Excretion of cholate glucuronide

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Abstract [3-^{3}H]Cholic acid glucuronide [7α,12α-dihydroxy-3α-O-(β-D-glucopyranosyluronate)-5β-cholan-24-oate] was synthesized and administered to rats prepared with either an external biliary fistula or a ligated bile duct. When bile fistula animals were given either microgram or milligram amounts of the glucuronide, biliary secretion of label was rapid and efficient: > 90% of the administered label was secreted within 60 min and total recovery of label in bile was 98.6 ± 1.2%. Studies in which [^{14}C]taurocholate was included in the dose indicated that this bile acid was secreted into bile significantly more rapidly than was the glucuronide. In animals with ligated bile ducts, urinary excretion was the major route of elimination: after 20 hr, 83.4 ± 9.3% of the administered dose had been excreted in urine. Urinary excretion of cholate glucuronide was significantly more rapid than that of taurocholate. Gas-liquid chromatographic analysis of the methyl ester acetate derivatives of labeled compounds isolated from bile and urine by chromatography established that the bulk (> 70%) of the administered material was secreted in bile or excreted in urine as the intact cholate glucuronide. From these results, we conclude that the glucuronidation of cholic acid produces a derivative which is rapidly and effectively cleared from the circulation and excreted. —**Little, J. M., M. V. Chari, and R. Lester.** Excretion of cholate glucuronide. *J. Lipid Res.* 1985. **26:** 583–592.

Supplementary key words bile acid • biliary secretion • bile flow

Glucuronides of all the common bile acids have been identified in human bile, plasma, and urine, most notably in patients with cholestasis (1–5). The kinetics of bile acid glucuronyl transferase have been studied in vitro (6, 7) and stimulation of bile acid glucuronidation by phenobarbital has been demonstrated in vivo (6, 8), however, little else is known about the metabolism of bile acid glucuronides. It has recently been shown that lithocholate glucuronide, a bile acid found in cholestatic urine, is a cholestatic agent itself, with a toxicity equal to or greater than that of free lithocholate or its taurine and glycine conjugates (9). Furthermore, even in animals with biliary obstruction, lithocholate glucuronide was minimally excreted in urine. Since glucuronidation has long been considered to be a detoxifying mechanism, these results raised the question of whether bile acid glucuronidation does or does not promote bile acid detoxification and elimination.

For purposes of comparison, the 3-O-β-D-glucuronide of [3-^{3}H]cholic acid was prepared and administered to rats prepared with either a biliary fistula or a ligated bile duct. The results indicate that both microgram and milligram quantities of cholate glucuronide are efficiently secreted in bile by fistula animals, with the larger doses producing a prompt and significant choleresis. When biliary secretion is blocked by bile duct ligation, cholate glucuronide is recovered in high yield in urine.

METHODS

Experimental design

The objective of the studies was to examine the hepatic metabolism and biliary secretion of the 3-O-β-D-glucuronide of cholic acid (hereafter referred to as cholate glucuronide). For this purpose, unlabeled and 3-^{3}H-labeled cholate glucuronide were synthesized and administered intravenously, in microgram and milligram amounts, to rats prepared with either an external biliary fistula or a ligated bile duct. In fistula rats, biliary secretion of label was measured for 20 hr and in bile duct-ligated animals, urinary excretion of label was followed for the same period. At the end of each study, label remaining in plasma, urine, and tissues was determined and the nature of the labeled steroid in bile and urine was characterized.

Synthesis of cholate glucuronide

Cholic acid (sodium salt; Calbiochem-Behring, La Jolla, CA) was converted to the 7,12-di-O-formate (10) and esterified with diazomethane to yield the methyl ester which was purified by column chromatography on silica gel G. Oxidation of the methyl ester with Jones' reagent yielded the 3-oxo derivative which, after purification by

Abbreviations: GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance spectrometry; MS, mass spectrometry; TLC, thin-layer chromatography; DEAP-LH20, diethylaminohydroxypropyl Sephadex LH-20.

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column chromatography, was characterized by NMR and MS. Reduction of the methyl-3-oxo-5β-7,12-di-O-formyl-cholate in isopropanol with NaB^3H_4 (sp act 350 mCi/mmol; ICN, Irvine, CA) gave a mixture of the 3-tritiated, 3α-, and 3β-hydroxy compounds as the major components of the product mixture. Preparative TLC of the product mixture on silica gel G plates (20 × 20 cm, 0.5 mm layer thickness; Supelco, Bellefonte, PA), using 1,2-dichloroethane–ether 1:1 as the solvent, resulted in the separation and purification of methyl-[3β-^{3}H]-cholate-7,12-diformate. This compound was condensed (20 hr) with methyl-2,3,4-tri-O-acetyl-1-bromo-1-deoxy-β-D-glucopyranuronate (11) in benzene solution using silver carbonate as acid acceptor and molecular sieve 3A (Aldrich Chemical Co., Milwaukee, WI) as desiccant. The silver salts and molecular sieve were filtered off; the solution was evaporated to dryness; the residue was dissolved in acetone and treated with 0.05 N H_2SO_4 (15 min) to decompose any ortho-ester side product formed. After neutralization with 1 N NaOH, the solution was evaporated to an oily residue which was subjected to preparative TLC on silica gel G plates as above. The zone corresponding to the protected [^{3}H]cholate glucuronide was scraped off, eluted with ethyl acetate, and evaporated to yield a colorless crystalline solid. The protected glucuronide was deesterified with 1 N NaOH in 72% ethanol at 55°C for 20 hr. The ethanol was evaporated, the residue was dissolved in water, acidified with 10% HCl to pH 4, and passed through an XAD-2 (Aldrich) column (1 × 20 cm). Elution with a solution of 2% ammonia in 72% ethanol gave the diammonium salt of [^{3}H]cholate glucuronide which was deionized with an Amberlyst A-15 (Aldrich) column with the effluent flowing directly onto a column (0.75 × 12 cm) of DEAP-LH20 prepared as described in (12). The latter column was washed with 72% ethanol to remove neutral compounds and the tritiated glucuronide was separated from residual free [^{3}H]cholate by stepwise elution: [^{3}H]cholate was eluted first with 0.1 M acetic acid, pH 3.8, after which the radioactive cholate glucuronide was recovered from the column in fractions of 1.5 ml with 0.3 M ammonium acetate buffer, pH 5.2. The eluate fractions containing the radioactive cholate glucuronide were treated with 2 drops of 10% ammonium hydroxide and the residues in each tube were lyophilized to remove all traces of ammonium acetate. The resultant [^{3}H]cholate glucuronide diammonium salt was > 95% radiochemically pure and had a specific activity of 58.2 μCi/mg.

