

Defective biliary secretion of bile acid 3-O-glucuronides in rats with hereditary conjugated hyperbilirubinemia¹

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Abstract Biliary secretion of bile acid glucuronides was studied in control rats and in rats with a congenital defect in hepatobiliary transport of organic anions (GY rats). In control animals, hepatobiliary transport of [³H]lithocholic acid 3-O-glucuronide and [³H]cholic acid 3-O-glucuronide was efficient (>95% in 1 h) and comparable to that of [¹⁴C]taurocholic acid. Secretion of both glucuronides was impaired in GY rats (24% and 71% at 1 h), whereas that of taurocholate was similar to control values. However, recovery of the glucuronides in bile was nearly complete within 24 h; virtually no radioactivity was found in urine. In control rats, biliary secretion of lithocholic acid 3-O-glucuronide, but not that of cholic acid 3-O-glucuronide or taurocholate, could be delayed by simultaneous infusion of dibromosulphthalein. In mutant rats, dibromosulphthalein infusion was also able to inhibit secretion of cholic acid 3-O-glucuronide. [³H]Hydroxyeticianic acid, a C₂₀ short-chain bile acid, was secreted by control rats as a mixture of 20% carboxyl-linked and 80% hydroxyl-linked (3-O-)glucuronide; secretion was very efficient (99% in 1 h). In GY rats, secretion was drastically impaired (16% at 1 h and 74% over a 24-h period). Initially, the mutant secreted more carboxyl- than hydroxyl-linked glucuronide, but the ratio reached that of control animals after 24 h. The rates of formation of both types of hydroxyeticianic acid glucuronide by hepatic microsomes from mutant rats were similar or even slightly higher than those of control microsomes. ■ These findings indicate that bile acid 3-O-glucuronides, but probably not carboxyl-linked glucuronides, are secreted into bile by a transport system shared with organic anions such as conjugated bilirubin and dibromosulphthalein, but different from that for amino acid-conjugated bile acids. — Kuipers, F., A. Radomska, P. Zimniak, J. M. Little, R. Havinga, R. J. Vonk, and R. Lester. Defective biliary secretion of bile acid 3-O-glucuronides in rats with hereditary conjugated hyperbilirubinemia. *J. Lipid Res.* 1989. 30: 1835-1845.

Supplementary key words taurocholic acid • lithocholic acid • cholic acid • hydroxyeticianic acid • etianic acid • transport • UDP-glucuronosyltransferase

Glucuronidation is a well established but quantitatively minor pathway in normal bile acid metabolism. Glucuronides of all common bile acids have been identified in human serum, bile, and urine (1-9). Relatively increased

amounts of these bile acid metabolites, in which the glucuronic acid is linked to the steroid moiety of the molecule, are found in urine and serum of patients with cholestatic liver disease (2, 4-9). In this case, glucuronidation may occur in addition to conjugation of the bile acid with glycine or taurine. It has recently been shown that, in the rat, glucuronidation is the only mode of conjugation for monohydroxylated short-chain bile acids, a group of acidic steroids abundant in the developing organism (10-15). Short-chain bile acids can be glucuronidated at the hydroxyl group of the steroid ring, the carboxyl group of the side chain, or both (14, 16-18).

It has been assumed that glucuronidation of bile acids prevents possible hepatotoxic effects and promotes urinary secretion. From recent findings, however, it is clear that these assumptions are an oversimplification. First, it was recently demonstrated that lithocholic acid 3-O-β-glucuronide (LCG) possesses cholestatic properties exceeding those of lithocholic acid itself (19). Second, it was found that the 3-O-β-glucuronide of lithocholic acid is secreted in bile, but is minimally secreted in urine even in the presence of complete biliary obstruction (19).

Abbreviations: LCG, lithocholic acid 3-O-glucuronide; CG, cholic acid 3-O-glucuronide; TC, taurocholic acid; hydroxyeticianic acid, 3α-hydroxy-5β-androstane-17β-carboxylic acid; GY, "Groningen Yellow" (mutants of Wistar rats with conjugated hyperbilirubinemia); HA, "high androsterone" glucuronidation capacity (Wistar rats with normal levels of 3-hydroxysteroid UDP-glucuronosyltransferase activity); LA, "low androsterone" glucuronidation capacity (mutants of Wistar rats with low levels of 3-hydroxysteroid UDP-glucuronosyltransferase activity); DBSP, dibromosulphthalein; TLC, thin-layer chromatography; UDPGT, UDP-glucuronosyltransferase.

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Similarly, the glucuronide of cholic acid (20), as well as the glucuronide(s) of the short-chain bile acid hydroxyeticianic acid (14), are rapidly secreted into bile but not into urine upon intravenous administration to bile fistula rats. In agreement with these findings, Stiehl and co-workers (21) found a bile:urine excretion ratio for glucuronidated bile acids of 226:1 in patients with alcoholic cirrhosis with moderate cholestasis.

Biliary secretion of cholephilic compounds involves uptake from sinusoidal blood by hepatocytes, intracellular transport, and secretion into bile at the canalicular pole of the cell. These processes have been studied in some detail for amino acid-conjugated bile acids, in particular for taurocholic acid (TC), and it is generally accepted that carrier-mediated transport processes are involved (22–28). It is not known whether glucuronidated bile acids are transported by the same systems that are operative for amino acid-conjugated bile acids. The present study was aimed at an initial characterization of the process of hepatobiliary transport of glucuronidated bile acids. For this purpose we used a Wistar rat strain (GY = Groningen Yellow) with a hereditary conjugated hyperbilirubinemia that is due to a defective hepatobiliary transport of organic anions such as conjugated bilirubin. We have recently demonstrated that the biliary secretion, but not the hepatic uptake, of 3 α -sulfated bile acids is impaired in GY rats, and that this inhibition becomes more pronounced with a decreasing number of hydroxyl groups on the bile acid's steroidal nucleus (29). In the present work, we studied the biliary secretion of lithocholic acid 3-O-glucuronide (LCG) and cholic acid 3-O-glucuronide (CG), as well as the short-chain bile acid hydroxyeticianic acid, and compared each with the secretion of TC. Since some of the above compounds are not secreted as such, but undergo metabolic conversions during their passage through the liver, the form (or forms) that are actually transported from the hepatocyte into bile have been analyzed and identified.

