

# Cholesterol crystallization-promoters in human bile: comparative potencies of immunoglobulins, $\alpha_1$ -acid glycoprotein, phospholipase C, and aminopeptidase N<sup>1</sup>

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**Abstract** Concanavalin A (Con A)-binding glycoproteins accelerate the rate of cholesterol crystal formation as a prelude to gallstone formation. Immunoglobulins (IgM, IgA, and IgG), aminopeptidase N (APN), phospholipase C (pcPLC), and  $\alpha_1$ -acid glycoprotein from this Con A fraction have all been proposed as candidate promoters. We immunopurified each of the six putative promoters and examined their comparative effects by adding equal amounts to a cholesterol crystal growth assay. The effects of immunoabsorptive removal of each of the specific candidate promoters from native bile were also compared. In additional studies, the potency of these proteins was in the following order: IgM > IgA = AAG > IgG. APN and pcPLC showed no effect on cholesterol crystal growth at their apparent physiological concentrations. In subtractive experiments, only a minor loss (<10%) of net promoting activity from that of the whole Con A-bound fraction was observed after immunoabsorptive removal of pcPLC, APN, or immunoglobulins. Total removal of AAG, however, showed a far greater loss (@33%) of the net promoting activity. These data indicate that AAG accounts for the greatest portion of net biliary Con A-bound promoting activity derived from currently defined and well-identified glycoproteins. However, more than 60% of total Con A-binding promoting activity remains unaccounted for, indicating the presence of other important and still unidentified promoters in human bile.—Abei, M., J. Schwarzendrube, H. Nuutinen, T. A. Broughan, P. Kawczak, C. Williams, and R. T. Holzbach. Cholesterol crystallization-promoters in human bile: comparative potencies of immunoglobulins,  $\alpha_1$ -acid glycoprotein, phospholipase C, and aminopeptidase N. *J. Lipid Res.* 1993. **34**: 1141–1148.

**Supplementary key words** gallstone • biliary protein • cholesterol nucleation • cholesterol immunoglobulins • orosomucoid • promoting factors • Concanavalin A

Supersaturation of cholesterol in bile is a necessary but not sufficient condition for the formation of cholesterol gallstones (1–3). Biliary proteins, which are capable of affecting the rate at which cholesterol crystallization occurs, are now recognized as important factors in the pathogenesis of cholesterol gallstone disease (4–15). Groen et al. (8, 9) found that bile from gallstone patients, as well

as from stone-free subjects, contains crystallization-promoting activity that binds to Concanavalin A (Con A). We and others (10, 11) have confirmed that this Con A-bound glycoprotein fraction (designated as CABG-fraction) is a major source of crystallization promoting activity in bile. Recently, several glycoproteins have been independently isolated from this CABG-fraction of human gallbladder bile and reported to have promoting activity (12–17). Among these are a 130-kDa glycoprotein (12), polymeric immunoglobulins (IgM and probably IgA) (13), phosphatidylcholine-specific phospholipase C (EC 3.1.4.3, designated here as pcPLC (14), and  $\alpha_1$ -acid glycoprotein (designated here as AAG) (15, 16). Also, the suggested identity of the 130-kDa glycoprotein of Groen et al. (12) as aminopeptidase N (EC 3.4.11.2, designated here as APN) has been reported (17) (pcPLC, AAG, APN are reviewed in references 18 through 20, respectively).

None of these reports, however, provide comparative data regarding the crystallization-promoting activity of these various putative promoters, nor do they examine results obtained by other groups. This lack of comparison is probably attributable to the striking differences in the protein purification procedures used by each group. In any case, whether all of these factors at their physiological level are more or less involved in *in vivo* cholesterol crystallization kinetics in bile (thus, multifactorial promotion) is unclear. Therefore, their relative significance in the pathogenesis of gallstone disease remains obscure.

The present study was undertaken to provide comparative data regarding the relative potency of these different glycoprotein promoters and to clarify the relative contri-

Abbreviations: AAG,  $\alpha_1$ -acid glycoprotein; APN, aminopeptidase N; CABG, Con A-bound glycoproteins; CSI, cholesterol saturation index; pcPLC, phosphatidylcholine phospholipase C.

<sup>1</sup>This paper is dedicated to Professor Gustav Paumgartner in honor of his sixtieth birthday.

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bution of these proteins to the net promoting activity in the biliary CABG-fraction. Using specific immunoaffinity columns, we isolated six different promoter candidates from human gallbladder bile (IgM, IgA, IgG, AAG, APN, and pcPLC). The promoting effects of these proteins on cholesterol crystallization were examined using a cholesterol crystal growth assay (21). Further, effects of immunoabsorption removal of these glycoproteins on the promoting activity of the CABG were compared.