The product of the analogous reaction using unlabeled methyl cholate-7,12-diformate and the bromo sugar was subjected to column chromatography on silica gel using toluene–ethyl acetate 1:1 as eluent. The protected cholate glucuronide was eluted as the first crystalline fraction and on recrystallization from 90% ethanol formed colorless needles (mp 230–233°C; 55% yield). The crystalline material was characterized by NMR and MS and deesterified as above. The solution was evaporated to dryness and the disodium salt separated from 72% ethanol as a colorless amorphous solid, decomposing above 260°C. The disodium salt of cholate glucuronide was characterized by its ^{1}H-NMR spectrum in D_2O at room temperature. In addition to the expected signals for the steroid moiety between 0.8 to 2.5 ppm, the spectrum exhibited signals for the sugar protons in the region 3.3 to 4.85 ppm, due to the glucuronide residue. The H-1' signal appeared as a doublet (J = 8 Hz) centered at 4.75 ppm, as expected for a β-D-glucopyranuronide.

Surgical procedures

Male Sprague-Dawley rats (180–300 g) were anesthetized with ether and the abdomen was incised along the midline. For bile fistula studies, a polyethylene catheter (PE-50, 0.58 mm i.d. × 0.965 mm o.d.; Clay-Adams, Parsippany, NY) was inserted into the common bile duct and exteriorized through a stab wound in the abdominal wall. For ligation studies, the common bile duct was ligated with two separate sutures placed well above the entry of the pancreatic ducts and the bile duct was severed between these sutures. In both series of studies, the abdominal incision was closed and a catheter (PE-10, 0.028 mm i.d. × 0.61 mm o.d.; Clay-Adams) was inserted into the femoral vein. Animals were then placed in Bollman restraining cages under a heat lamp and an infusion of saline (0.025 ml/min) was begun via the femoral vein catheter. Body temperature was maintained at 37 ± 1°C and the saline infusion was continued throughout the course of the study. Animals were allowed 2–3 hr to recover from anesthesia before the experiment was begun.

Experimental protocol

Labeled cholate glucuronide (0.25–1.0 μCi), with or without added carrier glucuronide (3.7–4.5 mg), was dissolved in saline (1.0–2.0 ml) and administered intravenously via the femoral vein catheter. Bile samples from bile fistula animals were collected in preweighed tubes at 10-min intervals beginning 1 hr before injection of the dose and continuing to 1 hr after injection. Subsequently, bile was collected at 30-min intervals for an additional 2 hr and as a single sample until the animals were killed at 20 hr. A single urine sample was collected over the experimental period. Urine samples from bile duct-ligated animals were collected and volumes were measured at hourly intervals, if possible, for the first 6–7 hr of the study and as a single sample thereafter until killing.

In three animals, [24-^{14}C]taurocholic acid (0.2–1.0 μCi; sp act 40 mCi/mmol; New England Nuclear, Boston, MA) was administered to fistula animals simultaneously with the [^{3}H]cholate glucuronide. In these studies, bile

was collected dropwise in individual scintillation vials for a period of 30 min, at 30-min intervals until 3 hr and as a single sample from 3 to 20 hr. In four additional animals with ligated bile ducts, [^{14}C]taurocholic acid (0.7 μCi) was included with [^{3}H]cholate glucuronide (0.7 μCi) in the administered dose. Urine was collected as described above.

In all studies, blood was collected from the abdominal aorta at the time of killing and plasma was separated and stored. Liver, kidneys, and small and large intestine were removed and frozen for later analysis.

ANALYTICAL TECHNIQUES

Determination of radioactivity

Bile and urine volumes were determined gravimetrically in tared tubes. Using bile flow rates and catheter volumes, collection times were adjusted to account for catheter dead space. Liver, kidneys, and small and large intestine contents and wall were homogenized in two vol of distilled water and aliquots (1 ml) of homogenates were extracted with 5 ml of methanol followed by centrifugation of the protein precipitates. Aliquots of bile, urine, plasma, and tissue extracts were analyzed for tritium (and ^{14}C) content in ACS scintillant (Amersham Corp., Arlington Heights, IL) in a Tracor Mark III, model 6882, liquid scintillation system (Tracor Analytic, Elk Grove, IL).

