Effect of cholesterol nucleation-promoting activity on cholesterol solubilization in model bile

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Abstract Human bile contains a factor with cholesterol nucleation-promoting activity that binds to concanavalin A-Sepharose. In this study we have investigated the effect of this activity on the dynamics of lipid solubilization in supersaturated model bile. A concanavalin A binding protein fraction of human bile was mixed with model bile and the effect on the distribution of cholesterol and phospholipid between mixed micelles and phospholipid/cholesterol vesicles was studied by means of density gradient ultracentrifugation. The nucleation-promoting activity containing fraction induced a transfer of cholesterol and phospholipid from the micellar to the vesicular phase. This led to a decrease in the density of the vesicular fraction. We have also studied the effect of promoting activity on the nucleation time of an isolated vesicle fraction. A decrease of the nucleation time of 10.7 ± 1.3 to 2.3 ± 0.3 days was observed. In conclusion, a concanavalin A binding protein fraction from human bile stimulated cholesterol nucleation via a double effect; it increased the amount of vesicular cholesterol and phospholipid, and it also directly induced nucleation of cholesterol from the vesicles. —**Groen, A. K., R. Ottenhoff, P. L. M. Jansen, J. van Marle, and G. N. J. Tytgat.** Effect of cholesterol nucleation-promoting activity on cholesterol solubilization in model bile. *J. Lipid Res.* 1989. **30:** 51–58.

Supplementary key words nucleation • vesicles

Recently it became clear that cholesterol in human bile is, to a significant extent, solubilized in cholesterol/lecithin vesicles (1–6). These vesicles play an important role in crystallization of cholesterol monohydrate crystals in supersaturated bile. Results of Halpern et al. (4) obtained by video-ennanced microscopy suggest that cholesterol crystals originate from these vesicles. Lee et al. (5) showed that nucleation of cholesterol decreased the amount of cholesterol in the vesicular phase but had no effect on the amount solubilized in mixed micelles. Harvey et al. (7) could demonstrate cholesterol nucleation in an isolated vesicle fraction. No crystal formation occurred from the isolated micellar phase. The nucleation time of bile from gallstone patients is significantly shorter than the nucleation time of normal bile (8–11). Hence it can be expected that there is a difference in the stability of vesicles in abnormal bile as compared to normal bile. Studies on the dynamic interchange of vesicular and micellar cholesterol in model bile systems by Halpern et al. (12) and Kibe et al. (13) have revealed that the amount of cholesterol in the vesicles is determined by the degree of supersaturation, the total lipid concentration, and the bile salt/lecithin ratio. However, bile from gallstone patients and bile from controls do not differ with respect to any of these variables (8). Therefore, other factors have to be responsible for the faster nucleation of bile from gallstone patients. Recently, several nonlipid factors have been described that influence the nucleation time. Holzbach et al. (14) reported the presence of biliary proteins, possibly apolipoproteins A-I and A-II (15), that are capable of inhibiting nucleation in supersaturated model bile systems. Preliminary evidence (16) indicates that these apolipoproteins stabilize the cholesterol/phospholipid vesicles. In addition to nucleation-inhibiting factors, nucleation-promoting factors have been postulated but there is some controversy as to the relative importance of these factors. Levy, Smith, and LaMont (17), Smith and LaMont (18), and Lee et al. (19–21) propose that biliary mucin is an important nucleation-promoting factor. This has been disputed by Gallinger et al. (22) who did not find an effect on the nucleation time when mucin was extracted from gallbladder bile. According to Burnstein et al. (11) the most important nucleation-promoting factor has a molecular weight of less than 300,000, which is much lower than the molecular weight of mucin or its subunits. Recently (23, 24), we have isolated a nucleation-promoting glycoprotein fraction which binds to concanavalin A (Con A). Possibly this fraction contains the factor proposed by Burnstein et al. (11).

Abbreviation: Con A, concanavalin A

The mechanism underlying the stimulation of cholesterol crystal nucleation is not understood. Smith (25) recently reported that mucin stimulates nucleation in isolated vesicles, indicating direct interaction of mucin with the vesicles. In the present study we studied the influence of the Con A-binding nucleation-promoting activity on the distribution of biliary lipid between the vesicular and micellar phase. The effect of the protein fraction on isolated vesicles was also investigated.

