Metabolism of lithocholic acid in the rat: formation of lithocholic acid 3-O-glucuronide in vivo

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Abstract Milligram amounts of [3β-^{3}H]lithocholic (3α-hydroxy-5β-cholanoic) acid were administered by intravenous infusion to rats prepared with a biliary fistula. Analysis of sequential bile samples by thin-layer chromatography (TLC) demonstrated that lithocholic acid glucuronide was present in bile throughout the course of the experiments and that its secretion rate paralleled that of total isotope secretion. Initial confirmation of the identity of this metabolite was obtained by the recovery of labeled lithocholic acid after β-glucuronidase hydrolysis of bile samples. For detailed analysis of biliary metabolites of [^{3}H]lithocholic acid, pooled bile samples from infused rats were subjected to reversed-phase chromatography and four major labeled peaks were isolated. After complete deconjugation, the two major compounds in the combined first two peaks were identified as murideoxycholic (3α,6β-dihydroxy-5β-cholanoic) and β-muricholic (3α,6β,7β-trihydroxy-5β-cholanoic) acids and the third peak was identified as taurolithocholic acid. The major component of the fourth peak, after isolation, derivatization (to the methyl ester acetate), and purification by high pressure liquid chromatography (HPLC), was positively identified by proton nuclear magnetic resonance as lithocholic acid 3α-O-(β-D-glucuronide). These studies have shown, for the first time, that lithocholic acid glucuronide is a product of in vivo hepatic metabolism of lithocholic acid in the rat. —**Little, J. M., P. Zimniak, K. E. Shattuck, R. Lester, and A. Radominska.** Metabolism of lithocholic acid in the rat: formation of lithocholic acid 3-O-glucuronide in vivo. *J. Lipid Res.* 1990. **31:** 615–622.

Supplementary key words bile acid • cholestasis • glucuronide

Lithocholic acid is a hydrophobic, toxic compound that has been shown to cause cholestasis in experimental animals (1–4). One result of cholestasis is a dramatic increase in the concentration of bile acids, including lithocholic acid, in serum and liver. The deleterious effects of this accumulation of lithocholic acid (and other bile acids) can be attenuated by two detoxification pathways available in the liver: oxidative reactions, mainly hydroxylations, and conjugation with sulfuric or glucuronic acids. The relative importance of these two pathways appears to be strongly species-specific.

In the human, the 3α-hydroxyl group of lithocholic acid is efficiently sulfated (5–9), but not glucuronidated (10). Alternatively, lithocholic acid can undergo hydroxylation in position 6α (11–13); the newly formed 6α-hydroxylated bile acids are rapidly conjugated with glucuronic acid in position 6 (10, 14, 15) and are efficiently excreted in urine (16). The conspicuous lack of the formation of lithocholic acid glucuronide is understandable in light of the somewhat unexpected finding that, at least in the rat, the compound is not less, but more toxic (cholestatic) than lithocholic acid itself (17).

The metabolism of lithocholic acid in the rat presents a more complex picture. In contrast to the human, formation of the 3-sulfate is a minor reaction (18–20). Lithocholic acid is known to be efficiently hydroxylated by the rat, both in vivo (19, 21) and in vitro by liver microsomes (22–24). Multiple hydroxylation reactions of various specificities occur, with 6β-hydroxylation predominating (24). The 6β-hydroxylation of lithocholic acid yields murideoxycholic acid (3α,6β-diOH) which by further hydroxylation can be converted to the muricholates (α-muricholic acid, 3α,6β,7α-triOH, and β-muricholic acid, 3α,6β,7β-triOH), normal constituents of rat bile which comprise approximately 20% of the bile acid pool (25). Therefore, 6β-hydroxylation in the rat not only detoxifies lithocholic acid but is also a salvage pathway that returns the additionally hydroxylated products to the bile acid pool.

The scheme of lithocholic acid metabolism in rat liver described above does not incorporate glucuronidation reactions. Indeed, the formation of bile acid glucuronides after administration of lithocholic acid to the rat has not been reported previously. However, studies from our and other laboratories have established that rat liver microsomes have a high capacity for the formation of lithocholic acid 3-O-glucuronide (26, 27), as well as glucuronides of

Abbreviations: TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance.