MATERIALS AND METHODS

Experimental design

The objective of the studies was to examine the biliary secretion of [³H]CG, [³H]LCG, and [¹⁴C]TC in GY and control rats. The biliary secretion of [³H]hydroxyeticianic acid, which gives rise to carboxyl- and hydroxyl-linked glucuronides in the rat, was also studied. Additionally, the effects of co-infusion of dibromosulphthalein (DBSP) on biliary secretion of glucuronidated lithocholic and cholic acid was investigated. For this purpose, unlabeled and labeled compounds were prepared and administered intravenously in trace amounts to unanesthetized control and GY rats prepared with a permanent external biliary fistula. The biliary and urinary secretion of radioactivity

was measured for 24 h, and the radioactive steroidal products in bile were characterized (virtually no radioactivity was found in urine).

The specific activities of bile acid-dependent UDP-glucuronosyltransferases were also determined using hepatic microsomes from control and GY rats.

Chemicals

Standard C₂₄ bile acids were obtained from Calbiochem-Behring (La Jolla, CA). Hydroxyeticianic acid (3 α -hydroxy-5 β -androstane-17 β -carboxylic acid) and 3-oxo-5 β -androstane-17 β -carboxylic acid were purchased from Steraloids (Wilton, NH). DBSP was from SERB (Societe d'Etudes et de Recherche Biologique, Paris, France). [24-¹⁴C]Taurocholic acid, sp act 56 Ci/mol, [2,4-³H]cholic acid, sp act 25 Ci/mmol, and tritium-labeled water, sp act 1.8 Ci/mol, were obtained from New England Nuclear (Boston, MA). [3 β -³H]-3 α -Hydroxy-5 β -cholanoic acid (tritium-labeled lithocholic acid) was synthesized as described previously (30).

The hydroxyl-linked glucuronides of unlabeled hydroxyeticianic, lithocholic, and cholic acids, as well as hydroxyl-linked glucuronides of tritium-labeled lithocholic and cholic acids were chemically synthesized as described previously (14, 17, 19, 20).

[2,2,4,4-³H₄]-3 α -hydroxy-5 β -Androstane-17 β -carboxylic acid ([³H]hydroxyeticianic acid) was prepared by enolization exchange as follows: 3 mg 3-oxo-5 β -androstane-17 β -carboxylic acid in 2 ml diethyl ether was mixed with 10 μ l (150 μ Ci) [carboxyl-³H]acetic acid containing 5% (w/w) ³HCl and allowed to stand for 40 h. ([Carboxyl-³H]acetic acid containing 5% ³HCl was prepared by mixing 0.362 ml acetic anhydride, 0.05 ml acetyl chloride, and 0.1 ml (10 mCi) ³H₂O for 1 h). The sample was extracted with 4 \times 1 ml aliquots of water acidified to pH 2. The pooled aqueous extracts were back-washed with 2 \times 2 ml ether. All ether extracts were pooled and dried; the residue was dissolved in 2 ml isopropanol containing 2 mg sodium borohydride and the reduction was allowed to proceed for 1 h. The reaction mixture was acidified with 6 N HCl, 1 ml of water was added, and the alcohol was evaporated off under nitrogen. The aqueous solution was extracted with ether, the extract was dried, and the residue was dissolved in ether-methanol (9:1). The sample, a mixture of 3 α - and 3 β -hydroxy acids, was methylated with diazomethane and dried; the methyl esters of the 3 α - and 3 β -hydroxy compounds were separated by preparative thin-layer chromatography on LK5 plates (Whatman Chemical Separation Inc., Clifton, NY), developed with benzene-acetone 95:5. Radioactive bands were localized using a Berthold Tracemaster 20 TLC analyzer and visualized by spraying the plates with water (31). The silica gel containing the 3 α -hydroxy compound was transferred to a column and the radioactive material was eluted with benzene-acetone 95:5. The recovered

material was checked by TLC in the same solvent and found to be >99% pure. The methyl ester was hydrolyzed by dissolving the sample in 2 ml methanol, adding 0.2 ml 5 N NaOH and leaving the mixture at 65°C for 24 h. The methanol was evaporated under N₂, water was added, and the sample was extracted with ether as described above. TLC in isoctane-ethyl acetate-acetic acid 10:10:0.2 (v/v), followed by scanning for radioactivity and visualization with Krowicki's reagent (1% phosphomolybdic acid and 5% ceric sulfate in 10% sulfuric acid) (32) demonstrated that the [³H]hydroxyeticianic acid was >99% chemically and radiochemically pure. The specific radioactivity was 1 Ci/mol.

Animals

Wistar rats with congenital conjugated hyperbilirubinemia (GY = Groningen Yellow), extensively characterized previously (29), were bred at the Central Animal Laboratory, University of Groningen. Biochemically and in the pattern of inheritance, GY rats show great resemblance to the mutant rat strain described by Jansen et al. (33, 34). Cross-breeding experiments have been recently performed and showed that both strains carry the same genetic defect.