Thin-layer chromatography (TLC)

Whatman LK-5 thin-layer plates (Pierce Chemical Co., Rockford, IL) were used for all TLC analyses. Solvent systems were as follows: system 1 (for free bile acids): isooctane–ethyl acetate–acetic acid 5:5:1; system 2 (for bile acid conjugates): chloroform–methanol–acetic acid–water 65:25:2:4; system 3: ethanol–ethyl acetate–ammonium hydroxide 45:45:15. Plates were developed to a distance of 15 cm from the origin and dried. Standards of free and conjugated bile acids (Calbiochem-Behring) and cholate glucuronide were visualized by spraying the plates with phosphomolybdic acid (3.5% in isopropanol) and heating at 110°C for 20 min. To localize radioactivity in chromatographed samples, each sample lane was divided into 1-cm segments from origin to front and the gel in each segment was transferred to a scintillation vial. One ml of methanol was added to each vial; samples were allowed to stand for 30 min and were then counted in ACS.

β-Glucuronidase hydrolysis

Bile and urine samples were diluted with 0.075 M sodium phosphate buffer, pH 6.8, and 50 units of *E. coli* β-glucuronidase (Sigma Chemical, St. Louis, MO) were added. Samples were incubated at 37°C for 18 hr and were then acidified and extracted with ethyl ether. For enzyme inhibition, incubation mixtures included 1.0 mM saccharo-1,4-lactone (Sigma). Ether extracts were dried and the residues were dissolved in methanol for TLC analysis or for measurement of total bile acid content.

Preliminary purification of urine samples

Prior to TLC and β-glucuronidase hydrolysis, urine samples were partially purified and concentrated by passing them through a reversed-phase silica cartridge (Bond-Elut C-18, 6 ml capacity; Analytichem International, Harbor City, CA) following the procedure described by Setchell and Worthington (13). Methanol eluates from the cartridges were dried and redissolved in methanol, for direct TLC analysis, or in buffer, for enzymatic hydrolysis, as described above.

Total bile acid analysis

Total 3α-hydroxy bile acid concentration in native bile and urine and in samples subjected to β-glucuronidase hydrolysis were assayed with 3α-hydroxysteroid dehydrogenase (Worthington Biochemical Corp., Freehold, NJ) using the method of Talalay (14) as modified by Admirand and Small (15).

Gas–liquid chromatography (GLC)

To purify cholate glucuronide in bile or urine prior to GLC analysis, a modification of the method of Almé et al. (12) was used, thus effecting group separation of bile acid conjugates. Preparation of the ion-exchanger, DEAP-LH20, and all stages of the isolation procedure up to and including separation of the fraction of neutral compounds were carried out as described by Almé et al. (12). Final group separation of bile acid conjugates from the DEAP-LH20 column (0.5 × 73 cm) was accomplished by gradient rather than stepwise elution. With a starting buffer of 0.2 M acetic acid, pH 3.8, in 72% ethanol, a linear pH gradient was generated with 0.2 M ammonium acetate buffer, pH 9.5, in 72% ethanol. A flow rate of 5–6 ml/hr was maintained by a peristaltic pump (Harvard Apparatus, Millis, MA). Aliquots of fractions (4 ml each) were checked for radioactivity by scintillation counting and those fractions containing significant label were pooled, checked for bile acid content by TLC in system 2, and then taken to dryness. The dry residues were methylated and acetylated and analyzed by GLC as previously described (9).

RESULTS

Biliary secretion and recovery of [^{3}H]cholate glucuronide

In studies in which microgram amounts of cholate glucuronide were administered intravenously to animals prepared with biliary fistulas (**Table 1,** 1–6), significant

TABLE 1. Experimental conditions and isotope recoveries

Animal No.	Surgical Procedure	Animal Wt. (g)	Dose (mg)	% Recovery				
				Bile	Urine	Plasma	Tissues[a]	Total
1	Bile fistula	190	0.013	97.1	0.3	0	0	97.4
2	Bile fistula	276	0.017	96.9	1.2	0	0	98.1
3	Bile fistula	186	0.018	103.5	0.1	0	0	103.6
4[b]	Bile fistula	295	0.016	96.2	0.6	0	0	96.8
5[b]	Bile fistula	226	0.017	96.8	0.1	0	0	96.9
6[b]	Bile fistula	197	0.017	101.2	0.3	0	0	101.5
7	Bile fistula	290	3.75	94.4	1.3	0	0	95.7
8	Bile fistula	202	4.56	97.4	0.1	0	0	97.5
9	Bile fistula	203	4.24	98.7	0.2	0	0	98.9
10	Bile duct ligation	251	0.017		66.0	2.4	1.2	69.6
11	Bile duct ligation	262	0.016		107.9	0.2	1.4	109.5
12	Bile duct ligation	227	1.06		87.2	0.1	0.4	87.7
13	Bile duct ligation	279	4.40		72.4	0.1	0.4	72.9
14[b,c]	Bile duct ligation	269	0.012		97.3	0	0	97.3
					(69.2)	(8.1)	(7.5)	(84.8)
15[b,c]	Bile duct ligation	268	0.011		100.5	0.3	0	100.8
					(79.6)	(4.5)	(2.6)	(86.7)
16[b,c]	Bile duct ligation	278	0.011		99.8	1.4	0	101.2
					(84.9)	(3.9)	(3.2)	(92.0)
17[b,c]	Bile duct ligation	217	0.012		80.4	0.6	0	81.0
					(70.9)	(7.3)	(7.7)	(85.9)

[a]Includes liver, kidneys, small and large intestine.
[b]Doses for these animals also contained tracer amounts of [24-^{14}C]taurocholic acid.
[c]Values in parentheses are the corresponding recoveries of [^{14}C]taurocholate.

label (40–60% of the administered dose) was detected in bile within the first 10 min (**Fig. 1,** open symbols). In all animals, more than 90% of the label had been secreted in bile after only 60 min and only small additional amounts of tritium were recovered in bile between 1 and 20 hr. After 20 hr of bile collection, the secreted bile contained 98.6 ± 1.2% of the administered label.

