Bile acid sulfates. I. Synthesis of lithocholic acid sulfates and their identification in human bile

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ABSTRACT Sulfate esters of lithocholic, glycolithocholic, and taurolithocholic acids were synthesized using sulfur trioxide in pyridine; they were purified by crystallization from methanol or ethanol as the diammonium salts, and their chemical compositions, infared spectra, and chromatographic behavior were determined. Strong alkaline hydrolysis of these sulfates, as commonly performed during quantitative and qualitative analyses of conjugated bile salts, was found to result in a number of degradation products, presumably through disruption of the C–O bond of the hydroxyl group and conversion of the original steroid to isolithocholate and other (possibly olefinic) compounds. After oral administration of lithocholate-^{14}C to three patients with cholelithiasis, radioactive metabolites having the chromatographic properties of sulfated lithocholates were isolated from bile and, confirming a preliminary report (1), were identified as sulfated glycolithocholate and taurolithocholate by their characteristic chromatographic mobilities during a series of specific hydrolytic procedures and by crystallizing them to constant specific activities with the synthetic sulfates. The fraction of endogenous lithocholate present in bile as the sulfate was calculated for two patients by isotope dilution and was shown to be 41% and 75% of the total. Sulfation can be expected to affect the physiological and pharmacological properties of lithocholates and may, therefore, influence the toxic properties of these compounds.

SUPPLEMENTARY KEY WORDS diammonium lithocholate-3-sulfate · diammonium glycolithocholate-3-sulfate · diammonium taurolithocholate-3-sulfate

Previous studies on the metabolism of orally administered lithocholic acid-24-^{14}C in humans revealed at least two unidentified biliary compounds that were more polar than the expected taurine and glycine conjugates (2). Sulfuric acid, glucuronic acid, and glutathione are commonly used to conjugate neutral steroids and other relatively insoluble compounds, and could be used to conjugate lithocholates. The existence of bile alcohol sulfates in lower animals and the polarity characteristics of the unknown compounds led us to synthesize several lithocholic acid sulfates and to compare their properties with those of the previously described metabolites. This report describes the synthesis of lithocholic, glycolithocholic, and taurolithocholic acid sulfates, their degradation by various hydrolytic and solvolytic procedures, and their identification as biological metabolites of endogenously formed lithocholic acid in human bile.

METHODS

Thin-layer Chromatography

TLC was performed using 0.2–1.0-mm layers of silica gel H (Brinkmann Instruments, Inc., Des Plaines, Ill.) and the following solvent systems: S-VIII (3), *n*-propanol–propionic acid–isoamylacetate–water 10:15:20:5; Butanol 1 (4), *n*-butanol–acetic acid–water 50:5:5; Bu-

A preliminary report of this work has been published (1).

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The following trivial names and abbreviations have been employed: TLC, thin-layer chromatography; lithocholic acid, 3α-hydroxy-5β-cholanic acid; isolithocholic acid, 3β-hydroxy-5β-cholanic acid; glycolithocholic acid, 3α-hydroxy-5β-cholanoyl-glycine; taurolithocholic acid, 3α-hydroxy-5β-cholanoyl-taurine; etiocholanolone, 3α-hydroxy-5β-androstane-17-one. "Sulfate" refers to the 3-sulfate ester of the corresponding bile acid unless otherwise indicated.

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tanol 3, *n*-butanol–0.01 M Tris buffer–propionic acid 50:9.25:0.75; and T40 (2), trimethyl pentane–ethyl acetate–acetic acid 40:20:0.5.

Solvolysis

Solvolysis was performed using a modification of the methods of Burstein and Lieberman (5) and Kornel (6). The sulfate ester was dissolved in ethanol, acidified to pH 1 or less with 2 N HCl, and diluted with 9 vol of acetone. The mixture was incubated at room temperature for 1–3 days, evaporated to dryness in vacuo, and refluxed in 5% methanolic KOH for 2 hr to hydrolyze the ethyl esters formed during solvolysis. The solutions were neutralized by batch treatment with Dowex 50, filtered, and evaporated.

Alkaline Hydrolysis

Strong alkaline hydrolysis of the amino acid conjugates were performed in Teflon test tubes by autoclaving for 19 hr at 130°C in 5 N NaOH. Enzymatic hydrolysis of the amide linkage was performed using cholylglycine hydrolase (Mann Research Laboratories, New York) as described by Nair, Gordon, and Reback (7).