MATERIALS AND METHODS

Materials

Egg yolk phosphatidylcholine and α-D-methylmannopyranoside were obtained from Sigma Chemical Co., St. Louis, MO. Cholesterol was purchased from Merck, Darmstadt, FRG. Pronase was from Boehringer, Mannheim, FRG. Taurocholate was purchased from Fluka, Buchs, Switzerland. Concanavalin A-Sepharose was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were of analytical grade.

Preparation of model bile

Model bile with a cholesterol saturation index of 1.2 was prepared as described previously (26). Briefly, taurocholate in methanol–water 85:15 and cholesterol and lecithin in chloroform were mixed thoroughly. The organic solvents were evaporated by flushing with nitrogen and subsequently the mixture was lyophilized. The dry lipid film was then solubilized in Tris–HCl buffer (50 mM, pH 8.1) and incubated at 56°C for 24 hr. The final lipid concentrations were: taurocholate, 129 mM; lecithin, 31 mM; cholesterol, 13 mM. Nucleation time was determined as described previously (24).

Isolation of nucleation promoting activity

T-tube bile (100 ml) or gallbladder bile (20 ml) obtained from cholesterol gallstone patients was loaded on a column (1.6 × 20 cm) containing 20 ml packed concanavalin A-Sepharose. The column was washed with 200 ml Tris–HCl buffer (10 mM, pH 7.4) containing 0.2 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$. Subsequently the column was eluted with 100 ml Tris-HCl buffer (10 mM, pH 7.4) containing 0.2 M NaCl and 0.1 M α-D-methylmannopyranoside, and the eluted material was dialyzed against distilled water for 48 hr at 4°C and concentrated to a volume of 10 ml. The protein content of this fraction was 0.5–2 mg/ml; the content of bile acid, cholesterol, and lecithin was lower than 0.1 mM. This fraction is called Con A-eluate. Pronase digestion of Con A-eluate was performed as described previously (24). Control Con A-eluate was obtained by loading the column with 20 ml of model bile instead of human bile.

Density gradient ultracentrifugation

Bile samples were centrifuged in a KBr density gradient essentially as described by Terpstra, Woodward, and Sanchez-Munir (27). Briefly, 2 ml of sample was mixed with 944 mg KBr and 50 mg sucrose. On this mixture 2.4 ml of a KBr solution with a density of 1.26 g/ml was layered, followed by 3.6 ml of a KBr solution with a density of 1.225 g/ml and 2.4 ml of KBr with a density of 1.1 g/ml. Finally, 2.4 ml of double-distilled water was layered and the tubes were centrifuged for 22 hr at 170,000 *g* and 20°C in a Beckman SW 41 Ti rotor. In this way a linear gradient was obtained with density limits of 1.045 and 1.339 g/ml. The tubes were fractionated by hand into eleven fractions of 1.17 ml each. Prospholipid/cholesterol vesicles were isolated as follows. Five ml of model bile was mixed with 100 mg sucrose and 1.357 g KBr. On this mixture 4.8 ml of KBr solution with a density of 1.082 g/ml was layered. Finally, 2.4 ml of double-distilled water was layered and the tubes were centrifuged as described above.

Transmission electron microscopy

About 5 μl of gradient fraction was layered on Formvar-coated 400 mesh grids. After 15 sec of incubation, excess fluid was removed and 5 μl of 1% ammonium molybdate was layered on the grid. After 60 sec incubation, excess fluid was eliminated. The grid was dried under air and then examined using a Philips EM 420 electron microscope.

Chemical analysis

Biliary lipids were determined using standard enzymic techniques (28–30). Free fatty acids were measured with acyl-CoA synthetase using the NEFA-C test of WAKO Chemicals, Neuss, FRG. Protein was determined as described by Lowry et al. (31).

Statistical methods

Statistical significance of the results was assessed using the Mann-Whitney test.

