Hydrophobic/hydrophilic balance of proteins: a major determinant of cholesterol crystal formation in model bile

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Abstract Biliary proteins are speculated to play an important role in modulating nucleation of cholesterol crystals from supersaturated gallbladder bile, acting either as pro- or antinucleating agents. However, conflicting results have been obtained in attempts to isolate specifically active proteins. Our hypothesis, therefore, was that this may be a nonspecific effect of proteins, related to their secondary structure or to their overall hydrophobic/hydrophilic balance. We studied the effect of a number of "nonspecific" proteins with different secondary structures on cholesterol crystal formation in model bile. Their relative hydrophobic/hydrophilic indices were experimentally determined by measuring their retention time on a phenyl-agarose column (hydrophobic ligand). The potency of each protein in enhancing or inhibiting crystal formation was ranked according to the lowest protein concentration capable of significantly influencing crystal formation by comparison with control using model bile with cholesterol saturation index (SI) of 1.2 and 1.5. Some of these proteins (chymotrypsin, IgA, myoglobin) significantly enhanced crystal formation, while some (apolipoproteins A-I, A-II, and B) inhibited it, and others (IgG, chymotrypsinogen) showed no effect. The different effects were not related to their secondary structure but to their hydrophobic/hydrophilic index, with the most hydrophilic proteins showing maximal pronucleating potency and vice versa ($r = 0.93$; and 0.97, $P < 0.005$ for SI = 1.2 and 1.5, respectively). Pronucleating proteins enhanced, while antinucleating proteins inhibited, the transfer of cholesterol and phospholipid from micellar to vesicular forms. We conclude that the effect of proteins on cholesterol crystal formation in model bile is nonspecific and mainly related to their hydrophobic indices rather than to their secondary structures.—**Ahmed, H. A., M. L. Petroni, M. Abu-Hamdiyyah, R. P. Jazrawi, and T. C. Northfield.** Hydrophobic/hydrophilic balance of proteins: a major determinant of cholesterol crystal formation in model bile. *J. Lipid Res.* 1994. **35:** 211–219.

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Cholesterol nucleation time has a close relation to the pathogenesis of cholesterol gallstone disease (1–5). It has been shown that human bile contains proteins that can enhance (5, 6) or inhibit (2) cholesterol crystal formation. Many attempts have been made to separate specific highly active protein(s). Groen and colleagues (1) have reported that a non-mucous glycoprotein fraction that can bind to concanavalin-A accounts for the pronucleating activity observed in gallbladder bile, and that it is especially prominent in cholesterol gallstone patients. This report has been followed by a number of studies (7–11) each describing a different biliary protein as the putative pronucleating agent in bile. Immunoglobulins (8), phospholipase C (7), fibronectin (9), and mucous glycoproteins (12) have been reported to have such a role. However, the results of these studies have been conflicting, e.g., the pronucleating role for phospholipase C in bile from gallstone subjects (7) has not been confirmed by another study (13), nor was that for immunoglobulins (10) or mucous glycoproteins (14).

Antinucleating proteins have been less extensively studied. Recently, a concanavalin-A bound, 42-kDa lipoprotein has been found to have antinucleating activity (15). Interestingly, the apolipoproteins A-I, A-II, and C-I have been shown to inhibit cholesterol crystal formation in model bile (16). However, their concentration in bile seems not to differ between normal and gallstone subjects (17). These proteins are known to have large hydrophobic domains.

The mechanism for cholesterol crystal formation in human bile seems to follow a specific pathway, in which cholesterol is transported from micellar to vesicular form (18), which is the immediate supplier of cholesterol for crystal formation (19–23). It would be logical for pronucleators to enhance this transfer, and for antinucleators to inhibit or even reverse it.

We hypothesized that the phenomenon could be nonspecific, related to the overall physico-chemical characteristics of biliary proteins. We therefore studied

Abbreviations: CSI, cholesterol saturation index; HIC, hydrophobic interaction chromatography.

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the effect of a group of nonspecific proteins on crystal formation in model bile having different SI values. Our results support the concept that the effect of proteins on crystal promotion or inhibition is nonspecific, related to the hydrophobic/hydrophilic character of the protein and not related to the secondary structure of the proteins.

