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Cholesterol microcrystals associated with concanavalin A-binding glycoproteins contribute artifactually to nucleating activity assays

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Abstract Concanavalin A (Con A) affinity chromatography is the standard procedure to separate cholesterol nucleating biliary proteins from lipids and pigment. We observed that even after extensive washing following application of bile, lipid contaminants remain. We have determined the contribution of lipid contamination to cholesterol nucleation and assessed a modified procedure to remove lipids from the column. Human gallbladder bile was spiked with [^{3}H]cholesterol and [^{14}C]phospholipid and applied to two sets of Con A-Sepharose columns. One column was washed in the usual manner with Tris-HCl buffer and the other with buffer containing 10 mM taurocholate prior to eluting bound glycoproteins with α-D-methylmannopyranoside. Eluted proteins were added to heated abnormal bile at a final concentration of 250 μg/ml to study the effect on cholesterol nucleation. A separate aliquot (20 μl) of the protein solutions was counted for radioactivity. Cholesterol nucleating activity was less in samples from columns washed with 10 mM taurocholate than in samples from columns not washed with the bile salt. Lipid radioactivity was associated with Con A-binding proteins prepared without taurocholate, but not in those prepared with taurocholate wash. Light microscopy revealed the presence of cholesterol microcrystals and vesicles in some Con A-binding protein preparations prepared without a taurocholate wash. However, pellets from ultracentrifuged Con A preparations prepared without a bile salt wash revealed cholesterol crystals in all samples (n = 6). Washing with taurocholate did not affect recovery of protein mass and appearance of bands on SDS-PAGE gel showed an identical pattern in the two groups. This modified procedure to prepare potential nucleating proteins from gallbladder bile should eliminate erroneous results that may arise due to lipid contamination.—**Harvey, P. R. C., G. Aravinda Upadhya, and S. M. Strasberg.** Cholesterol microcrystals associated with concanavalin A-binding glycoproteins contribute artifactually to nucleating activity assays. *J. Lipid Res.* 1995. **36:** 2661-2669.

Supplementary key words cholesterol nucleation • biliary proteins • concanavalin A affinity chromatography

Biliary proteins are now recognized as important factors in the pathogenesis of cholesterol gallstone disease. A number of nucleating proteins (1–7) as well as anti-nucleating proteins (8, 9) have been identified in gallbladder biles. Nucleating proteins appear to be responsible for the rapid nucleation of cholesterol from bile of gallstone patients. Many of these potential pronucleating proteins have been isolated using concanavalin A lectin affinity chromatography that separates biliary glycoproteins from biliary lipids and pigments. We observed that even after extensive washing following the binding of biliary proteins to concanavalin A, lipid contaminants still remain. These contaminants could affect the nucleating activity of putative pronucleating proteins. The purpose of this study was to investigate the contribution of lipid contamination to cholesterol nucleation and to compare the standard isolation method to a modified one that completely removes all the lipids from the isolated biliary proteins.

MATERIALS AND METHODS

Concanavalin affinity chromatography

Gallbladder bile was obtained from nine cholesterol gallstone patients by needle aspiration of the gallbladder (10) at surgery. A mixture of proteolytic inhibitors was added to gallbladder bile samples to give a final concentration of phenylmethylsulfonyl fluoride 100 μM, pepstatin 1 μM, and iodoacetamide 1 mM. Three to four milliliters of each sample was directly applied at 22°C to two concanavalin A-Sepharose columns (0.7 × 13 cm; 5.0 ml of Con A; Pharmacia, Uppsala, Sweden). In seven of the nine bile samples [^{3}H]cholesterol and [^{14}C]phospholipid (each approximately 10^6 dpm per ml of bile) were used to spike the bile prior to addition to the column. One column was washed with approximately 20

Abbreviations: Con A, concanavalin A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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column volumes (100 ml) Tris-HCl buffer (100 mM, pH = 7.4) containing 0.5 M NaCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, and 0.02% NaN_3. Further washes were performed to remove biliary contaminants as completely as possible. Columns were inverted about ten times with the addition of 10 ml Tris-HCl buffer each time and gently rocked to wash out entrapped lipids and this was continued until eluents were visibly free of lipids and pigment. Columns were left to stand overnight at room temperature and washing was continued the next day. The Con A binding glycoproteins were then eluted with the same buffer (30 ml) after adding 0.2 M α-D-methylmannopyranoside. The second column and sample were treated identically, except that after completing the washing of the column with Tris buffer, the column was washed with 300 ml of a Tris buffer containing sodium taurocholate at a concentration of 10 mM. After standing overnight, the column was washed with another 100 ml of the taurocholate-containing buffer followed by 50 ml of the standard Tris buffer without taurocholate.

