papers and notes on methodology

Critical evaluation of the existence of so-called tissue-bound lithocholate in human liver tissue by selected ion monitoring

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Abstract Monohydroxy bile acids in liver tissue may be of importance because of their hepatotoxicity and strong cholestatic effects. Recently, the existence of lithocholate in liver tissue in two forms was suggested by Nair et al. (*Lipids.* 1977. **12:** 922–929) i.e., either in free form or as so-called tissue-bound lithocholate released exclusively by cholylglycine hydrolase treatment. The presence of the latter aroused much interest in relation to its hepatotoxicity and possible role in tumor induction. In the present investigation lithocholyl-ε-L-lysine, proposed as the predominant tissue-bound bile acid, was synthesized and its metabolic behavior was tested. Lithocholyl-ε-L-lysine was not deconjugated by cholylglycine hydrolase treatment but only by alkaline hydrolysis. Bile acids in seven cirrhotic and three noncirrhotic liver samples were extracted with 95% ethanol–0.1% ammonium hydroxide. The bile acids in the extract and residue were quantified by glass capillary gas–liquid chromatography using selected ion monitoring. The presence of so-called tissue-bound lithocholate could not be substantiated in either cirrhotic or noncirrhotic liver tissues. Nearly complete extraction of lithocholate was achieved by the use of organic solvent alone. Therefore, tissue-bound lithocholate, if it exists at all, may be attached to tissue by a physical linkage which can be disrupted by the use of conventional organic solvent.—**Yanagisawa, J., Y. Akashi, H. Miyazaki, and F. Nakayama.** Critical evaluation of the existence of so-called tissue-bound lithocholate in human liver tissue by selected ion monitoring. *J. Lipid Res.* 1984. **25:** 1263–1271.

Supplementary key words gas–liquid chromatography–mass spectrometry • deuterated bile acid • lithocholyl-ε-L-lysine

Considerable attention has been focused on the role of monohydroxy bile acids in connection with the etiology of intrahepatic cholestasis and its possible contribution to the occurrence of primary biliary cirrhosis (1–4). For the purpose of studying the hepatic phase of bile acid metabolism in these disease states, accurate quantitation of bile acid in liver tissue is necessary. One of the difficulties expected is in the completeness of the extraction of bile acid from liver. Okishio and Nair (5) employed 95% ethanol containing ammonium hydroxide for extraction of bile acid from homogenized liver tissue, a procedure which gained wide popularity. However, Nair et al. (6, 7) threw doubt upon the completeness of extraction with an organic solvent and postulated the presence of tissue-bound lithocholate (LCA), resistant to organic solvent extraction, which accounted for more than 50% of the total LCA present in liver tissue especially in cirrhotic liver at autopsy. They claimed so-called tissue-bound LCA could be extracted from liver tissue only after cholylglycine hydrolase treatment. Lithocholyl-ε-L-lysine was held to be a predominant "tissue-bound LCA." Furthermore, the same group (8, 9) reported recently that "tissue-bound LCA" in liver tissue of rat is increased by methylazoxymethanol treatment and stressed its implication in carcinogenesis. Thus, the possible relation of "tissue-bound LCA" to hepatotoxicity and tumor induction has aroused much interest recently. Therefore, the present study was undertaken

Abbreviations: LCA, lithocholate; GLCA, glycolithocholate; BSA, bovine serum albumin; GLC–MS–SIM, gas–liquid chromatography–mass spectrometry–selected ion monitoring; DMES, dimethylethylsilyl; DCA, deoxycholate; CDCA, chenodeoxycholate; UDCA, ursodeoxycholate; CA, cholate; TLC, thin-layer chromatography; FD–MS, field desorption–mass spectrometry; EDTA-2Na, ethylenediaminetetraacetic acid disodium salt; DMESOH, dimethylethylsilanol.

to carefully evaluate whether LCA is bound chemically to tissue. In order to search for optimal conditions of deconjugation of lithocholyl-ε-L-lysine, both the ^{14}C-labeled and nonlabeled compounds were synthesized. Analytical methods capable of detecting tissue-bound LCA, if such exists, were established using lithocholyl-ε-L-lysine and glycolithocholate (GLCA)-bovine serum albumin (BSA) conjugate as model compounds. Bile acids remaining in the residue after repeated extraction with an organic solvent were determined, after alkaline hydrolysis, by gas–liquid chromatography–mass spectrometry–selected ion monitoring (GLC–MS–SIM) as described earlier (10).

