# A novel vesicular assay to study factors affecting cholesterol crystallization in vitro

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**Abstract** We present an assay to study cholesterol crystallization that is fast, facile, and highly reproducible. Cholesterol crystallization from metastable vesicles was induced upon addition of bile salts and depended on the hydrophobicity of the bile salt used and the cholesterol-to-phospholipid ratio of the vesicles. Bile salt-induced crystallization was stimulated upon addition of Con A-binding glycoproteins (CABGs), comparable to the results of the same CABGs in a crystal growth assay. The onset time and total measuring time, however, were much shorter. This assay might, moreover, provide a tool to study the mechanism of cholesterol crystallization in more detail.—**Zijlstra, A. I. M., B. J. M. Van de Heijning, M. van Overveld, and A. K. Groen.** A novel vesicular assay to study factors affecting cholesterol crystallization in vitro. *J. Lipid Res.* 1996. **37:** 877–883.

**Supplementary key words** cholesterol gallstones • bile • hydrophobicity • cholesterol nucleation • nucleation factors

Several methods have been described for the measurement of cholesterol crystallization in vitro. The two most commonly used methods are the nucleation time assay and the crystal growth assay. The nucleation time assay, as developed by Holan et al. (1), detects cholesterol crystals that form in a previously ultracentrifuged isotropic bile sample by microscopic examination. The interval to the first detection of cholesterol crystals is called the nucleation time, which ranges as high as 21 days or more in normal control bile. Cholesterol crystals smaller than several microns, however, are not detectable with conventional light microscopy and, hence, a better terminology for nucleation time would be crystal observation time (2, 3).

Busch, Tokumo, and Holzbach (4) introduced an assay in which cholesterol crystallization was studied in model bile systems using turbidimetric detection. The development of turbidity with time yields a sigmoidal crystal growth curve that reveals the onset time, the crystal growth rate, and the maximal final crystal concentration at equilibrium. The successive stages in the curve probably reflect the formation of unstable vesicles from mixed micelles, and the concomitant initiation of cholesterol nucleation, succeeded by a crystal growth phase and followed by an equilibrium stage. Although the crystal growth assay is a useful tool to study the effect of factors influencing nucleation/crystallization, it is rather time-consuming as the process requires at least 3 or 4 days.

Neither of these assays give information about the actual mechanism by which cholesterol nucleates. To study the sequence of events leading to cholesterol crystallization, Van de Heijning et al. (5) incubated vesicles isolated from model bile with different bile salts. Upon investigation of the dynamics of the bile salt/vesicle interaction, it was shown that an instantaneous micellization of vesicular cholesterol (CH) and phospholipid (PL) occurred. The start of micellization was accompanied by an immediate rise of the vesicular CH/PL ratio due to the preferential solubilization of phospholipid into de novo formed mixed micelles. The nucleation of cholesterol, probably originating from the highly unstable vesicles, started within 0.5 h after the exposure to bile salt (5). In the present study we modified the vesicular nucleation system of Van de Heijning et al. (5) in order to investigate protein-induced cholesterol crystallization. This assay might provide us with a tool to study the mechanism of cholesterol nucleation in more detail.

## MATERIALS AND METHODS

### Materials

Egg yolk lecithin (99%), cholesterol (99+%), taurocholate (ca. 98%), taurodeoxycholate (98%), deoxycholate (minimum 95%), and horseradish peroxidase for the

Abbreviations: CABG, concanavalin A-binding glycoprotein fraction; CH, cholesterol; Con A, concanavalin A; CSI, cholesterol saturation index; DC, deoxycholate; PL, phospholipid; TC, taurocholate; TDC, taurodeoxycholate.

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phospholipid assay were purchased from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase for the cholesterol assay, cholesterol oxidase, choline oxidase, and phospholipase D were from Boehringer Mannheim BmbH (Mannheim, Germany). Homovanillic acid was purchased from Merck (Darmstadt, Germany). Millex-HA 0.45 μm filter units and Millex-GS 0.22 μm filter units were obtained from Millipore (Molsheim, France) and concanavalin A Sepharose was from Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade.

