Detoxification of lithocholic acid. Elucidation of the pathways of oxidative metabolism in rat liver microsomes[1]

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Abstract The hydroxylation of lithocholic acid (3α-hydroxy-5β-cholanoic acid) by adult male Sprague-Dawley rat liver microsomes supplemented with NADPH was studied. Metabolites were separated by a combination of thin-layer chromatography and high pressure liquid chromatography, both with and without prior methylation and acetylation of the samples. The resulting products were characterized by thin-layer, gas-liquid, and high pressure liquid chromatography by comparison with authentic bile acid standards; final structure determination was by proton nuclear magnetic resonance spectroscopy and by mass spectrometry. The following reaction products were found: 3α,6β-dihydroxy-5β-cholanoic acid (80% of total metabolites) and 3α,6α-dihydroxy-5β-cholanoic, 3α,7α-dihydroxy-5β-cholanoic, 3α,6β,7β-trihydroxy-5β-cholanoic, and 3α-hydroxy-6-oxo-5β-cholanoic acids (≤5% each). In addition, one unidentified trihydroxylic bile acid and several minor compounds were present. It is concluded that four different hydroxylation reactions of lithocholic acid, namely the predominant 6β as well as the minor 6α, 7α, and 7β hydroxylations, are catalyzed by rat hepatic microsomes; 7β-hydroxylation may occur only with dihydroxylated bile acids but not with lithocholate itself. The presence of the 6-oxo bile acid can be explained either by direct oxidation of a hydroxyl group by cytochrome P-450, or by the action of microsomal dehydrogenase(s) which could also catalyze the epimerization of hydroxyl groups via their oxidation. The results form the basis of a proposed scheme of the oxidative metabolism of lithocholic acid in rat liver microsomes.—**Zimniak, P., E. J. Holsztynska, R. Lester, D. J. Waxman, and** A. Radominska. Detoxification of lithocholic acid. Elucidation of the pathways of oxidative metabolism in rat liver microsomes. *J. Lipid Res.* 1989. **30:** 907–918.

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Lithocholic acid (3α-OH), a minor bile acid formed predominantly by bacterial 7α-dehydroxylation of chenodeoxycholate (3α,7α-diOH) in the gut (1, 2), is a hydrophobic compound of considerable hepatotoxicity. Generally, two major biochemical strategies evolved for the reduction of hydrophobicity and toxicity, as well as for a more rapid excretion of such compounds: hydroxylation and conjugation (phase I and II detoxification reactions). In the case of lithocholic acid, conjugation with amino acids or with glucuronic or sulfuric acids fails partially or completely to eliminate the cholestatic potential of the compound (3–6); indeed, the 3-O-glucuronide of lithocholic acid is a more potent cholestatic agent than the parent compound (4). On the other hand, hydroxylation converts lithocholic acid into more hydrophilic bile acids, either of a type commonly present in the organism, thus returning the compound into the general bile acid pool, or into a bile acid with an atypical hydroxylation pattern, which appears to signal further metabolism and rapid clearance (7). In the rat, hydroxylation constitutes a major pathway of detoxification of lithocholate and other hydrophobic bile acids. Multiple reactions are involved, resulting in a complicated pattern of products. Even though the hydroxylation of lithocholic acid in the rat has been studied previously (8–13), most of the investigations

Abbreviations: NMR, nuclear magnetic resonance; MS, mass spectrometry; RDA, retro-Diels-Alder; s.c., side chain; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography; GLC, gas–liquid chromatography; TMS, trimethylsilyl derivative. The following trivial names for bile acids were used: lithocholic acid, 3α-hydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; murideoxycholic acid, 3α,6β-dihydroxy-5β-cholanoic acid; hyodeoxycholic acid, 3α,6α-dihydroxy-5β-cholanoic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; α-muricholic acid; 3α,6β,7α-trihydroxy-5β-cholanoic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanoic acid. For clarity, the positions of hydroxyl substituents are occasionally added in parentheses after the trivial name. "Methyl ester/acetate" refers to a bile acid derivative in which the carboxyl group is methylated and all hydroxyl groups are acetylated.

[1]One of the authors (P. Z.) dedicates this work to Professor Efraim Racker on the occasion of his recent seventy-fifth birthday.

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were completed two decades ago and are limited by the methodology available at that time. More recent relevant studies deal with related but not identical topics, such as bile acid hydroxylation in the human fetal liver (14–17), in human liver disease (18), or in cultured cells (19, 20). References in the above publications to the metabolism of lithocholic acid in the adult rat are brief and fragmentary. The present work is intended to elucidate the pathways of oxidative metabolism of lithocholate catalyzed by rat liver microsomes using rigorous spectral techniques to identify definitively the metabolites formed, and thus constitutes the basis for further investigations of the enzymology of these processes.

MATERIALS AND METHODS

Chemicals

[3β-^{3}H]-3α-Hydroxy-5β-cholanoic acid ([^{3}H]lithocholic acid) was synthesized and purified as described previously (21); [24-^{14}C]lithocholic acid (55 Ci/mol) was from Amersham Corp., Arlington Heights, IL. 3α-Hydroxy-6-oxo-5β-cholanoic and β-murichocholic acids were from Steraloids, Inc., Wilton, NH, and α-murichocholic acid was a generous gift from Drs. A. K. Batta and G. Salen, VA Medical Center, East Orange, NJ. The sources of other bile acids were as given in (7). Dithiothreitol, NADPH, and Brij 58 were from Sigma Chemical Co., St. Louis, MO.

Preparation of rat liver microsomes

Rats (Sprague-Dawley, male, 180–230 g) were used in all experiments. Hepatic microsomes were prepared as previously described (22), and were stable with respect to the measured enzymatic activity profiles for at least 6 months at −80°C.

Enzymatic hydroxylation reactions

The incubations contained 0.1 M HEPES-NaOH, pH 7.4, 0.1 mM EDTA, 2 mM dithiothreitol, 2 mM NADPH, 1.25 mg microsomal protein/ml (protein was measured using the Bio-Rad kit based on the Bradford method (23) with bovine serum albumin as standard), 50 μM [^{3}H]- or [^{14}C]lithocholic acid, as well as 0.006% (w/v) Brij 58 introduced together with lithocholic acid, which was added as mixed micelles with this mild detergent, as previously described in detail (22). Briefly, a 1 mM stock solution of lithocholic acid was prepared as follows: 1 μmol of lithocholic acid of the desired specific radioactivity was dissolved in methanol (typically 200–300 μl) and mixed with 120 μl 1% (w/v) aqueous Brij 58 and 20 μl aqueous 0.1 N NaOH. The mixture was evaporated at 40°C under a stream of nitrogen, and 1 ml water was added to the dry residue. The resulting micellar solution was optically clear; occasionally, brief sonication was required for clarification. Alternatively, lithocholic acid was introduced into the reaction mixture as a 10 mM methanolic solution (final concentration of methanol in the reaction mixture: 0.5%). Both methods gave identical results, and the micelle method was used in all experiments reported in this communication. Analytical reactions were carried out in a total volume of 80 μl. After 20 min at 37°C, the reactions were stopped with 20 μl ethanol, and an aliquot of 60 μl was directly applied to the preadsorbent layer of a TLC plate (see below). The elimination of a solvent extraction step resulted in a greater accuracy and in time saving. The formation of all seven products investigated (see below) was linear with time up to 40 min and linear with protein concentration up to 2.5 mg/ml.

