Factors affecting cholesterol monohydrate crystal nucleation time in model systems of supersaturated bile[1]

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Abstract We explored the influence of several compositional factors considered capable of influencing the nucleation time of model biles supersaturated in cholesterol. In addition to the classical techniques, e.g., electron microscopy and quasielastic light scattering, employed for size measurement and structural assessment, we employed a novel technique, i.e., video-enhanced microscopy, for particle evaluation in these polydisperse systems which often may simultaneously contain isolated small vesicles, their complex aggregates, and small cholesterol monohydrate crystals. The factors we studied included dilution, degree of cholesterol supersaturation, bile salt/lecithin molar ratio, and Ca^{2+} concentration. Dilution markedly raised the degree of cholesterol saturation, prolonged nucleation time for cholesterol monohydrate crystals, and favored formation of metastable small unilamellar vesicles. Increasing the degree of cholesterol supersaturation as an independent variable in more concentrated systems both shortened the nucleation time and favored spontaneous formation of a relatively small number of isolated vesicles. A decrease in bile salt/lecithin molar ratio within the physiologically relevant range was accompanied by a prolonged nucleation time and favored spontaneous vesicle formation. Large numbers of small unilamellar vesicles were observed even in concentrated model bile solutions (total lipids: 20 g/dl) when the bile salt/lecithin molar ratio was 1.9 or less. At physiological concentrations, Ca^{2+} promoted nucleation of cholesterol monohydrate crystals *only* in vesicle-containing solutions. Taken together, the following conclusions can be drawn. First, spontaneous vesicle formation in dilute systems prolongs solid cholesterol crystal nucleation. It can thus provide a supplementary *non-micellar* mode of cholesterol transport in micellar systems of supersaturated human bile. Second, dilution, degree of cholesterol supersaturation, and a decrease in bile salt/lecithin ratio prolong cholesterol crystal nucleation time and favor spontaneous vesicle formation. With increasing calcium concentrations, opposite effects are observed. Third, the presence of vesicles may help to account for the frequently observed and otherwise unexplained remarkable degree of metastable supersaturation and prolonged metastability (delayed nucleation time) for cholesterol in human bile. —**Kibe, A., M. A. Dudley, Z. Halpern, M. P. Lynn, A. C. Breuer, and R. T. Holzbach.** Factors affecting cholesterol monohydrate crystal nucleation time in model systems of supersaturated bile. *J. Lipid Res.* 1985. **26:** 1102–1111.

Supplementary key words nucleation • vesicle • liposome • bile • cholesterol • lecithin • cholesterol gallstone • calcium • video-enhanced microscopy

There is now general agreement that the pathogenesis of cholesterol gallstone disease is importantly related to the capacity of bile to maintain cholesterol in micellar solution and that the predominant driving force for cholesterol crystal nucleation and crystal growth in bile is the absolute degree of cholesterol supersaturation (2–4). Supersaturated bile does not, however, necessarily result in the formation of cholesterol crystals or gallstones; for example, bile supersaturated in cholesterol is found in more than half of all subjects who do not have gallstones (4, 5). Moreover, it is impossible to distinguish patients with cholesterol stones from controls merely by determining the absolute degree of cholesterol supersaturation. The nucleation time (the time required for earliest detection of cholesterol monohydrate crystals) of supersaturated normal bile is consistently prolonged when compared with bile from patients with cholesterol gallstones (6–8). Cholesterol monohydrate crystal nucleation time provides a sharper discrimination between patients with cholesterol gallstones and normals than the cholesterol saturation index (CSI) (6). Although a clear distinction between cholesterol crystal growth from nuclei versus true nucleation is often difficult to establish, recent observations support the view that a difference in the ability to form cholesterol crystals between supersaturated gallbladder bile resides at the nucleation step rather than that of crystal growth (9).

Abbreviations: HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; STC, sodium taurocholate; CSI, cholesterol saturation index; VEM, video-enhanced microscopy, video-enhanced contrast-differential interference microscopy.

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The only factors, other than the degree of cholesterol saturation, presently known to affect cholesterol nucleation are biliary proteins. Crude preparations of biliary proteins are capable of inhibiting cholesterol crystal nucleation in human gallbladder bile (10). Furthermore, apolipoproteins A-I and A-II present in bile are closely associated with the most potent nucleation-inhibiting crude protein fraction, and themselves prolong cholesterol crystal nucleation (11, 12). In contrast, evidence for the presence of nucleation-promoting factors in the gallbladder bile of animal models or of patients with cholesterol gallstones has been recently demonstrated (13, 14). Thus, cholesterol crystal nucleation and growth may represent a process reflecting a balance between nucleating and antinucleating factors in the milieu of gallbladder bile.

There is evidence, however, that other phenomena must be involved. For example, a prolonged nucleation time has been found in dilute supersaturated human hepatic bile (8). That vesicles containing cholesterol and lecithin may constitute a nonmicellar mode of cholesterol transport in human hepatic bile and in dilute supersaturated model (artificial) bile has been demonstrated independently by two groups using quasielastic light scattering (15, 16). More recently, the presence of vesicles has been shown not only in human hepatic bile but also in human gallbladder bile using video-enhanced microscopy (VEM) (17). Thus, it seems that the presence of vesicles may constitute yet another phenomenon relevant to the nucleation process. If vesicles are important to nucleation, other factors may in turn influence their properties, e.g., stability, thus affecting nucleation time. Factors capable of altering the properties of vesicles might, for example, include dilution, the bile salt/lecithin ratio, and the calcium ion concentration.