### Bile collection and processing

Hepatic bile was collected from two patients with indwelling T-tubes after cholecystectomy for cholesterol gallstone disease. Patient 1 was a single-stone male patient, age 60, and patient 2 was a multiple stone female patient, age 58. The gallstones were classified as cholesterol gallstones based on visual inspection and chemical analysis (more than 70% cholesterol by weight). Informed consent was obtained from both patients. Bile was stored at -20°C until processing.

### Vesicle preparation

Cholesterol and phospholipid mixtures were prepared in different molar ratios (1:1 to 2:1) in chloroform. The organic solvent was evaporated in a rotary evaporator and the residual solvent was eliminated completely by 2 h lyophilization. The dried lipid film was resuspended in aqueous buffer (50 mM HEPES, 100 mM NaCl, pH 7.4) to yield final concentrations of about 10–15 mM cholesterol and 10 mM phospholipid. The preparation was sonicated for 15 min with a Vibra cell sonicator (Sonics & Materials, Danbury, CT) set at 80% output control and pulsed sonication (4 sec off, 1 sec on). To minimize degradation of lipids, the sonication was performed on ice under a gentle stream of nitrogen. After sonication the vesicles were passed through successively smaller pore filters of 0.45 μm and 0.22 μm. Subsequently, the cholesterol concentration was measured with an enzymatic colorimetric method in accordance with a Boehringer Mannheim protocol (Mannheim, Germany) and the phospholipid concentration in accordance with a Wako protocol (Neuss, Germany).

### Cryoelectron microscopy of vesicles

Thin hexagonal 700-mesh grids were dipped into a vesicle suspension (CH = 13.1 mM, PL = 8.2 mM, CH/PL ratio = 1.6), blotted between filter paper, and shot into liquid ethane (100 K) with a gravity powered guillotine as described by Frederik et al. (6). After vitrification the specimen was transferred to the vacuum of an EM-420

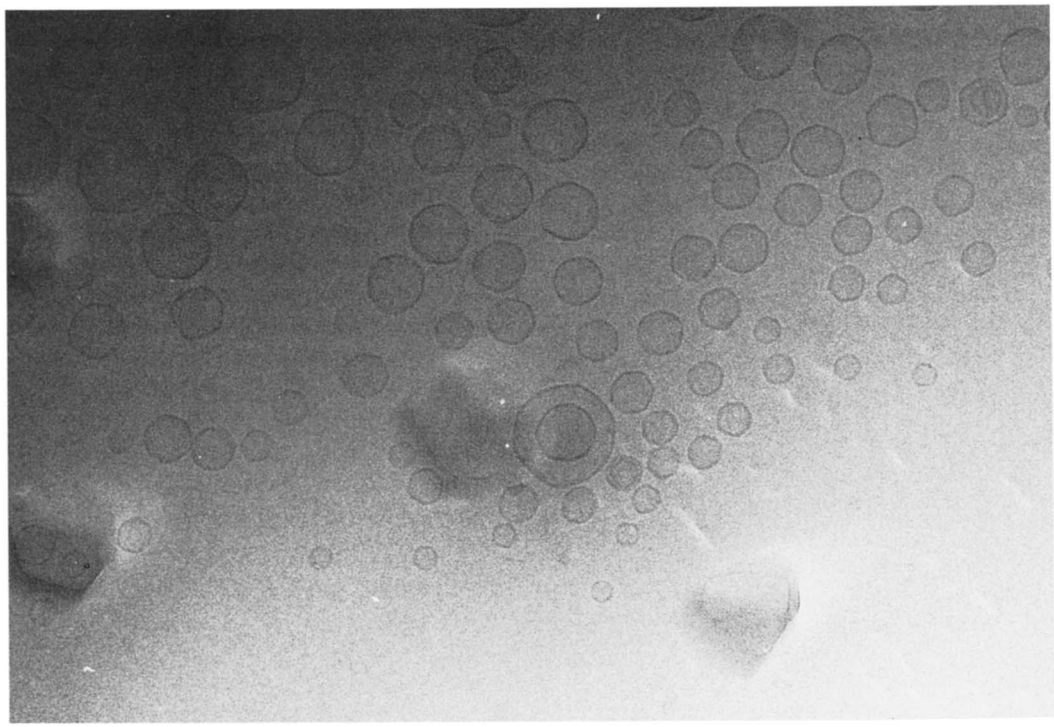

**Fig. 1.** Electron micrograph of vesicles composed of cholesterol (13.1 mM) and phospholipid (8.2 mM), CH/PL ratio = 1.6. The vesicle suspension contained mainly unilamellar vesicles, varying in size from 55 nm to 215 nm, and an occasional multilamellar vesicle. Magnification = 200,000 ×.

