

## Biliary Proteins

### Unique Inhibitors of Cholesterol Crystal Nucleation in Human Gallbladder Bile

R. T. Holzbach, A. Kibe, E. Thiel, J. H. Howell, M. Marsh, and R. E. Hermann

Gastrointestinal Research Unit, Department of Gastroenterology and Department of Surgery, Cleveland Clinic Foundation, Cleveland, Ohio 44106

**A**bstract. The onset time for cholesterol crystal nucleation of supersaturated normal human gallbladder biles is consistently prolonged when compared with biles from patients with cholesterol gallstone disease. Investigation of the factor(s) responsible for the suspended supersaturation (metastability) of normal human biles revealed that model bile solutions of cholesterol saturation index (CSI) and molar lipid composition identical to individual gallbladder bile specimens had much shorter crystal nucleation times, i.e., exhibited decreased metastability. Unsaturated normal biles, after supplementation with lecithin, cholesterol, and sodium taurocholate to a 'standard' supersaturated lipid composition, also demonstrated nucleation times three- to 15-fold longer than the comparable standard model bile. Total lipid extracts of normal biles, however, when similarly supplemented, did not differ in nucleation time from the control model solution. Gallbladder biles were fractionated by gel chromatography and the eluted fractions were pooled into two fractions. The fractions eluting in about the first 25% of the included volume when mixed with the supersaturated standard model bile induced a modest increase in nucleation time of ~1.5 times the control value. The fractions eluting in the second 25% of the included volume and which contained all of the bile lipids, were concentrated and supplemented with lipids to the standard composition. The nucleation times of these sup-

plements were 3–10 times longer than the control nucleation times. Delipidated bile protein mixtures, purified by discontinuous sucrose gradient centrifugation, were recombined with purified lipids at the standard composition used previously. The nucleation times of these mixtures were significantly prolonged to the same extent as those associated with the second chromatographic fraction. These observations demonstrate that the delayed onset (inhibition) of cholesterol crystal nucleation observed in normal human gallbladder bile is produced by a factor(s) present in the biliary protein fraction.

#### Introduction

In a previous report (1) the onset time for cholesterol crystal nucleation in human gallbladder biles was established as an index of differences in metastability (suspended supersaturation). Nucleation times of supersaturated normal biles were consistently prolonged when compared with biles from patients with cholesterol gallstone disease (abnormals). These findings have been confirmed by two other groups (2, 3). This distinction in nucleation times could not have been predicted by using differences in cholesterol saturation indices (CSI)<sup>1</sup> derived from observations with artificial biles (4). This finding suggested that a nonlipid component might stabilize the supersaturated normal biles. A comparison of solute compositions of normal and abnormal biles, however, revealed no evident difference in the nonlipid components (1), so the factor(s) responsible for the observed difference in metastability remained obscure.

A possible explanation for the observed difference in metastability has been the recent finding of nucleating (or nucleation-promoting) factor(s) that could account for observed rapid onset time for cholesterol crystal nucleation in biles associated with cholesterol gallstone disease (5–7). Although possibly accounting in part for the difference found between health and disease, the presence of nucleating factors in biles of cholesterol gallstone patients would not explain the prolonged metastability of biles from healthy controls.

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Address reprint requests to Dr. R. T. Holzbach.

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1. Abbreviations used in this paper: CSI, cholesterol saturation index; HBS, Hepes-buffered saline; PEG 8000, polyethylene glycol.

In this paper, there are two main objectives. First, experiments were done to unambiguously establish or exclude the presence of metastability-enhancing (nucleation-inhibiting) factors in the bile from healthy controls with biliary cholesterol supersaturation and prolonged metastability. An exact normalization was incorporated into these studies for two important determinants of nucleation time, i.e., percent total solids and CSI. The result of these carefully controlled studies confirm the presence of a nucleation-inhibiting effect conferred by some unidentified factor in normal bile. Second, using conventional fractionation procedures, studies were performed for the preliminary isolation and biochemical characterization of the nucleation-inhibiting factor. Bile proteins were found to consistently exhibit the inhibiting effect, and therefore to contain the factor, whereas bile lipids did not.

## Methods

**Clinical.** Fresh human gallbladder bile specimens were obtained at surgery as previously described (1) or were collected in a similar manner from legally dead, living donors for a renal transplant program. Prior approval of the protocol used in this investigation was obtained from the Clinical Research Projects and Institutional Review Committee regarding human studies. No difference in results was observed between either group of living subjects. The presence of gallstones in these patients was excluded on the basis of surgical palpation and the absence in the sample of cholesterol crystals by polarizing microscopy (2).

Gallbladder aspirates were either immediately fractionated or, after removal of aliquots for quantitative analysis, were frozen at  $-80^{\circ}\text{C}$  under nitrogen for storage. Cryoprecipitates were not observed after thawing bile samples stored up to 4 mo.

**Nucleation times.** Nucleation times were determined essentially as previously described (1). Each heated isotropic mixture was filtered through a detergent-free 0.22- or 0.8- $\mu\text{m}$  filter (Millipore Continental Water Systems, Bedford, MA) into a 2-ml capped glass microvial, gassed with  $\text{N}_2$ , sealed, and placed in a  $37^{\circ}\text{C}$  incubator. The time zero for the nucleation time determination was arbitrarily established at 30 min after an initial thermal equilibration. At time zero, 8–12 h later, and daily thereafter, one drop of solution was placed on a slide and into the temperature-controlled stage of a polarizing microscope and carefully scanned. The interval between time zero and the first detection of solid cholesterol crystal precipitate formation was designated the nucleation time. The mixtures were incubated in a nitrogen atmosphere throughout this procedure.

**Total lipid extraction.** Total lipid extracts of native bile were made by organic solvent extraction with either 2:1,  $\text{CHCl}_3/\text{MeOH}$  or 1:1, ethanol/diethyl ether. 20 vol of organic solvent was added to the bile, which was incubated with shaking for an hour and refrigerated overnight. The mixture was centrifuged (600  $g$  for 30 min), the supernatant collected, and the precipitate washed twice with a small volume of solvent. The pooled supernatants were dried by a stream of nitrogen and lyophilized. The samples were dissolved in methanol to their original volume and assayed for each lipid.

**Fractionation of native bile by gel chromatography.** Native bile, <12 h old, was fractionated by gel exclusion chromatography at  $20^{\circ}\text{C}$ , on a  $2.6 \times 70\text{-cm}$  column of Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, NJ). Bile was eluted with buffered saline (0.01 M HEPES [Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA],

0.15 M NaCl, pH 7.5), containing 5 mM sodium taurodeoxycholate, 0.15%  $\epsilon$ -amino caproic acid, and 0.15% EDTA (Fisher Scientific Co., Pittsburgh, PA). A flow rate of  $\sim 100$  ml/h was used and the totally excluded and totally included volumes of the column were determined with Blue Dextran 2000 (Sigma Chemical Co., St. Louis, MO) and [ $^{14}\text{C}$ ]sucrose (New England Nuclear, Boston, MA), respectively. Essentially all of the biliary lipids and  $\sim 25\%$  of the protein in this sample eluted at a  $K_{av}$  of  $\sim 0.45$  (Fig. 1). The lipids in other bile samples eluted similarly under these conditions. 90–100% of the proteins in the native bile sample were recovered. In each experiment the fractions eluting before the lipid peak and containing only protein were pooled, concentrated to the volume of the original bile sample, and designated as fraction A. The native lipid-containing fractions were also pooled, concentrated as above, and these were designated as fraction B. The proportion of the native biliary proteins recovered in fraction A varied from 25 to  $\sim 75\%$  (0.26–1.95 mg/ml) among the samples tested.

