Acyl chain unsaturation modulates distribution of lecithin molecular species between mixed micelles and vesicles in model bile. Implications for particle structure and metastable cholesterol solubilities

David E. Cohen and Martin C. Carey[1]

Department of Medicine, Harvard Medical School, Brigham and Women's Hospital and Harvard Digestive Diseases Center, Boston, MA 02115

Abstract We determined the distribution of lecithin molecular species between vesicles and mixed micelles in cholesterol supersaturated model biles (molar taurocholate–lecithin–cholesterol ratio 67:23:10, 3 g/dl, 0.15 M NaCl, pH ~6–7) that contained equimolar synthetic lecithin mixtures or egg yolk or soybean lecithins. After apparent equilibration (48 h), biles were fractionated by Superose 6 gel filtration chromatography at 20°C, and lecithin molecular species in the vesicle and mixed micellar fractions were quantified as benzoyl diacylglycerides by high performance liquid chromatography. With binary lecithin mixtures, vesicles were enriched with lecithins containing the most saturated *sn*-1 or *sn*-2 chains by as much as 2.4-fold whereas mixed micelles were enriched in the more unsaturated lecithins. Vesicles isolated from model biles composed of egg yolk (primarily *sn*-1 16:0 and 18:0 acyl chains) or soy bean (mixed saturated and unsaturated *sn*-1 acyl chains) lecithins were selectively enriched (6.5–76%) in lecithins with saturated *sn*-1 acyl chains whereas mixed micelles were enriched with lecithins composed of either *sn*-1 18:1, 18:2, and 18:3 unsaturated or *sn*-2 20:4, 22:4, and 22:6 polyunsaturated chains. Gel filtration, lipid analysis, and quasielastic light scattering revealed that apparent micellar cholesterol solubilities and metastable vesicle cholesterol/lecithin molar ratios were as much as 60% and 100% higher, respectively, in biles composed of unsaturated lecithins. Acyl chain packing constraints imposed by distinctly different particle geometries most likely explain the asymmetric distribution of lecithin molecular species between vesicles and mixed micelles in model bile as well as the variations in apparent micellar cholesterol solubilities and vesicle cholesterol/lecithin molar ratios. These results strongly suggest that modifications of biliary lecithin species may profoundly influence the physical chemistry of native bile as well as gallstone formation and prevention. —**Cohen, D. E., and M. C. Carey.** Acyl chain unsaturation modulates distribution of lecithin molecular species between mixed micelles and vesicles in model bile. Implications for particle structure and metastable cholesterol solubilities. *J. Lipid Res.* 1991. **32:** 1291–1302.

Supplementary key words bile salts • fatty acyl chain • high performance liquid chromatography • quasielastic light scattering • high performance gel filtration chromatography

Compared to the diverse nature of phospholipid classes present in hepatocyte membranes, biles of humans and most laboratory animals are selectively enriched (up to 95%) in lecithins with approximately 5% being other classes, particularly phosphatidylethanolamines (1–3). The molecular species of biliary lecithins and phosphatidylethanolamines are highly specific. The acyl chain in the *sn*-1 position is generally a saturated C-16 or C-18 fatty acid with an unsaturated C-18 or C-20 acyl chain occupying the *sn*-2 position (1, 4–6). Although high performance liquid chromatography (HPLC) has revolutionized the accuracy of phospholipid separation and quantitation (7, 8), the influence of phospholipid classes and molecular species on the physical chemistry of biliary lipid aggregates and the pathophysiology of bile has remained largely unexplored (9–11).

Recent studies from our laboratory (12–14) have suggested that, during early bile formation, bile salts structurally alter lecithin-rich vesicles in a step-wise series of physical-chemical interactions that ultimately result in the dispersion of secreted biliary lipids as mixed micelles or mixed micelles plus cholesterol-rich vesicles. Kinetics of bile salt-vesicle interactions were most rapid with unsaturated lecithin-rich vesicles (13), suggesting that lecithin molecular species may strongly modulate physical-chemical interactions among bile salts and other biliary lipids.

In cholesterol supersaturated biles, mixed micelles and vesicles differ strikingly in their lipid class compositions.

Abbreviations: HPLC, high performance liquid chromatography; QLS, quasielastic light scattering.

[1]To whom correspondence should be addressed at: Department of Medicine, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115.

Mixed bile salt-lecithin-cholesterol micelles have low cholesterol contents (cholesterol/lecithin ratios ≤ 0.3) and their principal components are bile salt molecules, in rapid equilibrium with intermicellar bile salt monomers and simple bile salt micelles (15–17). In contrast, vesicles are highly enriched in cholesterol (cholesterol/lecithin ratios up to 2 or greater) and contain essentially no bile salts (18, 19). In the present work, we employed high performance gel filtration chromatography (19) and state-of-the-art HPLC (7, 8) to demonstrate a marked asymmetric distribution of different lecithin molecular species between vesicles and mixed micelles in cholesterol supersaturated model biles. Vesicles were enriched in more saturated lecithin species whereas mixed micelles were enriched in more unsaturated lecithin species. Our results also demonstrate that in cholesterol supersaturated model biles individual molecular species of lecithin dramatically influence metastable micellar cholesterol solubilities as well as cholesterol to lecithin ratios of vesicles. Because molecular species of lecithin in native bile may be altered by diet (3, 6), exogenously administered bile acids (20), and disease (5, 21–23), these results hold important implications for furthering our understanding of the physical chemistry of bile as well as the pathophysiology of gallstone disease.

MATERIALS

The sodium salt of taurocholate (Calbiochem, San Diego, CA) was recrystallized to achieve greater than 99% purity (17). [^{3}H(G)]-taurocholate (sp act 8.1 Ci/mmol) and [4-^{14}C]cholesterol (sp act 60.0 mCi/mmol) were obtained from New England Nuclear (Boston, MA) and were the highest radiopurities available. Grade I egg yolk and soy bean lecithins (Lipid Products, South Nutfield, Surrey, U.K.) were >99% pure by high performance liquid chromatography (7) and thin-layer chromatography (200 μg applications). Synthetic molecular species of lecithin [*sn*-1 palmitoyl, *sn*-2 oleoyl (16:0–18:1), *sn*-palmitoyl, *sn*-2 arachidonoyl (16:0–20:4), *sn*-1 stearoyl, *sn*-2 oleoyl (18:0–18:1), *sn*-1 stearoyl, *sn*-2 linoleoyl (18:0–18:2), *sn*-1 stearoyl, *sn*-2 linolenoyl (18:0–18:3), dioleoyl (18:1–18:1), dilinoleoyl (18:2–18:2), dilinolenoyl (18:3–18:3)] were purchased from Avanti Polar Lipids (Birmingham, AL). Each lecithin species was >99% pure by HPLC (8) and gas-liquid chromatography of the fatty acid methyl esters. The supplier ascertained by specific phospholipase hydrolysis that each lecithin molecular species exhibited less than 20% fatty acyl chain racemization (13). All experiments were performed at a temperature (20°C) well in excess of the order-disorder transition temperatures of each lecithin species (24). Cholesterol was obtained from Nu-Chek-Prep (Elysian, MN) and found to be 99% pure by gas-liquid chromatography. All other chemicals and solvents were ACS or reagent grade purity (Fisher Scientific Co., Medford, MA). ACS grade NaCl was roasted at 600°C for 4 h in a muffle furnace to oxidize and remove organic impurities. Pyrex brand glassware was alkali-alcohol-washed for 24 h (EtOH–2 M KOH 1:1, v:v) followed by 24 h acid washing (1 M HNO_3) and rinsed thoroughly with purified water prior to drying. Water was filtered, ion-exchanged and glass-distilled (Corning Glass Works, Corning, NY).

METHODS

Model biles

After coprecipitation from stock solutions ($CHCl_3$-MeOH 1:1, v:v) (17), dried lecithin–taurocholate–cholesterol films (lecithin–taurocholate molar ratio = 0.43, 0–10 moles % cholesterol) were resuspended in aqueous solution (0.15 M NaCl, pH ~6–7) that included 3.0 mM NaN_3 as an antimicrobial agent. Final lipid compositions of model biles were determined analytically to be within 3% of the intended compositions.

