Molecular species of lecithins in human gallbladder bile[1]

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Abstract Using a precise high performance liquid chromatography (HPLC) technique, we identified the molecular species of lecithins in gallbladder biles from patients with cholesterol gallstones (n=29), pigment gallstones (n=9), morbid obesity (n=5), and "controls" (n=10). The major lecithin species identified in all groups, in descending rank order as represented by the fatty acids in the *sn*-1 and *sn*-2 positions, were 16:0–18:2, 16:0–18:1, 16:0–20:4, 18:0–18:2, and 18:1–18:2. Lecithin species were found to be more numerous and in substantially different proportions than reported by previous investigators. No significant differences were found between any biliary lecithin species in the cholesterol and pigment stone groups. However, compared with controls, both cholesterol and pigment stone patients had smaller proportions of 16:0–20:4, the principal arachidonyl lecithin species. Using the HPLC elution sequence for quantifying the hydrophilic–hydrophobic balance, we developed a Hydrophobic Index for lecithin species in each bile based upon the principles proposed by D. M. Heuman for bile salt species. Hydrophobic indices of bile salts and lecithin were positively correlated ($r=0.48$, $R^2=0.23$, $P=0.0002$) suggesting that more hydrophobic bile salts were associated with biliary secretion of more hydrophobic lecithins. The most hydrophobic major lecithin species, 18:0–18:2, was present in greater proportions in biles with cholesterol monohydrate crystals in their sediments and in those with cholesterol saturation indices greater than one. This work provides rigorous separation, identification, and quantitation of the lecithin species in human gallbladder bile from a large cohort of patients but, apart from a more hydrophobic bile salt pattern coupling more hydrophobic lecithins, we fail to identify any relationships of biomedical importance between lecithin species and other major biliary constituents.—**Hay, D. W., M. J. Cahalane, N. Timofeyeva, and M. C. Carey.** Molecular species of lecithins in human gallbladder bile. *J. Lipid Res.* 1993. **34:** 759–768.

Supplementary key words phospholipids • bile salts • arachidonic acid • hydrophilic–hydrophobic balance • cholesterol saturation index

Cholesterol gallstone formation is a process mediated by physical-chemical and functional derangements of both liver and gallbladder (1). Supersaturation of bile with cholesterol (2, 3), nucleation defects (4), and gallbladder hypomotility (5) are considered principal abnormalities culminating in cholesterol stone formation (6). Bile salts, phospholipids (> 95% lecithins), and cholesterol are the principal lipids in bile (7). The variations in bile salt species in humans and their importance in a variety of biliary tract diseases have received great attention (8–10). In contrast, relatively little attention has been paid to the molecular species of phospholipids in bile (7). The lecithin species of bile represent the most hydrophilic of the hepatic phospholipids and, as in other tissues, differ from one another in composition of the fatty acyl chains. Studies of the molecular species of lecithin in humans are few and limitations with previously available techniques have precluded precise molecular species identification (7). While changes in the molecular species of lecithin may be pathogenetically important in gallstone formation (11), the lecithin species of human bile have not been accurately analyzed and comparisons between patients with different pathologic conditions have been limited.

This study examines the molecular species of lecithin in human bile using a state-of-the-art technique (12) not previously used in the study of human bile. The relationships between lecithin species and lipid concentrations, cholesterol saturation, cation concentrations, molecular species of bile salts, and microscopy are delineated for gallbladder biles from a large cohort of cholesterol stone, pigment stone, and morbidly obese patients (a pre-stone condition (13)) as well as in patients with other intra-abdominal conditions necessitating laparotomy ("controls"). By means of this holistic study, the molecular species of lecithin are defined with precision in a variety of

Abbreviations: CSI, cholesterol saturation index; GC, gas chromatography; HPLC, high performance liquid chromatography; *sn*-1, stereospecific number 1; *sn*-2, stereospecific number 2; TUDC, tauroursodeoxycholate; STLC, sulfotaurolithocholate; TC, taurocholate; TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate; TLC, taurolithocholate; for glycine-conjugated bile salts, the letter G is substituted for the T in the aforementioned abbreviations; for unconjugated bile salts the T is absent.

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conditions but no relationships of evident pathophysiologic significance are identified.

METHODS

Materials

Molybdenum trioxide, molybdenum metal, Fiske and Subbarow Reducer, 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* were obtained from Sigma Chemical (St. Louis, MO). Stigmasterol was supplied by Steraloids (Wilton, NH) and >99% purity was established by silica and reverse phase thin-layer chromatography. Phospholipase C from *Bacillus cereus* was supplied by Boehringer Mannheim Biochemicals (Indianapolis, IN). Sulfoglycolithocholate was obtained from Calbiochem-Behring (LaJolla, CA). Other bile salt standards were obtained as previously described (14). Lysolecithin (>99% purity) derived from egg yolk lecithin was supplied by Avanti Polar Lipids (Pelham, AL) and Grade 1 egg yolk lecithin was supplied by Lipid Products (Surrey, England). Mixtures of fatty acid methyl esters for use as gas chromatography standards were obtained from Nu-Chek Prep (Elysian, MN, standards O6A, 68A, 68B,) and from Supelco (Bellefonte, PA, standards PUFA-2 and Qualmix-M). Sodium methoxide solution was obtained from Supelco and ammonium molybdate (ACS grade) was purchased from Aldrich Chemical (Milwaukee, WI). Reagent quality absolute ethanol was supplied by Florida Distillers (Lake Alfred, FL). Solvents of HPLC or ACS grade and all other chemicals of ACS or reagent grade were obtained from Fisher Scientific (Medford, MA).