A representative double isotope study comparing the biliary secretion of [^{3}H]cholate glucuronide and [^{14}C]taurocholate after intravenous administration is shown in **Fig. 2.** Bile from these animals (Table 1, 4–6) was collected dropwise and each drop was assayed for ^{3}H and ^{14}C content (Fig. 2A). Measurable amounts of both isotopes were detected in bile drop number 5 (less than 3 min after administration of the dose); however, ^{14}C secretion reached a maximum of 8.7% of the dose in bile drop number 8 (approx. 4.5 min after injection) while ^{3}H secretion achieved a maximum of 6.9% of the dose in bile

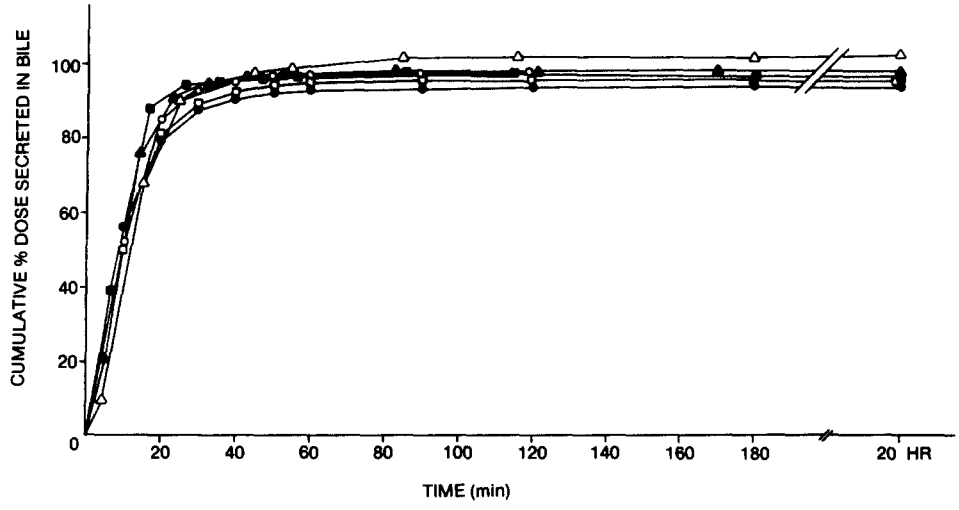

Fig. 1 Biliary secretion of [^{3}H]cholate glucuronide administered in microgram (open symbols) and milligram (solid symbols) amounts to rats with biliary fistulas.

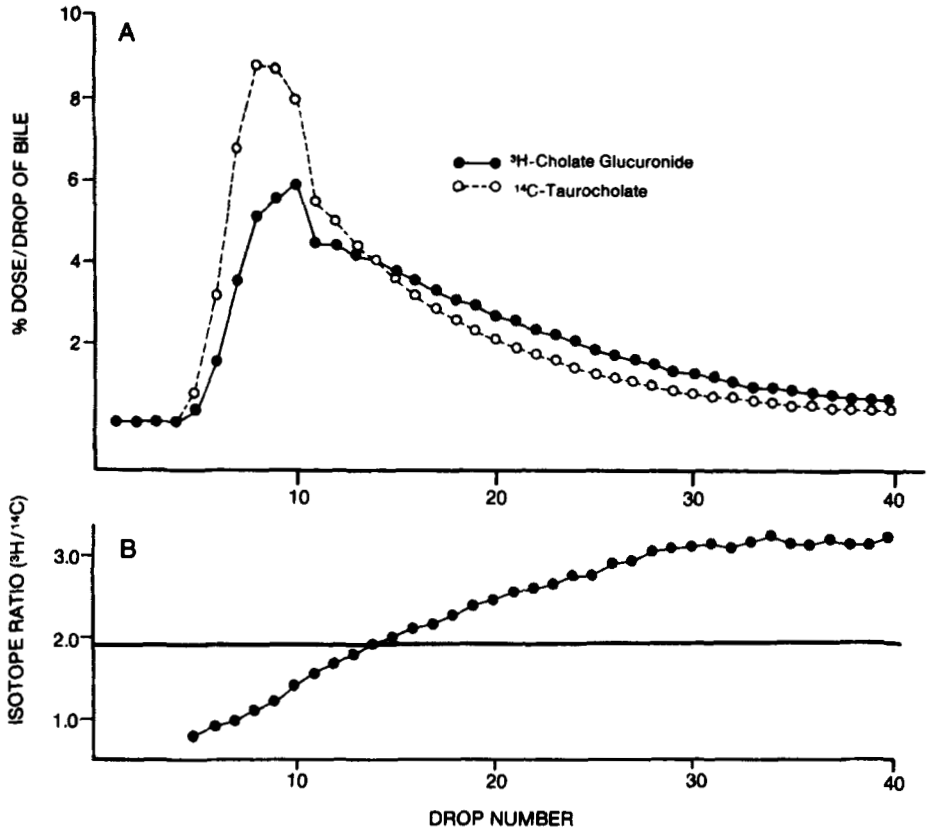

Fig. 2 Biliary secretion of [^{3}H]cholate glucuronide and [^{14}C]taurocholate. Both isotopes were administered simultaneously to a rat with a biliary fistula and bile was collected dropwise. ^{3}H/^{14}C isotope ratios (A) and percent secretion of the dose (B) were calculated for each drop of bile.