Preparative reactions were scaled-up (to 6 ml) versions of analytical reactions, except that the incubation was continued for 90–120 min, during which time the reactants lithocholic acid and NADPH were replenished every 30 min, each time increasing the lithocholate concentration by 16.7 μM and the NADPH concentration by 0.5 mM. In some cases, only NADPH was replenished (see text). The reactions were stopped by tenfold dilution with ice-cold 0.1 M glycine-trichloroacetate buffer, pH 2.8. The solution was passed through solid phase extraction cartridges (Bond Elut C_{18}, size: 6 cc; Analytichem International, Harbor City, CA), the cartridges were washed with 10 ml each of the above buffer and water, and bile acids were eluted with 5 ml methanol. Usually, twelve parallel preparative incubations were carried out, only one of which contained the labeled lithocholic acid (ca. 2×10^7 dpm) and was used for monitoring the progress of the reaction; the remaining incubation flasks contained unlabeled lithocholic acid at the same concentration. At the end of the reaction, all incubations were pooled, resulting in a specific radioactivity of all reaction products of ca. 2800 dpm/nmol. The qualitative pattern of products of preparative reactions was identical to that of analytical reactions, even though the relative amounts of the individual metabolites differed (see below).

Thin-layer chromatography

Silica gel plates with a layer thickness of 0.25 mm, type K5 or LK5 (Whatman, Clifton, NJ) or Si250-PA(19C) (19-channel plates with preadsorbent area; J. T. Baker, Phillipsburg, NJ) were used. Underivatized bile acids were separated in isooctane–ethyl acetate–acetic acid 10:10:2 (solvent system S11 in ref. 24); plates were developed twice. Bile acid methyl ester/acetates (see below) were chromatographed in benzene–acetone 98:2 (one development). Spots were localized by spraying with Krowicki's reagent (25) and heating on a hot plate at approximately 100°C or, for preparative separations, nondestructively by spraying with water and observing the transient spots that appear during drying (26); the latter procedure

also caused a deactivation of the silica gel and therefore facilitated subsequent elution of the chromatographed material (see below). Radioactive compounds were localized on the plate by scanning with a Tracemaster 20 windowless gas flow counter (Berthold Analytical Instruments, Nashua, NH), and/or by autoradiography at −80°C. Plates with tritium-labeled compounds were lightly sprayed with Enhance (Du Pont/NEN, Boston, MA) prior to autoradiography.

Compounds were recovered from preparative TLC plates by scraping the spots or bands, packing the silica gel in the form of a small column in a Pasteur pipette or placing it in a small sintered glass funnel, and eluting with an appropriate solvent. For underivatized bile acids, dichloromethane–methanol 1:1 was used. The eluate usually contained foreign material, probably binder from the plate, which could be removed by taking up the residue left after evaporation of the solvents in 0.1M glycine–trichloroacetate buffer, pH 2.8, passing it through a Bond Elut C_{18} cartridge, washing the cartridge with the above buffer and with water, and eluting the bile acids with methanol. In recovering derivatized (i.e., methylated/acetylated) bile acids from silica gel, ethyl acetate or benzene was used as eluant. No foreign material was extracted from the silica gel under these conditions, and the recovered compounds were essentially pure.

High pressure liquid chromatography (HPLC)

Preparative reverse phase HPLC was carried out using a μBondapack C_{18} column (0.78 × 30 cm, Waters, Milford, MA) with a CM400 gradient pump (Milton Roy/LDC, Riviera Beach, FL) and a model 1840 variable wavelength detector (ISCO, Lincoln, NE) set for 205 nm. For underivatized bile acids, the separation protocol was as follows: 0–40 min, 75% methanol–25% 5 mM potassium phosphate, pH 5.0; 40–55 min, linear gradient of 75 to 90% methanol in the above buffer; 55–70 min, 90% methanol in the above buffer; 70–80 min, linear gradient of 90 to 100% methanol in the above buffer; after 80 min, 100% methanol. The solvent flow rate was 2 ml/min, and 2-ml fractions were collected. Small aliquots of the fractions were used for radioactivity determination by liquid scintillation counting. Peaks were pooled, concentrated under a stream of nitrogen to remove methanol, acidified with 1 N HCl, and extracted 3 times with ethyl acetate.

Methyl/acetyl derivatives of bile acids were purified by reverse phase HPLC at 1 ml/min using methanol–water 65:35 (0–25 min) followed by a linear gradient of 65 to 100% methanol in water (25–40 min).

Derivatization of samples

Bile acids were methylated/acetylated as follows: purified products of enzymatic reactions as well as standards dissolved in methanol were treated with diazomethane in methyl *tert*-butyl ether and dried under a stream of nitrogen. The samples were then dissolved in 1 vol dry pyridine and 1.1 vol acetic anhydride was added. After 12 h at 65°C or, alternatively, after 3 days at room temperature, the samples were dried under nitrogen. When necessary, further purification was achieved by TLC.

For some GLC–MS analyses, the samples were silylated by treatment with 1 drop of dry pyridine and 2 drops of *bis*(trimethylsilyl)trifluoroacetamide for 30 min at 60°C. An aliquot of the reaction mixture was then injected, without further purification, onto the GLC column.

Gas–liquid chromatography

GLC separation and identification of methylated/acetylated bile acids were carried out in a Hewlett-Packard 5880A gas chromatograph equipped with a 11 m capillary DB-1 column, 0.25 mm i.d., 0.1 μm (J & W Scientific Inc., Rancho Cordova, CA), and a flame ionization detector. The following separation protocol was used: carrier gas, N_2 at 5 psi; temperature profile: 180°C for 2 min, linear increase to 250°C at 20°/min (3.5 min), 250°C for 30 min.

Mass spectrometry and NMR spectroscopy

Electron impact mass spectra of methyl/acetyl or methyl/silyl derivatives of bile acids were obtained on a Finnigan Incos-50 instrument at 70 eV. When a GLC inlet was used, the column was a 15 m SE-54, with the following temperature profile of the chromatographic separation: 2 min at 180°C, linear increase to 280°C at 20°/min (5 min), 30 min at 280°C.

Proton NMR spectra of methyl/acetyl derivatives of bile acids were obtained at 300 MHz under conditions described previously (27).