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the 6-hydroxylated bile acids that are the products of microsomal hydroxylation of lithocholic acid (24, 28). Other bile acids that are substrates of UDP-glucuronosyltransferases in vitro, when administered intravenously to rats, have been shown to be secreted in bile as glucuronide conjugates (29, 30). Faced with this apparent contradiction, we considered the possibility that the UDP-glucuronosyltransferase activities seen by us in hepatic microsomes may be latent or otherwise down-regulated in vivo. Alternatively, it was conceivable that bile acid glucuronides have not been found previously because methods of isolation and analysis were, in many cases, not devised for the identification of glucuronides. To examine these possibilities, we used methodology developed specifically for the purpose of analysis of bile acid glucuronides to evaluate the role of the glucuronidation reaction in the hepatic response to a lithocholic acid overload.

METHODS AND MATERIALS

Experimental design

The major objective of the studies was to determine whether lithocholic acid glucuronide is a product of the hepatic metabolism of lithocholic acid in the rat and, if so, whether its presence could be correlated with cholestasis. For this purpose, rats prepared with a biliary fistula and femoral vein catheter were infused with [^{3}H]lithocholic acid. Two groups of rats were used. In the first group (Group 1), bile was drained overnight to deplete the endogenous bile acid pool before beginning infusions; in the second group (Group 2), infusions were started 1–2 h after completion of surgery. Biliary secretion of label and bile flow were measured throughout the period of study. In several experiments, labeled biliary metabolites in individual bile samples were examined by thin-layer chromatography (TLC). Detailed analysis and identification of metabolites was carried out with bile pools from several animals.

Experimental protocol

The surgical preparation of the animals (male Sprague-Dawley rats, 200–350 g) and the synthesis of [3β-^{3}H]lithocholic acid have been described previously (17, 31). Lithocholic acid (both labeled and cold carrier) was dissolved in methanol, converted to the sodium salt by addition of an equimolar amount of aqueous NaOH, and dried under N_2. The dry residue was suspended in 7.5% bovine serum albumin (in 5% glucose/0.45% NaCl) by sonication and the bile acid was solubilized, at a maximum concentration of 5.3 μmol/ml (2 mg/ml), by heating this suspension at 45°C for 1–2 h.

After surgery, rats were placed in Bollman restraining cages under heat lamps and an infusion of 5% glucose in 0.45% saline (0.025 ml · 100 g body weight^{-1} · min^{-1}) was begun and was maintained throughout the study, except for the period of lithocholic acid infusion. In Group 1, bile was drained overnight before beginning the experiment. Bile was collected for three 10-min periods to establish basal bile flow rates after which the lithocholic acid was infused for 90 min at a rate of 0.2 μmol · 100 g body weight^{-1} · min^{-1}. Bile was collected in tared tubes at 10-min intervals through the infusion period, at 30-min intervals for the next 3–4 h, and as a single sample until the animal was killed at 24 h. With Group 2, bile was drained only for the time needed for the rats to recover from anesthesia (1–2 h) before beginning the infusion of lithocholic acid at rates from 0.05–0.4 μmol · 10 g body weight^{-1} · min^{-1}. Control rats were infused with the albumin solution over the same time period and at the same flow rates as the lithocholic acid. Bile was collected at intervals as above during the infusion and until the time of killing. In all studies, a single urine sample was collected from 0–24 h. At the time of killing, blood was drawn from the abdominal aorta with the plasma being separated and saved; liver and kidneys were removed and frozen for later analysis.

Analytical techniques

Bile and urine volumes were determined gravimetrically in tared tubes. Aliquots of bile, urine, and plasma were analyzed for radioactivity in Liquiscint scintillation cocktail (National Diagnostics, Manville, NJ) using an LKB 1214 liquid scintillation counter (Pharmacia-LKB, Gaithersburg, MD). Total plasma isotope was calculated by using 5% of body weight as an estimate of total plasma volume. Tissue samples were analyzed for isotope content as previously described (31).