Surgical procedures

Male mutant and normal Wistar rats, 275–325 g, were equipped with permanent silastic catheters in the bile duct and the heart (35). For a number of experiments, an additional, thinner heart catheter was inserted to allow simultaneous blood sampling and continuous intracardiac infusions (36). The animals were housed in individual Plexiglass cages and kept on a normal laboratory diet. Bile was led outside the cages through a polyethylene tubing with a swivel joint, which permitted the animals to move freely. Experiments were performed at least 8 days after surgery, to allow recovery from the operation and the establishment of a new steady state in bile acid synthesis (35). During experiments, the rats were placed in metabolic cages for simultaneous collection of urine and bile.

Experimental procedures

Tracer amounts of the bile acids were dissolved in a small volume of 0.1 M sodium hydroxide and added to 2–4 ml of a 4% bovine albumin solution (fraction V, Sigma, St. Louis, MO) in saline. The solution was neutralized by addition of 0.1 M acetic acid, and passed through a 0.22- μ m filter (Schleicher and Schuell, Den Bosch, The Netherlands). Rats received 0.4–1 μ Ci of bile acid as a bolus injection via the heart catheter. Bile was collected continuously in preweighed test tubes at 10-min intervals, beginning 30 min before injection to establish basal bile flow rates, and continuing for 2 h after the injection. Subsequently, bile was collected as a single sample for 2

h. A single urine sample was collected over the 24-h experimental period. Plasma disappearance of [³H]LCG was examined simultaneously with bile secretion in a number of experiments. For this purpose, blood samples of 0.15 ml were taken at 1, 3, 5, 10, 15, 30, 45, 60, 90, 120, and 180 min after injection. Blood was immediately transferred to heparinized test tubes and centrifuged to obtain plasma. D-Saccharic acid-1,4-lactone (Sigma) was added to bile and urine samples to prevent hydrolysis of bile acid glucuronides.

In a second set of experiments, mutant and control rats received a bolus injection of 7.7 μ mol of DBSP, followed by a constant infusion of 23.1 μ mol/h of the dye via the smaller heart catheter for 3 h. This infusion rate equals the estimated transport maximum for biliary secretion of DBSP in normal Wistar rats (29). After 1 h of DBSP infusion, 0.5 μ Ci of [³H]CG or 0.5 μ Ci of [³H]LCG was injected via the larger heart catheter. In all experiments, 0.2 μ Ci of [¹⁴C]TC was injected simultaneously with the glucuronides. Bile and urine were collected as described above. In addition, blood samples of 0.15 ml were taken at 0, 15, 30, 60, 120, and 180 min after the start of the DBSP infusion, to determine the plasma concentration of the dye.

Bile and urine volumes were determined gravimetrically in tared test tubes. The collection time of bile samples was adjusted to account for the dead space of the tubing system (35). Radioactivity in bile and plasma, in 10- μ l and 50- μ l aliquots, respectively, was determined in an LKB scintillation counter equipped with external standards to correct for quenching. Bile was decolorized with 50 μ l of hydrogen peroxide prior to counting.

The concentration of DBSP in bile and plasma was determined, after appropriate dilution of the samples, as described previously (37).

Isolation and identification of radioactive products in bile

Aliquots of bile from animals that received radiolabeled LCG or CG were diluted with 5 ml of 0.1 M glycine-trichloroacetate buffer, pH 2.8, and the samples were applied to Bond Elut C₁₈ cartridges (size: 6cc; Analytichem, Harbor City, CA). The cartridges were washed with 5 ml buffer and 2 \times 5 ml water, and bile acids were eluted with 2 \times 5 ml methanol. Methanol eluates were dried, residues were dissolved in methanol, and aliquots were chromatographed on Whatman LK5 thin-layer plates in chloroform-methanol-acetic acid-water 65:25:2:4 (v/v). Aliquots of bile from animals given hydroxyeticianic acid were acidified to pH 2 with 6 N HCl and extracted with 3 \times 4 ml ether. Ether extracts were taken to dryness and residues were redissolved in methanol. On several occasions, the solid phase extraction method described above for LCG and CG was used with identical results. An ali-

quot of each sample was chromatographed on TLC plates in ethyl acetate-absolute ethanol-concentrated ammonium hydroxide 45:45:15 (v/v).

An additional aliquot of each extract was dried, dissolved in 1 ml of 0.1 M sodium phosphate buffer, pH 6.8, and treated with 100 units of *E. coli* β -glucuronidase (Sigma Chemical Co., St. Louis, MO). Samples were incubated overnight at 37°C, extracted with C₁₈ cartridges as described above, and subjected to TLC in chloroform-methanol-acetic acid-water 65:25:2:4 (v/v).

Following β -glucuronidase treatment, extracts of bile from animals given LCG were dried, redissolved in 1 ml 0.1 M sodium acetate buffer, pH 5.6, and 25 units of cholyglycine hydrolase (Sigma) was added. Samples were incubated at 37°C for 18 h, extracted with C₁₈ cartridges, and subjected to TLC analysis in iso-octane-ethyl acetate-acetic acid 10:10:2 (v/v).

Selective hydrolysis of the carboxyl-linked glucuronide of hydroxyetianic acid in bile extracts was carried out in 0.1 N NaOH for 3 h at room temperature. For verification of the selectivity of the procedure, a standard mixture of tritium-labeled hydroxyl- and carboxyl-linked glucuronides of hydroxyetianic acid was simultaneously subjected to the same hydrolytic treatment. Following hydrolysis, bile acids were extracted with C₁₈ cartridges and analyzed by TLC in ethyl acetate-absolute ethanol-concentrated ammonium hydroxide 45:45:15 (v/v).

