Identification of Gallbladder Mucin-Bilirubin Complex in Human Cholesterol Gallstone Matrix

Effects of Reducing Agents on In Vitro Dissolution of Matrix and Intact Gallstones

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Abstract

The goals of this study were to isolate and characterize the nonlipid matrix of human cholesterol gallstones. The lipid portion of gallstones was dissolved in ethanol/ether, leaving an insoluble, granular, brown-black matrix that constituted 12.5% of solitary large stones and 3.5% of multiple small stones. The matrix was partially solubilized by sonication and studied by exclusion gel chromatography and density gradient ultracentrifugation. On Sepharose 2B column chromatography, bile pigment eluted with glycoprotein in the void volume, suggesting the presence of a high molecular weight complex ($M_r > 2 \times 10^6$). The identity of mucin in this complex was confirmed by its typical buoyant density during ultracentrifugation. The major bile pigments in the matrix were identified as bilirubin (84%) and bilirubin monoglucuronide (15%) by thin-layer chromatography.

Because of their ability to solubilize mucin-type glycoproteins, we tested the ability of the reducing agents 2-mercaptoethanol (2ME) and *N*-acetylcysteine (NAcCys) to solubilize gallstone matrix. Both reducing agents caused a two- to threefold enhancement of matrix dissolution after 4 d compared to aqueous buffer alone ($P < 0.01$). Sepharose 2B chromatography revealed that 2ME released a high molecular weight mucin-bilirubin complex as well as unbound pigment from the insoluble matrix. We also tested the effect of reducing agents on dissolution of matched cholesterol gallstones by monooctanoin, a cholesterol solvent. Both 2ME and NAcCys significantly accelerated gallstone dissolution in monooctanoin. Matched human cholesterol stones ($n = 10$) incubated for 4 d in monooctanoin plus either 2ME or NAcCys (1 M final concentration) weighed approximately half as much ($P < 0.01$ for each) as stones incubated in monooctanoin alone. This study describes, for the first time, the isolation of a bilirubin-mucin complex in the insoluble matrix of human cholesterol gallstones. The ability of reducing agents to dissolve the matrix and thereby accelerate gallstone dissolution by monooctanoin in vitro may be relevant to gallstone dissolution in humans.

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Introduction

Gallbladder mucin, a high molecular weight glycoprotein secreted by the epithelial cells of the gallbladder, has been implicated in the pathogenesis of cholesterol gallstones. Hypersecretion of mucin and accumulation of a thick mucus gel in the gallbladder lumen precedes the development of cholesterol gallstones in several experimental animal models (1, 2). Inhibition of mucin hypersecretion by aspirin prevented gallstone formation in the cholesterol-fed prairie dog but did not alter the lipid composition of bile (3). In addition, recent ultrasonographic studies in man have documented the accumulation of biliary sludge, consisting of gallbladder mucin and bile pigments, before gallstone formation in patients on prolonged total parenteral hyperalimentation (4). These observations suggest that gallbladder mucin may be important in the initial stage of cholesterol gallstone formation, that is the heterogenous nucleation of cholesterol crystals from supersaturated bile. Support for this mechanism is provided by our recent demonstration that purified gallbladder mucin accelerated the heterogenous nucleation of cholesterol monohydrate crystals from supersaturated native (1) and model (5) biles.

In addition to being an important factor in gallstone formation, gallbladder mucin is also believed to be a structural component of human cholesterol gallstones. Most cholesterol gallstones contain a matrix that typically is visualized in bisected gallstones as a central pigmented nidus with either radial or lamellar pigmented bands alternating with layers of crystalline cholesterol (6). Although glycoproteins (6, 7), bilirubin (8, 9), and inorganic calcium salts (10) have been identified in the matrix, its origin and composition is poorly understood. The insoluble nature of the gallstone matrix has hampered the elucidation of its structure by standard biochemical techniques. Thus previous studies have largely depended on indirect physical-chemical techniques such as solid-phase infrared spectroscopy (11, 12), micro-x-radiography (13), and scanning electron microscopy (14).

In this study we have isolated the gallstone matrix and studied its composition and structure by standard biochemical techniques. Our results indicate that the cholesterol gallstone matrix contains a macromolecular complex of mucin and bilirubin that has a composition similar to that reported for biliary sludge (15). In addition, we have found that the reducing agents 2-mercaptoethanol (2ME)[1] and *N*-acetylcysteine (NAcCys) partially solubilize the gallstone matrix and accelerate

1. *Abbreviations used in this paper:* 2ME, 2-mercaptoethanol; NAcCys, *N*-acetylcysteine; PBS, 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4.

the dissolution of cholesterol gallstones by the solvent monooctanoin.