MATERIALS AND METHODS

Materials

Cholesterol (> 99% pure), phosphatidylcholine (99% pure), sodium taurocholate (98% pure), phenyl-agarose (cross-linked 4% beaded agarose), albumin (from bovine serum 96–99% pure, essentially fatty acid-free), myoglobin (from horse skeletal muscle, 95–100% pure), lectin concanavalin-A (type IV, from jack beans, 95% pure), α-chymotrypin (type II, from bovine pancreas), α-chymotrypsinogen A (type II, from bovine pancreas), IgG (from human serum, 95% pure), IgA (from human colostrum, 98% pure), IgM (from human serum, approx. 80% pure), apolipoprotein A-I (from human plasma, 96% pure), apolipoprotein A-II (from human plasma, 97% pure), apolipoprotein B (from human plasma, 97% pure) were all purchased from Sigma Chemical Co. (St. Louis, MO). All the proteins were in the form of lyophilized, crystalline powder except IgM, which was in a buffer solution (0.05 M Tris-HCl, 0.15 M NaCl, 0.01 M glycine containing 0.1% sodium azide, pH 7.4). All other chemicals used were of analytical grade. Organic solvents were HPLC grade. [^{3}H]cholesterol and [^{14}C]phosphatidylcholine were purchased from Amersham International plc (Aylesbury, Bucks, UK); their specific activities were 40–60 Ci/mmol (1.5–2.2 TBq/mmol) and 50–60 mCi/mmol (1.85–2.2 GBq/mmol), respectively. Sepharose 6-B was purchased from Pharmacia LKB (Uppsala, Sweden).

Model bile preparation

Model bile was prepared according to Kibe et al. (24) substituting 25 mM Tris-HCl, pH 7.4 (37°C), containing 145 mM sodium chloride, 5 mM calcium chloride, and 3 mM sodium azide for the HEPES buffer and adjusting the total lipids to 200 g/l (to be diluted 1:1 in buffer at the time of use). The final concentrations of phosphatidylcholine, cholesterol and sodium taurocholate were 79, 29, and 235 mmol/l to give a final cholesterol SI of 1.2; and 79, 37, and 235 mmol/l to give a final SI of 1.5, respectively (25). All model bile samples were initially isotropic (crystal-free) on microscopical examination (400 ×) under polarized light using a heating stage (37°C). Under these experimental conditions, the nucleation time of model bile ranged between 7 and 8 days for the bile with SI = 1.2, and between 5 and 6 days for the bile with SI = 1.5.

Nucleation time determination

Cholesterol nucleation time was determined in model bile as described (26) by daily checking of a 10-μl sample for the appearance of cholesterol monohydrate crystals up to 21 days, using the above mentioned technique. Samples that did not nucleate by 21 days were arbitrarily considered to have nucleation time of 22 days. The effect on nucleation time of nonspecific proteins (albumin, concanavalin-A, myoglobin, α-chymotrypsinogen, α-chymotrypsin, IgA, IgM, IgG, apolipoproteins A-I, A-II and B) at different concentrations (100, 20, 5, 1 μg/ml) was checked in six samples for each protein. The model bile was mixed 1:1 with buffer containing double the desired concentration of each of the proteins studied. The effect on crystal promotion or inhibition was expressed as percent of nucleation time of control bile as described (1).

Hydrophobic interaction chromatography (HIC)

Hydrophobicity index for the various proteins was determined by measuring their retention time on phenyl-agarose column (27). The protein (2 mg) was loaded on 5.0 ml of phenyl-agarose and eluted using ammonium sulfate gradient (2.0 to 0.0 M in 0.01 to 0.05 M sodium phosphate, pH 7). Proteins were ranked according to their elution volume.

Gel-permeation chromatography

Micelles and vesicles in model bile were separated by gel-permeation chromatography as described (28), with slight modification. One hundred fifty μCi [^{3}H]cholesterol and 100 μCi [^{14}C]phosphatidylcholine were added to biliary lipids at the time of model bile preparation, and processed as usual. The model bile (with or without proteins) was incubated at 37°C and 0.5 ml aliquots were taken out at time zero and after 2, 7, and 30 days to be fractionated on Sepharose 6B column (60 × 1 cm), using 50 mM Tris-HCl (pH 8), 150 mM sodium chloride, 1.5 mM sodium EDTA, 10 mM sodium cholate, and 0.03% sodium azide as elution buffer. Fractions (3 ml) were collected and 50-μl aliquots were added to 10 ml scintillation liquid (Beckmann Ready-Solv HPb, Beckman, High Wycombe, England) and the dpm values from [^{3}H]cholesterol and [^{14}C]phosphatidylcholine were determined in a liquid scintillation counter (Beckman LS 6800). The vesicular and micellar cholesterol distribution was expressed as percent area under the curve. The data at time zero for the three studied proteins (myoglobin, albumin, and apolipoprotein A-I) and for control bile were based on a single gel filtration experiment. The experiment was performed once for each protein at the above specified times using a protein concentration of 20 μg/ml. The recovery of phospholipid ranged between 90 and

95% in all experiments for the different proteins and control bile, whereas the recovery of cholesterol dropped from 95% at day 2 to about 80% at 7 and 30 days for control bile and with albumin. With myoglobin, the cholesterol recovery dropped to 65% at 7 and 30 days, while the recovery for apolipoprotein A-I stayed virtually unchanged at about 95%.

Statistical analysis

Results are expressed as mean ± SEM. Correlation coefficients were calculated using Spearman's rank correlation (R_S). Nucleation times are expressed as percent of control values and an unpaired Student's t test was used to compare the different proteins. Differences were considered significant for a P value of < 0.05. The area under the curve was used to calculate % cholesterol distribution in the vesicular and micellar fractions.