Sodium chloride was roasted in a baffle furnace at 600°C for 4 h to remove organic impurities. Water was purified by filtration, ion-exchange, and distillation in an all-glass apparatus (Corning Glass Works, Corning, NY). All glassware was cleaned by soaking for 24 h in alkali–alcohol solution (EtOH–2 M KOH 1:1, v:v) and for 24 h in acid solution (1 M HNO_3) followed by thorough rinsing with purified water and oven drying.

Subjects and collection of bile and gallstones

Protocols for obtaining bile and gallstones were approved by the human subjects committee at each participating institution. Gallbladder bile was obtained at laparotomy from patients undergoing *1)* cholecystectomy for biliary pain without evidence of prior or current complications, *2)* gastroplasty for morbid obesity (in the absence of stones)[3], or *3)* laparotomy for the variety of non-stone conditions as detailed below.

Gallbladder bile from patients with cholelithiasis or morbid obesity was obtained by anaerobic aspiration through a 16-gauge needle into a 50-ml syringe, protected from light with aluminum foil, and cultured aerobically and anaerobically. As determined from chart review, intraoperative findings, pathological examination, and bile culture, biles from patients with clinical or laboratory evidence of acute cholecystitis, pancreatitis, bacterial infection, or biliary obstruction were excluded from further study. Biliary pH, ionized calcium, bilirubin conjugate species, and unconjugated bilirubin were determined within 30 min of collection of fresh samples and atomic absorption spectrophotometry for total calcium, magnesium and sodium was performed on frozen samples as described elsewhere (M. J. Cahalane, D. W. Hay, E. B. Cabot and M. C. Carey, unpublished results). Stones were washed and stored in distilled water until analysis of cholesterol content was carried out generally within 48 h (see below). Bile was centrifuged at 100,000 *g* for 20 min and the sedimented pellet was examined by direct and polarizing light microscopy for cholesterol monohydrate crystals, liquid crystals, calcium bilirubinate granules, and cell debris (3, 15, 16). All other analyses were carried out using portions of bile that had been stored at −20°C, under argon, and in darkness.

Gallbladder bile from "controls" was obtained in a similar fashion (generous gifts from S. Strasberg, Toronto, Canada) and was stored in vacuum tubes at −70°C[4]. The conditions that necessitated surgery in this "control" group included cancer of the colon (n=5), inflammatory bowel disease (n=3), ulcerative colitis complicated by cancer (n=1), and pancreatic cyst (n=1). These patients had no clinical, laboratory, or intraoperative evidence of biliary tract disease.

High performance liquid chromatography

HPLC was used for the analysis of lecithin species and bile salt species using equipment supplied by Beckman Instruments (Fullerton, CA). Two systems were used, each controlled by an AI406 analog-digital interface and Beckman System Gold software. One system used Model 110A pumps, a Z-10 sample injector, and a Hitachi 100-40 variable wavelength detector; the other system used Model 110 B pumps, a 210A sample injector, and a Model 167 Integrator.

Gas chromatography

GC was used to determine cholesterol concentrations in stones and bile and to identify methyl esters of fatty acids

[3]The surgeon performing gastroplasty in one of our institutions aspirates gallbladder bile to facilitate manual palpation of the gallbladder so that patients with cholelithiasis can be identified.

[4]Frozen samples of portions of "control biles" used in studies by Dr. Strasberg's laboratory (Toronto, Canada) were later made available to us. Since biles supersaturated with cholesterol were used preferentially in Dr. Strasberg's studies, our "control" population (see Table 1) may disproportionately represent biles unsaturated with cholesterol.

derived from lecithin as described fully below. The apparatus used was a Shimadzu GC-9A gas chromatograph with a flame-ionization detector coupled to a Shimadzu CR1A integrator (Shimadzu Instruments, Kyoto, Japan).

Lipid analysis

Biliary cholesterol concentrations were determined by GC using stigmasterol as an internal standard (17). Total bile salt concentration was determined enzymatically using the 3α-hydroxysteroid dehydrogenase method (15). Lecithin was determined as total inorganic phosphorus by the method of Bartlett (18). Total lipid concentration (g/dl) was determined from the sum of the concentrations of cholesterol, phospholipid, and bile salt. The cholesterol saturation index was calculated for each bile from the critical tables of Carey (19).

To assess possible lecithin hydrolysis due to storage, biliary lysolecithin concentrations were determined in all samples immediately prior to extraction of phospholipids into organic solvents for determination of lecithin molecular species. Aliquots of bile and standards of lecithin and lysolecithin containing approximately 10 μg of phosphorus were applied in quadruplicate to silica gel thin-layer plates (J. T. Baker Chemical, Phillipsburg, NJ) and developed for 2 h in chloroform–methanol–water 65:25:4 (by vol). Phospholipids were visualized using a molybdenum spray (20) and total phosphorus in lecithin and lysolecithin spots was determined (18).