**Delipidation of biliary proteins by ultracentrifugation.** Gallbladder biles were dialyzed (Spectrapor 2, Spectrum Medical, Los Angeles, CA) for 3 d under running cold tap water. Less than 0.1 mM bile salts remained in the dialyzed bile. All buffers used in subsequent fractionations contained 0.15%  $\epsilon$ -amino caproic acid and 0.13% EDTA. In control nucleation experiments, it was ascertained that neither  $\epsilon$ -amino caproic acid (0.15%) nor EDTA (0.15%) as well as polyethylene glycol (PEG-8000) (10–100  $\mu\text{g}/\text{ml}$ ) (Fisher Scientific Co.) had any effect on the nucleation time of the standard model bile.

Detergent-delipidated proteins were prepared using a modification of the method described by Helenius et al. (8). Octylglucoside (Sigma Chemical Co.) was added to the dialyzed bile at 100 mM, approximately fourfold in excess of the phospholipid concentration. This mixture was incubated with shaking at  $37^{\circ}\text{C}$  for 24–36 h. 1 ml of the mixture was layered over 10 ml of 25% sucrose, 0.14 M NaCl, 10 mM HEPES-buffered saline (HBS), 30 mM octylglucoside, pH 7.5, in an ultracentrifuge tube and centrifuged at 200,000  $g$  for 36 h using a SW-41 rotor in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The detergent delipidated proteins sedimented in the bottom two-thirds of the tubes and contained  $<0.1$  mM phospholipids. The phospholipid and protein profile of a typical experiment is shown in Fig. 2. This profile is representative of those obtained from many delipidation experiments. The cholesterol was always associated with the phospholipid peak. However, the recovery of sedimenting (detergent delipidated) protein varied from 16 to 78% (0.62–5.20 mg/ml) of the protein initially present in the dialyzed bile samples.

The delipidated proteins were dialyzed with HBS, concentrated to original volume using PEG-8000 and stored at  $-85^{\circ}\text{C}$  until use (typically within 1 or 2 mo). Other detergents used in this procedure included 1–10% Triton X-100 (Fisher Scientific Co.), 2% Tween-80 (Fisher Scientific Co.), 100 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (Calbiochem-Behring Corp.) and 1–4% octa-ethylene glycol mono-*n*-dodecyl ether ( $\text{C}_{12}\text{E}_8$ , Nikko Chemicals Co., Tokyo, Japan). The protein-lipid profiles obtained with Triton X-100 and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate were identical to the profile on Fig. 2, whereas less protein sedimented when Tween-80 and  $\text{C}_{12}\text{E}_8$  were used with the same dialyzed bile. Higher concentrations of octylglucoside or Triton X-100, up to a 10-fold molar excess with respect to phospholipid concentration produced no significant increase in sedimenting protein.

Detergent delipidated proteins were hydrolyzed by pronase (Pronase-CB, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) according to the procedure reported by Hoenders et al. (9) except that  $\sim 8$  protease U/mg of detergent delipidated proteins was used to

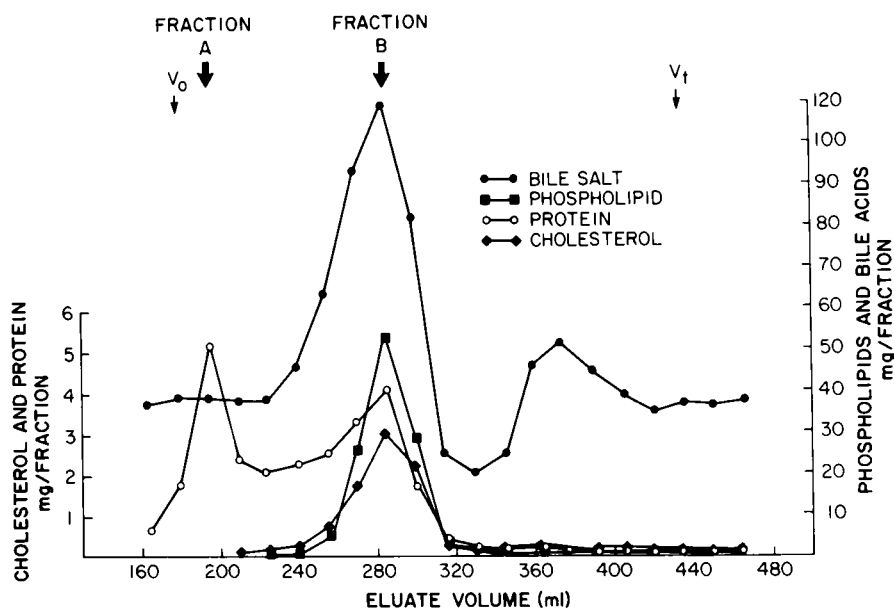


Figure 1. Gel chromatography of normal human gallbladder bile on a Sephacryl S-200 ( $2.6 \times 70$  cm) column. 5 ml of bile was eluted at 100 ml/h in a buffer containing 5 mM sodium taurodeoxycholate, 10 mM HBS, 0.14 M NaCl, 0.02%  $\text{NaN}_3$ , pH 7.5. Fractions of 15 ml were collected and assayed for phospholipid ( $\blacktriangle$ ), cholesterol ( $\circ$ ), bile salts ( $\square$ ), and protein ( $\bullet$ ). The totally excluded ( $V_0$ ) and totally included ( $V_t$ ) volumes are indicated by arrows.

ensure complete degradation of the proteins. The nucleation time of standard model bile lipids recombined with this concentration of pronase was not significantly different from control model biles containing only HBS (14–17 h).

**Lipid analyses.** Aliquots of homogeneously mixed human or model biles were diluted and stored in methanol (nanograde quality) (Mallinckrodt Inc., St. Louis, MO). Cholesterol was assayed as described by Alain (10, 11), lecithin was assayed as inorganic phosphorus (12, 13), and bile salts by the hydroxysteroid dehydrogenase method (14) using a  $3\alpha$ -hydroxysteroid dehydrogenase (Sigma Chemical Co., St. Louis, MO). Model bile lipids were analyzed by thin-layer chromatography according to conventional procedures (15).