RESULTS

Recently we have shown that 5–8% of protein in human gallbladder bile and T-tube bile binds to concanavalin A-Sepharose (23, 24). This fraction can be eluted with α-D-methylmannopyranoside and contains cholesterol nucleation-promoting activity (24). To investigate the effect of this fraction on the dynamics of cholesterol solubilization, Con A-eluate was mixed 1:1 with model bile and separated by density gradient ultracentrifugation either directly or after 48 hr incubation at 37°C. Controls contained Tris-HCl buffer instead of Con A-eluate and were subjected to the same procedure. The experiment was carried out in triplicate. With this technique an excel-

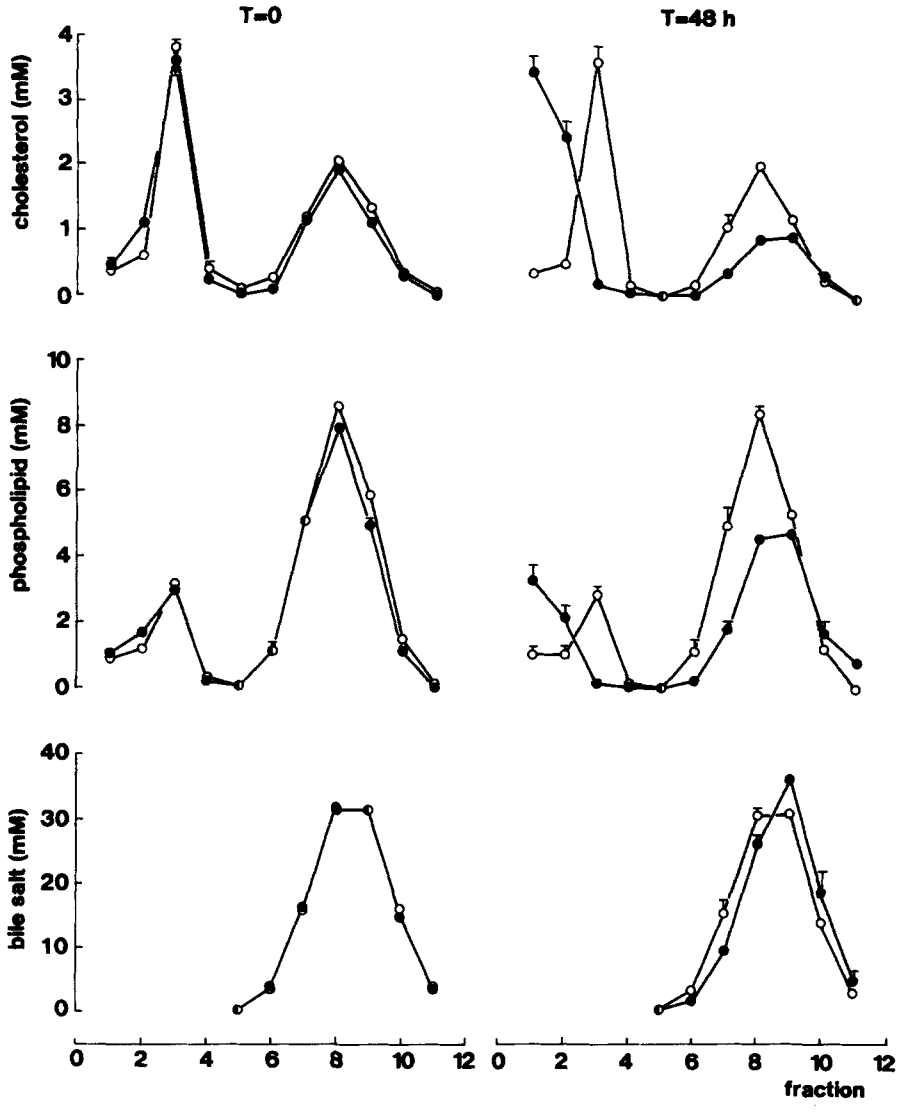

Fig. 1. The effect of Con A-binding nucleation-promoting activity on the distribution of biliary lipid in model bile. Nucleation-promoting activity containing Con A-eluate was isolated as described in Methods. Con A-eluate (2.98 mg/ml protein) was mixed with model bile (1:1, v/v) and centrifuged either directly or after incubation for 48 hr at 37°C. In the control incubation model, bile was mixed 1:1 (v/v) with Tris buffer (50 mM, pH 8.1). After the centrifugation, the tubes were fractionated into 11 fractions of 1.17 ml each and the biliary lipids were assayed in these fractions. Fraction 1 represents the top fraction (low density) and fraction 11 the bottom fraction. Open circles represent controls and closed circles represent tubes containing Con A-eluate. Data are presented as mean ± SD; n = 3.

