Biliary haptoglobin, a potent promoter of cholesterol crystallization at physiological concentrations

Gunji Yamashita, Stefano Ginanni Corradini, Roger Secknus, Arimachi Takabayashi; Carlumandarlo Williams, Leigh Hays, Ann L. Chernosky, and R. Thomas Holzbach[1]

Gastrointestinal Research Unit, Research Institute, and Department of Gastroenterology, Cleveland Clinic Foundation, One Clinic Center, 9500 Euclid Avenue, Cleveland, OH 44195-5218

Abstract Background/Aims: Several proteins present in human bile have been reported to promote cholesterol crystallization and thus are potentially important in the formation of cholesterol crystals as the initial stage in gallstone pathogenesis. To be physiologically relevant, such proteins must either be present in high concentration in bile or have a potent promoting activity. The current study explored several of the more abundant but unexamined biliary proteins based upon their also having sufficiently high serum concentrations that antibodies were available for both their isolation and quantitation. Methods: Protein purification was accomplished by immunoaffinity chromatography of bile followed by delipidation. Con A affinity chromatography of bile was used to obtain the bound fraction, a portion of which was delipidated. Crystallization-promoting activity of both the purified proteins and Con A-bound glycoprotein fractions (CABG) was measured by a photometric crystal growth assay. A competitive antibody-capture ELISA assay was developed to measure concentrations of α_1-antitrypsin, transferrin, and haptoglobin in native bile. Results: At their relevant physiological concentrations, biliary haptoglobin (15 μg/ml) had a crystallization-promoting activity twice that of the biliary IgM (75 μg/ml) used as a reference standard ($P < 0.05$). Biliary transferrin (20 μg/ml) had only modest promoting activity ($P < 0.05$). Biliary α_1-antitrypsin (50 μg/ml), by contrast, showed no promoting activity. Delipidation of the CABG fraction decreased its promoting activity by 75%. Biliary haptoglobin accounts for about 30% of delipidated total CABG-promoting activity. ■ Conclusions: Biliary haptoglobin at its physiological concentration has a highly potent crystallization-promoting activity and thus becomes a candidate for major attention in understanding gallstone pathogenesis. Biliary lipids associated with CABG account for a major portion of the cholesterol-crystallization-promoting activity of this fraction. **—Yamashita, G., S. G. Corradini, R. Secknus, A. Takabayashi, C. Williams, L. Hays, A. L. Chernosky, and R. T. Holzbach.** Biliary haptoglobin, a potent promoter of cholesterol crystallization at physiological concentrations. *J. Lipid Res.* 1995. **36:** 1325–1333.

Supplementary key words cholelithiasis • biliary haptoglobin • cholesterol-crystallization promoting activity • enzyme-linked immunosorbent assay

It is well known that human bile contains several cholesterol crystallization-promoting non-mucin glycoproteins; the first step in purification of these glycoproteins is conventionally achieved by concanavalin A (Con A) Sepharose chromatography. Con A Sepharose chromatography with subsequent steps has been used to isolate and identify the immunoglobulins IgG, IgM, and IgA, α_1-acid glycoprotein, aminopeptidase N, and other possible crystallization promoters (1–8). But, the various post-purification steps involved in separation and isolation of the complex biliary protein mixture found in the Con A-bound fraction have proved to be complicated and difficult. Perhaps in part because of this complexity, the major contributing proteins or other components necessary to explain most of the promoting activity of the total Con A fraction remain unknown. Consequently, either nonspecifically Con A-bound lipids or a recently reported but as yet incompletely characterized lipoprotein particle derived from the bound-Con A fraction may possibly account for this large discrepancy (9).

In light of these considerations, we decided to try an empirically based method to isolate and study several previously unexamined human biliary proteins on the basis of the following rationale. First, almost all biliary glycoproteins are also found in serum, but in much greater relative concentrations, on the order of about 50:1 (mass:mass). For several of these relatively more abundant serum proteins, antibodies are conveniently available. These proteins can also reasonably be expected to be

Abbreviations: Con A, concanavalin A; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; M_r, relative molecular mass; STDC, sodium taurodeoxycholate; CCG, cholesterol crystal growth assay; CABG, concanavalin A-bound glycoprotein.

[1]To whom correspondence should be addressed.

present in bile in concentrations approximately proportionate to those found in serum; consequently, the comparative abundance of these proteins in serum should also reflect those of comparatively greater abundance in bile and therefore be of potentially greater pathophysiologic importance. The issue of protein concentration versus potency is obviously a crucial consideration in attempting to rank the relative importance of putative crystallization promoters.

Therefore, in the present study we first directly immunoisolated three human biliary proteins that have a comparatively high serum abundance. Next, we developed an enzyme-linked immunosorbent assay (ELISA) method for immunoquantitation of the "physiological" concentrations for each of these proteins in bile. Last, we measured their comparative potencies with a kinetic photometric assay for crystal growth. The three proteins obtained and assayed were found to show crystallization-promoting activity at their physiological concentrations, ranging from minimal to marked, as follows: haptoglobin >>> transferrin >> α_1-antitrypsin. Delipidation of the CABG fraction decreased its promoting activity by 75%. Biliary haptoglobin was found to account for about 30% of the delipidated total CABG-promoting activity.

MATERIALS AND METHODS

Chemicals and antibodies

Sodium taurocholic acid and sodium taurodeoxycholic acid were purchased from Calbiochem (San Diego, CA). Egg yolk phosphatidylcholine was obtained from Lipid Products (S. Nutfield, Surrey, UK). Cholesterol was purchased from Eastman Kodak Co. (Rochester, NY). AminoLink™ coupling gel was purchased from Pierce Chemical Co. (Rockford, IL). Concanavalin A (Con A) Sepharose columns were purchased from LKB/Pharmacia Biotechnology Inc. (Piscataway NJ). Human serum α_1-antitrypsin, transferrin, and haptoglobin standards were obtained from Sigma Chemical (St. Louis, MO), as was *p*-nitrophenyl phosphate. Four to 20% gradient SDS-polyacrylamide gels were obtained from Bio-Rad Laboratories (Richmond, CA). All other chemicals were of the highest grade commercially available.

Polyclonal antibodies to human α_1-antitrypsin, transferrin, haptoglobin, IgM (μ chain), complement 3, ceruloplasmin, and α_2-macroglobulin were purchased from Sigma. Alkaline-phosphatase-labeled antibodies to rabbit and goat IgG were purchased from Sigma.