In the present work three things are done. First, the effects on nucleation time of dilution, bile salt/lecithin ratio, and calcium ion concentration are examined; second, the relation of these factors to vesicles is explored; third, the lipid composition of vesicles present in one system is measured. Our findings indicate that the presence of an appreciable number of vesicles accounts for the paradoxical coexistence, where observed, of high levels of supersaturation and prolonged nucleation times (increased metastability). Conversely, the addition of calcium leads to aggregation and precipitation of vesicles with a rapid nucleation time. Thus, vesicles seem to play a key role in the nucleation process.

EXPERIMENTAL PROCEDURES

Preparation of model bile solutions

Cholesterol was purchased from Eastman Kodak Co. (Rochester, NY) and stored under nitrogen at 4°C. Egg lecithin was obtained from Lipid Products (Surry, U.K.) and stored at −80°C. Sodium taurocholate (STC) (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) was twice recrystallized by the method of Pope (18). The procedures for model bile preparation were slight modifications of those previously described (5, 10). Briefly, stock model biles were prepared as follows. Aliquots of STC in methanol, lecithin in chloroform–methanol 2:1 (v/v), and cholesterol in methanol were added to a vial equipped with a Teflon-faced septum and cap to yield the desired amount of these three lipids (**Table 1**). After flushing with nitrogen, the mixture was shaken at 37°C for 2 hr and the organic solvent was evaporated under a stream of nitrogen until the mixture had condensed to a viscous paste. To achieve complete solvent removal, the mixture was then lyophilized. Lastly, the required volume of HEPES saline (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid–NaCl 10 mM:140 mM, pH 7.5) containing 0–20 mM calcium chloride was added to the preheated lyophilized lipids. When $CaCl_2$ was added to the hydration buffer, sufficient NaCl was removed so as to maintain the ionic strength of the solution. For several studies, dilution of the model biles in Table 1 was required and was performed by adding sufficient additional HEPES-saline to lyophilized lipids to reach the desired concentration (g/dl). The pH of each solution was then adjusted to 7.5 and the mixture was flushed with nitrogen. The resultant suspension (stock model bile or a dilution of the stock) was shaken at a constant speed of 100 rpm in a model G-24 Environmental

TABLE 1.

Reference Solution	Total Lipids	Moles % Cholesterol	Bile Salt/ Lecithin	Bile Salt	Phospholipid	Cholesterol	Cholesterol Saturation Index
	g/dl				*mM*		
A	20	7.2	5.7/1	285	50	26	1.2
B	20	8.5	4.0/1	258	65	30	1.2
C	20	10.0	3.0/1	235	79	35	1.2
D	20	10.8	2.3/1	213	93	37	1.2
E	20	11.2	1.9/1	197	103	38	1.2
F	8	5.0	2.3/1	88	38	7	0.7
G	8	9.6	2.3/1	86	37	13	1.2

Incubator Shaker (New Brunswick Scientific, New Brunswick, NJ) at 55°C until microscopically isotropic. For nucleation studies, the model biles were filtered through a pre-heated Swinney filter assembly and a 0.22-μ filter (Millipore Continental Water Systems, Bedford, MA), then flushed with nitrogen and incubated at 37°C without shaking. Initial (zero) time for a nucleation study was 30 min after cooling to 37°C. Nucleation studies were conducted as previously described (6). Lipid determinations were performed on each model bile (10, 19–23) and the cholesterol saturation indices for the solutions were calculated from tables provided by Carey (24).

Formation and characterization of spontaneously formed vesicles in model bile

Vesicles analyzed in these studies formed spontaneously in a supersaturated isotropic model bile by simply cooling from 55°C to 37°C for 6 hr. With time, the solution became visibly turbid and vesicle formation was monitored by absorbance at 300 nm. For the purpose of determining the cholesterol:phospholipid molar ratio of spontaneously formed vesicles as well as the relative vesicular/micellar distribution of cholesterol in a model bile, a procedure for isolation of the vesicles was employed. A supersaturated vesicle-containing model bile and an undersaturated vesicle-free model bile of identical total lipid and bile salt/lecithin molar were both centrifuged at 90,000 *g* for 18 hr at 37°C using a Beckman model L5-50 centrifuge (Beckman Instruments, Palo Alto, CA). The contents of the centrifuge tube were fractionated into 0.6-ml fractions and assayed for lipids (10, 19–23). Reproducibility of measurements was determined in replicate experiments.