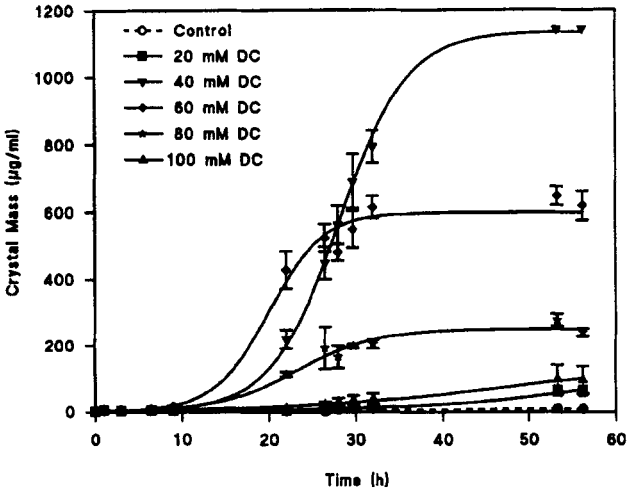


**Fig. 2.** Cholesterol crystallization from unstable vesicles (CH/PL ratio = 1.6) upon incubation with different concentrations of deoxycholate (DC). Controls were vesicles incubated with HEPES buffer only. Optimal crystallization occurred at a DC concentration of 40 mM; higher and lower concentrations induced less cholesterol crystallization. No spontaneous crystallization from the vesicles occurred without added bile salt. [CH] = 13.7 mM, [PL] = 8.8 mM. Results are expressed as the mean ± SD of four experiments.

electron microscope with a Philips PW6599/00 cryospecimen holder. The vitrified film was studied at 110 K with a vacuum of at least $0.2 \times 10^{-3}$ Pa. Micrographs were taken at a magnification of 18,000 ×.

### Preparation of Con A-binding glycoprotein fraction (CABG)

CABG from human hepatic T-tube bile was prepared as described previously (7). In brief, native T-tube bile of patient 1 was centrifuged at 3000 *g* for 5 min and incubated with concanavalin A (Con A)-Sepharose 4B for 2 h at room temperature. The column was pre-equilibrated with 10 mM Tris-HCl, pH 7.4, containing 0.2 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$ plus 0.02% azide as an antimicrobial agent. Bound glycoproteins were eluted with 0.1 M methyl α-D-mannopyranoside containing 0.02% azide. The eluate was dialyzed for 3 days at 4°C against 10 mM ammoniumbicarbonate containing 0.02% azide, and then concentrated 10-fold using an Amicon-8400 ultrafiltration cell with an YM-10 filter (Beverly, MA). This CABG of patient 1 was called CABG 1. The procedure was repeated with T-tube bile of patient 2 which provided an eluate CABG 2.

### Analytical procedures for lipids in CABG

The cholesterol content of the CABGs was determined by an enzymatic assay using fluorometric detection by a modification of the procedure described by Huang, Kuan, and Guilbault (8). The reaction medium contained 1 mM homovanillic acid, 1 U/ml horseradish peroxidase, and 0.15 U/ml cholesterol oxidase in 50 mM MOPS (pH 7.7) with 0.05% Triton X-100. The change of fluorescence was measured at 425 nm (lambda ex = 327 nm) in a Cobas Bio centrifugal analyzer (Roche Diagnostica, Basel, Switzerland). The cholesterol content of the CABGs was calculated by referring to a standard calibration curve.

With a similar approach, the phospholipid content of the CABGs was determined. The reaction mixture contained 1 mM homovanillic acid, 0.21 U/ml phospolipase D, 38 U/ml horseradish peroxidase, and 50 mU/ml choline oxidase in 50 mM MOPS (pH 8.0) with 10 mM $CaCl_2$, and 0.05% Triton X-100.