Methods

Human cholesterol gallstones were obtained at elective cholecystectomy or autopsy, washed carefully in distilled water to remove bile and debris, and then dried in a vacuum desiccator until constant weight was achieved. Ethyl alcohol (absolute), ethyl ether (anhydrous), chloroform, methanol, and glacial acetic acid were obtained from the Fisher Scientific Co. (Fairlawn, NJ). Silica gel G thin-layer chromatography (TLC) plates (20 × 20 cm, 250/μm) were obtained from Analtech Co. (Newark, DE) and activated by heating to 125°C for 1 h. Cesium chloride, density gradient grade, was purchased from Gallard Schlesinger (Carle Place, NY). Pronase *Streptomyces griseus* type VI, 2ME, *N*-acetylcysteine, sucrose, and Sepharose 2B were purchased from Sigma Chemical Co. (St. Louis, MO). Monooctanoin as Capmul 8210 was a generous gift from Ascot Pharmaceuticals, Inc. (Skokie, IL). Capmul 8210 was diluted with deionized water 10% (vol/vol), heated to 37°C and stirred for 4 h before use. Bilirubin was obtained from Porphyrin Products (Logan, UT) and the IXαisomer was purified according to the method of McDonagh and Assisi (16). This preparation contained >98% of the IXαisomer by high performance liquid chromatography analysis (17) (courtesy of Dr. Carl Goresky, McGill Medical School, Montreal, Canada).

Isolation of gallstone matrix. Cholesterol gallstones from 43 patients were dissolved separately in 200 ml of ethanol/ether (3:2, vol/vol) at 4°C for 72 h in the dark with vigorous mixing. The mixture was then centrifuged at 1,500 *g* for 1 h in a Beckman model J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, CA). The colorless supernatant was decanted and the insoluble pigmented residue was washed twice with 50 ml ethanol/ether (3:2, vol/vol) and recentrifuged. On average the ratio of stone weight to dissolution solvent was 15 mg/ml. The supernatant and washings were combined and assayed for cholesterol (18). The insoluble pigmented residue (matrix) was dried under N_2 in the dark, lyophilized, and weighed. The matrix was stored under N_2 at 4°C in the dark until further analyzed.

Bile pigment analysis. Bile pigments were extracted from 5 to 10 mg of lyophilized matrix into 2 ml of chloroform after solubilization by alkaline methanolysis according to the method of Fevery et al. (19). Pigments were separated and identified by TLC. 25–50 μl of the chloroform phase was spotted on activated silica G plates (125°C for 1 h), and developed in chloroform/methanol/acetic acid (97:2:1) in the dark. Fresh human hepatic bile and purified bilirubin were used as standards after extraction into chloroform by alkaline methanolysis. Total bilirubin content of the matrix was estimated spectrophotometrically by measuring the absorbance of the extracted pigments at 453 nm in chloroform. Since this extraction does not yield pure solutions of the pigments, quantitation by A_{453} may overestimate the concentration of bilirubin and should be interpreted as an approximate value. The relative quantities of unconjugated bilirubin, bilirubin monoglucuronides, and bilirubin diglucuronide were determined by scraping pigments from the TLC plates and eluting the pigments into chloroform. The absorbance of each pigment at 453 nm was determined and expressed as a percent of the total. The relative quantities of the IIIα, IXα, and XIIIα isomers in the bilirubin (unconjugated) fraction were determined by TLC on activated silica G plates developed in chloroform:acetic acid (100:1, vol/vol) according to the method of McDonagh and Assisi (20). The pigment bands were scraped and eluted into chloroform and their absorbance at 453 nm determined and expressed as a percent of the total.

Mucin analysis. Isolated gallstone matrix was highly insoluble in aqueous buffer, thus preventing direct biochemical analysis. To determine its composition, we placed samples of matrix (5–10 mg) from individual stones in 5 ml of 0.2 M NaCl and solubilized them by sonication with a Heat Systems–Ultrasonics Model W220F sonicator (Heat Systems–Ultrasonics, Inc., Farmingdale, NY). Samples were sonicated on ice for six 30-s intervals at 30 W. After sonication, samples were centrifuged at 2,000 rpm for 30 min to separate solubilized matrix (supernatant) from the insoluble residue (pellet). The pellets were then washed three times in de-ionized water, lyophilized, and weighed to determine percent original weight remaining after sonication. Total mucin content of solubilized matrix was determined by a modification of the periodic acid–Schiff assay of Mantle and Allen (21) previously reported from our laboratory (22).

The structure of the gallstone matrix solubilized by sonication was studied by Sepharose 2B exclusion gel chromatography and cesium chloride equilibrium density gradient ultracentrifugation. We have previously used these techniques to study mucin in gallbladder bile (23) and gallbladder mucosal homogenates (24). In addition, the binding of bile pigment to mucin was studied by sucrose density gradient ultracentrifugation.