drop number 10 (ca. 5.5 min after injection). Comparison of the rates of secretion of ^{14}C and ^{3}H over the initial linear portion of the curve (drops 5 through 8) demonstrated that [^{14}C]taurocholate secretion was significantly more rapid than that of [^{3}H]cholate glucuronide ($P < 0.01$). In the study shown in Fig. 2, the rate of secretion of of [^{14}C]taurocholate exceeded that of [^{3}H]cholate glucuronide by 78% over the initial 5 min. In two other studies, ^{14}C secretion rates were 73 and 30% greater than those of tritium. The ^{3}H/^{14}C isotope ratio calculated for each drop (Fig. 2B) was initially considerably lower than that of the administered material (0.55 for bile drop number 8 vs. 1.20 for the dose) and this ratio increased steadily throughout the period of peak isotope secretion to a maximum of 1.5 at 12 min. The same pattern of isotope ratios was seen in the other two double isotope studies.

In all animals, small and variable amounts of label were excreted in urine (0.4 ± 0.2% of the dose) but there was no measurable activity at the end of the study in plasma or in any of the analyzed tissues (Table 1). Overall recovery of the administered label averaged 99.0 ± 1.2%.

Intravenous administration of milligram quantities of [^{3}H]cholate glucuronide (Table 1, 7–9) resulted in comparable rates of biliary secretion and comparable recoveries to those observed in tracer studies. The secretion of the administered label in bile was 96.8 ± 1.3% over 20 hr and the pattern of secretion (Fig. 1, solid symbols) was identical to that of a tracer amount. As with the previous series, variable small amounts of label were excreted in urine (0.5 ± 0.4%) for a total recovery of 97.4 ± 1.0% of the dose. Milligram amounts of cholate glucuronide, however, produced a significant choleresis which was not observed in the tracer studies. Within 10 min after injection of the glucuronide, bile flow increased an average of 40% (range 29–55%) over the preinjection levels (**Fig. 3A**). 3α-Hydroxysteroid dehydrogenase was used to measure the total bile acid content of pre- and post-injection bile samples before and after β-glucuronidase hydrolysis. As shown in Fig. 3B, no increase in bile acid secretion was seen in native, unhydrolyzed bile during the choleretic period. However, following β-glucuronidase hydrolysis, post-injection bile samples were shown to have significantly increased levels of 3α-hydroxy bile acid (mean increase: 80%; range: 57–104%) which paralleled the increases in bile flow and secretion of label.

Urinary excretion and recovery of [^{3}H]cholate glucuronide

Cholate glucuronide was administered to animals with ligated bile ducts (Table 1, 10–13) in both microgram and

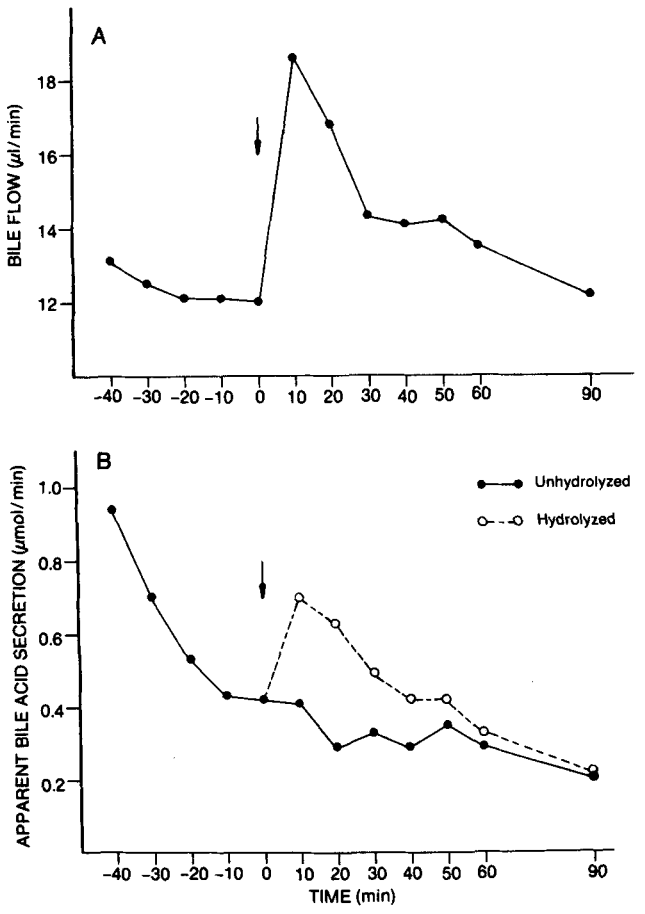

Fig. 3 Effect of cholate glucuronide on bile flow and bile acid secretion. A, Bile flow. B, Total bile acid secretion before (solid line) and after (dotted line) β-glucuronidase hydrolysis of bile samples. In both figures, the arrow indicates the time of administration of 3.75 mg of cholate glucuronide to a rat with a biliary fistula.

milligram amounts. The major portion of the administered isotope (83.4 ± 9.3%) was recovered in urine over the 20 hr of study (Table 1). Urinary excretion of label did not appear to be affected by the quantity of cholate glucuronide administered but did vary considerably (**Fig. 4**), paralleling differing urine outputs of the individual animals. In all but one of the animals, excretion of label in urine proceeded in a relatively linear fashion. The one exception had a urine output in the first 4 hr (9.7 ml) that was nearly twice that of any of the other animals (4.9, 5.1, and 4.7 ml). Smaller amounts of label were found in plasma (0.7 ± 0.6%) and tissue (0.5 ± 0.3%) at the end of study, resulting in an overall recovery of 84.9 ± 9.1% of the dose.