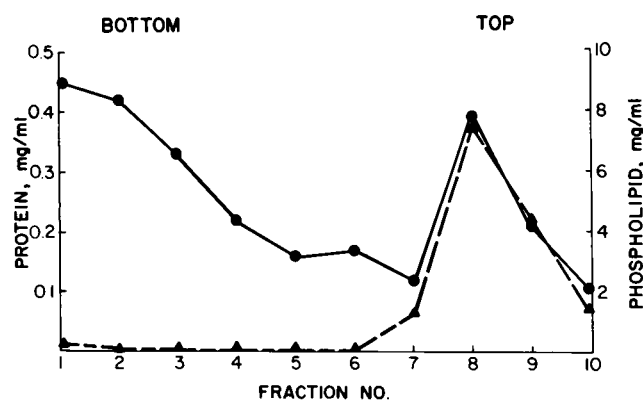


Figure 2. Discontinuous sucrose density ultracentrifugation of proteins and lipids from 1 ml of normal bile. Dialyzed bile was dispersed in 100 mM octylglucoside, layered above a discontinuous sucrose gradient containing 30 mM octylglucoside, and centrifuged at 200,000  $g$  for 36 h. Fractions of 1 ml were collected from the bottom and phospholipid ( $\blacktriangle$ ) and protein ( $\bullet$ ) was determined in each fraction.

Cholesterol saturation indices were calculated from critical tables (16). Weight average molecular weights of 492 and 759 were used for human bile salts and biliary phospholipid, respectively.

**Protein assay.** Biliary proteins were quantitated fluorometrically by a modified fluorescamine assay (17). Bile proteins and serum albumin standards were precipitated with 7% trichloroacetic acid (TCA) in 1.5-ml microfuge tubes and the TCA and residual lipids were removed by washing the precipitates twice with ethanol/diethyl ether (1:1). The precipitates were hydrolyzed in 2.5 N NaOH for 15 h at  $110^\circ\text{C}$  and neutralized with concentrated HCl. The standards and samples were diluted to a concentration from 10 to 100  $\mu\text{g}/\text{ml}$  with borate buffer (0.2 M sodium borate, 0.5% sodium dodecyl sulfate [SDS], pH 8.5). Between 0.025 and 0.100 ml of diluted sample or standard in a  $12 \times 75$ -mm borosilicate glass tube was vortexed and 0.100 ml of fluorescamine solution (0.3 mg/ml) (Sigma Chemical Co.) was added during mixing. The mixtures were brought to a total volume of 3.0 ml, mixed, and the relative fluorescence was measured using a Turner model III fluorometer (Turner Designs, Mountain View, CA) equipped with a 405-nm excitation filter and a Wratten No. 3 emission filter. This procedure was the only protein assay that provided an accurate quantitation (by weight) of protein in bile and was calibrated by amino acid analysis. All other conventional protein assays were affected by interference or cross-reactivity with phospholipids, bile salts, or bile pigments. The total protein concentration of the native gallbladder biles in this study had a mean concentration ( $\pm\text{SD}$ ) of  $7.1 \pm 5.5$  mg/ml ( $n = 12$ ).

**Protein electrophoretic studies.** SDS-polyacrylamide slab gels were run in the buffer system described by Laemmli (18). The apparatus was similar in design to that of Reid and Bielecki (19). 7–20% exponential polyacrylamide gradient gels were prepared and run at 30-mA constant current (Regulated Power Supply, EC-500, E-C Apparatus Corp., St. Petersburg, FL). Isoelectrofocusing and two-dimensional electrophoresis were carried out by the method of O'Farrell (20) using a different composition of ampholytes (1% pH 3.5–10, 0.75% pH 4–4.5, and 0.25% pH 2.5–4, final concentrations). Isoelectric focusing gels were stained with Coomassie Blue. All other gels were fixed in 14% methanol and 10% acetic acid overnight and stained with silver nitrate by the method

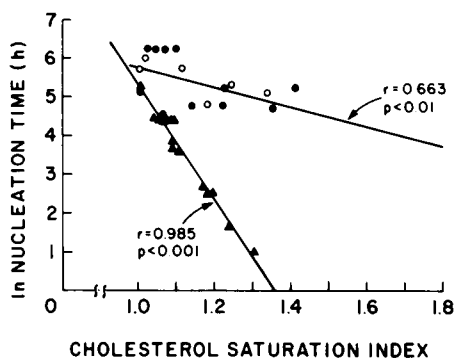


Figure 3. Log nucleation time as a function of CSI for a series of model biles (20 g/dl [ $\blacktriangle$ ] ( $n = 15$ )) and for normal gallbladder biles ( $n = 18$ ) as reported in an earlier study from this laboratory (study 1 [ $\circ$ ] (1) and from another group study (study 2 [ $\bullet$ ] (3)). (Note the interrupted scale on the abscissa.) For the grouped native bile data, the linear regression equation is  $Y = 2.63x + 8.38$  where  $Y$  represents  $\ln$  nucleation time and  $x$  represents CSI. For the model biles, the linear regression equation is  $Y = 14.8x + 20.1$ .

of Merrill et al. (21). Purified proteins were used as standards (Pharmacia Fine Chemicals, Piscataway, NJ).

**Experimental protocol.** Three different in vitro functional assay approaches were utilized in this study, matching supplementation and recombination. In the matching experiments, model biles were constructed from purified cholesterol, phosphatidylcholine, and bile salt having an identical molar lipid composition and percent total lipids to that of a given supersaturated normal human bile. The nucleation times of the native and matched model biles were then determined. In the supplementation experiments native bile samples were supplemented with sufficient purified model bile lipids to achieve a final lipid composition consisting of 20% total solids and a CSI of 1.17 in the physiologic range of biliary lipid composition. The nucleation times under these conditions were rigorously observed between the supplemented native biles and control model biles of the same lipid composition. The third approach involved a recombination of delipidated native bile proteins with a standard model bile as described below. Nucleation times were then compared between the recombined and control samples.

**Matching experiments.** A model bile was constructed using a combination of methods previously described (18, 19), with recent refinements recommended by Carey and Small (4), to match the previously determined molar lipid composition of each supersaturated normal gallbladder bile using stock solutions of pure egg lecithin (Lipid Products, Surrey, England), cholesterol (Eastman Kodak Co., Rochester, NY), and sodium taurocholate (Calbiochem-Behring Corp.) twice recrystallized by the Pope method (24). Appropriate quantities of each lipid were mixed, incubated for several hours in a 2-ml vial, dried under nitrogen, and lyophilized. At an initial temperature of at least 55°C (see Results, Matching Experiments), HBS was added to the dried lipids to a final volume of 1.0 ml and the solutions were tightly capped under nitrogen and incubated with shaking at the elevated temperature, usually at 55°C, until they were microscopically isotropic (no birefringent crystals observed under polarized light). Following rapid cooling to 37°C, nucleation times were then measured as described above.

Another series of model bile solutions (20 g/dl) was prepared in a similar manner, however, CSI values were chosen to encompass the range normally encountered in human biles (Fig. 3).

**Supplementation experiments.** The cholesterol phospholipid and total bile acid composition of unsaturated normal biles, total lipid extracts, and chromatographic fraction B (see below) were first determined. A lipid supplement mixture of sodium taurocholate, egg lecithin, and cholesterol stock solutions was placed in a 3-ml capped glass vial, dried, and lyophilized. The composition of the supplement lipids was calculated so that, when brought to a final volume of 1.0 ml, using the native sample as a modification of diluent, the final total lipid composition of each mixture would be: 8.5 mol% cholesterol, 18.3 mol% phospholipid, 73.2 mol% bile salts, with a total lipid concentration of 20 g%.