Solubilization of gallstone matrix by reducing agents. Three identical samples of lyophilized matrix (10 mg) were obtained from each of 10 different gallstones and incubated in 5 ml of either 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS), PBS plus 2ME, or PBS plus NAcCys. The final concentration of the reducing agents was 1.0 M and the final pH of all solutions was adjusted to 7.4 with the addition of small amounts of 1.0 M NaOH. The mixtures were incubated at 37°C in the dark for 96 h with gentle agitation provided by a rocker platform (Bellco Glass Co., Vineland, NJ; model 3774, eight oscillations per minute). Solubilization of the matrix was determined by monitoring the release of pigment from the insoluble residue into the supernatant. Each mixture was centrifuged daily at 1,000 *g* for 20 min and an aliquot of the supernatant was obtained for determination of pigment (A453 nm). Results were compared for statistical significance with *t*-test. In addition, we analyzed the structure of the solubilized matrix using exclusion gel chromatography. Supernatants (3 ml) from matched samples of isolated gallstone matrix incubated for 4 d in either PBS alone or PBS and 2ME were chromatographed on a Sepharose 2B column as described above.

Effect of 2ME and NAcCys on the dissolution of cholesterol gallstones in vitro by monooctanoin. The effect of 2ME and NAcCys on cholesterol gallstone dissolution was examined by incubating matched whole gallstones in 5 ml of the cholesterol solvent monooctanoin (as Capmul 8210) with or without the addition of either reducing agent. The composition of solvents used in these experiments were as follows: solution A contained 3.5 ml monooctanoin + 1.5 ml ethanol; solution B contained 3.5 ml monooctanoin + 1.1 ml ethanol + 0.4 ml 2ME; solution C contained 3.5 ml monooctanoin + 1.5 ml of NAcCys in PBS. Ethanol was added to solutions A + B to achieve a final volume of 5 ml as in solution C and to compensate for the effect of 2ME on the viscosity of monooctanoin. The final concentrations of 2ME and NAcCys were 1.0 M. The apparent viscosities of these solutions were measured using a low shear falling ball viscometer (25), which was calibrated with glycerol standards of known viscosities. The relative ability of these solutions to solubilize purified cholesterol monohydrate crystals was determined by incubating an excess of purified cholesterol monohydrate crystals (500 mg) in 2 ml of solution A, B, or C at 37°C in the dark for 96 h with gentle agitation provided by a rocker platform. The solutions were filtered through a 0.45 U Gelman filter, centrifuged at 2,000 *g* for 60 min, and the cholesterol content of the supernatant determined (18).

To study the effect of reducing agents on stone dissolution, we obtained gallstones from patients whose gallbladders contained multiple stones of similar size and shape. Recent studies have demonstrated that matched gallstones from the same gallbladder have nearly identical compositions and dissolution rates in cholesterol solvents (26). Matched stones were incubated separately in 5 ml of solution A, B, or C at 37°C with gentle agitation provided by a Belles Rocker platform at eight oscillations per minute. The initial stone weights were 339±59 mg (solution A), 330±59 mg (solution B), and 368±61 mg (solution C). Dissolution rates were determined by removing stones from their incubation vials daily, air drying for 1 h and then weighing. Results are expressed as the percent of initial weight remaining at daily intervals. The solutions were not changed during the 4-d incubation.

Results

Isolation and composition of gallstone matrix. All the cholesterol gallstones studied dissolved within 72 h in an ethanol-ether (3:2 vol/vol) solution. After dissolution, centrifugation yielded an optically clear supernatant and a granular black-brown sediment representing the pigmented matrix. Solitary large gallstones ($n = 20$, diameter > 1.0 cm, average weight 5,640±578 mg) contained significantly more matrix and significantly less cholesterol than multiple small stones that were morphologically similar ($n = 23$; average 59 stones per patient; average weight 243±35 mg). Matrix contributed 12.5±3.2% vs. 3.5±0.7% ($P < 0.02$) as percent of initial weight and cholesterol contributed 79.6±4.2% vs. 85.8±2.2% ($P < 0.05$) of initial weight for solitary large and multiple small stones, respectively. Approximately 10% of stone weight for each group was not accounted for after totalling the cholesterol and matrix content. This probably reflects losses due to handling large solvent volumes for dissolution, as well as small amounts of bile salts, phospholipid, and calcium salts that were not measured.

Bile pigments in the matrix were examined by extraction using alkaline methanolysis and separation by TLC (Fig. 1). The major pigment in matrix was unconjugated bilirubin (83.4%) with smaller amounts of bilirubin monoglucuronides (15.0%) and bilirubin diglucuronides (1.4%). Trace amounts (<1% of total) of more polar pigments were present but not further characterized. These probably represent oxidative degradation products of the native bile pigments. Analysis of unconjugated bilirubin isomers by TLC (not shown) revealed the following distribution: IIIα, 7.9%; IXα, 86.4%; and XIIIα, 5.7%. These figures are mean values for cholesterol gallstones from 15 patients. The predominance of the naturally occurring IXα isomer suggests that the extraction and separation procedures resulted in only minor amounts of degradation of the pigments.