MATERIALS AND METHODS

Chemicals

All solvents used were of analytical grade or distilled prior to use. Bond-Elut, reverse-phase octadecylsilane bonded silica (500 mg absorbent), was obtained from Analytichem International, Harbor City, CA; it was washed successively with water, ethanol, ethyl acetate, *n*-hexane, ethanol, and water, prior to use. Three different batches of cholylglycine hydrolase were obtained from two sources, partially purified (Catalog no; C-4018, Lot no; 20F-6833) and crude powder (Cat. no; C-3636, Lot no; 105C-6800) from Sigma Chemical Co., St. Louis, MO, and the other (Cat. no; 822213, Lot no; GB-1215) from Schwarz-Mann, Orangeburg, NY. Dimethylethylsilyl (DMES) imidazole was purchased from Tokyo Kasei Kogyo Co., Tokyo, Japan and Sephadex LH-20 (25–100 μm) was from Pharmacia Fine Chemicals AB, Uppsala, Sweden. LCA and [carboxy-^{14}C]LCA (145 μCi/mg) were obtained from Tokyo Kasei Kygyo Co. and Amersham International Ltd., Amersham, UK, respectively. Glycine-conjugated deuterated bile acids, i.e., glyco[6,6,7,7-$^{2}H_4$]lithocholate, glyco[6,6,7,7,8-$^{2}H_5$]deoxycholate, glyco[11,11,12,12-$^{2}H_4$]chenodeoxycholate, glyco[11,11,12,12-$^{2}H_4$]ursodeoxycholate, and glyco[11,11,12β-$^{2}H_3$]cholate were synthesized using coupling reagent (11) from the corresponding deuterated unconjugated bile acids used in a previous study (10). The glycine-conjugated bile acids were checked for purity by thin-layer chromatography (TLC) in two of the following systems: isopropanol–isooctane–dioxane–acetic acid 7:10:6:2 (v/v/v/v) (12), *n*-butanol–acetic acid–water 10:1:1 (v/v/v) (13), and isooctane–isopropyl ether–acetic acid 10:5:5 (v/v/v) (14). GLCA-BSA conjugate was a gift from Dr. S. Yamauchi, Kyushu University Faculty of Medicine, Department of Surgery I; it was synthesized using the method of Erlanger et al. (15) using BSA (Sigma Chemical Co., Cat. no; A 7368) and LCA. The LCA content was 0.40 μmol per mg dry weight or 32 molecules per molecule of albumin.

Preparation of lithocholyl-ε-L-lysine

Ethyl dimethylaminopropylcarbodiimide · HCl (5 mmol, Bio-Rad Laboratories, Richmond, CA) in aqueous tetrahydrofuran (5 ml + 5 ml) was added to a chilled solution of LCA (5 mmol), N-α-benzyloxycarbonyl-L-lysine benzyl ester benzenesulfonate (5 mmol) (Kokusan Chemical Works Ltd., Tokyo, Japan) and triethylamine (5 mmol) in tetrahydrofuran (30 ml) below 0°C. The resulting solution was left to react overnight at room temperature. The reaction mixture was concentrated under reduced pressure to yield an oily material. To the residue was added chloroform (300 ml), and the solution was washed with saturated aqueous $NaHCO_3$ solution, water, 1 N HCl, and water, successively. The organic layer was dried over anhydrous $MgSO_4$. After filtration, the filtrate was evaporated to dryness. The crude material (3.48 g) was dissolved in chloroform (25 ml) and the solution was subjected to silica gel column chromatography using chloroform–methanol 25:1 (v/v) as an eluent. The eluate was fractionated in 14-ml portions. Fractions from 420 ml to 980 ml were collected and evaporated to yield a crude product (1.50 g). This material was recrystallized from ethyl acetate and *n*-hexane to give 1.44 g of protected lithocholyl-ε-lysine in a 41.2% yield; mp 131–132°C. One gram of protected lithocholyl-ε-lysine was hydrogenated over palladium black in methanol (100 ml) for 4.5 hr. The crude lithocholyl-ε-L-lysine was dissolved in a mixture of *n*-butanol–acetic acid–water 4:1:1 (v/v/v) and the resulting solution was subjected to silica gel column chromatography using the same solvent as an eluent. The eluate was fractionated in 7-ml portions. Fractions from 147 ml to 196 ml were collected and evaporated to give 420 mg of a colorless powder. Two hundred and eighteen mg was redissolved in methanol (3 ml) and passed through a column packed with Sephadex LH-20 to remove silica gel. The eluate was fractionated in 6.5-ml portions and the fractions 10 to 13 were collected and concentrated to dryness. The residues were triturated with ethyl ether to give 106 mg of pure lithocholyl-ε-L-lysine in an overall yield of 8.0%; mp 175–189°C (dec.). Nair et al. (6) reported some characteristics of synthetic ε- or α-lithocholyl-L-lysine on TLC. The former compound was ninhydrin-positive with R_f values of 0.49 and 0.73 in the solvent systems of *n*-butanol–acetic acid–water 4:1:1 (v/v/v) and ethanol–ammonium hydroxide–water 6:1:2 (v/v/v), respectively. The latter compound was ninhydrin-negative with R_f values of 0.54 and 0.45 in the same solvent systems. The product prepared in

the present paper showed a single ninhydrin-positive spot with R_f values of 0.47 and 0.72 in the respective solvent systems. The product was further examined by field desorption–mass spectrometry (FD–MS) on a JEOL JMS-D300 mass spectrometer equipped with a FD ion source and a JMA-2000 data processing system (JEOL, Tokyo, Japan). Emitter current was programmed with a digital emitter current programmer at a rate of 0.1 mA/sec. FD–MS analysis (**Fig. 1**) showed the presence of $[M + H]^+$ ion at m/z 505 as the base peak accompanied by $[M + Na]^+$ ion at m/z 527. The intense ion at m/z 461 was considered to be the $[M - COO + H]^+$ ion. Electron impact ionization–mass spectrometry following alkaline hydrolysis and derivatization of lithocholyl-ϵ-L-lysine prepared showed the identical mass spectrum to that of the ethyl ester-DMES ether derivative of authentic LCA.