RESULTS

Effect of nonspecific proteins on nucleation of model bile in relation to their secondary structure

We studied the effect of a group of proteins having different secondary structures (**Table 1**) (29–31) at different concentrations (1, 5, 20, 100 μg/ml) on cholesterol nucleation in model bile having CSI of 1.2 and 1.5 (**Fig. 1** and **Fig. 2**). Some of the proteins (IgA, chymotrypsin, myoglobin) showed statistically significant pro-nucleator activity down to 5 μg/ml for both model bile preparations. Concanavalin-A showed significant pronucleator effect down to 5 μg/ml only with model bile with CSI = 1.5; and down to a concentration of 20 μg/ml for model bile with CSI = 1.2. This was also true for albumin but at a lower significance level. IgM showed significant pronucleating activity down to a concentration of 20 μg/ml using the two model bile preparations. All the pronucleator proteins lost their effect at the lowest concentration studied (1 μg/ml). The apolipoproteins A-I, A-II, and B showed antinucleating activity at all the concentrations studied, using the two model bile preparations. For these proteins there was no nucleation up to 21 days, the conventional time to stop checking for cholesterol monohydrate crystals. The remaining proteins (IgG and chymotrypsinogen) did not show any difference from control at most concentrations tested, with the exception of a slight pronucleating effect at 100 μg/ml (CSI = 1.2) for IgG, and a slight antinucleating effect at 1 μg/ml (CSI = 1.2) for chymotrypsinogen.

Protein hydrophobicity in relation to nucleating activity

We ranked the proteins experimentally according to their retention on a hydrophobic ligand column (phenyl-agarose) (**Table 2**) (27, 32). Interestingly, the effect of these proteins as pro- or anti-nucleators at a concentration of 5 μg/ml was significantly correlated with the hydrophobic/hydrophilic index of the proteins, as determined by measuring their retention times (expressed as elution volume) on phenyl-agarose column (**Fig. 3-A:** $r = 0.93$, $P < 0.005$; Fig. 3-B: $r = 0.97$, $P < 0.005$ for CSI = 1.2 and 1.5, respectively). The most potent pronucleator proteins (IgA, chymotrypsin, myoglobin) were the most hydrophilic, while the antinucleator proteins (apolipoproteins B, A-I, and A-II) were the most hydrophobic. The intermediate-rank proteins for effect on nucleation time (albumin, chymotrypsinogen, IgG) were also intermediate according to their hydrophobic indices. As the values for the effect of apolipoproteins on nucleation time were arbitrarily calculated for a nucleation time of 22 days, we also plotted the same relationship for the other proteins excluding the calculated values for apolipoproteins (Fig. 3-C) and the correlations were statistically significant at both CSI ($R_S = 0.85$, $P < 0.03$ for CSI = 1.2; and $R_S = 0.95$, $P < 0.02$ for CSI = 1.5).

TABLE 1. Secondary structure of the proteins used (%) (29–31)

Protein	α-Helix	β-Pleated Sheets	Random Coil
Concanavalin-A	0	70–74	26–30
Albumin (bovine)	66	3	31
Chymotrypsin	0–8	82–90	10
Chymotrypsinogen	9	36	55
Myoglobin	77	2	21
IgA	mostly β-sheets		
IgG	mostly β-sheets		
IgM	mostly β-sheets		
ApoA-I	70	10	20
ApoA-II	69	11	20
ApoB	25	37	37

Effect of nonspecific proteins on macromolecular cholesterol and phospholipid aggregates in model bile

We studied the effect of some of these nonspecific proteins (at a concentration of 20 μg/ml) on the relative distribution of cholesterol and phospholipid between the micellar and vesicular phases in model bile with CSI = 1.2, over a period of 30 days (0, 2, 7, and 30 days). The two phases were clearly separated on the Sepharose 6-B column (**Fig. 4**), and this allowed the expression of results as percentage of the area under the curves. We found that the pronucleating protein (myoglobin) accelerated the rate of migration of cholesterol from the micellar to the vesicular phase over the observation period. The vesicular:micellar cholesterol ratio (**Table 3**) increased from the initial 0.19 to 1.09 and then to 1.57 at 7 and 30 days, respectively. The antinucleating protein (apolipoprotein A-I) showed a drop in the vesicular:micellar cholesterol ratio from the initial 0.19 to 0.10 and 0.08 at 7 and 30 days, respectively. Thus, apolipoprotein A-I seemed to enhance the migration of model bile cholesterol from vesi-