Determination of molecular species of lecithin

Biliary phospholipids were separated into classes by the HPLC method of Patton, Fasulo, and Robins (21). Lipids were extracted from bile by the method of Folch, Lees, and Sloane Stanley (22) and dried under a stream of nitrogen. The extracts were dissolved in absolute ethanol and chromatographed on a 250 × 4.6 mm Hibar column packed with 5 μ LiChrospher Si-100 (EM Science, Cherry Hill, NJ). The elution solvent consisted of isopropanol–hexane–ethanol–25 mM phosphate buffer (pH 7.00)–glacial acetic acid 495:367:100:57:0.3 (by vol) that was filtered prior to use through a 0.2-μm polycarbonate filter (Nucleopore, Pleasanton, CA). Absorbance was monitored at 205 nm, flow rate was 1 ml/min, and the eluant containing lecithin was collected, dried under a stream of nitrogen, and then extracted into chloroform and washed three times with methanol–0.15 M NaCl 1:1 (by vol) (22). Lecithins were then subjected to phospholipase C hydrolysis (23) and the resultant diglycerides were benzoylated (24). Benzoyl derivatives were chromatographed on a 250 × 2.5 mm Ultrasphere 5 μ ODS column (Beckman) using an elution solvent of methanol–water–acetonitrile 937:38:25 (by vol) (12). The flow rate was 0.3–0.4 ml/min and absorbances were monitored at 230 nm.

Peak identity of benzoyl derivatives was established by analogy to previously published chromatograms of rat bile (12) and from plots of logarithm of the relative retention time versus effective carbon number in the *sn*-1 position (12). Assignment to the *sn*-1 or *sn*-2 position was made by analogy to studies of Cantafora et al. (25) who used specific phospholipase hydrolysis of fatty acids in lecithin. To confirm peak identities, a single bile sample from each patient group was chromatographed on a semi-preparative column (250 × 10 mm Ultrasphere 10 μ ODS column from Beckman), at a flow rate of 10 ml/min, with the same solvent as used in the analytical work. The solvent containing lipid was collected in individual peaks, dried under a stream of nitrogen, and stored up to 7 days in chloroform under a blanket of argon. The benzoyl derivatives were hydrolyzed and transesterified in 0.5 ml of sodium methoxide for 15 min at 50°C. After addition of 0.5 ml of 5 M HCl, the fatty acid methyl esters were extracted with 4 ml and 2 × 3 ml aliquots of hexane. The pooled hexane fractions were washed with 2 ml of 0.1 M Na_2CO_3 and then dried for 12 h over anhydrous sodium sulfate. Fatty acid methyl esters derived from each peak were chromatographed on a 30-meter fused silica capillary column with a 0.2 μm SP-2380 stationary phase (Supelco, Bellefonte, PA) using helium carrier gas (99.995%) at 0.5 ml/min, with a column temperature of 185°C, and with detector and injector temperatures of 275°C. Fatty acid methyl ester peaks were identified and quantified using commercially prepared external standards (26).

Determination of the molecular species of bile salts

Bile salt species were determined by HPLC using the solvent and monitoring system of Rossi, Converse, and Hofmann (27). In brief, approximately 50–100 μl of bile was dissolved in 1 ml methanol and centrifuged for 10 min with a table-top centrifuge to remove precipitated protein. Aliquots of the supernatant were chromatographed on a μBondapak C18 column (Waters Associates, Milford, MA) equipped with a Pecosphere C18 guard column (Perkin Elmer, Norwalk, CT). The elution solvent ran at 0.7 ml/min and was prepared by the addition of 4.2 ml 5 M NaOH to a liter of 75:25 (v/v%) methanol–0.01 M KH_2PO_4 adjusted to an apparent pH of 5.35 with the addition of approximately 1 ml of 85% H_3PO_4. Calibration curves at 200 nm and relative retention time for each bile salt species were established using authentic standards (27).

Statistical analysis

Comparisons between means of values determined in cholesterol stone patients, pigment stone patients, morbidly obese patients, and controls were carried out by analysis of variance (28). Where the F-test was significant at the $P < 0.05$ level, the significance of intergroup differences was established using multiple comparison tests (either the Fisher Protected Least Significant Difference or the Scheffé F tests) (28, 29). When means in only two groups were compared, Student's one-tailed unpaired *t*-

test was used. All analyses were performed with a commercially produced statistics computer program (Statview SE + Graphics, supplied by Abacus Concepts, Berkeley, CA).

RESULTS

Definition of patient groups

Patients with gallstones were divided into two groups on the basis of gallstone composition. Gallstones composed of greater than 50% cholesterol by weight were designated cholesterol stones and those with less than 20% cholesterol by weight were designated pigment stones. No so-called "mixed" stones were found in this study in agreement with other series of sterile biles (30, 31). Subsequent comparisons were then made between four patient groups: cholesterol stone patients (n=29), pigment stone patients (n=9), morbidly obese patients without stones (n=5), and "controls" with a variety of non-biliary tract diseases (n=10).

Biliary lipid compositions and cholesterol saturations

Table 1 indicates that bile from patients with cholesterol stones showed greater saturation with cholesterol than bile from pigment stone patients or controls. Bile from cholesterol stone patients demonstrated a lower total lipid concentration than that of controls. Absolute lecithin concentrations were lowest in cholesterol and pigment stone patients though no differences were seen in the mole percentage of lecithin among the four patient groups. The mole percentage of bile salts was lower in the morbidly obese group than in either the pigment stone or control groups. The pathophysiologically important cholesterol to lecithin coupling ratios (32) show cholesterol enrichment of bile relative to lecithin in cholesterol stone patients (ratio=0.40) compared with pigment stone patients (ratio=0.30) and intermediate values for obese subjects (ratio=0.36). In contrast, the lecithin to bile salt coupling ratio is elevated only in the obese group.