Preparation of [carboxy-^{14}C]lithocholyl-ϵ-L-lysine

Synthesis of [carboxy-^{14}C]lithocholyl-ϵ-L-lysine was carried out using 10 μCi of [^{14}C]LCA and 8 mg of LCA with smaller amounts of other reagents, as in the synthesis of nonlabeled compound. Because of the small amount of material synthesized, TLC (solvent system, isooctane–ethyl acetate–acetic acid 5:5:1, v/v/v (16)) was used for separating protected lithocholyl-ϵ-lysine from unreacted compounds and by-product instead of column chromatography. The hydrogenated product showed a single peak with an R_f value similar to that of nonradioactive compound with the two solvent systems described above. Its identity was confirmed by FD–MS.

Hydrolysis of lithocholyl-ϵ-L-lysine

In order to study the effect of aqueous 1 M NaOH at 80°C for 30 min and subsequent ultrasonification, used in the preliminary extraction of liver tissue, on the peptide linkage of lithocholyl-ϵ-L-lysine, 0.2 μCi (0.4 μmol) of [^{14}C]lithocholyl-ϵ-L-lysine was added to approximately 50 mg of liver tissue in 1 ml of 1 M NaOH, heated to 80°C, and ultrasonified. Since the solubility of lithocholyl-ϵ-L-lysine in 0.1 M acetate buffer (pH 5.6) was poor, lithocholyl-ϵ-L-lysine solution was prepared by heating for a few minutes in ethanol and an aliquot of 0.1 ml was used.

Approximately 0.4 μmol of lithocholyl-ϵ-lysine or 0.2 μCi of [^{14}C]lithocholyl-ϵ-L-lysine and crude liver extract was treated with one of the three preparations of cholylglycine hydrolase; 5 U of partially purified or crude enzyme from Sigma or 5 μl (ca. 66 U) of the enzyme from Schwarz-Mann. In another experiment, the amounts of the enzymes from Sigma were increased to 20 U and the Schwarz-Mann enzyme was increased to 100 μl (ca. 1322 U). Incubation was carried out at 37°C for 18 hr in the presence of 40 μmol each of β-mercaptoethanol and ethylenediaminetetraacetic acid disodium salts (EDTA-2Na) in 4 ml of 0.1 M acetate buffer (pH 5.6) (17–19). In a separate experiment, alkaline hydrolysis was also evaluated. To lithocholyl-ϵ-L-lysine or 0.2 μCi of [^{14}C]lithocholyl-ϵ-L-lysine and crude liver extract was added 4 ml of 1.25 M NaOH. The hydrolysis was done in a sealed Teflon tube at 120°C for 7 hr.

After addition of few drops of 1.25 M NaOH (pH 8–9), the mixture treated with cholylglycine hydrolase was applied onto Bond-Elut. The reaction mixtures following mild alkaline treatment or alkaline hydrolysis were applied directly onto the cartridge. Good recovery (97–101%) of lithocholyl-ϵ-L-lysine on Bond-Elut extraction under the conditions described above was observed in our preliminary experiments. Liberated LCA and lithocholyl-ϵ-L-lysine were extracted with ethanol and separated from each other by TLC with a mixture of

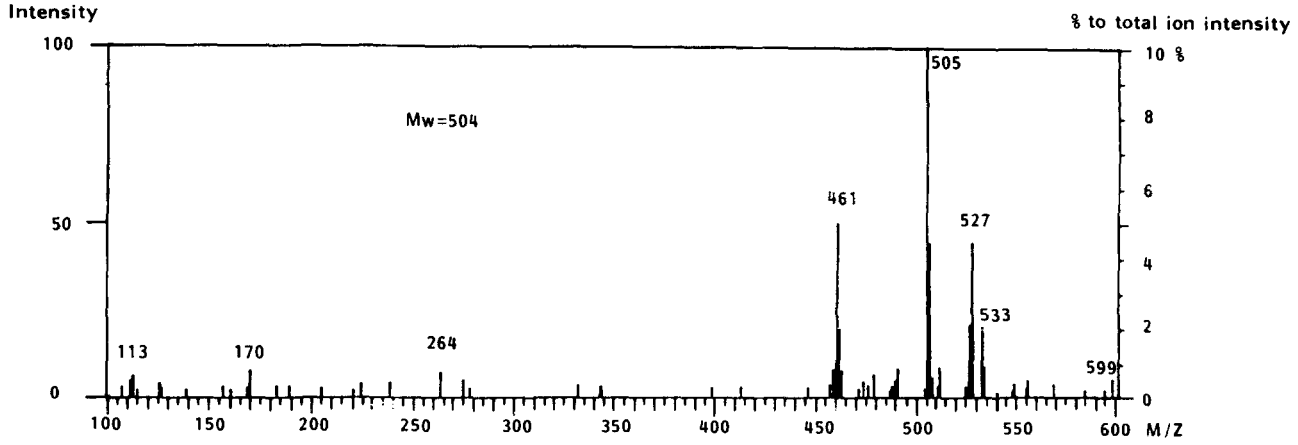

Fig. 1. Mass spectrum of synthetic lithocholyl-ϵ-L-lysine obtained by field desorption–mass spectrometry (FD–MS).