Isotopic Techniques

Radioactive compounds on thin-layer chromatograms were detected by radioautography using RP-54 X-Omat medical X-ray film (Eastman Kodak), or by scanning with a Vanguard 885 glass plate scanner (Vanguard Instrument Co., LaGrange, Ill.). After chromatography, thin-layer plates were exposed to ammonia vapors to diminish solvolysis during the elution process. The silica gel containing radioactivity was scraped from the plate, moistened with water, and refluxed in chloroform–methanol 1:1. A few drops of ammonium hydroxide were added, the solution was filtered, and the filtrate was evaporated in a flash evaporator. Radioactive samples were counted in a Nuclear-Chicago liquid scintillation counter; corrections for quenching were made using ^{14}C-labeled toluene as an internal standard.

Clinical

Lithocholic acid-24-^{14}C (10–50 μCi, 7.58 mCi/mmole; New England Nuclear Corp.), shown to be pure by TLC and radioautography, was administered orally to three patients with functioning gallbladders 36 hr (patients A and B) and 19 hr (patient C) prior to elective cholecystectomy for cholelithiasis. The patients were euthyroid clinically and without signs or symptoms of biliary tract obstruction. At operation, the bile obtained from the gallbladder contained approximately 10% of the administered radioactivity. Bile specimens were stored in the freezer prior to analysis.

Miscellaneous

Enzymatic assays of bile acids were performed as previously described (8). Infrared spectra were obtained through the courtesy of Dr. Josef Fried. Samples were pelleted in potassium bromide, and the spectra were determined in a Perkin-Elmer model 137 infrared spectrophotometer.

RESULTS

Synthesis of Bile Acid Sulfates

The 3α-sulfate esters of bile acids were prepared with sulfur trioxide, according to Fieser (9). A 25-ml stoppered conical tube containing 1 ml of dry pyridine was held slanting in ice while 0.1 ml of chlorosulfonic acid was added in small drops. The resulting suspension was homogenized with a stirring rod. 100 mg of bile acid was dissolved in 0.5–1.0 ml dry pyridine with warming and transferred to the reaction tube; a second equal portion of pyridine was used to rinse the rest of the bile acid into the reaction tube. The reaction mixture was allowed to stand at room temperature for 3–7 days until the reaction was more than 90% complete, as judged by small thin-layer chromatograms. Water was added to terminate the reaction.

The mixture was evaporated in vacuo, to remove excess pyridine, until the pH became acid (ca. 3). The mixture was transferred to a separatory funnel, quickly extracted twice with 2 vol of *n*-butanol, and the butanol was backwashed twice with 0.5 vol of water. A few drops of concentrated ammonium hydroxide were immediately added to the butanol, which was then evaporated at 37–40°C in vacuo.

Significant loss of the steroid sulfate may occur in alcoholic solutions, presumably by transesterification, as in methanolysis or ethanolysis (5, 10). Excess ammonium hydroxide was therefore added as soon as the steroid had been extracted from the acid aqueous phase into butanol. (The diammonium salts are not readily extracted from the aqueous phase with butanol.)

Purification of Bile Acid Sulfates

Bile acid sulfates were crystallized from methanol or ethanol as the diammonium salts. The use of excess ammonia, easily removed subsequently, prevented the solvolysis that frequently occurred during other crystallization procedures; attempts to prepare potassium pyridinium and tetramethyl ammonium salts were not successful.

Diammonium Lithocholate-3-sulfate. Diammonium lithocholic acid-3-sulfate was crystallized from methanol–ethyl acetate in the presence of excess ammonium hydroxide. Further crystallization from ethanol in the presence of excess ammonium hydroxide resulted in larger

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crystals, mp 181.0–181.5°C. The crystals were hygroscopic, and drying to constant weight resulted in the variable loss of nitrogen; however, elementary analysis (MicroTech Laboratories, Skokie, Ill.) showed a carbon/sulfur ratio of 9.10 (calculated, 8.98).

Diammonium Glycolithocholate-3-sulfate. Glycolithocholic acid-3-sulfate was dissolved in ethanol and a small amount of concentrated ammonium hydroxide. The volume was reduced by boiling, with the loss of ammonia, to a concentration of about 65 mg/ml. The addition of a few drops of concentrated ammonium hydroxide then resulted in crystallization of the diammonium salt in about 80% yield. Repeated crystallization resulted in small crystals, mp 183.5–184.0°C. Elementary analysis showed:

Analysis: $C_{26}H_{49}O_7S_1N_3$;
calculated: C, 57.0; H, 9.0; N, 7.7; S, 5.9
found: C, 57.0; H, 8.9; N, 7.2; S, 5.9