Spots on TLC plates were localized by scanning for radioactivity using a Berthold Tracemaster 20, by non-destructive visualization through spraying with water (for preparative plates) (31), or by visualization through spraying with Krowicki's reagent and heating on a hot plate at 100-120°C (32).

In vitro glucuronidation of bile acids

Microsomes of livers from control and mutant Wistar rats were prepared as previously described (17) and stored at -80°C. Bile acid:UDP-glucuronosyltransferase (UDP-GT) activity was assayed as previously described (17) except that the reactions were carried out in 100 mM Na-HEPES buffer, pH 6.5 for carboxyl-linked, and pH 7.5 for hydroxyl-linked glucuronide formation. The glucuronosyltransferase reaction was linear with protein concentration up to 1.5 mg/ml and with time for 15 min for both the control and GY rats. The concentration of hydroxyetianic acid was 100 μ M in all experiments; this concentration was determined to be saturating but not inhibitory for both the control and GY rats. Separation of carboxyl- and hydroxyl-linked glucuronides and unreacted material was performed by TLC (17).

A mutation greatly reducing the 3-hydroxysteroid UDPGT activity is known to occur in Wistar rats with an incidence of about 50% (38-40). Microsomal preparations derived from all rats were therefore tested with

lithocholic acid (41) in order to classify them as LA (mutant) or HA (normal phenotype).

The specific activity of enzymes is expressed as nmol of bile acid glucuronide formed per mg protein and per min, and is reported as mean \pm SD. Protein was measured using the Bio-Rad kit based on the Bradford protein assay (42), with bovine serum albumin as standard.

RESULTS

Biliary secretion of bile acid 3-O-glucuronides

Fig. 1 shows the appearance of radioactivity in bile after intravenous administration of [³H]LCG, [³H]CG

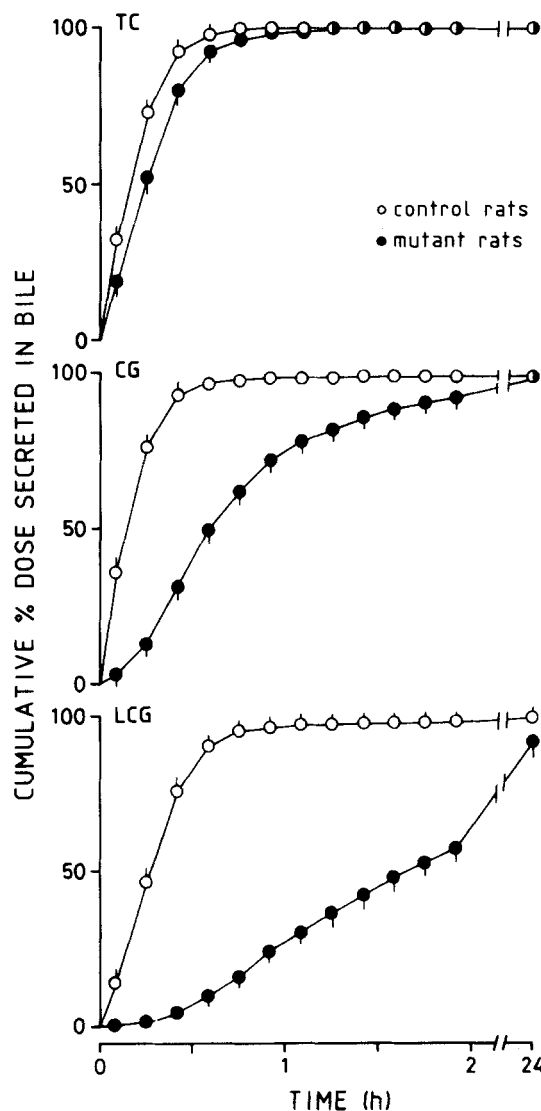


Fig. 1. Biliary secretion of radioactivity after intravenous administration of tracer amounts of [¹⁴C]taurocholic acid (top), [³H]cholic acid glucuronide (middle), and [³H]lithocholic acid glucuronide (bottom) to unanesthetized control (open symbols) and GY rats (closed symbols) with permanent biliary drainage. Values represent means \pm SEM of five-six separate experiments for each bile acid, both in control and GY rats.

and, for comparison, of [^{14}C]TC in control and GY rats. Secretion of [^{14}C]TC was slightly delayed in the GY rats when compared to controls, but almost all of the administered label had been secreted into bile within only 60 min. In contrast, biliary secretion of [^3H]CG and [^3H]LCG in GY animals was significantly impaired. The degree of impairment was different for each compound. The recoveries of [^3H]CG and [^3H]LCG in bile of GY rats were $71 \pm 4\%$ and $24 \pm 3\%$, respectively, at 60 min after injection, compared to $98 \pm 1\%$ and $96 \pm 2\%$, respectively, in controls. However, the administered dose was almost completely recovered from bile collected over a 24-h period; urinary excretion of these compounds was negligible in both controls and GY rats.

The disappearance of [^3H]LCG from plasma was clearly delayed in GY rats in comparison to controls. **Fig. 2** shows the plasma disappearance of LCG in representative experiments in which 2 mg (1 μCi) of the compound was injected. Pharmacokinetic analysis of these curves using the curve-stripping computer program RUGFIT (43), as described in detail for sulfated glycolithocholic acid (29) and a number of other organic anions (33, 34), revealed that the delayed plasma disappearance is attributable to defective transport from liver to bile; the rate constant for this process was reduced from 0.211 min^{-1} to 0.013 min^{-1} . At 3 h after injection, the liver of the GY rat still contained 13.6% of the administered dose, compared to 0.9% in the control rat. Biliary recoveries at 3 h were 60.0% and 92.8%, respectively.

