No pathophysiologic relationship of soluble biliary proteins to cholesterol crystallization in human bile[1]

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Abstract **This study explores the pathophysiologic effects of soluble biliary glycoproteins in comparison to mucin gel and cholesterol content on microscopic crystal and liquid crystal detection times as well as crystallization sequences in lithogenic human biles incubated at 37°C. Gallbladder biles from 13 cholesterol gallstone patients were ultracentrifuged and microfiltered (samples I). Total biliary lipids were extracted from portions of samples I, and reconstituted with 0.15 m NaCl (pH 7.0) (samples II). Portions of samples II were supplemented with purified concanavalin A-binding biliary glycoproteins (final concentration = 1 mg/mL) (samples III), or mucin gel (samples IV), respectively, isolated from the same cholesterol gallstone biles. Samples V consisted of extracted biliary lipids from uncentrifuged and unfiltered bile samples reconstituted with 0.15 m NaCl (pH 7.0). Analytic lipid compositions of samples I through IV were identical for individual biles but, as anticipated, samples V displayed significantly higher cholesterol saturation indexes. Detection times of cholesterol crystals and liquid crystals were accelerated in the rank order of samples: IV > V > I = II = III, indicating that total soluble biliary glycoproteins in pathophysiologic concentration had no appreciable effect. Crystallization sequences (**D. Q-H. Wang and M. C. Carey. ***J. Lipid Res.*** **1996.** 37: **606–630; and 2539–2549) were similar among samples I through V. Crystal detection times and numbers of solid cholesterol crystals were accelerated in proportion to added mucin gel and the cholesterol saturation of bile only. For pathophysiologically relevant conditions, our results clarify that mucin gel and cholesterol content, but not soluble biliary glycoproteins, promote cholesterol crystallization in human gallbladder bile.—**Wang, D. Q-H., D. E. Cohen, F. Lammert, and M. C. Carey. **No pathophysiologic relationship of soluble biliary proteins to cholesterol crystallization in human bile.** ***J. Lipid Res.*** **1999.** 40: **415–425.**

Supplementary key words gallstone • phase diagram • bile salt species • nucleation • microscopy • phospholipid • concanavalin A-affinity chromatography

Rapid in vitro crystallization of cholesterol monohydrate (ChM) crystals from the "isotropic phase" of gallbladder bile appears to discriminate lithogenic gallbladder biles of Ch gallstone patients from Ch-supersaturated biles of controls (1). Although it is highly likely (2–6) that mucin gel is a strong pro-nucleation/crystallization factor in lithogenic biles, the pathophysiological role, if any, of soluble biliary glycoproteins in the pathogenesis of Ch gallstone formation remains unclear. A large number of workers (7–16) have demonstrated that soluble biliary glycoproteins could be pro-nucleating/crystallizing factors although this has been challenged by other reports (17–20) suggesting that these glycoproteins have no effects on ChM crystal detection times in bile. Proteolysis of soluble biliary glycoproteins (21) does not influence the speed of ChM crystal detection either in normal or abnormal gallbladder and hepatic biles, which suggests that soluble biliary proteins may not be important pathophysiological factors affecting Ch crystallization (21). Despite extensive in vitro work using purified proteins added to model bile systems (for summary, see ref. 22), little attention has been paid to investigating whether the pro-nucleating/crystallizing capacity of mixed proteins in pathophysiological concentrations might influence Ch crystallization sequences in native biles (22). In recent work, we characterized all Ch crystallization pathways in model biles (23), and illustrated their pathophysiologic importance by showing corresponding pathways in native human (24) and lithogenic mouse biles (25, 26). Furthermore, using concanavalin A (Con A) affinity chromatography, Harvey, Upadhya, and Strasberg (27) discovered residual microcrystals retained in purified biliary proteins and showed that these microcrystals could accelerate ChM crystal detection times in human biles. Therefore, this study ex-

Abbreviations: ACh, anhydrous cholesterol; BS, bile salt; Ch, cholesterol; ChM, cholesterol monohydrate; Con A, concanavalin A; CSI, cholesterol saturation index; HPLC, high performance liquid chromatography; L, lecithin; [TL], total lipid concentration.

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plored *i*) the effects of mucin gel, Ch content, and purified soluble Con A-binding biliary glycoproteins free of Ch crystals in (patho)physiological concentrations on Ch crystallization times and sequences in lithogenic human gallbladder biles; and *ii*) tested whether ultracentrifugation plus microfiltration was sufficient to eliminate the putative residual microcrystals influencing Ch crystallization times compared to the reconstituted gallbladder biles. This study showed that rapid Ch crystallization from lithogenic human gallbladder biles is not affected by a purified mixture of soluble Con A-binding biliary glycoproteins in (patho)physiological concentrations but Ch crystallization was promoted solely by added mucin gel and the Ch content of bile.

MATERIALS AND METHODS

Materials

Grade I taurocholate (Sigma Chemical Co., St. Louis, MO) was recrystallized (28) and found to be >99% pure by high performance liquid chromatography (HPLC). For HPLC analyses of bile salt (BS) molecular species, all reagents were HPLC grade and were purchased from Fisher Scientific Co. (Medford, MA). Purity of individual BS was at least >98% by HPLC (23, 29). Other chemicals and solvents were American Chemical Society (ACS) or reagent grade quality from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Medford, MA). Water was filtered, ion exchanged, and glass distilled (Corning Glass Works, Corning, NY). Pyrex brand glassware were washed for 24 h in a solution of 2 m KOH in 50% ethanol, followed by 24 h washing in 3 m HNO_3 and thorough rinsing in glass-distilled, deionized H_2O prior to drying.

Collection and preparation of gallbladder biles

Fresh gallbladder biles together with all gallstones were obtained from 13 gallbladders of patients undergoing elective laparoscopic cholecystectomy. One representative gallstone was kept for analysis. All patients had normal liver function tests and functioning gallbladders by ultrasonography and no historical, clinical, or laboratory evidence of present or prior hepatobiliary complications. All gallbladder biles were aspirated from the gallbladders of patients with Ch gallstones as defined by chemical analysis of gallstones (see below). To obtain all phase-separated material, a large-bore hypodermic needle and syringe was used at the beginning of the operative procedure and prior to gallbladder manipulation or dissection. All 13 patients had multiple gallstones: 4 had two or three stones, and 9 had four or more stones. In general, only gallbladders that provided at least 12 mL of bile and showed complete gallbladder emptying prior to surgery were entered into the study. Each bile sample was cultured to exclude aerobic and anaerobic infection. Written informed consent was obtained according to a protocol approved by the Institutional (BWH) Human Subjects Committee.