Lysolecithin concentrations were similar in all four groups (Table 1) and although these concentrations are higher than seen with fresh biles (refs. 33, 34 and N. Timofeyeva and D. W. Hay, unpublished observations) only a small portion of the lecithin was hydrolyzed during storage.

Molecular species of lecithin

Fig. 1 shows a typical HPLC chromatogram of benzoyl derivatives of lecithins from human bile. After reviewing all chromatograms 28 peaks were identified, but individual biles displayed only 24–27 of them. **Table 2** displays the identities of the principal peaks and their proportions in each patient group; the lecithin pattern was similar in all. The major lecithin species present in the cholesterol stone group was 16:0–18:2 (44.1%) which was threefold greater in concentration than any other lecithin species. Found in roughly equal proportions were 16:0–18:1 (13.8%) and 16:0–20:4 (12.6%) lecithins. Most lecithin species had a 16:0 fatty acid in the *sn*-1 position but 18:0–18:2 (5.3%) and 18:1–18:2 (4.4%) lecithins were present in appreciable quantities. A variety of minor species, which included 14:0–20:4, 16:1–16:1, 16:1–20:4, 18:1–20:4, 17:0–18:2, 18:0–22:6, were identified but they comprised less than 0.4% of total lecithins.

There were no significant differences between the individual lecithin species of biles in cholesterol and pigment stone patients. When compared to controls, both cholesterol and pigment stone patients had smaller proportions of 16:0–20:4 lecithin and larger proportions of 18:0–18:2 lecithin. Morbidly obese subjects had greater proportions of 16:0–20:4 lecithin than either group of stone patients but the difference reached statistical

TABLE 1. Biliary lipid composition and weight in each patient group[a]

Biliary Lipids	Cholesterol Stones	Pigment Stones	Morbidly Obese	Controls
Bile salt (mM)	114 ± 15	131 ± 18	136 ± 25	172.0 ± 14
Lecithin (mM)	37.6 ± 3.4[b,c]	40.6 ± 4.0[d]	65.0 ± 11[b,d]	56.4 ± 5.6[c]
Cholesterol (mM)	14.1 ± 1.4	12.2 ± 2.0	23.3 ± 5.4	17.5 ± 3.6
Bile salt (mole %)	66.8 ± 1.4	70.3 ± 3.0[d]	60.3 ± 1.5[d,e]	70.4 ± 1.8[e]
Lecithin (mole %)	23.8 ± 1.0	22.9 ± 2.2	29.6 ± 1.4	22.8 ± 0.9
Cholesterol (mole %)	9.40 ± 0.7	6.80 ± 1.0	10.10 ± 0.6	6.80 ± 1.1
% Lysolecithin[g]	1.6 ± 0.3	1.9 ± 0.5	3.0 ± 1.0	1.2 ± 0.2
Total lipids (g/dl)	9.16 ± 1.0[c]	10.16 ± 1.0	12.69 ± 2.3	13.61 ± 1.1[c]
CSI	1.34 ± 0.09[c,f]	0.96 ± 0.12[f]	1.20 ± 0.05	0.88 ± 0.12[c]
Weight (kg)	77 ± 5[f]	56 ± 4[d,f]	133 ± 10[b,d,e]	69 ± 5[e]

[a] Values are means ± SE.
[b,c,d,e,f] $P < 0.05$ for intergroup comparisons.
[g] Lysolecithin expressed as percent of total phospholipids.

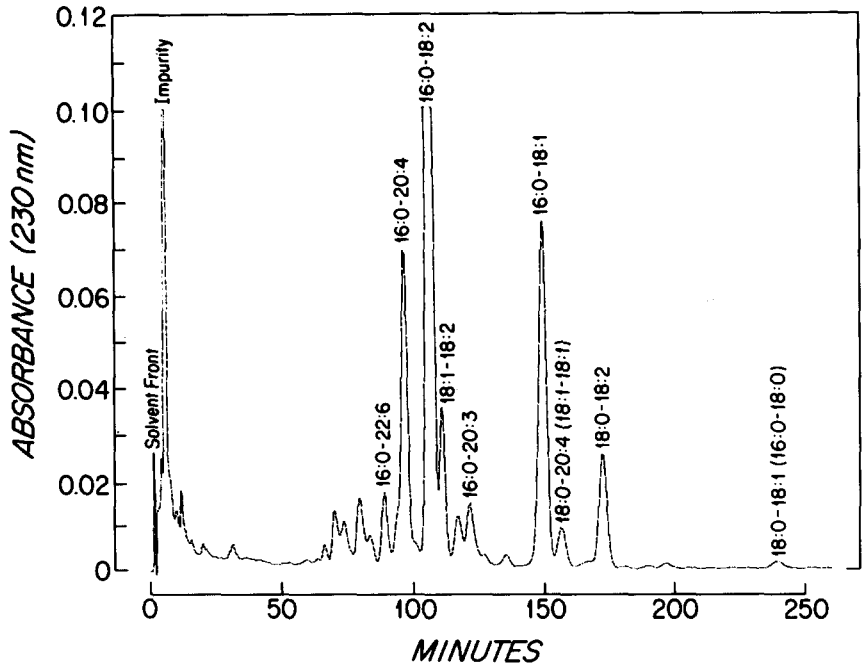

Fig. 1. Molecular species of lecithins in human bile separated by HPLC as their benzoyl diglyceride derivatives. Peaks are identified by the carbon number and double bond number in the *sn*-1 and *sn*-2 positions, respectively. Individual biles contain 24–28 peaks, but not all molecular species of lecithins were evident in all biles.

significance only when morbidly obese subjects were compared with pigment stone patients.