This lipid composition is referred to as the standard lipid composition and its CSI is 1.17. The final mixtures were incubated at 55°C, with shaking, until they were isotropic, typically within 48 to 72 h. Nucleation time was then measured following rapid cooling to 37°C.

**Recombination experiments.** For the recombination experiments samples were prepared by adding a solution of biliary proteins to the above standard model bile. All proteins were in HBS solution and had been previously concentrated to a volume equal to the original volume of the native gallbladder bile. Incubation at 55°C for 48–72 h, with shaking, was generally necessary to completely dissolve the cholesterol. Fraction A proteins and detergent delipidated proteins were recombined with standard model biles in concentrations ranging from 0.25 to 1.95 mg/ml and 0.62 to 5.20 mg/ml, respectively.

## Results

**Model bile studies.** A series of model biles ( $n = 15$ ) were constructed from purified sodium taurocholate, egg lecithin, and cholesterol with CSI between 1.0 and 1.3. The nucleation times of these model solutions were determined and are shown on Fig. 3 in a plot of natural logarithm ( $\ln$ ) (nucleation time) vs. CSI. Linear regression analysis of this data shows that it fits the equation,  $y = 14.8x + 20.1$  ( $r = 0.985$ ) where  $y$  represents  $\ln$  (nucleation time) and  $x$  represents the CSI. Nucleation times and lipid compositions for 18 supersaturated normal human gallbladder biles are available from two previous studies (1, 3), and are also plotted in the graph on Fig. 3. The linear regression equation,  $y = 2.63x + 8.38$ , provided the best fit ( $r = 0.663$ ) for this native bile data. The greatly decreased slope exhibited by the native biles when compared with the model solutions indicates the presence of substantially increased metastability (nucleation inhibition) in native biles compared with model biles at equivalent CSI and solute concentrations.

**Matching experiments.** The lipid composition and nucleation time of aliquots of fresh normal human gallbladder biles ( $n = 8$ ) was determined at 37°C. The nucleation times of these biles ranged from 55 to 200 h. Model biles, consisting of purified lecithin, cholesterol, and sodium taurocholate, were then prepared in order to match each human bile for molar composition of the three lipids. A comparison of the nucleation times for each matched pair is shown in Fig. 4. The lipid compositions of the native and model biles that correspond in numerical sequence to the nucleation times of Fig. 4 are given in Table I. All model bile samples dissolved at 55°C except for model biles, No. 3, 5, and 8, which required incubation at 95°C for complete dissolution. Model bile No. 4 did not become isotropic after 5-d incubation (with periodic mixing) at 95°C.

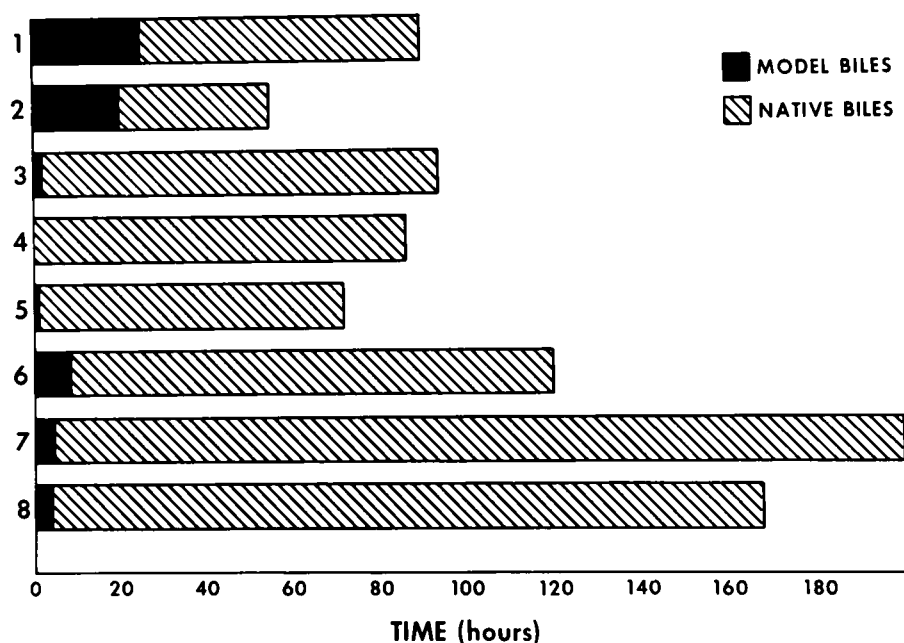


Figure 4. Nucleation times of eight normal human gallbladder bile (hatched columns) and eight matching model bile (solid columns) at 37°C.

The nucleation times of the native bile were from three- to 40-fold longer than those of the matched model bile.

*Supplementation experiments.* An alternative procedure for evaluation of metastability was to supplement biliary lipid-containing samples with purified model bile lipids to achieve a similar standard lipid composition. This approach is especially useful and even essential with human bile samples that are undersaturated with cholesterol. Supplementation of normal bile to equivalent CSI and total lipid concentration (Table II A) was achieved and resulted in solutions of comparable degrees of supersaturation. The nucleation times of these solutions, however, were strikingly prolonged when compared with a stan-

dard model bile of similar composition (Fig. 5). Nucleation times of the supplemented normal bile shown in Fig. 5 correspond in numerical sequence to the compositional data shown in Table II A).

Bile can be deproteinized by extraction of the lipids with 2:1 chloroform/methanol or 1:1 ethanol/ether, drying, and reconstitution in aqueous solution. Lipid extracts from normal gallbladder bile were thus prepared and supplemented to the standard lipid composition as previously described (Table II B). The nucleation times of the resulting supplements were determined. Table II C represents the compositions of supplemented lipid extracts that correspond in numerical sequence to the data

Table I. Lipid Compositions of Normal Gallbladder Biles and Matched Model Biles

	Normal biles					Matching model biles				
	Moles percent			Percent total lipids	CSI	Percent moles			Percent total lipids	CSI
	Chol	Lec	BS			Chol	Lec	BS		
1.	9.3	22.0	68.6	15.6	1.19	9.4	21.8	68.8	15.0	1.22
2.	9.2	27.1	63.7	12.9	1.11	9.8	25.3	64.9	11.9	1.22
3.	11.8	29.2	59.0	7.1	1.51	11.8	25.0	63.1	6.7	1.60
4.	10.9	16.0	73.1	7.0	1.98	—	—	—	—	—*
5.	12.3	21.6	66.1	8.4	1.76	9.9	19.2	70.9	9.0	1.50
6.	8.7	19.3	71.9	16.6	1.20	8.4	18.4	73.2	17.0	1.19
7.	9.9	21.8	68.3	17.5	1.24	9.8	20.9	69.3	17.5	1.26
8.	9.7	19.6	70.6	15.0	1.35	9.7	18.9	71.3	15.2	1.35

Chol, cholesterol; Lec, lecithin; BS, bile salts.