We have previously reported that Tris buffer leaches Con A from the columns (1). In order to determine whether added bile salts affected leaching, concanavalin A columns were washed with increasing concentrations of sodium taurocholate (0, 10, 20, and 50 mM) in Con A buffer and effluents were collected for measurement of Con A by PAGE. There was no increase in leaching in any of the concentrations of bile salt tested (data not shown).

Protein addition studies

Eluted protein fractions were dialyzed, filtered (Corning syringe filters 0.2 μm, Corning NY) and concentrated to 1.0 ml by centrifugation at 1600 rpm (1000 *g*) in a Sorvall centrifuge at 4°C, using Centricon-10 concentrators (Amicon). Preparations were stored at 4°C until addition studies were performed. Protein was determined by the assay of Lowry et al. (11). To determine the effect of the eluted material on cholesterol nucleation time, 100 μg of protein obtained by each of the methods from the nine samples was added to a "heated abnormal" bile sample (cholesterol saturation of 1.2; total lipid concentration of 11.0 gm/dl) to give a final concentration of added protein of 250 μg/ml. Heated abnormal samples are bile samples from patients with cholesterol gallstones that have been heated to 100°C for 60 min. These samples nucleate slowly but respond to pronucleating agents (12). The number of cholesterol crystals formed over the observation period was recorded.

To quantify lipids remaining with the Con A-binding proteins, we initially attempted direct measurements of cholesterol (13) (Boehringer Mannheim, Indianapolis, IN) and phospholipids (14) (Wako Chemical Industries, Osaka, Japan) by enzymatic assays in two samples. Lipids were detectable, but values were at the lower end of detection. Therefore, to quantify the lipids more accurately, we added radioactive cholesterol and phospholipid to the gallbladder bile in the next seven samples, as noted above. The mass of lipid remaining in the Con A protein fraction was calculated based on the amount of lipid radioactivity in the Con A protein preparations, assuming radioactivity added represented the initial lipid mass of the bile samples. Aliquots (20 μl) of the eluates were counted in a liquid scintillation counter (Beckman LS 9000) after addition of 3.0 ml of scintillation fluid (Ready Solve HP/b, Beckman Instruments, Fullerton, CA).

To determine whether modifying the technique affected the protein pattern of the eluates, slab sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) were performed on the protein fractions using a gradient of 5–20% with a 4% stacking gel (15). A constant mass of protein (10 μg) as determined by the Lowry procedure

TABLE 1. Concentration of lipids and proteins in the nine gallbladder bile samples studied

Patient	1	2	3	4	5	6	7	8	9	Mean (± SD)
Bile salts (mM)	181.9	126.2	127.1	108.7	211.7	145.3	41.2	81.1	93.2	124.04 (48.75)
Phospholipid (mM)	54.4	44.7	27.1	36.2	79.6	29.1	10.8	18.9	29.5	36.7 9 (19.46)
Cholesterol (mM)	23.7	18.8	11.5	16.2	32.8	19.5	4.09	8.44	15.6	16.74 (8.00)
Total lipid conc. (gm/dl)	14.06	10.39	8.79	8.77	17.83	10.14	3.02	5.77	7.47	9.58 (4.12)
Chol/PL ratio	0.44	0.42	0.42	0.45	0.41	0.67	0.38	0.45	0.53	0.46 (0.08)
CSI	1.22	1.30	1.53	1.39	1.18	1.78	1.40	1.39	1.65	1.43 (0.19)
Protein (mg/ml)	6.80	1.59	3.86	2.23	0.55	ND	1.31	1.3	1.64	2.42 (1.89)

was applied to each lane and gels were silver stained for protein by the Bio-Rad technique.

We studied the effect of ultracentrifugation of Con A preparations prepared by standard technique on cholesterol nucleation as, our hypothesis was that cholesterol microcrystals contaminate Con A glycoproteins prepared by the standard procedure. In this study Con A-binding glycoprotein solutions were prepared as described above without a bile salt wash and were ultracentrifuged to remove any microcrystals. Briefly, the eluted bound proteins contained in 20–25 ml of buffer were dialyzed to remove the salts and sugar and concentrated by Amicon filtration to 3.0 ml. Half the sample (1.5 ml) was ultracentrifuged (200,000 *g* for 2.0 h at 4°C) using a Beckman TLX table-top ultracentrifuge (Beckman Instruments, Palo Alto, CA). The pellets were examined for cholesterol crystals and the supernatants were filtered (0.2 μm filter) and added to heated abnormal biles for nucleation studies. Nucleation results were compared to untreated samples and Con A standard preparations that were only filtered (0.2 μm filter).