## MATERIALS AND METHODS

### Chemicals and antibodies

Sodium taurocholic acid and sodium taurodeoxycholic acid were purchased from Calbiochem (San Diego, CA). Egg yolk phosphatidylcholine (grade I) 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). Bacterial pcPLC (*Bacillus cereus*, grade 1-2000 IU/0.5 ml) was from Boehringer Mannheim (Indianapolis, IN). Human serum IgG, IgA, IgM, and AAG standards were obtained from Sigma Chemical (St. Louis, MO). All other chemicals were of the highest grade commercially available.

The following seven specific antibodies were used in this study. Polyclonal antibody to human generic immunoglobulins (anti-Ig, which reacts with IgG, IgM, and IgA) and polyclonal antibodies to three individual immunoglobulins [anti-human IgM ( $\mu$  chain), anti-human IgG ( $\gamma$  chain), and anti-sIgA ( $\alpha$  chain + secretory component)] were purchased from Boehringer Mannheim. Polyclonal antibody to human AAG was obtained from Sigma. Monoclonal antibody to APN was a gift from Dr. Ward A. Olson, Department of Gastroenterology, University of Wisconsin. Polyclonal antibody to pcPLC was a gift from Dr. I. Moraru, Department of Surgery, University of Connecticut. The latter antibody has been shown to crossreact and inhibit pcPLC activity in human tissue (22–24).

### Cholesterol crystal growth assay

Supersaturated model bile was prepared, and the subsequent cholesterol crystal growth assay was performed, as described previously (21). Briefly, appropriate aliquots of cholesterol, phosphatidylcholine, and sodium taurocholic acid solutions in methanol-CH<sub>2</sub>Cl were mixed to construct a supersaturated model bile with a cholesterol saturation index (CSI) of 1.4, a total lipid concentration (TL) of 12.5 g/dl, and a bile acid/phospholipid molar ratio (BA/PL) of 4.4. The lipid mixture was evaporated to dryness and then resolubilized with 25 mmol/l Tris-HCl and 150 mmol/l NaCl at pH 7.4 (TBS) and filtered through a 0.22- $\mu$ m pore filter. Aliquots (325  $\mu$ l) of this model bile

were distributed in vials and mixed with either a protein sample (50  $\mu$ l in TBS) or a control solution (50  $\mu$ l of TBS). The vials were incubated at 37°C with shaking. The crystal mass in the vials was measured at various times by diluting a 25- $\mu$ l aliquot with 10 mmol/l sodium taurodeoxycholic acid-TBS (475  $\mu$ l) solution for 20 min, followed by absorbance measurement at 900 nm. The cholesterol crystal growth curves of supersaturated model bile without (control) and with (experimental) protein samples were thus obtained.

The crystal growth curves usually consist of three components: the initial metastable phase (from time zero until the onset of crystal nucleation), the growth phase (from crystal nucleation until the equilibrium phase), and the equilibrium phase (when the growth of the crystal mass reaches a plateau). Effects of protein samples on these three phases of cholesterol crystal growth were assessed by the nucleation time index ( $I_t$ ), the growth index ( $I_g$ ), and the equilibrium crystal index ( $I_c$ ) as described previously (11).

### Bile collection

Human gallbladder bile samples were obtained at cholecystectomy from patients with cholesterol cholelithiasis identified by defined morphological criteria (3). Gallbladder aspirates were pooled and stored at -80°C.

### Chemical and electrophoretic analysis

Total bile acid concentrations were measured by 3 $\alpha$ -hydroxysteroid dehydrogenase method (25). Phospholipid concentrations were determined by the method of Bartlett (26), and cholesterol concentrations were quantified by a cholesterol oxidase method (27). Biliary proteins were measured fluorometrically after delipidation (28, 29) or in delipidated samples by the method of Bradford (30). SDS-PAGE (4%–20%) under nonreduced and reduced conditions was performed as described by Laemmli (31) with silver stain by the method of Morrissey (32).

### Isolation of cholesterol crystallization-promoting glycoproteins

Pooled native gallbladder bile was ultracentrifuged (Model L5-50, with 50.3 Ti rotor, Beckman Instruments, Fullerton, CA) at 100,000 *g* for 1 h to remove solid crystals as well as mucus glycoprotein. The samples were then concentrated and dialyzed against 25 mmol/l ammonium bicarbonate, pH 8.

### Immunoaffinity chromatography

Six antibody affinity columns (anti-IgM, anti-IgG, anti-IgA, anti-AAG, anti-PLC, anti-APN) were prepared to isolate immunoglobulins, AAG, APN, and PLC from bile samples. Approximately 5–10 mg of each antibody was coupled according to manufacturer's specifications to

2 ml of AminoLink™ gel to make these antibody columns (0.5 cm × 2 cm). The AminoLink™ gel contains aldehydes that react with the primary amine groups of antibodies and thus can form a stable covalent linkage with minimal leakage of immobilized antibodies.