Sonication solubilized an average of 35.6% of the isolated gallstone matrix. Mucin contributed 11% and bilirubin 1.3% of the weight of the solubilized stone matrix. These values may not be representative of the whole matrix, however, because of incomplete solubilization by sonication. Attempts to characterize the nonmucin proteins of the gallstone matrix were unsuccessful because SDS polyacrylamide gel electrophoresis failed to demonstrate any discrete protein bands despite loading up to 200 mg of matrix per lane (not shown). The composition of the remainder of the partially solubilized matrix is unknown. Calcium salts may contribute significantly to the matrix but were not analyzed in this study.

Analysis of gallstone matrix structure. Sepharose 2B exclusion gel chromatography of sonicated gallstone matrix demonstrated a large glycoprotein peak eluting in the void volume of the column with an apparent molecular weight in excess of 2×10^6. As shown previously (24, 27), this is a typical elution profile for gallbladder mucin. The pigment in the matrix eluted in two peaks, one co-eluting with the glycoprotein in the void volume and a second smaller peak eluting at the bed volume. An average of 75.4% of the pigment eluted in the void volume and 24.6% is the bed volume when the solubilized matrix of 11 different cholesterol stones were analyzed by Sepharose 2B column chromatography. A representative column chromatogram is shown in Fig. 2.

Cesium chloride equilibrium density gradient ultracentrifugation of the solubilized matrix revealed two glycoprotein peaks (Fig. 3). The major glycoprotein peak (representing >80% of the glycoprotein on analysis of five different stones) eluted at a buoyant density of 1.45–1.50 g/ml, which is typical

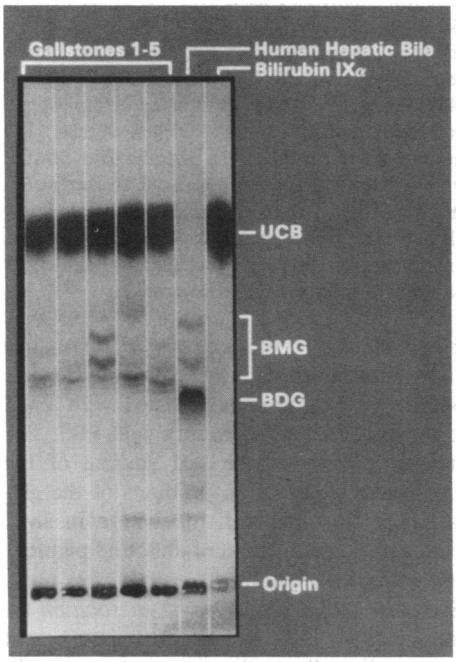

Figure 1. TLC of pigments in gallstone matrix. Pigments in the isolated gallstone matrix of five different stones were extracted into chloroform after alkaline methanolysis (see Methods). Samples of human hepatic bile and purified bilirubin IXα were extracted in the same manner and used as standards. 25–50 μl of the chloroform phase of each sample were spotted on an activated Silica G plate and eluted in chloroform/methanol/acetic acid (97:2:1) under N_2 in the dark. Unconjugated bilirubin (UCB), bilirubin monoglucuronides (BMG), and bilirubin diglucuronides (BDG) were identified in the gallstone matrix by comparison with the standards of human hepatic bile and bilirubin IXα.

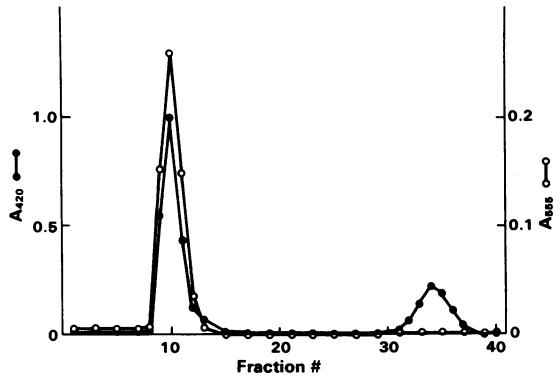

Figure 2. Sepharose 2B column chromatography of gallstone matrix. The matrix was isolated from cholesterol gallstones as described in Methods and solubilized by sonication in 0.2 M NaCl and 0.03% sodium azide. The solubilized matrix (3 ml) was loaded on Sepharose 2B column 40 × 1.0 cm^2 and eluted with 0.2 M NaCl, 0.03% sodium azide with a flow rate of 10 ml/h at 4°C. Fractions (2 ml) were analyzed for pigment by A420 and for mucin by the periodic acid–Schiff method (A555).

for gallbladder mucin. The large apparent molecular weight (>2 × 10^6 [Fig. 2]) and the high buoyant density (1.45–1.50 g/ml, Fig. 3) are highly diagnostic of mucous-type glycoprotein from gallbladder (24, 27) and other epithelia. Bile pigments eluted exclusively in the low density fractions of cesium chloride gradient (Fig. 3, fractions 1–3) separate from the mucin peak (fractions 5 and 6). A small glycoprotein peak was also present in the low density fraction, but was not further analyzed.