Bile collection and processing

With the approval of the Cleveland Clinic Foundation's Research Projects and Institutional Review Committee Regarding Human Studies, human gallbladder bile samples from 17 gallstone-free patients (controls) and 18 patients with cholesterol gallstones were obtained by aspiration of the gallbladder during surgery for cholelithiasis or during laparotomy for a variety of nonbiliary conditions; the collection procedures used and stone criteria are described in previous studies (10, 11). None of the cholesterol gallstone patients had signs of acute cholecystitis during the 4 months before surgery. Bile was stored at −80°C until processing.

Assay procedures

Protein concentrations of the native bile samples were measured by amino acid analysis using a Beckman amino acid analyzer, model 7300, (Beckman Instruments Inc., Fullerton, CA). The concentrations of purified proteins were measured by the Coomassie blue dye-binding assay using a commercially available kit (Bio-Rad Laboratories, Richmond, CA); bovine serum albumin was used as a standard (12). Because reactivity of different purified proteins with the Coomassie blue dye-binding assay varies and can be considerably different from that of albumin used as a standard, the data from use of the conventional Coomassie blue assay using an albumin standard seldom represents the actual protein concentration. Therefore, protein concentrations estimated by the dye-binding assay were adjusted to results obtained with use of amino acid analysis (n = 3) on identical samples of each purified protein. From these comparative studies, correction factors based on amino acid analysis values divided by Coomassie blue dye-binding assay values for each of the proteins were obtained. For α_1-antitrypsin, transferrin, haptoglobin, and IgM, the derived correction ratios were 2.68, 0.89, 1.04, and 1.96, respectively. The concentration of 100 μg/ml for IgM by the Coomassie-blue assay, for example, was 196 μg/ml. Such derived correction ratios were then applied to all estimates of purified proteins used in the present studies, including all ELISA data.

Total bile acid concentrations were measured by the 3α-hydroxysteroid dehydrogenase method (13). Phospholipid concentrations were determined by a kit modification of the enzymatic method of Takayama (PL-Kit K, Nippon Shoji Kaisha, LTD, Osaka, Japan) (14, 15). Cholesterol concentrations were determined enzymatically using a commercially available assay kit (Boehringer-Mannheim Corp., Indianapolis, IN) (16). When the cholesterol and phospholipid concentrations of samples for the cholesterol crystal growth assay were measured, samples were concentrated 40-fold. Maximal sensitivities for the cholesterol and phospholipid concentration measurements were less than 1 μmol/l and 8 μmol/l, respectively, when applied to the solution used in the cholesterol crystal growth assay.

Immunoaffinity chromatography

Pooled native gallbladder bile from three gallstone-free controls and three cholesterol gallstone patients was

ultracentrifuged (Model L5-50, with a 50.3 Ti rotor, Beckman Instruments, Fullerton, CA) at 100,000 *g* for 1 h to remove solid crystals as well as mucus glycoprotein.

Seven immunoaffinity columns (anti-haptoglobin, anti-transferrin, anti-α_1-antitrypsin, anti-ceruloplasmin, anti-α_2-macroglobulin, anti-complement-3, and anti-IgM) were prepared. Approximately 5 mg to 10 mg of each antibody was coupled to 5 ml of AminoLink™ gel according to manufacturer's specifications.

Three ml of pooled bile was applied to 5 ml of each antibody column, which had been equilibrated with Tris-buffered saline ([TBS], 25 mmol/l, Tris-HCl, 150 mmol/l NaCl, pH 7.4). Each column was allowed to equilibrate overnight at 4°C to completely absorb the antigen from the applied sample. Each column was then washed with 20 column volumes of TBS containing 10 mmol/l of sodium taurocholate and next washed with 10 column volumes of TBS without 10 mmol/l sodium taurocholate. The bound proteins (haptoglobin, transferrin, α_1-antitrypsin, ceruloplasmin, α_2-macroglobulin, complement-3, and IgM) were then eluted from the column with 5 column volumes of 100 mmol/l glycine-HCl, pH 2.6, into tubes containing 1 mol/l Tris-HCl, pH 9.5, to immediately neutralize the acidic elution buffer. The bound fractions were then diafiltered with TBS by Amicon ultrafiltration (Amicon, Beverly, MA). After filtration with a 0.22-μm filter, for comparison the same protein solutions were delipidated by performing Centricon-30 ultrafiltration (Amicon, Beverly MA) 10 times with the following: TBS, TBS containing 10 mmol/ml sodium taurodeoxycholate (STDC), and 50% *tert*-butanol. The buffer was then changed to 2.5 mmol/l Tris-HCl, 15 mmol/l NaCl, pH 7.4, using Centricon-30 ultrafiltration. After additional filtration with a 0.22-μm filter, the solutions were concentrated using a Speedvac (Savant Instruments Inc., Farmingdale, NY) just before assaying for cholesterol crystal growth. The concentration of inorganic salts in the protein solution was then adjusted to TBS with distilled water and TBS. The content of cholesterol and bile acid in the protein solutions was below the limit of detectability.

Electrophoresis and Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% gradient gels was performed using the standard technique described by Laemmli (17). After fixation, the gels were stained with silver by the method of Morrissey (18).

Western blot analysis for each protein was performed using a polyclonal antibody to each protein. After each protein was separated by SDS-PAGE, it was transferred to a polyvinylidene difluoride membrane (Immobilion P, Millipore Corp., Bedford, MA) in 48 mmol/l Tris, 39 mmol/l glycine, and 20% methanol, pH 9.2, using a semi-dry blot electrophoretic transfer system (Trans-Blot SD) (Bio-Rad Laboratories, Richmond, CA). After the transfer, the membranes were blocked with 3% bovine serum albumin in TBS. The membranes were then incubated in anti-protein antibody solution followed by incubation in an alkaline phosphatase-labeled secondary antibody solution, with extensive washing in TBS between each incubation. Finally, the blot was developed by exposing the membrane to 154 mmol/l 5-bromo-4-chloro-3-indolyl phosphate, 77 mmol/l nitroblue tetrazolium in 100 mmol/l Tris-HCl, 100 mmol/l NaCl, and 5 mmol/l $MgCl_2$, pH 9.5.

