzed far more slowly than their corresponding taurine or glycine analogues in vitro (54) and in vivo (55–57). 23(*R*)OH-CDCA has the potential to form additional hydrogen bonds by means of its 23-hydroxy group with either water or amino acid residues of the enzyme, which could also result in inhibition of the deconjugation process.

During their enterohepatic cycling, bile acids undergo bacterial deconjugation in both the small intestine (reviewed in ref. 58) and the large intestine (reviewed in ref. 59). Resistance to deconjugation should promote a high intraluminal concentration, as conjugated bile acids undergo little passive absorption from the small intestine (60). Even if unconjugated 23(*R*)OH-CDCA were formed in the intestinal lumen by bacterial enzymes, its behavior should differ from that of CDCA. The 23(*R*)OH-CDCA is soluble at pH values above pH 5.3, whereas CDCA has maximal aqueous solubility only above pH 6.8. In addition, 23(*R*)OH-CDCA is likely to undergo a much slower passive absorption from the small intestine for two reasons. The first is because its lower pK_a results in greater ionization at intestinal pH, and little passive absorption of the ionized molecule will occur (cf 61). Second, even when protonated, the passive membrane permeability of the α-hydroxy bile acid should be less because of its lower lipid/water partition coefficient. A high intraluminal concentration of bile acids is considered desirable for the solubilization of dietary lipids in the form of mixed micelles (62). The CMC of 23(*R*)OH-CDCA-taurine was only slightly higher than the taurine amidate of CDCA. Whether 23(*R*)OH-CDC-tau has the ability to solubilize fatty acids and monoglycerides as well as CDC-tau has not been examined.

Resistance to deconjugation also results in a slower production of 7-deoxy bile acids, as 7-dehydroxylation of conjugated bile acids does not occur (56, 57, 63, 64). When CDCA undergoes 7-dehydroxylation, it forms lithocholic acid, a bile acid with severe hepatotoxicity (65).

Functional superiority of 23(*R*)OH-CDCA

We conclude that from a physiological point of view, the insertion of an α-hydroxy group on the side chain of a bile acid results in a detergent that is more resistant to bacterial degradation and whose unconjugated derivative is more soluble and has less passive membrane permeability. As noted, the course of evolution in ducks appears to select for such bile acids.

The formation of 23(*R*)OH-CDCA is likely to be especially useful in animals in whom conjugated bile acids are exposed to considerable bacterial deconjugation in the small intestine during their enterohepatic cycling. It will be of interest to compare the metabolism of 23(*R*)OH-CDC-tau with that of CDC-tau in animals such as wading birds and sea mammals in which both conjugates are present in bile.

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