General protocols for separation and purification of enzymatically hydroxylated bile acids

Protocol A. Reaction products obtained as described earlier from a set of preparative reactions (starting with 7.2 μmol of lithocholic acid) were applied to the preadsorbent layers of six 20 × 20 cm TLC plates, and the plates were developed twice in isooctane–ethyl acetate–acetic acid 10:10:2. Bands localized by radioactivity scanning and spraying the plates with water were scraped, and the compounds were recovered as described above. The individual fractions were methylated and, without further purification, acetylated. The derivatized samples were purified either by preparative TLC or by chromatography on 0.3 × 10 cm silica gel columns (columns were prepared and samples applied in benzene; after washing with the same solvent, the radioactive material was eluted with 2% acetone in benzene).

Protocol B. Reaction products obtained as in protocol A were divided into two portions and subjected to HPLC in

the methanol–phosphate buffer system (see section on HPLC). After initial characterization by TLC, the fractions were methylated, acetylated, and further separated by HPLC using methanol–water as the solvent.

RESULTS

Reaction products

The hydroxylation of lithocholic acid (3α-OH) by hepatic microsomes from the rat was studied. The initial overall rate of the reaction was 0.8–1.2 nmol/min · mg protein. Multiple metabolites were formed. The qualitative pattern of products was identical in short-term (analytical) and long-term (preparative) reactions, even though the relative amounts of the metabolites changed, as will be discussed later. Since the main objective of the present work was the elucidation of the chemical structure of the products, preparative reactions that were continued for 90 to 120 min were utilized in order to maximize the yield of metabolites.

A typical pattern of hydroxylation products of lithocholic acid (3α-OH) by rat liver microsomes, as revealed by thin-layer chromatography, is shown in **Fig. 1.** The assignment of structures to the spots seen on the TLC plate is based on spectral analysis of isolated compounds (see below) rather than on co-migration with standards alone. 6β-Hydroxylation is by far the most active reaction (70 to 80% of total), leading to the formation of murideoxycholic (3α,6β-diOH) and β-muricholic (3α,6β,7β-triOH) acids.

Isolation and identification of reaction products

The major products of microsomal hydroxylation of lithocholic acid were purified by either of the two protocols described in Methods. In protocol A, the fractions were obtained from preparative TLC plates similar to that shown in Fig. 1. The results of the HPLC separation used in protocol B are shown in **Fig. 2.** After preliminary identification by TLC and HPLC, the free bile acids obtained by these methods were subjected to methylation and acetylation. The methyl ester/acetates were used for initial characterization by HPLC (not shown) and by GLC, which was followed by a rigorous structure determination by proton NMR and by MS.

TLC comparison of the underivatized reaction products with bile acid standards yielded results consistent with the structure assignment shown in Fig. 1. It is noteworthy that compound 3 did not co-migrate with α-muricholate (3α,6β,7α-triOH); the latter standard had an R_f similar or identical to that of compound 1. HPLC of free and methylated/acetylated reaction products and standards confirmed the conclusions drawn from the TLC data.

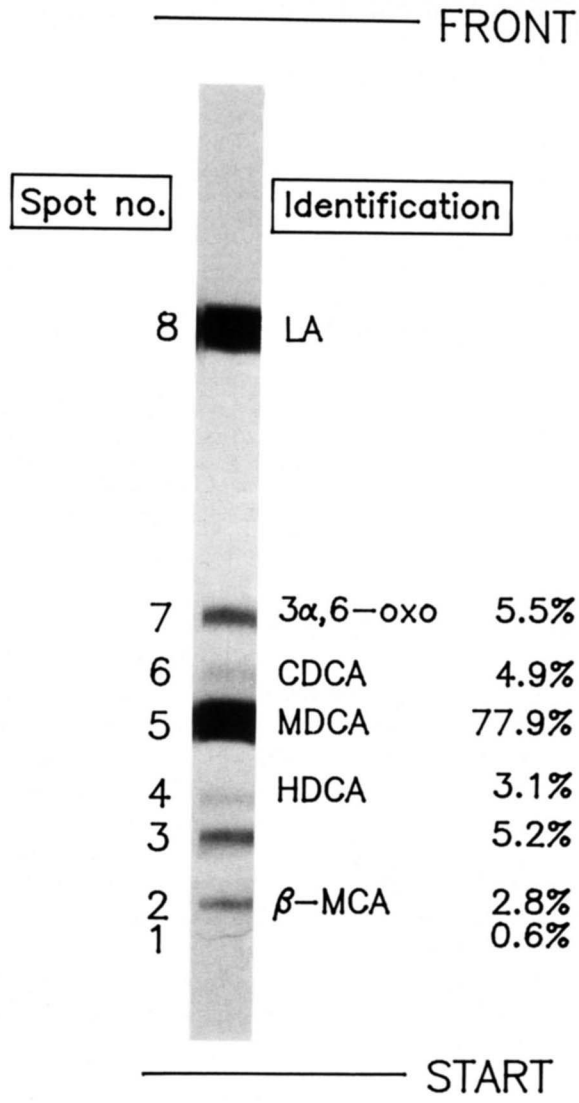

Fig. 1. Products of microsomal hydroxylation of lithocholic acid. Reaction products were applied to a silica gel TLC plate at the start line and the plate was developed twice in isooctane–ethyl acetate–acetic acid 10:10:2. An autoradiogram of the plate is shown. Abbreviations: β-MCA, β-muricholic acid (3α,6β,7β-triOH); HDCA, hyodeoxycholic acid (3α,6α-diOH); MDCA, murideoxycholic acid (3α,6β-diOH); CDCA, chenodeoxycholic acid (3α,7α-diOH); 3α,6-oxo, 3α-hydroxy-6-oxo-5β-cholanoic acid; LA, lithocholic acid (3α-OH). The percentages shown refer to the sum of hydroxylation products, i.e., they exclude unreacted lithocholic acid, and were obtained from an analytical reaction in which the formation of all products was approximately linear with time.