Superose 6 gel filtration chromatography

A prepacked and factory calibrated (i.e., with specified V_o and V_t) HR10/30 Superose 6 column (Pharmacia, Piscataway, NJ) was used for high performance separation of lipid particles as previously described (19). A Pharmacia P-500 pump, in continuous mode, delivered eluant at a flow rate of 30 ml/h. Fractions were collected using a Pharmacia-LKB Frac-100 fraction collector. Lipid analysis of fractions from the column revealed that recoveries exceeded 90% uniformly and were close to 100% in most experiments.

Quasielastic light scattering (QLS)

QLS measurements of model biles and individual column fractions were performed on a home-built apparatus (25) at a scattering angle of 90° and sample temperature of 20°C. Mean diffusion coefficients of particles in solution were derived by cumulants analysis of intensity autocorrelation functions (26). The mean diffusion coefficient of each particle population was then translated into the mean hydrodynamic radius by the Stokes-Einstein relationship utilizing the viscosity of the solvent (18).

EXPERIMENTAL DESIGN

Distribution of lecithin molecular species between vesicles and mixed micelles

Cholesterol-supersaturated model biles (taurocholate–lecithin–cholesterol 63:27:10, 3 g/dl, 0.15 M NaCl, 3.0 mM NaN_3 pH ~6–7) were prepared containing either binary

(1:1) mixtures of synthetic lecithins or natural lecithin mixtures (egg yolk or soy bean). Model biles were equilibrated in the dark at 20°C for 48 h with periodic vortex mixing under an atmosphere of argon and then fractionated into vesicles and micelles using Superose 6 gel filtration (19). Prior to gel filtration, Superose 6 columns were pre-equilibrated with 50 ml (equivalent to two column volumes) of 7.5 mM taurocholate, 0.15 M NaCl, and 3.0 mM NaN_3 at 20°C, and eluted with the same solution. This taurocholate concentration was shown elsewhere (19) to correspond closely to the intermicellar-intervesicular concentration of the system. Equal volumes of model biles (0.5 ml) were applied to columns and 1.0-ml fractions were collected. Separation of vesicles and mixed micelles was verified by QLS analysis of individual column fractions (19). Molecular species of lecithin were determined for vesicle and mixed micellar fractions as well as whole model biles (see Analytical procedures). To achieve sufficient quantities of vesicle and micellar lecithin for molecular species analysis, each model bile was fractionated four times by gel filtration. Corresponding fractions, as determined by lipid analysis (see below) and QLS analysis (see above), were pooled in preparation for HPLC analysis (see below).

Influence of lecithin molecular species on cholesterol solubilities in mixed micelles

The apparent solubility of cholesterol in mixed micelles was determined by two independent methods. The qualifier "apparent" is used here because, when prepared by co-precipitation and reconstituted with aqueous solvent, dilute (3 g/dl) cholesterol supersaturated model biles form mixtures of cholesterol-rich vesicles and mixed micelles (18) that remain in a supersaturated state for prolonged periods of time (27).

Gel filtration analysis. Cholesterol-supersaturated model biles (taurocholate–lecithin–cholesterol 63:27:10, 3 g/dl, 0.15 M NaCl, 3.0 mM NaN_3, pH ~6–7) were prepared containing either a single synthetic lecithin molecular species, binary (1:1) mixtures of synthetic lecithin molecular species, or natural lecithin mixtures (egg yolk or soy bean). Model biles were equilibrated and fractionated by high performance gel filtration chromatography as described above. Cholesterol concentrations were then determined for individual column fractions. The solubility of cholesterol in mixed micelles was defined as the product of the fraction of total eluted cholesterol present in the mixed micellar peak and the moles percent cholesterol present in the unfractionated model bile (10 mol%).

QLS analysis. Model biles of constant lecithin–taurocholate molar ratio (0.43) but with systematic increases in cholesterol content (0–10 mol%) were prepared as described above (3 g/dl, 0.15 M NaCl, 3.0 mM NaN_3, pH ~6–7). Mean hydrodynamic radii of unfractionated model biles were monitored as functions of time until values stabilized. When plotted as functions of increasing cholesterol content, mean hydrodynamic radii increased sharply from micellar to vesicle sizes when the cholesterol concentration exceeded micellar solubility (18). The solubility of cholesterol in mixed micelles was taken as the highest mol% cholesterol where stable micellar sizes were observed (18).

Influence of lecithin molecular species on vesicle cholesterol/lecithin molar ratios

Cholesterol-supersaturated model biles were fractionated into vesicles and micelles as described above. Taurocholate, lecithin and cholesterol concentrations were serially determined in individual fractions eluting from the column. Cholesterol–lecithin molar ratios of vesicles were determined from the ratios of integrated peak areas of cholesterol and lecithin, respectively.

ANALYTICAL PROCEDURES

Lipid analysis

Concentrations of taurocholate were determined by the 3α-hydroxysteroid dehydrogenase method (17) and by liquid scintillation counting using a Beckman (Fullerton, CA) model LS-5000 TD scintillation counter and a hydrofluor scintillant (National Diagnostics, Highland Park, NJ). We determined lecithin concentrations by both an inorganic phosphorus procedure (17) and liquid scintillation counting, and cholesterol concentrations were assayed by gas–liquid chromatography (Shimadzu model GC9a, Kyoto, Japan) (28) and liquid scintillation counting.

Molecular species of lecithin

Lecithin plus cholesterol in model biles and in vesicle and mixed micellar fractions were extracted into chloroform according to the method of Folch, Lees, and Sloane Stanley (29). To separate lecithin from cholesterol, extracts were dried under N_2, resuspended in ethanol, and chromatographed on a Hibar 5 μm 250×4.6 mm LiChrospher Si-100 silica HPLC column (EM Science, Cherry Hill, NJ) in filtered (0.2 μm polycarbonate filter, Nucleopore, Pleasanton, CA) isopropanol–hexane–ethanol–25 mM phosphate buffer (pH 7.0)–glacial acetic acid 495:367:100:57:0.3 by vol according to the method of Patton, Fasulo, and Robins (7). A Beckman Instruments (Fullerton, CA) Model 110B HPLC pump provided a solvent flow rate of 1.0 ml/min and absorbance was monitored at 205 nm using a Beckman Model 166 detector and Model 167 integrator. The HPLC pump, detector, and integrator were all controlled by a Beckman AI406 analog-digital interface and Beckman System Gold Software. The silica column eluant containing lecithin was dried under a stream of N_2, dissolved in chloroform, and then washed

three times with methanol–0.15 M NaCl (29). Using phospholipase C from *Bacillus cereus* (Boehringer Mannheim Biochemicals, Indianapolis, IN), lecithins were hydrolyzed to diglycerides from which benzoate derivatives were prepared (30). These were fractionated by reverse phase HPLC using a Beckman Ultrasphere 5 μm ODS 250 × 2.5 mm column and a mobile phase consisting of methanol–water–acetonitrile 937:38:25 by vol (8) at a flow rate of 0.4 ml/min. Relative peak areas of benzoate absorbances monitored at 230 nm directly represented mole fractions of individual lecithin molecular species present in the original mixture. Peak identities were established according to Patton and Robins (8). In preliminary experiments, we determined that lecithin molecular species in vesicle and mixed micellar fractions containing the highest (peak) lipid concentrations were, within experimental error, representative of other vesicle or mixed micellar fractions, respectively. We therefore present in the results section, our determinations of lecithin molecular species in peak vesicle and mixed micellar fractions only.

In the cases of egg yolk and soy bean lecithins, HPLC peak identities were confirmed by capillary gas chromatography of fatty acid methyl esters after preparative HPLC (31). Briefly, benzoate derivatives of egg yolk and soy bean lecithin were chromatographed using a Beckman Ultrasphere 10 μm ODS 250 × 10.0 mm column and the solvent described above at a flow rate of 10 ml/min. Fatty acid methyl esters were prepared from individual lecithin (benzoate) peaks by incubation with 0.5 ml sodium methoxide at 50°C for 15 min followed by addition of 0.5 ml of 5 M HCl. Fatty acid methyl esters were extracted with hexane, washed with 0.1 M Na_2CO_3, dried over sodium sulfate, and separated on a Supelco (Bellefonte, PA) 30m SP-2380 0.2 μm capillary column using a fused silica stationary phase. Peak identities were established by comparison with commercially available standards (31). Stereospecific identities of *sn*-1 and *sn*-2 fatty acyl chains were based on published studies using specific phospholipase hydrolysis of egg yolk (4) and soy bean lecithins (32, 33).