Techniques for preliminary purification, hydrolysis, and TLC of bile samples were as previously reported (17, 31). The solvent systems used for TLC were as follows: system 1: 2,2,4-trimethylpentane–ethyl acetate–glacial acetic acid 5:5:1, developed twice; system 2: chloroform–methanol–glacial acetic acid–water 65:25:2:4; system 3: absolute ethanol–ethyl acetate–concentrated ammonium hydroxide 45:45:15. The distribution of radioactivity on analytical TLC plates was evaluated using a Berthold Tracemaster 20 TLC scanner (Berthold Analytical Instruments, Nashua, NH). Purification and separation for the preliminary survey of labeled metabolites was carried out using a modification of the extraction technique; extracts of previously analyzed individual bile samples were pooled and readsorbed on a C_{18} cartridge and bile acids were eluted with a stepwise gradient of methanol in water (3 × 3 ml each of 5, 10, 15, 20, 30, 40, 50, 60, 80, and 100% methanol). Each 3-ml fraction was checked for label and surveyed for bile acid content by TLC; fractions were pooled accordingly and analyzed in more detail by TLC, before and after various hydrolytic procedures.

For hydrolysis of glucuronides, bile samples diluted with 0.075 M sodium phosphate buffer, pH 6.8, were incubated with 50 units of *E. coli* β-glucuronidase for 24 h at 37°C. Cholylglycine hydrolase hydrolysis was carried out in 0.1 M sodium acetate, pH 5.6, with 25–100 units of enzyme, incubated at 37°C for 24 h. For alkaline hydrolysis, samples were dissolved in 2 N NaOH and heated at 120°C for 16 h. Where necessary, samples were solvolyzed using the method described by Hirano et al. (32). Products of all hydrolyses were recovered from incubation mixtures by passage through C_{18} cartridges.

Tritiated water content was determined by evaporation; the aqueous effluent containing label not retained by the C_{18} cartridge or the methanol extract of tissue was taken to dryness, the residue was redissolved in water and counted, and the difference between pre- and post-evaporation values was taken as the ^3H_2O content.

Large scale purification of biliary metabolites of lithocholic acid

Bile samples collected over the first 3–4 h from Group 2 rats (see Table 1, nos. 5–10) were pooled into a single sample and labeled metabolites were extracted as previously described (31). The resulting crude fraction (210 mg) was dissolved in 30 ml of 0.1 M glycine-trichloroacetate buffer, pH 2.8, and applied to a modified C_{18} cartridge (1.5 × 4 cm) prepared by combining the packing from four standard 6-ml cartridges. The sample was eluted with a continuous linear gradient of 0 to 75% methanol in water (600 ml total volume). Fractions (5 ml) were collected, assayed for radioactivity, and pooled accordingly. TLC analysis (in systems 2 and 3) of each labeled peak recovered from the fractionation provided a preliminary characterization of labeled metabolites. The fraction found to contain the putative lithocholic acid glucuronide was further purified and analyzed in detail as described below. The other fractions were subjected to solvolysis and cholylglycine hydrolase or alkaline hydrolysis and the free bile acids thus liberated were analyzed by TLC.

The eluate containing the glucuronide was evaporated to dryness and the methyl ester-acetate derivative, prepared as described before (27), was isolated by high pressure liquid chromatography (HPLC). Briefly, the derivatized glucuronide was purified by isocratic elution from a μBondpack C_{18} column (0.78 × 30 cm, Waters, Milford, MA) with methanol–water 75:25 at a flow rate of 2.0 ml/min. A refractive index detector (Model 771, Micrometrics, Norcross, GA) was used to detect peaks. The purified product was dissolved in deuterated chloroform and analyzed by 300 MHz ^{1}H-NMR using a General Electric QE-300 instrument (28) to provide confirmation of the assigned structure.

RESULTS

Bile flow and biliary secretion of [^{3}H]lithocholic acid

In our hands, cholestasis could be consistently produced only in rats whose endogenous bile acid pool had been depleted by overnight biliary drainage. The lack of a consistent effect of lithocholic acid on bile flow in Group 2 rats made any correlation of bile flow and biliary metabolite secretion difficult, but maintenance of bile flow in these animals allowed for infusion of larger amounts of lithocholic acid and higher recovery of labeled metabolites in bile. Therefore, bile from Group 2 animals was used for the large scale purification of metabolites and bile flow data obtained from Group 1 rats only are presented here.