Microscopic studies

Eluates were examined by light microscopy using polarizing filters to determine whether they contained particulate lipids such as vesicles or cholesterol microcrystals. Reconstituted lipids used for lipid addition studies were also examined in this way.

Lipid addition studies

Lipids associated with Con A-bound proteins obtained by the standard technique would be expected to affect nucleation. In an attempt to determine directly the effect of such lipids, we isolated them from the eluates of the taurocholate washes in three samples. The lipids were extracted by the procedure of Folch, Lees, and Sloane Stanley (16) into chloroform–methanol 2:1 from the taurocholate washes of the modified procedure and added to the model bile system (n = 3). To determine the appropriate mass for the addition, the lipid mass present in the protein fractions of the Con A eluates obtained by the standard method was calculated using the data available from the measurement of lipid radioactivity in these eluates. Lipid extracts were also added to Con A protein preparations obtained by the modified method and nucleation activity was investigated.

RESULTS

Table 1 shows the concentrations of biliary lipids and protein in the nine gallbladder bile samples that were used to prepare the Con A-binding proteins by the standard and modified procedures. All biles were supersaturated and the lipid and protein concentrations were in the range expected for specimens from patients with cholesterol gallstones. **Table 2** shows the quantity of lipids that remained associated with the Con A-binding

TABLE 2. Lipid and protein concentrations in samples eluted by the standard and modified methods

Patient	3	4	5	6	7	8	9	Mean (± SD)
Standard method								
Phospholipid (nmol/ml)	95.8	90.7	306.7	25.1	34.1	26.6	58.4	91.06 (92.07)
% of total PL to column	0.12	0.19	0.13	0.03	0.10	0.04	0.07	0.097 (0.052)
Cholesterol (nmol/ml)	39.9	29.6	140.8	35.3	13.8	10.5	19.6	41.36 (41.83)
% of total Chol to col	0.12	0.06	0.14	0.06	0.11	0.04	0.04	0.081 (0.038)
Protein (mg/ml)	2.0	1.95	1.36	2.87	0.72	1.5	1.6	1.71 (0.61)
% of Protein to column	17.87	29.15	82.42	ND	18.32	32.9	32.5	35.53 (21.85)
Modified method								
Phospholipid (nmol/ml)	4.45	3.81	7.13	2.51	0.63	0.76	3.3	3.23 (2.08)
% of total PL to column	0.006	0.003	0.003	0.003	0.002	0.001	0.003	0.003 (0.001)
Cholesterol (nmol/ml)	0.88	1.04	1.32	0.80	0.14	0.21	0.92	0.76 (0.40)
% of total Chol to col	0.003	0.002	0.001	0.001	0.001	0.001	0.002	0.002 (0.001)
Protein (mg/ml)	2.1	2.65	1.28	2.65	0.46	1.5	1.35	1.71 (0.74)
% of Protein to col	18.77	39.61	77.58	ND	11.70	32.9	27.4	34.66 (21.22)

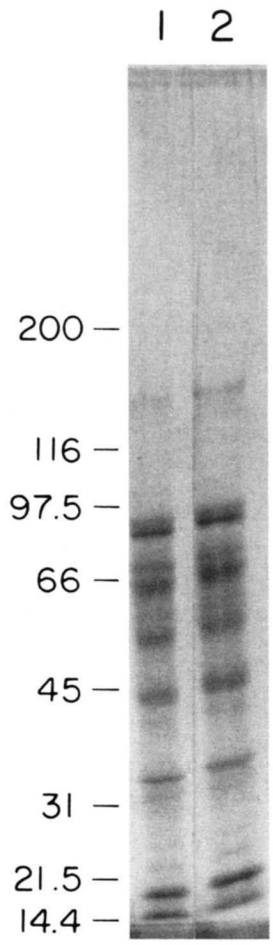

Fig. 1. SDS-polyacrylamide gel electrophoresis comparing concanavalin A-bound proteins prepared with and without bile salt wash. Con A glycoproteins (10 μg) were applied to each lane and the gel was run under reducing conditions and silver stained by the Bio-Rad technique. Lane 1 represents proteins eluted from concanavalin A without a taurocholate wash and lane 2 are proteins eluted after a taurocholate wash. The pattern of bands on SDS-PAGE gel was identical in the two groups. Migration of molecular weight markers are indicated.