lent separation between the vesicular phase and micellar phase was obtained (**Fig. 1**). Fractions 1–4 contained the vesicular phase. The size of the vesicles varied between 50 and 200 nm. Probably some aggregation and fusion took place during the centrifugation procedure (**Fig. 2**). The bile salt concentration in these fractions was lower than 0.1 mM. About 45% of cholesterol and 20% of lecithin was present in the vesicular phase. By comparing left-hand with right-hand panels, the effect of Con A-eluate on the distribution of biliary lipid can be appreciated. After incubation for 48 hr in the presence of Con A-eluate, a significant shift of cholesterol and phospholipid from the micellar to the vesicular phase had occurred. In addition to this shift it was observed that the vesicular peak moved to lower density. Concomitantly, the cholesterol/phospholipid ratio in the top fraction (fraction 1) increased from 0.4 to 1.2. The recovery of cholesterol was 93% in these experiments. The recovery of phosphatidylcholine was somewhat lower (78%). To investigate whether this was due to phospholipid breakdown during the incubation, we determined lecithin recovery and degradation products directly after the 48 hr of incubation.

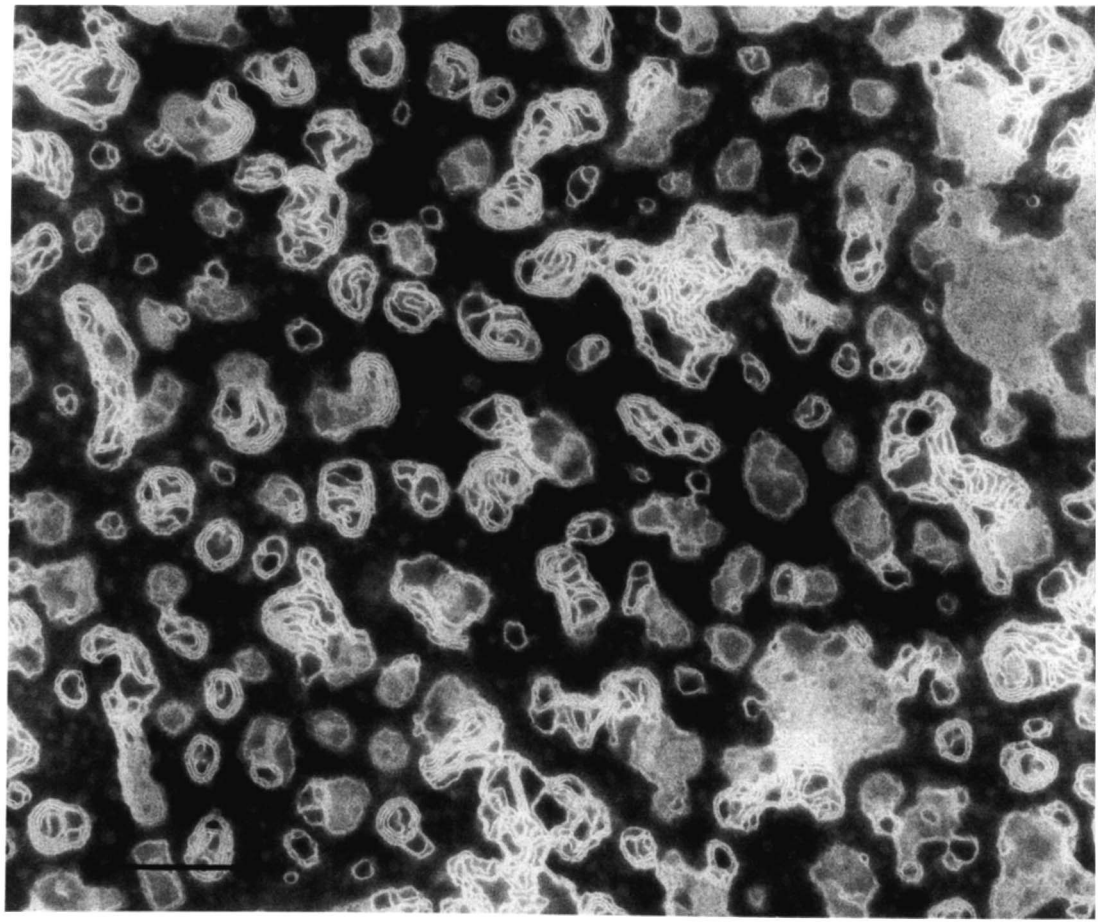

Fig. 2. Transmission electron micrograph of the vesicular fraction in model bile. The vesicular fraction was isolated from model bile as described in the text. The vesicles were negative stained with ammonium molybdate. Bar indicates 0.2 μm.