Diammonium Taurolithocholate-3-sulfate. The taurolithocholic acid sulfate, contaminated with unreacted taurolithocholate, was dissolved in hot methanol in the presence of ammonium hydroxide. Addition of ethyl acetate resulted in crystallization of the diammonium sulfate salt in approximately 50% yield, with elimination of most of the taurolithocholate in the filtrate. Subsequent crystallizations from hot ethanol, in the presence of ammonium hydroxide, with ethyl acetate gave better yields of small crystals, mp 189–190°C. Elementary analysis showed:

Analysis: $C_{26}H_{51}O_8S_2N_3$;
calculated: C, 52.2; H, 8.6; N, 7.0; S, 10.7
found: C, 52.3; H, 8.6; N, 7.0; S, 10.7

Infrared spectra were obtained from the three sulfated lithocholates, and are shown in Fig. 1.

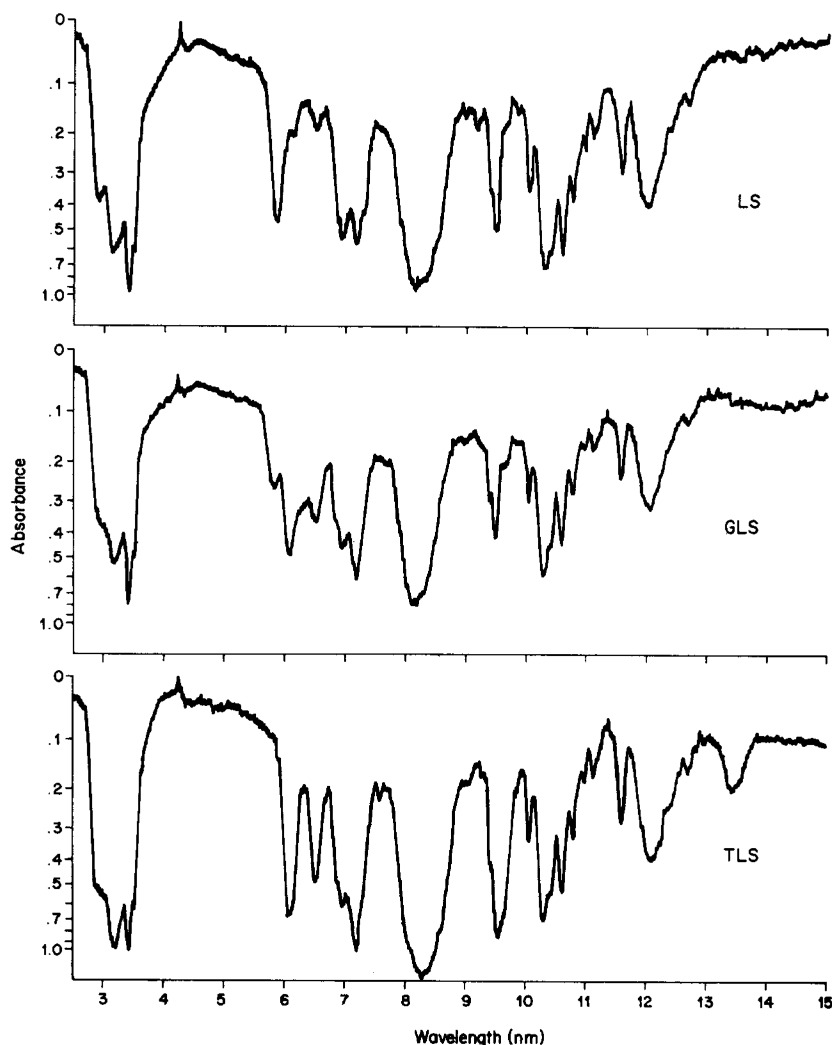

Fig. 1. Infrared spectra of sulfated lithocholates. *LS*, diammonium lithocholate-3-sulfate; *GLS*, diammonium glycolithocholate-3-sulfate; *TLS*, diammonium taurolithocholate-3-sulfate.