proteins prepared by the standard procedure and the modified procedure using sodium taurocholate as calculated from the retained radioactivity in seven patients. When Con A-binding proteins were prepared by the standard method, significantly more cholesterol and phospholipid remained associated with the Con A-binding proteins than when the modified method was used. Con A-binding proteins prepared by the standard method contained, on average, 28 times more phospholipid and 54 times more cholesterol compared to Con A proteins prepared with a taurocholate wash (Table 2). Actually, the calculated radioactivity (dpm/min) was so low in the modified method that the samples were not different from background radioactivity; in 20-μl aliquots, dpm for [^{3}H]cholesterol was 2 ± 1 and that for [^{14}C]phospholipid was 2 ± 1. However, to complete the table and obtain comparisons between the two Con A preparations, these values were used in the calculations. The radioactivity in 20-μl aliquots associated with Con A proteins prepared by the standard method was 69 ± 39 dpm for [^{3}H]cholesterol and 65 ± 40 dpm for [^{14}C]phospholipid (n = 7 for both).

The protein mass recovered from the bile salt-washed Con A columns was similar to that recovered by the standard method (Table 2). Con A bound proteins in both preparations represented 35 ± 21% of total biliary proteins. There was no difference in the appearance of eluted proteins in the two methods when examined by SDS-PAGE electrophoresis (**Fig. 1**).

In seven samples we calculated the mass of lipid associated with 100 μg of protein obtained in the standard method using the data in Table 2 and this information is presented in **Table 3.** The values obtained were used to determine how much lipid should be added to model bile to simulate the effect on nucleation of the lipid retained by the standard method. There was considerable variability in the amount of retained lipid and in the C:P ratio of the retained lipid. The greatest amount of lipid per mass of protein was obtained in patient 5 who had high lipid levels and low protein levels in the initial bile sample. Similarly, the high cholesterol to phospholipid ratio in the native bile of patient 6 was reflected in the C:P ratio of the retained proteins.

Protein addition studies

Cholesterol nucleation time was less and crystal number was greater in all samples prepared by the standard method than in sample pairs prepared by the modified method using sodium taurocholate washes. **Figure 2** shows the number of cholesterol crystals formed in 10 μl of heated abnormal bile over the observation period. Note that on day 1 there was a significant difference. On this day all nine samples, to which proteins obtained by the standard method had been added, nucleated cholesterol crystals. Only three of nine sam-

TABLE 3. C:P ratio and calculated lipid mass per 100 μg protein of lipids associated with proteins eluted in the standard method

Patient	Phospholipid	Cholesterol	C/P Ratio
	nmol/100 μg protein		
3	4.79	2.00	0.42
4	4.63	1.51	0.33
5	22.7	10.42	0.46
6	0.88	1.24	1.41
7	4.77	1.93	0.40
8	1.76	0.69	0.39
9	3.68	1.23	0.33

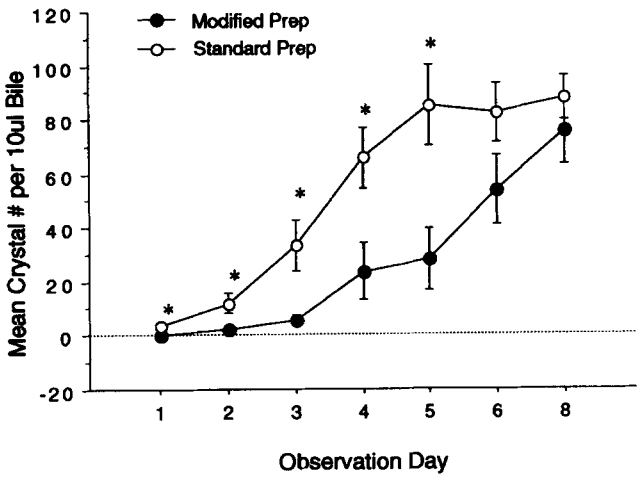

Fig. 2. Comparison of nucleating activity of concanavalin A-binding proteins prepared from columns that were washed with (modified prep) or without (standard prep) 10 mM taurocholate. Mean number (± SE) of cholesterol crystals formed per 10 µl of heated abnormal bile over the observation period ($P < 0.003$, Scheffe's considering all time points; *$P < 0.05$ for individual time points, Scheffe's, n = 9 except day 5 n = 3). No crystals formed in the control incubations without added protein until day 8 at which time a mean of 2 crystals per 10 µl aliquot was observed.

ples, to which proteins obtained by the modified method had been added, nucleated cholesterol crystals on this day, i.e., the crystal observation time was more rapid when proteins obtained by the standard method were used (Fisher's Exact test, $P < 0.005$). Note also that there were significantly greater numbers of crystals on days 2–5 in sample pairs prepared by the standard method (ANOVA, Scheffe's, $P < 0.05$).