Figure 1 displays a flow chart of in vitro preparation of gallbladder biles after collection. Each bile sample was divided into five equal portions. Gallbladder bile specimens from Ch gallstone patients were ultracentrifuged at 100,000 *g* for 30 min at 37°C and filtered through a preheated (37°C) Swinnex-GS filter assembly containing a 0.22-μm filter (Millipore Products Divi-

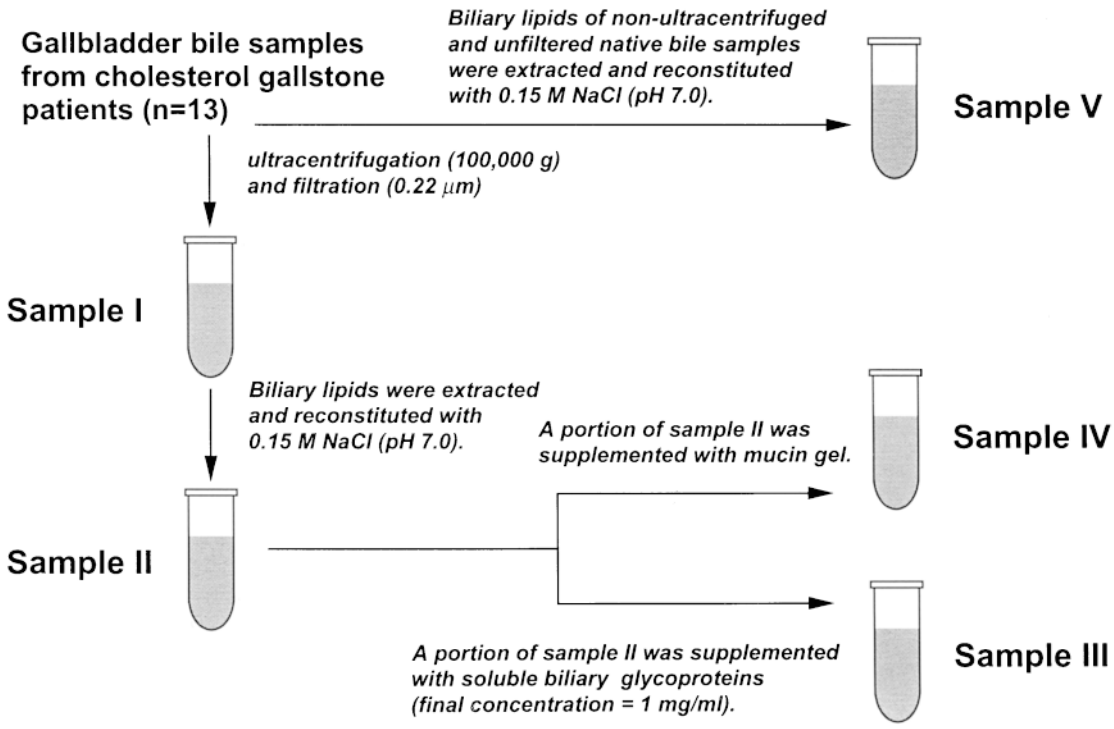

Fig. 1. Thirteen gallbladder bile specimens from Ch gallstone patients were ultracentrifuged (100,000 *g*) and filtered (0.22 μm) (samples I). Biliary lipids were extracted from samples I, and reconstituted with 0.15 m NaCl (pH 7.0) (samples II). Portions of samples II were supplemented with biliary glycoproteins (final concentration = 1 mg/mL) purified from identically matched Ch gallstone biles using concanavalin A-affinity chromatography (27) (samples III), or with mucin gel (samples IV), respectively. Biliary lipids of uncentrifuged and unfiltered native bile samples were extracted and reconstituted with 0.15 m NaCl (pH 7.0) (samples V). The crystal and liquid crystal detection times as well as crystallization pathways of all biles were examined by polarizing light microscopy (magnification ×400) at 37°C.

sion, Bedford, MA) to remove mucus gel (see below) and to generate a bile specimen that was verified by polarizing light microscopy (magnification ×800) to contain no solid/liquid crystals or other particulate matter (samples I). Biliary lipids were extracted from samples I. The lipids were first frozen with acetone in dry ice, and freeze-dried for 9–12 days. After dissolution to achieve complete molecular mixing in $CHCl_3$–MeOH 2:1 (vol/vol) and drying under a stream of N_2 at reduced pressure to constant weight, each lipid film was dissolved in an appropriate volume of aqueous 0.15 m NaCl plus 3 mm NaN_3 to achieve total lipid concentrations ([TL]) identical to those of the original bile samples (samples II). Portions of samples II were supplemented with purified soluble Con A-binding biliary glycoproteins (final concentration = 1 mg/mL, see rationale below) from Ch gallstone biles using Con A-affinity chromatography (see below) (samples III). To another portion of samples II, mucin gel (see below) obtained from the identical gallbladder bile was added (samples IV). For preparation of samples V, biliary lipids of non-ultracentrifuged and unfiltered bile samples were extracted and reconstituted according to the method described for samples II above. In the addition experiments, an identity for each patient's bile between protein extracted and reconstituted lipids was maintained throughout the experimental period.

Con A-affinity chromatography and preparation of soluble biliary glycoproteins

Soluble Con A-binding biliary glycoprotein purification was performed according to the method of Harvey and colleagues (27) with minor modifications. In brief, 4 mL of gallbladder bile from each sample was applied directly to a Con A-Sepharose column (1 × 13 cm; 5 mL of Con A; Pharmacia, Uppsala, Sweden) and eluted at 4°C. The column was washed with 300 mL of 100 mm Tris buffer (pH 7.4) containing 10 mm taurocholate, 0.5 m NaCl, 1 mm $CaCl_2$, and 0.02% NaN_3. After 12 h, the column was washed with another 100 mL of Tris buffer without taurocholate until the eluent was free of lipids and biliary pigment (27). After addition of 0.2 m α-d-methylmannopyranoside, the Con A-binding glycoproteins were eluted with 30 mL of Tris buffer without taurocholate. Eluted protein fractions were filtered through a Swinnex-GS filter assembly containing a 0.22-μm filter, and concentrated to 10 mL by centrifugation at 1,600 rpm (1,000 *g*) at 4°C. Glycoproteins were then dialyzed, and freeze-dried overnight. Preparations were stored at 4°C until the protein additions for crystallization studies were performed.