isooctane–ethyl acetate–acetic acid 5:5:1 (v/v/v) (16). The spots were visualized with 50% H_2SO_4 in ethanol. In order to ensure detection of liberated LCA, approximately 50–100 μg of lithocholyl-ε-L-lysine was applied onto the TLC plate. The detection limit of LCA on TLC was found to be 1–2 μg as a spot. In experiment using a ^{14}C-labeled bile acid, hydrolysis of lithocholyl-ε-L-lysine was evaluated by the liberation of LCA by direct scanning of the plate using Aloka Thin-Layer Chromatogram Scanner (Aloka, Tokyo, Japan).

Hydrolysis of glycolithocholate-bovine serum albumin conjugate

To approximately 10 mg of GLCA-BSA conjugate was added 10 ml of 95% ethanol–0.1% ammonium hydroxide. The mixture was heated at 80°C for 30 min and immersed in an ultrasonic bath for 10 min. After concentration, an aliquot of the suspension was subjected to TLC with a solvent system of isopropanol–isooctane–dioxane–acetic acid 7:10:6:2 (v/v/v/v) (12). GLCA-BSA conjugate in 1 M NaOH was heated at 80°C for 30 min or in 1.25 M NaOH at 120°C for 7 hr.

Enzymatic hydrolysis of GLCA-BSA conjugate was also tested using 5 U of partially purified cholylglycine hydrolase (Sigma) in 4 ml of 0.1 M acetate buffer (pH 5.6) and 0.2 ml ethanol or the buffer alone. The reaction mixture was applied to Bond-Elut. LCA and GLCA released from GLCA-BSA conjugate were recovered with 10 ml of ethanol and subjected to TLC.

Sample preparation

Liver specimens were obtained from three patients with hepatolithiasis during partial hepatic resection. Seven samples of cirrhotic liver were obtained at autopsy. Immediately after the resection, a part of the tissue was removed, rinsed with chilled saline, sliced into small blocks corresponding to about 10 mg of fresh liver tissue, rinsed again, briefly dried on a filter paper, weighed, and stored at −20°C until analyzed.

Sample preparation is outlined in **Fig. 2.** Liver tissue corresponding to about 100 mg of wet liver tissue was homogenized in 2 ml of 95% ethanol containing 0.1% ammonium hydroxide using a Teflon pestle homogenizer driven by a motor at about 700 rpm for 5 min on ice. The homogenate was transferred into a centrifuge tube and washed with three 3-ml portions of 95% ethanol–0.1% ammonium hydroxide with the aid of ultrasonification. The combined washings (ca. 11 ml) were heated in a water bath at 80°C for 10 min under continuous stirring, and then centrifuged in the cold for 10 min at 5,000 *g*. After removal of the supernatant, the residue was resuspended in 12 ml of 95% ethanol–0.1% am-