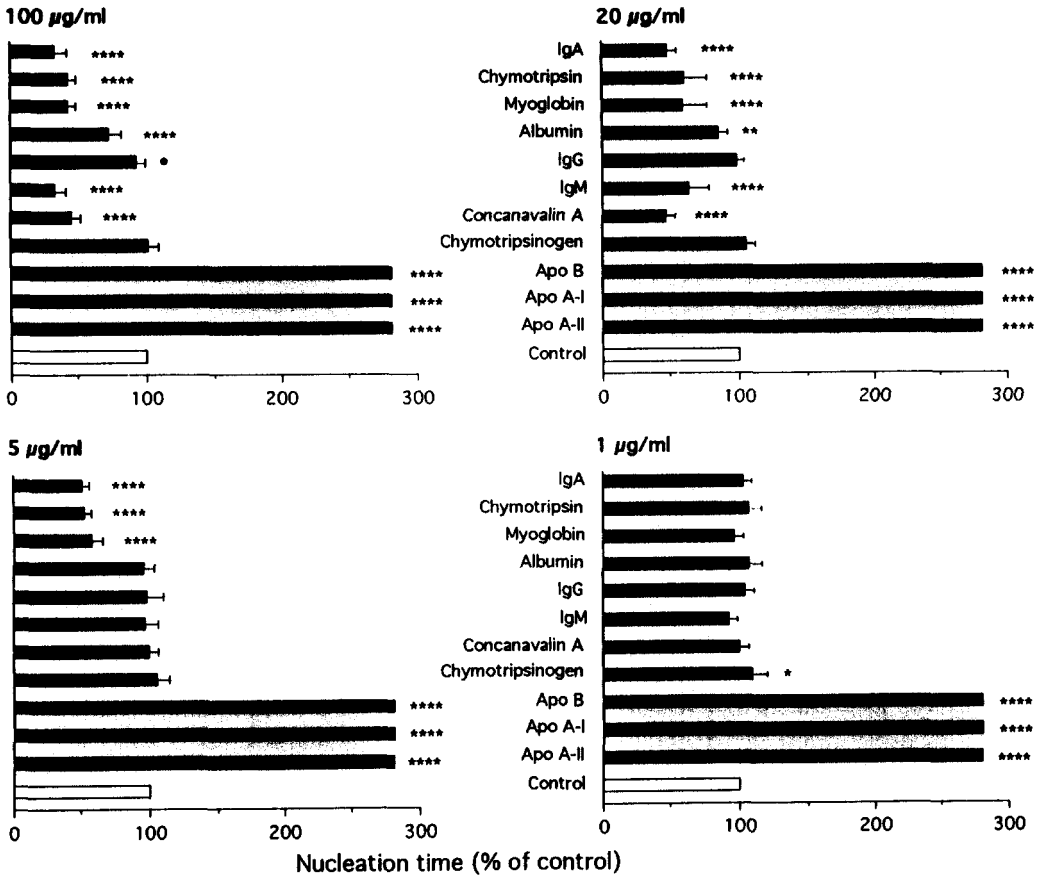

Fig. 1. Effect of nonspecific proteins on cholesterol nucleation in model bile with CSI = 1.2. Results expressed as % of control: pronucleators had values less than 100%, while antinucleators had values more than 100%. Statistical significance levels (vs. control) are expressed as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.005$; $****P < 0.001$.

cles into micelles, i.e., opposite to the effect of myoglobin. The intermediate-rank protein (albumin) showed a pattern similar to that of control, where the vesicular:micellar cholesterol ratio increased from the initial 0.19 to 0.47 and 0.56, at 7 and 30 days, respectively. That effect was in the same direction as that of myoglobin, though the rate of change was clearly slower.

As phospholipid coexists with cholesterol in both micelles and vesicles, we looked into the effect of the same three proteins on the cholesterol:phospholipid molar ratio in these two fractions from model bile with CSI = 1.2 (**Table 4**). With myoglobin, the vesicular cholesterol:phospholipid ratio increased from the initial 2.1 to 3.3 in 2 days, and there was virtually no further rise at 7 and 30 days, while the micellar cholesterol:phospholipid ratio dropped from the initial 0.54 to 0.31 in 2 days, and stayed virtually unchanged thereafter. By contrast, with apolipoprotein A-I, the vesicular cholesterol:phospholipid ratio increased to 2.4 at 2 days, remained virtually unchanged at 7 days, and to 2.6 at 30 days, i.e., a rise of 24%, compared to 62% for myoglobin. The micellar cholesterol:phospholipid ratio dropped from the initial 0.54 only to 0.52 at 2 days (in contrast to 0.31 with myoglobin) with little or no change thereafter. Albumin showed an effect similar to control, where vesicular cholesterol:phospholipid ratio increased from the initial value of 2.1 to 2.9 at 2 days, and to 3.2 at 7 days, with little change thereafter. The main increase in the vesicular cholesterol:phospholipid ratio occurred over the first 7 days with albumin and in control bile, but over the first 2 days for myoglobin. The micellar cholesterol:phospholipid ratio dropped from the initial value of 0.54 to 0.46 at 2 days and continued to decrease over 7 and 30 days.