For GLC, a temperature program was chosen to obtain good separation of derivatized standards of a variety of bile acids, ranging from the monohydroxylated lithocholic acid (3α-OH) to trihydroxylated compounds. **Fig. 3** shows the GLC separation and retention times of standards and of methylated/acetylated fractions obtained from the hydroxylation reaction. Compound 6 (refer to Fig. 1 for numbering of fractions), which had a tendency to decompose during acetylation, yielded multiple peaks in gas

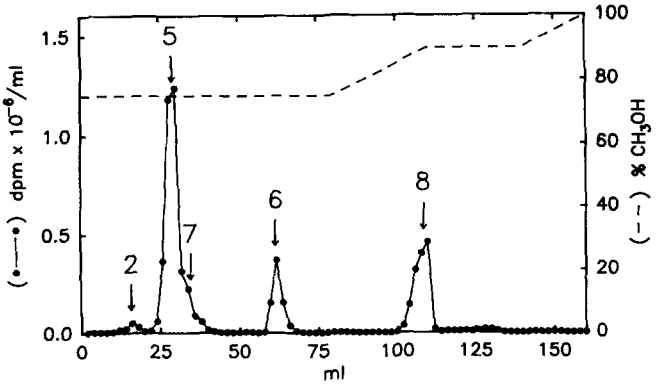

Fig. 2. HPLC separation of products of enzymatic hydroxylation of lithocholic acid. The separation of underivatized compounds was carried out on a C_{18} reverse phase column with a gradient of methanol in 5 mM potassium phosphate, pH 5.0 (dashed line) under conditions described in Methods. The numbers represent identified compounds and correspond to the nomenclature used in Fig. 1.

chromatography; the retention time of the major peak is shown in Fig. 3. In cases where the retention time of one of the isolated compounds fell within 2 min of a standard, the possible identity was further investigated by co-injection. In this way, it was established that compound 2 co-migrates with β-muricholic acid (3α,6β,7β-triOH), compounds 4 and 5 with hyodeoxycholic (3α,6α-diOH) and murideoxycholic (3α,6β-diOH) acids, respectively, compound 6 with chenodeoxycholic acid (3α,7α-diOH), and compound 8 with lithocholic acid (3α-OH). It was further established that compound 7 does not co-chromatograph with cholic acid (3α,7α,12α-triOH). No standard with a retention time similar to that of compound 3 was available.

^{1}H-NMR of methyl ester/acetate derivatives of reaction products

The small amount of available material (less than 100 μg for all fractions except murideoxycholic acid (3α,6β-diOH), of which larger amounts could be isolated) imposed some limitations on the use of this technique. Nevertheless, adequate spectra could be obtained for most fractions, thus making possible an unequivocal and direct structure determination of these compounds. The spectra are summarized in **Table 1.** For all compounds listed in Table 1, analysis of the NMR spectra confirmed the preliminary identification based on gas chromatographic behavior. The main criteria in structure elucidation were the chemical shifts of protons geminal to acetoxy groups, the coupling patterns of the signals, the number of acetoxy signals, and the chemical shifts of the angular methyl groups 18 and 19. The theoretical analysis was subsequently confirmed by matching the spectra of the isolated fractions with reference spectra of methyl ester/acetates of authentic β-muricholic (3α,6β,7β-triOH), hyodeoxycholic (3α,6α-diOH), murideoxycholic (3α,6β-diOH), 3α-hydroxy-6-oxo-5β-cholanoic, and lithocholic (3α-OH) acids, which were identical to spectra of compounds 2, 4, 5, 7, and 8, respectively (not shown). The analysis of fraction 8 served the purpose of ascertaining that it represents unreacted lithocholic acid and contains no other compounds with similar chromatographic mobility. Specifically, no isolithocholic acid (3β-hydroxy-5β-cholanoic acid) has been found at the sensitivity level of the method (approx. 5% of the main compound).

Fraction 1 was not characterized because of the small amount available. The NMR spectrum of fraction 3 indicated a trihydroxylated bile acid, in agreement with mass spectrometric data (see below). The identity of this compound remains unknown. The instability of the acetylated compound 6 (chenodeoxycholic acid, 3α,7α-diOH), a minor metabolite (less than 5% of total), prevented us from obtaining an interpretable NMR spectrum. Therefore, chenodeoxycholic acid was identified by its chromatographic behavior in TLC, HPLC, and GLC, as well as by the MS spectrum of its methyl/silyl derivative (see below).

Mass spectrometry of derivatized reaction products

For selected compounds, the structure assignment obtained by NMR was confirmed by mass spectrometry. The interpretation of mass spectra followed the principles laid out in references (28) and (29). For compound 5

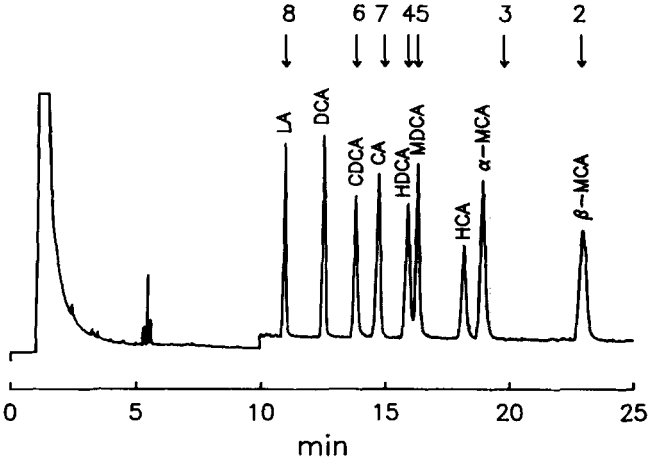

Fig. 3. GLC separation of methyl/acetyl derivatives of bile acid standards and of products of microsomal hydroxylation of lithocholic acid. The chromatogram represents the separation of a mixture of derivatized bile acid standards (see Methods for conditions). The numbered arrows represent a compilation of retention times of purified methyl/acetyl derivatives of reaction products obtained under identical chromatographic conditions. The numbers correspond to the nomenclature introduced in Fig. 1. The abbreviations are: α-MCA, α-muricholic acid (3α,6β,7α-triOH); CA, cholic acid (3α,7α,12α-triOH); DCA, deoxycholic acid (3α,12α-diOH); the remaining abbreviations are as in Fig. 1. The sensitivity of the recorder was increased fourfold at 10 min after injection.

TABLE 1. ^{1}H-NMR (300 MHz) spectra of methyl/acetyl derivatives of products obtained from lithocholic acid incubated with rat liver microsomes and NADPH

Protons	Comp. 2 (β-MCA)	Comp. 4 (HDCA)	Comp. 5 (MDCA)	Comp. 7 (3α-OH,6-oxo)	Comp. 8 (LA)
H-3	4.66br (H-3β)	4.69br (H-3β)	4.69br (H-3β)	4.69br (H-3β)	4.72br (H-3β)
H-6	4.97dd (H-6α) ($J_{6\alpha,7\alpha}$ = 3.5, $J_{6\alpha,5\beta}$ = 2.1)	5.16m (H-6β)	4.72m (H-6α)		
H-7	4.91dd (H-7α) ($J_{7\alpha,8\beta}$ = 10.8, $J_{7\alpha,6\alpha}$ = 3.5)				
Me-18	0.74s	0.66s	0.70s	0.66s	0.65s
Me-19	1.09s	1.00s	1.03s	0.86s	0.93s
Me-21	0.94d (J = 6.8)	0.93d (J = 6.8)	0.93d (J = 6.3)	0.93d (J = 6.7)	0.91d (J = 5.7)
MeO	3.68s	3.68s	3.68s	3.68s	3.67s
AcO	1.96s, 2.04s, 2.09s	2.04s, 2.07s	2.03s, 2.05s	2.04s	2.03s

The abbreviations used for bile acids are the same as in the legend to Fig. 1.