To confirm the presence of a mucin bilirubin complex in the isolated gallstone matrix, we studied the solubilized matrix by sucrose gradient ultracentrifugation with and without protease digestion. Sucrose density ultracentrifugation of solubilized matrix incubated with heat-killed pronase (Fig. 4, top) demonstrated a single glycoprotein peak eluting in the most dense fraction. Two pigment peaks were present, one eluting with mucin (Fig. 4, fraction 10) and a second peak eluting in the least dense fractions. The co-elution of mucin and bilirubin in Sepharose 2B column chromatography (Fig. 2) and during sucrose density gradient ultracentrifugation confirms the presence of a mucin-bilirubin complex in the gallstone matrix. Incubation of the sonicated matrix with active pronase (Fig. 4, bottom) resulted in a fourfold decrease in the amount of mucin and bilirubin eluting in the most dense fraction of the gradient (fraction 10). Concomitantly, a glycoprotein peak appeared in the low density fraction of the gradient (fractions 2–4) with a proportional rise in the amount of bilirubin eluting in the low density fractions. This finding indicates that the mucin-bilirubin complex in the solubilized matrix is susceptible to proteolytic degradation.

Solubilization of the gallstone matrix by reducing agents. Previous studies of tracheal (28, 29), gastrointestinal (30, 31), and gallbladder (24, 27) mucins indicate that exposure of these glycoproteins to thiol-reducing agents causes solubilization of mucus gel and disaggregation of the soluble mucin polymer. We therefore examined the ability of two reducing agents, 2ME and NAcCys, to solubilize the isolated gallstone matrix. Both reducing agents significantly increased the solubilization of the isolated gallstone matrix when compared to the control solution of PBS (Table I). This effect was evident after the first day and increased progressively over the subsequent 4 d of observation. Sepharose 2B column chromatography of the

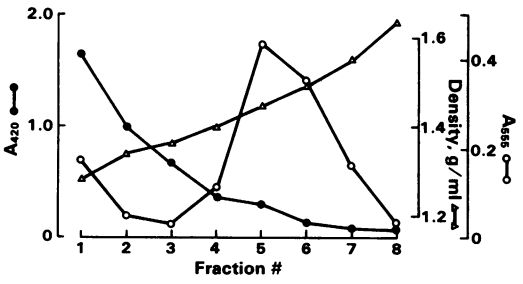

Figure 3. Cesium chloride equilibrium density gradient ultracentrifugation of gallstone matrix. Cesium chloride was added to samples of solubilized gallstone matrix to achieve a final concentration of 60 g/100 ml. Samples (8 ml) were centrifuged at 300,000 *g* for 24 h at 4°C in a Ti 70.1 fixed angle rotor (Beckman Instruments, Inc.). The gradients were fractionated by aspiration of 1-ml samples from the top of the gradient which were analyzed for pigment (A420), mucin by periodic acid–Schiff (A555). Densities were determined gravimetrically.