The data in **Table 1** show that about 15% of phosphatidylcholine is degraded during the 48-hr incubation with Con A-eluate. Small amounts of free choline and free fatty acids were formed. Since this could influence the lipid distribution in model bile, we also determined the effect of pronase-digested Con A-eluate on lecithin breakdown. In an earlier study it was shown that pronase digestion of Con A-eluate has no effect on its nucleation-promoting effect (24). It can be seen in Table 1 that in the presence of pronase-digested Con A-eluate no lecithin breakdown takes place. The effect of pronase-digested Con A-eluate on the lipid distribution in model bile is given in **Table 2**. Pronase digestion does not significantly change the transfer of cholesterol and phospholipid from the micellar to the vesicular fraction.

During Con A-Sepharose fractionation, some of the

TABLE 1. Effect of Con A-eluate and pronase-digested Con A-eluate on the concentration of phosphatidylcholine, free choline, and free fatty acid in model bile

Addition	n	Phosphatidylcholine	Free Choline	Free Fatty Acid
			mM	
None	3	15.8 ± 0.9	0.04 ± 0.04	0.44 ± 0.05
Con A-eluate	3	13.3 ± 1.4[a]	0.53 ± 0.12[a]	0.54 ± 0.2
Con A-eluate + pronase	3	15.9 ± 0.5	0.16 ± 0.09	0.46 ± 0.05

Model bile was mixed 1:1 with Tris buffer, Con A-eluate, or pronase-digested Con A-eluate. After 48 hr of incubation at 37°C, a sample of the incubation was assayed for phosphatidylcholine, free choline, and free fatty acids. Con A-eluate derived from three different gallstone patients was used in the experiments. Data are presented as means ± SD.

[a] $P < 0.05$.

TABLE 2. The effect of Con A-eluate, pronase-digested Con A-eluate, and control Con A-eluate on the lipid distribution in model bile

Addition	n	Vesicles			Micelles		
		Ch	PL	Ch/PL	Ch	PL	BA
		mg				*mg*	
Tris buffer	9	1.86 ± 0.11	4.55 ± 0.40	0.84 ± 0.05	2.36 ± 0.39	20.0 ± 2.3	69.9 ± 4.9
Control Con A-eluate	3	1.76 ± 0.10	4.55 ± 0.12	0.77 ± 0.02	2.60 ± 0.13	21.5 ± 1.1	74.2 ± 0.4
Con A-eluate	5	2.57 ± 0.22[a]	5.34 ± 0.43[a]	1.03 ± 0.07[a]	1.54 ± 0.54[a]	13.7 ± 2.0[a]	67.3 ± 0.4
Con A-eluate + pronase	4	2.50 ± 0.42[a]	5.37 ± 0.49[a]	0.93 ± 0.07[b]	2.01 ± 0.24[b]	19.8 ± 1.4	71.0 ± 0.9

Con A-eluate, pronase-digested Con A-eluate, control Con A-eluate, or Tris buffer were mixed with model bile (1:1, v/v). After 48 hr incubation at 37°C, 2 ml of the mixtures was separated by density gradient ultracentrifugation as described in Methods. Fractions 1–4 (vesicular fraction) and 6–11 (micellar fraction) were pooled and the concentration of biliary lipids was assayed in these pooled fractions. Data are given as means ± SD; Ch, cholesterol; PL, phospholipid; BA, bile acid.

[a] $P < 0.01$ versus Tris buffer.

[b] $P < 0.05$ versus Tris buffer.

Con A dissociates from the column and is recovered in the eluate (P. R. C. Harvey, personal communication). To check whether this influences the results we carried out a control fractionation by eluting model bile on a Con A-Sepharose column. This control Con A-eluate was supplemented with 2 mg/ml albumin and the effect on the lipid distribution in model bile was determined as described above. As shown in Table 2, control Con A-eluate had no significant effect on the lipid distribution in model bile.