(murideoxycholate, 3α,6β-diOH), the methyl ester/*bis*(trimethylsilyl) ether was prepared and analyzed by GLC–MS (see Methods). This derivative yielded the following mass spectrum: MS (70 eV, GLC inlet), *m/z* (% int.): 550 (0.3, $M^{+\cdot}$), 535 (1.0, M–Me), 460 (41, M–TMS-OH), 445 (6, M–TMS-OH–Me), 405 (35, M–TMS-OH–(C1..C4)–H, RDA fragment), 370 (34, M–2×TMS-OH), 355 (13, M–2×TMS-OH–Me), 323 (17, M–TMS-OH–(C1..C5, C9..C12, C19)), 316 (11, M–2×TMS-OH–(C1..C4), RDA fragment), 315 (7, fragment "405"–TMS-OH), 263 (3.3, M–(C1..C6, C10, C19)), 262 (2.1, M–(C1..C6, C10, C19)–H), 255 (20, M–2×TMS-OH–s.c.), 249 (7, M–(C1..C7, C10, C19)), 228 (16, fragment "255"–(C16..C17)), 213 (40, fragment "255"–(C15..C17)), 73 (100). This spectrum agrees well with a reference spectrum of derivatized authentic murideoxycholic acid (3α,6β-diOH) obtained under identical conditions. Because of the difficulty of separating compound 6 (chenodeoxycholate) from compound 5 (murideoxycholate; see Fig. 1), compound 6 was contaminated with murideoxycholate. The gas chromatographic mobilities of the methyl ester/trimethylsilyl ether derivatives of these compounds on the column used for GLC/MS were also very similar; therefore, only a spectrum of the mixture could be obtained. The spectrum was that of a dihydroxylated bile acid and resembled in its overall characteristics the spectrum of murideoxycholate. However, certain features of the spectrum, such as the presence of an ion at *m/z* 243 (7.0, M–rings C,D–(C1..C2)–(C8..C10)), and the low intensity of the signal at *m/z* 405 as compared to that at *m/z* 370, are characteristic for chenodeoxycholate (3α,7α-diOH) as opposed to murideoxycholate (3α,6β-diOH) (ref. (28, 29) and spectra of authentic reference compounds obtained under identical conditions). For compound 7 (3α-hydroxy-6-oxo-5β-cholanoic acid), the following spectrum of the methyl/acetyl derivative was obtained: MS (70 eV, GLC inlet), *m/z* (% int.): 446 (3.6, $M^{+\cdot}$), 386 (40, M–HAc), 371 (18, M–HAc–Me), 313 (16, M–s.c.–H_2O), 271 (60, M–s.c.–HAc), 253 (35, M–s.c.–HAc–H_2O), 244 (15, M–s.c.–HAc–(C16..C17)), 229 (33, M–s.c.–HAc–(C15..C17)), 55 (100). This spectrum was consistent with the structure of 3α-hydroxy-6-oxo-5β-cholanoic acid assigned to compound 7 on the basis of its NMR spectrum. Finally, the following mass spectrum was recorded for the methyl ester/peracetate of compound 3: MS (70 eV, probe), *m/z* (% int.): 446 (0.6, M–HAc–"42"(=ketene)), 428 (1.1, M–2×HAc), 392 (3.4, fragment "446"–(C1..C4), RDA fragment), 386 (12, M–2×HAc–ketene), 373 (0.6, M–s.c.–HAc), 371 (1.7, fragment "386"–Me), 368 (9, M–3×HAc), 353 (2.0, M–3×HAc–Me), 332 (13, fragment "386"–(C1..C4), RDA fragment), 331 (2.3, M–s.c.–HAc–"42"(=ketene or (C15..C17)), 317 (1.2, fragment "332"–Me), 314 (0.6, M–3×HAc–(C1..C4), RDA fragment), 313 (6, M–s.c.–2×HAc), 303 (1.6), 271 (14, M–s.c.–2×HAc–"42"(=ketene or (C15..C17)), 253 (12, M–s.c.–3×HAc), 226 (9, M–s.c.–3×HAc–(C16..C17)), 217 (8, fragment "332"–s.c.), 211 (13, M–s.c.–3×HAc–"42"(=C15..C17)), 55 (100). The spectrum is compatible with a methylated and acetylated trihydroxy-bile acid. As mentioned previously, compound 3 remains unidentified; some possibilities for its structure will be discussed later.

Oxidation reactions

In addition to hydroxylation reactions, oxidations of hydroxyl groups to ketones also took place during the incubation of bile acids with rat liver microsomes and NADPH. These oxidations could have been catalyzed by a cytochrome P-450, leading to a ketone either via the formation of a geminal diol followed by its dehydration or by a direct dehydrogenation of the hydroxyl group by peroxycytochrome P-450 (30–32). An alternative mechanism of the formation of oxo-bile acids is via a dehydrogenase reaction utilizing $NADP^+$ formed from NADPH during

cytochrome P-450-catalyzed hydroxylations, or present in the NADPH preparation as a contaminant. In support of this possibility, we found that in preparative reactions with lithocholic acid that was tritium-labeled in position 3 ([3β-^{3}H]-3α-hydroxy-5β-cholanoic acid), only 80% of the radioactivity could be recovered in nonpolar compounds by solid phase extraction. Of the residual 20% that remained in the aqueous phase, two-thirds was volatile and was thus presumed to be in tritiated water. The remaining one-third was associated with a nonvolatile substance that was not extractable from either acidic or alkaline aqueous milieu with diethyl ether, ethyl acetate, or n-butanol; the substance may correspond to NADP^{3}H. The steroidal product of the oxidation would be unlabeled and thus not detectable by autoradiography of the TLC plate. However, when [^{14}C]lithocholic acid was used for the incubation, TLC separation of products showed a band with an R_f slightly higher than that of lithocholic acid but lower than that of 3-oxo-5β-cholanoic acid (data not shown). Treatment of this compound with diazomethane led to an increase of mobility in TLC, consistent with the formation of a methyl ester. Thus, the original compound possessed a free carboxyl group. The identity of this material remains unknown, although the above findings could indicate 3-oxochol-4-enoic acid. No further studies have been performed on this fraction.

Lithocholic acid is not the only bile acid that was shown to undergo oxidation under the conditions used in this work. The accumulating hydroxylation products of lithocholic acid can serve as substrates for oxidation. Thus, compound 7 was identified as 3α-hydroxy-6-oxo-5β-cholanoic acid, an oxidation product of one or both of the 3,6-dihydroxylated bile acids formed in the incubation.