Samples were applied to each antibody column, which had been equilibrated with 25 mmol/l Tris-HCl, 150 mmol/l NaCl, pH 7.4 (TBS). 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 to remove unbound materials, which were collected for subsequent analysis. The unbound fraction was concentrated and examined by SDS-PAGE stained with silver and by Western blot (see below). When the unbound fraction still contained bands corresponding to either IgM, IgG, or IgA, by either method, this fraction was then re-loaded onto the affinity column. This procedure was repeated as necessary until a completely Ig- or AAG-free fraction was obtained. In all cases, not more than a single recycling was found necessary for complete removal by use of these criteria. The bound proteins were then eluted from the columns by 5 column volumes of 0.1 mol/l glycine-HCl, pH 2.6, into tubes containing 1 mol/l Tris-HCl, pH 8.0, which immediately neutralized the acidic elution buffer.

#### Immunoabsorption removal of different crystallization-promoters from the CABG-fraction

The CABG-fraction, which possesses cholesterol crystallization-promoting activity, was isolated from bile as originally described by Groen et al. (8, 9). Briefly, pooled native bile was dialyzed in 10 mmol/l Tris-HCl, 0.5 mol/l NaCl, 1 mmol/l CaCl<sub>2</sub>, 1 mmol/l MgCl<sub>2</sub>, 1 mmol/l MnCl<sub>2</sub>, 4 mmol/l sodium taurocholic acid, and 1.5 mmol/l NaN<sub>3</sub>, pH 7.4 (starting buffer) and applied to a Con A Sepharose (LKB/Pharmacia Biotechnology Inc., Piscataway, CA) column (1.6 cm × 10 cm). After extensive washing with the starting buffer, glycoproteins bound to the column were eluted with 0.2 mol/l  $\alpha$ -D-methylmannopyranoside (Sigma) in the starting buffer.

Polyclonal antibody to human generic immunoglobulins (anti-Ig) was coupled to 2 ml of AminoLink™ gel as described above. Approximately 10 mg of the CABG-fraction was applied to this column as well as to the anti-AAG, anti-PLC, anti-APN columns. The proteins that did not bind to these columns (the unbound fractions) were collected for subsequent analysis.

#### Western blotting procedure

Aliquots of the biliary CABG-fraction obtained both before and after immunosorptive removal of the three Ig classes, AAG, APN, and pcPLC were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride

membrane (Immobilon P™, Millipore Corp., Bedford, MA) in 48 mM TRIS, 39 mM glycine, 20% methanol, pH 9.2, using a Trans-Blot SD semi-dry blot electrophoretic transfer system (Bio-Rad). Electrophoretic transfer was completed in 36 min at constant voltage of 20 V. After the transfer, immunostaining (33) of the membrane was performed using rabbit polyclonal antibody against human generic immunoglobulins (reacting with IgG, IgM, and IgA) as well as antibodies to human AAG (polyclonal), human APN (monoclonal), and bacterial pcPLC (polyclonal). In addition to these blotting studies, which were performed to estimate completeness of immunosorption removal of the various fractions, more quantitative assessments of this were undertaken by nephelometric immunoquantitation (Igs) (34) and by ELISA (AAG) (35) of the total CABG fraction as well as of the derived bound and unbound fractions from each of the four different immunoaffinity columns during chromatographic separations.

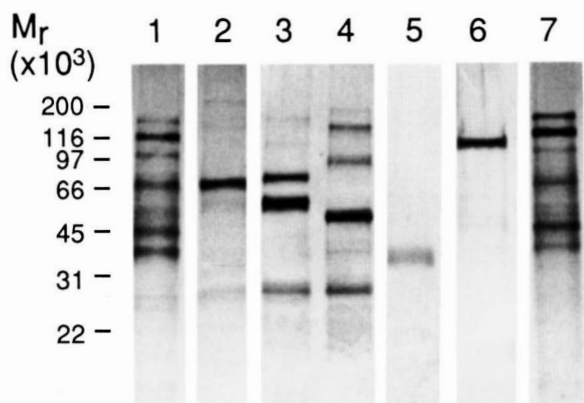
#### Statistical analysis

Statistical analysis of the cholesterol crystal growth curves was performed using analysis of variance (ANOVA) at each time to determine whether differences existed between the study groups. When the ANOVA was statistically significant ( $P < 0.05$ ), the Dunnett's multiple comparison procedure was used to compare each of the study groups to the control group (36).