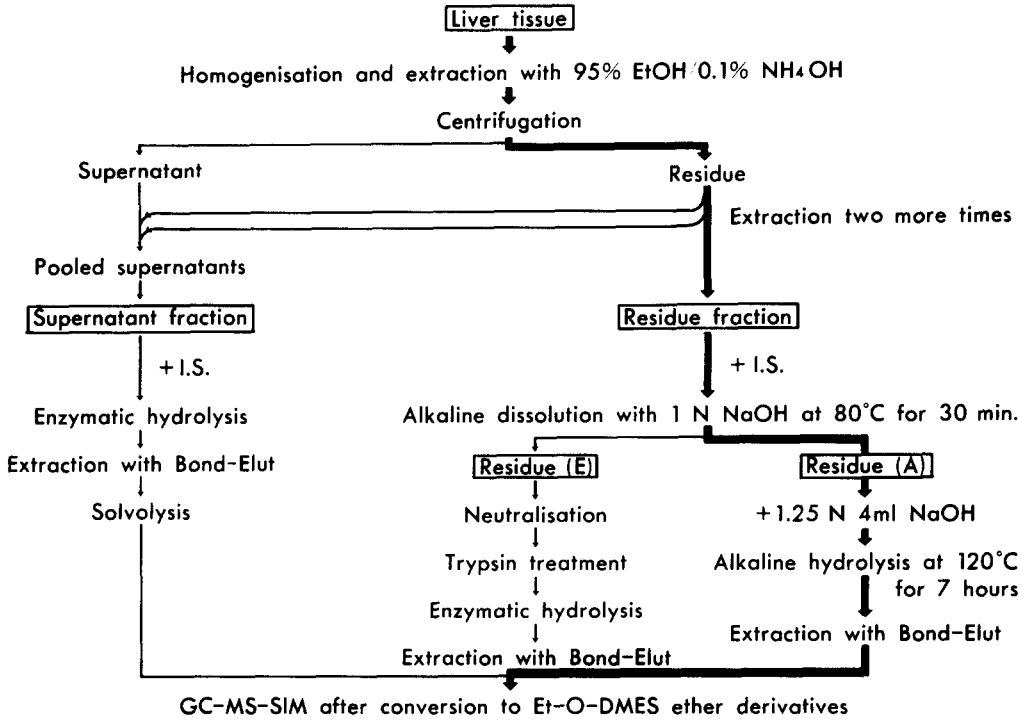

Fig. 2. Outline of sample preparation procedure for GLC–MS. The broad solid line indicates the stream along which tissue-bound lithocholate, if it exists, would be recovered and subsequently determined. I.S., internal standard; Et-O-DMES ether, ethyl ester dimethylethylsilyl ether.

monium hydroxide. The extraction procedure was repeated twice with the same solvent.

Supernatant fraction. To an aliquot of the pooled supernatants, the following internal standards were added; 0.1 nmol glyco[$^2H_4$]LCA, 0.5 nmol glyco-[$^2H_5$]DCA, 6.0 nmol glyco[$^2H_4$]CDCA, 0.1 nmol glyco[$^2H_4$]UDCA, and 3.6 nmol glyco[$^2H_3$]CA. After evaporating to dryness, the residue was dissolved in 0.2 ml of ethanol and 4 ml of 0.1 M acetate buffer (pH 5.6) with the aid of ultrasonification, and subjected to enzymatic hydrolysis with 2 U of partially purified cholylglycine hydrolase (Sigma Chemical Co.) and other cofactors described above. After the removal of the organic solvent in the supernatant fraction, the yellowish oily residue left in the bottom of the flask was found to be insoluble in 0.1 M acetate buffer (pH 5.6). Therefore, a small amount of ethanol was added (6). Our preliminary experiment showed no inhibitory effect of ethanol on the deconjugation of authentic tauro- or glyco-bile acids by partially purified cholylglycine hydrolase, when the amount added was less than 14% by volume. Non-amidated bile acid was extracted from the hydrolysate on Bond-Elut (20). Following solvolysis in equilibrated ethyl acetate (with 2 M H_2SO_4)-ethanol (21, 22), the bile acid was converted into ethyl ester–DMES ether as described previously (10).

Residue fraction. To the residue formed after repeated solvent extraction was added 1 ml of 1 M NaOH. The residue was dissolved by heating up to 80°C for 30 min. Following addition of internal standards, the crude extract was divided into two parts. One portion was transferred into a Teflon tube. After the addition of 4 ml of 1.25 M NaOH, alkaline hydrolysis was carried out at 120°C for 7 hr. The deconjugated bile acid in the residue was recovered on Bond-Elut and derivatized for GLC–MS. The other half of the solubilized residue pellet was treated in the manner reported by Turjman and Nair (8), i.e., neutralized with 1 M HCl and predigested at 37°C for 2 hr with 10 mg of trypsin (Merck, Darmstedt, West Germany) suspended in 1 ml of 0.06 M phosphate buffer (pH 7.5). The reaction was terminated by boiling for 5 min. To the resultant solution (about 2 ml) was added 10 ml of 0.1 M acetate buffer (pH 5.6), 80 μmol each of cofactors, and 5 U of partially purified cholylglycine hydrolase (Sigma Chemical Co.). After enzymatic treatment, the bile acid in the residue was recovered on Bond-Elut and derivatized for GLC–MS.

Gas–liquid chromatography–mass spectrometry–selected ion monitoring

Gas–liquid chromatography–mass spectrometric analysis was performed using a Shimadzu Auto GC–MS 9020DF equipped with a data processing system, Shimadzu SCAP 1123 (Shimadzu Seisakusho, Kyoto, Japan). Bile acid derivative was chromatographed on a glass capillary column, 25 m × 0.35 mm i.d., coated with SE-30 (LKB-Productor, Stockholm, Sweden). Mass spectrometric resolution was approximately 1000 at m/z 693.

The DMES ether derivative of LCA ethyl ester is characterized by the appearance of base peak at m/z 461 (M-29), with intense ions at m/z 386 (M − DMESOH), m/z 341 (M − DMESOH − OC_2H_5), m/z 257 (M − DMESOH − side chain), and m/z 215 (M − DMESOH − side chain − D ring). When LCA present in the sample was minute, especially in the residue, ions at lower m/z values were interfered by those from large amounts of coexisting contaminants. Therefore, usual identification of LCA in the sample was carried out by monitoring two sets of ions, i.e., m/z 461 and 386 for LCA and m/z 465 and 390 for [$^2H_4$]LCA, and for quantitation a pair of $[M - 29]^+$ ions was used. The ions used for quantitation of other bile acids and other operating conditions were similar to those in the previous study (10).