Time course of formation of hydroxylation products

The time course of product formation in a preparative incubation in which NADPH, but not lithocholic acid, was replenished every 30 min is illustrated in **Fig. 4.** The reaction products can be divided into two groups. In one group, the rate of formation of the compound remained approximately constant over the time span of the experiment; β-muricholic acid (3α,6β,7β-triOH), 3α-hydroxy-6-oxo-5β-cholanoic acid, and the unidentified compound 3 belong to this class. In the other group, the reaction rates leveled off; in fact, in some cases the amount of the compound in question diminished after prolonged incubation. The major product of lithocholate (3α-OH) hydroxylation, murideoxycholic acid (3α,6β-diOH), as well as hyodeoxycholic (3α,6α-diOH) and chenodeoxycholic (3α,7α-diOH) acids belong to this second category. These results provided supportive information for metabolite identification and were helpful in establishing the biosynthetic relationships between the various metabolites (see Discussion).

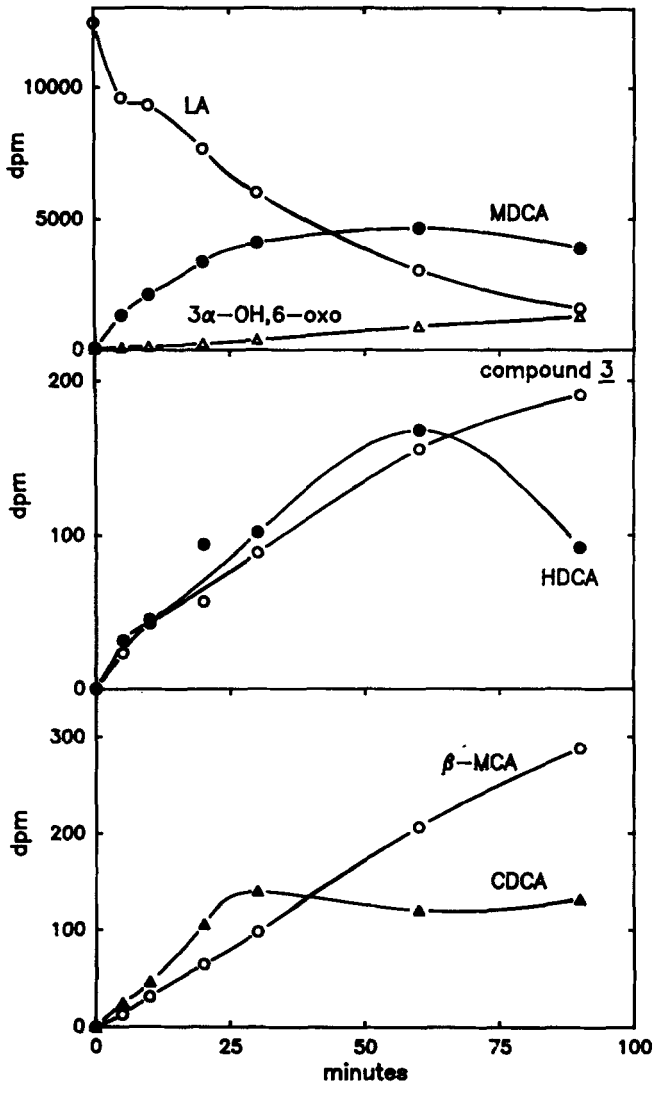

Fig. 4. Time course of microsomal hydroxylation of lithocholic acid. A preparative reaction (total volume: 6 ml), in which NADPH but not lithocholic acid was replenished by additions every 30 min, was sampled at the times indicated. Each sample (50 μl) was applied to a TLC plate, and the plate was developed twice in isooctane–ethyl acetate–acetic acid 10:10:2. Radioactive spots were localized (compare Fig. 1) and scraped; the radioactivity in each spot is shown on the ordinate and represents a mean from two identical plates. The six metabolites shown are grouped according to similar total radioactivity and graphed separately on the three panels of the figure for clarity only. Abbreviations are the same as used in Figs. 1 and 3.

DISCUSSION

The in vitro incubation of lithocholic acid with rat liver microsomes in the presence of NADPH leads to the formation of a large number of metabolites. Since our main interest was in the hydroxylation reaction, we focused our attention on compounds more polar than the starting material, operationally defined by an R_f lower than that of lithocholic acid in thin-layer chromatography. Even in

this subset of reaction products, seven distinct compounds could be identified by chromatographic techniques. Spectral identification of these compounds demonstrated that hydroxylation was not the only reaction occurring; oxidation of hydroxyl groups to keto functions was also present as a competing process, contributing to the variety of metabolites found.

The methodology used was based on a two-step approach to the identification of the isolated metabolites. A comparison of the chromatographic behavior of the fractions, in their free or derivatized form, with a battery of bile acid standards constituted the first step and yielded valuable information concerning the structure of the compounds. However, chromatographic properties of a compound are insufficient to provide definitive identification. For this, the second step of the analysis, namely a positive structure elucidation from spectral data, is necessary. Compound 7 can serve an an illustration of this point. Its R_f in the TLC system used was identical to that of deoxycholic acid (3α,12α-diOH), a possible product of lithocholic acid (3α-OH) hydroxylation. However, the mass of the molecular ion of the methylated/acetylated compound 7 , 446, suggested a dihydroxylated but monoacetylated bile acid with one double bond in the molecule; the double bond could be located either between two carbon atoms or between a carbon and an oxygen atom. It is difficult to derive the position of the double bond from the mass spectrum unless further chemical transformations are carried out; in this case, NMR yields more structural information than MS. The presence of only one signal in the range characteristic for protons geminal to acetoxy groups, and of one acetoxy signal, indicated that only one of the hydroxyl groups of the molecule was available for acetylation. The absence of signals in the area characteristic for protons geminal to free hydroxyl groups indicated that the second hydroxyl had undergone oxidation, with a loss of the adjacent hydrogen. This localized the position of the double bond, the presence of which was already inferred from the MS spectrum, to a keto function. The chemical shift of the 4.69 ppm signal, as well as its characteristic shape resulting from a complicated coupling pattern, identified it as being due to proton 3β adjacent to an acetoxy group in position 3α. Therefore, the hydroxyl group that underwent oxidation was not the 3α-OH originally present, but a group introduced by enzymatic hydroxylation of lithocholic acid. This assignment is consistent with the fact that compound 7 is radioactive; oxidation of position 3 in [3β-^{3}H]3α-hydroxycholanoic acid would lead to a loss of tritium. Finally, the position of the keto function was inferred from the chemical shift of the angular methyl group 19 in the NMR spectrum: the relatively strong upfield shift of this signal (Table 1) suggested, by inspection of Zürcher constants (33), that the oxo group is in position 6. This assignment was confirmed by comparison of the spectrum with that of an authentic sample. Thus, the identity of compound 7 with 3α-hydroxy-6-oxo-5β-cholanoic acid was concluded mostly from spectral data, and corrected the initial erroneous identification as deoxycholic acid (3α,12α-diOH) based on chromatographic mobility.