## RESULTS

#### Isolation of different crystallization-promoters from cholesterol gallstone-associated gallbladder bile

Six different candidate glycoprotein promoters were purified from gallbladder bile using specific antibody columns. From 2 ml of native bile, the following approximate amounts of specific glycoprotein were thus obtained: IgM (100  $\mu$ g), IgA (200  $\mu$ g), IgG (200  $\mu$ g), AAG (100  $\mu$ g), and APN (15  $\mu$ g). These proteins, as well as the CABG-fraction, were run on SDS-PAGE. As shown in Fig. 1, each of these respective proteins was thus isolated with apparent purification to homogeneity;  $\mu$  chain + light chain for IgM (lane 2),  $\alpha$  chain + light chain + secretory component for IgA (lane 3),  $\gamma$  chain + light chain for IgG (lane 4), a 42-kDa band for AAG (lane 5), a 130-kDa band for APN (lane 6) are identifiable. On the other hand, no pcPLC could be identified by either protein assay ( $< 0.1 \mu$ g) or by Western blot. Because no pcPLC was recovered from 2 ml of bile sample, the experiment was repeated with more than 10 times the volume of bile sample (25 ml), but the result was the same. High catalytic activity was retained in the immunopurified APN fraction indicating that the structural integrity of this protein must have been mostly intact.



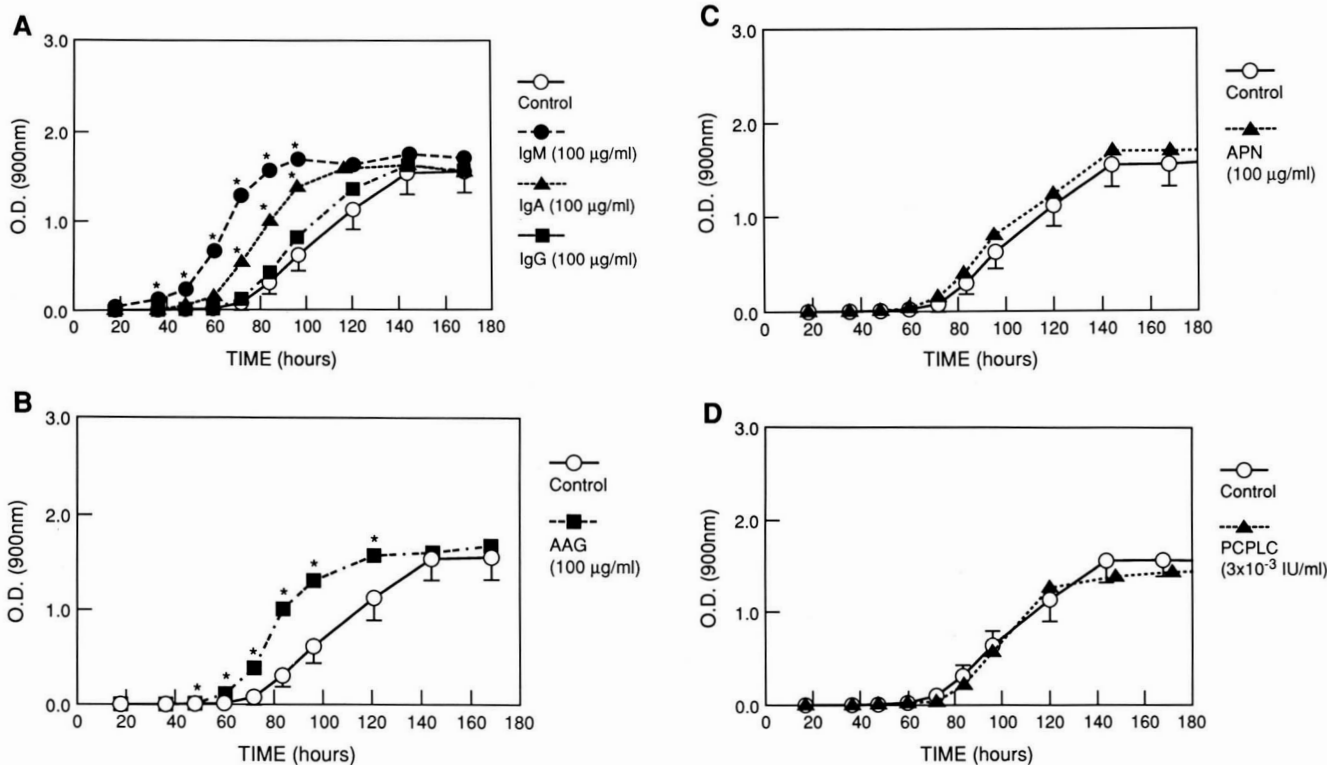
**Fig. 1.** SDS-PAGE (reducing conditions) of isolated biliary immunoglobulins, AAG, and APN. Lanes 1 and 7, the biliary CABG-fraction; lane 2, Ig M ( $\mu$  chain and light chain); lane 3, Ig A ( $\alpha$  chain, light chain, and secretory component); lane 4, Ig G ( $\gamma$  chain and light chain); lane 5, AAG (42 kDa); lane 6, APN (130 kDa).