RESULTS

Cleavage of peptide linkage of lithocholyl-ε-L-lysine

After liver tissue with added [^{14}C]lithocholyl-ε-L-lysine was subjected to mild alkaline treatment, i.e., 1 M NaOH at 80°C for 30 min, and subsequent ultrasonification, nearly all radioactivity was found to be present in the region of lithocholyl-ε-L-lysine on a TLC plate with only a trace of radioactivity in the region of LCA (**Fig. 3a**), indicating that lithocholyl-ε-L-lysine was not hydrolyzed under these conditions.

Enzymatic hydrolysis with partially purified cholylglycine hydrolase (Sigma) resulted in no deconjugation of [^{14}C]lithocholyl-ε-L-lysine added to the crude extract of liver tissue (Fig. 3b). Further experiments were carried out using the non-radioactive compound. Although bile acid conjugates such as glycine- or taurine-conjugated DCA, CDCA, or CA were completely hydrolyzed to give a single spot of free bile acid by TLC, no sign of cleavage of peptide linkage of this bile acid conjugate, i.e., lithocholyl-ε-L-lysine was found with three preparations of cholylglycine hydrolase. Hydrolysis was also tried in buffer of different pH values, i.e., 0.1 M acetate buffer at pH 4.5, 5.2, 5.4, 5.6, 5.8, 6.0, 6.2, 6.6, 7.1, and 8.1 or in the presence of an increased amount of the enzyme. However, all these changes failed to liberate free LCA. Since an excess amount of lithocholyl-ε-L-lysine was applied onto the TLC plate, absence of the

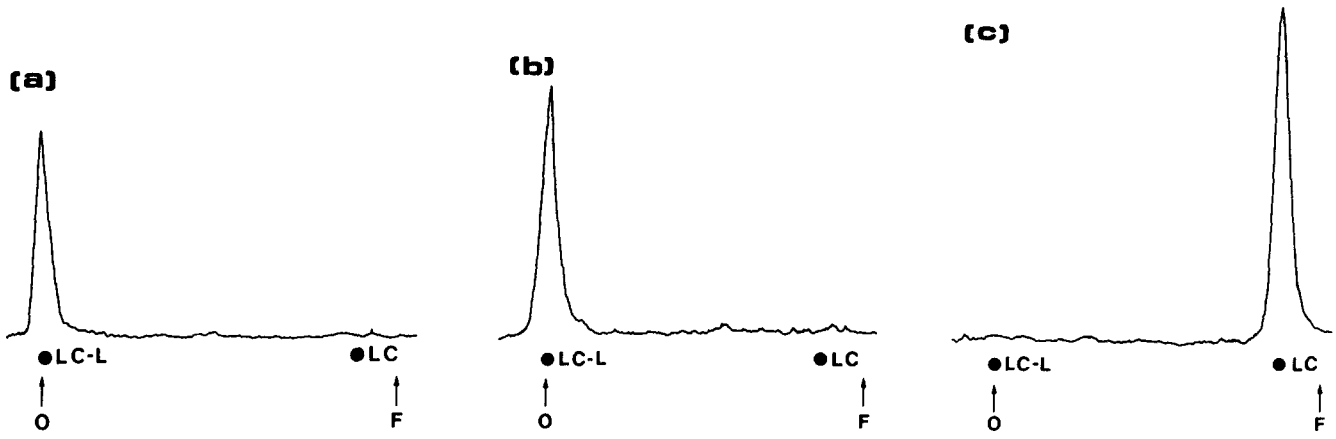

Fig. 3. Radiochromatogram of [^{14}C]lithocholyl-ε-L-lysine added to liver tissue or crude liver extract after (a) mild alkaline treatment (1 M NaOH at 80°C for 30 min); (b) enzymatic hydrolysis with partially purified cholylglycine hydrolase (Sigma Chemicals); and (c) alkaline hydrolysis (1.25 M NaOH at 120°C for 7 hr). Solvent system; isooctane–ethyl acetate–acetic acid 5:5:1 (v/v/v) (16). Almost all radioactivity remained in the zone of lithocholyl-ε-L-lysine (●LC-L) in (a) and (b), whereas it shifted to the zone of unconjugated lithocholate (●LC) in (c). O, origin; F, solvent front.

spot with the same mobility as LCA suggests that hydrolysis of lithocholyl-ε-L-lysine does not occur significantly, or that LCA, even if formed, must have been less than a few percent of lithocholyl-ε-L-lysine. On the other hand, alkaline hydrolysis at 120°C for 7 hr resulted in the appearance of unconjugated LCA without any trace of lithocholyl-ε-L-lysine remaining on the TLC plate (n = 3) (Fig. 3c).

Hydrolysis of glycolithocholate-bovine serum albumin conjugate

LCA and GLCA were not released significantly from GLCA-BSA conjugate during ethanol–ammonia extraction and the subsequent ultrasonification or mild alkaline treatment. However, both enzymatic hydrolysis with or without addition of ethanol and alkaline hydrolysis resulted in appearance of the LCA spot without the GLCA spot, indicating that both hydrolysis steps are effective for hydrolysis of GLCA-BSA conjugate.