\* Anisotropic sample at up to 95°C.

Table II. Lipid Compositions of Supplemented Normal Human Gallbladder Biles and Controls

	Original bile				Supplemented bile				
	Moles percent			Percent total lipids	Moles percent			Percent total lipids	CSI
	Chol	PL	BS		Chol	PL	BS		
<b>A. Normal gallbladder bile</b>									
1.	9.3	22.0	68.6	15.6	8.2	18.7	73.1	20.0	1.11
2.	5.7	17.4	77.0	18.9	7.8	18.4	73.8	22.1	1.05
3.	9.2	27.1	63.7	12.9	9.2	19.1	71.7	20.5	1.23
4.	6.0	12.5	81.5	9.9	8.7	19.9	71.4	17.7	1.16
5.	6.5	18.2	75.3	12.9	8.8	18.1	73.1	17.3	1.24
<b>B. Standard model bile</b>									
	—	—	—	—	8.5	18.3	73.2	20.0	1.17
<b>C. Lipid extracts</b>									
1.	5.0	24.6	70.4	28.1	8.0	19.4	72.6	20.6	1.08
2a.	12.2	19.1	68.7	6.9	8.6	19.7	71.8	20.7	1.12
2b.*	12.3	22.3	65.4	6.1	8.7	19.4	71.9	20.4	1.14
3.	12.7	28.5	58.8	6.9	8.7	19.8	71.5	19.0	1.14
4.	8.6	14.3	41.7	10.7	8.8	19.5	71.7	21.7	1.12
5.*	3.8	13.0	83.2	2.5	9.1	20.2	70.6	16.3	1.22
6.*	7.8	22.3	69.8	10.2	8.3	18.7	72.9	18.8	1.14

Chol, cholesterol; PL, phospholipids; BS, bile salts.

\* These lipid fractions were 1:1 ethanol/ether (vol/vol) extracts of the native biles. All other lipid fractions were obtained with 2:1 chloroform/methanol (vol/vol).

in Fig. 5. These data (Fig. 5) also show both that the supplemented lipid extracts had essentially similar CSI and that their nucleation times were not different from that of the standard model biles. Thus, deproteinization of normal human bile greatly

decreases the metastability (nucleation inhibition) of supersaturated lipids derived from bile.

Pooled, concentrated lipid-containing B fractions derived from gel chromatography of normal gallbladder biles, were sup-

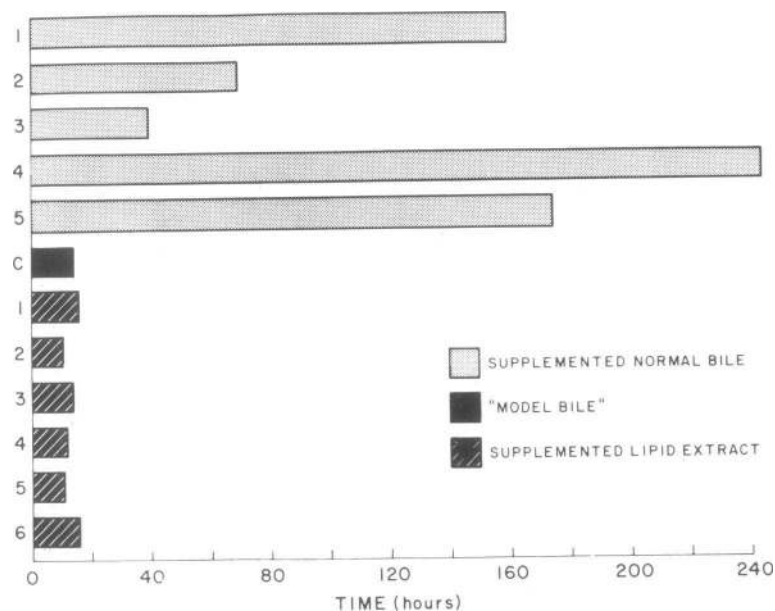


Figure 5. Nucleation times of a standard control model bile (black bar), five normal human gallbladder biles supplemented to the standard lipid composition (stippled columns) and of lipid fractions (diagonally striped columns) extracted from six normal human gallbladder biles (data from Table II).

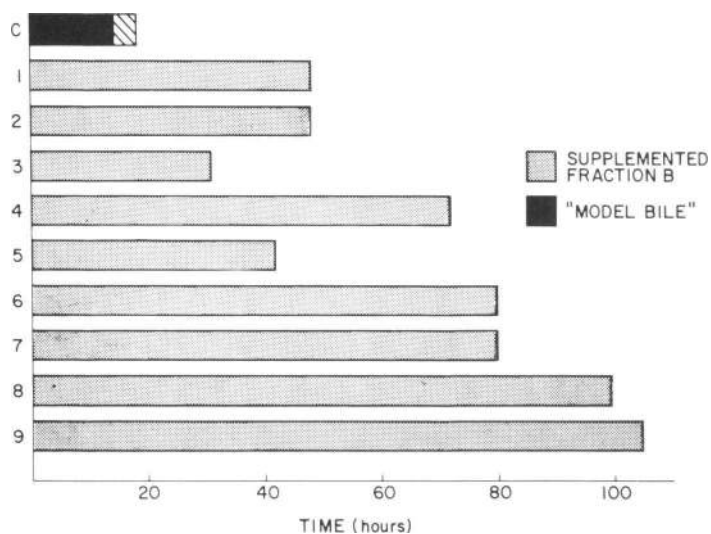


Figure 6. Nucleation times of control model bile (solid column) and of fraction B from normal gallbladder bile supplemented to the control lipid composition (stippled columns). Hatched areas at the end of control results denote the range of variability of the observed nucleation times.

plemented to the standard lipid composition (Table II B), as previously described, and their nucleation times were determined (Fig. 6). The lipid compositions of the B fractions and of the final supplements are shown in Table III, the numerical sequence of which correspond to the nucleation time observations indicated in Fig. 6. All such supplements demonstrated prolonged nucleation times compared with the controls.

**Recombination experiments.** Sedimented (detergent delipidated) proteins were prepared from normal gallbladder biles that were dialyzed free of detergent and concentrated to the original sample volume. These fractions were recombined with standard model biles and the nucleation times of the mixtures were determined. All samples had substantially longer nucleation times when compared with the control model bile (Fig. 7). When detergent delipidated bile proteins were preincubated with pronase, and subsequently recombined with standard model bile

lipids, the observed nucleation time for the mixture was that of the control (12 h). The nucleation times of the recombined mixture containing unpretreated protein and of the lipids recombined only with saline were 41 and 17 h, respectively. Pronase alone did not affect the nucleation time of the standard model bile. As a further protein control, human serum albumin (Calbiochem-Behring Corp.) was recombined with standard model bile using the bile protein recombination technique previously described in a concentration series encompassing the range of 50 to 2,000  $\mu\text{g}/\text{ml}^{-1}$ . The observed nucleation time for these mixtures was not different from that of the control.