Preparation of mucin gel

Gallbladder bile was ultracentrifuged (see above), and mucin gel was harvested from the sediment with a pasteur pipette. Fresh mucin gel obtained from all patients with Ch gallstones contained ChM crystals with or without liquid crystals as detected by polarizing light microscopy (magnification ×200). Accordingly, mucin gel was suspended in 10 volumes of 100 mm Tris buffer (pH 7.4) containing 10 mm taurocholate to dissolve crystalline and bound lipids, and was shaken at 100 rpm overnight at room temperature (22 ± 1°C) using a model 75 Wrist Action Shaker (Burrell Corporation, Pittsburgh, PA). Mucin gel was washed 3 times with 100 mm Tris buffer containing taurocholate by centrifugation at 10,000 *g* for 1 h. Then, mucin gel was washed 3 times with 100 mm Tris buffer (pH 7.4) without taurocholate by centrifugation at 10,000 *g* for 1 h, followed by dialysis and freeze-drying. Mucin gel was verified to be free of phase-separated lipids by polarizing light microscopy (magnification ×800), and was stored at 4°C until crystallization experiments were executed.

Detection times of Ch crystals, including liquid crystals and crystallization sequences

Bile samples were flushed with N_2, sealed with Teflon-lined caps, and incubated individually at 37°C. Detection of Ch crystals and liquid crystals as well as crystallization sequences was performed by polarizing light microscopy at ×400 magnification according to previous methods (23). Arc (putatively anhydrous Ch), filamentous, tubular and helical crystals (intermediate forms), plate-like ChM crystals as well as small, aggregated, and fused liquid crystals were defined according to published criteria (23). Crystal numbers were counted in 5-μL bile samples for 5 days using a microscopic field of 1 cm^2 (×200 magnification) (24).

Chemical analyses

Small aliquots from each bile sample were stored at −20°C for lipid analysis. Total BS were quantified by the 3α-hydroxysteroid dehydrogenase method (30), lecithin (L) by the inorganic phosphorus procedure (31), and Ch enzymatically (32). Individual BS were determined by the HPLC method of Rossi, Converse, and Hofmann (33). Ch saturation indexes (CSI) of native biles were calculated from the critical tables (34). Gallstones were washed with distilled water, dried at room temperature, ground to a powder in an agate mortar, and dissolved in isopropanol. Ch content was measured enzymatically (32) and by HPLC (35) and expressed as percent of gallstones (wt/wt) in the extract. Stones with a Ch content of >80% by weight were classified as Ch gallstones. Biliary proteins were precipitated from bile by the addition of 7% trichloroacetic acid. The precipitated proteins were washed with ethanol–ether 1:1 (vol/vol) to remove biliary lipids. The organic solvents were decanted and the protein pellet was dissolved in 0.2 m borate buffer (pH 8.5). Protein concentration was determined by the assay of Bradford (36). Mucin gel was observed as non-birefringent amorphous strands (24,25), and verified by periodic acid/Schiff (PAS) staining (37).

Statistics

Data are expressed as means ± SD. Statistically significant differences among groups of bile samples were assessed by Student's *t*-test or by Mann-Whitney U tests for parametric and nonparametric data, respectively. Using *SuperANOVA* software (Abacus Concepts, Inc., Berkeley, CA), parameters significantly associated in linear regression analyses with detection times of Ch crystals and liquid crystals were further assessed by a stepwise multiple regression analysis to identify the independence of the association between variables and crystal detection times. Statistical significance was defined as a two-tailed probability of less than 0.05.

RESULTS

Microscopic studies, lipid compositions, and Ch crystallization pathways

After aspiration, all fresh gallbladder biles, i.e., before centrifugation and filtration, were examined immediately by polarizing light microscopy. ChM crystals, liquid crystals, and mucin gel were observed in most bile samples. It was our impression, but not quantified by microscopy, that mucin gel contained more ChM crystals and liquid crystals than bulk bile.

Table 1 lists mean lipid compositions of gallbladder biles in all studied samples (see Fig. 1). This demonstrates that analytic lipid compositions were identical in samples I through IV. As expected for lipids extracted from non-

TABLE 1. Biliary lipid compositions of gallbladder biles

Sample	%Ch	%L	%BS	[TL]	CSI[a]
I	11.36 ± 2.23	19.32 ± 4.25	69.32 ± 6.09	11.94 ± 3.55	1.66 ± 0.23
II	11.17 ± 2.09	19.28 ± 4.43	69.55 ± 6.22	11.87 ± 3.56	1.63 ± 0.19
III	11.31 ± 2.13	19.29 ± 4.32	69.40 ± 6.11	11.85 ± 3.56	1.67 ± 0.21
IV	11.30 ± 2.01	19.30 ± 4.33	69.44 ± 6.15	11.90 ± 3.46	1.66 ± 0.20
V	15.00 ± 1.74[b]	20.10 ± 3.25	64.90 ± 4.31	13.18 ± 3.63	2.04 ± 0.22[b]

Data are expressed as means ± SD.

[a] CSI values of gallbladder biles were calculated from the critical tables (34); Ch, cholesterol; L, lecithin; BS, bile salts; [TL], total lipid concentration; CSI, cholesterol saturation index.

[b] $P < 0.05$, sample V vs. samples I–IV, N.S. among samples I, II, III, and IV.

ultracentrifuged and unfiltered biles, samples V had significantly higher ($P < 0.05$) mole % Ch and CSI values.

Figure 2 plots the relative lipid compositions of individual gallbladder biles of samples I and V on a condensed phase diagram for the mixed Ch–L–BS systems (pH 7.0, 37°C, 0.15 m NaCl) (23, 38) using a mean [TL] = 12 g/dL (see Table 1). The appropriate micellar phase boundary (23, 38) and Ch crystallization pathways (23, 24) denoted by diagonal dashed and solid lines are given for model biles with mixed BS (23). The relative lipid compositions of all gallbladder biles taken from samples I (closed circles) plotted within crystallization pathways denoted by regions B, C, and D, with most samples falling in region C (see also ref. 23). Although samples V (closed squares) contained significantly ($P < 0.05$) higher Ch content than samples I, their lipid compositions invariably located within the same crystallization regions.