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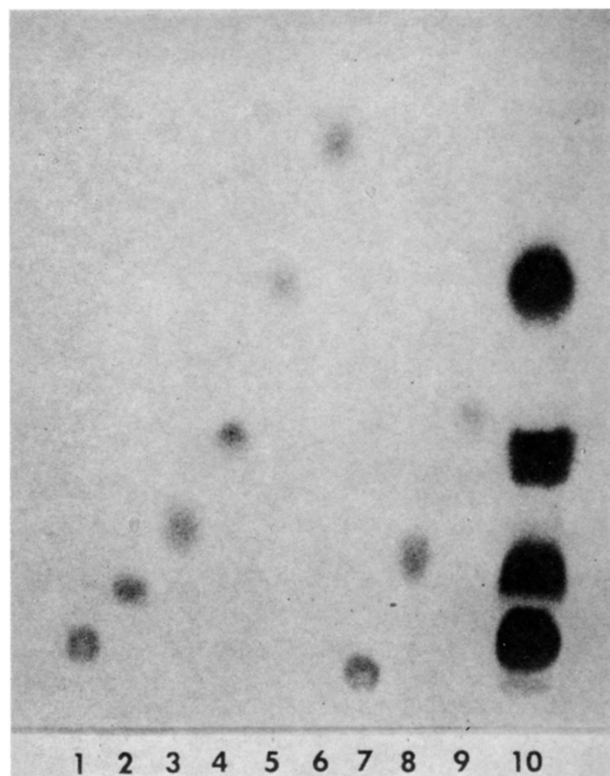

Fig. 2. Chromatographic mobility of bile acid sulfates on silica gel H using system S-VIII (3). *1*, taurocholic acid; *2*, taurodeoxycholic acid; *3*, taurolithocholic acid; *4*, glycocholic acid; *5*, glycodeoxycholic acid; *6*, glycolithocholic acid; *7*, taurolithocholic acid sulfate; *8*, glycolithocholic acid sulfate; *9*, lithocholic acid sulfate; *10*, human bile.

Properties of Bile Acid Sulfates

The diammonium salts described above ran as single spots on thin-layer chromatography; a chromatogram using Hofmann's S-VIII system (3) is shown in Fig. 2. The R_F values for these and other conjugated bile salts in several common chromatographic systems are listed in Table 1. The sulfated compounds were considerably more polar than their unsulfated precursors and by gross inspection were much more water-soluble. They did not appear to form micelles in aqueous solutions at room temperature, as indicated by their failure to cause a color shift when added to a solution of pinacyanol chloride (11). Small and Admirand have characterized some of the physical properties of these sulfates in greater detail (12).

The bile acid sulfates were not oxidized by incubation with the enzyme 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50), thus confirming the complete esterification of the 3α-hydroxyl group.

Incubation of taurine or glycine conjugated bile acid sulfates with cholylglycine hydrolase resulted in significant hydrolysis of the amide linkage; the sulfate ester group was not affected. The reaction was slower than with the unsulfated compounds, however, and the incubation conditions necessary for complete hydrolysis have not been determined.

TABLE 1 Thin-layer Chromatographic Mobilities of Sulfated Lithocholates

Bile Salt	Chromatographic System		
	S-VIII	Butanol 1	Butanol 3
Taurolithocholate-3-sulfate	0.02	0.23	0.17
Taurocholate	0.07	0.25	0.24
Taurodeoxycholate	0.15	0.41	0.39
Glycolithocholate-3-sulfate	0.20	0.50	0.14
Taurolithocholate	0.24	0.53	0.50
Glycocholate	0.39	0.55	0.20
Lithocholate-3-sulfate	0.41	0.66	0.60
Glycodeoxycholate	0.61	0.73	0.32
Glycolithocholate	0.83	0.83	0.40

The chromatographic systems are given in the section on Methods. The chromatograms were allowed to develop until the solvent front had reached a line 15 cm from the origin. The mobilities are tabulated as R_F values, relative to the front.

Effect of Hydrolytic Procedures on Bile Acid Sulfates

Strong alkaline hydrolysis of bile acid sulfates, as commonly used in the hydrolysis of bile acid conjugates, resulted in hydrolysis of amide linkages, partial disruption of the sulfate esters, and alterations in the steroid nuclear configuration (Figs. 3 and 4). The latter alterations gave rise to several compounds less polar than lithocholic acid; the predominant one had the chromatographic mobility of isolithocholic acid, while another major one was much less polar and ran near cholesterol or cholanic acid. No further effort was made to identify these compounds.

Isolation and Identification of ^{14}C-labeled Bile Acid Sulfates in Human Bile

In a previous study, the oral administration of lithocholic acid-24-^{14}C gave rise to four biliary conjugates: glycolithocholic acid, taurolithocholic acid, and two unidentified, more polar compounds, I and II (2). Bile from all three patients in this study showed the same four labeled components, with the unidentified compounds together comprising 44, 61, and 82% of the recovered radioactivity from each patient.