Molecular species of bile salts

The proportions of the 12 major conjugated bile salts present in each patient group are presented in **Table 3.** The major differences between groups are accounted for by the significantly higher proportion of cholates and the lower proportion of deoxycholates in the controls than in the cholesterol and pigment stone patients. The bile salt species were similar in the morbidly obese and control groups.

Hydrophobic indices of lecithin and bile salt mixtures

The hydrophilic–hydrophobic balance of a bile salt mixture has been quantified as a "hydrophobic index" (35). The hydrophobic index of an individual bile salt is determined from its HPLC retention time compared to that of other bile salt species and the hydrophobic index

TABLE 2. Molecular species of lecithins in human bile[a]

Peak #	Lecithin Species	Cholesterol Stones	Pigment Stones	Morbidly Obese	Controls
4	14:0–18:2; others[b]	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.4 ± 0.1
5	16:1–18:2; 18:2–20:4; others	1.5 ± 0.1	1.9 ± 0.3	1.5 ± 0.2	1.4 ± 0.2
6	16:0–20:5; others	2.1 ± 0.1	2.6 ± 0.5	2.5 ± 0.3	1.9 ± 0.2
7	16:0–18:3; 16:0–20:5; others	2.7 ± 0.1	2.9 ± 0.2	2.6 ± 0.2	2.2 ± 0.2
8	15:0–18:2; 18:1:18:3; others	0.8 ± 0.1[c]	0.9 ± 0.1[d,e]	0.6 ± 0.1[d]	0.5 ± 0.1[c,e]
9	16:0–22:6	2.7 ± 0.2	3.0 ± 0.3	2.4 ± 0.4	2.6 ± 0.2
10	16:0–16:1	0.8 ± 0.3	0.9 ± 0.3	0.7 ± 0.3	0.1 ± 0.1
11	16:0–20:4	12.6 ± 0.5[c]	11.3 ± 0.7[d,e]	14.5 ± 0.9[d]	14.9 ± 1.2[c,e]
13	16:0–18:2	44.1 ± 1.2	42.5 ± 1.8	43.3 ± 1.4	44.6 ± 1.5
14	18:1–18:2	4.4 ± 0.2	5.0 ± 0.4	4.1 ± 0.2	3.7 ± 0.2
15	16:0–20:3; others	2.0 ± 0.1	2.6 ± 0.3	1.9 ± 0.1	1.7 ± 0.2
16	16:0–20:3	3.0 ± 0.2	2.8 ± 0.4	3.2 ± 0.2	2.5 ± 0.2
20	16:0–18:1	13.8 ± 0.6	13.6 ± 0.4	13.0 ± 0.8	16.2 ± 0.8
21	18:0–20:4; 18:1–18:1	1.5 ± 0.1	1.7 ± 0.2	1.7 ± 0.1	1.5 ± 0.2
23	18:0–18:2	5.3 ± 0.2[c]	5.2 ± 0.2[e]	5.3 ± 0.1[f]	4.3 ± 0.3[c,e,f]
28	18:0–18:1; 16:0–18:0	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.0	0.4 ± 0.0
	All other peaks	1.7	2.3	1.7	1.2

[a] Species comprising less than 0.4% of the total are not shown. Peak numbers correspond to consecutive peaks in Fig. 1. Values expressed as mole% lecithin species (means ± SE).
[b] The term "others" denotes species present in amounts <0.2% of the total but not precisely quantified.
[c,d,e,f] $P < 0.05$ for intergroup comparisons.

TABLE 3. Bile salt species present in gallbladder biles[a]

Bile Salts	Cholesterol Stones	Pigment Stones	Morbidly Obese	Controls
TUDC	1.5 ± 0.2	1.9 ± 0.9	1.0 ± 0.3	1.6 ± 0.6
STLC	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.2
GUDC	2.2 ± 0.4	4.4 ± 2.3	2.0 ± 0.9	0.4 ± 0.1
TC	10.6 ± 1.2	9.6 ± 1.5	11.1 ± 1.5	12.0 ± 3.0
SGLC	3.4 ± 0.4	2.3 ± 0.4	1.7 ± 0.3	1.9 ± 0.8
GC	21.2 ± 1.1[b,c]	23.4 ± 1.8[d]	29.5 ± 2.5[b]	30.9 ± 3.3[c,d]
TCDC	11.3 ± 1.1	10.6 ± 1.2	10.8 ± 1.0	11.2 ± 2.6
TDC	6.7 ± 0.7[c]	5.4 ± 1.1[d]	3.9 ± 1.1	2.2 ± 0.7[c,d]
GCDC	22.7 ± 1.4	25.5 ± 2.5	26.2 ± 2.4	29.3 ± 2.1
GDC	19.2 ± 1.9[c]	15.5 ± 2.5	12.9 ± 3.1	9.3 ± 2.6[c]
TLC	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.3 ± 0.1
GLC	0.6 ± 0.1	0.7 ± 0.3	0.3 ± 0.2	0.4 ± 0.2
Total UDC	3.7 ± 0.5	6.3 ± 2.6	3.0 ± 1.2	2.0 ± 0.6
Total cholate	31.7 ± 1.8[b,c]	33.0 ± 2.8[d]	40.6 ± 3.4[b]	42.9 ± 2.5[c,d]
Total CDC	33.9 ± 1.9	36.1 ± 1.8	37.0 ± 2.2	40.5 ± 1.7
Total DC	26.0 ± 2.4[c]	20.9 ± 3.3	16.9 ± 4.1	11.4 ± 2.9[c]
Total SLC	3.8 ± 0.5	2.7 ± 0.5	2.2 ± 0.3	2.4 ± 1.0
Total LC	0.9 ± 0.1	1.0 ± 0.3	0.3 ± 0.2	0.7 ± 0.3