Rates of biliary secretion of isotope and of bile flow from a representative experiment using a bile acid-depleted rat (**Table 1**, no. 1) are shown in **Fig. 1**. Although the time of onset and the extent of cholestasis varied from rat to rat, the overall pattern was similar in all animals. Infusion of lithocholic acid in albumin produced an initial choleresis with bile flow increasing 32.0 ± 8.5% (n = 4) above basal levels over the first 20–40 min of infusion. (In two control rats, infusion of the albumin vehicle alone increased bile flow 28–32%.) This choleretic phase was followed by cholestasis during which bile flow decreased to minimum rates that averaged 10% of the initial rates (range: 2–25%). The nadir of flow was reached 2–3 h after the start of the experiment (or 0.5–1.5 h after termination of the infusion); after this, flow rates increased slowly but were still below basal levels at the time the animals were killed (at 24 h, flow rates were 57.2 ± 21.2% of pre-infusion values). Over this same period, biliary secretion of isotope more or less paralleled changes in bile flow with the highest rates of secretion found during the choleretic phase. However, recovery of isotope in bile (see Table 1) was low and highly variable: after 4 h, 26.9 ± 16.4% of the administered dose was recovered in bile while the corresponding figure at 24 h was 32.9 ± 16.6%. Significant isotope was found in urine (12.8 ± 6.0%), liver (2.8 ± 0.8%), and plasma (2.3 ± 0.5%) and total recovery averaged only 51.2 ± 21.3% of the dose.

Bile samples, urine, plasma, liver, and kidney from two animals with significantly different recoveries (Table 1, nos. 1 and 3) were analyzed for tritiated water content. Measurable tritiated water was present in bile samples within 20 min after the start of the infusion. The proportion of total biliary radioactivity represented by water increased with time but the amounts in samples collected over the first 4 h were relatively small with cumulative excretion equal to less than 10.0% of the recovered label. In the overnight bile collections (4–24 h), however, a larger portion of biliary isotope (32.8 and 22.4%) was present as water. More than half of activity recovered from liver

TABLE 1. Experimental conditions and isotope recoveries

Exp. No.	Rat Wt.	Total Dose	Infusion Period	% Recovery					
				Bile	Urine	Plasma	Liver	Kidney	Total
	g	*μmol*	*min*						
Group 1[a]									
1	204	36.0	90	24.9	7.6	2.5	3.7	0.5	39.2
2	253	45.5	90	14.4	8.3	2.3	3.1	0.4	28.5
3	264	45.6	90	52.1	15.0	1.6	1.9	0.3	70.9
4	210	40.2	90	40.1	20.4	2.7	2.6	0.5	66.3
Group 2[b]									
5	259	66.2	120	58.8	0.6	0.8	nd[c]	nd	60.2
6	349	66.2	120	48.0	5.8	2.7	nd	nd	56.5
7	304	69.5	180	63.0	0.1	nd	nd	nd	63.1
8	324	69.5	180	75.6	2.1	1.1	nd	nd	78.8
9	314	71.7	150	47.9	0.1	nd	nd	nd	48.0
10	272	84.2	150	84.4	1.8	0.2	nd	nd	86.4

[a]Overnight biliary drainage.
[b]One to 2 h biliary drainage.
[c]nd, not done.

(53.7 and 60.2%) and almost all of that found in kidney (88.7 and 89.0%) was present as water. All of the isotope recovered in urine (7.6 and 15.0% of the dose) and plasma (2.5 and 1.6%) was in the form of tritiated water.