An examination of the reaction products (**Fig. 5**) revealed that at least three, and probably four, different kinds of steroid hydroxylation reactions were catalyzed by rat liver microsomes: at positions 6α, 6β, 7α, and probably 7β (the possibility of epimerization of hydroxyl groups will be addressed later). Of these, the 6β-hydroxylation was clearly the predominant type: it accounted for almost 80% of all metabolites formed. 6α- And 7α-hydroxylations were quantitatively minor, 5% or less each, but were nevertheless clearly discernible. They led to the formation of hyodeoxycholate (3α,6α-diOH) and chenodeoxycholate (3α,7α-diOH), respectively. Thus, in the rat the formation of the primary bile acid chenodeoxycholate (3α,7α-diOH) from the secondary lithocholate (3α-OH) is feasible, unlike in other mammals, such as the guinea pig and probably human, in which the reaction proceeds from 3β-hydroxychol-5-enoic acid bypassing lithocholate (34). We found no product of direct 7β-hydroxylation of lithocholic acid; however, β-muricholic acid (3α,6β,7β-triOH), a compound carrying a 7β-hydroxyl group, was present. Although we consider the formation of β-muricholic acid (3α,6β,7β-triOH) via murideoxycholic acid (3α,6β-diOH) as the most likely route (compare Fig. 5), its formation via 3α,7β-dihydroxy-5β-cholanoic acid cannot be ruled out: the latter compound would not accumulate if it were used up rapidly in a subsequent 6β-hydroxylation reaction. Experiments with murideoxycholate (3α,6β-diOH) and 3α,7β-dihydroxy-5β-cholanoate as substrates, rather than with lithocholic acid (3α-OH) itself, will be necessary to decide whether the order of 6β and 7β hydroxylation is random or whether an obligatory pathway is followed. This question is of potential interest because of the therapeutic use of 3α,7β-dihydroxy-5β-cholanoic acid (ursodeoxycholic acid) in the treatment of gallstones and of primary biliary cirrhosis (35, 36) in humans.

Secondary oxidation reactions introduced a further degree of complexity into the metabolite pattern, since each of the hydroxyl groups present in a bile acid molecule could, in principle, be further metabolized to a keto group by cytochrome P-450 or by an NADP$^+$-dependent dehydrogenase. Since [3β-^{3}H]lithocholic acid was used in most experiments, oxidation of the 3-hydroxyl group would lead to a loss of radiolabel; products of such reaction would thus become undetectable by the methods used. Of other potential target groups, the 6β-hydroxyl occurs in largest amounts and appears to be the most likely site of oxidation. In fact, we identified 3α-hydroxy-6-oxo-5β-cholanoic acid among the metabolites formed

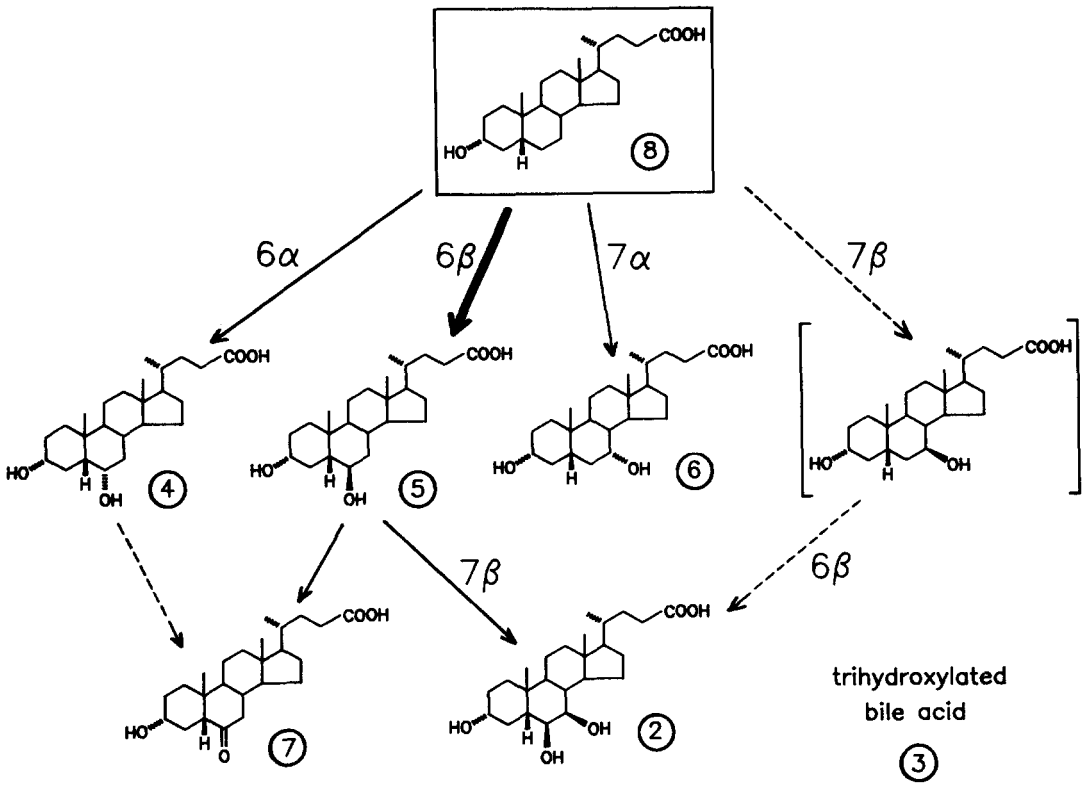

Fig. 5. Proposed relationships between the various products of microsomal metabolism of lithocholic acid. The circled numbers correspond to the nomenclature introduced in Fig. 1. The site of hydroxylation is noted at the arrows. The major reaction, 6β-hydroxylation, is depicted with a heavy arrow; less likely or hypothetical pathways are shown as dashed arrows. The product of 7β-hydroxylation of lithocholic acid, 3α,7β-dihydroxy-5β-cholanoic (ursodeoxycholic) acid, has not been found among the analyzed metabolites; it is included in the figure because of its possible role as an intermediate (see Discussion). Compound 3 is a trihydroxylated bile acid in which the position of the additional hydroxyl groups is not known.

from lithocholate (3α-OH). Even though the concentration of murideoxycholic acid (3α,6β-diOH) in the reaction mixture was much higher than that of hyodeoxycholic acid (3α,6α-diOH), without the knowledge of the stereospecificity of the secondary oxidation step it is not possible to decide definitively which of these two bile acids is the precursor for the oxidation.

In some experiments, [^{14}C]lithocholic acid was used as the substrate for hydroxylation. In these cases, additional reaction products appeared which were not detectable when [3β-^{3}H]lithocholic acid was utilized. This indicates an oxidation of the 3α-hydroxyl group with the concomitant loss of the 3β-^{3}H. The identity of these metabolites was not further investigated.