Quantitation of specific proteins by ELISA

Optimal conditions for the ELISAs were determined by multistep titration of different reagents in the assay (19). Briefly, microELISA plates (Corning Glass Works, Corning, NY) were coated with each antigen (5 μg/ml) and incubated for 2 h at room temperature. The purity of the protein standards was assessed by SDS-gel using silver staining. No contaminating protein bands were detectable. These antigen-coated plates were then blocked overnight with blocking buffer consisting of phosphate-buffered saline and Tween (150 mmol/l NaCl, pH 7.4; 0.2% Tween 20). Each antigen standard or diluted (1:70 using blocking buffer) bile sample was added to the wells, followed by the primary antibody (rabbit anti-human haptoglobin, rabbit anti-human transferrin, and rabbit anti-human α_1-antitrypsin) and incubated for 2 h at 37°C. The concentration range of the standards used was between 0.015 μg/ml and 2.5 μg/ml. A 1:1000 dilution of the labeled secondary antibody (goat anti-rabbit IgG alkaline phosphatase conjugate or rabbit anti-goat IgG alkaline phosphatase conjugate) was then added to the wells and incubated for 2 h at 37°C. After incubation, absorbance was read with an automatic plate reader (Microplate Autoreader EL311, Biotek Instruments Inc., Windooski, VT), using a reference wavelength of 405 nm. All assays were performed in triplicate. Addition "spiking" experiments were performed to determine the accuracy for each of the ELISAs using bile samples previously depleted of the specific protein being measured. Each protein-depleted bile sample aliquot was obtained by loading pooled native bile onto an immunoaffinity column for each specific protein. Each "depleted" unbound fraction was then concentrated to its original volume. This procedure was then repeated. Each of the specific proteins was no longer detectable in the final "depleted" unbound fraction using Western blotting. Twenty μg of each specific protein was added to 1 ml bile from which it had been previously depleted. Each "spiked" bile sample was then measured by ELISA using three different dilutions (1:35, 1:70, and 1:140) (n = 3) to assess the effect of potential interfering substances contained in bile, e.g., lipids. The recovery of each of the specific proteins in the addition "spiking" experiments ranged between 85 and 115%.

When protein concentrations obtained by use of the ELISAs were compared, the protein concentration found in a given sample was divided by its total lipid concentration to derive an operational relative (%) value for the protein concentration. This was necessary in order to minimize the effect of the known wide physiological intersample variability of total solute concentrations reflecting differences in sample dilution. This variable affects all solutes, including those of greatest relevance in this study, i.e., proteins and lipids. The absolute concentration of a given specific protein or total biliary lipids is strongly affected by this variable. For this reason, as the protein-to-lipid ratio is most likely the key determinant of interactions important to the proteins as measured in this study, we believe that this relative (%) value is of greater importance than the absolute protein value on which it is based.

Cholesterol crystal growth (CCG) assay

The effects of immunopurified proteins on cholesterol crystallization kinetics were assessed with a photometric assay (20). Aliquots of stock solutions of cholesterol, egg lecithin, and sodium taurocholate were mixed to construct a supersaturated model bile having a cholesterol saturation index (20) of 1.4, a total lipid concentration of 10–12.5 g/dl, and a bile acid-to-phospholipid ratio of 4.4. The lipid mixture was evaporated until completely dry and then lyophilized and resolubilized in TBS that included 3 mmol/l NaN_3 as an antimicrobial agent. The suspension was then incubated at 55°C for 6 h. After filtration with a 0.22-μm filter, aliquots (130 ml) of this model bile were mixed with either the purified protein samples obtained from pooled native gallbladder bile in 20 μl of TBS or control solutions of 20 μl of TBS and were then incubated at 37°C with gentle shaking. The cholesterol crystal concentration in the mixture was sequentially monitored by obtaining 10 μl aliquots and diluting each aliquot with 10 mmol/l STDC in 270 μl of TBS, waiting 20 min, and then measuring absorbance at 900 nm.

Con A affinity chromatography

Con A-bound proteins were isolated essentially as described by Groen et al. (21). Pooled native bile, immunopurified biliary and serum haptoglobin were each individually applied to a small Con A Sepharose column (1.6 cm × 5.0 cm) that was equilibrated with a starting buffer of 10 mmol/l Tris-HCl, 0.5 mmol/l NaCl, 1 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 1 mmol/l $MnCl_2$, and 1.5 mmol/l NaN_3. The column was washed with 20 column volumes of the starting buffer to remove all Con-A unbound proteins. The proteins bound to the column were then eluted with 0.2 mmol/l α-D-methyl-mannopyranoside (Sigma Chemical Co., St. Louis, MO) in the starting buffer. The Con A-bound and unbound fractions were diafiltered with TBS by Amicon ultrafiltration. After filtration with a 0.22-μm filter, Con A-bound fractions from native bile were then delipidated by Centricon-10 ultrafiltration with TBS (× 5), or with TBS containing 10 mmol/l STDC (×10). The sample that was delipidated by TBS containing STDC was buffer exchanged. This was done by Centricon ultrafiltration-10 with TBS alone to completely remove the STDC. The residual content of bile salts in the buffer exchanged Con A-bound fractions was below the limit of detectability. Both nondelipidated and delipidated Con A-bound fractions were prepared identically to the other fractions obtained by immunoaffinity chromatography for the cholesterol crystal growth assay.

Delipidated Con A-bound fraction was applied to an anti-haptoglobin immunoaffinity column to obtain a haptoglobin-depleted fraction as previously described (8, 22). After this step, using Western blotting, haptoglobin was no longer detectable in the Con A-bound fraction.

Statistical analysis

The differences between protein concentrations and cholesterol crystal growth data were analyzed with a two-tailed Wilcoxon rank-sum test for comparison of groups comprised of nonparametric data (23). Comparisons for the data from CCG assays were performed using the unpaired *t*-test for each point. The significance level was set at 0.05.

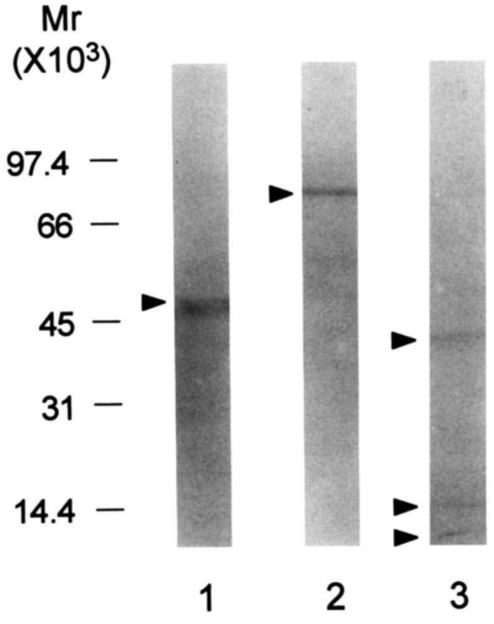


Fig. 1. Pooled native bile was run on SDS-PAGE under reducing conditions and Western blots were performed using anti-α_1-antitrypsin, anti-transferrin, and anti-haptoglobin. Lane 1: α_1-antitrypsin (50-kD); lane 2: transferrin (76-kD); lane 3: haptoglobin multimer with 3 subunits, i.e., 40-kD (β subunit), 17-kD (α_2 subunit) and 9-kD (α_1 subunit). No cross-reactivity can be observed on any of the Western blots.