DISCUSSION

Our results concerning the effect of proteins on cholesterol crystal formation in the two model bile preparations studied are consistent with the hypothesis that the activity of proteins as pro- or antinucleators is a nonspecific phenomenon related to the physico-chemical properties of the proteins rather than a specific, enzyme-substrate-like mechanism. The group of proteins studied

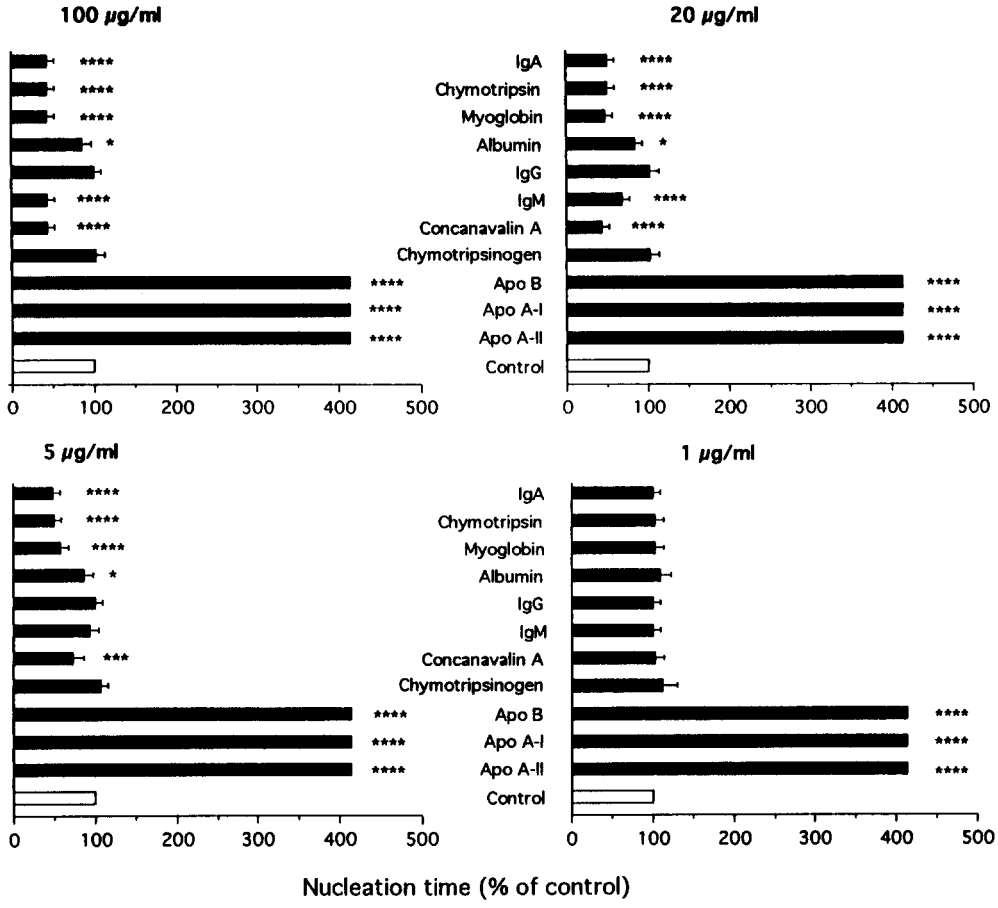

Fig. 2. Effect of nonspecific proteins on cholesterol nucleation in model bile with CSI = 1.5. Results expressed as % of control: pronucleators had values less than 100%, while antinucleators had values more than 100%. Statistical significance levels (vs control) are expressed as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; $^{****}P < 0.001$.

were different in their hydrophobic indices as measured by retention on phenyl-agarose and in their secondary structure. Previous experiments on the interactions of long-chain alcohols with proteins showed a difference in the interaction between proteins that are mostly α-helical in structure and those with mostly β-pleated structure (33). As cholesterol, bile acids, and phospholipid, like long-chain alcohols, are all amphipathic molecules, it was appropriate to investigate the effect of proteins with different secondary structures on cholesterol nucleation. Some of these proteins exist in normal bile, e.g., albumin, immunoglobulins, and apolipoproteins (34), while others do not. Myoglobin, chymotrypsin, and IgA were the most potent pronucleators, while the apolipoproteins A-I, A-II, and B were clearly antinucleators, under the experimental conditions. Other proteins showed less pronounced pronucleator or antinucleator activity (Fig. 1 and 2). The effect of proteins on cholesterol nucleation time was similar in both model bile preparations, despite the different CSI used, the only exception being two of the intermediate-rank proteins (albumin and concanavalin-A) which were more active at the higher CSI, for which they showed a significant pronucleator effect down to a concentration of 5 μg/ml.