RESULTS

Separation of coexisting vesicles and micelles in cholesterol supersaturated model biles

To determine the lecithin composition of vesicles and mixed micelles, cholesterol-supersaturated model biles were first fractionated using a well-characterized high performance gel filtration technique (19). As in our published work (see Fig. 5A of reference 19), we demonstrated that Superose 6 gel filtration of a typical model bile used in this study (taurocholate–egg yolk lecithin–cholesterol 63:37:10, 3 g/dl, 0.15 M NaCl, 20°C) using an intermicellar-intervesicular concentration of 7.5 mM taurocholate as eluant, yielded vesicles (mean hydrodynamic radius of approximately 320 Å) composed of cholesterol and lecithin (but no bile salt) eluting at the position of the void volume (V_o); mixed micelles containing taurocholate, lecithin, and cholesterol eluting within the included volume (mean hydrodynamic radii of 40–50 Å); and simple micelles (mean hydrodynamic radii of 11–15 Å) composed of pure taurocholate (i.e., no detectable lecithin or cholesterol) eluting at the total volume (V_t). Based upon replicate (n = 5) gel filtration analyses of model biles containing egg yolk, 16:0–20:4, and 18:0–18:1 lecithins, we determined that the standard deviations for cholesterol and lecithin concentrations in separated vesicles and mixed micelles were 2% or less.

Lecithin compositions of vesicles and mixed micelles

Binary mixtures of synthetic lecithins. **Table 1** lists the concentrations of synthetic lecithin species in vesicles and in mixed micelles separated from model biles. In each case, the concentration of a synthetic lecithin species was obtained from the product of the total lecithin concentration in vesicles or mixed micelles (determined by gel filtration chromatography) and the fraction of the lecithin molecular species present in each particle (determined by HPLC analysis). In all separations, mixed micelles contained the higher proportions (89–96%) of total lecithin concentration and only small, statistically insignificant differences were demonstrated in micellar concentrations of each lecithin molecular species.[2] On a mass basis, however, small differences in mixed micellar compositions corresponded to substantial changes in the lecithin composition of vesicles. Whereas vesicles contained only 4–11% of the total lecithin concentration, there were large, statistically significant increases in absolute vesicle concentrations of more saturated compared with less saturated lecithin molecular species. For purposes of comparison with unfractionated model biles, these results are graphically illustrated in Figs. 1 and 2.

Fig. 1 demonstrates the influence of *sn*-1 fatty acyl chain unsaturation on lecithin compositions of vesicles and mixed

[2]The statistical variability of the lecithin molecular species analysis was assessed using biles containing egg yolk and soy bean lecithins. Standard deviations of HPLC peak areas were determined to be 2% for benzoate derivatives of each lecithin species in vesicle fractions (n = 4 egg yolk lecithin, n = 2 soy bean lecithin), micellar fractions (n = 4 egg yolk, n = 3 soy bean), and unfractionated biles (n = 2 egg yolk, n = 3 soy bean). Because total as well as individual vesicle and mixed micellar lecithin concentrations were reproducible to within 2% (see above) and because the concentrations of lecithin molecular species in vesicles and mixed micelles (Table 1) represent the product of these independent determinations, we calculated that the overall standard deviations were 3% (the square root of the sum of the squares of the individual standard deviations). A significant change is reported only when a difference in two measured values was greater than twice the sum of the individual standard deviations.

micelles in model biles. The vertical axis plots molar ratios of lecithin species for unfractionated model biles, as well as the vesicle and mixed micellar fractions. For model biles containing 18:0–18:1 plus 18:1–18:1 and 18:0–18:2 plus 18:2–18:2 lecithins, both the molar ratios of 18:0–18:1 to 18:1–18:1 and 18:0–18:2 to 18:2–18:2 lecithin in vesicles increased by a factor of 1.8 compared with the equimolar mixtures in the model biles. The lower vesicle concentrations of 18:0–18:2 and 18:2–18:2 lecithin compared with 18:0–18:1 and 18:1–18:1 lecithin (Table 1) yielded an identical molar ratio because there was a tendency for the more unsaturated *sn*-2 18:2 lecithins to partition into mixed micelles compared with the *sn*-2 18:1 lecithins. A further increase in the degree of fatty acyl unsaturation in the binary mixture 18:0–18:3 plus 18:3–18:3 lecithin resulted in enrichment of vesicles with lecithin containing the saturated *sn*-1 fatty acyl chain (18:0–18:3 lecithin) as reflected by a 2.4-fold increase in the 18:0–18:3 to 18:3–18:3 lecithin ratio. Table 1 demonstrates that both 18:0–18:3 and 18:3–18:3 lecithin species were dispersed in vesicles to a lesser extent than was observed for the more saturated mixtures.

Fig. 2 demonstrates the influence of variations in *sn*-1 fatty acyl chain length and *sn*-2 fatty acyl chain unsaturation on the distribution of lecithin molecular species between vesicles and mixed micelles in otherwise identical model biles. Compared with the original equimolar binary mixture, the molar ratio of 16:0–18:1 to 16:0–20:4 lecithin in vesicles increased to 1.8. Increasing the length of the *sn*-1 fatty acyl chain of one of these lecithins to 18 carbons (i.e., 18:0–18:1 plus 16:0–20:4 lecithins) resulted in

TABLE 1. Distributions of synthetic lecithin molecular species between vesicles and mixed micelles in model biles[a]

Lecithin Mixtures	Concentration in Vesicles[b,c]	Concentration in Micelles[b,c]
binary	*mM*	*mM*
18:0–18:1[d]	0.58	6.27
18:1–18:1	0.33	6.86
18:0–18:2	0.49	6.48
18:1–18:2	0.27	7.57
18:0–18:3	0.41	6.19
18:3–18:3	0.17	6.70
16:0–18:1	0.67	6.22
16:0–20:4	0.37	6.59
18:0–18:1	0.67	6.31
16:0–20:4	0.34	6.75
18:0–18:1	0.91	6.11
16:0–18:1	0.82	6.22

[a]Unfractionated model bile composition and conditions identical throughout: taurocholate–lecithin–cholesterol molar ratio 63:27:10, 3 g/dl, pH 6–7, 20°C, 0.15 M NaCl, 48 h equilibration.

[b]Data are calculated based on the distribution of lecithin molecular species in vesicles and mixed micelles (determined by HPLC analysis) together with the distribution of total concentration between vesicles and mixed micelles (determined by gel filtration chromatography).

[c]As determined from replicate analyses of representative samples (see text, footnote 2); data are precise to ± 3% (SD).

[d]*sn*-1–*sn*-2 Fatty acid composition of biliary lecithin mixtures: number antecedent to colon represents number of carbons in the fatty acid chain; number following colon represents number of double bonds in the chain.

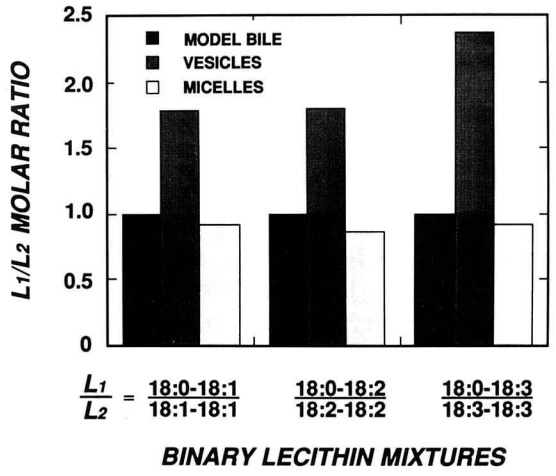

Fig. 1. Influence of *sn*-1 fatty acyl chain unsaturation on distribution of lecithin molecular species between vesicles and mixed micelles in cholesterol-supersaturated model biles (taurocholate–lecithin–cholesterol molar ratio 63:27:10; 3 g/dl total lipid concentration, 0.15 mM NaCl, pH ~6–7). Equimolar lecithin (L) concentrations (L_1/L_2 molar ratio = 1.0) were present in unfractionated model biles. L_1/L_2 molar ratios of vesicles and mixed micelles were obtained by HPLC analysis of vesicle and mixed micellar peaks obtained by Superose 6 fractionation of the model biles (19). Values plotted are means of two determinations. As inferred from replicate analyses of representative samples,[2] standard deviations were ≤3%.