Fig. 3 shows a dose–response effect of Con A-eluate on the nucleation time and the lipid distribution in model bile. Serial dilutions of Con A-eluate were added to model bile. The nucleation times of the mixtures were determined and the effect of Con A-eluate in various dilutions on the distribution of cholesterol and lecithin over the micellar and vesicular fractions was measured after 24 hr incubation at 37°C. Undiluted Con A-eluate (0.32 mg/ml protein) decreased the nucleation time of model bile from 5 days to 1 day and induced a shift in the cholesterol distribution from 55% micellar and 45% vesicular to 43% micellar and 57% vesicular. Concomitantly, a 5% shift in phospholipid from the micellar to the vesicular phase occurred. Both the effect on nucleation time and lipid distribution decreased when diluted Con A-eluate was added.

The kinetics of the effect of Con A-eluate were studied by following the distribution of cholesterol and phospholipid for 9 days both in a control incubation and in the presence of a limited amount of Con A-eluate. In the left panel of **Fig. 4,** the result for the control incubation is shown. There was no effect on the lipid distribution during this time interval. The nucleation time of the incubation was 8 days. In the presence of Con A-eluate (0.05 mg/ml protein) the nucleation time decreased to 3 days. As shown in the right panel of Fig. 4, the amount of vesicular cholesterol and phospholipid increased before the onset of crystal formation and continued thereafter almost linearly.

Is the nucleation-promoting effect of Con A-eluate solely a consequence of the shift in the cholesterol and phospholipid distribution? To address this question we have added promoting activity to an isolated vesicle fraction and determined the effect on the nucleation time. Cholesterol/phospholipid vesicles were isolated from a model bile by density gradient ultracentrifugation. Fractions 3 and 4 were collected and pooled and the vesicles

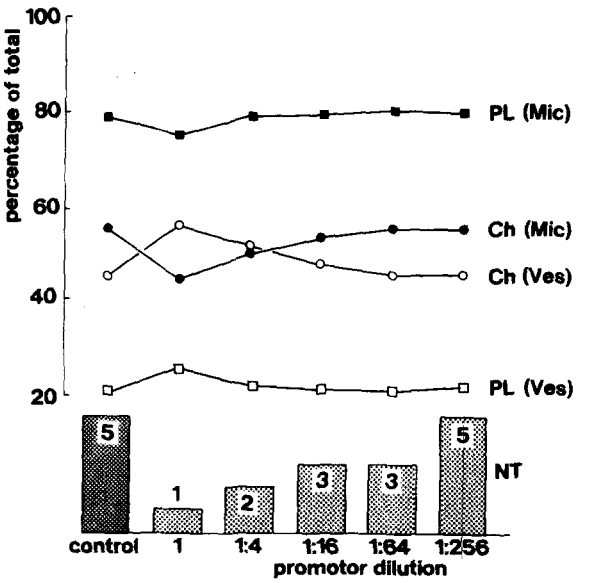

Fig. 3. Dose response effect of nucleation-promoting activity containing Con A-eluate on lipid distribution and nucleation time of model bile. Con A-eluate (0.32 mg/ml protein) in various dilutions was mixed with model bile (1:1, v/v) and incubated for 48 hr at 37°C. After incubation, 2 ml of the mixtures was centrifuged as described in Methods. After centrifugation, the tubes were fractionated in 11 fractions of 1.17 ml each. Fractions 1–4 and 6–11 were pooled and contained the vesicular (Ves) and micellar (Mic) phases. The nucleation time of the mixtures was determined as described previously (24). The total concentration of cholesterol (Ch) and phospholipid (PL) was 12.7 and 32 mM, respectively.