Fraction A proteins from six normal biles were dialyzed and recombined with standard model bile lipids and the nucleation times of the mixtures are given in Fig. 8. There is a modest but consistent prolongation of nucleation time in the mixtures ranging up to threefold the control nucleation time. No correlation

Table III. Lipid Compositions of B Fractions and of Supplemented B Fractions from Normal Human Gallbladder Biles

	Fraction B				Supplemented fraction B				
	Moles percent			Percent total lipids	Moles percent				CSI
	Chol	PL	BS		Chol	PL	BS	Percent total lipids	
Control	—	—	—	—	8.6	18.2	73.3	19.6	1.18
1.	3.6	21.3	75.1	6.6	8.52	18.8	72.7	19.2	1.15
2.	6.1	16.6	77.3	15.4	8.4	18.5	73.0	17.9	1.16
3.	7.9	12.5	81.8	6.6	8.4	18.8	72.9	15.8	1.19
4.	7.1	16.8	48.5	13.2	8.7	17.9	73.3	17.6	1.25
5.	1.2	8.9	89.8	7.6	9.1	19.3	71.6	18.0	1.22
6.	3.7	21.5	74.7	6.6	8.5	18.3	73.2	19.2	1.17
7.	5.1	17.2	77.6	12.4	8.3	20.4	71.2	19.8	1.08
8.	2.6	10.2	87.2	4.0	8.2	18.8	73.0	19.1	1.12
9.	5.8	17.1	77.1	14.2	8.5	17.6	73.9	18.2	1.22

Chol, cholesterol; PL, phospholipids; BS, bile salts.

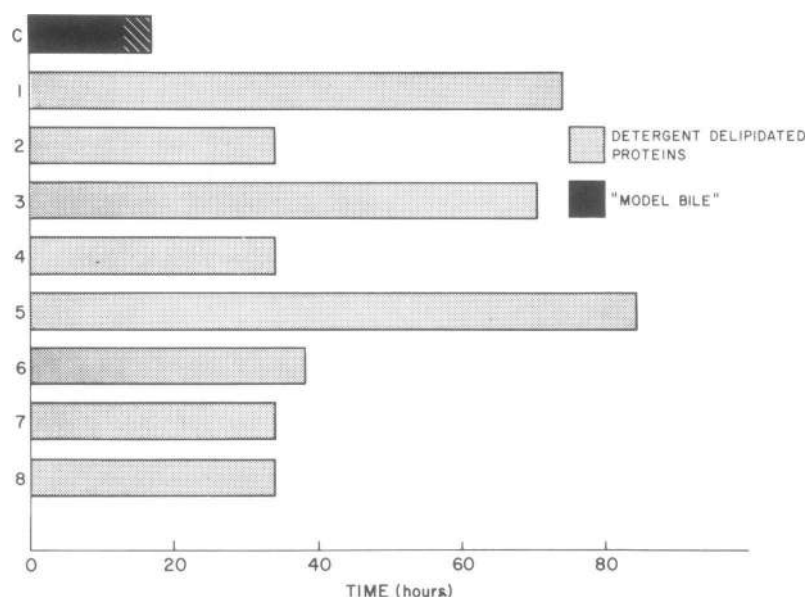


Figure 7. Nucleation times of control model bile (solid column) and of model bile lipids recombined with a bile protein fraction from normal human biles obtained by detergent delipidation (stippled columns). Hatched areas at the end of control results denote the range of variability of the observed nucleation times.

was found between protein concentration (weight per volume) of the mixtures and nucleation times in any of the recombination experiments.

Qualitative analysis, by thin-layer chromatography of model bile lipids, following recombination experiments in which inhibition of nucleation time was observed, showed no significant differences between lipids exposed to delipidated proteins and to saline control solutions. Thus, model bile lipid degradation

does not occur to an appreciable extent during the course of nucleation time assays.

*Characterization of the bile proteins.* Differences in the character of the proteins found in fractions A and B are demonstrated in the electrophoretic gel shown in Fig. 9. Although there is overlap, the proteins found in fraction A show a distinct trend toward larger molecular weights when compared with those in fraction B. From an overview standpoint, in Fig. 10 A is shown an analytical isoelectrofocusing gel of delipidated fraction B proteins. Further, in Fig. 10 B is shown a two-dimensional gel of delipidated fraction B proteins. Clearly de-

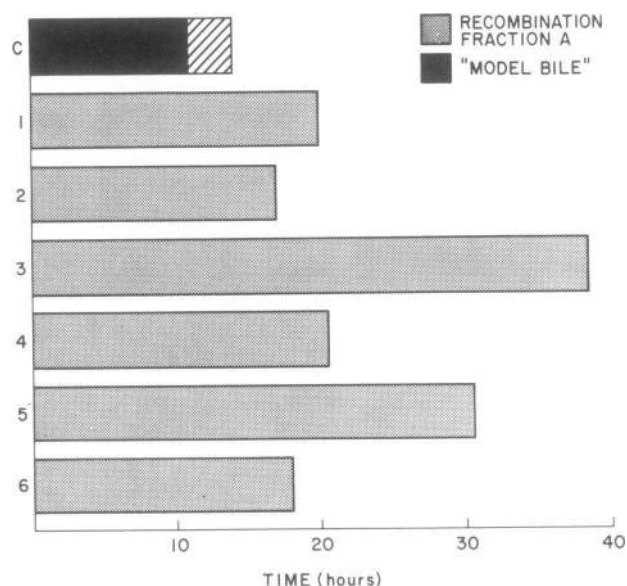


Figure 8. Nucleation times of control model bile (solid column) and of model bile lipids recombined with fraction A proteins from normal human gallbladder biles (stippled columns). Hatched areas at the end of control results denote the range of variability of the observed nucleation times.

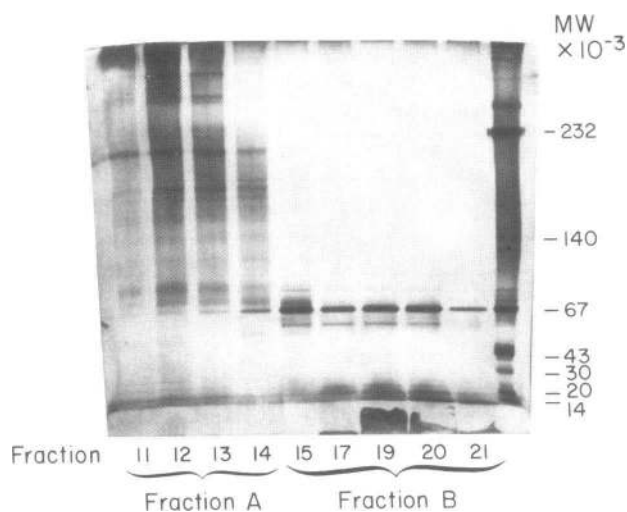


Figure 9. SDS-polyacrylamide gel electrophoresis under nonreducing conditions of proteins present in human gallbladder bile after fractionation by Sephacryl S-200 gel filtration chromatography. Fractions are as in Fig. 1.