Figure 3 shows that filtration of Con A proteins prepared by the standard method through a 0.2-µm filter significantly delayed nucleation compared to the untreated Con A samples, while ultracentrifugation and filtration delayed nucleation to the greatest extent (ANOVA, Scheffe's, $P < 0.001$). Furthermore, the three untreated samples contaminated with more cholesterol crystals nucleated significantly more crystals than the samples containing fewer cholesterol crystals, reaching 100 cholesterol crystals per 10 µl bile at a mean of 3 days compared to 6 days for the less contaminated samples (results not shown).

Microscopic examination

Light microscopy of the protein fractions prepared by the standard technique revealed the presence of vesicles and all six samples of Con A preparations were contaminated to some degree with cholesterol crystals (**Fig. 4** and **Fig. 5**). All ultracentrifuged pellets contained cholesterol crystals. Examination of untreated samples revealed obvious cholesterol crystals in three samples. The other three samples had such a low quantity of cholesterol crystals that light microscopy of only one 10 µl aliquot failed to reveal their presence. However, multiple examinations of these three samples did reveal at least one cholesterol crystal per 10 µl aliquot and, as noted above, the ultracentrifuged pellets of these same samples all contained cholesterol crystals. No such structures were observed in Con A glycoproteins prepared by the modified procedure. The presence of microcrystals appeared to have no relationship to the total lipid concentration or the cholesterol saturation index of native bile. The cholesterol microcrystals shown in Figs. 4 and 5 correspond, respectively, to patients 6 and 7 in Table 1.

Lipid addition studies

Addition of isolated lipids to control bile at the same concentration as that associated with Con A-bound proteins prepared by the standard procedure was done in three samples. The addition did not significantly accelerate cholesterol nucleation (**Fig. 6**). The addition of lipid to lipid-free Con A-bound proteins prepared by the modified method, which was done to simulate Con A-bound samples prepared by the standard technique, also did not accelerate nucleation (Fig. 6).

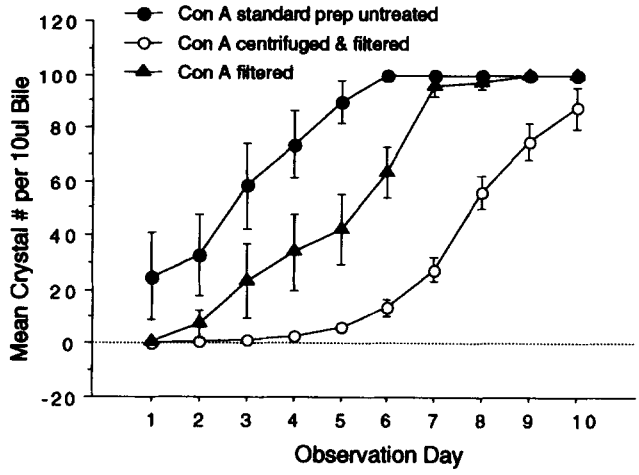

Fig. 3. Nucleating activity of concanavalin A-binding proteins prepared from columns without 10 mM taurocholate wash (standard prep). Nucleating activity of untreated Con A-bound glycoproteins were compared to preparations that were filtered (0.2 µm) or ultracentrifuged and filtered (0.2 µm). Mean number (± SE) of cholesterol crystals formed per 10 µl of heated abnormal bile over the observation period. Compared to untreated Con A preparations, nucleating activity was significantly less for filtered samples (ANOVA, Scheffe's $P < 0.008$; n = 6) and for ultracentrifuged and filtered samples (ANOVA, Scheffe's $P < 0.0001$; n = 6). For practical reasons, crystals were not counted beyond 100 per 10 µl bile. No crystals formed in the control incubations without added protein until day 8 at which time a mean of 2 crystals per 10 µl aliquot was observed and reached 5 crystals per 10 µl aliquot by day 10.