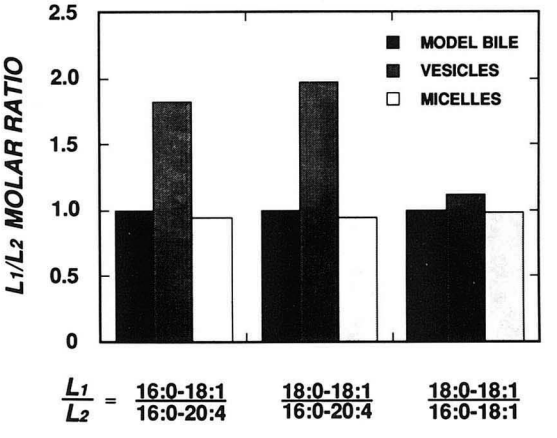

Fig. 2. Influence of *sn*-1 fatty acyl chain length and *sn*-2 fatty acyl chain unsaturation on distribution of lecithin species between vesicles and mixed micelles in cholesterol supersaturated model bile (taurocholate–lecithin–cholesterol molar ratio 63:27:10; 3 g/dl total lipid concentration, 0.15 mM NaCl, pH ~6–7). Equimolar lecithin (L) concentrations (L_1/L_2 molar ratio = 1.0) were present in unfractionated model biles. L_1/L_2 molar ratios of vesicles and mixed micelles were obtained by HPLC analysis of vesicle and mixed micellar peaks obtained from Superose 6 fractionation of the model biles (19). Values plotted are means of two determinations. As inferred from replicate analyses of representative samples,[2] standard deviations were ≤3%.

further enrichment of vesicles with that lecithin as evidenced by a twofold increase in the vesicle 18:0–18:1 to 16:0–20:4 lecithin ratio (Fig. 2, Table 1). When the model bile contained 18:0–18:1 plus 16:0–18:1 lecithins (Fig. 2, Table 1), vesicles contained a more even distribution of the two lecithin species. However, the lecithin with the 18:0 *sn*-1 fatty acyl chain was slightly but, on a statistical basis, significantly enriched in vesicles (vesicle 18:0–18:1 to 16:0–18:1 lecithin molar ratio = 1.1) compared with the original model bile. As shown in Table 1, the vesicle concentrations of both 16:0–18:1 and 18:0–18:1 lecithins were increased compared with either binary mixture containing 16:0–20:4 lecithin.

Natural lecithin mixtures. We examined the vesicle and mixed micellar distribution of individual lecithin molecular species in model biles containing complex mixtures of lecithin species, i.e., either egg yolk or soy bean lecithins. **Fig. 3** demonstrates the HPLC elution profiles of benzoyl derivatives of diglycerides derived from egg yolk lecithin (Fig. 3A) and soy bean lecithin (Fig. 3B). In each case, lecithin molecular species accounting individually for 1% or more of total lecithin are identified with carbon length

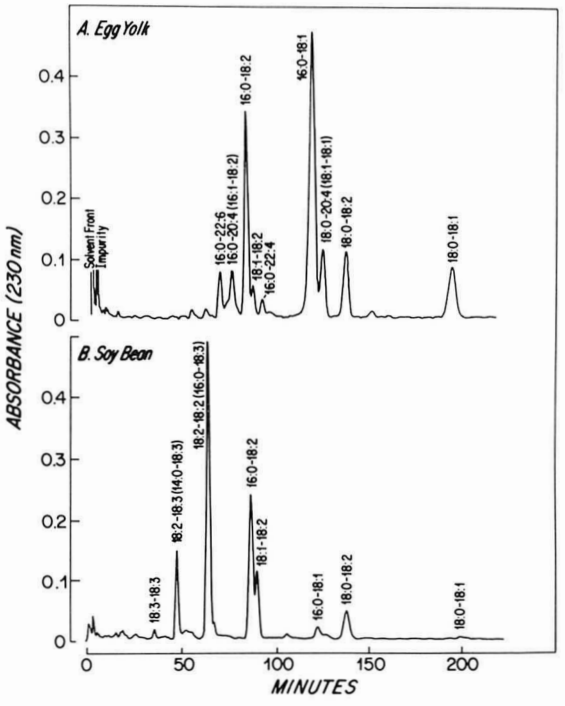

Fig. 3. HPLC analysis of benzoate derivatives of (A) egg yolk lecithin and (B) soy bean lecithin. Absorbance values at 230 nm result in peak areas of benzoate derivatives that directly represent the mole fractions of lecithin species present in the original mixture. Lecithin species are identified by carbon chain length and number of unsaturation of fatty acyl chains in the *sn*-1 and *sn*-2 positions for peak areas representing 1% or greater.[3] For peaks containing two lecithin species, the quantitatively lesser species is given in parentheses. Peak identities were confirmed by capillary gas chromatography analysis of methyl esters prepared from the lecithin's fatty acids following saponification.

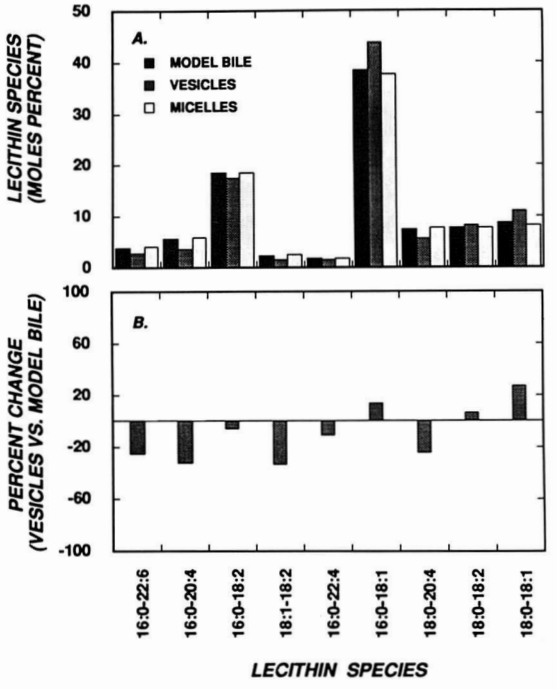

Fig. 4. Distribution of egg yolk lecithin species between vesicle and mixed micelles in cholesterol supersaturated model biles (model bile of identical composition to that in Figs. 1 and 2). (A) Moles percent lecithin is plotted for unfractionated model biles, separated vesicles, and mixed micelles for each lecithin species contained in the egg yolk lecithin. From left to right on the horizontal axis, lecithin species are represented according to increasing HPLC retention times (as in Fig. 3). (B) Percent change in each egg yolk lecithin species in vesicles compared with unfractionated model bile. A positive deflection represents vesicle enrichment with the indicated lecithin species whereas a negative deflection represents vesicles depleted in that lecithin species. Standard deviations of HPLC peak areas were determined to be 2% for benzoate derivatives of each lecithin species in vesicle fractions (n=4), micellar fractions (n=4), and unfractionated biles (n=2).

and number of unsaturations in *sn*-1 and *sn*-2 positions.[3] Fig. 3A shows that egg yolk lecithin was composed principally (94%) of nine molecular species, eight of which had saturated *sn*-1 fatty acyl chains. As determined by capillary gas chromatography, two lecithin peaks contained appreciable amounts of a second lecithin species identified in parentheses: 16:1–18:2 lecithin comprised 34% of the 16:0–20:4 lecithin peak and 18:1–18:1 lecithin comprised 34% of the 18:0–20:4 lecithin peak. Fig. 3B shows that soy bean lecithin was composed principally (93%) of eight molecular species. Two peaks contained appreciable amounts of a second lecithin species: 14:0–18:3

[3]Lecithins with peak area cutoffs of greater than 1% were chosen for analysis because, in general, peak areas of less than 1% were not reproducible with the same high degree of accuracy (<2% SD) due to contamination from other small peaks. An exception was 18:0–18:1 lecithin, which was included in our analysis. Although accounting for only 0.5% of soy bean lecithin (Fig. 3B), this molecular species eluted in a position that was far removed from all other peaks and therefore reproducible to the same high degree of accuracy as peaks of 1% or greater.

lecithin constituted 10% of the 18:2–18:3 lecithin peak and 16:0–18:3 lecithin accounted for 6% of the 18:2–18:2 lecithin peak. In contrast to egg yolk lecithin, which had a predominance of saturated *sn*-1 fatty acyl chains, soy bean lecithin contained lecithin molecular species with both saturated and unsaturated *sn*-1 fatty acyl chains.