Preliminary identification of biliary metabolites of lithocholic acid

Individual bile samples from the same two animals used above (Table 1, nos. 1 and 3) were examined by TLC to investigate the time course of metabolite secretion. Extracts of bile samples were chromatographed first in solvent system 3 which separates bile acid glucuronides from other conjugated and free bile acids. Scans of plates run in this system demonstrated a labeled component in each bile sample which chromatographed with the same mobility (R_f) as authentic lithocholic acid 3-O-glucuronide. Further validation of the identity of this compound will be presented later. The amount of isotope in each spot was determined and the results are shown, compared to total isotope secretion, in **Fig. 2**. Glucuronide secretion in both animals paralleled total isotope secretion and represented an average of 10% of each sample. In overnight bile samples, the glucuronide was the predominant component, accounting for 32.1 and 51.8% of the isotope in the two samples. Thus, over 24 h, 20.5 and 16.1% of the label recovered in bile was present as lithocholate glucuronide.

Aliquots of bile extracts were also chromatographed in system 2. Qualitatively, each bile sample contained 3–4 significant labeled components in varying proportions. These were preliminarily identified as taurolithocholic acid, a taurodihydroxy bile acid overlapping with lithocholic acid glucuronide, and a taurotrihydroxy bile acid.

To facilitate further analysis, a partial fractionation of pooled bile samples from both animals was carried out as described in Methods. Pooled fractions from the stepwise elution of bile acids from the C_{18} cartridge were analyzed by TLC in systems 2 and 3. This survey showed that the putative lithocholic acid glucuronide was recovered in relatively pure form (ca. 70% of the fraction) in the 80% methanol fraction. The major component of the fraction co-chromatographed with authentic lithocholic acid glucuronide in both systems (R_f = 0.28 and 0.02, in systems 2 and 3, respectively) and was well separated in both

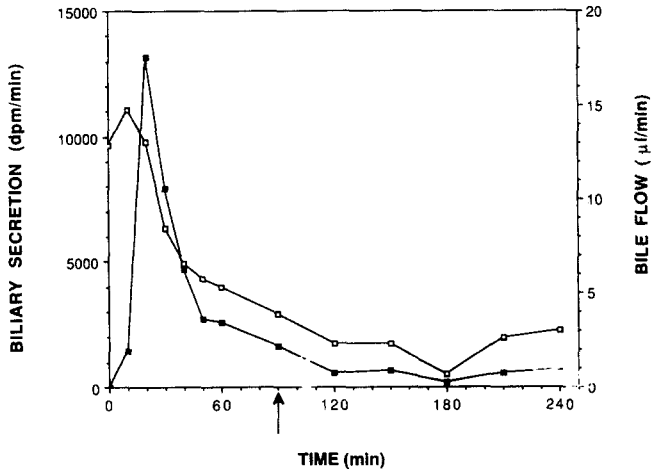

Fig. 1. Rates of biliary secretion of isotope (closed symbols) and bile flow (open symbols) from a representative experiment in which [^{3}H]lithocholic acid was administered by intravenous infusion to a bile acid-depleted rat with a biliary fistula. The arrow indicates the end of the infusion period.

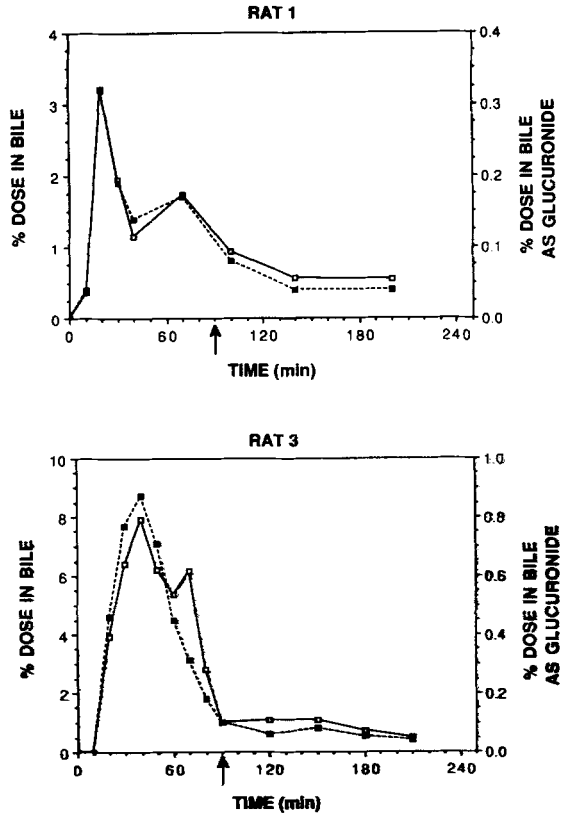

Fig. 2. Pattern of total isotope secretion (open symbols) as compared to secretion of labeled lithocholic acid glucuronide (closed symbols) in two rats infused with [^{3}H]lithocholic acid.