Video-enhanced microscopy studies

A Zeiss Axiomat microscope in the inverted configuration equipped with Nomarski differential interference contrast optics was used. Model bile stock solutions (Table 1), or dilutions thereof, containing spontaneously formed vesicles were mounted between two No. 0 coverglasses (Gold Seal, Clay Adams Division, Becton, Dickinson and Co., Parsippany, NJ) and observed at 37°C using a 50-watt DC powered mercury arc and Zeiss heat reflection and green interference filters. All specimens were viewed using a high numerical aperture (NA) Achromatic-Aplanatic oil-immersed differential interference contrast condenser (NA 1.4) and 100× planapochromat (NA 1.3) POL oil-immersed objective. Images were recorded using a Hamamatsu C-1000 Chalnicon head video camera on a 3/4" Panasonic NV-9240-XD videotape unit using a previously described video-enhanced contrast method (25–27). The gain and offset features of the Hamamatsu Camera Control Unit were adjusted to optimize contrast and detection of small structures in the video-enhanced image. The illustrations presented in this paper were obtained by photographing still frames from a television monitor of images recorded on videotape following background mottle subtraction by digital image processing. Television raster lines were blurred by diffraction using a 50-lines ronchi grating (Rolyn Optics Co., Covina, CA) attached in front of the camera lens.

Electron microscopy studies

Transmission electron microscopy. Model solutions containing spontaneously formed vesicles were studied using formvar coated grids. Equal volumes of 1% phosphotungstic acid and the specimen were mixed on the grid. After eliminating excess liquid, the grid was allowed to dry and was examined with a JEOL 100S microscope.

Freeze fracture electron microscopy. Model bile solutions containing spontaneously formed vesicles were immediately plunge-frozen in Freon 22 at −150°C. Fractures were performed with a BALZERS BAF 301 freeze fracture instrument at −150°C in a vacuum (1×10^7 Torr) using platinum-carbon shadowing at 45°C. Replicas were cleaned with bleach (Clorox) solution and mounted on copper grids with formvar support film and observed with the above JEOL 100S instrument.

Quasielastic light scattering

Quasielastic light scattering measurements of the autocorrelation function of light scattered from a supersaturated model bile (Table 1, G) and its dilutions were performed at 37°C by use of a digital photon correlation spectrometer incorporating a Coherent Radiation Model 52 Ar^+ laser (28, 29). The degree of polydispersity was estimated from both the measurement uncertainty and the second moment using the method of cumulants (30). From these measurements, determinations of the mean translational diffusion coefficient (D) and the mean hydrodynamic radius (R) were obtained. R was measured by using the Stokes-Einstein relation, $R = kT/6\eta D$ where k is Boltzmann's constant, T is the absolute temperature and η is the viscosity of the solvent. Because imaging by VEM entails use of differential interference contrast (Nomarski optics) which overestimates actual size (31), particle size determinations were derived from a combination of electron microscope techniques and from quasielastic light scattering.

RESULTS

Dilution and cholesterol saturation index (CSI)

Dilution of supersaturated model bile B (Table 1) containing 20 g/dl total lipids to a final concentration of 15, 12, 8, 4, or 2 g/dl, despite raising the CSI from 1.2 to 2.1, produced microscopically isotropic solutions after shaking at 55°C. The sole exception was the case of the 2 g/dl solu-

tion. The latter remained turbid even when heated to 95°C for 1 hr. Unlike the isotropic model biles, this solution was not filtered prior to starting the nucleation study.

Nucleation time studies revealed a direct correlation in dilute model bile with the degree of dilution. Nucleation times were prolonged from 12 hr at 20 g/dl to 15, 200, or 400 hr with increasing dilutions of the same system (**Fig. 1**). Furthermore, dilution to a total lipid concentration of 2 or 4 g/dl, i.e., solutions mimicking native human hepatic bile, abolished the relationship between nucleation time and CSI which was previously reported for 20 g/dl systems (**Fig. 2**) (10). At these total lipid concentrations, cholesterol monohydrate crystal nucleation was consistently delayed and was independent of CSI. In contrast, as seen in Fig. 2, when the total lipid concentration was greater than 8 g/dl, the logarithm of nucleation time correlated inversely with the CSI.

Although initial examinations (i.e., zero-time) using polarizing microscopy revealed no cholesterol monohydrate crystals, non-birefringent spontaneously formed vesicles exhibiting rapid, random (Brownian) movement were seen by video-enhanced microscopy. Regardless of lipid concentration, vesicles were initially present in *all* model solutions studied. In the more concentrated systems (e.g., 15 or 20 g/dl) only isolated, widely separated

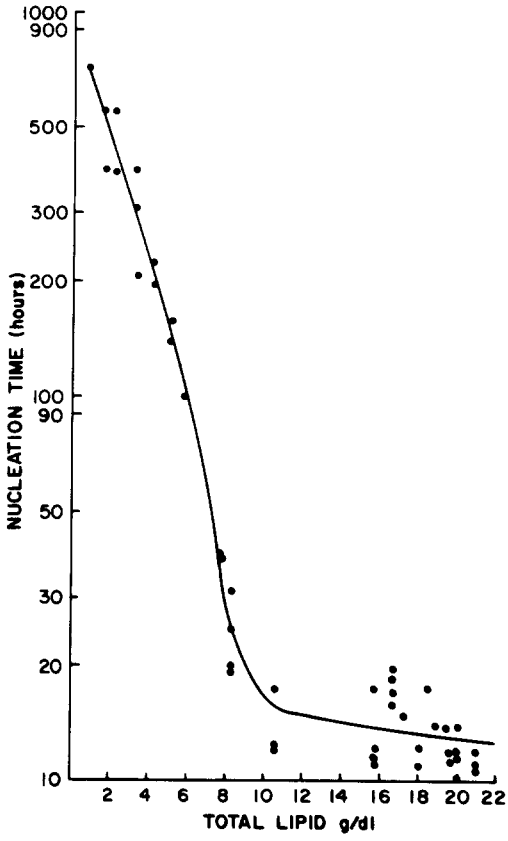

Fig. 1. Effect of dilution on nucleation time.