Inhibition by DBSP of hepatobiliary transport of bile acid 3-O-glucuronides

Tracer amounts of tritium-labeled LCG or CG, together with a tracer of [^{14}C]TC, were injected during continuous infusion of DBSP, at a rate of $23.1 \mu\text{mol/h}$, or of saline both in control and GY rats. Biliary secretion of the dye reached the level of $92.0 \pm 4.5 \text{ nmol/min}$ and 100 g body weight in controls and $43.4 \pm 3.5 \text{ nmol/min}$ and 100 g body weight in GY rats, values comparable to the recently reported transport maxima for biliary secretion of DBSP after an intravenous bolus injection (29). Plasma levels of DBSP at the end of infusion were $0.34 \pm 0.02 \text{ mM}$ in controls and $0.65 \pm 0.03 \text{ mM}$ in jaundiced rats.

Radiolabeled bile acids were injected at 1 h after the start of DBSP or saline infusion. **Fig. 3** gives the results of representative experiments in which a mixture of [^3H]LCG and [^{14}C]TC was administered to control rats. DBSP infusion caused an almost twofold increase in bile flow, delayed the biliary secretion of [^3H]LCG, but did not affect [^{14}C]TC secretion. Data from similar experiments with the different bile acids studied are summarized in **Fig. 4**. These data show that DBSP did not inhibit biliary secretion of [^3H]CG or [^{14}C]TC in controls, whereas it significantly impaired the secretion of [^3H]LCG. On the other hand, secretion of both glu-

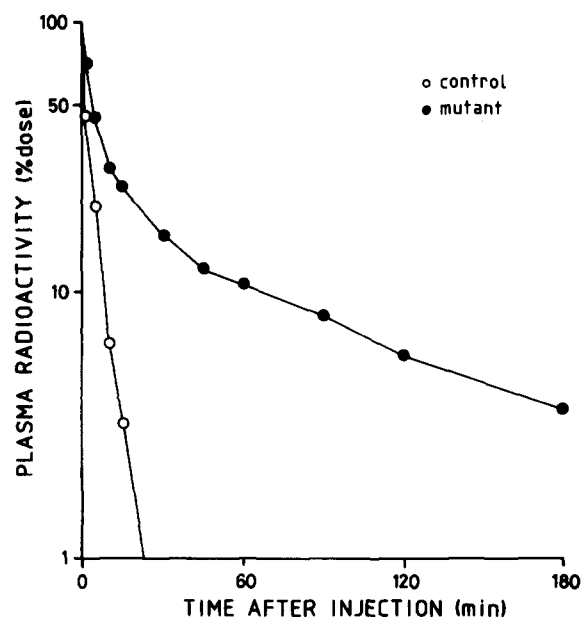


Fig. 2. Plasma disappearance of radioactivity after intravenous administration of 2 mg (1 μCi) of [^3H]lithocholic acid glucuronide to a control and a GY rat.

curonides was significantly inhibited by DBSP in the GY rats, but under these conditions the dye still did not impair secretion of [^{14}C]TC.

Biliary secretion of hydroxyeticianic acid

Biliary secretion of radioactivity was very rapid after intravenous administration of [^3H]hydroxyeticianic acid to control rats, the recovery in bile being virtually complete within 60 min after injection (**Fig. 5**). Biliary secretion was very markedly impaired in mutant rats; only $15.7 \pm 0.5\%$ of the dose was found in bile at 1 h after injection and $73.6 \pm 3.4\%$ at 24 h.

Characterization of labeled compounds in bile

Identification of chemical forms of the label secreted in bile was accomplished by TLC before and after β -glucuronidase hydrolysis. Chemically synthesized compounds were used as reference compounds.

Extracts of bile from control and GY rats injected with [^3H]CG showed a single radioactive peak with an R_f identical to that of authentic CG. After β -glucuronidase hydrolysis, a single peak co-chromatographing with free cholic acid was found in all samples. The major compound in bile of control rats injected with [^3H]LCG had the same chromatographic mobility as the administered material, with a smaller amount (25% of biliary radioactivity) of a highly polar metabolite. From the results of sequential hydrolysis with β -glucuronidase and cholyglycine hydrolase, the latter compound was tentatively identified as tauroolithocholic acid glucuronide. The relative