### Comparative effect of adding crystallization-promoters on cholesterol crystal growth

Because approximately 10  $\mu\text{g/ml}$  to 200  $\mu\text{g/ml}$  of IgM, IgA, IgG, AAG, and APN were recovered from native bile, 100  $\mu\text{g/ml}$  each of these isolated proteins was chosen for equivalent mass comparison and mixed with an identical

supersaturated model bile (CSI = 1.4, TL = 12.5 g/dl, BA/PL = 4.4). Despite our failure to detect any amount of biliary pcPLC, a minimal amount of pcPLC could still be present below the detectable threshold. The activity of pcPLC in gallbladder bile has been estimated by others at 19.1 nmol/h per mg bile protein ( $1.2 \times 10^{-3}$  IU/mg protein) (35), and at 0.1  $\mu\text{mol}/24$  h per ml ( $6.9 \times 10^{-5}$  IU/ml) (36). Accordingly, to ensure that a sufficient activity was used, a supraphysiologic amount of  $3.0 \times 10^{-3}$  IU/ml of bacterial pcPLC (approximately 50 times the apparent physiological level in human bile) was added to model bile to examine its effect on cholesterol crystallization.

The results of these addition experiments are shown in **Fig. 2**. IgM showed the strongest promoting effects among the three immunoglobulins on both initial crystal detection and maximal growth rate (Fig. 2A). IgA also enhanced cholesterol crystallization, whereas the promoting effect of IgG was only minimal (Fig. 2A). Biliary AAG also showed a potent promoting effect similar to that of IgA (Fig. 2B). Neither immunoglobulins nor AAG showed any effect on crystal mass at equilibrium. Adding APN had no effect on the cholesterol crystal growth curve (Fig. 2C). Adding pcPLC likewise had no effect on the cholesterol crystal growth curve (Fig. 2D). The results of



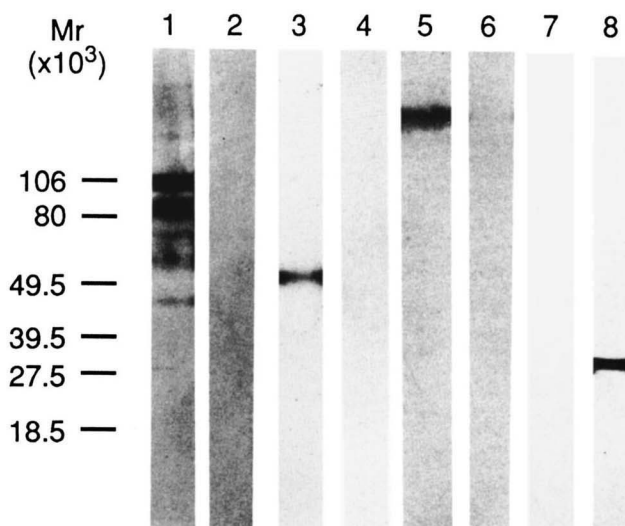
**Fig. 2.** Comparative promoting effect of isolated biliary immunoglobulins, AAG, APN, and pcPLC on the cholesterol crystal growth curve. Isolated biliary IgM, IgA, IgG, AAG, and APN (each 100  $\mu\text{g/ml}$  model bile) were mixed with an identical supersaturated model bile solution (CSI = 1.4, TL = 12.5 g/dl, BA/PL = 4.4). A) Immunoglobulins. The promoting effects were in the following order: IgM > IgA > IgG. B) Adding AAG produced a significant promoting effect. C) Adding APN produced no effect on cholesterol crystal growth. D) Adding bacterial pcPLC ( $3 \times 10^{-3}$  IU/ml) likewise had no promoting effect. The control curve is given as the mean  $\pm$  SD,  $n = 4$ . Each of the experimental curves is given as mean,  $n = 2$ . \*:  $P < 0.01$  compared to control value at each time.

these addition experiments regarding specific effects on the crystal growth curve are summarized in **Table 1**. The promoting effects on crystallization (decreased  $I_t$  and increased  $I_g$ ) of these proteins were in the following order:  $IgM > IgA = AAG > IgG$ . APN and pcPLC failed to show any effect at their estimated physiological concentration.