Urinary excretion of [^{3}H]cholate glucuronide and [^{14}C]taurocholate administered simultaneously to bile duct-ligated rats (Table 1, 14–17) is shown in **Fig. 5.** Excretion of cholate glucuronide was more rapid than that of taurocholate in the earlier portion of the study. Two hours after administration of the bile acids, urine contained 10 times more cholate glucuronide than taurocholate. After 20 hr of study, total urinary recovery of cholate glucuronide was 94.5 ± 4.7% of the administered material and that of taurocholate was 76.2 ± 3.7%. While only a minor portion of the administered tritium was found in plasma (0.6 ± 0.3%) and none was detected in tissues, significant amounts of ^{14}C were retained in plasma (6.0 ± 1.0%) and tissues (5.2 ± 1.4%). Total recoveries of [^{3}H]cholate glucuronide and [^{14}C]taurocholate were, respectively, 95.1 ± 4.8% and 87.4 ± 1.6% of the administered dose.

Characterization of the labeled compound(s) in bile and urine

Data obtained from the analysis of both bile and urine samples are summarized in **Table 2.**

Initial identification of the chemical form of the label secreted in bile and excreted in urine was accomplished by TLC before and after β-glucuronidase hydrolysis. Aliquots of bile were chromatographed directly or hydrolyzed and chromatographed, whereas urine samples were partially purified before hydrolysis or chromatography. Unhydrolyzed bile and urine, chromatographed in system 2, each had a major radioactive peak which contained more than 95% of the applied label and had an R_f of 0.28, identical to that of authentic cholate glucuronide. Urine samples also showed a minor radioactive peak (< 2% of the total) at R_f 0.91, corresponding to free cholic acid. This second peak was not found in bile samples. Samples of urine from bile duct-ligated animals given both [^{3}H]cholate glucuronide and [^{14}C]taurocholate were chromatographed in system 3 which produces a wide separation between the two conjugates. In this system, more than 95% of all tritium migrated as a single band with the R_f of cholate glucuronide (R_f 0.08) while at least

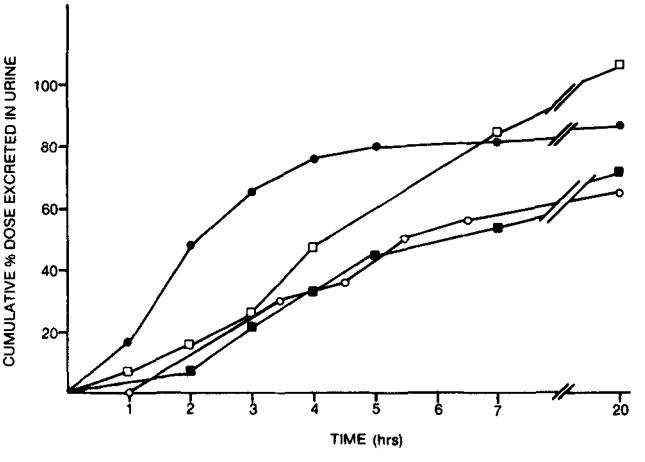

Fig. 4 Urinary excretion of [^{3}H]cholate glucuronide by rats with ligated bile ducts. Rat 10: ○—○; Rat 11: □—□; Rat 12: ●—●; Rat 14: ■—■.

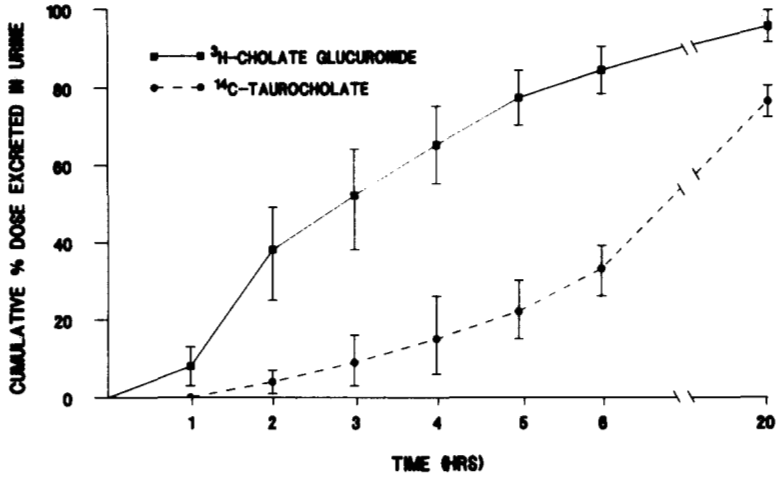

Fig. 5 Urinary excretion of simultaneously administered [^{3}H]cholate glucuronide (■—■) and [^{14}C]taurocholate (●- -●). Each point represents the mean of four experiments and the brackets give the standard error.

85% of the ^{14}C co-chromatographed with taurocholate (R_f 0.55). A small amount of ^{14}C activity (1.0–3.4%) was recovered with a faint band with an R_f of 0.35, similar to that of taurocholate-3-sulfate (R_f 0.40). No other discrete bands of ^{14}C activity were detected.

Following β-glucuronidase hydrolysis and TLC in system 1, more than 90% of the applied isotope in both bile and urine samples from animals given [^{3}H]cholate glucuronide co-chromatographed with free cholic acid (R_f 0.20). In urine, but not in bile, there was a smaller amount of the label (< 5%) that remained at the origin.