### Rapid vesicular crystallization assay

The hydrophobic bile salt deoxycholate, or the taurine-conjugated form taurodeoxycholate (TDC), or the more hydrophilic bile salt taurocholate were added to the vesicles (1:1) in different concentrations from 20 to 250 mM. The samples were incubated at 37°C and measured at different time intervals for the formation of cholesterol crystals using a Behring nephelometer as described by De Bruijn et al. (9). Crystallization from more stable vesicles (CH/PL ratio = 0.9) upon incubation with 40 mM deoxycholate (DC) was compared to the crystallization upon incubation with 40 mM DC in the presence of 100 µg/ml CABG.

The crystallization curves were analyzed with a curve-fitting program (Fig. P, Biosoft, Cambridge, UK) using the equation for a logistic sigmoid curve. The maximal slope of the curve provided the crystal growth (µg/ml • $h^{-1}$) parameter and the time point at which the curve was

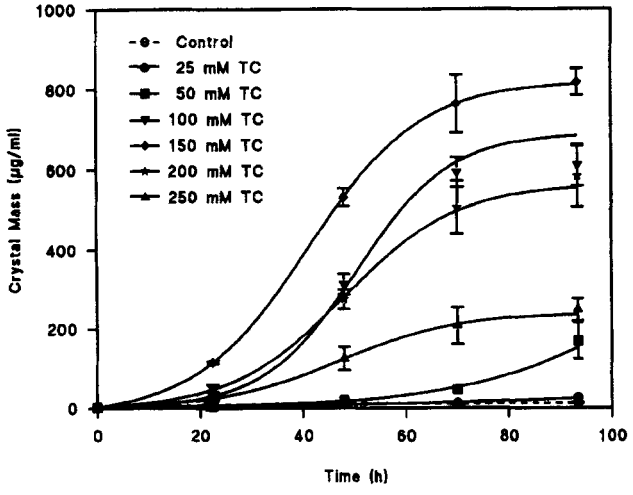


**Fig. 3.** Cholesterol crystallization in unstable vesicles (CH/PL ratio = 1.6) upon incubation with different concentrations of taurocholate (TC). Control were vesicles incubated with HEPES buffer only. Maximal crystallization was induced upon addition of 150 mM TC. Higher and lower concentrations of TC gave rise to less cholesterol crystallization. [CH] = 15.4 mM, [PL] = 9.6 mM. Results are expressed as the mean ± SD of four experiments.

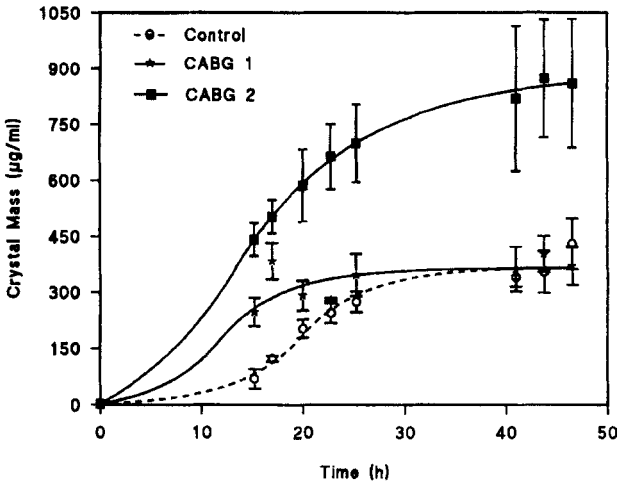


**Fig. 4.** Cholesterol crystallization from more stable vesicles (CH/PL ratio = 0.9) upon incubation with 40 mM DC (control) or upon incubation with 40 mM DC with the addition of two different CABGs (100 µg/ml). CABG 2 strongly stimulated cholesterol crystallization when compared to the control incubation. CABG 1 showed little effect on cholesterol crystallization. [CH] = 7.9 mM, [PL] = 8.7 mM. Results are expressed as the mean ± SD of four experiments.

significantly (95% confidence interval) elevated above the x-axis yielded the onset time (h).