### Comparative effect of removing crystallization-promoters on the promoting activity of the CABG-fraction

Approximately 10 mg of the CABG-fraction was loaded onto the anti-Ig column, the anti-AAG column, and the anti-APN column, respectively. When the unbound fractions from these columns were examined by Western blots using these specific antibodies, no immunoreactive proteins were detected (**Fig. 3**). Furthermore, only minimal amounts i.e., < 5%, of immunoglobulins of the three major classes and no detectable amounts of AAG were observed in the unbound fractions using immunochemically based methods; upon recycling, less than 1% of each of the Igs was still present in the unbound fraction by immuno-quantitation. Thus, it appears that the corresponding antigens were completely removed from the applied sample protein mixtures by applying them to these antibody columns. The promoting activities of these relevant unbound fractions were compared with that of the untreated CABG-fraction. As shown in **Fig. 4**, adding the CABG-fraction to the model bile caused a promoting effect on all three of the crystallization curve components. The crystal growth curve with the CABG-fraction depleted of immunoglobulins was not significantly different from that of the untreated CABG-fraction (**Fig. 4A**). On the other hand, the growth curve with the CABG-fraction devoid of AAG showed a clear reduction in crystal growth rate (@33% by planimetric measurement of curve-defined area) compared with that of the untreated CABG-fraction (**Fig. 4B**). Nevertheless, a major residual promoting effect was still present in the CABG-fraction after removal of AAG.

Removing APN (detectable) and pcPLC (undetectable) from the CABG-fraction did not alter the promoting



**Fig. 3.** Western blots of the biliary CABG-fraction using anti-Ig, anti-AAG, anti-APN, anti-pcPLC antibodies, before and after immunosorptive removal of Ig, AAG, APN, and pcPLC. Lanes 1, 3, 5, and 7, biliary CABG-fractions, before immunosorption; lanes 2, 4, and 6, CABG-fractions from the anti-Ig, the anti-AAG, and the anti-APN columns, respectively, after immunoabsorption chromatography. Lane 8; bacterial pcPLC. Note that complete removal from the CABG-fraction of the relevant proteins as defined by immunoblotting is demonstrable in each case after immunosorption (lanes 2, 4, and 6) and that in lane 7, by contrast no pcPLC can be observed by use of the identical immunoblotting procedure prior to immunosorption. The result shown in lane 8 merely serves as a control for the ability to detect pcPLC if it were present in the bile samples by use of the immunoblotting technique.

effect of the CABG-fraction. The CABG-fraction had no effect on equilibrium crystal mass upon the removal of any of these specific promoter glycoproteins. Results of removal experiments from the CABG-fraction and the effects of the whole Con A-bound glycoproteins are compared in **Table 2**.

## DISCUSSION

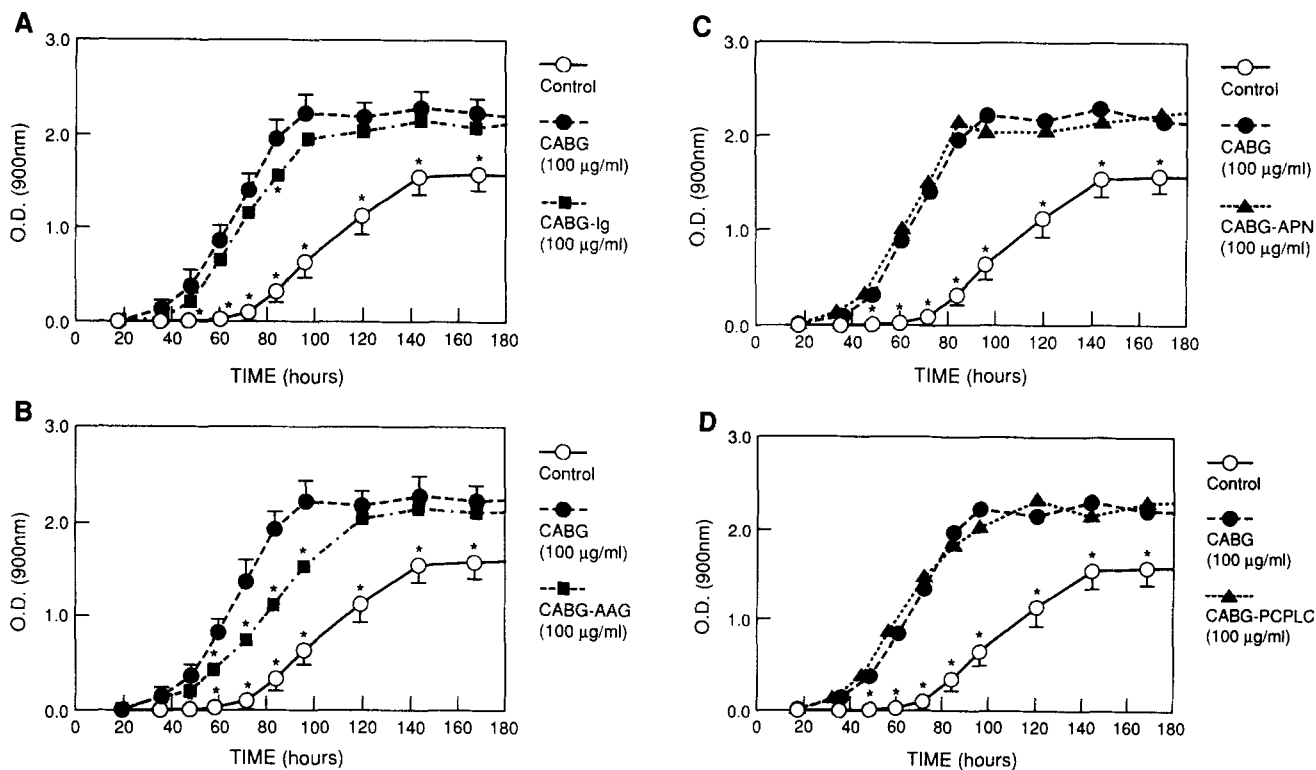
The present study is the first attempt, as far as we know, to clarify the relative potencies of various cholesterol crystallization-promoter candidates of possible relevance to gallstone pathogenesis. These candidates have all recently been identified individually or as protein classes (i.e., immunoglobulins) in the CABG-fraction by different investigators (12–17). Two reciprocal experimental design approaches have been used: specific addition and subtraction. In the addition experiments, of the immunoglobulin classes examined, IgM and IgA showed a readily detectable effect on cholesterol crystallization in supersaturated model bile at their apparent physiological concentrations. This effect, however, was not found for IgG.