RESULTS

Specific of antibodies

Aliquots from pooled native bile were run on SDS-PAGE under reducing conditions and Western blots were subsequently performed using anti-α_1-antitrypsin, anti-transferrin, and anti-haptoglobin. As in **Figure 1,** Western blots using anti-α_1-antitrypsin (lane 1) and anti-transferrin (lane 2) showed only a single band at 50 kD and 76 kD, representing α_1-antitrypsin and transferrin, respectively, and using anti-haptoglobin (lane 3), showed a 40 kD (β-subunit), a 17 kD (α_2-subunit) and a 9 kD (α_1-subunit) band, representing haptoglobin. No antibody cross-reactivity was observed.

Immunoisolation of various different biliary proteins

Three proteins, α_1-antitrypsin, transferrin, and haptoglobin (each of which are relatively abundant in serum) were purified from gallbladder bile using specific antibody columns. These proteins were then individually run on SDS-PAGE under reducing conditions and the gels were stained with silver. As shown in **Figure 2,** lane 1 (α_1-antitrypsin), lane 2 (transferrin), lane 3 (haptoglobin) showed M_r bands identical to those found in the reference lanes shown in Fig. 1. No contaminating protein bands were detectable.

Recovery for ceruloplasmin, complement-3 and α_2-macroglobulin in each case was less than 3 μg/ml; purity after isolation with immunoaffinity chromatography was not demonstrable on SDS-PAGE (data not shown). Therefore, further studies concerning these three unpurified proteins were discontinued.

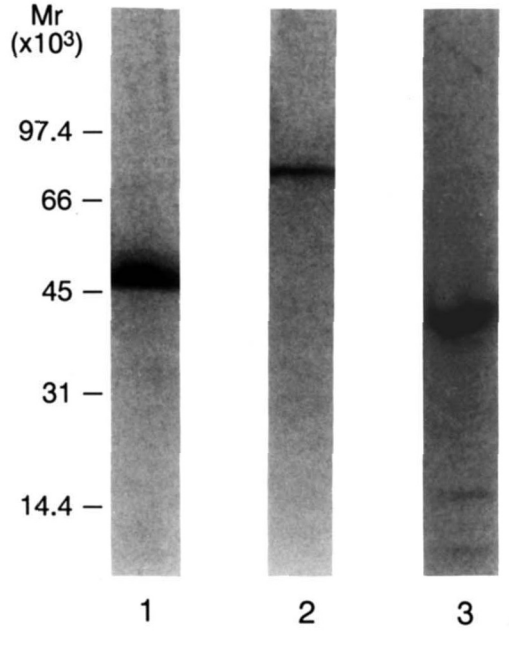


Fig. 2. SDS-PAGE (reducing conditions) of immunoisolated biliary proteins. Lane 1: α_1-antitrypsin; lane 2: transferrin; lane 3: haptoglobin. Gels were stained with silver. Bands found in each lane are identical to those shown in Fig. 1. No contaminating proteins can be detected.

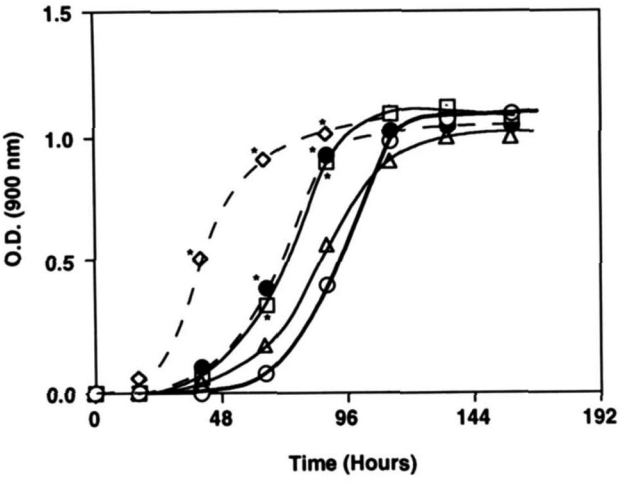


Fig. 3. Comparison of relative promoting activities for α_1-antitrypsin, transferrin, haptoglobin, and IgM at their physiological concentrations in bile. Haptoglobin showed, by far, the most potent activity; IgM and transferrin showed almost the same level of promoting activity; and α_1-antitrypsin showed no significant promoting activity. Each point on the control and experimental curves represents a mean value (n = 4). Standard deviations were omitted on the graph for purposes of clarity. Symbols: ---○---, control; ---△---, α_1-antitrypsin; ---□---, transferrin; ---◇---, haptoglobin; ---●---, IgM. $^*P < 0.05$ versus control.

Effect of adding immunoisolated biliary proteins to the cholesterol crystal growth assay

The results of addition experiments at physiological concentrations found in bile are shown in **Figure 3.** Biliary haptoglobin (15 μg/ml) clearly showed the most potent promoting activity. Biliary transferrin (20 μg/ml) also enhanced cholesterol crystallization to nearly the same magnitude as that seen with the 75 μg/ml of biliary IgM. No significant promoting effect for biliary α_1-antitrypsin, however, could be detected. Although the cholesterol and phospholipid amount of each protein test solution was well below the detection limit of our cholesterol measurement assay, the possibility that some cholesterol crystals or vesicles could still have been present in these samples could not be excluded. We therefore compared effects of delipidation of the isolated proteins on promoting activity by the crystal growth assay without and with exposure to 50% *tert*-butanol. As delipidation with 50% tert-butanol had no discernible effect on measured promoting activity (data not shown), the comparative crystallization promoting effects of these biliary proteins could be ranked by potency in the following order: haptoglobin >>> IgM = transferrin >> α_1-antitrypsin (no effect).

As shown in **Figure 4,** increasing concentrations (3.75, 7.5, and 15 μg/ml) of haptoglobin showed promotion of cholesterol crystallization to be concentration-dependent.