Final identification of the material in bile and urine from animals given milligram amounts of cholate glucuronide was accomplished by GLC analysis of the methyl ester acetates of labeled peaks eluted from DEAP-LH20 columns (see Methods). **Fig. 6** shows the pattern of gradient development and elution of the labeled peaks obtained from the ion-exchange chromatography of bile (Fig. 6A) and urine (Fig. 6B). A single peak of radioactivity was recovered from bile at an elution volume (192 ml) and pH (6.25) similar to those of authentic cholate glucuronide (184 ml, pH 6.35). This peak contained 81.5% of the activity applied to the column and no other radioactive peaks were detected. Chromatography of urine produced two peaks: peak A was eluted at the very beginning of the gradient at a volume and pH (75 ml, pH 5.2) equivalent to that of the free bile acids (72 ml, pH 5.2) and represented 7.9% of the total radioactivity. The major peak, B, with 73.6% of the applied label, had an elution volume (190 ml) and pH (6.3) equivalent to those of the biliary steroid and of cholate glucuronide.

When fractions from the peak areas were pooled and checked by TLC, the bile sample peak and peak B from the urine sample contained material chromatographing with an R_f identical to that of cholate glucuronide, while peak A from urine co-chromatographed with free cholic acid. These identifications were confirmed by GLC analysis of the methyl ester acetates of each of the samples. Peak A from the DEAP-LH20 chromatography of urine was composed of a single GLC peak with a retention time (R_t) of 7.13 min, the same as that of cholic acid methyl ester triacetate. The single bile peak and peak B from urine both chromatographed as single major peaks with an R_t of 18.90 min, equivalent to that of the dimethyl ester pentaacetate of the synthetic cholate glucuronide (R_t 18.82 min).

DISCUSSION

It is well established that bile acids are secreted in bile as taurine and glycine conjugates (16). In recent years, it

TABLE 2. Identification of labeled compounds in bile and urine

Samples	TLC R_f[a] System 1	R_f[a] System 2	DEAP-LH-20[b] Vol	DEAP-LH-20[b] pH	GLC[c] R_t
					min
Standards					
Cholate glucuronide	0	0.28	184	6.35	18.82
Cholic acid	0.21	0.89	72	5.20	7.13
Bile					
Native		0.28			
Hydrolyzed[d]	0.20				
DEAP-LH-20-peak		0.29	192	6.25	
Urine					
Native		0.28			
		0.91			
Hydrolyzed[d]	0.20				
DEAP-LH-20-peak A	0.21	0.92	75	5.20	7.13
DEAP-LH-20-peak B	0	0.29	190	6.30	18.90

[a]System 1 separates free bile acids; system 2 separates conjugated bile acids; see text for solvent composition.
[b]Values are given for peak radioactive fractions; see text for details.
[c]As methyl ester acetate derivatives; see text for conditions.
[d]β-Glucuronidase hydrolysis; see text for details of incubation.

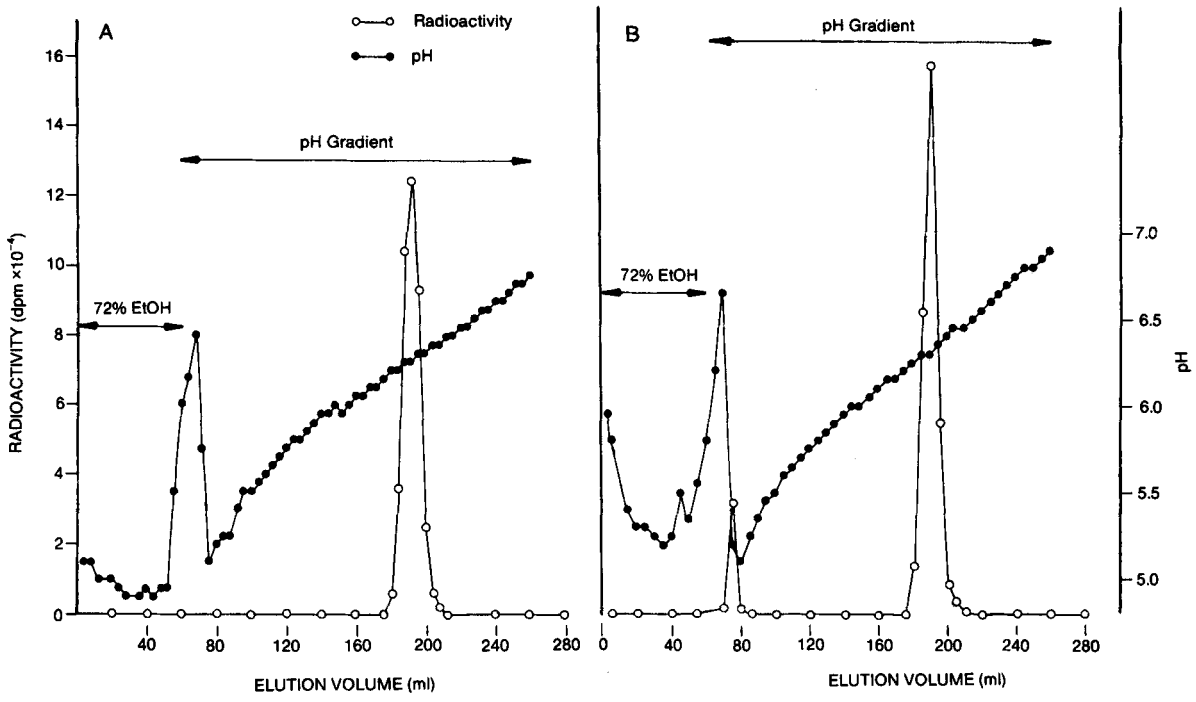

Fig. 6 DEAP-LH20 chromatographic separation of labeled compounds in bile (A) and urine (B). Each column was eluted with 35 ml of 72% ethanol (bracket) and then a pH gradient was developed as shown by the solid symbols and the right ordinate. The tritium content of each fraction is given by the open symbols and the left ordinate.