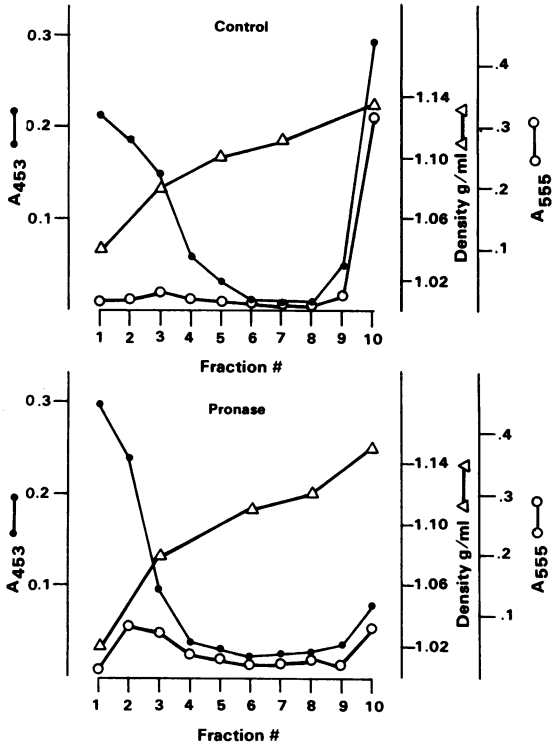

Figure 4. Sucrose density gradient ultracentrifugation of gallstone matrix. Solubilized gallstone matrix was incubated with either heat-killed or active pronase (*Streptomyces griseus* type VI) in 0.1 M sodium acetate, 0.03% Na-azide buffer, pH 6.5, at 37°C for 72 h. Samples (0.8 ml) were then layered on a 25% (wt/vol) sucrose step gradient (4.2 ml) and centrifuged in a SW55 swinging bucket rotor at 40,000 *g* for 4 h at 4°C. The gradient was fractionated by aspiration of 0.5 ml samples from the top of the gradient which were then analyzed for pigment (A420) and mucin by the periodic acid–Schiff method (A555) after dialysis against deionized water. Densities were determined gravimetrically. The top panel shows the results obtained with a sample of gallstone matrix incubated with heat-killed pronase, while the bottom panel shows the results after incubation of an identical aliquot of solubilized matrix with active pronase.

incubation supernatant (Fig. 5) showed an approximately sixfold increase in the amount of mucin recovered in the void volume of the column in the sample incubated with 2ME when compared to the sample incubated only with PBS. The small glycoprotein peak present at the bed volume of the column probably represents degradation products of the glycoprotein, but was not further analyzed. Incubation in 2ME also caused a large increase in the amount of unbound pigment eluting at the bed volume of the columns.

Effect of 2ME and NAcCys on gallstone dissolution by monooctanoin. Monooctanoin (as Capmul 8210) is a potent cholesterol solvent used clinically to dissolve cholesterol gallstones impacted in the common bile duct. Capmul 8210 contains 70% glyceryl-1-monooctanoate, 30% glyceryl-dioctonoate, and trace amounts of glyceryl trioctanoate and octanoic acid (32). Glyceryl-1-monoctanoate (monooctanoin) is primarily responsible for the cholesterol solubilizing ability of Capmul 8210. In view of the ability of 2ME and NAcCys to solubilize the gallstone matrix, we tested their ability to enhance dissolution of intact gallstones in monooctanoin. Table II shows our results when ten sets of matched stones were incubated in either monooctanoin, monooctanoin plus 2ME, or monooc-

Table I. Effect of 2ME and NAcCys on Solubility of Cholesterol Gallstone Matrix

Solvent	Absorbance at 453 nm of supernatant			
	Day 1	Day 2	Day 3	Day 4
PBS	0.387±0.098	0.599±0.164	0.658±0.176	0.793±0.155
PBS plus 2ME (1 M)	1.243±0.473	2.373±0.791*	2.752±0.738‡	3.136±0.732§
PBS plus NAcCys (1 M)	1.007±0.294*	1.521±0.398‡	1.766±0.408§	2.143±0.425§

Three identical samples of the lyophilized matrix (10 mg) from cholesterol gallstones of 10 separate patients were incubated in 5 ml of solvent as described in Methods. At daily intervals the supernatants were examined spectrophotometrically (A_{453}) to determine release of pigment from the insoluble matrix. Values represent the mean±SEM. * $P < 0.05$ vs. PBS. ‡ $P < 0.02$ vs. PBS. § $P < 0.01$ vs. PBS.

tanoin plus NAcCys. Both reducing agents caused a statistically significant increase in dissolution of ten sets of matched cholesterol stones, despite the fairly rapid dissolution of stones in monooctanoin alone.

Bogardus (33) has reported that decreasing the viscosity of monooctanoin by addition of water significantly increases its solvent property for cholesterol. We therefore measured the effect of reducing agents on viscosity of monooctanoin and also on the ability of monooctanoin to dissolve pure cholesterol monohydrate. Addition of 2ME to monooctanoin had no significant effect on apparent viscosity or cholesterol solubility when compared to the control solution of monooctanoin containing ethanol (viscosity: 4.2 vs. 4.1 centipoise; cholesterol solubility 62 vs. 64 mg/ml for 2ME and control, respectively).