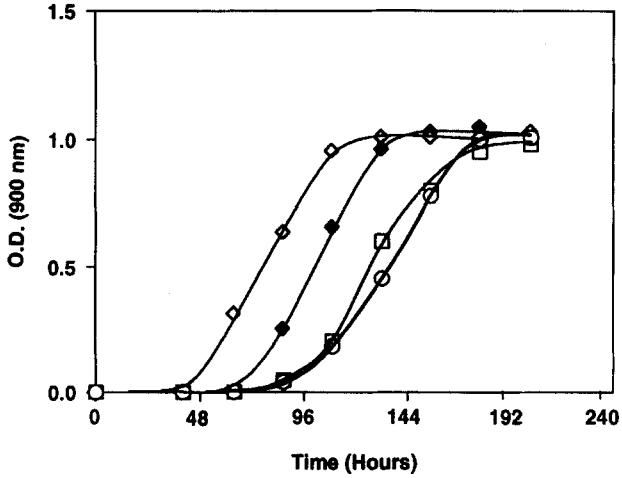


Fig. 4. Concentration-dependence for the promoting activity of biliary haptoglobin as measured by the cholesterol crystal growth assay. Three concentrations of the purified haptoglobin (3.75, 7.5, 15 μg/ml) were used in the crystal growth assay. A concentration-dependent increase in promoting activity can be seen. Each point on the control and experimental curves represents a mean (n = 4). Symbols: ---○---, control; ---□---, 3.75 μg/ml; ---◆---, 7.5 μg/ml; ---◇---, 15 μg/ml.

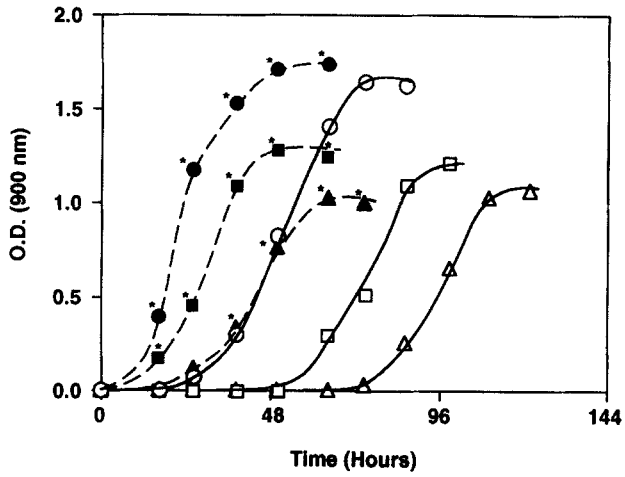


Fig. 5. Comparison of relative promoting activities for Con A-bound fractions with (open symbols) or without (closed symbols) delipidation at three different concentrations. The promoting effect (onset time) of 100 μg/ml of delipidated Con A-bound fraction and 25 μg/ml of non-delipidated Con A-bound fraction were almost identical. The final optical density (i.e., crystal mass) was not different between delipidated and non-delipidated samples. Each point on the experimental curves represents a mean (n = 5). Symbols; ---●○---, 100 μg/ml; ---■□---, 50 μg/ml; ---▲△---, 25 μg/ml. $^*P < 0.05$ versus a delipidated Con A-bound fraction having the same protein concentration.

Con A affinity chromatography

After the Con A-bound fractions were prepared, sample solutions were delipidated by STDC treatment. As precipitation occurred during exposure to 50% *tert*-butanol of the Con A-bound fractions, this convenient approach could not be used for delipidation (24). Protein concentration in the Con A-bound fractions was in the range of 150 to 250 μg/ml. The cholesterol and phospholipid concentrations of Con A-bound fractions treated by ultrafiltration with TBS alone were 10 to 20 μmol/l and below the detection limit, respectively. When ultrafiltration was performed using TBS and STDC, the concentration of cholesterol was less than 1 μmol/l. The cholesterol crystallization-promoting activity of these two types of samples was measured by the CCG assay. **Figure 5** shows the CCG results from delipidated and nondelipidated Con A-bound fractions. The promoting activity decreases by 75% upon delipidation with TBS-STDC, but the final optical density measurement (i.e., final cholesterol mass) was unchanged after delipidation. To determine the affinity to Con A lectin of immunoisolated biliary haptoglobin, this purified protein was chromatographically separated into Con A-bound and -unbound fractions (25). About 90% of biliary haptoglobin was found in the Con A-bound fraction. To determine the relative contribution of the promoting activity of haptoglobin to the total activity of delipidated Con A-bound fractions at several dilutions and for comparison purposes, the activities of 14 μg of haptoglobin and of 186 μg (200 μg–14 μg) of a haptoglobin-depleted Con A-bound fraction were concomitantly tested by CCG assay. **Figure 6** shows a typical example of the promoting activities observed with the CCG assays of delipidated Con A-bound fractions compared with that of delipidated haptoglobin. These demonstrate two things. First, the promoting effect of the delipidated

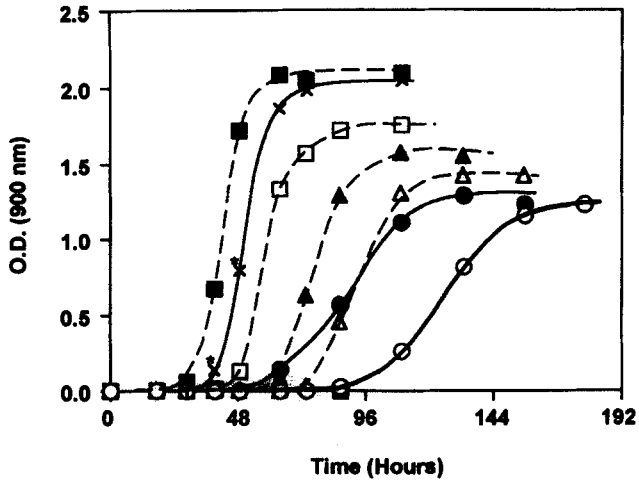


Fig. 6. Comparison of relative promoting activities for delipidated Con A-bound fraction at different concentrations compared with that of 14 μg/ml (physiological concentration) of immunoisolated biliary haptoglobin and 186 μg/ml of haptoglobin depleted (by immuno-extraction) Con A-bound fraction (200 μg/ml–14 μg/ml). The promoting activity (onset time) of 50 μg/ml of Con A-bound fraction and that of biliary haptoglobin is nearly the same. The final optical density (i.e., crystal mass) was comparatively less with haptoglobin. The cholesterol crystallization activity of Con A-bound fraction is significantly decreased by removal of haptoglobin. Each point on the control and experimental curves represents a mean (n = 5). Symbols; ---○---, control; ---●---, haptoglobin; ---X---, haptoglobin-depleted Con A-bound fraction; ---■---, 200 μg/ml; ---□---, 100 μg/ml; ---▲---, 50 μg/ml; ---△---, 25 μg/ml of the Con A-bound fraction. $^*P < 0.05$ comparison between 200 μg/ml of Con A-bound fraction and haptoglobin-depleted Con A-bound fraction.