systems from the only other labeled material. Following β-glucuronidase hydrolysis of this fraction and TLC in system 1, more than 80% of the activity on the plate was associated with a spot with the R_f (0.72) of authentic lithocholic acid. Material eluted with 10–30% methanol appeared to contain taurine-conjugated di- and trihydroxylated bile acids and possibly sulfate conjugates. Rechromatography of this fraction after solvolysis resulted in no significant change in the distribution of activity, eliminating sulfation as a major form of conjugation. Elution with 40–60% methanol recovered mainly a compound chromatographing in system 2 with the R_f (0.37) of taurolithocholic acid. After cholylglycine hydrolase hydrolysis of the remainder of the 10–30% and 40–60% methanol fractions, the resulting free bile acids were identified by their chromatographic mobilities in system 1. These results are summarized in **Fig. 3** with tentative assignment of form of conjugation.

Large scale purification and identification of biliary metabolites

The results of the continuous gradient fractionation of Group 2 pooled bile extracts are shown in **Fig. 4**. Four major peaks were recovered; fractions comprising A and B, which were incompletely resolved, were combined and accounted for 41% of the total label; fractions in C and D contained 24 and 27%, respectively, of the recovered isotope. Preliminary TLC analysis of each fraction indicated that peak A + B contained a multiplicity of taurine and/or sulfate conjugates of additionally hydroxylated metabolites, peak C had only one major component with the chromatographic mobility of taurolithocholic acid, and peak D contained, in relatively pure form, a labeled compound that co-chromatographed with lithocholic acid 3-O-glucuronide.

Solvolysis and cholylglycine hydrolase hydrolysis of peak A + B, followed by TLC of the free acids and of their methyl esters showed that the major bile acid present was murideoxycholic (3α,6β-diOH) acid. Also identified were a significant amount of β-muricholic (3α,6β,7β-triOH) acid and small amounts of ursodeoxycholic (3α,7β-diOH), hyodeoxycholic (3α,6α-diOH), and chenodeoxycholic (3α,7α-diOH) acids. Similar hydrolysis and analysis of peak C resulted in the recovery of a single major component which had the chromatographic mobility of lithocholic acid. After β-glucuronidase hydrolysis of material from peak D, more than 80% of the isotope was found in the spot having the R_f of free lithocholic acid. The inclusion of 1 mM saccharo-1,4-lactone in the incubation mixture completely inhibited β-glucuronidase hydrolysis; no free lithocholic acid (only intact lithocholic acid glucuronide) was detected in chromatograms of these samples.

Unequivocal identification of structure was carried out only on peak D. After derivatization and isolation by HPLC, the purified metabolite, as the methyl ester acetate, was analyzed by NMR. Spectra were taken at 300 MHz in $CDCl_3$ and chemical shifts were calculated