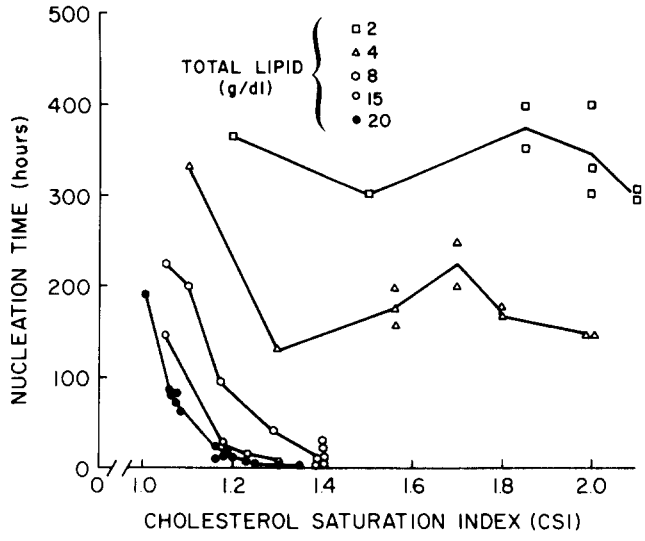

Fig. 2. Effect of total lipid concentration and CSI on nucleation time (NT).

vesicles were observed (**Fig. 3a**). Solutions containing 10 to 12 g/dl contained greatly increased numbers of vesicles. At dilutions representing 8 g/dl or less, vesicles were present in such abundance that it became impossible to perceive numerical differences (Figs. 3b and 3c).

Electron microscopy by both techniques revealed that the vesicles in model bile G (Table 1) were unilamellar with a diameter of approximately 100 nm. This measurement was also confirmed by quasielastic light scattering [i.e., 97.3 ± 12.3 nm (SEM)] using a supersaturated model bile (Table 1, G) with a linear dilution series extrapolation. Relative monodispersity of the vesicle size range was reflected in the comparatively small magnitude of the second moment or cumulant (K_2) (30).

At the time that cholesterol monohydrate crystals were first detected in each model bile (i.e., the nucleation time), the solutions were again examined by video-enhanced and electron microscopy. Their appearance was strikingly different (**Figs. 4a and 4b**). Many of the unilamellar vesicles were transformed into larger, apparently fused vesicles (2–5 times the diameter of unilamellar vesicles) or to large multilamellar and multivesicular forms. Other unilamellar vesicles had aggregated and contained cholesterol monohydrate crystals. The latter were observed floating free in the medium as well as protruding from aggregated vesicles.

Bile salt/lecithin molar ratio

The effect on nucleation time and spontaneous vesicle formation of varying the bile salt to lecithin molar ratio within the physiologic range of human gallbladder bile was examined using model biles having a constant total lipid concentration (20 g/dl) and a CSI of 1.2 (Table 1, A, B, C, D, E). As in the dilution studies, at initial (i.e., zero-