Molecular species of BS in gallbladder biles

Proportions of the 12 common conjugated BS species in the gallbladder biles as determined by the highly sensitive HPLC (33) revealed that all molecular species of BS were similarly distributed among samples I–V. All gallbladder biles showed the same distributions of BS compositions as found in previous studies from this laboratory (24, 39). Concentrations of glycine-conjugated BS species as percent of total BS were in decreasing rank order: glycocholate (24.7–25.7%), glycochenodeoxycholate (22.6–23.3%), glycodeoxycholate (21.0–22.3%), glycolithocholate sulfate (3.0–3.1%), glycoursodeoxycholate (1.6–1.9%), and glycolithocholate (0.4–0.5%), as well as concentrations of taurine-conjugated BS species decreased in the rank order: taurocholate (7.9–8.5%), taurochenodeoxycholate (7.5–9.0%), taurodeoxycholate (5.2–6.0%), taurolithocholate sulfate (1.5–1.7%), tauroursodeoxycholate (1.2–1.3%), and taurolithocholate (0.04–0.12%).

Influence of soluble Con A-binding glycoproteins, mucin gel, and Ch content on Ch crystallization

After removing mucin gel and presumably some insoluble large-molecule proteins from bile by ultracentrifugation and microfiltration, mean total protein concentra-

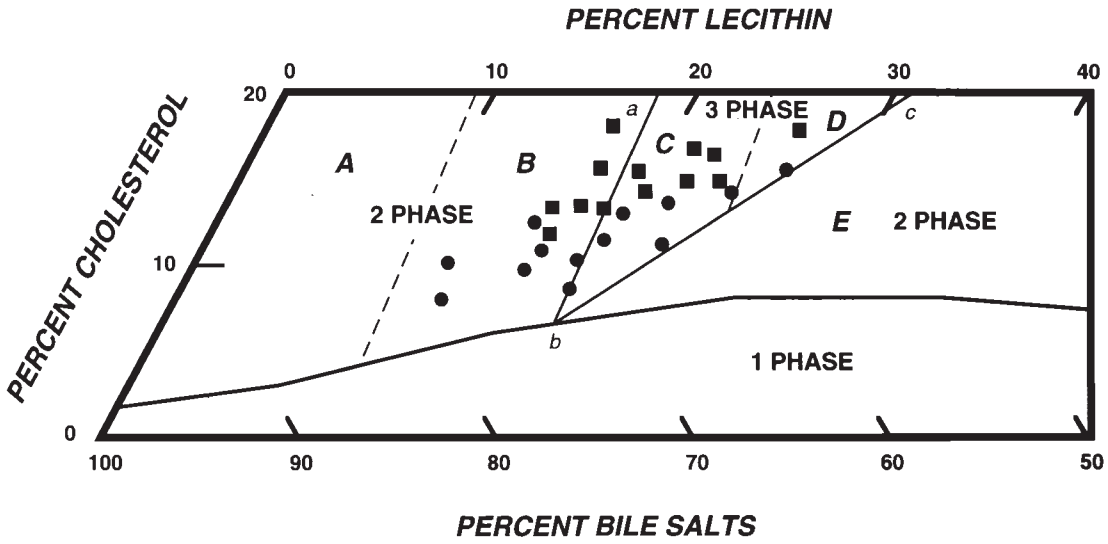

Fig. 2. Individual relative lipid compositions (moles per 100 moles) of gallbladder biles from samples I and V (see Fig. 1) of 13 Ch gallstone patients plotted on a condensed phase diagram appropriate for a mixed Ch-L-BS system (pH 7.0, 37°C, 0.15 m NaCl) using an average total lipid concentration of 12 g/dL (see Table 1). The one-phase micellar zone (at bottom) is enclosed by a solid curved line. Two solid and two dashed lines divide the remnant of the condensed phase diagram into regions A to E with different crystallization sequences (see refs. 23 and 24). The relative lipid compositions of all biles plot within crystallization pathways (23) denoted B, C, and D, with most in region C. Although samples V have higher (mole %) Ch compared with samples I, lipid compositions of biles invariably located within the same crystallization pathways. Symbol ■ represents samples V; and ● samples I. See text for further description.

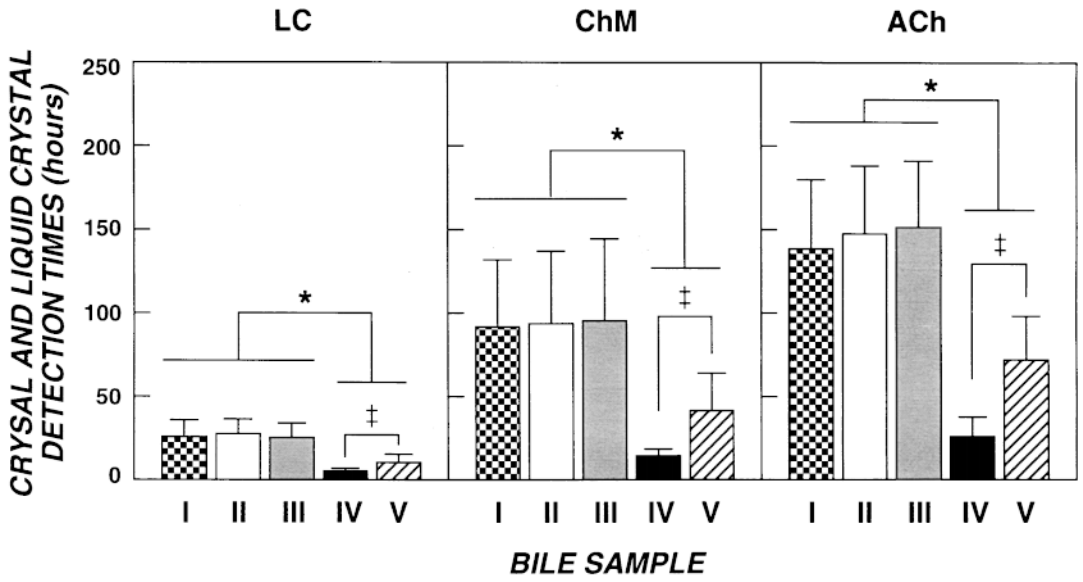

Fig. 3. Ch crystal and liquid crystal detection times for bile samples I through V. Note that detection times of liquid crystals (LC) (left panel), ChM crystals (middle panel), and ACh crystals (right panel) were accelerated in the rank order of samples IV (with addition of mucin gel) > samples V (with higher Ch content) > samples I (ultracentrifugation and filtration) = samples II (lipids-extracted and reconstituted) = samples III (with addition of purified soluble Con A-binding biliary glycoproteins). *$P < 0.05$, samples I–III compared with samples IV and V; ‡$P < 0.05$, samples IV compared with samples V.