TLC of bile with the Butanol 3 system (Fig. 5) separated the unidentified ^{14}C-labeled compounds (area *A*) from the ^{14}C-labeled taurine and glycine conjugates (area *B*). Areas *A* and *B* were eluted and rechromatographed with the Butanol 1 system (Fig. 6). Area *A* contained two components (compounds I and II) with the chromatographic mobilities of glycolithocholic acid sulfate and taurolithocholic acid sulfate, respectively. On solvolysis, they gave corresponding labeled compounds with the mobilities of glycolithocholic acid and tauro-

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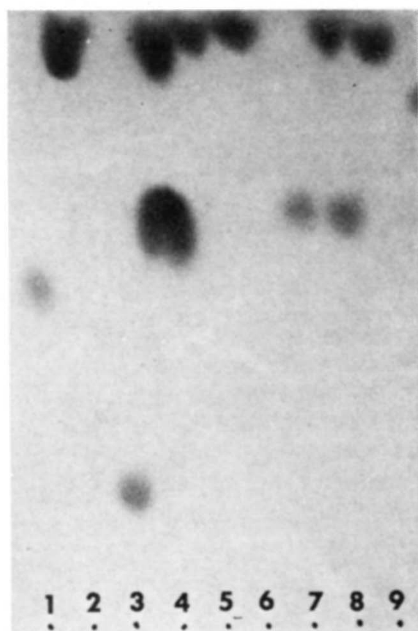

FIG. 3. Effect of strong alkaline hydrolysis on bile acid conjugates. TLC on silica gel H using Butanol 1 (4). *1*, taurolithocholic acid; *2*, taurolithocholic acid after strong alkaline hydrolysis; *3*, taurolithocholic acid sulfate; *4*, taurolithocholic acid sulfate after strong alkaline hydrolysis; *5*, taurolithocholic acid sulfate after solvolysis and subsequent strong alkaline hydrolysis; *6*, lithocholic acid; *7*, lithocholic acid sulfate; *8*, lithocholic acid sulfate after strong alkaline hydrolysis; *9*, lithocholic acid sulfate after solvolysis and subsequent strong hydrolysis. Notice that strong alkaline hydrolysis of taurolithocholic acid sulfate (*4*) has failed to remove approximately one-half of the sulfate, as shown by the large spot with the mobility of lithocholic acid sulfate (*7*).

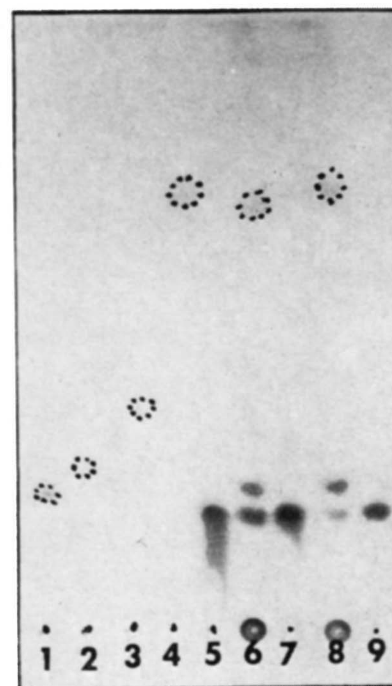

FIG. 4. Effect of strong alkaline hydrolysis on the nuclear configuration of bile acid sulfates. TLC on silica gel H using T40 system (2). *1*, lithocholic acid; *2*, isolithocholic acid; *3*, 3-ketocholanic acid; *4*, cholesterol; *5*, taurolithocholic acid after strong alkaline hydrolysis; *6*, taurolithocholic acid sulfate after strong hydrolysis; *7*, glycolithocholic acid after strong hydrolysis; *8*, glycolithocholic acid sulfate after strong alkaline hydrolysis; *9*, lithocholic acid after strong alkaline hydrolysis. The large spots at the origin (*6* and *8*) presumably represent lithocholic acid sulfate (see Fig. 2). Spots not showing well in the photograph have been outlined by dots.

lithocholic acid. Area *B* contained two components with mobilities similar to the material in solvolyzed area *A*. The individual spots were eluted, subjected to strong hydrolysis, and chromatographed with system T40; each gave a single radioactive spot with the mobility of lithocholic acid.

Substantial amounts of compounds I and II were isolated by preparative TLC (Butanol 3 followed by Butanol 1). Compound I was added to unlabeled glycolithocholic acid sulfate, compound II was added to unlabeled taurolithocholic acid sulfate, and the two compounds were crystallized to constant specific activity as the diammonium salts (Tables 2 and 3).