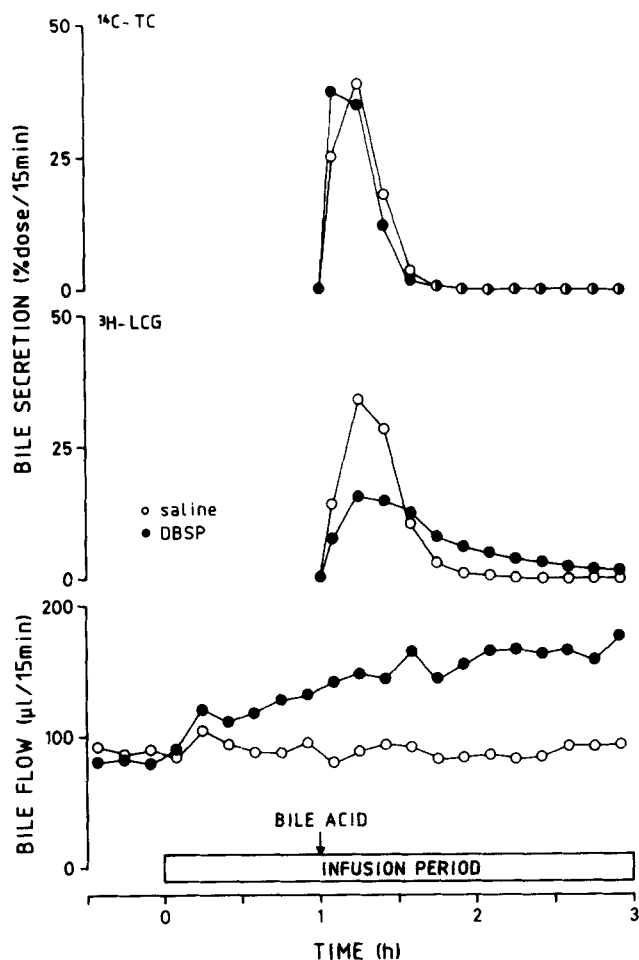


Fig. 3. Biliary secretion of [^{14}C]taurocholic acid (top) and [^3H]lithocholic acid glucuronide (middle) and bile flow (bottom) in control Wistar rats infused with either dibromosulphthalein (closed symbols) or saline (open symbols). Both bile acids were administered simultaneously to unanesthetized rats with permanent biliary drainage at 1 h after initiation of an intravenous infusion of the dye ($23.1 \mu\text{mol/h}$) or its vehicle.

proportion of the tauro-conjugate was higher in bile of GY rats than in that of controls, and increased even further during the course of the experiment; 59% of radioactivity present in bile collected during the initial 2 h of the experiment and 78% in samples from 2 to 24 h was in the form of taurolithocholic acid glucuronide.

Bile of three out of the four control rats, collected during the initial 2 h after injection of [^3H]hydroxyeticianic acid, contained the hydroxyl-linked glucuronide and the carboxyl-linked glucuronide of [^3H]hydroxyeticianic acid in a 4:1 ratio (Table 1), as shown by TLC (see ref. 17 and Methods). The identity of the carboxyl-linked glucuronide was further confirmed by selective alkaline hydrolysis under mild conditions (see Methods); this procedure led to the disappearance of the carboxyl-linked glucuronide on the TLC plate with a concomitant appearance of an equivalent amount of free hydroxyeticianic acid, but left

the hydroxyl-linked glucuronide unchanged. One control rat secreted radioactivity predominantly as the carboxyl-linked glucuronide of hydroxyeticianic acid (88%), the remainder being in the form of a very polar, as yet unidentified, metabolite; no hydroxyl-linked glucuronide was present. This rat is probably a representative of the low 3-hydroxysteroid UDPGT mutation (LA mutation) mentioned in the Methods section; the latter enzyme is responsible for the formation of the hydroxyl-linked (3-O-), but not of the carboxyl-linked glucuronide of hydroxyeticianic acid (41). Bile of GY mutant rats collected within the first 2 h after injection was enriched in the carboxyl-

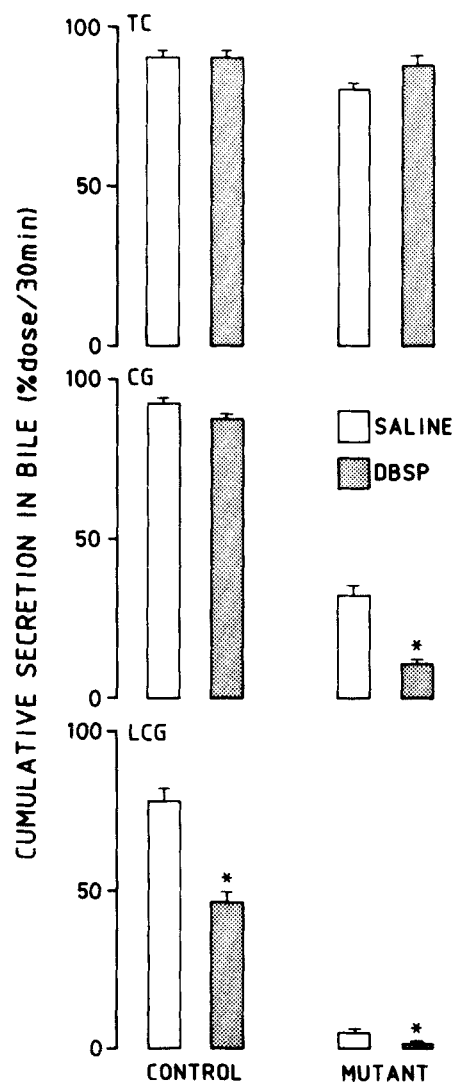


Fig. 4. Biliary recovery at 30 min after injection (% dose) of [^{14}C]taurocholic acid (top), [^3H]cholic acid glucuronide (middle), and [^3H]lithocholic acid glucuronide (bottom) in control and GY rats. The compounds were administered intravenously to unanesthetized rats with permanent biliary drainage at 1 h after initiation of an intravenous infusion of dibromosulphthalein (23.1 mmol/h) or its vehicle (saline). Values are means \pm SEM of four to six separate experiments.

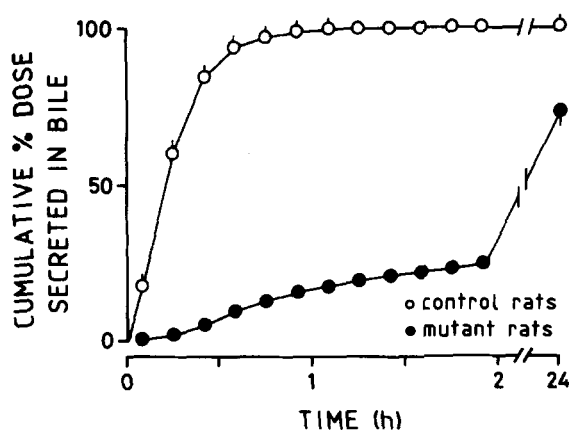


Fig. 5. Biliary secretion of radioactivity after intravenous administration of a tracer amount of [³H]hydroxyeticianic acid to unanesthetized control (open symbols) and GY rats (closed symbols). Values represent means \pm SEM of four separate experiments in both control and GY rats.

linked glucuronide, as compared with bile of control rats from the same time span. However, when calculated over the total 24 h of the experiment, i.e., the time in which the cumulative secretion in the mutant was similar to that in control animals, both types of glucuronide were present in a ratio close to that found for control rats: 76% hydroxyl-linked and 24% carboxyl-linked glucuronide in the mutant versus 81% and 19%, respectively, in controls (Table 1).