Fig. 4 shows the distribution of egg yolk lecithin molecular species for unfractionated model biles as well as for separated vesicles and mixed micelles. The cholesterol supersaturated model bile had total and relative lipid compositions identical to those used in the synthetic lecithin study. Fig. 4A plots mol% of each lecithin molecular species in the model bile as well as that in separated vesicles and mixed micelles. Molecular species of lecithin are arranged from left to right on the horizontal axis in order of increasing retention times on the reverse phase HPLC column (Fig. 3). Fig. 4B displays the moles percent change of each lecithin molecular species in vesicles compared with unfractionated model bile: a positive deflection represents a relative enrichment of vesicles with a lecithin molecular species, whereas a negative deflection represents a relative depletion of a lecithin molecular species. Each positive or negative bar corresponds to a relative depletion or enrichment, respectively, of a lecithin in mixed micelles, but data are not shown for purposes of clarity. Vesicles were enriched with lecithins in the rank order 18:0–18:1 > 16:0–18:1 > 18:0–18:2 and depleted in lecithins in the rank order 16:0–18:2 < 16:0–22:4 < 18:0–20:4 (18:1–18:1) < 16:0–22:6 < 16:0–20:4 (16:1–18:2). For a given *sn*-1 fatty acyl chain composition (16:0 or 18:0), vesicles became progressively enriched in lecithin species when the degree of saturation of the *sn*-2 fatty acyl chain increased: With *sn*-1 16:0 fatty acyl compositions, vesicles contained lecithins in the rank order 16:0–18:1 > 16:0–18:2 > 16:0–22:4 > 16:0–22:6 and similarly, lecithins with *sn*-1 18:0 fatty acyl composition became enriched in vesicles in the rank order 18:0–18:1 > 18:0–18:2.[4]

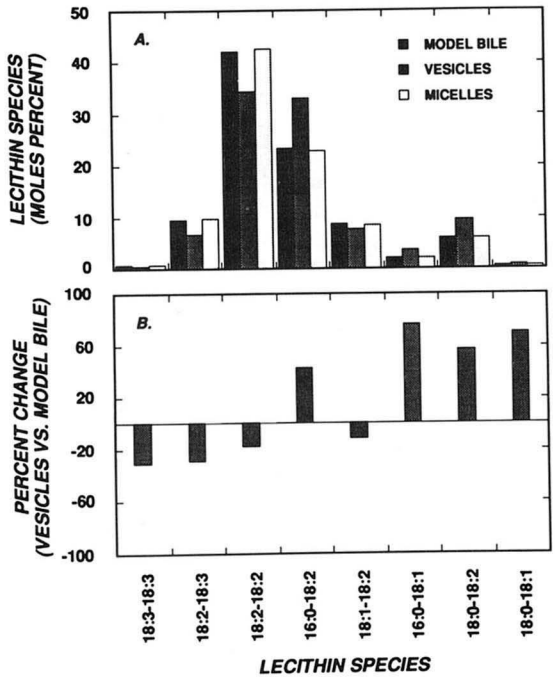

Fig. 5. Distribution of soy bean lecithin species between vesicles and mixed micelles in cholesterol-supersaturated model bile (molar taurocholate–soy bean lecithin–cholesterol ratio 63:27:10; 3 g/dl total lipid concentration). (A) Moles percent lecithin is plotted for unfractionated model biles, separated vesicles, and mixed micelles for each lecithin species contained in the soy bean lecithin. From left to right on the horizontal axis, lecithin species are represented according to increasing HPLC retention times (as in Fig. 3). (B) Percent change in each soy bean lecithin species in vesicles compared with unfractionated model biles. A positive deflection represents vesicle enrichment with the indicated lecithin species whereas a negative deflection represents vesicles depleted in that lecithin species. Standard deviations of HPLC peak areas were determined to be 2% for benzoate derivatives of each lecithin species in vesicle fractions (n = 2), micellar fractions (n = 2), and unfractionated biles (n = 3).

Fig. 5 shows the vesicle and mixed micellar distribution of soy bean lecithin molecular species in cholesterol-supersaturated model biles containing the same total and relative lipid compositions as employed in the experiments depicted in Fig. 4. Compared with model biles, vesicles were enriched with lecithins that contained an *sn*-1 saturated fatty acyl chain in the rank order: 16:0–18:1 > 18:0–18:1 > 18:0–18:2 > 16:0–18:2 lecithin, and depleted in lecithins that contained an *sn*-1 unsaturated chain in the rank order: 18:1–18:2 < 18:2–18:2 (16:1–18:2) < 18:2–18:3 (14:0–18:3) < 18:3–18:3 lecithin. As was observed for egg yolk lecithin, when the *sn*-1 fatty acyl chain remained the same, vesicles became more enriched in lecithin species with decreasing degrees of *sn*-2 unsaturation. In the case of lecithins containing an *sn*-1 16:0 fatty acyl chain, vesicles were enriched in 16:0–18:1 more than 16:0–18:2 lecithin, and in the case of lecithins containing an *sn*-1 18:0 or 18:2 fatty acyl chain, vesicles were enriched in the rank order 18:0–18:1 > 18:0–18:2 > 18:2–18:3 lecithin.[4] Similarly, for a constant *sn*-2 fatty acyl composition, vesicles were enriched with lecithins in the rank order 18:0–18:2 > 18:1–18:2 > 18:2–18:2, demonstrating that the same direct correlation between partitioning into vesicles and degree of saturation of either *sn*-1 or *sn*-2 fatty acyl chains applied.

[4]The HPLC peaks that represented 16:0–20:4 and 18:0–20:4 lecithins in the egg yolk mixture (Fig. 3A) each contained smaller quantities of a second lecithin species (34% in each case) which could influence the rank ordering of the major lecithin species (Fig. 4). Therefore, these lecithins were excluded from our analysis. In contrast, the 18:2–18:2 and 18:2–18:3 lecithins of the soy bean mixture (Fig. 3B) contained much smaller quantities of a second lecithin species (6% and 10%, respectively). Because the contributions of these lecithins to the peak areas were minor and should not influence rank ordering of the major lecithin species (Fig. 5), the latter were included in our analysis.

Influence of lecithin species on cholesterol solubilities of vesicles and mixed micelles

Table 2 summarizes the influence of lecithin molecular species on apparent micellar cholesterol solubilities and vesicle cholesterol/lecithin molar ratios determined for a variety of individual synthetic lecithin molecular species as well as binary mixture of synthetic lecithins and natural lecithin mixtures. Values for apparent micellar cholesterol solubilities and vesicle cholesterol/lecithin molar ratios were determined after Superose 6 fractionation of cholesterol supersaturated model biles, all for the same relative and total lipid concentrations as used throughout this study. Apparent solubility values were independently verified to within ± 0.5 mol% by QLS analysis of model biles prepared with each lecithin or lecithin mixture (lecithin–taurocholate molar ratios of 0.43) but with systematic increases in cholesterol content (see Methods section) (18). As the overall degree of unsaturation of a synthetic lecithin or lecithin mixture in model bile increased, apparent micellar cholesterol solubilities as well as vesicle cholesterol/lecithin molar ratios also increased. **Fig. 6** plots the significant positive correlation (r = 0.69, P = 0.02) between vesicle cholesterol/lecithin molar ratios and apparent micellar cholesterol solubilities.