Determination of bile acid in supernatant and residue fractions of liver tissue

As shown in **Table 1,** LCA concentrations in the supernatant of ten different liver specimens varied considerably in the range from 0.28 to 12.1 pmol/mg liver. In the residue LCA was found to be present in a trace amount after alkaline or enzymatic hydrolysis, i.e., less than 0.2 pmol/mg of liver which was near the lower limit of quantitation. Representative selected ion recording of LCA (from patient 5) was shown in **Fig. 4.** Thus, only a trace amount, if any, of LCA unextractable with the organic solvent was found to be present in the

TABLE 1. Lithocholate in supernatant and residue fractions of liver tissue

Patient				Lithocholate Concentration (pmol/mg liver)			$\frac{LCA(S) \times 100^c}{LCA(S) + LCA(RA)}$ (%)
No	Age	Sex	Diagnosis	Supernatant	Residue(A)[a]	Residue(E)[b]	
1	40	M	Chronic hepatitis	0.28	0.19[d]	0.18[d]	59.6
2	62	F	Chronic hepatitis	12.10	0.20	0.20	98.4
3	74	F	Chronic hepatitis	0.44	0.05	0.03	89.8
4	51	M	Liver cirrhosis	1.48	0.04	0.03	97.4
5	55	M	Liver cirrhosis	0.87	0.02	0.02	97.8
6	58	F	Liver cirrhosis	7.00	0.10	0.10	98.6
7	58	F	Liver cirrhosis	1.11	0.06	0.06	94.9
8	60	M	Liver cirrhosis	3.26	0.03	0.02	99.1
9	61	F	Liver cirrhosis	3.71	0.07	0.06	98.1
10	64	M	Liver cirrhosis	1.18	0.08	0.06	93.7

[a] Residue(A); residue fraction treated by alkaline hydrolysis.
[b] Residue(E); residue fraction treated by enzymatic hydrolysis.
[c] LCA(S); lithocholate in supernatant, LCA(RA); lithocholate in Residue(A).
[d] The value may be overestimated due to the presence of a coeluting peak.

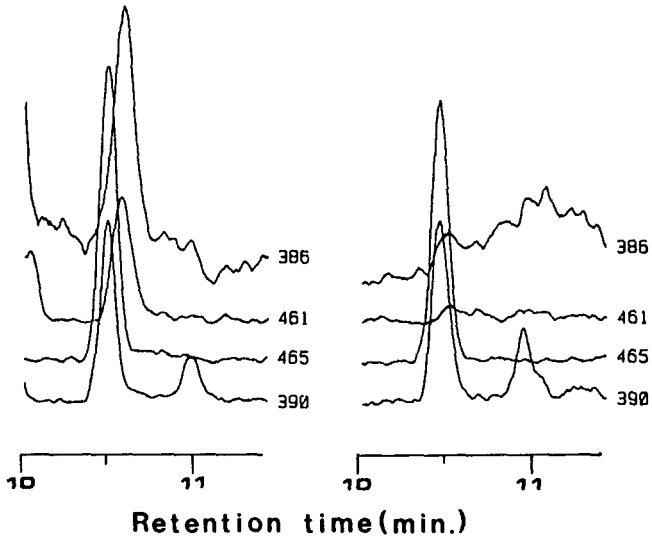

Fig. 4. Selected ion recording of lithocholate in liver tissue (patient 5). Lithocholate (LCA) was monitored at m/z 461 (M − 29) and 386 (M − DMESOH) and [$^2H_4$]LCA at m/z 465 and 390. The LCA peak emerged slightly later than that of [$^2H_4$]LCA due to the isotopic effect. Left panel, LCA in supernatant fraction; right panel, LCA in residue fraction.

residue, although there was precaution to add only one-fifth of the amount of the internal standard to the residue compared to the supernatant to enhance relative intensity of LCA peak as much as possible for better quantitation. Although the amount of LCA found in the residue was more after alkaline hydrolysis than after enzymatic hydrolysis, the difference seemed to be not significant because of the limitation imposed by the inaccuracy of quantitation near the detection limit. LCA extracted into the supernatant was found to be over 90% of total LCA present in liver tissue in all but patient 1, in whom approximately 40% of LCA seemed to remain in the residue. The LCA peak in GLC–MS–SIM in this case was broad, suggesting the presence of coeluting substances resulting in an overestimation of LCA. The mass spectrum of this peak showed the presence of intense ions at m/z 503, 462, 385, 264, and 193 and very weak ions at m/z 465, 461, 390, 386, 219, and 215. A series of the former ions was probably from an unidentified compound that interfered with quantitation of LCA. Statistical analysis using non-parametric rank correlation (23) showed that the proportion of LCA extracted into the supernatant correlated significantly with the sum of LCA levels in the supernatant and residue ($P < 0.01$).