[a] Values expressed as mole% of the total (means ± SE).
[b,c,d] $P < 0.05$ for intergroup comparisons.

of a mixture is determined by summation of the products of the mole fraction and hydrophobic index of each bile salt in the mixture (35). Heuman (35) determined experimentally the hydrophobic indices of all major conjugated bile salts in human bile with the exception of sulfated lithocholates. Based on our HPLC data we have determined that the hydrophobic indices of sulfotaurolithocholate and sulfoglycolithocholate are −0.36 and −0.32, respectively. Using these values a hydrophobic index of biles salts was calculated for each bile in the present study.

We then developed a hydrophobic index for mixtures of lecithins based on the same principles as for bile salts (35). Using chromatograms of four different biles, relative retention times were established for each benzoyl diglyceride peak. The hydrophobic indices of 16:0–18:2 (peak 13, Table 2) and 18:0–18:2 (peak 23, Table 2) were arbitrarily set to 0 and 1, respectively, and the hydrophobic indices for lecithins corresponding to each peak were calculated (35). Species in peak 1 (Table 1) were the least hydrophobic (index of 1.73). A lecithin hydrophobic index was then calculated for the lecithin mixtures in each bile.

Fig. 2 displays the significant but weak positive correlation between the hydrophobic indices of bile salts and lecithins in each bile (r=0.48, R^2=0.23, P = 0.0002). The characteristics of bile with more hydrophobic lecithins were compared to bile with less hydrophobic lecithins. **Table 4** shows that biles with a hydrophobic index below the median value differed in several characteristics from those with a high hydrophobic index. Biles with more hydrophobic lecithins not only had a higher hydrophobic index of bile salts, but also a greater percentage of their total lipids were lecithins, a smaller percentage of their bile salts were the relatively hydrophilic cholate species, and they came from heavier patients.

Table 5 compares the characteristics of biles with more hydrophilic bile salts to those with more hydrophobic bile salts in a fashion similar to that carried out for lecithin hydrophobic index. Biles with a more hydrophobic bile salt mixture had a higher pH, lower total sodium, contained less of the relatively hydrophilic 16:0–18:2 lecithin, more of the relatively hydrophobic 16:0–18:1 lecithin, and had a higher lecithin hydrophobic index. Data on pH, calcium, and sodium contents will be presented in a future paper (M. J. Cahalane, D. W. Hay, E. B. Cabot and M. C. Carey, unpublished results).

Microscopic findings

Table 6 demonstrates significant differences between biles with and without cholesterol monohydrate crystals. Biles with crystals were less concentrated, more saturated with cholesterol, had a greater proportion of the more

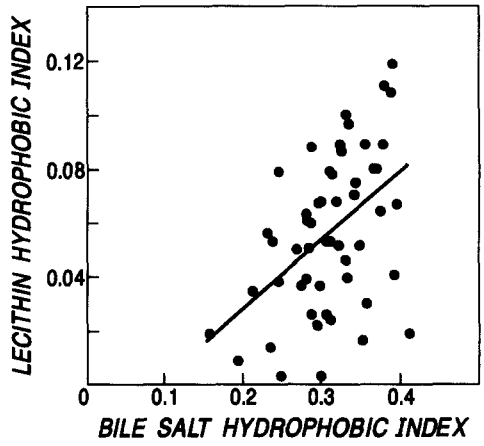

Fig. 2. Dependence of lecithin hydrophobic index on bile salt hydrophobic index for all biles in the study. The correlation is weak (r=0.48, R^2=0.23) but highly significant (P=0.0002).

TABLE 4. Parameters that varied with lecithin hydrophobic index[a]

	Low Lecithin Hydrophobic Index	High Lecithin Hydrophobic Index	*P* value
% Total lipid as lecithin	22.8 ± 1.2	25.3 ± 0.9	0.050
% Bile salts as taurolithocholate	0.18 ± 0.04	0.37 ± 0.08	0.022
% Bile salts as cholate	37.4 ± 1.8	32.4 ± 1.8	0.028
Bile salt hydrophobic index	0.488 ± 0.015	0.530 ± 0.012	0.016
Weight (pounds)	154 ± 11	188 ± 15	0.035

[a]Values are means ± SE.

hydrophobic 18:0-18:2 lecithin species, had smaller proportions of chenodeoxycholates and greater proportions of deoxycholate and sulfolithocholate conjugates. The differences between biles with a CSI ≥ 1.0 and those with a CSI <1.0 were similar to the differences seen between biles with and without cholesterol crystals (data not shown).

Lecithin patterns and body weight

There was a very weak ($r=0.31$) but significant ($P=0.04$) relationship between body weight and the proportion of 16:0-20:4 lecithin, the predominant arachidonyl-containing lecithin in human bile. This weak relationship was evident when all the biles were examined as a single group, but a stronger positive relationship was demonstrated when the control group was examined separately. There was a positive correlation between the proportion of arachidonyl lecithin in gallbladder bile and body weight ($r=0.91$, $P=0.001$) and the inverse relationship between body weight and the proportion of 16:0-18:2 lecithin ($r=-0.87$, $P=0.005$) in the control group.