tion was 1.12 ± 0.62 mg/mL. The mass of soluble biliary glycoproteins recovered from the Con A column was 33 ± 15% of total proteins. For addition studies, we used a final protein concentration of 1 mg/mL, which was similar to the mean pathophysiological level in the biles. After purified soluble Con A-binding glycoproteins were added, the biles remained transparent for 12 h. For mucin gel addition experiments, the lipid-free mucin gel was added into the reconstituted bile samples to keep its concentration similar to the original biles. After mucin gel was reconstituted in the bile samples, a viscous translucent gel formed immediately. After a few hours incubation at 37°C, we observed several liquid crystals with Maltese crosses within the mucin gel.

As displayed in **Fig. 3**, detection times of liquid crystals (26.6 ± 9.3 h), ChM (94 ± 43 h), and ACh crystals (144 ± 41 h) were essentially indistinguishable in bile samples I, II, or III, respectively. Even though samples III contained added purified soluble Con A-binding biliary glycoproteins, the detection times (liquid crystals = 25.5 ± 7.0 h; ChM crystals = 96 ± 48 h; ACh crystals = 151 ± 41 h) fell within a few hours of the aggregate mean values. However, samples IV and V displayed significantly ($P < 0.05$) faster detection times of Ch crystals and liquid crystals than samples I–III. In particular, samples V with higher Ch content displayed faster detection times (liquid crystals = 10.3 ± 4.9 h; ChM crystals = 41 ± 26 h; ACh crystals = 72 ± 26 h) than samples I–III. Moreover, after mucin gel was added to bile lipids (samples IV), detection times of Ch crystals and liquid crystals were the fastest observed (liquid crystals = 5.6 ± 1.2 h; ChM crystals = 14 ± 5 h; ACh crystals = 26 ± 12 h) and accelerated significantly ($P < 0.05$) compared to samples V. In general, detection times of Ch crystals and liquid crystals were accelerated in the rank order: samples IV (with addition of mucin gel) > samples V (with higher Ch content) > samples I (ultracentrifugation and microfiltration) = samples II (lipids extracted and reconstituted) = samples III (with addition of purified soluble Con A-binding biliary glycoproteins).

Figure 4 shows Ch crystal and liquid crystal detection times as functions of Ch content expressed as CSIs of individual gallbladder biles that plotted in regions B, C, and D of the phase diagram (Fig. 2). Closed squares, triangles, and circles represent ChM crystal, ACh crystal, and liquid crystal detection times, respectively. The insets in the middle and right panels (Fig. 4) show effects of Ch content on liquid crystal detection times in regions C and D. Note that no liquid crystals were observed in region B only, and no ACh crystals were found in region D (23, 24). It is obvious that there are significant inverse linear relationships between CSI values and detection times of ChM crystals, ACh crystals, and liquid crystals, particularly marked in region B for ChM and ACh detection times. Nevertheless, it is to be noted that there were no statistical differences among the slopes of the regressions in regions B, C, and D.

Ch crystallization sequences are uninfluenced by biliary proteins or Ch content

Figure 5 shows Ch crystallization sequences in samples I. Figure 5 (top panel) depicts the crystallization sequence in region B (Fig. 2). The first precipitates (arrowed) were ChM crystals, followed by arc ACh crystals including the intermediate (helical and tubular) crystals (24, 25). Figure 5 (middle panel) displays that in region C (Fig. 2), liquid crystals including small, aggregated, and fused liquid crystals (24, 25) appeared initially, followed by ChM crystals as indicated by an arrow. After ChM crystals became visible, the number of liquid crystals decreased and arc