These effects did not seem to relate to the secondary structure of the proteins (Table 1), as myoglobin (77% α-

TABLE 2. Hydrophobic/hydrophilic rank of nonspecific proteins (retention on phenyl-agarose)

Protein	Elution Volume	Rank
	ml	
IgA	29.7	1
Chymotrypsin	29.7	1
Myoglobin	39.6	3
Concanavalin-A	47.2	4
Albumin	49.5	5
IgG	49.5	5
IgM	56.1	7
Chymotrypsinogen	59.4	8
ApoB	75.9	9
ApoA-I	85.8	10
ApoA-II	89.1	11

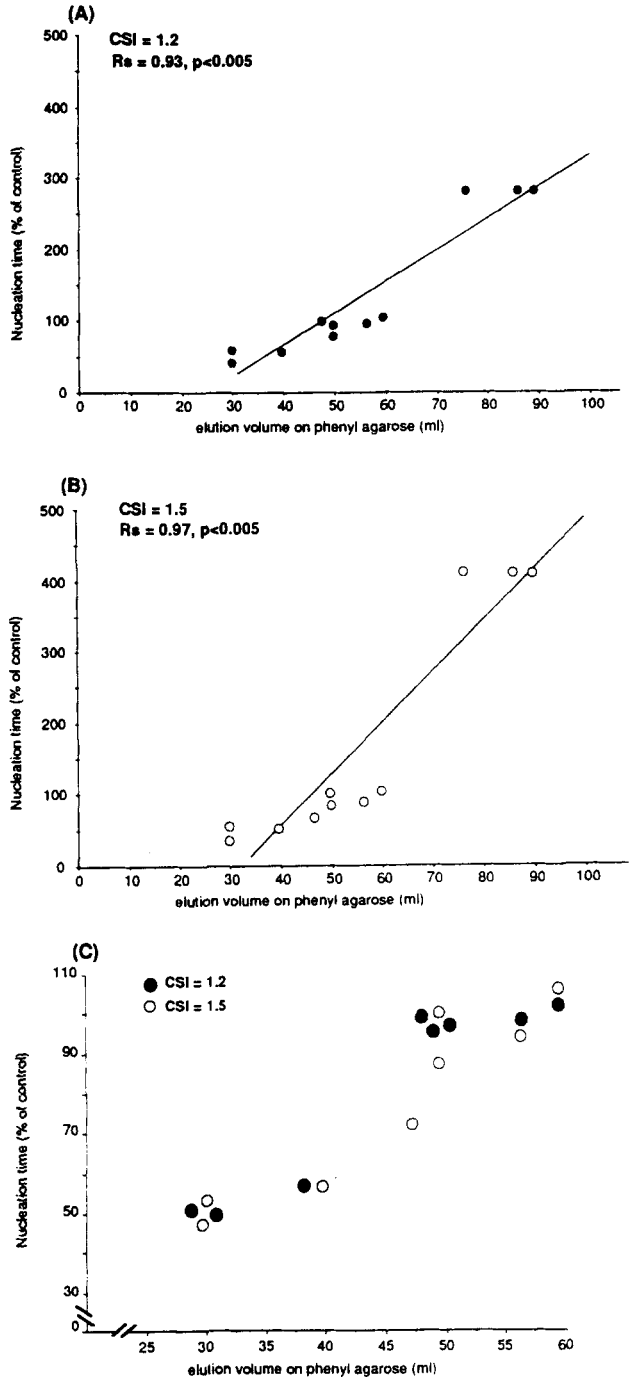

Fig. 3. Correlation of nucleation time (% of control) in model bile having CSI = 1.2 (A) and 1.5 (B) for a protein concentration of 5 μg/ml with the elution volume (ml) of proteins from phenyl-agarose column as an index of protein hydrophobicity. Fig. 3(C) shows the same correlations omitting the arbitrary values for apolipoproteins. R_S indicates Spearman's rank correlation coefficient.

helix) and chymotrypsin (> 80% β-pleated sheets) were both potent pronucleators. All three apolipoproteins studied were effective antinucleators with a mixed secondary structures, having different proportions of α-helix, β-pleated sheets, and random coils. Both types of proteins, predominantly α-helix and predominantly β-pleated sheets, as well as mixed-structure proteins, showed pronucleating or antinucleating activity irrespective of the secondary structure.

The proteins were then examined with respect to the relationship between their individual hydrophobicity and the effect on cholesterol nucleation time. The proteins were ranked according to their retention on a hydrophobic ligand column (phenyl-agarose), which, in contradistinction to reverse-phase HPLC, does not entail the use of organic solvents, and thus the proteins are kept in the native state during the chromatographic procedure (32). The data obtained are more reliable than the calculated hydrophobic indices, which do not take into account the folded nature of the protein structure and assume that values predicted from amino acid hydrophobicity can be extrapolated to protein hydrophobicity (35). We found that the more hydrophobic proteins (apolipoprotein A-I, A-II, and B) inhibited, while the more hydrophilic proteins (chymotrypsin, myoglobin, and IgA) enhanced the process of cholesterol nucleation in model bile, by comparison with control values. Intermediate-rank proteins for effect on nucleation time were also intermediate on the hydrophobic scale (Fig. 3). These results were largely consistent for the two model bile preparations studied.