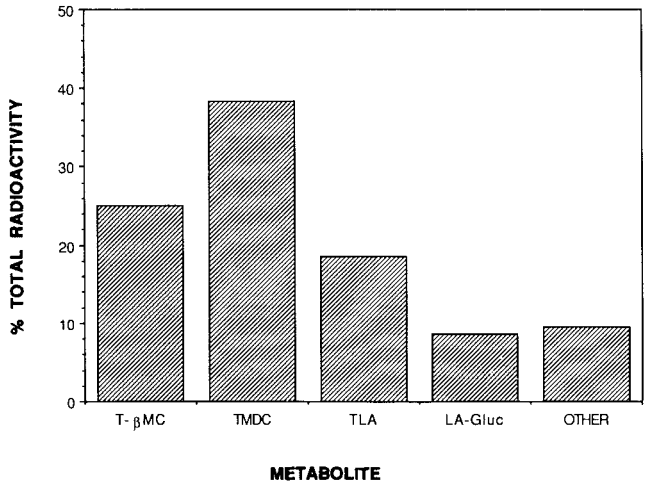

Fig. 3. Distribution of radioactivity in metabolites of lithocholic acid recovered in bile from rats infused with [^{3}H]lithocholic acid. Abbreviations: T-β-MCA: tauro-β-muricholic (3α,6β,7β-triOH) acid; TMDCA: tauromurideoxycholic (3α,6β-diOH) acid; TLA: taurolithocholic (3α-OH) acid; LAGluc: lithocholic acid 3-glucuronide.

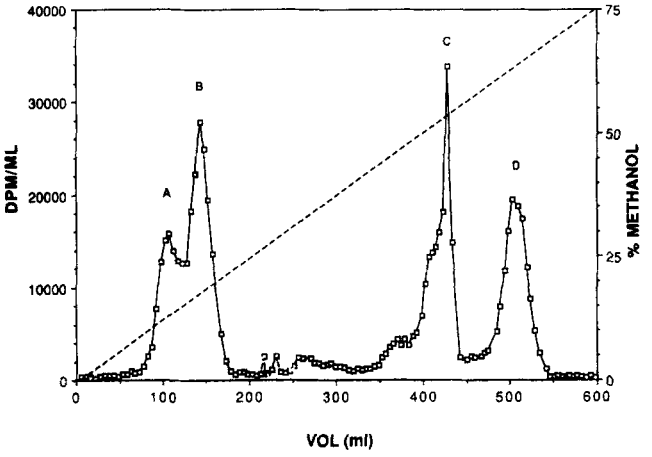

Fig. 4. Fractionation of labeled metabolites recovered in bile of rats infused with [^{3}H]lithocholic acid. A crude extract of bile acids from pooled bile samples was applied to a modified C_{18} reversed-phase cartridge and metabolites were eluted with a continuous gradient of methanol in water (dotted line). Major components: peaks A + B: TMDCA and T-β-MCA; peak C: TLA; peak D: LAGluc. Abbreviations as in Fig. 3.

relative to the $CHCl_3$ singlet (7.26 ppm). The spectrum of the isolated material was identical in all diagnostically important signals to that of chemically synthesized, methylated, and acetylated 3-O-glucuronide of lithocholic acid (27). Points of identity included the presence of two methyl ester and three acetate singlets and the chemical shifts of the geminal proton H-3β in the steroid and proton H-1' and H-2' (and others) in the sugar. All of these confirm the structure of an ether-linked β-glucuronide on the 3α-hydroxyl group. No signals characteristic of an ester (carboxyl-linked) glucuronide or of additional hydroxyl groups on the steroid moiety, either glucuronidated or acetylated, were observed. Therefore, the NMR spectrum provided an unequivocal identification of the original compound as lithocholic acid 3α-O-(β-D-glucuronide).

DISCUSSION

In spite of the demonstrated toxicity of lithocholic acid glucuronide (17) and the presence in rat liver of mechanisms for glucuronidation of lithocholic acid (26, 27) and biliary secretion of the glucuronide conjugate (17), formation of this compound after administration of lithocholic acid has not been reported. This could be because the focus of most previous studies has been induction of cholestasis per se or changes to the steroid nucleus, not patterns of conjugation. Also, bile acid glucuronides could easily have gone undetected if appropriate analytical techniques were not used. To clarify the situation, we have administered milligram amounts of [^{3}H]lithocholic acid to rats with a biliary fistula and have characterized the biliary metabolites with methods developed specifically for the isolation and analysis of bile acid glucuronides.

Initial identification of the glucuronide conjugate was accomplished by TLC analysis of individual bile samples or small bile pools from Group 1 rats. All samples analyzed contained a labeled metabolite with the chromatographic mobility of authentic lithocholic acid glucuronide which was recovered as lithocholic acid after β-glucuronidase hydrolysis.