### Crystal growth assay

Model biles with a cholesterol saturation index (CSI) of 1.61 were prepared according to Kibe et al. (10) with slight modifications. Briefly, Na-taurodeoxycholate and Na-taurocholate were dissolved in methanol–water 85:15 and mixed with a solution of egg yolk lecithin and cholesterol in chloroform. The mixture was dried under nitrogen for 2 h at 50°C and lyophilized overnight. The lyophilisate was reconstituted in an aqueous buffer (50 mM HEPES, 100 mM NaCl containing 0.04% azide, pH 7.4) and incubated at 56°C for 2 h to obtain an isotropic phase. Crystal growth induction by CABG was determined according to Busch et al. (4) as modified by De Bruijn et al. (9). In brief, 300 µl of a model bile solution was added to either 300 µl of a protein sample (final concentration 100 µg/ml) or 300 µl of the HEPES buffer as a control. Total lipid concentration was 7.1 g/dl, containing 48.4 mM TC, 48.4 mM TDC, 23.3 mM PL, and 12.6 mM CH. The samples were incubated at 37°C after filtration through a 0.22 µm filter. Daily measurement of crystal mass and curve analysis were done as described for the vesicular assay.

## RESULTS

**Figure 1** shows a micrograph of a vesicle preparation with a CH/PL ratio of 1.3 as examined with cryo-electron microscopy. The vesicle suspension contained mainly unilamellar vesicles with a sporadic multilamellar vesicle. To test the potential of bile salts to induce cholesterol crystallization from vesicles with a high CH/PL ratio, the vesicles were incubated with deoxycholate (DC) at concentrations from 0 to 100 mM (**Fig. 2**). A prolonged sonciation period, which gave a homogeneous unilamellar vesicle suspension, did not affect the crystallization curves significantly (results not shown). As shown in Fig. 2, the deoxycholate-induced crystallization was concentration-dependent with maximal crystallization upon incubation with 40 mM DC. The onset time of crystallization of the 40 mM DC incubation was 7.5 h, the crystal growth was 72.0 µg/ml • $h^{-1}$. Further increases in DC concentrations (60, 80, and 100 mM) resulted in a concentration-dependent decrease in the crystal growth parameter (47.3, 13.8, and 3.1 µg/ml • $h^{-1}$, respectively) and a concomitant decrease in total crystal mass. The effect on the onset time of crystallization was less marked, being 6.0, 8.5, and 29.9 h, respectively. Lower DC concentrations (20 mM) barely induced crystallization. Above 100 mM DC no crystallization could be observed using this detection technique (results not shown). No spontaneous cholesterol crystallization from the vesicles occurred without the addition of bile salt. With the taurine-conjugated bile salt, taurodeoxycholate, almost the same results were obtained as with deoxycholate (results not shown) which might be expected because their hydrophobicity differs only slightly (5). For economical reasons, DC was used in the rest of the experiments.

To investigate the effect of a difference in hydrophobicity of the bile salt, the same experiments were re-

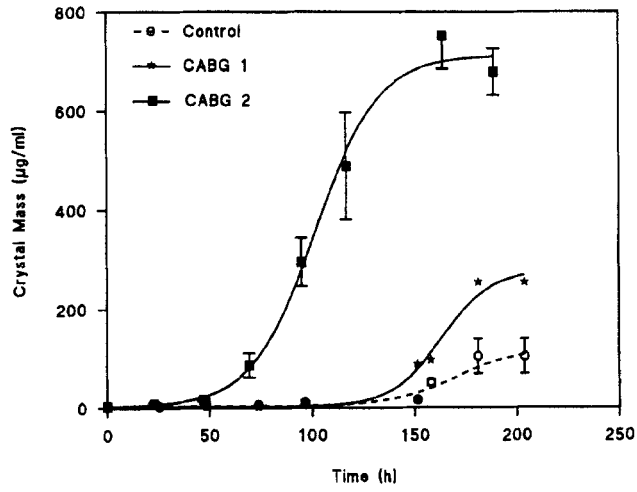


**Fig. 5.** Cholesterol crystal growth assay, using the same CABGs as in Fig. 3. Only CABG 2 induced cholesterol crystallization to a significant extent, which is in accordance with its effect in the vesicular assay. [CH] = 12.6 mM, [PL] = 23.2 mM, [TC] = 48.4 mM, [TDC] = 48.4 mM (CSI 1.61). Results are expressed as the mean ± SD of four experiments, except CABG 1 which represents the results of one typical experiment.