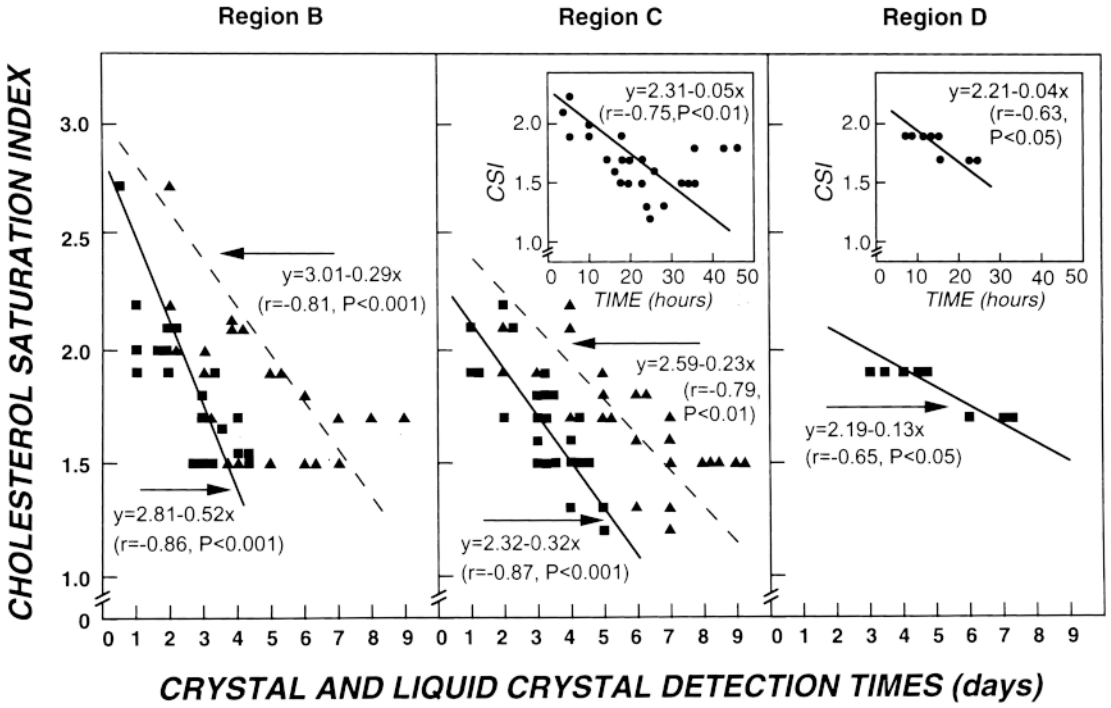

Fig. 4. Influence of Ch content expressed as Ch saturation index (CSI) on Ch solid and liquid crystal detection times for regions B, C, and D (see Fig. 2) at constant [TL] of 12 g/dL (range 11.9–13.2 g/dL). The insets in the middle and right panels, respectively, show effect of Ch content on liquid crystal detection times in regions C and D. Note that no liquid crystals were observed in region B; and no ACh crystals were found in region D. Detection times of ChM crystals, ACh crystals, and liquid crystals become shorter with increasing CSI values. The equations in the panels describe the inverse linear relationships between CSI values and detection times of ChM crystals, ACh crystals and liquid crystals, particularly marked in region B. There are no statistically significant differences between the regressions in regions B, C, and D. Symbols ■ represent ChM crystal detection times; ▲ ACh crystal detection times; and ● liquid crystal detection times. See text for further description.

ACh crystals including helical and tubular intermediate crystals appeared. Liquid crystals disappeared after 1–2 days and only ChM and ACh crystals remained. Figure 5 (bottom panel) shows that in region D (Fig. 2), liquid crystals appeared first, followed by ChM crystals only. Not shown are data for samples II through V, as their crystallization sequences were similar to the plotted samples I. Our observations demonstrate that addition of purified Con A-binding biliary glycoproteins or mucin gel does not influence Ch crystallization sequences.

High Ch content and mucin gel increase solid Ch crystal number

Figure 6 shows ACh and ChM crystal numbers for samples I, II, III, and V, which plotted in crystallization regions B, C, and D. Data for samples IV are insetted in the right panel (Fig. 6). Closed symbols represent numbers of ChM crystals and open symbols represent numbers of ACh crystals. For clarity, liquid crystals are omitted in the middle and right panels of Fig. 6. CSI values (see Table 1) were significantly higher ($P < 0.05$) in samples V (CSI = 2.0) compared with samples I–IV (CSI = 1.6) and accordingly the numbers of solid Ch crystals were significantly ($P < 0.05$) greater in samples V than samples I–III. There were no significant differences in the numbers of solid Ch crystals among samples I–III. With addition of mucin gel we observed that samples IV became very sticky and Ch crystallization occurred rapidly (liquid crystals within 6 h and ChM crystals within 14 h). Although mean CSI value of samples IV was 1.6, mucin gel increased solid Ch crystal number appreciably over the next 24 h. Individual ChM crystals enlarged in size and became consolidated by mucin gel as agglomerates of 1–3 μm diameter.

DISCUSSION

Soluble Con A-binding biliary glycoproteins do not influence detection times of Ch solid and liquid crystals

Using Con A-affinity chromatography (7) and Ch-supersaturated model biles as the assay medium, several putative pro-nucleating/crystallizing proteins have been isolated from the Con A-binding fraction of bile, which include IgA, IgG, and IgM (12, 13), fibronectin (14), α1-acid glycoprotein (16, 17), phospholipase C (10), aminopeptidase N (11), haptoglobin (40), anionic polypeptide fraction (15, 41), and a 130-kilodalton glycoprotein (9). Harvey and co-workers (12, 13) showed that IgM, IgA, and IgG possessed pro-nucleating/crystallizing activities, and their activities were in the rank order IgM > IgG > IgA. Meanwhile, other groups (17–19) found that Igs did not affect ChM crystal detection times in model biles. Abei et al. (17) reported that α1-acid glycoprotein accounted for up to 30% of total accelerating activity in

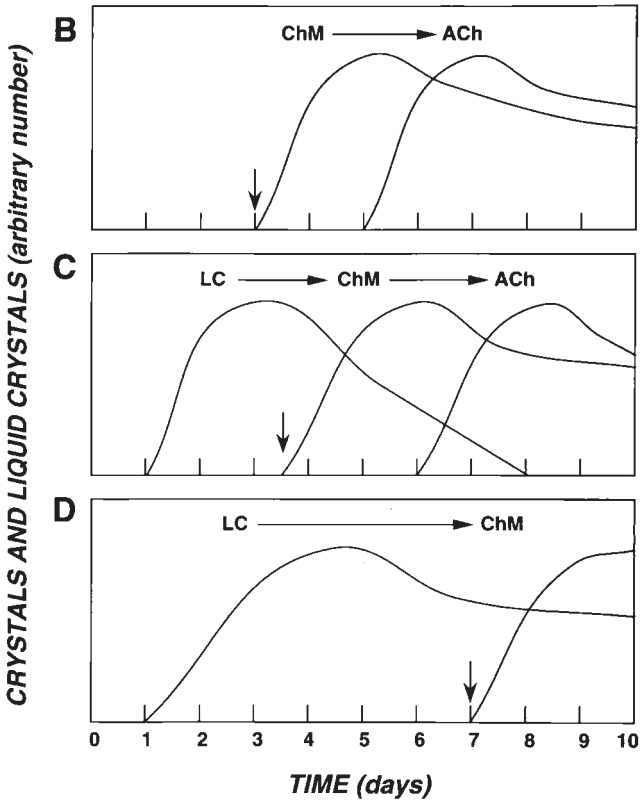

Fig. 5. Panels show crystallization sequences of samples I. The vertical axes represent arbitrary numbers of crystals and liquid crystals per high power microscopic field, all normalized to the same maximum. LC, ChM, and ACh on curves represent the appearance and persistence times of small, aggregated, and fused liquid crystals (LC), ChM crystals, and arc ACh followed by intermediate (helical and tubular) crystals, respectively (see refs. 23 and 24). Vertical arrows indicate the first appearance of ChM crystals. Because of daily sampling, relative numbers of solid and liquid crystals decreased artifactually at later time points (23, 24). Data for samples II through V are not shown here, as their crystallization sequences were identical to the plotted samples I.