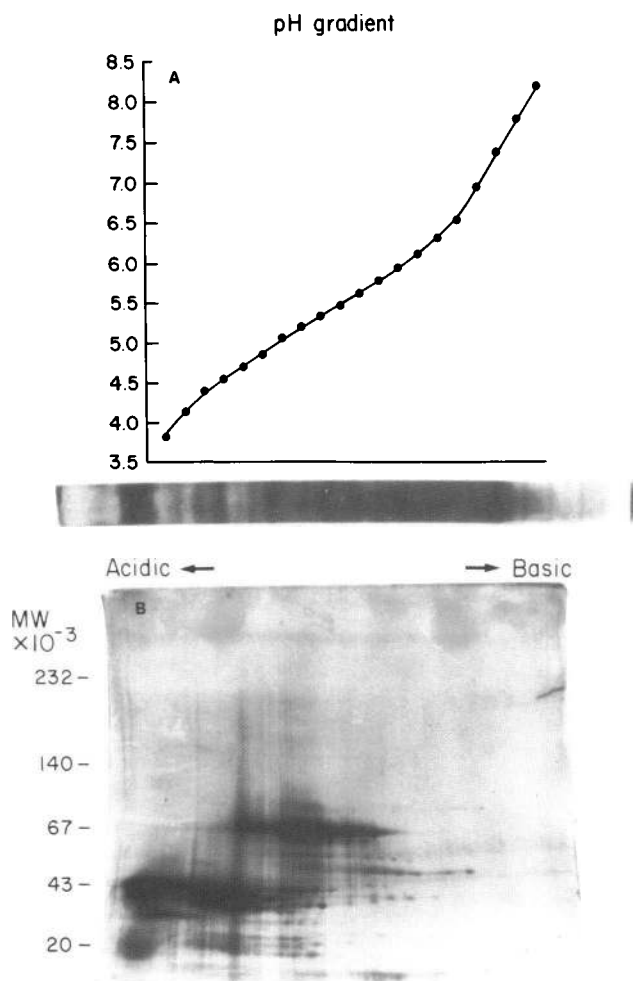


Figure 10. (A) Isoelectrofocusing of delipidated fraction B proteins from human gallbladder bile. (B) Two-dimensional electrophoresis of delipidated fraction B proteins from human gallbladder bile.

monstrable from the combination of these techniques is the inherent complexity of the bile proteins.

## Discussion

Cholesterol crystal nucleation, the crucial first step in the process of gallstone formation in bile, does not occur simply as predicted from studies of model solutions (4). In normal gallbladder bile there is a significant retardation or inhibition of nucleation as indicated by observations of its delayed onset time when compared with control model systems (Fig. 3). These observations are definitively established in the matching and supplementation studies of the present work (Figs. 4 and 5).

**Nucleation inhibitory factor.** Since the crystal nucleation inhibitory effect is not found in model biles composed only of the primary biliary lipids, a reasonable deduction has been that the chemical nature of the inhibitor is not that of a lipid. Such an expectation was borne out in the present studies by a failure

to demonstrate an inhibitory effect using the present *in vitro* functional assay system with total biliary lipid extracts under controlled conditions (Fig. 5).

Although bile proteins comprise <5% of the total biliary solid content, as a class they represent the next most abundant solute, second only to the biliary lipids (1). Thus, despite their comparatively low concentration in bile, it was natural to wonder whether the bile proteins could have a crystal nucleation inhibitory effect. If the proteins of bile play a role in metastable supersaturation of cholesterol, the first requirement is that they form complexes or otherwise physically interact in some way with bile lipids. This is suggested by analogy with serum lipoproteins. The existence of lipoprotein complexes in bile has been previously suggested but not proven by a number of workers (25–34). The presence of an inhibitory effect was demonstrable in the present study in several fractions of delipidated bile proteins (Figs. 7 and 8) as well as a fraction of lipid-containing bile protein (Fig. 6). These findings neither conclusively demonstrate the presence of a bile lipoprotein complex as a valid entity nor exclude the possibility that there could also be some additional contribution to the naturally occurring nucleation-inhibiting effect seen in bile from some as yet unidentified solute. They do indicate, however, that the predominant nucleation inhibitory factor probably represents a subfraction of bile proteins interacting with bile lipids. This view is further strengthened by the observations that pronase treatment completely abolished the nucleation inhibitory effect. Regarding the presently demonstrated nucleation inhibitory effect of proteins, one could reasonably wonder whether this is merely a general effect of proteins or a specific effect of bile proteins. The lack of a demonstrable effect for albumin indicated as a control for the detergent delipidation protein recombination experiments (Fig. 7) supports the view that the bile protein effect is probably specific.

Of the gel chromatography fractions, a less potent inhibitory effect was found in the fraction eluting between the excluded volume limit and the biliary lipids (Figs. 1 and 8). A more potent inhibitory effect was found in the bile protein fraction exhibiting an apparently greater degree of lipid association during elution (Figs. 1 and 6). Because the nucleation time observations suggest some form of functional protein-lipid interaction, these findings are not too surprising. The protein fraction obtained by detergent ultracentrifugation delipidation exhibited an inhibitory effect resembling, in relative potency, that of the more closely lipid-associated chromatographic protein fraction (fraction B) (Figs. 6 and 7). The comparative equivalency in potency between the detergent delipidated proteins and the lipid-associated chromatographic fractions suggests that they contain a roughly equivalent amount of the as yet unidentified effector proteins. No correlation was found in the matching and supplementation studies between protein concentration and nucleation times. Similarly, despite great variability in recovery of bile proteins for the recombination studies, no correlation was observed between protein recovery and the degree of inhibition of crystal nucleation. Albumin, as we have indicated, demonstrates no nucleation inhibitory effect and is the predominant component of bile proteins. A correlation between nucleation time and total

bile proteins would therefore not necessarily have been expected.

**Relevance to other nucleation studies.** The observation of a sharp discrimination between normal and abnormal biles in the basis of nucleation time originally reported by us has now been confirmed by others (2, 3, 5). Much of the focus of attention in these studies has been on the potential presence of nucleating agents in the abnormal biles to account for the remarkably shortened nucleation times observed. A recent study, moreover, has demonstrated that the potency of this apparent nucleating factor is great (5). Although the biochemical nature of this factor remains to be determined, it is assumed to be produced within the gallbladder and appears not to be a gallbladder mucosa-derived mucin (3). By contrast, in the prairie dog model, recent studies have suggested a role for mucosal-derived gallbladder mucin as a nucleating agent. The relevance of these findings to the pathogenesis of human gallstones, however, remains unestablished (6, 7). Evidence provided in this study for a crystal nucleation inhibitor normally present in human bile justifies speculation that in disease the inhibitor could be either absent, blocked, or inactivated. Several papers have appeared in the literature that describe specific proteins having an effect on crystallization phenomena in solutions of biological interest (35–38). The mechanism(s) whereby these proteins exert their effects probably differ considerably from the effect of bile proteins. This is because they affect ionic electrolyte solutions rather than predominantly micellar solutions and because their effects are more probably on inhibition of crystal growth, rather than on crystal nucleation per se.