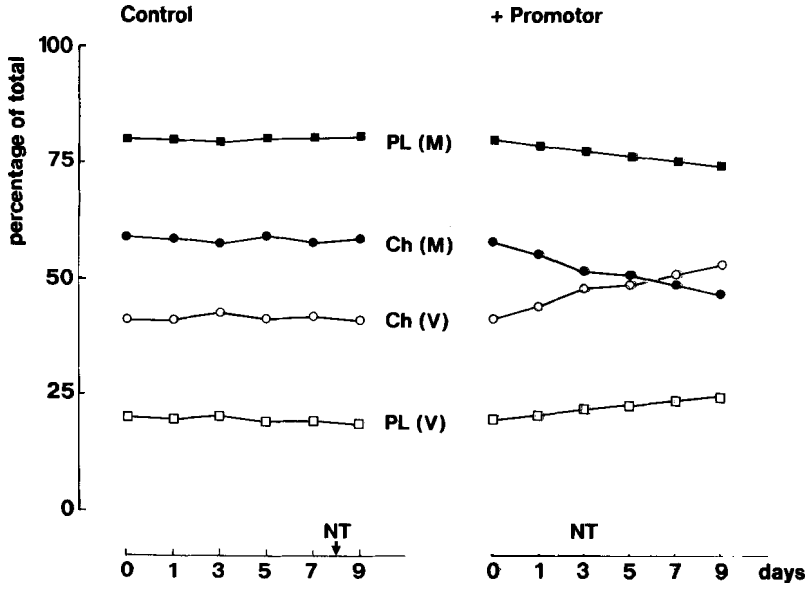

Fig. 4. Kinetics of the effect of nucleation-promoting activity on the lipid distribution in model bile. Model bile was mixed 1:1 with Con A-eluate (0.05 mg/ml protein) or Tris buffer (50 mM, pH 8.1). The mixtures were centrifuged directly and after incubation for 1, 3, 5, 7 and 9 days at 37°C. Vesicular (V) and micellar (M) cholesterol (Ch) and phospholipid (PL) were measured as described in the legend to Fig. 2. The total concentration of cholesterol and phospholipid was 11.7 and 33.9 mM, respectively.

were incubated in the presence of Con A-eluate. The lipid content of the vesicular fraction isolated in this manner was 5 mM cholesterol and 5.5 mM lecithin. This was similar to the lipid content of the vesicular fraction of the model bile used in this study. **Fig. 5** shows that the nucleation time of the isolated vesicles was 10.7 ± 1.3 days (n = 3). In the presence of Con A-eluate this nucleation time decreased to 2.3 ± 0.3 days ($P < 0.001$). In addition to the effect on the nucleation time, Con A-eluate increased the turbidity of the incubation. This indicates that fusion or aggregation of the vesicles had occurred.

DISCUSSION

A significant part of nucleation-promoting activity in human bile binds to Con A-Sepharose (24). In this report we have studied the mechanism by which this activity induces crystallization of cholesterol in supersaturated model bile. To investigate the interaction of nucleation-promoting activity with biliary lipid without the possible interference of other biliary components, we used model bile in these studies.

Nucleation of cholesterol monohydrate crystals is a multistep process. The sites at which nucleation-promoting factors could influence nucleation are schematically represented in **Fig. 6**. To discriminate between these possibilities one must be able to study the influence of promoting factors on micelles and vesicles in bile separately. Three different techniques have been used to study micelles and vesicles in bile: quasi-elastic light scattering (1, 32), gel permeation (2, 3, 33), and density gradient ultracentrifugation (2, 33). With quasi-elastic light scattering it is difficult to visualize micelles in the presence of vesicles (1). Gel permeation and density gradient ultracentrifugation have the disadvantage that shifts from the micellar phase to the vesicular phase and vice versa can occur during the procedure (3, 33). Since we were primarily interested in relative changes in the lipid distribution induced by the nucleation-promoting activity in Con A-

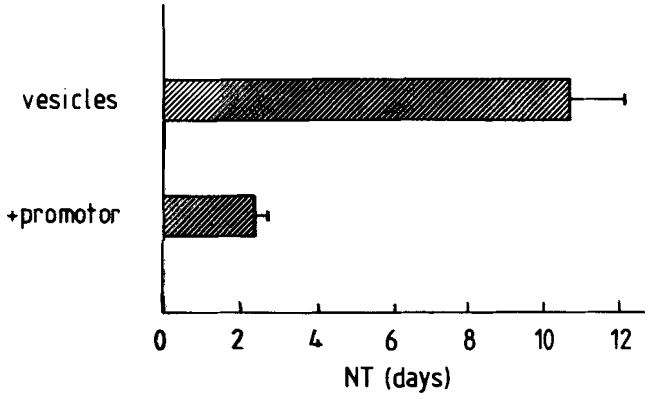

Fig. 5. The effect of nucleation-promoting activity on the nucleation time of isolated cholesterol/phospholipid vesicles. Model bile was separated by density gradient ultracentrifugation as described in Methods. Fractions 3 and 4 were collected and pooled. This pooled fraction was either mixed 1:1 with Tris-HCl buffer or with Con A-eluate. The experiment was carried out three times with three different batches of Con A-eluate, containing, respectively, 2, 2.3, and 1.9 mg/ml protein.