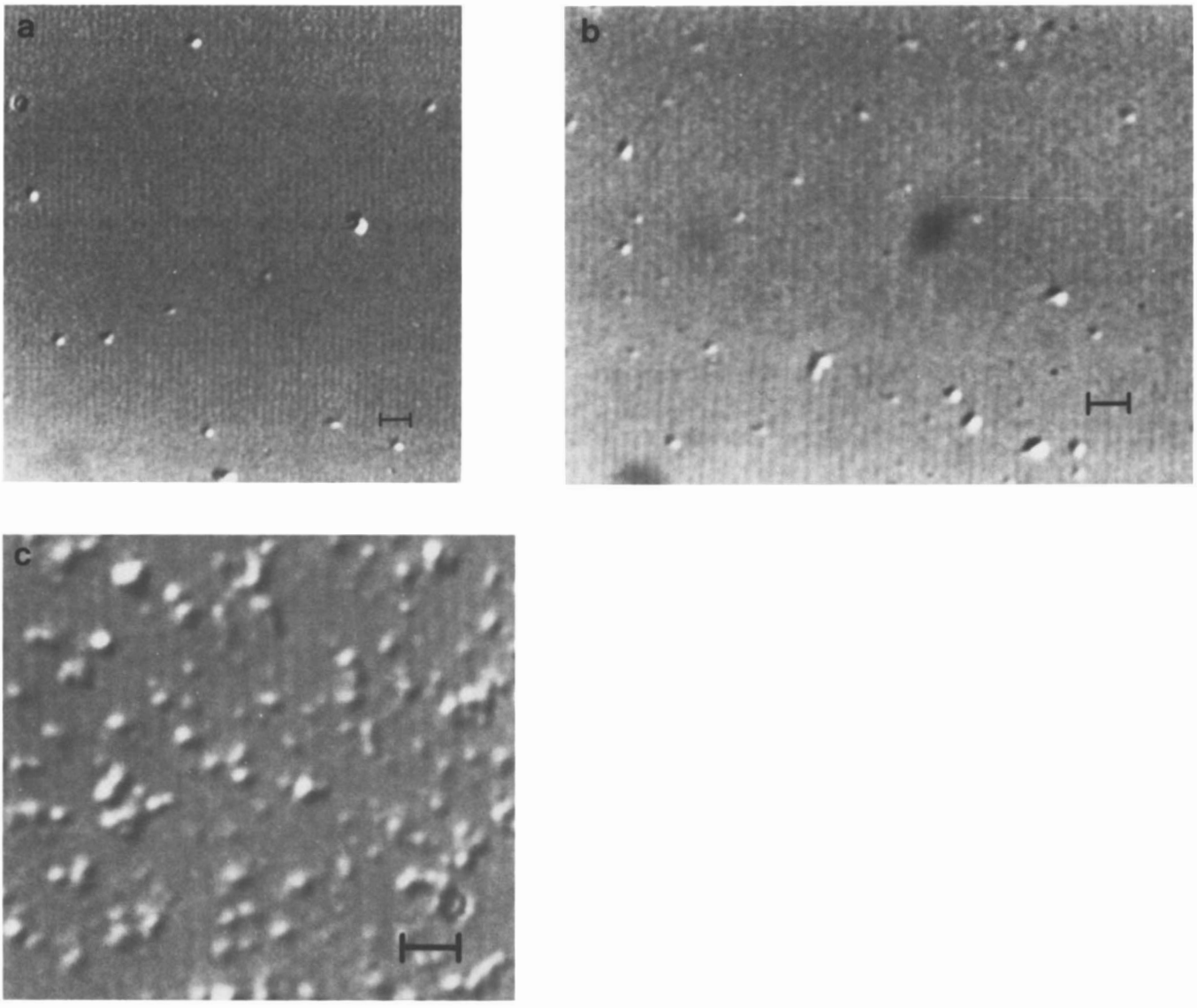

Fig. 3. Effect of dilution on spontaneously formed vesicles observed by VEM at zero-time a) 20 g/dl; b) 15 g/dl; c) 8 g/dl (bar = 2500 nm).

time) examination of the model biles, no cholesterol monohydrate crystals were observed. Although all solutions used in this study appeared initially to be isotropic by polarizing light microscopy, all demonstrated unilamellar vesicles by video-enhanced microscopy. Further, the lower the bile salt/lecithin ratio, the greater the apparent number of unilamellar vesicles in the solution.

As the bile salt/lecithin molar ratio of these solutions fell from 5.7:1 to 2.3:1, their nucleation times increased slowly (**Fig. 5**). Not until the ratio fell to 1.9:1, however, was the cholesterol monohydrate nucleation time significantly prolonged ($P < 0.001$). This lecithin-rich system slowly became turbid. By microscopy, under these conditions, both spontaneous vesicle and lecithin-cholesterol liquid crystal formation were greatly enhanced.

Calcium concentration

The addition of Ca^{2+} to model biles or their dilutions affected nucleation time only when the model biles were of sufficient dilution (at zero-time) (e.g., total lipid 8, 4, and 2 g/dl) that they contained numerous isolated small vesicles. Under these conditions using model bile B (Table 1) at dilutions representing total lipid of 8, 4, and 2 g/dl, Ca^{2+} consistently promoted rapid nucleation of cholesterol monohydrate crystals (**Fig. 6**). Electron microscopy and video-enhanced microscopy examination at the time of nucleation revealed that many isolated small vesicles had fused, aggregated, or formed multilamellar vesicles. In contrast, the addition of Ca^{2+} to either undiluted model bile B (Table 1) or at only minimal dilution of model bile B (e.g., total lipid concentrations: 15 g/dl), solutions