Other bile acids, i.e., DCA, CDCA, UDCA, and CA, were extracted with organic solvent in a range of 97 to 100%. DCA present in residue after alkaline hydrolysis tended to be lower than after enzymatic hydrolysis. However, the values were minute and near the detection limit of the procedure and therefore the difference was considered to be negligible.

DISCUSSION

The existence of tissue-bound LCA advocated by Nair et al. (6, 7) was mainly based on two pieces of experimental evidence. When bile acid in the residue after extraction of homogenized liver tissue with ethanol–ammonium hydroxide was quantitated by GLC, a larger amount of LCA was recovered from the residue after treatment with cholylglycine hydrolase as compared to that of the supernatant. Second, the residue treated with 6 M HCl under partial vacuum showed a ninhydrin-positive spot with the same R_f value as synthetic lithocholyl-ε-L-lysine on the TLC plate. As the cholylglycine hydrolase was thought to be capable of releasing LCA from synthetic lithocholyl-ε-L-lysine, LCA-BSA conjugate, and lithocholyl polylysine, tissue-bound LCA seemed to be LCA conjugated with the lysine residue of tissue protein via ε-amino groups. Later, the same group reported that pretreatment of the residue with trypsin improved the yield of tissue-bound LCA by as much as 20–30-fold (14).

In order to assess the behavior of tissue-bound LCA during the whole sample preparation step of liver tissue, GCLA-BSA conjugate and lithocholyl-ε-L-lysine were tested as the model compounds. Hydrolysis of both compounds by organic solvent extraction and ultrasonification was not found, suggesting that tissue-bound LCA, if it exists, would remain in the protein-bound form during the initial extraction procedure. As the alkaline hydrolysis of lithocholyl-ε-L-lysine was readily effected, the present method for analysis of bile acid in liver tissue (Fig. 2) involving an alkaline hydrolysis step is expected to yield a good recovery with the possible presence of lithocholyl-ε-L-lysine and tissue-bound LCA, if it exists.

The absence of the enzyme activity with three different preparations of cholylglycine hydrolase toward lithocholyl-ε-L-lysine is in apparent conflict with the previous report by Nair et al. (6). Lowered enzyme activity in the present preparations was unlikely because hydrolysis of usual bile acid conjugates readily proceeded to completion. One possible reason for the discrepancy between our results and those of others may be due to the substrate specificity of the enzyme used. The enzyme used in the present investigation was too pure (as cholylglycine hydrolase) to act on unusual substrate, lithocholyl-ε-L-lysine, as a nonspecific peptidase, although one of the preparations used (Schwarz-Mann) was the

same as that used by Nair et al. (6, 7). Nair et al. (6) reported that synthetic poly-ϵ-deoxycholyllysine was cleaved only in a trace amount with highly purified cholylglycine hydrolase.

For the determination of LCA in the residue, two different methods of sample preparation were compared. Half of the residual pellet solubilized in 1 M NaOH was subjected to alkaline hydrolysis to ensure hydrolysis of lithocholyl-ϵ-L-lysine. The other half was predigested with trypsin followed by cholylglycine hydrolase treatment in order to reproduce the procedure described by Turjman and Nair (8) as close as possible. However, both methods showed the presence of only a trace amount of LCA in the residue and more than 90% of LCA was present in the supernatant, irrespective of disease. This finding is in sharp contrast to that reported by Nair et al. (7); they described that LCA in the supernatant constituted less than 50% of the total LCA present in liver tissue in half of the cases and no LCA was extracted in one case. Using GLC, they found so-called tissue-bound LCA ranged from 4 to 116 μg, i.e., 11 to 308 pmol/mg liver and total LCA 29–493 pmol/mg liver. Whereas, in our hands, with the more reliable GLC–MS–SIM procedures, such a high level of total LCA was never encountered. It ranged only from 0.5 to 12 and at the most 20 pmol/mg liver. LCA separated from liver tissue is usually highly contaminated with other coexisting materials which interfere with quantitation, even by the use of capillary GLC–MS as in a case of patient 1 in the present study, let alone by conventional GLC using a packed column as in a case in previous reports (6, 7).

Cleavage of the amide linkage in proteins has been accomplished by treating protein with 4.2 M NaOH at 110°C for 16 hr (24) or with 6 M HCl at 110°C for 20 hr (25). The former procedure was applied to the analysis of the LCA content in the residue or whole liver tissue from two patients. A similar amount of LCA was found in the sample subjected to the usual conditions of alkaline hydrolysis, providing further evidence of lack of tissue-bound LCA in any significant amount.

The amount of LCA in Residue(A) and (E) (Table 1) from the same patient did not differ significantly, suggesting a small amount of LCA found in the Residue(A) after organic solvent extraction was mainly due to incomplete extraction. The sensitivity of GLC–MS–SIM is so high (5–10 pg) that the very small amount of LCA remaining in the residue after incomplete extraction could be detected.

In conclusion, the results of the present study do not support the presence of a quantitatively significant amount of LCA bound to liver tissue via a peptide bond. When the extraction procedure was done repeatedly, LCA in liver tissue seemed to be extracted to a large extent in the organic solvent.

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