has been recognized additionally that bile acids are metabolized to sulfates (17–19), and that sulfates are the predominant bile acid conjugates in the urine of patients with cholestasis (19–22). The role of bile acid glucuronidation is less widely appreciated. Only minute amounts of bile acid glucuronides are found in normal biologic materials, but these levels become greatly increased in the plasma and urine of patients with cholestasis (2–5). Additionally, atypical bile acids of conventional and short chain-length have been shown to form glucuronide conjugates (23–25).

The presence of bile acid glucuronides in the plasma and urine in cholestatic liver disease suggests that these compounds are normally secreted in bile. In addition, recent results in studies with lithocholate glucuronide (9), which were different from what might have been predicted of bile acid glucuronide behavior based on available clinical observations, mandated a detailed study of the metabolic behavior of additional bile acid glucuronides.

The present results establish that cholate glucuronidation, when compared to taurine or glycine conjugation, does not alter the fundamental pattern of bile acid turnover and elimination. Microgram ("tracer") and milligram ("load") quantities of intravenously administered cholate glucuronide were rapidly and efficiently secreted in bile: more than 80% of the administered dose appeared in the bile of fistula rats within 20 min of injection. Cholate glucuronide administered in milligram quantities produced a choleretic effect within 10 min of injection, with bile flow increases averaging 40% and paralleling cholate glucuronide secretion. Thus, the rate and configuration of secretion and the choleretic properties of cholate glucuronide closely resembled those of the amino acid conjugates.

Differences in conjugate secretion were examined more closely in bile fistula rats injected simultaneously with [^{3}H]cholate glucuronide and [^{14}C]taurocholate. Each drop of bile was counted separately during the period of rapid initial secretion. With this rapid sampling, fine differences in secretory patterns could be observed which might be missed or ignored if studies were performed in separate animals or with longer sampling intervals. These studies established a small but measurable retardation of the biliary appearance time of the glucuronide as compared to the taurine conjugate of cholic acid. The present studies do not establish at what stage in the hepatobiliary secretion of cholate glucuronide the delay occurs.

Cholate glucuronide was secreted in bile intact and unaltered when administered in either microgram or milligram amounts. This was shown enzymatically, using the principle that 3α-hydroxysteroid dehydrogenase will react with cholate glucuronide only after cleavage of the conjugate with β-glucuronidase. It was further documented by direct TLC comparison of the product of biliary secretion with appropriate standards, and by GLC of suitably purified and derivatized samples from bile.

Biliary fistula rats excreted little intravenously administered cholate glucuronide in urine, but bile duct-obstructed animals averaged 83% urinary excretion. Again, TLC and GLC analysis confirmed that the radiolabeled material excreted was predominantly intact cholate glucuronide. A minor fraction (approximately 7%) was shown by GLC to be free cholate. Since less than 2% free cholate was evident on TLC analysis, it is probable that the major portion of free cholate was generated as an artifact of the analytical process, rather than by renal excretion of free cholate or by glucuronide hydrolysis in bladder urine. Urine collected from obstructed animals given both the glucuronide and taurine conjugates of cholic acid contained a larger amount of cholate glucuronide than of taurocholate in the first few hours after intravenous injection, indicating more efficient renal clearance of the glucuronide conjugate. Less effective excretion of taurocholate was also shown by the higher recovery of this conjugate from plasma and tissues at the end of the experiment. TLC analysis showed that both conjugates were excreted predominantly in intact form.

Therefore, while cholate glucuronide is secreted intact in urine under conditions of biliary obstruction or cholestasis, the process of renal clearance is less efficient than is biliary secretion. However, the urinary route of excretion is more effective for the glucuronide than for the taurine-conjugate of cholic acid. Insofar as the bile duct-obstructed rat simulates clinical conditions in humans, the results suggest that glucuronide formation would promote urinary cholate excretion in cholestatic liver disease.

Finally, the physical and biological properties of cholate glucuronide present an interesting counterpoint to those of lithocholate glucuronide. While both compounds are water-soluble and readily secreted in bile in trace amounts, administration of milligram quantities of cholate glucuronide produced a choleresis at concentrations which with lithocholate glucuronide resulted in partial or complete cholestasis (9). When complete cholestasis was mimicked by bile duct ligation, cholate glucuronide was effectively cleared from the plasma by urinary excretion and did not accumulate to a significant degree in tissues. When, however, lithocholate glucuronide was administered intravenously to bile duct-ligated animals, urinary excretion was modest and the material accumulated in body tissues.

The results suggest that glucuronidation confers certain properties on the bile acid molecule distinctive from those conferred by amino acid conjugation. Even among bile acid glucuronides, however, certain physiologic properties of the molecule are influenced primarily by the steroid moiety, rather than by the form of conjugation.

This work was partially supported by grants from the National Institutes of Child Health and Human Development, Grant No. HD14198, and the March of Dimes Birth Defects Foundation, Grant No. 6-305.

Manuscript received 16 August 1984.

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