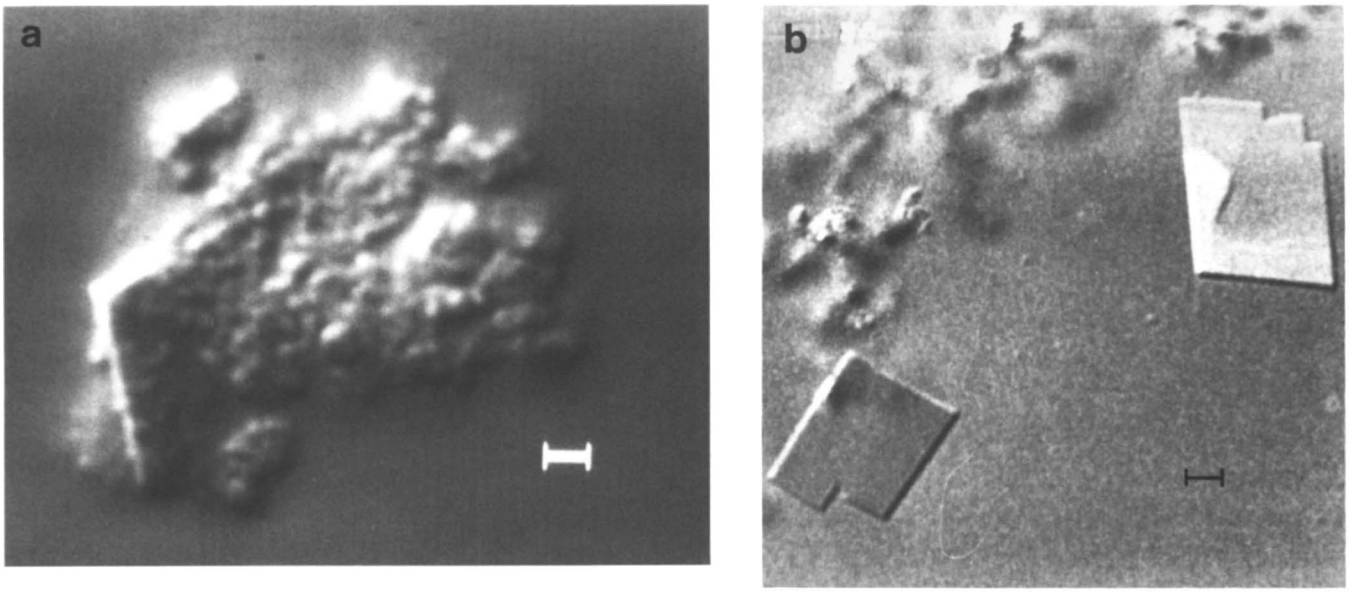

Fig. 4. Model bile seen by VEM at the time of cholesterol monohydrate nucleation. a) Fused vesicles with a protruding cholesterol crystal; b) aggregated vesicles and free-floating cholesterol crystals (bar = 2500 nm).

which contained only a few small vesicles, failed to show an effect on nucleation time.

Characterization of spontaneously formed vesicles

Vesicles were isolated by ultracentrifugation from a supersaturated model bile (Table 1, G). For control purposes, the lipid profile of an undersaturated model bile was also determined in order to illustrate the distribution of lipids in a solution without vesicles (Table 1, F). In the supersaturated model bile, fractions 5–7 were visually turbid and contained an abundance of unilamellar vesicles when examined by video-enhanced microscopy (**Fig. 7**). The comparative lipid profiles obtained from fractionation of the two model biles showed that, in the same turbid fractions indicated above, the cholesterol and lecithin concentrations were raised only in the supersaturated model bile (**Fig. 8**). The lipid concentration of the micelles within the system was estimated by drawing a baseline between the lipid values of fractions 5 and 8. The area above baseline was considered representative of vesicles and the area below baseline was considered representative of micelles. An approximate composition for the vesicle fraction based upon this assumption could then be determined using the lipid concentration of the fraction containing the largest number of vesicles (fraction 7) and subtracting the lipid concentrations for lecithin and cholesterol below baseline from the respective total lipid concentrations for each. The resulting cholesterol:lecithin molar ratio in the vesicular fraction was 1.85 ± 0.05 (n = 7) whereas the ratio of cholesterol to phospholipid was less than 1 in the micellar phase. Furthermore, using area triangulation, the size of the vesicle fraction under these conditions was estimated. Approximately 14% of the total cholesterol in model bile G was present in vesicles; the remainder was incorporated in micelles.

DISCUSSION

In the present study, we have explored various factors that can affect nucleation time for cholesterol monohydrate crystals and spontaneous vesicle formation in model solutions of supersaturated bile. The effect of *simple dilution* is to promote spontaneous vesicle formation in metastably supersaturated model bile and to prolong nucleation time. This finding alone can account for the recently

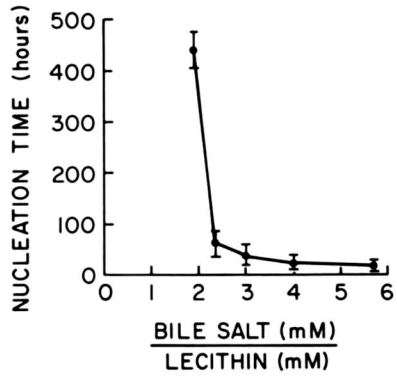

Fig. 5. Effect of bile salt (STC)/lecithin molar ratio on nucleation time. Each point represents the mean ± SD of four observations.

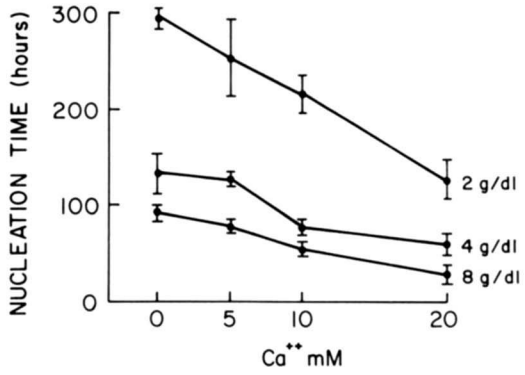

Fig. 6. Effect of Ca^{2+} on nucleation time. Each point represents the mean ± SD of three observations.