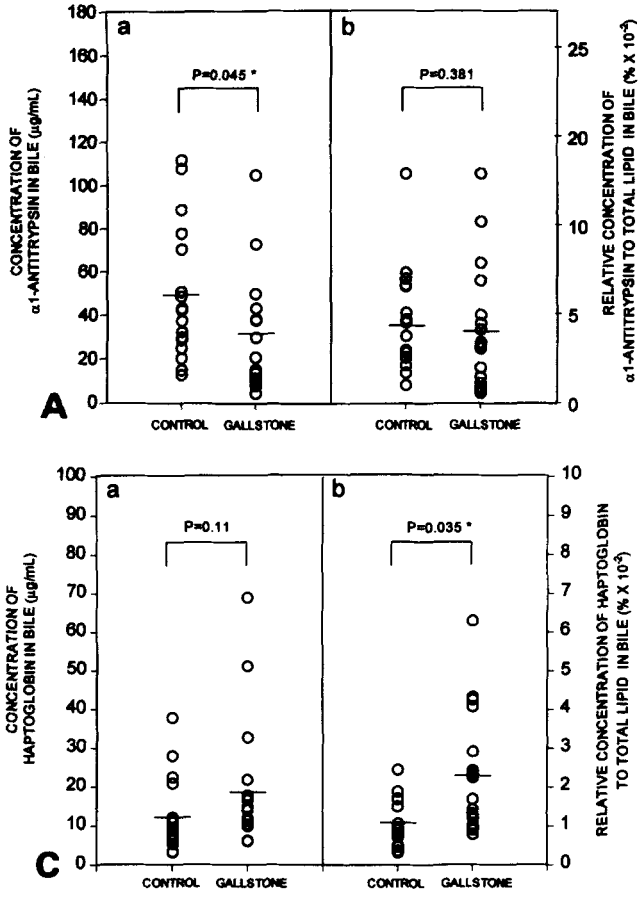
Con A-bound fractions is concentration-dependent. Second, by comparing the activity of immunopurified haptoglobin with that of the delipidated Con A-bound fraction, it can be seen that the contribution of haptoglobin to the fractional promoting activity (onset time) represents about 30% of the physiologically relevant concentration of the delipidated Con A-bound fraction (200 μg/ml). Thus, even with the delipidated Con A-bound fraction, the observed final cholesterol mass cannot be accounted for by the effect of biliary haptoglobin. The promoting activity of the delipidated Con A-bound fraction was significantly decreased on depletion of haptoglobin by immuno-extraction. The contribution of the haptoglobin-specific promoting activity in the Con A-bound fraction, however, cannot be accurately estimated by use of this immuno-extraction approach. This is because the dose-dependence of promoting activity of the Con A-bound fraction is not linear, especially the range between 100 μg/ml and 200 μg/ml as shown in Figure 6.

Concentrations of the biliary proteins

Concentrations of the three immunoisolated biliary proteins in gallbladder bile samples from gallstone-free controls and of randomly chosen individual samples from cholesterol gallstone patients were compared. As shown in **Figure 7A (a),** concentrations of α_1-antitrypsin were significantly lower in the gallstone group ($P < 0.05$). The concentration of biliary haptoglobin tended to be higher in the gallstone group than in the controls; however, neither the concentrations of biliary haptoglobin ($P = 0.11$) nor biliary transferrin ($P = 0.87$) were significantly different when compared to controls as shown in Figs. 7B (a), 7C (a). In Figs. 7A (b), 7B (b), and 7C (b), the data indicate that only for biliary haptoglobin is there a significant increase in its relative concentration for the cholesterol gallstone-associated samples compared to the gallstone-free controls ($P < 0.05$).

DISCUSSION

Biliary haptoglobin showed the most potent promoting activity of the three immunoisolated biliary proteins even at its comparatively low physiological concentration of 15 μg/ml (Fig. 3). Based on published data, biliary IgM at its physiological concentration of 75 μg/ml must be considered the most potent single biliary promoting protein identified prior to the present work (3). As can be seen in Fig. 3, biliary haptoglobin at its physiological concentration has about twice the promoting potency of IgM



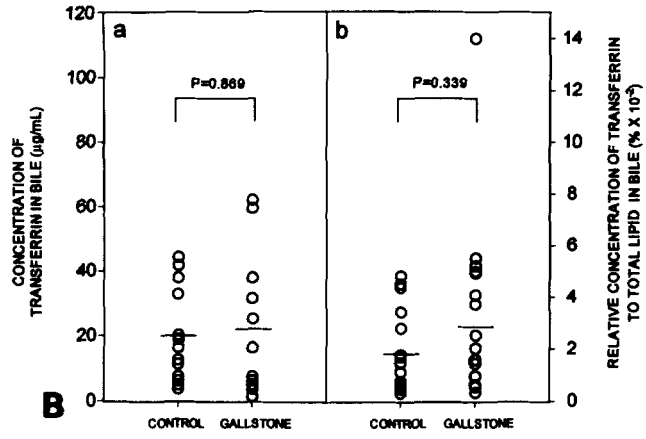


Fig. 7. (A) Absolute ($P = 0.045$*) and relative concentrations ($P = 0.381$) of biliary α_1-antitrypsin; (B) absolute ($P = 0.869$) and relative concentrations ($P = 0.339$) of biliary transferrin; (C) absolute ($P = 0.11$) and relative concentrations ($P = 0.035$*) of biliary haptoglobin, in individual bile samples from stone-free controls and cholesterol gallstone patients. Absolute protein concentrations are shown in panel (a). Relative protein concentrations to total biliary lipid are shown in panel (b). Means are shown as horizontal lines. *$P < 0.05$ comparison between cholesterol gallstone and gallstone-free control groups.

and thus replaces IgM as the biliary promoting protein with greatest potency at its physiological concentration. In contrast to this observation, biliary α_1-antitrypsin, when assayed at its physiological concentration, showed no significant promoting activity. The apparent conflict between this observation and that of a recent preliminary report by Zijlstra et al. (26) may be related to differences in the protein concentrations used in the crystal growth assay. For example, we found that promoting activity for α_1-antitrypsin was roughly equivalent to that of biliary transferrin at its physiological concentration of 20 μg/ml but only when the assay concentration of α_1-antitrypsin was increased by threefold to 150 μg/ml.