As is evident from the results using albumin and concanavalin-A, a protein could be a potent pronucleator at a relatively high concentration, but lose this property at a lower concentration (Fig. 1), as if an effective increase in the hydrophobicity occurs. It has been shown that hydrophobic interactions between some proteins (β-lactoglobulin, bovine and human serum albumin, human transferrin, lysozyme) and long-chain alcohols (C_{8-12}) are increased on protein dilution (33, 36). This might explain the loss of pronucleator activity at the low concentrations studied. Alternatively, a critical protein concentration might be required to demonstrate the pronucleator effect under the experimental conditions. On the other hand, with apolipoprotein dilution the absolute concentration of the protein decreases but the degree of self-association also decreases, thus exposing more hydrophobic surfaces originally used in self-association (37). These effects are likely to counterbalance each other, thus preserving the antinucleator effect of the apolipoproteins studied at the lowest concentrations used.

Most workers in the area agree that cholesterol is present in bile mainly in micellar and vesicular forms, with the latter working as the immediate supplier of cholesterol for crystal formation (5, 19–23). Pronucleating proteins are expected to enhance the transfer of cholesterol from the more stable form (micelles) into the less stable form (vesicles), while the antinucleating proteins inhibit or possibly reverse this process. In our results (Fig. 4, Table 3), the pronucleating protein (myoglobin)

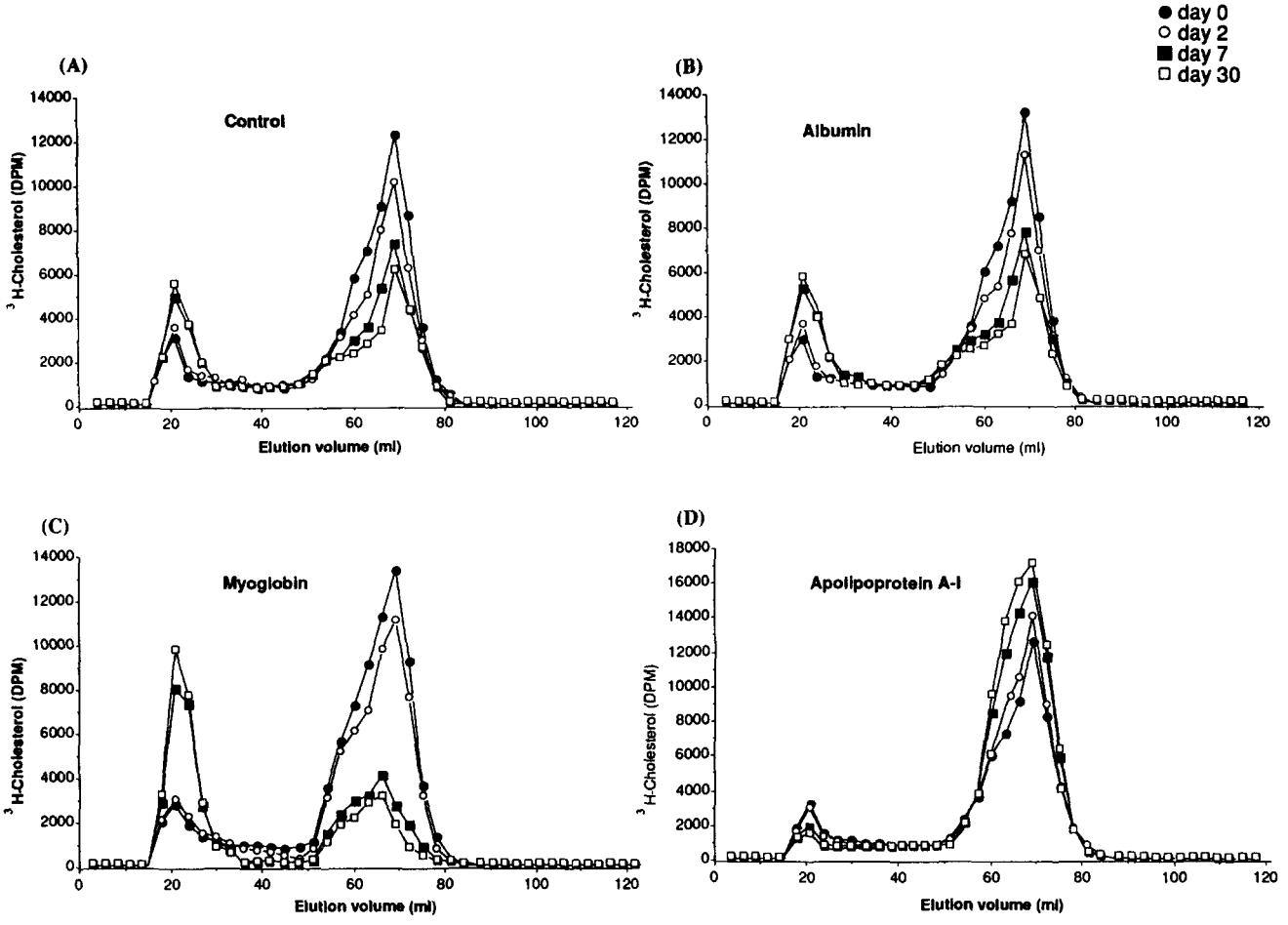

Fig. 4. Separation of vesicular and micellar phases in model bile (CSI = 1.2) on Sepharose 6-B after 0, 2, 7, and 30 days of incubation for control, albumin, myoglobin, and apolipoprotein A-I. The experiment was performed once for each protein at the specified times using a protein concentration of 20 μg/ml. Myoglobin enhanced the transfer of cholesterol from vesicles to micelles to a greater extent than albumin and control, while apolipoprotein A-I stimulated the transfer in the opposite direction.