terms of ChM crystal detection times, however, De Bruijn and colleagues (19) could not find any pro-nucleating/crystallizing activity with α1-acid glycoprotein. Chijiiwa et al. (14) proposed that fibronectin, also a Con A-binding protein, was a pro-nucleating/crystallizing factor; whereas, two other groups (20, 21) obtained results inconsistent with any nucleation/crystallization activity of fibronectin on ChM crystal detection times. In the extensive literature (summarized in ref. 22), most of the studies of biliary proteins used super-physiological concentrations of individual proteins for investigating their effects on Ch crystallization. Moreover, some biliary proteins, such as IgA, IgM, α1-acid glycoprotein, haptoglobin, and aminopeptidase N, do not display any differences in protein concentrations between Ch gallstone patients and controls (18). In past years, the effect of individual biliary proteins of the Con A-binding fraction on Ch crystallization has been studied extensively, and these investigations used Ch-supersaturated model bile systems. However, little information is available regarding the effect of total soluble biliary glycoproteins on Ch crystallization in native human biles. Pattinson and Willis (10) observed that proteolysis of soluble biliary glycoproteins did not affect Ch crystal detection times of either normal gallbladder or abnormal gallbladder and hepatic biles, which suggests that total biliary proteins may not be important factors influencing Ch crystallization. Recently, using univariate and multivariate statistical analyses, Miquel et al. (18) found that biliary Ch saturation, but not proteins, was the crucial factor for accelerating Ch crystallization in native biles from Ch gallstone patients. Taken together, these observations on Con A-binding glycoproteins are confusing and contradictory, and led to the present systematic evaluation on the effects of total soluble biliary proteins in pathophysiological concentrations on Ch crystallization in reconstituted human biles in vitro.

Several investigators (42–45) have determined the mucin content of human biles, but the reports encompassed enormous differences in gallbladder concentrations, ranging from 0.2 to 10 mg/mL probably due to the polydispersity, insolubility, and complex sol–gel transition range of mucin glycoproteins (46). In this study, after mechanically removing mucin gel cleanly with some insoluble large-molecule proteins by ultracentrifugation and microfiltration, mean total protein concentration in bile was 1.12 ± 0.62 mg/mL, which is consistent with the literature on the soluble fraction of glycoproteins (1.7 to 2.7 mg/mL) (5, 27, 47). Con A-affinity chromatography was first proposed by Groen et al. (7), and later modified by Harvey, Upadhya, and Strasberg (27) to purify soluble biliary glycoproteins. Our results show that the soluble glycoprotein mass recovered from the Con A column was 33 ± 15% of total biliary proteins comparable to the modified method (36%) of Harvey, Upadhya, and Strasberg (27). In the literature, final concentrations of added Con A-binding glycoproteins ranged from 0.25 to 2 mg/mL (19, 20, 27). We added a final protein concentration of 1 mg/mL, which was similar to the pathophysiological levels in the gallbladder biles used in this study. Our major finding is that purified soluble Con A-binding biliary glycoproteins at 1 mg/mL concentration did not affect detection times of Ch solid and liquid crystals in reconstituted biles, each from the same patient. Also, the freeze-drying methods have been used extensively by other groups (9, 12) and do not influence protein activity in the protein addition experiments.

Mucin gel and high Ch content accelerate Ch crystallization in lithogenic gallbladder biles

It is believed (2–6) that mucin gel may be of crucial importance in the pathogenesis of Ch gallstones and play a role as a pro-nucleating/crystallizing factor for Ch crystallization. This concept is based principally on in vitro studies of model biles (3, 4), and in vivo studies of animal biles such as prairie dogs (2), hamsters (48), and mice (25, 49, 50). It has been well documented that gallbladder mucin hypersecretion and accumulation precede the formation of Ch crystals and the development of gallstones in lithogenic mice (25, 49, 51), prairie dogs (2), and hamsters (48). Our current in vitro experiments (Figs. 3 and 4)

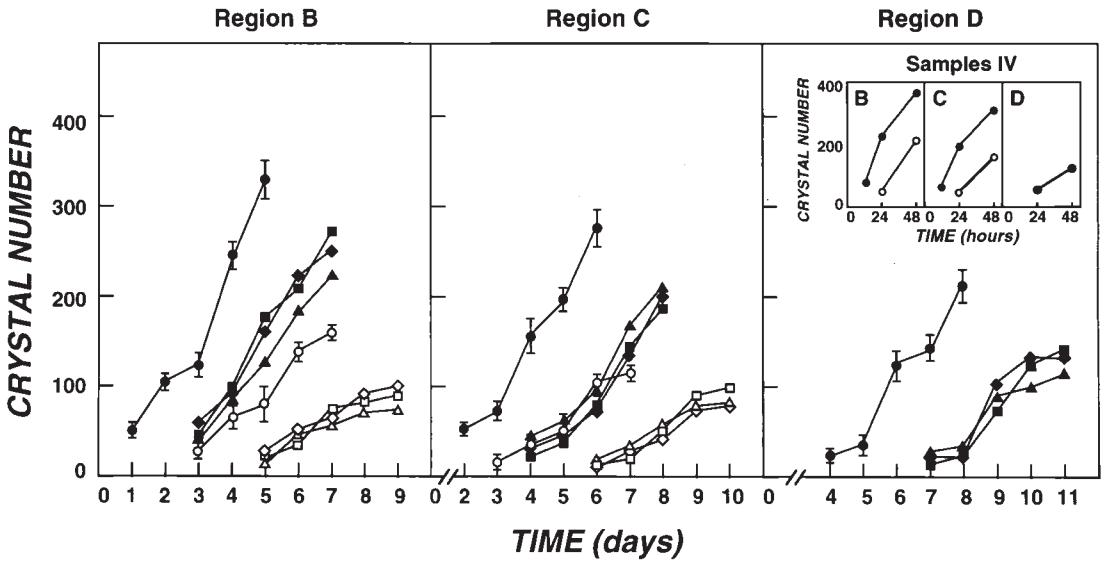

Fig. 6. Effects of Ch content and mucin gel on numbers of ChM plate-like crystals as well as arc ACh and intermediate helical and tubular crystals. Closed symbols represent numbers of ChM crystals; ■ samples I; ▲ samples II; ◆ samples III; and ● samples V. Open symbols represent numbers of arc ACh and intermediate crystals; □ samples I; △ samples II; ◇ samples III; and ○ samples V. The inset in the right panel shows the effect of Ch content on ChM (●) and ACh (○) crystal numbers for samples IV. Because samples IV with addition of mucin gel became very sticky and Ch crystallization occurred rapidly (liquid crystal within 6 h and ChM crystal within 14 h), solid Ch crystal number increased appreciably over the following 24 h. CSI values (see Table 1) were significantly higher ($P < 0.05$) in samples V (CSI = 2.0) than samples I–IV (CSI = 1.6). Ch content of reconstituted bile influences not only crystal numbers of both ACh (plus intermediate crystals) and ChM crystals but also their detection times. However, soluble biliary proteins do not influence either numbers or detection times of solid Ch crystals. For clarity, only mean Ch solid crystal numbers for samples V are shown at each time point. See text for additional details.

show that mucin gel acts as a strong pro-nucleating/crystallizing factor for accelerating solid Ch and liquid crystal detection times, and as a nucleus for holding ChM crystals and liquid crystals. These results are in agreement with our previous in vivo and in vitro observations on mouse gallbladder biles (25) as well as prairie dogs (2).