These results are consistent with the original report by Harvey, Upadhy, and Strasberg (13), who separated the

**TABLE 1.** Effect of adding immunoglobulins, AAG, APN, and pcPLC on cholesterol crystal growth

Protein ( $\mu\text{g/ml}$ )	Activity Indices <sup>a</sup>		
	$I_g$	$I_c$	$I_t$
IgM (100)	1.96	1.13	0.52
IgA (100)	1.67	1.07	0.78
IgG (100)	1.25	1.00	0.94
AAG (100)	1.60	1.10	0.72
APN (100)	1.00	1.07	1.00
pcPLC	1.00	0.90	1.00

<sup>a</sup> $I_g$ , growth index;  $I_c$ , equilibrium crystal index;  $I_t$ , nucleation time index.



**Fig. 4.** Effect of removal of immunoglobulins and AAG on promoting activity of the biliary CABG-fraction. The crystal growth curves with the CABG-fractions that were depleted of immunoglobulins(A), APN (C), or pcPLC (D), did not differ significantly from those with the untreated CABG-fraction. On the other hand, the immunoabsorption removal of AAG from the CABG-fraction significantly reduced the promoting effect of the CABG-fraction (33% decrease in growth rate) (B). The curves for control and for the untreated CABG-fraction are given as the mean  $\pm$  SD,  $n = 4$ . The rest are given as the mean,  $n = 2$ . \*:  $P < 0.01$  compared to the values for the untreated CABG-fraction at each time point.

CABG-fraction into three subfractions by gel filtration HPLC and found that the high-molecular-weight subfraction containing IgM and IgA showed much promoting activity, whereas the intermediate molecular weight subfraction, containing IgG, showed little activity. The findings suggest the possibility that the immunoglobulin effect somehow depends on molecular size because of the regularity of the direct relationship for immunoglobulins between size and potency. Despite these results, our experiments showed that removing all major immunoglobulin classes (IgM, IgA, and IgG) from the CABG-fraction does not significantly reduce its net promoting activity. Thus, while certain biliary immunoglobulins (i.e., IgM and IgA) are capable of expressing significant promoting activity, they cannot account for much, if any, of the potent promoting activity of the CABG-fraction. Most likely, most of the immunoglobulins, that is, 66% of biliary IgA and 95% or more of total biliary IgM and IgG, do not bind to Con A (37).