enhanced the transfer of cholesterol from micellar to vesicular form to a greater extent compared to albumin and control bile, while the antinucleating protein (apolipoprotein A-I) reversed this process. Earlier work from Groen et al. (38), using a density-gradient centrifugation technique carried out at a single time interval (48 h), suggested that the concanavalin-A bound fraction of bile from cholesterol gallstone patients increases the amount of vesicular cholesterol and phospholipid, and also induces nucleation of cholesterol from the vesicular fraction. More recently, Ginanni Corradini et al. (3) showed an inverse correlation between the amount of native gallbladder bile proteins (in both cholesterol gallstone patients and controls) and the cholesterol nucleation of both whole bile and isolated micellar fractions. Using a technique similar to the one we used, they also showed that native biliary proteins induce phasing out and redistribution of micellar cholesterol and lecithin towards vesicles.

The effect of myoglobin, albumin, and apolipoprotein A-I on the cholesterol:phospholipid molar ratio in vesicular and micellar fractions from model bile (CSI = 1.2) was studied (Table 4). With myoglobin the vesicular cholesterol:phospholipid ratio increased rapidly over the first 2 days of incubation and stayed stationary thereafter, while with albumin and control the increase occurred over the first 7 days. With apolipoprotein A-I the vesicular

TABLE 3. Effect of proteins on vesicular:micellar cholesterol distribution, expressed as ratio of % area under the curve, in model bile at CSI = 1.2

Incubation Time	Control	Albumin	Myoglobin	Apolipoprotein A-I
days				
0	0.19	0.19	0.19	0.19
2	0.27	0.24	0.23	0.16
7	0.47	0.47	1.09	0.10
30	0.54	0.56	1.57	0.08

The experiment was performed once for each protein at the specified times using a protein concentration of 20 μg/ml.

TABLE 4. Effect of proteins on the cholesterol:phospholipid molar ratio (peak values) in vesicular (V) and micellar (M) fractions in model bile (CSI = 1.2)

	Control		Albumin		Myoglobin		Apolipoprotein A-I	
Incubation Time	V	M	V	M	V	M	V	M
days								
0	2.1	0.54	2.1	0.54	2.1	0.54	2.1	0.54
2	2.7	0.49	2.9	0.46	3.3	0.31	2.4	0.52
7	3.3	0.40	3.2	0.37	3.4	0.32	2.3	0.50
30	3.2	0.33	3.3	0.30	3.2	0.30	2.6	0.51

cholesterol:phospholipid ratio increased to a lesser extent and apparently over the whole 30 days of study. As cholesterol was shown to be transferred from micellar to vesicular form with myoglobin, albumin, and control bile, the increase in cholesterol:phospholipid ratio can be explained by increased vesicular cholesterol content. However, with apolipoprotein A-I, the cholesterol was shown to be transferred from the vesicular to the micellar form. Thus, the small increase in the vesicular cholesterol: phospholipid ratio is likely to be due to faster transfer of phospholipid from the vesicles to the micelles; however, this needs to be investigated further. The micellar cholesterol:phospholipid ratio was decreasing throughout the time course of the experiment for myoglobin, albumin, and control bile, although the drop was quicker for myoglobin. With apolipoprotein A-I there was a little decrease over the time course of the experiment, though to a much lesser extent than the other proteins and control. It has already been shown that apolipoproteins are essential for the stability of lipid aggregates in plasma lipoproteins (39). Thus, it could be suggested that the anti-nucleating proteins, possibly through co-solubilization at the micellar-water interphase, can stabilize micellar aggregates.

In conclusion, our experiments are consistent with the hypothesis that the effect of proteins on cholesterol nucleation in supersaturated model bile is nonspecific and related to the individual protein hydrophobicity. This effect is mediated through alteration of the transfer of cholesterol from micellar to vesicular form. One can speculate that in vivo, the overall hydrophobic/hydrophilic balance of biliary proteins would control the vesicular:micellar cholesterol equilibrium. If the overall balance shifts towards predominant hydrophilicity, cholesterol crystal formation is likely to occur, provided that the absolute and relative concentration of biliary lipids and other factors (e.g., gallbladder emptying) permit this event. On the other hand, if the balance shifts towards predominant hydrophobicity, cholesterol crystal formation is not likely to occur even in the presence of supersaturation.

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