DISCUSSION

Our laboratory was the first to implicate biliary proteins other than mucous glycoprotein in nucleation of cholesterol in bile (17, 18). Biliary proteins were shown to be qualitatively different from the proteins isolated from patients without cholesterol gallstones with regard to the nucleation of cholesterol from native bile (18). Purification of biliary proteins was technically difficult and laborious. Many investigators now use the lectin concanavalin A to purify biliary proteins free of lipids and pigment. The technique first introduced by Groen et al. (19) was a significant advance in preparation of biliary proteins for investigation of their effect on nucleation of cholesterol from bile. However, our study indicates that lipids remain with the Con A preparations, when standard procedures are used, and these can significantly contribute to nucleation of cholesterol. The forms in which the lipids exist in the bound fraction from concanavalin A columns without the taurocholate wash are vesicular structures of approximately 300 nm in diameter as determined under oil with the light microscope. However, cholesterol microcrystals are also detectable by light microscopy in all samples. Sensitivity of detecting cholesterol crystals was greatly enhanced by observing the pellets of ultracentrifuged standard Con A preparations. It is conceivable that these microcrystals could form upon storage of the Con A preparations prior to addition studies by interaction of pronucleating proteins with cholesterol vesicles. The presence of such crystals could provide false information in the nucleation assay either by being detected directly upon addition to model bile, or by acting as a seed crystal and accelerating the nucleation of cholesterol independently of the added protein. The fact that all samples prepared by the standard method nucleated more rapidly with greater crystal formation compared to ultracentrifuged samples suggests that microcrystals are responsible for nucleation in untreated Con A preparations. Recently, Yamashita et al. (20) also reported the appearance of cholesterol microcrystals in some Con A-binding protein preparations. Previous investigators have shown that the addition of microcrystals to model biles has a profound effect on cholesterol nucleation (21, 22). Busch, Tokumo, and Holzbach (22) added seed crystals to model bile solutions at a concentration of 1.5