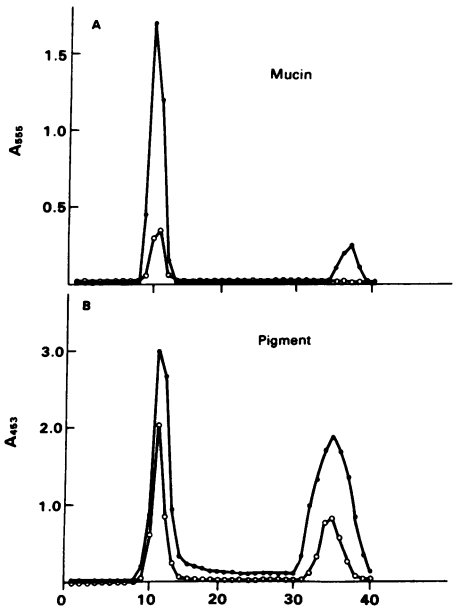

Figure 5. Sepharose 2B column chromatography of gallstone matrix after incubation with 2ME. Samples of lyophilized gallstone matrix from the same stone were incubated in 5 ml of 0.01 M sodium phosphate 0.15 M NaCl, pH 7.4 (PBS), or PBS plus 1.0 M 2ME. Samples were incubated at 37°C in the dark under N_2 for 96 h and then centrifuged at 1,000 rpm for 20 min. Supernatants (3 ml) from the incubation mixtures were then chromatographed on a Sepharose 2B column 40 × 1 cm^2 eluted with 0.2 M NaCl and 0.3% sodium azide at a flow rate of 10 ml/h at 4°C. Fractions (2 ml) were analyzed for mucin by the periodic acid–Schiff method (A555) (*top*) and pigment (A420) (*bottom*). ○, PBS; ●, PBS + 2ME.

NAcCys, on the other hand, increased viscosity (17.9 centipoise) and decreased cholesterol solubility (48 mg/ml) in monooctanoin. Thus, the two reducing agents had opposite effects on the viscosity and solvent properties of monooctanoin for cholesterol, but a similar effect on the solubilization of the isolated gallstone matrix (Table I) and on the dissolution of intact cholesterol gallstones (Table II).

Discussion

Previous studies of gallstone composition and structure have relied primarily on indirect physical chemical techniques (11, 12). This study presents a novel biochemical analysis of the composition and structure of the cholesterol gallstone matrix. Three major findings are presented: (*a*) a macromolecular complex of mucin and bilirubin has been identified as a structural component of the gallstone matrix; (*b*) the thiol-reducing agents 2ME and NAcCys partially solubilize the isolated gallstone matrix; and (*c*) these agents accelerate the dissolution of intact cholesterol gallstones by the solvent monooctanoin.

Absolute quantitation of the composition of the gallstone matrix was not possible because sonication solubilized only one third of the isolated matrix by weight. Mucin and bilirubin contributed a significant portion of the matrix solubilized by sonication (11.0% and 1.3%, respectively), but their relative contribution to the total matrix could not be determined. Although we were unable to identify discrete protein bands on SDS polyacrylamide gel electrophoresis, our results do not exclude the presence of other proteins in the matrix, particularly that portion not solubilized by sonication.

TLC (Fig. 1) of bile pigments in the gallstone matrix confirmed previous observations (8, 9) that bilirubin was the major pigment in cholesterol gallstones. The relative amounts of bilirubin, bilirubin monoglucuronides, and bilirubin diglucuronide in gallstone matrix was remarkably similar to the distribution of bilirubin and bilirubin conjugates reported in biliary sludge (15). The close similarity of bile pigment composition in sludge and matrix gives further support to the speculation that biliary sludge is a precursor of gallstones (4), and may serve as the nidus for cholesterol crystal nucleation and gallstone formation. Inasmuch as gallbladder sludge is also rich in gallbladder mucin, we speculate that the pigmented matrix of cholesterol gallstones may originate from biliary sludge.

The cause for the grossly disproportionate amounts of unconjugated and monoconjugated bilirubin in biliary sludge

Table II. Effect of 2ME and NAcCys on Ability of Monooctanoin to Dissolve Human Cholesterol Gallstones

Solvent	% Initial weight			
	Day 1	Day 2	Day 3	Day 4
Monooctanoin	65.85±0.06	43.24±0.06	30.41±0.05	21.38±0.04
Monooctanoin plus 2ME	59.55±0.06*	33.15±0.05‡	19.43±0.04§	10.02±0.03§
Monooctanoin plus NAcCys	68.66±0.06	35.15±0.08	19.80±0.06‡	10.38±0.05§

Matched human cholesterol gallstones (three stones each from 10 separate patients) were incubated in 5 ml of each solvent as described in Methods. The stones were weighed each day after drying, and the results are expressed as the percent of initial stone weight remaining at each time point. Values represent the mean±SEM. * $P < 0.05$ vs. monooctanoin. ‡ $P < 0.02$ vs. monooctanoin. § $P < 0.01$ vs. monooctanoin.

(15) and in gallstone matrix (Fig. 1) when compared to the bulk aqueous phase of bile is unknown. Both increased excretion of bilirubin monoglucuronide (34) and deconjugation of bilirubin diglucuronide in the biliary tract (35) have been proposed to explain this phenomenon. We have previously reported that purified bovine gallbladder mucin bound bilirubin IXα, and that this mucin bilirubin complex was dissociated by ultracentrifugation in 60% cesium chloride but not by ultracentrifugation in 10–40% linear sucrose gradients (23). In addition, we showed that bilirubin competitively displaced the fluorescent hydrophobic probe 1-anilino-8-naphthalenesulfonate from a hydrophobic domain on the nonglycosylated, protease-sensitive portion of the mucin peptide core (24). In the presence of gallbladder stasis, mucus gel in the gallbladder lumen may selectively sequester the more hydrophobic bile pigments (unconjugated bilirubin and bilirubin monoglucuronides) from the bulk aqueous phase of bile. Womack et al. (6) reported a similar sequestration of cholesterol in gallbladder mucus gel compared to the aqueous phase of bile.