In vitro formation of hydroxyeticianic and lithocholic acid glucuronides

Glucuronidation of bile acids in vitro, using microsomes isolated from livers of control and GY rats, was studied under previously described assay conditions (17). Table 2 shows the synthesis rates for the hydroxyl-linked and carboxyl-linked glucuronides in control and GY rats. The glucuronidation of lithocholic acid was measured in

order to ascertain whether LA mutants (mutants with a low level of 3-hydroxysteroid UDPGT) were present. One such mutant was found within the control group, but none was present among the five GY rats used. It should be noted that microsomes were prepared from animals of the same breeding colony, but not from the same individual rats that were used for the in vivo transport studies. Therefore, the fact that the in vivo and in vitro control groups each included one LA animal, but no LA phenotype was observed among GY rats, was coincidental. For hydroxyeticianic acid, the rate of synthesis of both types of glucuronide was approximately 40% higher in GY mutant rats than in HA controls. However, the ratio in which both types of glucuronide were formed was very similar in GY mutants and controls. In addition, this ratio was similar to that observed in bile of these animals (Table 1). For microsomes obtained from the LA animal, the ratio of the two glucuronides was reversed (approx. 90% carboxyl-linked glucuronide) and corresponded closely to the biliary pattern of hydroxyeticianic acid glucuronides found in vivo in the single putative LA rat (see previous section).

DISCUSSION

Glucuronidation has been recognized as a pathway in bile acid metabolism of importance in relation to specific bile acid substrates and in relation to cholestatic liver disease. During the past few years, the enzymology of the formation of bile acid glucuronides has been studied extensively. Several UDPGT activities specific for various bile acids have been demonstrated in liver, intestine, and kidney of humans (44-47) and experimental animals (48-50). One of these enzymes, the 3-hydroxysteroid UDPGT, has been purified to homogeneity from rat liver (49), and its gene has been cloned and sequenced (51, 52). This enzyme catalyzes the glucuronidation of bile acids at the 3-OH position in the A-ring. The specificity of bile

TABLE 1. The relative amounts of hydroxyl-linked and carboxyl-linked glucuronides of [³H]hydroxyeticianic acid in bile, after intravenous administration of tracer amounts of [³H]hydroxyeticianic acid to control and GY Wistar rats

Rat	n	Time after Injection	Type of Glucuronide	
			Hydroxyl-Linked	Carboxyl-Linked
		h	%	
Control (HA)	3	0-2	81.1 \pm 1.6	18.9 \pm 1.6
Control (LA)	1	0-2	0	88.0
GY	4	0-2	32.2 \pm 4.7	67.8 \pm 4.7
		2-24	90.3 \pm 0.1	10.0 \pm 0.1
		0-24	76.4	23.6

The values are means \pm SEM. HA, mutant with high 3-hydroxysteroid UDPGT activity; LA, mutant with low activity of this enzyme; GY, hyperbilirubinemic mutant. The values for the total 0-24 h bile for the GY mutant have been calculated from the measured values for partial collections (0-2 h and 2-24 h).

TABLE 2. Specific activities of UDP-glucuronosyltransferase(s) toward hydroxyetianic and lithocholic acid in hepatic microsomes from control Wistar and GY Wistar rats

Microsomes	n	Hydroxyetianic Acid Glucuronide		Lithocholic Acid Glucuronide	
		Hydroxyl-Linked	Carboxyl-Linked	Hydroxyl-Linked	Carboxyl-Linked
		<i>nmol/mg · min</i>			
Control (HA)	5	2.39 ± 0.49*	0.98 ± 0.21*	1.85 ± 0.33*	0
Control (LA)	1	0.11	0.70	0	0
GY (HA)	5	3.67 ± 0.35*	1.36 ± 0.12*	3.07 ± 0.37*	0

The values are means ± SD; n denotes the number of individual rats; determinations were each done in quadruplicate. Reactions were carried out as described in Methods, and products were separated and identified (using appropriate synthetic glucuronide standards) by TLC as hydroxyl- or carboxyl-linked glucuronides (17). HA, mutant with high 3-hydroxysteroid UDPGT activity; LA, mutant with low activity of this enzyme; GY, hyperbilirubinemic mutant.

*Difference between HA control and GY mutant rats is statistically significant by both the Mann-Whitney and the *t*-test ($P < 0.001$).

acid UDPGT activities for the site of glucuronidation, the configuration of the bile acid's hydroxyl groups, and the length of the side chain have recently been studied extensively, using microsomes prepared from human (47) and rat liver (17, 41, 50). These studies revealed the existence of three types of bile acid glucuronides: hydroxyl-linked glucuronides at position 3 and at position 6 of the steroid nucleus, and a carboxyl-linked glucuronide in which the glucuronic acid is linked to the side chain. The latter type of glucuronide has been described for short-chain (C_{20} , C_{23}) bile acids (16, 17) as well as for a conventional (C_{24}) bile acid (50).