Although it was suggested originally that CSI values of biles are a good predictor for the rate of Ch crystallization (38), the effects of CSI values on detection times of Ch solid and liquid crystals have been neglected in recent years. We found that higher CSI values accelerated detection times of Ch solid and liquid crystals appreciably in model bile studies (23). Indeed, Holan and colleagues (1) also observed the inverse relationship between speed of onset of Ch crystal formation and the CSI values. Nevertheless, they argued that CSI values were non-discriminatory as their CSI values did not allow for a sharp distinction between bile samples of Ch gallstone patients and controls but mucin gel was also ignored in their study (1). The present study confirmed our previous observations (23) that when [TL] was kept constant, there was a positive correlation between higher CSI values and detection times of Ch solid and liquid crystals (23). This effect is especially strong in regions B and C of the phase diagram (Fig. 2) in which the relative compositions of most human gallbladder biles locate. Our results support the view that higher degrees of Ch supersaturation act as an independent pro-nucleating/crystallizing factor in native bile (18).

Furthermore, little attention has been paid to the relationship between CSI values and [TL]. Although dilute hepatic biles have higher CSIs than gallbladder biles, Ch crystal detection times are always slower in hepatic biles than gallbladder biles (52). In dilute model biles we (23) observed that with decreases in [TL], all physical states, and crystallization pathways are shifted to the left, i.e., to lower lecithin contents. Concomitantly, the right two-phase zone (region E) (see Fig. 2) extended between the micellar zone and the central three-phase zone and overlapped pathophysiological compositions (23). The expansion of region E, which contains at equilibrium liquid crystals and saturated micelles but not solid crystals, provides a physical–chemical explanation why Ch crystallization seldom occurs in dilute hepatic bile despite high CSI values. However, if [TL] is kept constant, there is a positive correlation between higher CSI values and shorter detection times of Ch solid and liquid crystals (23). Therefore, [TL] is a moderately strong and independent factor influencing Ch crystallization in bile (23, 52).

Ch content, mucin gel, and soluble biliary glycoproteins do not influence Ch crystallization sequences or phase boundaries

Although Ch content of bile had a major influence on crystal detection times (Fig. 4), it has no effect on crystallization sequences (Fig. 5). On the basis of published work of Ahmed et al. (53), we hypothesized (23) that hydrophilic or hydrophobic proteins, as determined by their elution times on a phenyl-agarose column, may produce pro- or anti-nucleating/crystallizing effects by shifting phase

boundaries and Ch crystallization pathways. Nonetheless, we found in the present work that mucin gel and soluble Con A-binding biliary glycoproteins in (patho)physiological concentrations did not influence crystallization pathways or phase boundaries. Afdhal and colleagues (54, 55) have suggested that mucin gel facilitates vesicle fusion leading to formation of multilamellar liposomes (liquid crystals), which may in turn act as a source of nucleating solid ChM crystals.

Ultracentrifugation plus microfiltration is sufficient to remove the putative residual microcrystals in bile

Only in the past few years has it been realized (1, 21, 27, 56) that it is critical to ensure complete removal of residual Ch microcrystals prior to incubation of bile samples for Ch nucleation/crystallization studies. After XM-300 filtration of bile compared to the 0.22 μm filtration, Kiyosawa and co-workers (56) found no differences in concentrations of total protein, soluble glycoproteins, or lipid compositions. However, using the XM-300 filtration procedure, they observed that [TL] decreased 20–40%, which resulted in prolonged Ch crystal detection times. As we showed previously, this dilution factor may move all phase boundaries to the left (see Fig. 2) retarding Ch crystallization (23). Furthermore, the filtration study of these authors (56) was consistent with our results in that soluble glycoproteins are not responsible for rapid Ch crystal detection times in human bile. In the present study, we excluded the effects of biliary proteins and putative residual Ch microcrystals on detection times by lipid extraction and reconstitution of bile. Using ultracentrifugation (100,000 *g*) and filtration (0.22 μm) methods, crystal detection times did not alter in our new approach. Therefore, our results showed that for in vitro Ch nucleation/crystallization studies, ultracentrifugation and microfiltration are sufficient to exclude the possibility of retaining Ch crystals that clearly influence detection times of Ch crystals and liquid crystals in model nucleation/crystallization studies (27). In the present study, preparation of gallbladder biles was similar to the method for preparing model biles as described in our previous paper (23); as verified by physical–chemical analyses, these techniques do not influence the overall lipid compositions.

To summarize, our results support the concept that mucin gel and high Ch content promote Ch nucleation/crystallization in human biles, but purified soluble Con A-binding biliary glycoproteins do not display any effect, at least in (patho)physiological concentrations matched precisely to the lipids of the bile from which they were extracted. Furthermore, irrespective of whether their roles in Ch nucleation/crystallization are pro-accelerant or neutral, neither mucin gel, Ch content, nor soluble Con A-binding biliary glycoproteins influence any of Ch crystallization sequences typical of the pathways of the phase diagram in which they plot (23). Although there are contradictory data, for example, concerning the effect of biliary IgA (13, 17–19, 57) on in vitro Ch crystallization in model bile, we (D. Q-H. Wang, F. Lammert, B. A. Hendrickson and M. C. Carey, unpublished observations) found no differences on Ch crystallization in vivo in J-chain-knockout mice (58, 59) when biliary IgA concentrations were markedly decreased compared to controls. Furthermore, in gallstone-susceptible mice, we observed (51) that high Ch saturation and mucin gel were the only factors exclusively associated with high genetic susceptibility to Ch gallstone formation. Therefore, to evaluate the importance of any putative biliary proteins on Ch crystallization and gallstone formation in the future, new animal models such as inbred, knockout, and transgenic mice should be studied to ascertain whether individual or total biliary proteins have in vivo effects on Ch crystallization and gallstone formation.

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