reported observations that human hepatic bile, which is invariably dilute, has a relatively longer nucleation time (enhanced metastability). This prolonged nucleation time is observed despite the presence of more marked supersaturation in cholesterol for the dilute system when compared to that of gallbladder bile having comparable proportionate lipid composition (8). The rare occurrence of de novo cholesterol gallstone formation in the absence of a gallbladder is also noteworthy given the marked degree of supersaturation of hepatic bile. The approximately threefold concentration of hepatic bile that occurs in the gallbladder considerably increases the solubilizing capacity for cholesterol. Despite the fact that this effect reduces the net degree of cholesterol supersaturation (CSI) from 2.5 to ~1.3, many healthy subjects still have gallbladder bile that is supersaturated in cholesterol (3, 5, 8). Thus, the concentrating effect on hepatic bile by the human gallbladder may be an important contributory factor for promoting cholesterol gallstone formation whenever the more concentrated gallbladder bile remains significantly supersaturated. Although dilution increases the degree of supersaturation in a given model (or native) bile, an increased degree of supersaturation (raised CSI) as an independent variable in more concentrated systems was found both to shorten nucleation time and promote spontaneous formation of vesicles. Similar, although not entirely comparable, observations were recently noted by Mazer and Carey (16). The clear relationship between shortened nucleation times and vesicle formation in the more concentrated systems is absent when there is significant dilution.

Within the physiological range of bile salt/lecithin molar ratios found in supersaturated human bile, a bile salt/lecithin ratio reduced sufficiently to produce a lecithin-rich system not only enhances the cholesterol solubilizing capacity of the bile salt-mixed lipid micelles, but also favors spontaneous vesicle formation resulting in a prolonged nucleation time (3). This observation suggests that, were it possible, some form of pharmacologic manipulation capable of enhancing phospholipid secretion into bile could have potential usefulness in prevention of cholesterol gallstone formation.

Divalent cations, in particular Ca^{2+}, have been known to have a strong interaction with negatively charged phospholipid vesicles, resulting in increased permeability as well as aggregation and fusion (32–37). It was, therefore, reasonable to examine the effect of Ca^{2+} on vesicle formation and the nucleation process in supersaturated model bile. As expected, Ca^{2+} addition to vesicle-containing model biles had a strong effect on vesicle structure and on the cholesterol nucleation process. With the addition of Ca^{2+}, vesicles became larger in size and smaller in number probably by fusion, aggregation, or clustering of small unilamellar vesicles concomitant with the promotion of cholesterol monohydrate nucleation. This observation agrees with a previous report that cholesterol monohydrate growth rate is accelerated by the presence of Ca^{2+} (38). These data suggest that Ca^{2+} might have a potential role in cholesterol gallstone pathogenesis because Ca^{2+} in bile is the cation second only to sodium in relative concentration (6) and because insoluble calcium salts are

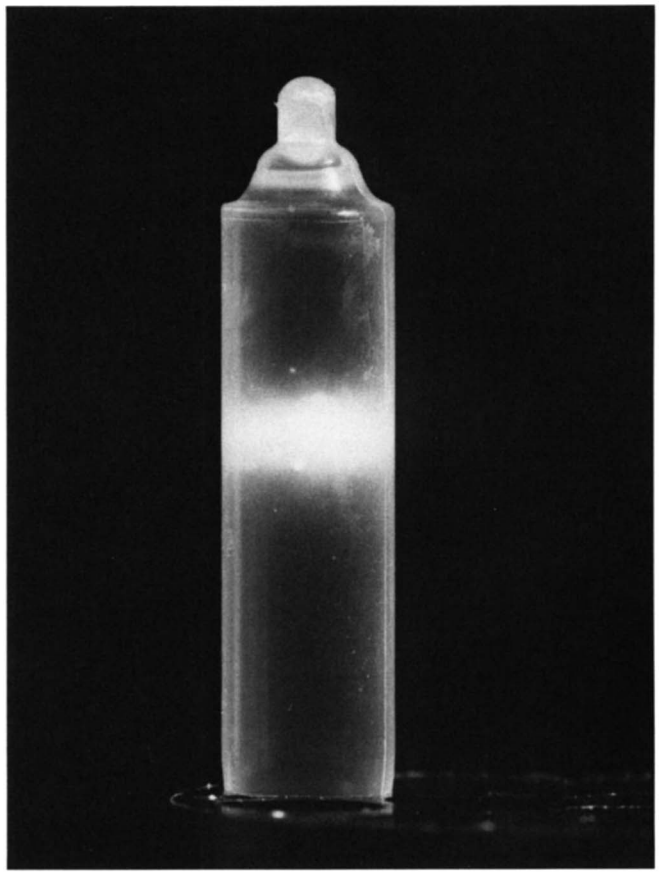

Fig. 7. Ultracentrifugation of supersaturated model bile containing spontaneously formed vesicles results in separation of vesicles into an opaque bond.