The Con A-bound fraction obtained from native bile is known to have strong cholesterol crystallization-promoting activity (21). Although several proteins derived from the Con A-bound fraction have been identified as crystallization promoters, the activity of these proteins is insufficient to account for most of the observed promoting activity (8). We found that cholesterol concentration in the Con A-bound fraction is in the range of 10 to 20 μmol/l. Despite the fact that this low level of cholesterol concentration increases the cholesterol saturation index of model bile by only 0.002, when even micromolar amounts of cholesterol are added to the system the effect is not negligible. Addition of only 1.2 μmol/l of cholesterol as seed crystals, for example, strongly accelerates cholesterol crystallization activity (20). The apparent promoting activity of the Con A-bound fraction decreased by about 75% after delipidation. Residual cholesterol, therefore, seems to be primarily responsible for this discrepancy. When the potential effect of small amounts of cholesterol on the apparent crystallization-promoting activity of immunopurified proteins was examined, a change in crystallization promoting activity of biliary haptoglobin was not associated with delipidation. Based on our results, the contribution of haptoglobin to the total promoting activity (onset time) in the delipidated Con A-bound fractions is about 30%. Nearly all of the Con A-bound glycoprotein-derived crystallization promoting activity can probably now be explained by the several proteins already reported as having promoting activity (1, 3–8). The enhanced effect of even the delipidated Con A-bound fraction on the final (equilibrium) cholesterol crystal mass, however, remains unexplained by our results. We had anticipated that the delipidation procedure that decreased promoting activity would also eliminate this effect. This unexpected result may indicate that there is some undefined Con A-bound promoter protein that may somehow have a unique effect on cholesterol crystal mass.

It has been proposed that one of the important criteria to establish the credibility of a putative promoter is that the concentration of the candidate promoter compared to that found in gallstone-free control samples should be increased in bile samples acquired from patients with gallstone disease (27). As the protein-to-lipid ratio is likely the key determinant of interactions between biliary proteins and lipids, we used protein concentrations normalized to individual sample total biliary lipid content to compare intergroup specific protein concentrations. As shown in Fig. 7, quantitative measurements using this basis for comparison show a twofold mean increase in concentration for biliary haptoglobin in the gallstone-associated samples compared to controls. In contrast, similar comparative measurements for biliary transferrin and α_1-antitrypsin failed to show such a pattern. It should be noted that haptoglobin is an inflammatory acute phase reactant as are α_1-antitrypsin and IgM, neither of which has been shown to increase in cholesterol gallstone disease (3). Thus, the most likely associated chronic inflammatory process associated with gallstones, i.e., cholecystitis, does not seem to provide as ready an explanation for disease-associated elevations as it probably does for elevated levels of biliary IgG (3).

Whereas the normalized concentration of haptoglobin shows a twofold mean increase in cholesterol gallstone patients compared to controls, it follows that there is a considerable overlap in biliary haptoglobin concentration within these groups. As the pathogenesis of gallstone disease is undoubtedly multifactorial, differences in crystallization effectors such as the various pronucleating proteins, inhibitor proteins and degree of biliary cholesterol supersaturation do not always clearly discriminate between samples from gallstone and control groups (28, 29). Because low concentration levels of haptoglobin lack potent crystallization-promoting activity as shown in Fig. 4, about half of the gallstone patients in this study may not have been strongly affected by biliary haptoglobin concentrations. Nevertheless, biliary haptoglobin, which at physiological concentrations is probably the most potent cholesterol crystallization-promoting protein thus far reported, should have a significant pathogenetic role in the major subgroup of cholesterol gallstone patients in whom its biliary concentration is increased.

Groen (30) showed that the Con A-bound fraction appears to shift biliary lipid from micelles to vesicles. Ahmed et al. (31) recently showed that promotor proteins tend to be relatively hydrophilic. The precise mechanism(s) of the cholesterol crystallization promoting activity of haptoglobin, however, is unclear. As haptoglobin is one of the several cholesterol crystallization promoters found in Con A-bound fractions (1–8) and is hydrophilic, a possible mechanism of the pronucleating activity of haptoglobin may be that of shifting biliary cholesterol from biliary micelles to vesicles (32).

The authors acknowledge the skillful assistance of Ms. Janeth Sperry in manuscript preparation. This work was supported by Research Grant DK-17562 from the USPHS National Institutes of Health. A portion of this work was presented in preliminary

form at the annual meeting of the American Gastroenterological Association, New Orleans, LA. May 15, 1994 and published as an abstract in *Gastroenterology* **106:** 781A, 1994. Drs. Yamashita and Secknus were supported in part by the Research Institute, Cleveland Clinic Foundation. Dr. Ginanni Corradini was the recipient of a National Institutes of Health Fogarty International Research Fellowship. Dr. Secknus was the recipient of a fellowship from the Feodor Lynen Program of the Alexander Von Humboldt Foundation of Germany. Dr. Ginanni Corradini's present address: Universita degli Studi di Roma I, Il Cattedra di Gastroenterologia, Instituto di Clinica Medica III, Universita' La Sapienza, Viale dell' Universita, 37, 00158 Rome, Italy. Dr. Takabayashi's present address: Department of Surgery, Kitano Hospital, Osaka, Japan. Dr. Takabayashi was supported in part by Shionogi and Co., Ltd., Osaka, Japan.

Manuscript received 11 November 1994 and in revised form 30 January 1995.