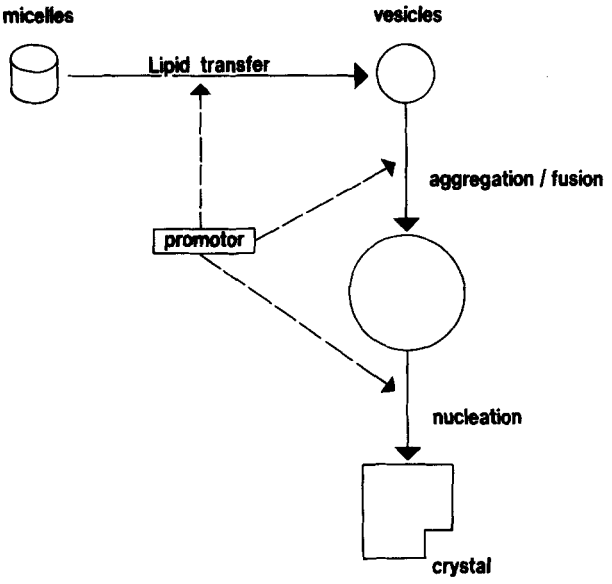

Fig. 6. Simplified scheme of cholesterol nucleation.

eluate, we have used density gradient ultracentrifugation for the separation. Compared to gel permeation, this technique has the advantage that a good separation of micelles and vesicles is obtained without the necessity of adding extra bile salt.

Addition of Con A-eluate to supersaturated model bile induced a shift in cholesterol and phospholipid from the micellar to the vesicular phase, indicating that the first step in the scheme in Fig. 6 is affected. The transfer of cholesterol occurred at a higher rate than that of phosphatidylcholine. This led to a significant increase in the vesicular cholesterol/phospholipid ratio. An explanation for this phenomenon could be that nucleation-promoting activity in Con A-eluate destabilizes mixed micelles and forces cholesterol and phospholipid to move to the vesicular phase. Whether this destabilization is caused by binding of promoting factor to micelles is as yet unknown.

In addition to the transfer of cholesterol and phospholipid from the micellar to the vesicular phase, Con A-eluate also decreases the density of the vesicular fraction. Whether this is caused by the transfer of lipid from the micellar phase or by a direct effect of nucleation promoting activity is not clear. That nucleation-promoting activity also interacts directly with vesicles was demonstrated by its ability to decrease the nucleation time of an isolated vesicle fraction by about 80%. The question arises whether the differential effects on micelles and vesicles are caused by a single factor or perhaps a combination of factors. Con A-eluate is not a pure fraction. As we have shown recently (24), it still contains a large number of proteins. Only a few of these are resistant to treatment with the proteolytic enzyme pronase. A pronase-resistant protein with an apparent molecular weight of 130,000 showed nucleation-promoting activity. The relative importance of this protein is not yet clear. The fact that both the transfer of lipid and the nucleation of isolated vesicles are also induced by pronase-digested Con A-eluate suggests that only one factor is involved. It seems unlikely that two factors share the very uncommon resistance to pronase.

What is the role of Con A binding promoting activity in the pathogenesis of gallstone disease? To answer this question directly, methods to quantify this activity in gallbladder bile have to be developed. However, there is some indirect evidence that the role of this activity could be important. In a careful study, Halpern et al. (12) quantified the amount of vesicular cholesterol in model bile at various levels of total lipid and cholesterol saturation index. When these results are compared to data of Lee et al. (5) for human bile from gallstone patients, it is striking that the amount of vesicular cholesterol in the human bile samples of comparable total lipid and cholesterol saturation index is in many cases much higher. Therefore a non-lipid factor must have induced a transfer of cholesterol from the micellar to the vesicular phase. We suggest that Con A-binding nucleation-promoting activity could be responsible for this transfer.

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