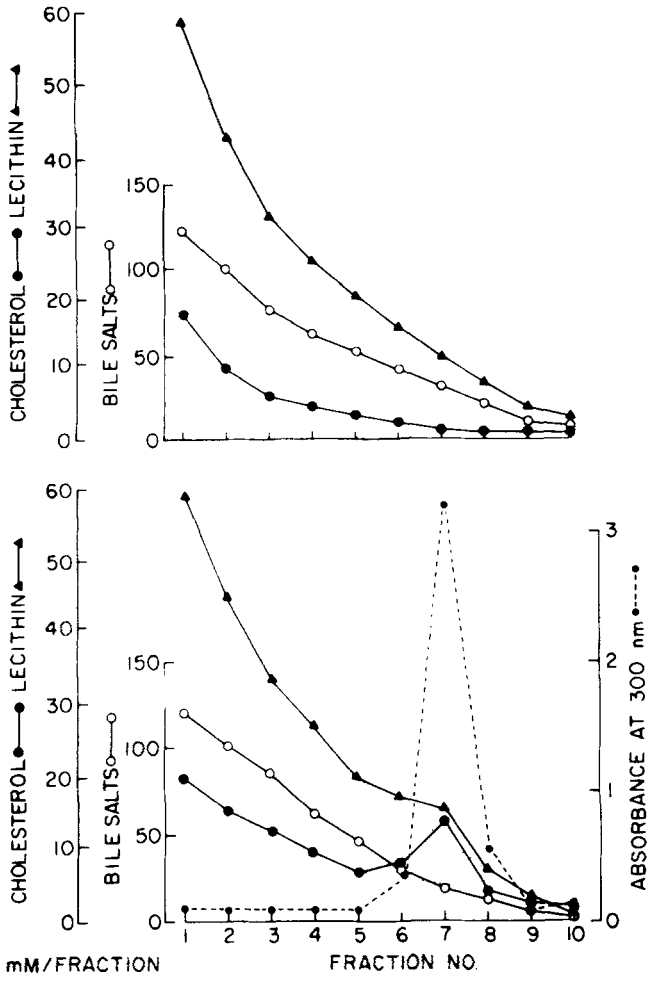

Fig. 8. Lipid profiles after ultracentrifugation of undersaturated model bile (Table 1, F) containing no vesicles (upper graph) and supersaturated model bile (Table 1, G) containing vesicles (lower graph). In the supersaturated model bile, fractions 6–8 contained the spontaneously formed vesicles as determined by VEM and absorbance at 300 nm. The dotted lines connecting the baseline values for lecithin and cholesterol from fractions 5 through 8 divide the vesicular and micellar phases.

known to be one of the main constituents of cholesterol gallstones (39). The binding of Ca^{2+} to micelles of conjugated bile salts has been studied by two groups (40, 41) using a Ca^{2+}-specific electrode. Both groups found that not only free anionic monomers of bile salts but also polyionic micelles had a strong interaction with Ca^{2+}, thus reducing the effective "free" concentration of Ca^{2+} in bile. This finding may explain why Ca^{2+} had little or no effect on cholesterol monohydrate crystal nucleation time in the more concentrated model biles of the present study.

The presence of vesicles similar to those described in the present work has recently been shown both in supersaturated human hepatic bile by Sömjen and Gilat (15) and in a systematic study of cholesterol precipitation phenomena in supersaturated model bile solutions of varied composition by Mazer and Carey (16). In both studies, particle size measurements were obtained primarily by quasielastic light scattering. Under conditions wherein comparability exists, the particle sizes reported in the present study are essentially in agreement with the data of these previous studies. The focus of the present work was to investigate compositional factors that could influence nucleation time of cholesterol monohydrate crystals in supersaturated systems at 37°C. The nucleation time measurements of the present work represent a semiquantitative approach to the long-term assessment of metastability in these systems. This approach differs from the greater attention to quantitation of shorter time or "labile" precipitation kinetics in the Mazer-Carey study. They assessed rapid onset of microprecipitate formation by monitoring the effects of fast temperature change through the ranges typically of 60°→10°C (16). In instances where the solution systems of the two studies were nearly comparable, the focus and design of the experiments differed significantly. Thus, our findings regarding nucleation (or degree of metastability) cannot be directly compared but are complementary to the earlier study (16). In addition, in the present work, we have introduced a novel method, i.e., video-enhanced microscopy, to the study of formation of microprecipitates such as isolated small vesicles, their complex aggregated forms, and small cholesterol monohydrate crystals. We have shown this to be a convenient tool for the rapid scanning of solutions containing a wide variety of precipitate forms simultaneously. For this reason, the method appears to be of particular utility in observing nucleation phenomena in these supersaturated colloidal systems, even though it does not replace more precise classical methods. As an example, we have recently reported observations on vesicles and other microprecipitates in a more concentrated and compositionally more complex solution such as human gallbladder bile supersaturated in cholesterol (17).

The cholesterol/lecithin molar ratio of vesicles in this study was approximately 2.0. This result is in agreement with data previously provided by Mazer and Carey, despite their employment of a somewhat different estimation technique (16). Several previous reports on the cholesterol solubilizing capacity of lecithin vesicles have demonstrated that the maximal molar ratio of cholesterol to lecithin is 2:1 in vesicles or bilayer membranes (42–46). In a recent study, Collins and Phillips (46) have shown that vesicles having this maximal level of cholesterol content are actually metastable and that a molar ratio of unity reflects the true equilibrium value. These data indicate that vesicles have a greater capacity for transport or storage of cholesterol when compared with that of biliary micelles. This could account for both the inexplicably high cholesterol level (mean CSI = 2.6) and the remarkably enhanced metastability (prolonged nucleation time) in dilute hepatic bile and model bile-containing vesicles. In addition, it is conceivable that the presence of cholesterol-rich lecithin vesicles might play an important role in

explaining, at least in part, the degree and duration of metastability in the supersaturated gallbladder bile frequently observed in healthy normal humans.

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