Sepharose 2B column chromatography (Fig. 2) demonstrated a macromolecular complex ($M_r > 2 \times 10^6$) of mucin and bile pigment in the void volume of the column. Mucin was further identified as a glycoprotein peak with a buoyant density of 1.45–1.50 g/ml on equilibrium density gradient ultracentrifugation in cesium chloride (Fig. 3). The dissociation of bilirubin and mucin during cesium chloride density gradient ultracentrifugation suggests that noncovalent interactions are involved in complex formation. The chaotropic effects of high salt concentration and the steep density gradient formed (1.35–1.75 g/ml) by ultracentrifugation of 60 g/100 ml cesium chloride are probably responsible in part for the disruption of mucin-bilirubin binding. On the other hand, the nonionic nature and shallow density gradients formed with sucrose ultracentrifugation do not disrupt the mucin-bilirubin complex (23). Using this technique, we have confirmed the presence of mucin-bilirubin complex in gallstone matrix and have demonstrated its degradation by prolonged proteolysis (Fig. 4). The susceptibility of the mucin-bilirubin complex in the isolated gallstone matrix to proteolysis appears to be analogous to the previously reported digestion of the matrix of common bile duct stores by proteolytic enzymes (36).

The thiol-reducing agents 2ME and NAcCys are known to solubilize native mucus gel, as well as to disaggregate the polymer structure of purified soluble mucin (37). Starkey et al. (30) have proposed that 2ME depolymerizes soluble gastric mucin by splitting disulfide bridges that cross-link mucin subunits. Our laboratory has reported that 2ME disaggregates purified soluble gallbladder mucin, although the presence of sulfhydryl bridges was not confirmed (24). The biochemical mechanism by which thiol-reducing agents solubilize native mucus gel is not known. We hypothesized that the poor aqueous solubility of the isolated gallstone matrix may in part be due to extensive polymerization of its mucin content, and we therefore examined the ability of 2ME and NAcCys to solubilize the matrix. Both reducing agents significantly ($P < 0.01$) increased the solubilization of the gallstone matrix (Table I) when compared to PBS. Analysis of the incubation supernatants by Sepharose 2B exclusion gel chromatography (Fig. 5) revealed that 2ME caused a sixfold increase in the amount of mucin eluting in the void volume as well as a large increase in the amount of unbound bilirubin eluting in the bed volume of the column. This effect of 2ME on the binding of mucin and bilirubin is analogous to, although not identical with, that which we described for highly purified soluble gallbladder mucin (23). After incubation with 2ME the soluble mucin polymer was disaggregated and eluted in the included volume of a Sepharose 2B column rather than in the void volume as in this study. The reason for the difference in response of soluble mucin and mucin in the insoluble matrix of gallstones to incubation with 2ME is not known. However, the biochemical basis for this difference may be of importance in the future understanding of polymerization in mucus gel and in the formation of biliary sludge.

Finally, we tested the hypothesis that the pigmented matrix was a functionally important component of cholesterol gallstone structure with reference to gallstone dissolution by cholesterol solvents. The dissolution rates of matched cholesterol gallstones incubated in monooctanoin were compared with or without the addition of a reducing agent. Both reducing agents significantly ($P < 0.01$) increased the dissolution rate of cholesterol gallstones (Table II). Moreover, a dark granular shell possibly representing layers of the gallstone matrix was visible on the surface of stones as they dissolved in monooctanoin but not on stones incubated with 2ME (not shown). This observation suggests that reducing agents may enhance gallstone dissolution by solubilizing layers of the gallstone matrix interposed between layers of crystalline cholesterol, thereby increasing the contact of the solvent with crystalline cholesterol. The apparent resistance of the gallstone matrix to dissolution in cholesterol solvents may be a possible explanation for the common clinical occurrence of partial gallstone dissolution (38).

In conclusion our results indicate that the human cholesterol gallstone matrix contains a macromolecular complex of mucin and bilirubin. This matrix can be partially solubilized by thiol-reducing agents and appears to have functional significance in overall gallstone structure. Previous studies of gallstone disso-

lution have focussed almost exclusively on the solubilization of crystalline cholesterol with bile salts (38) or other cholesterol solvents (32). Such strategies have yielded less than optimal results. We propose that future investigations should consider the composition and structure of the gallstone matrix when devising strategies for gallstone dissolution.

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