Adding AAG (100  $\mu$ g) to model bile resulted in a promoting effect about equivalent to that of IgA. The added AAG in the present work was obtained by immunoaffinity chromatography instead of by acetonitrile HPLC separation as in a recent study showing its promoting activity (15). Since the promoting activity with

either method was comparable, expressed concerns about solvent-induced partial denaturation and consequent partial loss of enhancement of function appear unwarranted (15, 38). The concentration of AAG in abnormal bile measured by ELISA is approximately 50  $\mu$ g/ml to 100  $\mu$ g/ml (35). AAG was shown by its removal to be responsible for a significant portion (about 1/3) of the total promoting activity of the CABG-fraction. Because the present study was conducted using pooled bile samples from patients with cholesterol gallstones, the amounts

**TABLE 2.** Comparative effect of adding the biliary Con A-bound glycoproteins with and without removing the immunoglobulins, AAG, APN, and pcPLC on the cholesterol crystal growth activity indices

Proteins ( $\mu$ g/ml)	Activity Indices <sup>a</sup>		
	$I_g$	$I_c$	$I_t$
CABG (100)	2.00	1.50	0.43
CABG-Igs (100)	1.85	1.43	0.54
CABG-AAG (100)	1.55	1.40	0.50
CABG-APN (100)	2.00	1.43	0.43
CABG-pcPLC (100)	2.00	1.48	0.43

<sup>a</sup> $I_g$ , growth index;  $I_c$ , equilibrium crystal index;  $I_t$ , nucleation time index.

of each protein added to model bile merely represent their mean concentrations in the stone-forming bile. Thus, the relative significance of immunoglobulins and AAG awaits quantitative information on the amount of these proteins present in stone-forming as well as in normal bile.


A potential effect of pcPLC on cholesterol solubility in model bile was first proposed by Neiderhiser, Roth, and Webster (39). Pattison and Willis (40) have recently identified pcPLC-like activity in human bile and estimated its activity in gallbladder bile to be @19.1 nmol/h per mg bile protein ( $1.2 \times 10^{-3}$  IU/mg protein). This estimated catalytic activity is uncharacteristically low for any form of enzyme kinetics; it more nearly resembles a non-enzymatic reaction. Moreover, it could not be purified and other enzyme properties such as saturation kinetics were not demonstrated. These workers have, however, also shown a promoting effect of pcPLC at 500 times the estimated activity given above on cholesterol nucleation using bacterial pcPLC (14). Thus, the physiological relevance of their observations remain uncertain. Moreover, Groen et al. (41) have also shown that pcPLC accelerates nucleation at high concentrations but not at the "physiological" concentration of 0.1  $\mu$ mol/24 h per ml (@ 4 nmol/h per mg protein) ( $6.9 \times 10^{-5}$  IU/ml) estimated in their study to be present in bile.

In the present study, we attempted to isolate pcPLC from bile using an antibody affinity column. No detectable pcPLC was observed. Also, adding bacterial pcPLC at 50 times more than the estimated biliary activity reported by Pattinson and Willis (40), or Groen et al. (41), revealed no effect on cholesterol crystallization. Further, immunoabsorptive removal of pcPLC (if present even in undetectable amounts) showed that it does not account for any promoting activity in the CABG-fraction. Therefore, our data support the view of Groen et al. (41) that pcPLC does not accelerate cholesterol nucleation at the estimated physiological level of activity present in bile.

APN also failed to show any effect on crystallization at its apparent physiological level in stone-forming bile. This result is simply in conflict with the result given by Offner, Gong, and Afdhal in a preliminary report (17). We have no explanation for this discrepancy. Either the 130-kDa potent promoting glycoprotein reported by Groen et al. (12) is not APN, contrary to that which has been suggested (17), or the assignment of promoting activity to a 130-kDa protein is somehow incorrect. At present, no choice can be made between these alternatives.

The major portion of crystallization-promoting activity still remained in the biliary CABG-fraction after each of the promoters examined in this study were removed. This finding points to the fact that at present the most potent promoting factor(s) present in the CABG-fraction remain unidentified. Other more recently described Con A-bound biliary glycoproteins that have been claimed to exert promoting activity include glycoproteins specifically

associated with biliary vesicles reported by Miquel et al. (42), and a unique biliary lipoprotein isolated from the Con A-fraction by Groen and co-workers (43) and de Bruijn et al. (44). One or several of these could account for the as yet unexplained major portion of total CABG promoting activity. The biochemical properties and identities of these hydrophobic proteins, however, are not as yet established.

The growth curve with the CABG-fraction shows not only enhanced crystal nucleation or growth but also an enhanced third component: an increased crystal mass at the equilibrium phase ( $i_c > 1$ ) (Fig. 4). This increased mass suggests that the CABG-fraction may also contain factors capable of altering the equilibrium cholesterol solubility in the supersaturated model bile. Indeed, it has been claimed that the CABG fraction not only promotes nucleation from the vesicular phase but may also enhance cholesterol transfer from micelles to vesicles (9). However, none of the promoters examined in this study appears to increase the crystal mass at equilibrium. This finding suggests that the other as yet unidentified promoting factors present in the CABG-fraction may also influence equilibrium solubility. 

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