Dependence of lecithin molecular species on bile salt molecular species

Taking all patients' biles as a group, there were no significant relationships between the proportions of any one bile salt species and any one major lecithin species. Only when the control group was considered separately were there correlations between the proportions of 16:0-20:4 or 16:0-18:2 lecithin and the proportion of glycocholate ($r=-0.82$, $P=0.004$ for 16:0-20:4 and $r=0.73$, $P=0.02$ for 16:0-18:2) and glycochenodeoxycholate concentration ($r=-0.86$, $P=0.001$ for 16:0-20:4 and $r=0.79$, $P=0.006$ for 16:0-18:2). In contrast, the proportion of 16:0-20:4 lecithin in the control group increased with the proportion of taurodeoxycholate ($r=0.75$, $P=0.01$).

DISCUSSION

This work confirms that biliary lecithin is a complex mixture of molecular species. However, using state of the art methodology we show for the first time that biliary lecithins of humans are composed of more than 30 identifiable molecular species. These species represent the least hydrophobic species of the hepatic lecithins. For example, at least 80% of biliary lecithins have a 16:0 moiety in the *sn*-1 position and an unsaturated acyl chain in the *sn*-2 position (Table 2). Next in importance are *sn*-1 18:0 lecithins. However, lecithin species with unusual fatty acids such as 15:0 and 17:0 were observed and we regularly identified species with highly unsaturated acyl chains such as 20:5 and 22:6.

A minor limitation of the current study is that lecithin species were determined on previously frozen samples of bile. Nonetheless, a small ($<2\%$) degree of hydrolysis of lecithin to form lysolecithin was detected in all patient groups. It is unlikely, therefore, that our results would have been substantially different had the analysis been done promptly on fresh specimens. When biles collected in the first half of the study were compared to those collected in the second half, no significant differences in the lecithin species were noted, suggesting that lecithin oxidation or hydrolysis, which would be expected to be time dependent, did not occur at a substantial rate following freezing.

There are substantial differences between the lecithin

TABLE 5. Biliary parameters that varied when groups with high and low hydrophobic indices were compared[a]

	Low Bile Salt Hydrophobic Index	High Bile Salt Hydrophobic Index	*P* value
pH	7.14 ± 0.07	7.32 ± 0.06	0.029
Total sodium (mmol/l)	205 ± 14	171 ± 14	0.049
mole% 16:1-18:2; 18:2-20:4	1.7 ± 0.1	1.4 ± 0.1	0.045
mole% 16:0-18:2	45.1 ± 0.9	42.4 ± 1.3	0.041
mole% 16:0-18:1	13.2 ± 0.5	15.1 ± 0.6	0.010
Lecithin hydrophobic index	0.064 ± 0.004	0.089 ± 0.006	0.001

[a]Values are means ± SE.

TABLE 6. Statistically significant differences in composition between biles with and without cholesterol monohydrate crystals[a]

Biliary Lipids	Cholesterol Crystals Absent	Cholesterol Crystals Present	*P* value
Bile salts (mM)	161 ± 15	101 ± 9	0.001
Lecithin (mM)	52.9 ± 3.7	35.9 ± 3.3	0.001
% Lipids as cholesterol	7.4 ± 0.7	9.6 ± 0.6	0.001
Total lipid concentration (g/dl)	12.7 ± 0.9	8.3 ± 0.7	0.009
Cholesterol saturation index	0.97 ± 0.08	1.37 ± 0.09	0.007
mole% 18:0-18:2	4.8 ± 0.2	5.4 ± 0.2	0.022
% Sulfoglycolithocholates	2.2 ± 0.4	3.3 ± 0.4	0.028
% Taurodeoxycholates	4.4 ± 0.7	6.3 ± 0.7	0.037
% Glycochenodeoxycholates	26.5 ± 1.4	23.0 ± 1.5	0.041
% Glycodeoxycholates	13.5 ± 1.8	18.7 ± 1.9	0.026
% Chenodeoxycholates	38.3 ± 1.6	33.5 ± 1.7	0.021
% Deoxycholates	17.9 ± 2.4	25.0 ± 2.4	0.021
% Sulfolithocholates	2.6 ± 0.4	3.8 ± 0.5	0.041

[a]Values are means ± SE.

patterns found in the current work and those identified by previous investigators. Ahlberg et al. (33) prepared the trimethylsilyl ether derivatives of biliary lecithins and separated them into six major peaks which were identified by GC-mass spectrometry. Minor species were mentioned as being present in all the major peaks but were not quantified or identified. Compared to the biles of gallstone patients in the current study, biles from patients studied by Ahlberg et al. (33) had much more 16:0-18:1 lecithin (32% vs. 14%), more 18:1-18:2 lecithin (8% vs. 4%), and more 16:0-16:1 lecithin (5% vs. 1%). Conversely, patients studied by Ahlberg et al. (33) had less 16:0-20:4 lecithin in their bile than those in any patient group in the present work (5% vs. 13%). The differences between these two studies are likely due to differences in technique, but differences based upon dietary habits, disease states, or ethnic background cannot be absolutely excluded.