TABLE 2. Influence of lecithin molecular species on apparent micellar cholesterol solubilities and vesicle cholesterol/lecithin molar ratios in model biles[a]

Lecithin Species	Micellar Cholesterol Solubility[b]	Vesicle Cholesterol/Lecithin Molar Ratio[c]
	moles %	
16:0-18:1 + 18:0-18:1[d]	5.0	1.2
16:0-18:1	5.0	1.5
18:0-18:1	5.6	1.3
Egg yolk (mixture)	5.9	1.6
18:0-18:2	6.0	n.d.[e]
18:0-18:1 + 16:0-20:4	6.1	1.7
16:0-18:1 + 16:0-20:4	6.2	1.8
18:0-18:3	7.0	n.d.[e]
18:0-18:1 + 18:1-18:1	7.1	1.4
18:0-18:2 + 18:2-18:2	7.1	1.5
18:0-18:3 + 18:3-18:3	7.5	1.8
Soy bean (mixture)	7.5	2.4
18:1-18:1	8.0	n.d.[e]
18:2-18:2	8.0	n.d.[e]
16:0-20:4	8.0	2.1

[a]Unfractionated model bile composition and conditions identical throughout: taurocholate–lecithin–cholesterol molar ratio 63:27:10, 3 g/dl, pH 6–7, 20°C, 0.15 M NaCl, 48 h equilibration.

[b]As determined by replicate (n = 5) analyses of representative samples (egg yolk, 16:0–20:4, and 18:0–18:1 lecithins), data are precise to ± 2% (SD). Other values represent single or means of duplicate determinations.

[c]As determined by replicate analyses (see footnote [b]); data are precise to ± 3% (SD).

[d]See footnote [d] of Table 1 for explanation of nomenclature.

[e]Not determined.

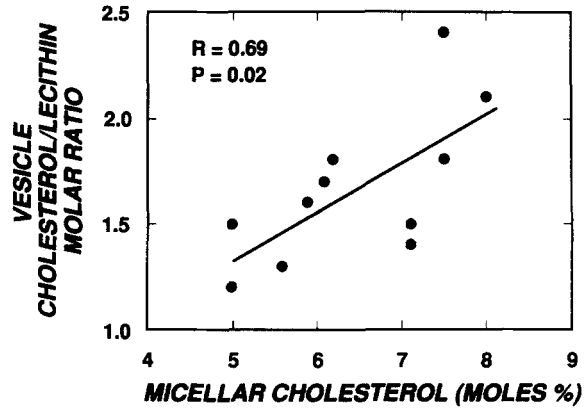

Fig. 6. Positive correlation between apparent micellar cholesterol solubilities and vesicle cholesterol/lecithin molar ratios (from Table 2). The solid line represents a linear least squares fit to the data. Increases in apparent micellar cholesterol solubility correlate positively with increasing cholesterol/lecithin ratio of vesicles and with increasing degree of lecithin fatty acyl chain unsaturation.

DISCUSSION

Based upon their relative abundance in bile and detergent-like properties, it has long been presumed that bile salt largely dictated biliary cholesterol solubility. On this basis, the physical-chemical properties of these organic anions have been the subject of more than 30 years of intense investigation (34). Nevertheless, it is appreciated that biliary phospholipids play a crucial role in cholesterol solubilization, making possible the formation of mixed bile salt–phospholipid–cholesterol micelles as well as cholesterol–phospholipid vesicles, the aggregates cooperatively involved in biliary cholesterol transport (35, 36). However, due to unsophisticated technology for accurate separation and quantitation of biliary phospholipids in the past, and to earlier observations that certain lecithins failed to influence overall cholesterol solubilities in model biles (9–11), the influence of both phospholipid classes and lecithin molecular species on the physical chemistry of bile has remained unstudied and frequently considered unimportant in the literature (9–11). The development of sophisticated HPLC techniques to separate individual molecular species from mixtures within a single lipid class has revolutionized our accuracy for phospholipid separation and quantitation (7, 8, 37). Accurate knowledge of the phospholipid composition of native human biles is rapidly emerging (1, 3, 5, 6), and a systematic definition of the physical-chemical properties of biliary phospholipids alone and in combination with bile salt and cholesterol is now necessary to define the roles of phospholipids in bile formation and in the solubility and precipitation of biliary cholesterol.

Using model systems, we have begun herein to explore the influence of molecular species of lecithin, the principal

biliary phospholipid, on the physical chemistry of bile. We focused on two closely related aspects of biliary lipid aggregation. We first examined the distribution of lecithin molecular species, as defined by fatty acyl chain composition, between vesicles and mixed micelles in cholesterol supersaturated model biles and then quantified the influence of lecithin molecular species on the physical chemistry of cholesterol solubilization in bile as reflected in apparent micellar cholesterol solubilities and cholesterol/lecithin molar ratios in biliary vesicles.

To characterize the distribution of lecithin molecular species between vesicles and mixed micelles, we studied dilute model biles with identical total and relative lipid compositions that were designed to have a "physiological" cholesterol saturation index of 1.7 (19). At equilibrium, biles of this composition contain coexisting cholesterol-saturated mixed micelles, lecithin-cholesterol lamellar liquid crystals, and solid cholesterol monohydrate crystals (17). However, as observed in native biles (38, 39), dilute model biles prepared by coprecipitation reach a metastable equilibrium state in which cholesterol-rich vesicles and mixed micelles coexist (18). With variations in lecithin molecular species only, we fractionated model biles using a high resolution gel filtration technique recently developed in our laboratory (19). We further simplified our investigation by focusing on model biles containing binary mixtures of synthetic lecithin species (Figs. 1 and 2) prior to characterizing the distributions of lecithin species found in more complex natural mixtures such as egg yolk and soy bean lecithins (Figs. 4 and 5). Because native biles contain complex mixtures of both bile salt and lecithin molecular species as well as heterogeneous proteins, bile pigments, and ions (2), highly accurate separations of biliary vesicles and mixed micelles have yet to be achieved (27). We have therefore limited our present study to model systems wherein lecithin molecular species could be varied systematically.

From observations on binary mixtures of synthetic lecithins, it is apparent that increasing fatty acyl chain saturation in either the *sn*-1 (Fig. 1) or *sn*-2 (Fig. 2) positions of lecithin favor vesicle enrichment with that lecithin. Although differences in micellar lecithin concentrations did not reach statistical significance, mixed micelles simultaneously became slightly enriched in more unsaturated lecithin molecular species as necessitated by conservation of mass. This influence of fatty acyl chain unsaturation observed in binary mixtures of synthetic lecithins was also manifest with more complex naturally occurring lecithin mixtures. As demonstrated by vesicle and mixed micellar distributions of individual lecithin molecular species from egg yolk (Fig. 4) and soy bean lecithins (Fig. 5), increases in *sn*-1 or *sn*-2 fatty acyl chain saturation favored enrichment of an individual lecithin species in vesicles whereas the converse held true for mixed micelles. Of note was that this partitioning effect was most pronounced when the transition was made from a single double bond in the *sn*-1 fatty acyl chain to a saturated fatty acyl chain rather than by a reduction in the number of polyunsaturations in the *sn*-1 fatty acyl chain.