Conjugates of Endogenous Lithocholic Acid in Human Bile

Bile from the second and third patients was analyzed to determine the extent to which endogenous lithocholic acid had undergone glycine conjugation, taurine conjugation, and sulfation. (Bile from the first patient had been completely used in initial experiments.) The method used was to calculate the concentration of labeled lithocholate (nCi/ml) for each of the four derivatives (glycolithocholate, taurolithocholate, and their sulfates) from (*a*) the total concentration of isotope in bile, and (*b*) the distribution of isotope among the chromatographic fractions. Lithocholic acid was then isolated from each fraction and the specific activity was determined. Dividing the concentration of isotope (nCi/ml) in each fraction by the specific activity (nCi/μmole) gave the concentration of unlabeled, endogenous derivative (μmoles/ml) in bile.

Specifically, sulfated and nonsulfated derivatives were separated by TLC (Butanol 3 system), detected by scanning, and eluted. After solvolysis of the sulfates, the taurine and glycine conjugates were separated (Butanol 1 system), scanned, and eluted. The distribtion of radioactivity was estimated from the areas under the peaks of the chromatogram scans, and the total radioactivity in each fraction was then calculated from the total amount present in bile. The four eluted fractions were then hydrolyzed and the free lithocholic acid was isolated (system T40). The specific activity of lithocholic acid derived from each fraction was then determined by enzymatic assay (8) and liquid scintillation counting as described under Methods. This then permitted calcula-

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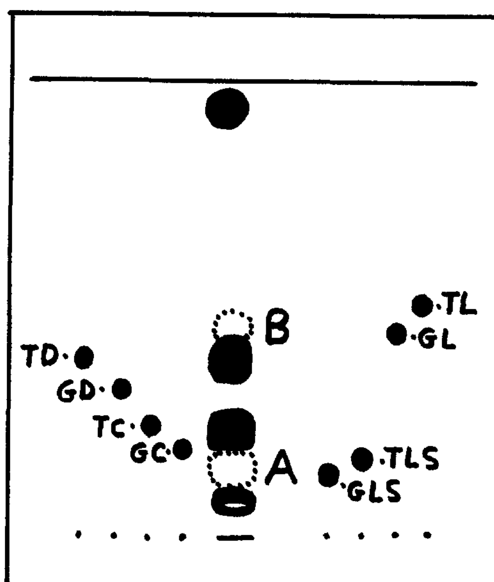

Fig. 5. Group separation of sulfated metabolites of lithocholate-^{14}C in human bile. TLC: silica gel H and Butanol 3 system. The areas containing labeled metabolites, detected by radioautography, are outlined by dots, *A* and *B*. Solid spots represent areas charred by concentrated sulfuric acid. Reference compound abbreviations: *T*, taurine; *G*, glycine; *S*, sulfate; *D*, deoxycholate; *C*, cholate; *L*, lithocholate.

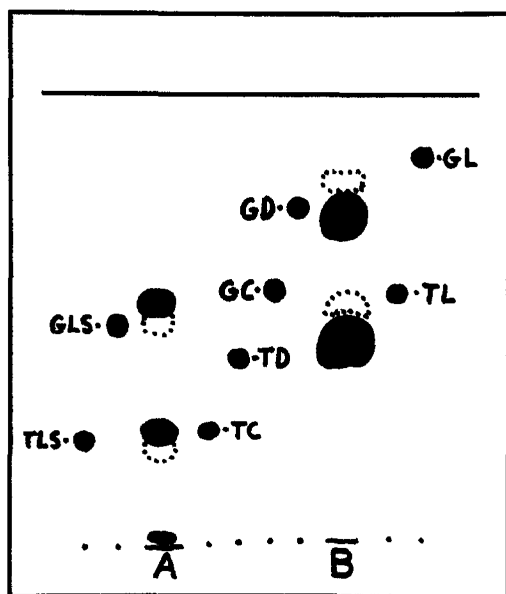

Fig. 6. Separation of individual radioactive compounds eluted from the sulfated (*A*) and nonsulfated (*B*) areas shown in Fig. 4. TLC: silica gel H and Butanol 1 system. Legend as in Fig. 4.

tion of the total amount of lithocholic acid originally present as each of the four derivatives in bile. The results are shown in Table 4. The total quantity of lithocholic acid derivatives calculated to be present in bile (B, 0.82 μmole/ml; C, 2.82 μmoles/ml) constituted about 0.6% (B) and 2.7% (C) of the total bile acids (B, 136 μmoles/ml; C, 104 μmoles/ml) measured enzymatically, figures consistent with others reported in the literature (13).