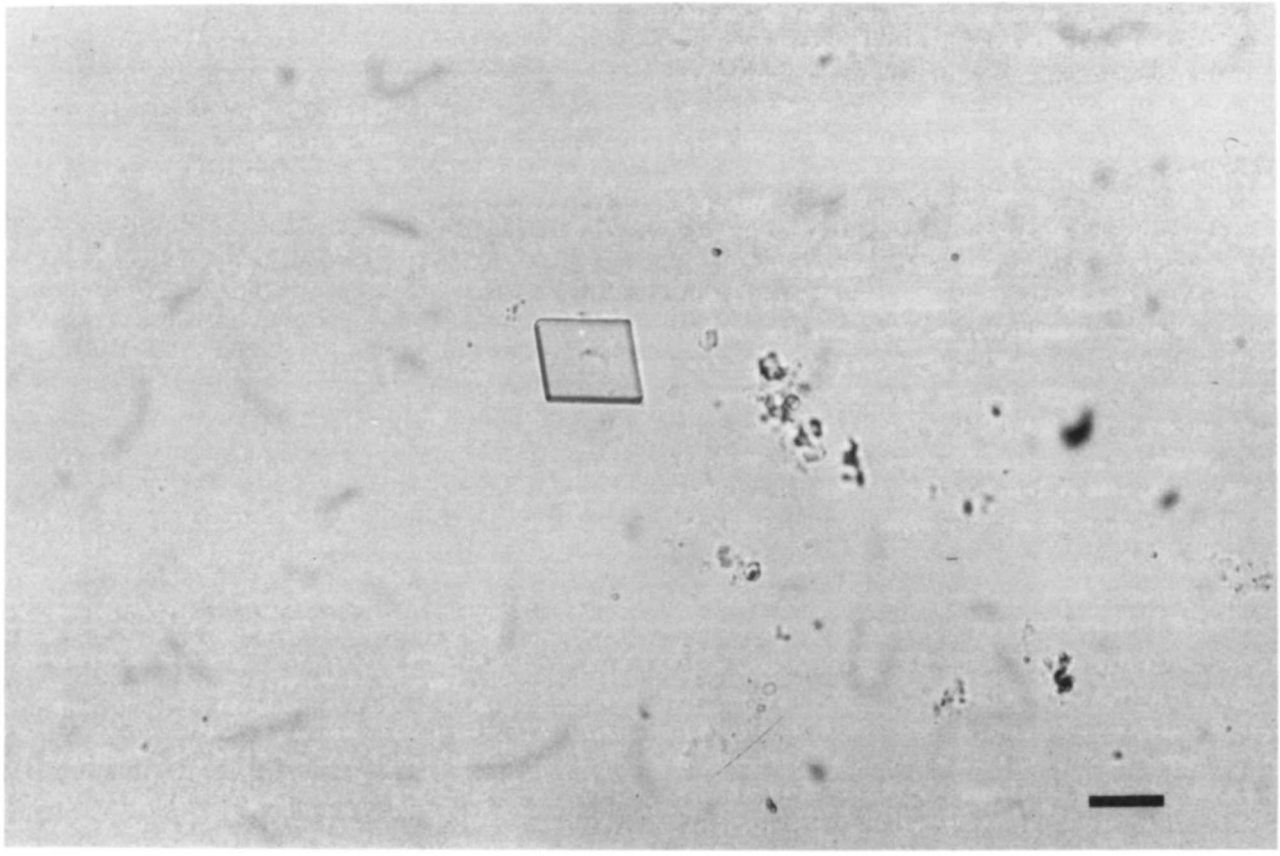

Fig. 4. Light microscopy of Con A-bound glycoproteins prepared by standard technique from patient # 6 showing presence of cholesterol microcrystal. Magnification × 2500 (bar = 4000 nm). Such well-formed microcrystals were not obvious in all samples.

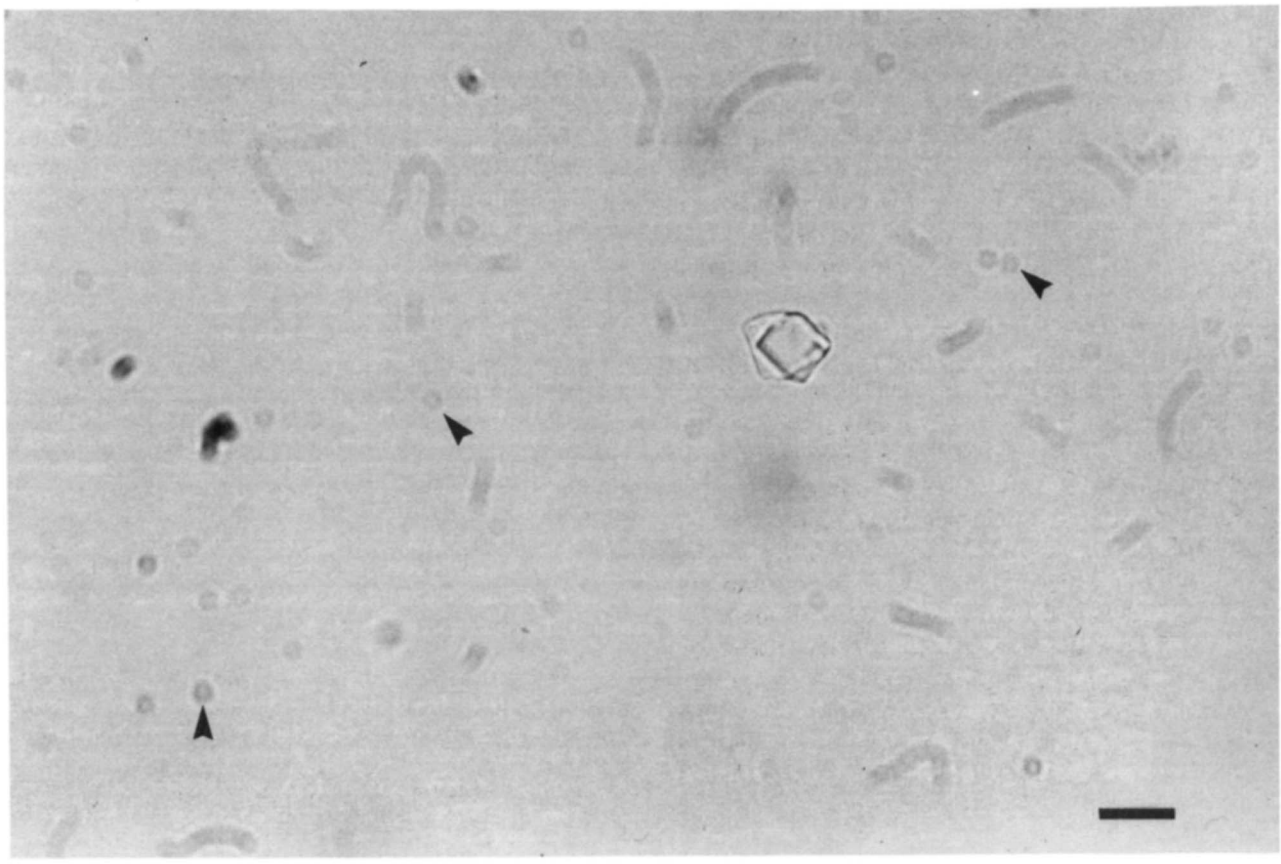

Fig. 5. Light microscopy under oil of Con A-bound glycoproteins prepared by standard technique from patient # 7. Two cholesterol microcrystal of approximately 1200 nm can be seen in center of photograph. Microcrystals of such size would be missed by normal light microscopy of 150 × magnification. Vesicles of approximately 300 nm can also be seen (arrow). Magnification × 6240 (bar = 1600 nm). Streaks represent vesicles in motion during time to photograph.