peated with the more hydrophilic bile salt taurocholate (TC). As shown in **Fig. 3,** compared to DC, a much higher TC concentration was required to induce precipitation of cholesterol crystals from the vesicles. As with deoxycholate, the taurocholate-induced cholesterol crystallization was concentration-dependent. Maximal crystallization occurred upon incubation with 150 mM TC. Lower and higher concentrations produced less cholesterol crystallization. Even with an optimal TC concentration (150 mM), however, the plateau phase of crystallization was reached at a later timepoint when compared to 40 mM DC. This was due to both a delay in onset time and a slower crystal growth rate (31.1 h and 12.8 $\mu g/ml \bullet h^{-1}$, respectively).

An important determinant of cholesterol crystallization is the cholesterol-to-phospholipid ratio of the vesicles (11–13). At a CH/PL ratio of 1, no crystallization could be induced by taurocholate (results not shown). Deoxycholate at a concentration of 40 mM, however, still induced crystallization, albeit at a lower total crystal mass when compared to the crystallization from vesicles with a higher CH/PL ratio. The vesicles with a CH/PL ratio of 1 were used to test the potency of added glycoproteins to stimulate cholesterol crystallization compared to vesicles in 40 mM DC only. The results for two different Con A-binding glycoprotein fractions are shown in **Fig. 4.** CABG 1 barely stimulated the deoxycholate-induced cholesterol precipitation from the vesicles. By contrast, CABG 2 gave a much higher total crystal mass as compared to the control incubation with deoxycholate only. The results match the results of the promoting activity of the same CABGs in the crystal growth assay (**Fig. 5**). The onset time for crystallization on addition of CABG 2 in the crystal growth assay was 36.0 h while in the vesicular assay the onset time was 2.5 h and the process was completed in 50 h. Measurement of the cholesterol and phospholipid contents of the CABGs, with extremely sensitive assays, revealed that CABG 1 contained 14.5 μM phospholipid and 23.3 μM cholesterol, whereas the more potent crystallization promotor CABG 2 contained 1.3 μM phospholipid and 16.6 μM cholesterol.

## DISCUSSION

During bile formation, vesicles are dissolved into mixed micelles composed of cholesterol, phospholipid, and bile salt. The bile salt micelles extract phospholipid in excess of cholesterol; as a result the residual vesicles are increasingly enriched in cholesterol (14, 15). Cholesterol-rich vesicles are thermodynamically unstable and it is shown that from these vesicles the crystallization of cholesterol could occur (12–14, 16, 17). The vesicular crystallization assay simulates this in vivo system. When vesicles with a CH/PL ratio of 1 or higher were incubated with concentrations of bile salts well above the critical micellar concentration, formation of cholesterol crystals ensued within hours. The onset, rate, and total amount of cholesterol crystallization from the vesicles was, however, dependent on both the concentration of the bile salt used and on its hydrophobicity. Three different situations could arise upon incubation of the vesicles with different bile salt concentrations. First, at low bile salt concentrations minimal micellization of vesicular lipid would occur, in which case the vesicle suspension would not destabilize sufficiently to initiate crystallization. Using vesicles with a CH/PL ratio of 1.6, this was observed when 20 mM DC, or 25 and 50 mM TC, was added (Figs. 2 and 3). Second, at very high bile salt concentrations the complete incorporation of vesicular lipid into mixed micelles would lead to too few remaining vesicles with a high CH/PL ratio to induce crystallization. This was observed upon incubations with 100 mM DC or 250 mM TC (Figs. 2 and 3). Third, at a moderate range of bile salt availability, only part of the cholesterol could be incorporated in micelles, with the remainder in increasingly metastable vesicles with higher CH/PL ratios. The presence of enough of these vesicles will lead to sufficient cholesterol nucleation. This occurred in incubations with 40 mM DC and 150 mM TC. This scenario likely simulates what occurs in supersaturated bile in vivo.