TABLE 2 Identification of ^{14}C-labeled Glycolithocholic Acid-3-sulfate by Recrystallization to Constant Specific Activity

Amount	Specific Activity
mg	*dpm/mg* $\times$ *10*$^{-2}$
74.1	3.07
61.9	3.83
45.7	3.77
30.5	3.96

Unlabeled diammonium glycolithocholate-3-sulfate (103.6 mg) was added to compound 1 (3.46×10^4 dpm) eluted from thin-layer plates, giving a calculated specific activity of 3.34×10^2 dpm per mg. The material was recrystallized several times from ethanol–ammonium hydroxide.

TABLE 3 Identification of ^{14}C-labeled Taurolithocholic Acid-3-sulfate by Recrystallization to Constant Specific Activity

Solvent	Amount	Specific Activity
	mg	*dpm/mg* $\times$ *10*$^{-3}$
Ethanol–ammonium hydroxide	87.0	2.18
	63.0	2.35
Ethanol–ammonium hydroxide–ethyl acetate	52.3	2.28
	46.9	2.26
	38.6	2.15

Unlabeled diammonium taurolithocholate-3-sulfate (191 mg) was added to compound II (3.85×10^5 dpm) eluted from thin-layer plates, giving a calculated specific activity of 2.01×10^3 dpm per mg.

DISCUSSION

The identification of glycolithocholic acid sulfate and taurolithocholic acid sulfate in human bile demonstrates a new pathway of bile acid metabolism in humans. These compounds were identified as biliary metabolites of orally administered lithocholic acid-24-^{14}C. After absorption, labeled lithocholate could theoretically enter at least six pools, depending on whether it remained free or was conjugated with taurine or glycine, and on whether or not it was sulfated. Since each compound could have its own pool size and turnover rate, it is not possible to assess the quantitative importance of these various pathways from the analysis of labeled metabolites present in bile at one point in time. However, data to be presented in the following paper (14) indicate that, in rats, sulfated lithocholates are less well absorbed from the intestine and more rapidly excreted than nonsulfated lithocholates, suggesting that the relative importance of sulfation in the metabolism of the labeled lithocholates (40–80% of the total recovered from bile) is more likely to be underestimated than overestimated from such analyses.

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TABLE 4 Lithocholic Acid Conjugates in Patients B and C

Conjugate	Lithocholic Acid-14C				Specific Activity		Total Lithocholic Acid			
	Distribution		Concentration				Concentration		Distribution	
	B	C	B	C	B	C	B	C	B	C
	%		μCi/ml		μCi/μmole		μmole/ml		%	
Glycolithocholic acid	8.5	9.8	0.0267	0.0304	0.123	0.076	0.217	0.399	22.4	14.1
Taurolithocholic acid	30.3	7.8	0.0950	0.0242	0.265	0.081	0.359	0.298	37.1	10.6
Glycolithocholic acid sulfate	36.1	57.9	0.1132	0.1792	0.533	0.122	0.212	1.473	22.0	52.2
Taurolithocholic acid sulfate	25.1	24.5	0.0787	0.0758	0.440	0.116	0.179	0.653	18.5	23.1
Totals	100.0	100.0	0.3136	0.3096			0.967*	2.823†	100.0	100.0

Lithocholic acid-24-14C (specific activity 7.58 μCi/μmole by direct assay) was administered orally to two human subjects (B and C) and gallbladder bile was recovered at operation 36 and 19 hr later. The distribution of radioactivity among the four conjugate fractions was measured, and the specific activity of lithocholic acid isolated from each fraction was determined. Values are averages of duplicate determinations. The amount of unlabeled, or endogenous, lithocholic acid present in each conjugate fraction was then calculated.

* Total bile acids, 136 μmoles/ml; 0.0414 μmole/ml added as the lithocholic acid-14C.

† Total bile acids, 104 μmoles/ml; 0.0409 μmole/ml added as the lithocholic acid-14C.

Another difficulty in interpreting results with labeled lithocholate is that the metabolism of orally administered lithocholate, presumably absorbed from the upper intestine, might not parallel the metabolism of endogenously formed lithocholate, which is presumably mainly reabsorbed from the colon.

Because of these considerations, the isotope dilution data in Table 4 are of interest. In the two patients examined, the concentration of endogenous, unlabeled lithocholate in each of the four major fractions was determined. Unlike the labeled lithocholates, the unlabeled lithocholates can be considered to be in a relatively steady metabolic state. Therefore, irrespective of turnover rates and other physiological considerations, sulfated lithocholates constituted 40–75% of the total endogenous lithocholate pool in gallbladder bile. Since gallbladder bile contains a large part of the total bile acid pool in humans, it is apparent that in these patients at least, sulfation was a physiologically important pathway for the metabolism of endogenous as well as exogenous lithocholate. It seems probable that the distribution of metabolites in liver bile would not have been greatly dissimilar, although it is possible that differential reabsorption from the gallbladder could have altered the biliary composition.