We conclude that patients without gallstone disease have a cholesterol crystal nucleation inhibitor that is predominantly derived from bile proteins. This finding affords an explanation for the prolonged metastability found in normal human bile, especially in the presence of marked biliary cholesterol supersaturation. The precise mechanism whereby the inhibitory effect is mediated remains unclear and this may be of fundamental importance to both the definition of health and the pathogenesis of cholesterol gallstone disease.

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## References

1. 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.
2. Sedaghat, A., and S. M. Grundy. 1980. Cholesterol crystals and the formation of cholesterol gallstones. *N. Engl. J. Med.* 302:1274–1277.
3. Gollish, S. H., M. J. Burnstein, R. G. Ilson, C. N. Petrunka, and S. M. Strasberg. 1983. Nucleation of cholesterol monohydrate crystals from hepatic and gallbladder bile of patients with cholesterol gallstones. *Gut*. 24:836–844.
4. Carey, M. C., and D. M. Small. 1978. The physical chemistry of cholesterol solubility in bile. *J. Clin. Invest.* 61:988–1026.
5. Burnstein, J. M., R. G. Ilson, C. N. Petrunka, and S. M. Strasberg. 1983. Evidence for a potent nucleating factor in gallbladder bile of patients with cholesterol gallstones. *Gastroenterology*. 85:801–807.
6. Lee, S. P., J. T. LaMont, and M. C. Carey. 1981. Role of gallbladder mucus hypersecretion in the evolution of cholesterol gallstones. Studies in the prairie dog. *J. Clin. Invest.* 67:1712–1723.
7. Lee, S. P., M. C. Carey, and J. T. LaMont. 1981. Aspirin prevention of cholesterol gallstone formation in prairie dogs. *Science (Wash. DC)*. 211:1429–1431.
8. Helenius, A., E. Fries, and J. Kartenbeck. 1977. Reconstitution of Semliki Forest virus membrane. *J. Cell Biol.* 75:866–880.
9. Hoenders, H. J., J. G. G. Schoenmakers, J. J. T. Gerding, G. I. Tesser, and H. Bloemendal. 1968. N-Terminus of alpha crystallin. *Exp. Eye Res.* 7:291–300.
10. Allain, C. C., L. S. Poon, C. S. G. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total serum cholesterol. *J. Clin. Chem.* 20:470–475.
11. Cholesterol (Enzymatic Method). 1979. Boehringer Mannheim Biochemicals, Indianapolis, IN. Third ed.
12. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* 66:375–400.
13. Barlett, G. R. 1959. Phosphorus assay by column chromatography. *J. Biol. Chem.* 234:466–468.
14. Turley, S. D., and J. M. Dietschy. 1978. Re-evaluation of the 3-hydroxysteroid dehydrogenase assay for total bile acids in bile. *J. Lipid Res.* 19:924–928.
15. Randerath, K. 1966. Thin-Layer Chromatography. Second ed. Academic Press, Inc., New York. 151–179.
16. Carey, M. C. 1978. Critical tables for calculating the cholesterol saturation of native bile. *J. Lipid Res.* 19:945–955.
17. Castell, J. V., M. Cervera, and R. Marco. 1979. A convenient micromethod for the assay of primary amines and proteins with fluorescamine. A re-examination of the conditions of reaction. *Anal. Biochem.* 99:379–391.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680–685.
19. Reid, M. S., and R. L. Bielecki. 1968. A simple apparatus for vertical flatsheet polyacrylamide gel electrophoresis. *Anal. Biochem.* 22:374–381.
20. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
21. Merril, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science (Wash. DC)*. 211:1437–1438.
22. 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.
23. Holzbach, R. T., and C. Corbusier. 1978. Liquid crystals and cholesterol nucleation during equilibration in supersaturated bile analogs. *Biochim. Biophys. Acta*. 528:436–444.
24. Pope, J. L. 1967. Crystallization of sodium taurocholate. *J. Lipid Res.* 8:146.
25. Verschure, J. C. M., P. H. Mijnlief, F. M. C. Heofsmitt, and

- A. E. Nooter Van Hoeven. 1956. The dominating macromolecular complex of human gallbladder bile. *Clin. Chim. Acta.* 1:154-166.
26. Verschure, J. C. M., J. De Wael, and P. F. Mijnlieff. 1956. Further investigations on the macromolecular complex in human bile. *Clin. Chim. Acta.* 1:511-518.
27. Nakayama, F., and H. Miyake. 1965. Cholesterol complexing by macromolecular fractions in human gallbladder bile. *J. Lab. Clin. Med.* 65:638-648.
28. Lairon, D. H., H. Lafont, and J. C. Hauton. 1972. Lack of mixed micelles: bile salt-lecithin-cholesterol in bile and the presence of a lipoprotein complex. *Biochimie (Paris).* 54:529-530.
29. Nalbone, G., H. Lafont, N. Domingo, D. Lairon, G. Pautrat, and J. C. Hauton. 1973. Ultramicroscopic study of the bile lipoprotein complex. *Biochimie (Paris).* 55:1503-1506.
30. Lafont, H., D. Lairon, N. Domingo, G. Nalbone, and J. C. Hauton. 1974. Does a lecithin-polypeptide association in bile originate from membrane structural subunits? *Biochimie (Paris).* 56:465-468.
31. Lafont, H., G. Nalbone, D. Lairon, J. C. Dagorn, N. Domingo, J. Amic, and J. C. Hauton. 1977. Zone electrophoresis study of the bile lipoprotein complex. *Biochimie (Paris).* 54:445-452.
32. Nalbone, G., H. Lafont, D. Lairon, J.-L. Vigne, N. Domingo, J. Leonardi, and J. C. Hauton. 1978. Immunogenicity of the apoprotein of the bile lipoprotein complex. *Biochimie (Paris).* 60:691-694.
33. Nalbone, G., H. Lafont, J.-L. Vigne, N. Domingo, D. Lairon, C. Chabert, P. Lechene, and J. C. Hauton. 1979. The apoprotein fraction of the bile lipoprotein complex: isolation, partial characterization, and phospholipid binding properties. *Biochimie (Paris).* 61:1029-1041.
34. Manzato, E., R. Fellin, G. Baggio, S. Walch, W. Neubeck, and D. Seidel. 1976. Formation of lipoprotein-X. Its relationship to bile compounds. *J. Clin. Invest.* 57:1248-1260.
35. Sutor, D. J., and J. M. Percival. 1976. Presence or absence of inhibitors of crystal growth in bile. *Gut.* 17:506-510.
36. Ito, H., and F. L. Coe. 1977. Acidic peptide and polyribonucleotide crystal growth inhibitors in human urine. *Am. J. Physiol.* 233:F455-463.
37. Coe, F. L., H. C. Margolis, L. H. Deutsch, and A. L. Strauss. 1980. Urinary macromolecular crystal growth inhibitors in calcium nephrolithiasis. *Miner. Electrolyte Metab.* 3:268-275.
38. Nakagawa, Y., H. C. Margolis, S. Yokoyama, F. J. Kezdy, E. T. Kaiser, and F. L. Coe. 1981. Purification and characterization of a calcium oxalate monohydrate crystal growth inhibitor from human kidney tissue culture medium. *J. Biol. Chem.* 256:3936-3944.