Using force-area curves, Demel and coworkers (40) studied the influence of lecithin unsaturation and cholesterol content on molecular packing in lecithin monolayers at an air–water interface. These authors demonstrated that varying degrees of unsaturation of either *sn*-1 or *sn*-2 fatty acyl chains in lecithin dramatically increased molecular areas; further, cholesterol "condensed" the lecithins that contained a saturated *sn*-1 acyl chain to an extent that varied inversely with the degree of unsaturation of the *sn*-2 acyl chain. However, cholesterol failed to condense lecithins with varying degrees of unsaturation in the *sn*-1 acyl chain. This suggests that the presence of a saturated *sn*-1 fatty acyl chain plays an important role in facilitating lecithin packing with cholesterol within lecithin-cholesterol monolayers and bilayers. Similarly, Patton et al. (41) observed that activation energies for lecithin exchange between surface monolayers of chylomicra and high density lipoproteins decreased with increasing degrees of *sn*-1 fatty acyl unsaturation. Taken together, these observations support the hypothesis that lecithin containing more saturated fatty acyl chains (particularly those with saturated *sn*-1 fatty acyl chains) pack more tightly and therefore partition better into cholesterol-rich vesicles. In contrast, lecithins that contain more highly unsaturated fatty acyl chains partition more favorably into mixed micelles, presumably because mixed micellar aggregates are characterized by a more loosely associated packing of lecithin molecules than in highly ordered bilayer structures such as vesicles. This hypothesis is supported by the liposome fluidity measurements of van Blitterswijk, van der Meer, and Hilkmann (42) who demonstrated that more unsaturated lecithins, which alone form highly fluid bilayers, appear to create fluid membrane domains within mixed cholesterol-lecithin bilayers because of their failure to associate strongly with cholesterol.

Vesicles and mixed micelles in bile may serve not only as morphologically separate lipid domains but as physicochemically distinct lipid milieus that might potentially modify the partitioning of other biliary molecules. Furthermore, vesicles with their high cholesterol contents will impart even stricter packing constraints within the mixed bilayers favoring further enrichment with more saturated lecithins. In contrast, we infer that mixed micelles become enriched with more unsaturated lecithins not only because of their low cholesterol contents but because the lipid organization in mixed micelles may be very different from that in a highly curved bilayer. Recent evidence from model systems (43, 44) suggests that bile salt–lecithin mixed micelles may possess a more disordered rod-like rather than organized disk-like morphology as was previously suggested (16). Nevertheless, it is clear that all

of these lecithins, irrespective of the *sn*-1 and *sn*-2 chain length and degree of saturation are capable of being solubilized completely by bile salts as a one phase micellar system. Such would also be the case in ternary lipid systems when cholesterol content is less than micellar saturation. This suggests that the internal structure as well as properties of bile salt–lecithin–cholesterol mixed micelles may vary considerably with the degree of cholesterol saturation of bile.

Metastable micellar cholesterol solubilities as well as vesicle cholesterol to lecithin ratios are clearly dependent on lecithin molecular species (Table 2). In studies that relied on turbidimetry (a less sensitive light-scattering technique compared with QLS) to assess the micellar phase limits of model bile, Saunders and Wells (11) found no differences in micellar cholesterol solubilities in the presence of different lecithin species that contained both saturated and unsaturated fatty acyl chains (16:0–18:1, 16:0–18:2, 18:0–18:1 and 18:0–18:2 lecithins). On this basis, these authors concluded that different lecithin species had no appreciable influence on biliary lipid aggregation and cholesterol solubility. Human bile, however, contains appreciable and often variable concentrations of highly unsaturated lecithin species particularly 16:0–20:4 lecithin (2). As demonstrated herein, different lecithin molecular species have very large effects on metastable cholesterol dispersion in bile. For example, there is a 60% increase in apparent micellar cholesterol solubility in model biles containing 16:0–20:4 lecithin compared with the more common 16:0–18:1 lecithin (Table 2).

As suggested above, the model bile composition used in this work was most likely not at equilibrium. Mazer and Carey (18) have indicated that in such dilute biles (3 g/dl), interfacial energy is minimized when excess cholesterol precipitates with lecithin, first as metastable cholesterol-rich vesicles, prior to the formation of cholesterol monohydrate crystals. Based on the preferential association between cholesterol and more saturated lecithins in monolayers (40), it is likely that larger quantities of more saturated lecithins precipitate with cholesterol to form vesicles in model biles. This would result in vesicles with lower cholesterol/lecithin molar ratios as well as apparently lower micellar cholesterol solubilities. In model biles containing more unsaturated lecithin molecular species, lecithin is preferentially solubilized in mixed micelles and becomes less available for vesicle formation. Consequently, fewer cholesterol-rich vesicles will form and these will have higher cholesterol/lecithin molar ratios. Under these circumstances, it appears that both vesicles and mixed micelles become more supersaturated with cholesterol (27).

Based upon our results, the pattern of fatty acids in the *sn*-1 and *sn*-2 positions of lecithin may have important functions in biliary lipid transport as well as cholesterol monohydrate precipitation in bile. Recent studies on gallbladder biles of prairie dogs (6) and African green monkeys (45) that were fed cholesterol-rich diets supplemented with fish (menhaden) oil demonstrated decreased cholesterol crystal nucleation times (6) and decreased incidence of gallstones (45). As shown by careful analysis of prairie dog biles, fish oil feeding resulted in biliary enrichment with *sn*-2 20:5, 22:5, and 22:6 lecithins and depletion in *sn*-2 18:2 and 20:4 lecithins (6). Of note is that after fish oil feeding, biles not only became highly enriched in unsaturated lecithin species, but the relative concentrations of total biliary lecithin significantly increased in both animal models. These observations, taken together with the present results, suggest that a detailed understanding of the influence of lecithin molecular species on the physical chemistry of lipid aggregation and cholesterol nucleation in native biles will likely improve our understanding of gallstone pathogenesis and yield important insights regarding prevention of gallstone disease.

Supported in part by Grant Nos. DK36588 and DK34854 from the National Institutes of Health (U.S. Public Health Service).

Manuscript received 6 February 1991, in revised form 7 May 1991, and in re-revised form 24 May 1991.

REFERENCES

1. Alvaro, D., A. Cantafora, A. F. Attili, S. G. Corradini, C. De Luca, G. Minervini, A. Di Biase, and M. Angelico. 1986. Relationships between bile salts hydrophilicity and phospholipid composition in bile of various animal species. *Comp. Biochem. Physiol.* **83B:** 551–554.
2. Hay, D. W., and M. C. Carey. 1990. Chemical species of lipids in bile. *Hepatology.* **12:** 6S–16S.
3. Booker, M. L., T. E. Scott, and W. W. La Morte. 1990. Effect of dietary cholesterol on phosphatidylcholines and phosphatidylethanolamines in bile and gallbladder mucosa in the prairie dog. *Gastroenterology.* **97:** 1261–1267.
4. Cantafora, A., A. Di Biase, D. Alvaro, M. Angelico, M. Marin, and A. F. Attili. 1983. High performance liquid chromatographic analysis of molecular species of phosphatidylcholine. Development of a quantitative assay and its application to human bile. *Clin. Chim. Acta.* **134:** 281–295.
5. Hay, D. W., M. J. Cahalane, N. Timofeyeva, and M. C. Carey. 1989. Molecular species of lecithins in human gallbladder bile: hydrophilicity of the bile salt pool determines hydrophilicity of the lecithins. *Hepatology.* **10:** 599.
6. Booker, M. L., T. E. Scott, and W. W. La Morte. 1990. Effects of dietary fish oil on biliary phospholipids and prostaglandin synthesis in the cholesterol-fed prairie dog. *Lipids.* **25:** 27–32.
7. Patton, G. M., J. M. Fasulo, and S. J. Robins. 1982. Separation of phospholipids and individual molecular species of phospholipids by high-performance liquid chromatography. *J. Lipid Res.* **23:** 190–196.
8. Patton, G. M., and S. J. Robins. 1987. HPLC of molecular species of glycerophospholipids in studies of lipoproteins and lipid transport. *In* Chromatography Library: Lipids in Biomedical Research and Clinical Diagnosis. A. Kuksis, editor. Elsevier, Amsterdam. 311–347.
9. Montet, J. C., and D. G. Dervichian. 1971. Solubilisation micellaire du cholestérol par les sels biliares et les lecithins