μg per model bile or approximately 4.0 nmol and observed a significant effect on cholesterol crystallization. This is in the range of the quantity of cholesterol that we have found associated with Con A-bound proteins prepared by the standard technique. The fact that there are cholesterol crystals in all samples obtained by the standard method means that this technique is not adequate for the purpose of isolating potential pronucleating proteins.

The modified method resulted in protein samples with virtually no detectable lipid by analysis of radioactivity and there were no lipid phases seen on light microscopy. Furthermore, the protein recovery was quantitatively and qualitatively unchanged. Therefore, the modified method would seem to be an acceptable means of proceeding with isolation and study of potential pronucleating proteins.

Delipidation of protein fractions by an organic extraction might denature the proteins. Therefore, we used a Folch wash to isolate lipids from the fractions eluted from the columns with sodium taurocholate. Such lip-

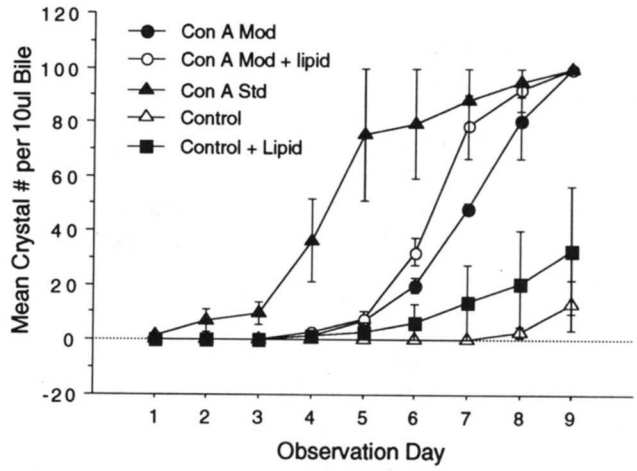

Fig. 6. Effect on cholesterol nucleation of adding lipid to control bile and to lipid-free Con A-bound proteins. Lipids were isolated from the taurocholate washes and added to model bile or to model bile with lipid-free Con A-binding glycoproteins (Con A Mod). Cholesterol added in a noncrystalline form at the concentration present in Con A-binding proteins prepared by the standard technique did not accelerate cholesterol nucleation (mean ± SE, n = 3).

ids, when reconstituted at concentrations calculated to be present in the protein eluates of samples isolated by the standard method, did not affect nucleation. The simplest explanation of this result is that the microcrystals were dissolved by the Folch wash and that on reconstitution in model bile buffer the cholesterol was all incorporated into vesicular form. These lipid extracts were examined by light microscopy and after reconstitution microcrystals were no longer present in any sample.

Studies to characterize the contribution of proteins contained in the Con A-binding fractions to cholesterol nucleation should be interpreted with caution. Some investigators have attempted to remove specific proteins from the Con A-binding fraction and then assess the nucleating activity of the remaining proteins (23, 24). A decrease in nucleating activity was attributed to the removal of specific proteins and the nucleating activity remaining was credited to those proteins not removed. Significant nucleating activity remaining may be due to contaminating cholesterol microcrystals and calculated percentage of nucleating activity attributed to those proteins removed must be reconsidered. Yamashita et al. (20) have reported that at least 75% of nucleation activity in the Con A-binding proteins is due to residual lipid.

Recently, investigators are using specific antibodies and generating affinity columns to remove selected biliary proteins. A taurocholate wash step should also be introduced for these affinity columns to remove traces of contaminating lipids. We routinely wash our affinity columns with bile salt before eluting biliary immunoglobulins (2) and have found that this procedure eliminates all lipids that may contaminate our protein preparations.

We conclude that lipids remain in the Con A preparations or other affinity chromatography procedures and can significantly contribute to the nucleation of cholesterol. Therefore, proteins prepared by affinity columns should be further delipidated prior to studies to assess their nucleation potential. Washing the column with 10 mM taurocholate appears to completely eliminate contaminating lipids. We strongly recommend that investigators purifying biliary proteins by affinity chromatography introduce a bile salt wash step prior to eluting the proteins from the column. This procedure, in contrast to organic washes after the protein is eluted from the column, is non-denaturing. A bile salt wash step prior to eluting proteins from affinity columns also removes lipids without affecting protein recovery.

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