Much less is known about the physiology of bile acid glucuronides. The presence of increased amounts of these compounds in urine of patients with cholestatic liver disease has led to the assumption that bile acids are removed from the body as glucuronides via the kidneys in situations when biliary secretion is impaired or blocked. As already pointed out in the Introduction, experimental data do not bear out this assumption for bile acids. It is now well established that bile is the main excretory route for bile acid 3-O-glucuronides (14, 16, 19–21). Yet, the mechanism(s) for the hepatobiliary transport of these bile acid metabolites have, to our knowledge, not been studied. In the present paper, we used GY rats to study this process. GY rats show a markedly reduced ability for biliary secretion of organic anions, such as conjugated bilirubin and DBSP (29). Jansen and co-workers found that secretion of a neutral steroid (ouabain) (34) and of cysteinyl leukotriene (53) is also impaired. In addition, glutathione is completely absent in bile of these animals, whereas it is present in bile of control Wistar rats in concentrations of 3–4 mM (54, 55). In contrast, hepatobiliary transport of amino acid-conjugated bile acids is not affected in mutant animals (29, 33, 34). The nature of the secretory defect in GY rats is not yet known.

In a previous study (29), we demonstrated that biliary secretion of a number of 3 α -sulfated bile acids is significantly impaired in GY mutant rats, whereas that of the unsulfated parent bile acids is not. We were able to demonstrate that the reduced biliary secretion of these sulfates is not due to an impaired hepatic uptake of the compounds from sinusoidal blood. These data were taken to indicate that, in the rat, separate transport systems exist for secretion of sulfated and unsulfated bile acids from hepatocyte into bile. The data presented in this report allow us to extend this conclusion to bile acid 3-O-glucuronides. Bile secretion of LCG, CG, and also of the 3-O-glucuronide of hydroxyetianic acid was very efficient in control rats but markedly impaired in GY rats. Apparently, the introduction of a negatively charged group to the part of a bile acid molecule opposite to the side chain turns it into a less favorable substrate for the "bile acid transporting system" and directs it to the "organic anion transporting system," which is deficient in GY rats. It should be noted that considerable amounts of the taurine conjugate of LCG are formed, especially in the GY strain of rats. However, taurine conjugation does not change the overall distribution of negative charges, i.e., the factor that appears to determine which transporter is used by the molecule, and is therefore of limited relevance to the present considerations. Direct studies on isolated membranes may be best suited for defining how LCG and tauro-LCG differ in transport kinetics or mechanism.

In analogy to bile acid sulfates (29), the presence of additional hydroxyl groups in CG as compared to LCG allowed for a more efficient hepatobiliary transport of CG in GY rats. In addition, secretion of CG, just as that of TC, was not inhibited by co-infusion of DBSP in controls, whereas secretion of LCG was. Only in GY rats was DBSP able to inhibit CG secretion under conditions which did not affect TC transport. Whether these obser-

vations reflect a more efficient transport of CG compared to LCG via the "organic anion" transport system, or additional transport of CG via the "bile acid" transport system, remains to be established.

In addition to experiments performed with bile acid 3-O-glucuronides, we also studied secretion of the short-chain bile acid hydroxyetianic acid. Using this compound, we were able to compare the secretion pattern of endogenously formed carboxyl- and hydroxyl-linked bile acid glucuronides. By direct TLC comparison of the radioactive products in bile it was found that the two classes of glucuronides were formed in both control and GY Wistar rats. In control animals exhibiting high hepatic androsterone glucuronidation capacity, i.e., the HA phenotype associated with normal levels of 3-hydroxysteroid UDPGT activity (38-41), the ratio of hydroxyl- to carboxyl-linked glucuronides in bile was 4:1. In an earlier work addressing the hepatic metabolism of hydroxyetianic acid (14), we were able to identify only the major metabolite, namely the hydroxyl-linked glucuronide. Improved methodology allowed us now to characterize unequivocally the second metabolite of hydroxyetianic acid. This was achieved not only through its chromatographic behavior and differential stability toward alkali (this communication), but primarily through isolation from bile and characterization by high-field proton magnetic resonance (not shown; the spectra were identical with those described previously (17) for metabolites obtained in vitro).

The secretion of hydroxyetianic acid in GY rats was significantly impaired, and the carboxyl-linked glucuronide was identified as the major radioactive component in bile during the first 2 h after injection. Initially it seemed possible that this difference was the result of abnormal bile acid glucuronidation in GY rats. However, a direct comparison of the pattern and of the specific activity of glucuronidation of hydroxyetianic acid by control and GY liver microsomes showed that the enzymatic activities were almost identical, or even slightly higher in GY rats. This suggests that the relative lack of the hydroxyl-linked glucuronide in bile immediately after injection of hydroxyetianic acid to GY rats is due to altered biliary secretion, rather than altered synthesis. While the transport of the hydroxyl-linked glucuronide is impaired in the GY mutant, the secretion of the carboxyl-linked glucuronide remains efficient. A carboxyl-linked glucuronide of a bile acid resembles structurally an amino acid-conjugated bile acid; in both cases, the single negative charge is associated with the side chain. Therefore, our transport studies lend support to the conclusion of Anwer et al. (56) that the presence of one or two charges in the side chain is a prerequisite for efficient secretion of a bile acid derivative from the hepatocyte into bile via the bile acid transporter. On the other hand, a hydroxyl-linked glucuronide of a bile acid bears one negative charge on the side chain and a second negative charge in the vicinity of ring A, i.e., at the

opposite end of the molecule. This structure makes it similar to bile acid 3-O-sulfates or to dianionic organic dyes, compounds that are poor substrates for the bile acid transporter but are secreted by the organic anion transporter. Increased serum levels of bile acid 3-O-glucuronides in cholestasis may therefore be, at least partly, the result of less efficient hepatobiliary transport due to competition for secretion with bilirubin conjugates. The formation of the carboxyl-linked glucuronide (16, 17, this work) would redirect the bile acid to a different transport system and thus facilitate secretion. ■■

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