REFERENCES

1. Harvey, P. R. C., G. A. Upadhya, and S. M. Strasberg. 1991. Immunoglobulins as nucleating proteins in the gallbladder bile of patients with cholesterol gallstones. *J. Biol. Chem.* **266:** 13996–14003.
2. Pattinson, N. R., and K. E. Willis. 1991. Effect of phospholipase C on cholesterol solubilization in model bile. A concanavalin A-binding nucleation-promoting factor from human gallbladder bile. *Gastroenterology.* **101:** 1339–1344.
3. Upadhya, G. A., P. R. C. Harvey, and S. M. Strasberg. 1993. Effect of human biliary immunoglobulins on the nucleation of cholesterol. *J. Biol. Chem.* **268:** 5193–5200.
4. Nuñez, L., L. Amigo, A. Rigotti, L. Paglielli, G. Mingrove, A. V. Greco, and F. Nervi. 1993. Cholesterol crystallization-promoting activity of aminopeptidase-N isolated from the vesicular carrier of biliary lipids. *FEBS Lett.* **329:** 84–88.
5. Offner, G. D., D. Gong, and N. H. Afdhal. 1994. Identification of a 130-kilodalton human biliary concanavalin A binding protein as aminopeptidase N. *Gastroenterology.* **106:** 755–762.
6. Abei, M., P. Kawczak, H. Nuutinen, A. Langnas, J. Svanvik, and R. T. Holzbach. 1993. Isolation and characterization of a cholesterol crystallization-promoter from human bile. *Gastroenterology.* **104:** 539–548.
7. Abei, M., H. Nuutinen, P. Kawczak, J. Schwarzendrube, S. P. Pillay, and R. T. Holzbach. 1994. Identification of human biliary α_1-acid glycoprotein as a cholesterol crystallization promoter. *Gastroenterology.* **106:** 231–238.
8. Abei, M., J. Schwarzendrube, H. Nuutinen, T. A. Broughan, P. Kawczak, C. Williams, and R. T. Holzbach. 1993. Cholesterol crystallization-promoters in human bile: comparative potencies of immunoglobulins, α_1-acid glycoprotein, phospholipase C, and aminopeptidase N. *J. Lipid Res.* **34:** 1141–1148.
9. de Bruijn, M. A. C., K. Mok, N. Domingo, H. Lafont, and G. N. J. Tytgat. 1992. Further characterization of a cholesterol crystallization promoting lipoprotein in biliary Con A-binding protein. *Gastroenterology.* **102:** 310A(Abstr.)
10. Busch, N., N. Matiuck, S. Sahlin, S. P. Pillay, and R. T. Holzbach. 1991. Inhibition and promotion of cholesterol crystallization by protein factors from normal human gallbladder bile. *J. Lipid Res.* **32:** 695–702.
11. Holan, K. R., R. T. Holzbach, R. E. Hermann, A. M. Cooperman, and W. J. Claffey. 1979. Nucleation time: a key factor in the pathogenesis of cholesterol gallstone disease. *Gastroenterology.* **77:** 611–617.
12. Yamazaki, K., S. P. Powers, and N. F. LaRusso. 1988. Biliary proteins: assessment of quantitative techniques and comparison in gallstone and nongallstone subjects. *J. Lipid Res.* **29:** 1055–1063.
13. Turley, S. D., and J. M. Dietschy. 1978. Re-evaluation of the 3α-hydroxysteroid dehydrogenase assay for total bile acids in bile. *J. Lipid Res.* **19:** 924–928.
14. Takayama, M., S. Itoh, T. Nagasaki, and I. Tanimizu. 1977. A new enzymatic method for determination of serum choline-containing phospholipids. *Clin. Chim. Acta.* **79:** 93–98.
15. Gurantz, D., M. F. Laker, and A. F. Hofmann. 1981. Enzymatic method of choline-containing phospholipids in bile. *J. Lipid Res.* **22:** 373–376.
16. Allain, C. C., L. S. Poon, C. S. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total serum cholesterol. *J. Clin. Chem.* **20:** 470–475.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature.* **227:** 680–685.
18. Morrissey, J. H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced sensitivity. *Anal. Biochem.* **117:** 307–310.
19. Harlow, E., and D. Lane. 1988. Antibodies, a Laboratory Manual. Cold Spring Harbor Laboratory, New York. 553 pp.
20. Busch, N., H. Tokumo, and R. T. Holzbach. 1990. A sensitive method for determination of cholesterol crystal growth using model solutions of supersaturated bile. *J. Lipid Res.* **31:** 1903–1908.
21. Groen, A. K., R. Ottenhoff, P. L. M. Jansen, J. van Marle, and G. N. J. Tytgat. 1989. Effect of cholesterol nucleation-promoting activity on cholesterol solubilization in model bile. *J. Lipid Res.* **30:** 51–58.
22. de Bruijn, M. A. C., K. S. Mok, T. Out, G. N. J. Tytgat, and A. K. Groen. 1994. Immunoglobins and α_1-acid glycoprotein do not contribute to the cholesterol crystallization-promoting effect of concanavalin A-binding biliary protein. *Hepatology.* **20:** 626–632.
23. Zar, J. H. 1984. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, NJ. 718 pp.
24. Pike, R. N., and C. Dennison. 1989. Protein fractionation by three phase partitioning (TPP) in aqueous/*t*-butanol mixtures. *Biotechnol. Bioeng.* **33:** 221–228.
25. Katnik, I. 1984. Studies on haptoglobin binding to concanavalin A. *Biochim. Biophys. Acta.* **790:** 8–14.
26. Zijlstra, A., A. K. Groen, N. H. Afdahl, and G. D. Offner. 1993. Identification of pronase-resistant Con A binding proteins in human bile. *Hepatology.* **18:** 95A(Abstr.)
27. Harvey, P. R. C., and S. M. Strasberg. 1993. Will the real cholesterol-nucleating and -antinucleating proteins please stand up? *Gastroenterology.* **104:** 646–650.
28. Holzbach, R. T., M. Marsh, M. Olszewski, and K. Holan. 1973. Cholesterol solubility in bile: evidence that supersaturated bile is frequent in healthy man. *J. Clin. Invest.* **52:** 1467–1479.
29. Sewell, R. B., S. J. T. Mao, T. Kawamoto, and N. F. LaRusso. 1983. Apolipoproteins of high, low, and very low density lipoproteins in human bile. *J. Lipid Res.* **24:** 391–401.
30. Groen, A. K. 1990. Nonmucous glycoproteins as pronucleating agents. *Hepatology.* **12:** 189S–194S.
31. Ahmed, H. A., M. L. Petroni, M. Abu-Hamdiyyah, R. P. Jazrawi, and T. C. Northfield. 1994. Hydrophobic/hydrophilic balance of proteins: a major determinant of cholesterol crystal formation in model bile. *J. Lipid Res.* **35:** 211–219.
32. Yamashita, G., R. Secknus, A. Chernosky, K. A. Krivacic, and R. T. Holzbach. 1995. Comparison of haptoglobin and apolipoprotein A-I on biliary lipid particles involved in cholesterol crystallization. *Gastroenterology.* (Abstr.)