Cantafora et al. (25) developed a less precise HPLC method for separating the molecular species of lecithins and applied it to bile from seven patients with cholesterol gallstone disease. Their technique, which monitored absorbance of fatty acyl double bonds at 205 nm, required response factors for each peak and is less precise than the method used here. In contrast to the current work, greater proportions of 16:0-18:2 and 16:0-18:1 lecithins were detected in the study by Cantafora et al. (25), although direct comparison is difficult as analysis of gallbladder biles from only three patients was reported.

There were substantial variabilities in the lecithin species from patient to patient in the present study. Not all of the minor species were observed in all patients. Presumably, lecithin species such as 17:0-18:2 were present only in patients with substantial quantities of the odd chain fatty acid in their diets.

It has been suggested that increases in the proportion of arachidonyl lecithin may be an important event in gallstone formation (11) and a recent study suggests that arachidonyl lecithin and PGE_2 synthesis increases in human bile prior to cholesterol crystal nucleation (36). When comparisons were made between the lecithin fatty acid composition or the lecithin molecular species of gallstone patients and controls, two groups of investigators have found increased arachidonyl lecithin in stone patients (37, 38) and two found no difference (33, 39). In the current work, morbidly obese patients had greater proportions of biliary 16:0-20:4, the major arachidonyl biliary lecithin, than did cholesterol or pigment stone patients, but statistical significance was reached only when the pigment and morbidly obese groups were compared (Table 2). These data initially suggested to us that in the presumptive "pre-stone state" (i.e., in the morbidly obese), the proportion of arachidonyl lecithin was increased but returned to baseline after stones were formed. This would parallel, therefore, the rise and fall in biliary arachidonyl lecithin seen in the cholesterol stone-forming golden hamster (40). However, data from our control group does not support this hypothesis. The control group had arachidonyl lecithin levels comparable to that of the morbidly obese group and significantly greater than either the cholesterol or pigment stone groups. However, several of our "control" group, though free of biliary tract disease, suffered from inflammatory bowel disease (known also to be a prestone condition (41)). Therefore, in the absence of a healthy control group, this study cannot support or refute the hypothesis that an increase in biliary arachidonyl lecithin is an important event leading to cholesterol stone formation in humans.

As the hydrophilic-hydrophobic balance of bile salts affects their equilibrium micellar cholesterol-solubilizing capacities (14), it was of interest to discover that there was a weak but highly significant correlation between the hydrophobic indices of bile salts and lecithin (Fig. 2), suggesting that the hydrophobicity of the bile salt species, in part, determines the hydrophobicity of the lecithin species. The R^2 or "predicted variance" of only 0.23 suggests that only 23% of variance can be explained by the regres-

sion line, a fact that underscores the weakness of the relationship. However, this finding is consistent with recent experimental work on model systems from this laboratory (42) suggesting that the *sn*-1 palmitoyl (16:0) species may be subselected in part for bile by physical-chemical interactions of intracellular bile salts with lecithin in hepatocyte membranes. Nevertheless, although prior studies suggested that bile acid feeding changed the fatty acid composition of the lecithin species (33, 43), animals with widely varying bile salt hydrophilic-hydrophobic balances have similar lecithin patterns (34) so that the mechanism by which changes in bile salt species influence the pattern of lecithin species is not clear.

As detailed in Results (Table 5) biles enriched in hydrophobic bile salts have less of the relatively hydrophilic 16:0-18:2 lecithin and more of the more hydrophobic 16:0-18:1 lecithin. Biles enriched in hydrophobic bile salts also appear to have a higher pH and lower total sodium but the physical-chemical basis of these findings is not evident. Biles with cholesterol crystals and those that were cholesterol supersaturated had greater proportions of the more hydrophobic 18:0-18:2 lecithin. This is also consistent with the predictions of recent experimental work (42) that suggests that biles derived by the dissolution of cholesterol-enriched membranes will have a greater proportion of 18:0-18:2 lecithin than those derived from cholesterol-poor membranes. Our observation of a greater proportion of deoxycholate and lithocholate conjugates in both cholesterol and pigment stone biles (Table 3) and in bile containing cholesterol crystals (Table 6) is in agreement with the hypothesis that stone formation and/or gallbladder dysmotility leads secondarily to increased exposure of the bile salt pool to the colonic bacterial flora (44). Supersaturation and rapid nucleation may in turn be promoted by conjugated deoxycholates through a variety of mechanisms (reviewed in ref 10).

Limited data have been available on the relationship between bile salt species and lecithin species in bile. In studies of the fatty acid composition of lecithin in the bile of gallstone patients and controls, Cantafora et al. (38) described a negative correlation between the percentages of arachidonate and biliary chenodeoxycholate in gallstone patients but not in controls. In a study of duodenal bile composition from healthy volunteers (45) a similar negative correlation between arachidonyl lecithins and chenodeoxycholate conjugates was described as well as a strong positive correlation between arachidonyl lecithin and deoxycholate conjugates. In the current work, relationships between bile salt species and lecithin species were evident only in controls, with negative correlations between the major arachidonyl-containing lecithin and the glycine conjugates of cholate and chenodeoxycholate and a positive correlation between arachidonyl lecithin and taurodeoxycholate. It is not evident why these relationships should exist and only in controls or why the relationships are not paralleled by both taurine and glycine conjugates of the same bile salt. Our results suggest that strong individual relationships between bile salt species and lecithin species do not exist in bile many years after gallstones have formed. Nevertheless, it is possible that in the pre-stone stage of active lithogenesis, important and transient alterations in lecithin molecular species may occur.

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