It can also be seen from Table 4 that, of the endogenous metabolites, relatively more glycolithocholate than taurolithocholate occurred as the sulfate (patient B, 49.5% vs. 33.3%; patient C, 78.7% vs. 68.6%). Furthermore, if the sulfated and nonsulfated fractions are compared, the glycine/taurine ratios of the former are seen to be roughly twice those of the latter (patient B, sulfated glycolithocholate/taurolithocholate = 1.19; nonsulfated glycolithocholate/taurolithocholate = 0.60; patient C, sulfated glycolithocholate/taurolithocholate = 2.26; nonsulfated glycolithocholate/taurolithocholate = 1.33). The same kinds of differences hold for the labeled metabolites. While other interpretations are possible, the data from these two subjects suggest that sulfate esterification may occur more readily with the less polar glycine conjugates than with the more polar taurine conjugates, a hypothesis consonant with the common occurrence of sulfate derivatives of many nonpolar neutral steroid metabolites.

The site of bile acid sulfation is a matter of some interest. Most sulfation is assumed to occur in the liver, although the sulfation of neutral steroids during passage through the intestinal wall has been demonstrated (15). Data presented in the following report (14) indicate that lithocholic acid can be sulfated during its passage through the rat liver, but other tissues, such as blood, intestine, or intestinal contents (bacteria) cannot be excluded as potential sulfating sites.

Strong alkaline hydrolysis of lithocholic acid sulfate was found unexpectedly to result in several degradation products, the major one of which appeared to be isolithocholic acid on the basis of TLC mobility and theoretical considerations. Inversion of the C-3 hydroxyl group and elimination of the hydroxyl group with the formation of olefinic compounds are well known side reactions in the strong acid hydrolysis of neutral steroid sulfates (16, 17), and apparently result from an attack on the C–O bond. A similar mechanism can be postulated to result in the formation of isolithocholic acid and a cholenic acid during the strong alkaline hydrolysis of sulfated lithocholates, although the identities of these breakdown products have not been established conclusively. Irrespective of the nature of these compounds, their formation during hydrolytic procedures frequently utilized for the analysis of bile acids in biological fluids suggests that the presence of sulfates should be considered as a possible source of error in ascertaining bile acid nuclear configuration following such procedures. However, solvolysis of steroid sulfates has been shown to attack the S–O bond rather than the C–O bond (5) and should not result in such artifact formation. Our studies indicate that mild acid solvolysis (room temperature in

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acetone–alcohol) also attacks only the S–O bond of bile acid sulfates, since strong alkaline hydrolysis following solvolysis does not result in the formation of artifacts (Figs. 3 and 4).[1] Thus, solvolysis followed by strong alkaline hydrolysis permits determination of the nuclear structure of bile acid sulfates and would seem to be essential for accurate quantitation of lithocholates.

The biological significance of bile acid sulfates will depend on their physiological and pharmacological properties. Physiological properties of lithocholic acid that might be altered by sulfation include the extent of intestinal absorption, the route of excretion, the metabolic transformations it undergoes in vivo, and its biological half-life. Observations on these parameters in rats are the subject of the following communication (14). Pharmacological properties of lithocholic acid include the capacity to produce intense fever (18), tissue damage (19), inflammation (18, 20), and proliferation of bile ducts and ductular cells (21–26). Sulfation may alter these toxic properties, as it has been shown to abolish the fever-producing activity of etiocholanolone (27), a related pyrogenic steroid. Bile acid sulfates, like other steroid metabolites, may also have new and important biological activities of their own. The pharmacological properties of lithocholic acid sulfates are under investigation and will be the subject of a subsequent report.

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[1] Under the conditions of solvolysis described here, a considerable proportion of the bile acid is converted to the ethyl ester, as a result of the acid–alcohol treatment. This ester can then be split by mild alkaline hydrolysis, as described under Methods, but this also results in variable hydrolysis of any glycine or taurine conjugates that may be present. The formation of esters is, of course, not important if strong alkaline hydrolysis is planned subsequently, but it could be of significance if enzymatic hydrolysis of the amino acid conjugates is contemplated. Unfortunately, the conditions for complete solvolysis of the sulfates or complete enzymatic hydrolysis of the amino acids have not been worked out in detail, and the interpretation of analysis of biological extracts still requires caution.

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