extraits de la bile humaine. *Biochimie.* **53:** 751–754.
10. Neiderhiser, D. H., and H. P. Roth. 1972. The effect of modifications of lecithin and cholesterol on the micellar solubilization of cholesterol. *Biochim. Biophys. Acta.* **270:** 407–413.
11. Saunders, D. R., and M. A. Wells. 1969. The cholesterol solubilizing capacity of lecithins in aqueous solutions of bile salt. *Biochim. Biophys. Acta.* **76:** 828–835.
12. Cohen, D. E., M. Angelico, and M. C. Carey. 1989. Quasielastic light scattering evidence for vesicular secretion of biliary lipids. *Am. J. Physiol.* **257:** G1–G8.
13. Cohen, D. E., M. Angelico, and M. C. Carey. 1990. Structural alterations in lecithin-cholesterol vesicles following interactions with monomeric and micellar bile salts: physical-chemical basis for subselection of biliary lecithin species and aggregative states of biliary lipids during bile formation. *J. Lipid Res.* **31:** 55–70.
14. Cohen, D. E., and M. C. Carey. 1990. Physical chemistry of lipids during bile formation. *Hepatology.* **12:** 143S–148S.
15. Donovan, J. M., N. Timofeyeva, and M. C. Carey. 1991. The hydrophilic-hydrophobic balance of bile salts (BS) determines the intermicellar-intervesicular concentration (IMC) of model and native biles. *Gastroenterology.* **100:** A737.
16. Mazer, N. A., G. B. Benedek, and M. C. Carey. 1980. Quasielastic light-scattering studies of aqueous biliary lipid systems. Mixed micelle formation in bile salt-lecithin solutions. *Biochemistry.* **19:** 601–615.
17. Carey, M. C., and D. M. Small. 1978. Physical chemistry of cholesterol solubility in bile. Relationship to gallstone formation and dissolution in man. *J. Clin. Invest.* **61:** 998–1026.
18. Mazer, N. A., and M. C. Carey. 1983. Quasielastic light scattering studies of aqueous biliary lipid systems. Cholesterol solubilization and precipitation in model bile systems. *Biochemistry.* **22:** 426–442.
19. Cohen, D. E., and M. C. Carey. 1990. Rapid (1 hour) high performance gel filtration chromatography resolves coexisting simple micelles, mixed micelles, and vesicles in bile. *J. Lipid Res.* **31:** 2103–2112.
20. Salvioli, G., M. Romani, P. Loria, N. Carulli, and J. M. Pradelli. 1985. Effect of acute administration of bile acids on fatty acid composition of biliary phosphatidylcholines in man. *J. Hepatol.* **1:** 291–300.
21. Ahlberg, J., T. Curstedt, K. Einarsson, and J. Sjövall. 1981. Molecular species of biliary phosphatidylcholines in gallstone patients: the influence of treatment with cholic acid and chenodeoxycholic acid. *J. Lipid Res.* **22:** 404–409.
22. Cantafora, A., M. Angelico, A. Di Biase, U. Pièche, F. Bracci, A. F. Attili, and L. Capocaccia. 1981. Structure of biliary phosphatidylcholine in cholesterol gallstone patients. *Lipids.* **16:** 589–592.
23. Kajiyama, G., S. Kubota, H. Sasaki, T. Kawamoto, and A. Miyoshi. 1980. Lipid metabolism in the development of cholesterol gallstones in hamsters. I. Study on the relationship between serum and biliary lipids. *Hiroshima J. Med. Sci.* **29:** 133–141.
24. Coolbear, K. P., C. B. Berde, and K. M. W. Keough. 1983. Gel to liquid-crystalline phase transitions of aqueous dispersions of polyunsaturated mixed-acid phosphatidylcholines. *Biochemistry.* **22:** 1466–1473.
25. Cohen, D. E., M. R. Fisch, and M. C. Carey. 1990. Principles of laser light scattering spectroscopy: applications to the physicochemical study of model and native biles. *Hepatology.* **12:** 113S–122S.
26. Koppel, D. E. 1972. Analysis of macromolecular polydispersity in intensity correlation spectroscopy: the method of cumulants. *J. Chem. Phys.* **57:** 4814–4820.
27. Donovan, J. M., and M. C. Carey. 1990. Quantitation and separation of cholesterol carriers in bile. *Hepatology.* **12:** 94S–105S.
28. Kern, F., Jr., H. Eriksson, T. Curstedt, and J. Sjövall. 1977. Effect of ethynylestradiol on biliary excretion of bile acids, phosphatidylcholines, and cholesterol in the bile fistula rat. *J. Lipid Res.* **18:** 623–634.
29. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
30. Blank, M. L., M. Robinson, V. Fitzgerald, and F. Snyder. 1984. Novel quantitative method for determination of molecular species of phospholipids and diglycerides. *J. Chromatogr.* **298:** 473–482.
31. Patton, G. M., S. Cann, H. Brunengraber, and J. M. Lowenstein. 1981. Separation of methyl esters of fatty acids by gas chromatography on capillary columns, including deuterated from nondeuterated fatty acids. *Methods Enzymol.* **72:** 8–20.
32. Nishihara, M., and M. Kito. 1978. Changes in the phospholipid molecular species composition of soybean hypocotyl and cotyledon after dedifferentiation. *Biochim. Biophys. Acta.* **531:** 25–31.
33. Terao, J., Y. Hirota, M. Kawakatsu, and S. Matsushita. 1981. Structural analysis of hydroperoxides formed by oxidation of phosphatidylcholine with singlet oxygen. *Lipids.* **16:** 427–432.
34. Carey, M. C. 1985. Physical-chemical properties of bile acids and their salts. *In* New Comprehensive Biochemistry. H. Danielsson and J. Sjövall, editors. Elsevier, Amsterdam. 345–403.
35. Cabral, D. J., and D. M. Small. 1989. The physical chemistry of bile. *In* Handbook of Physiology - The Gastrointestinal System. III. S. G. Schultz, J. G. Forte, and B. B. Rauner, editors. American Physiological Society, Bethesda. 621–662.
36. Carey, M. C. 1988. Lipid solubilization in bile. *In* Bile Acids in Health and Disease. T. C. Northfield, R. P. Jazrawi, and P. L. Zenter-Munro, editors. Kluwer Academic Press, Dordrecht, The Netherlands. 61–82.
37. Bernett, J. T., Jr., N. K. Meredith, J. R. Akins, and W. H. Hannan. 1985. Determination of serum phospholipid metabolic profiles by high-performance liquid chromatography. *J. Liq. Chromatogr.* **8:** 1573–1591.
38. Pattinson, N. R. 1985. Solubilisation of cholesterol in human bile. *FEBS Lett.* **181:** 339–342.
39. Sömjen, G. J., and T. Gilat. 1985. Contribution of vesicular and micellar carriers to cholesterol transport in human bile. *J. Lipid Res.* **26:** 699–704.
40. Demel, R. A., W. S. M. Geurts van Kessel, and L. L. M. van Deenen. 1972. The properties of polyunsaturated lecithins in monolayers and liposomes and the interactions of these lecithins with cholesterol. *Biochim. Biophys. Acta.* **266:** 26–40.
41. Patton, G. M., S. J. Robins, J. M. Fasulo, and S. B. Clark. 1985. Influence of lecithin acyl chain composition on the kinetics of exchange between chylomicrons and high density lipoproteins. *J. Lipid Res.* **26:** 1285–1293.
42. van Blitterswijk, W. J., B. W. van der Meer, and H. Hilkmann. 1987. Quantitative contributions of cholesterol and the individual classes of phospholipids and their degree of fatty acyl (un)saturation to membrane fluidity by fluorescence polarization. *Biochemistry.* **26:** 1746–1756.
43. Chamberlin, R., G. Thurston, G. Benedek, D. Cohen, and

M. Carey. 1989. Light scattering evidence for "worm-like" mixed detergent-diacylphosphatidylcholine (PC) micelles. *Materials Research Society Fall Meeting, Boston, MA, Abstract booklet.* 697.

44. Cohen, D. E., R. A. Chamberlin, G. M. Thurston, G. B. Benedek, and M. C. Carey. 1991. Cylindrical "worm-like" micelles in bile salt-lecithin solutions: implications for the earliest events in bile formation. *In* Bile Acids As Therapeutic Agents. G. Paumgartner, A. Stiehl, and W. Gerok, editors. Kluwer Academic Press, Lancaster, UK. 147–150.

45. Scobey, M. W., F. L. Johnson, J. S. Parks, and L. L. Rudel. 1988. Dietary fish oil reduces bile lithogenicity in the African green monkey. *Gastroenterology.* **94:** A588.