Rigorous proof of the structure of the putative glucuronide was carried out with material extracted, isolated, and purified from a pool of bile from Group 2 rats. Chromatographic identification of the glucuronide conjugate was used to monitor the isolation and purification procedure and, at several points, verification of the form of conjugation was obtained, as above, by recovery of lithocholic acid after β-glucuronidase hydrolysis (which was inhibited by saccharolactone) of a portion of the material being purified. Final assessment of structure was accomplished by NMR analysis of the methylated, acetylated compound. The spectrum of the purified, derivatized biliary metabolite was identical to that of the methyl ester acetate of lithocholic acid 3-O-glucuronide obtained by chemical or enzymatic synthesis with no evidence of any other metabolic alteration of the compound. Detailed analysis of the NMR spectrum provided unequivocal identification of the original compound as lithocholic acid 3α-O-(β-D-glucuronide).

Labeled metabolites of lithocholic acid other than the glucuronide conjugate also were identified by TLC. Since these products have been thoroughly examined and identified by others (9, 21–24), our proof of structure was confirmatory rather than absolute, with tentative assignment of conjugation based on results from various hydrolytic procedures. Thus, as has been reported before, the major biotransformations of lithocholic acid, other than glucuronidation, were additional hydroxylation (primarily in the 6β-position) and taurine conjugation. This resulted in the recovery of significant amounts of tauromurideoxycholic, tauro-β-muricholic, and taurolithocholic acids. Small amounts of other dihydroxylated products of lithocholic acid metabolism were also identified.

The presence of tritiated water in bile, urine, and plasma samples indicated that some metabolite(s) had not been detected due to loss of the 3β-^{3}H such as would occur upon oxidation of the 3-hydroxyl group. The fact that major amounts of tritiated water were found only in the overnight bile collection and in plasma and urine suggested that the transformation at the 3-hydroxy position was gradual and probably related to the length of time the substrate was exposed to hepatic enzyme systems.

Although other investigators have induced cholestasis with lithocholic acid in rats whose bile acid pools had not been depleted by overnight drainage (1–3, 33–36), our

success with this preparation was very limited, perhaps due to differences in rat strain, type of anesthesia, or other factors. In rats with depleted bile acid pools (Group 1), lithocholic acid infusion consistently produced cholestasis although the extent and time of onset were variable. Bile flow minima differed considerably but were all significantly lower (by 75–98%) than basal flow rates established before starting the infusion.

Lithocholic acid glucuronide was present in sequential bile samples throughout the experiments and its secretion more or less paralleled both total isotope secretion and bile flow. Correlation of glucuronide secretion and cholestasis was complicated by the formation and secretion in bile of significant amounts (as much as 50% of total label in some samples) of taurolithocholic acid which is itself cholestatic (3, 4, 33, 36, 37). In addition, recent studies using a mutant strain of rats in which biliary secretion of organic anions, such as bilirubin glucuronide and dibromosulphthalein, is defective have shown that biliary secretion of bile acid 3-O-glucuronides and sulfates is also significantly reduced (38–40). This suggests that these conjugates are secreted into bile by the "organic anion" transport system while other bile acids, including taurolithocholic acid, are secreted via the "bile acid" transport system. Thus, infusion of lithocholic acid results in the secretion of at least two cholestatic metabolites which are transported by two different mechanisms. Since the transport of non-bile acid organic anions is considered to be a major factor in bile acid-independent bile flow (41), it may be that lithocholic acid glucuronide contributes to cholestasis mainly via effects on this fraction of bile water secretion. The fact that, in the above-mentioned mutant rats, bile flow is significantly decreased despite normal biliary bile acid output (39) and that doses of lithocholic acid glucuronide which induce cholestasis in control animals had no effect on bile flow in mutant animals (38) lend support to this speculation. Other metabolites of lithocholic acid, e.g., taurolithocholic acid, may induce cholestasis by decreasing bile acid secretion, thereby reducing bile acid-dependent bile flow.

In summary, the major accomplishment of these studies was the unequivocal identification of lithocholic acid 3-O-glucuronide as a product of in vivo hepatic metabolism of lithocholic acid in the rat. This highly toxic metabolite may play a role in the initiation or, more likely, the maintenance of lithocholic acid-induced cholestasis in the rat.

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