Besides the bile salt concentration, bile salt hydrophobicity was an important determinant for cholesterol precipitation from the vesicles. The onset time of crystallization upon incubation with TC (Fig. 3) was about four times longer than the onset time of incubations with the more hydrophobic DC (Fig. 2) (31.1 vs. 7.5 h, respectively). Furthermore, the crystal growth in TC incubations was much slower than in DC incubations (12.8 vs. 72.0 $\mu g/ml \bullet h^{-1}$, respectively). These effects of bile salt hydrophobicity on cholesterol crystallization are in accordance with the results of other studies (18–20). The effect of bile salt hydrophobicity is twofold: the mode of bile salt/membrane interaction is dependent on the number of α-hydroxy groups on the hydrophilic hemisphere of the bile salt molecule (21). Schubert and Schmidt (22) showed that bile salts with two α-oriented hydroxy groups (i.e., DC and chenodexycholate) have a higher initial binding strength to phospholipid vesicles than the more hydrophilic trihydroxy bile salt TC. Although cholesterol protects the membrane against a penetration by bile salts, the induction of membrane disturbances would always occur if the concentration of bile salts is above a certain threshold (15, 22). Thus, hydrophobic bile salts, binding more firmly to lipid molecules, induce more membrane disruption than hydrophilic bile salts. Second, Donovan, Jackson, and

Carey (23) showed that in bile salt mixtures hydrophobic bile salts are preferentially associated with lecithin in mixed micelles and vesicles. Therefore, more hydrophobic bile salts might solubilize phospholipids better than more hydrophilic bile salts which would favor the micellization of phospholipid. This, in turn, may produce vesicles with a higher CH/PL ratio and for that reason stimulate the nucleation of cholesterol.

Added CABG rapidly and reproducibly stimulated vesicular cholesterol crystallization in systems containing 40 mM DC and vesicles with a CH/PL ratio of 0.9 (Fig. 4). The results were consistent with the ability of the CABGs to promote cholesterol precipitation in the crystal growth assay (Fig. 5), but the onset times of crystallization were four times faster in the vesicular assay. In a recent study Yamashita et al. (24) claimed that delipidation of the CABG fraction decreased its pronucleating activity by 75%. Therefore, we examined the lipid content of the CABGs used in the vesicular assay. The more potent crystallization promotor, CABG 2, contained less cholesterol and phospholipid than the less active CABG 1. Moreover, the total CH and PL content of the CABGs (maximal 38 μM) is negligible as compared to the total CH and PL content in the assay system (about 16 mM). Possibly, the delipidation procedure in the study of Yamashita et al. (24) removed a lipid-associated protein or a protein–lipid complex. We reported the presence of such a particle in CABG in a previous study (25). To anticipate possible artifacts, we prefiltered the samples with a 0.22 μm filter to remove submicroscopical cholesterol crystals, should they be present.

The vesicular assay might give more insight to the mechanism by which glycoproteins stimulate (or inhibit) cholesterol crystallization. In earlier studies (26) we claimed that concanavalin A-binding glycoproteins stimulated cholesterol crystallization by inducing a transfer of cholesterol and phospholipid from the micellar to the vesicular phase. The higher rate of cholesterol transfer would then lead to an increase in the vesicular CH/PL ratio and a concomitant increase in crystallization (26). In the present study, however, the addition of glycoproteins to vesicles with an already high CH/PL ratio stimulated cholesterol crystallization considerably (Fig. 4). Therefore, the glycoproteins must at least partly exert their effect on crystallization via a mechanism other than cholesterol transfer. Possibly a direct interaction of the glycoproteins with the vesicles induces the formation of cholesterol-rich domains from which the nucleation of cholesterol might preferentially occur.

In summary, cholesterol crystallization from unstable vesicles with a CH/PL ratio above or equal to 1 was dependent on both the concentration and the hydrophobicity of the bile salt used. The bile salt-induced crystallization could be stimulated by addition of Con A-binding glycoproteins. The vesicular assay can therefore be used as an alternative for the crystal growth assay with the major advantage that crystallization begins within minutes after addition of bile salt. Hence, the vesicular assay seems to be a promising alternative for the crystal growth assay. By inducing cholesterol crystallization upon bile salt incubation, the vesicular assay might also be useful for the investigation of cholesterol crystallization inhibitors.

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