The biogenetic relationships between the various metabolites of lithocholic acid (3α-OH) proposed in Fig. 5 were substantiated by analysis of the time course of their formation. The compounds fall into two groups. In the first group, which includes murideoxycholate (3α,6β-diOH), hyodeoxycholate (3α,6α-diOH), and chenodeoxycholate (3α,7α-diOH), the formation was initially rapid but slowed down later. In some cases, the amount of the compound even decreased with time (Fig. 4). In the second group, including 3α-hydroxy-6-oxo-5β-cholanoic acid, the unidentified trihydroxylated bile acid (compound 3), and β-muricholic acid (3α,6β,7β-triOH), the rate of synthesis remained relatively constant over the entire time of the measurement. Presumably, the first group corresponds to the set of primary metabolites of lithocholic acid, i.e., compounds that are formed from this substrate in a single hydroxylation reaction (Fig. 5). The compounds in the second group are synthesized from the primary metabolites and can thus be termed secondary metabolites of lithocholic acid.

The identity of compound 3 remains to be elucidated. Its mass spectrum indicated a trihydroxylated bile acid. Despite the limited amount of material available for NMR analysis, the intense singlets due to methyl groups were clearly discernible; the presence of three acetoxy signals is consistent with a trihydroxylated bile acid. However, the signals that are most useful for the determination of the position of hydroxyls, namely those of protons geminal to the acetoxy groups, were insufficiently resolved. Since the major hydroxylation found by us for rat liver microsomes was 6β-hydroxylation, with 7α-hydroxylation also present, we considered the possibility that compound

3 is α-muricholic acid (3α,6β,7α-triOH), a normal constituent of rodent bile that was reported to be formed from lithocholic acid (3α-OH) in the rat (10). However, the chromatographic properties of compound 3 in both TLC and GC (Fig. 3) preclude its identity with α-muricholic acid; if α-muricholic acid were present, it would be localized on the TLC plate in band 1. A different hydroxylation, perhaps in position 1β, as described for the human (14, 15, 18, 37, 38) as well as for cultured rat hepatoma cells (19), may be responsible for the formation of compound 3.

Our results generally confirm and substantiate, with rigorous spectral techniques, earlier identifications of metabolites of lithocholic acid formed by the rat. Thus, the major hydroxylation product of lithocholic acid (3α-OH) in the rat, murideoxycholic acid (3α,6β-diOH), has been found previously both in vivo (10) and in vitro (9, 12), and the involvement of cytochrome P-450 in this reaction has been demonstrated using partially purified P-450 fractions (39, 40). Similarly, the formation of chenodeoxycholate (3α,7α-diOH) and of β-muricholate (3α,6β,7β-triOH) has been described by earlier workers (8–10, 12, 13, 20, 41). However, some of the metabolites have not been identified previously. We found small but significant amounts of hyodeoxycholic acid (3α,6α-diOH), a product of 6α-hydroxylation that is known to occur in the fetal human liver (15, 42) and, to a smaller extent, in human adults (18, 43–45), as well as in other species, most notably the pig (46). This process is, however, uncommon in rodents, perhaps with the exception of 6α-hydroxylation specific for allo-bile acids (47); a bile acid that could be either hyodeoxycholate (3α,6α-diOH) or ursodeoxycholate (3α,7β-diOH) has been found in the bile of rats subjected to chronic administration of taurolithocholic acid (13), but a precursor–product relationship has not been directly established. We assume, mainly on the basis of the time course of its formation, that hyodeoxycholate (3α,6α-diOH) is a product of direct 6α-hydroxylation of lithocholic acid (3α-OH). However, the presence of 3α-hydroxy-6-oxo-5β-cholanoic acid, another metabolite of lithocholic acid that has not been previously described in the rat, suggests an alternative route of formation of hyodeoxycholate (3α,6α-diOH), namely by epimerization of murideoxycholate (3α,6β-diOH); the 6-oxo compound would be the intermediate of the oxidoreduction reaction. An analogous process, the formation of 6α-hydroxy-3-oxo-5β-cholanoic acid from hyodeoxycholate (3α,6α-diOH), with subsequent reduction to 3β,6α-dihydroxy-5β-cholanoic acid, has been recently described for human fetal liver microsomes (15). In addition to the unequivocal demonstration of the oxidation in position 6, we have suggestive evidence for oxidation in position 3 (see above). Finally, β-muricholic acid (3α,6β,7β-triOH) could be formed by epimerization in position 7 from α-muricholic acid (3α,6β,7α-triOH) (10, 11), which would in turn be derived from chenodeoxycholate (3α,7α-diOH) by 6β-hydroxylation. It has been pointed out (15) that epimerizations might have a physiological function, as α and β epimers of bile acids differ in their toxicity.

The results described above demonstrate that hydroxylation constitutes a major detoxification pathway for lithocholic acid in the rat. This conclusion is consistent with the results of in vivo studies which show that a considerable fraction, perhaps one-third, of the acutely administered compound is secreted in the form of rehydroxylated products (8, 10, 41, 48, 49); during chronic exposure to taurolithocholic acid, essentially all of the compound was rehydroxylated (13). For an assessment of the relative importance of hydroxylation in comparison to the alternative detoxification pathway, namely direct conjugation of lithocholic acid, several facts need to be considered. As mentioned previously, the 3-O-glucuronide and 3-O-sulfate of lithocholic acid are cholestatic. Since neither compound undergoes further hydroxylation (41, 48) and both are sufficiently hydrophobic to be poorly excreted in the urine ((48) and J. M. Little, unpublished results), they tend to accumulate in the circulation and thus exacerbate cholestasis. However, the re-uptake of both the 3-O-sulfate (50) and the 3-O-glucuronide (51) of lithocholic acid from the gut is impaired, so that whatever fraction of these compounds is still secreted into bile is efficiently removed with the feces. Generally, sulfation and hydroxylation are mutually exclusive: bile acid sulfates do not undergo hydroxylation, and di- or trihydroxylated bile acids are poor substrates for sulfation (41). Similarly, glucuronidation appears to preclude subsequent hydroxylation (but not vice versa: certain polyhydroxylated bile acids are efficiently glucuronidated (7)). Both modes of detoxification (hydroxylation and conjugation) are probably present in all mammals that have been studied, but their relative importance seems to be strongly species-specific. In the human, sulfation probably predominates (52), with 6α-hydroxylation, coupled to the formation of a 6-O-glucuronide and its rapid excretion (7), present as a pathway with a lower capacity but perhaps higher affinity for the substrate. In the rat, hydroxylation may be a more prominent detoxification pathway, as suggested previously (13, 53).

In summary, the detoxification of lithocholic acid by the rat via hydroxylation has been characterized by rigorous identification of reaction products. The knowledge of the identity of the metabolites, together with data on the rates of their formation, made it possible to propose a scheme of the oxidative metabolism of lithocholic acid by rat liver microsomes. This scheme contributes to a better understanding of both the relationships between the various metabolites and the reactions that lead to their formation.

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