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IMAGING AND MONITORING CHOLESTEROL CRYSTALLIZATION IN BILE

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Abstract

Characterizing the microstructural evolution of lipid aggregates and precipitates in lithogenic bile is pivotal for understanding the process of cholesterol crystallization, leading to cholesterol gallstone formation. We have studied cholesterol precipitation in bile models and in human bile samples by the combined use of light- and cryotransmission electron microscopy with density gradient separation of lipid aggregates and other physical chemical techniques. Supersaturated unilamellar vesicles and spherical micelles precede the formation of filamentous cholesterol crystals in a dilute bile model. The filaments are replaced by intermediate crystal structures, before the formation of thermodynamically stable plate-like cholesterol monohydrate crystals. In concentrated synthetic bile models and in native bile, cholesterol crystallization is preceded by and partly concurrent with the appearance of supersaturated oligo- and multilamellar vesicles of varying sizes and numbers of bilayers. The vesicular structures become desaturated while intermediate type crystals, and cholesterol monohydrate plates form. The available data indicate that biliary cholesterol crystallization is a multi-phase process, involving several intermediate lipid and microcrystalline structures.

Key Words: Cholesterol gallstones, nucleation, crystallization, bile, cryo-transmission electron microscopy, vesicles, micelles.

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Introduction

The vast majority (> 80%) of gallstones in the Western world are cholesterol gallstones [62]. These are composed of cholesterol crystals, held together by an organic matrix containing mainly mucin glycoproteins [63, 78]. The cholesterol content of the stones ranges from 50% to nearly 100% (by weight) [14]. Nucleation and crystallization of cholesterol within the gallbladder is considered to be the critical step in the formation of cholesterol gallstones [48]. However, despite some significant progress in recent years, our understanding of biliary cholesterol crystallization is still incomplete [28, 59]. Compared to crystallization occurring in a monocomponent system, such as cholesterol crystallizing from a melt [47], the crystallization process from a polycomponent system, such as bile, is much more complex. Hence, the pathway in supersaturated bile, leading from cholesterol being minimally soluble as monomers [24], and mainly solubilized within micelles, vesicles, and maybe some other aggregates [17, 54, 66, 71, 73], to the formation of cholesterol monohydrate crystals is still in many ways a black box [3, 64, 65] (Fig. 1).

Our knowledge of the process of biliary cholesterol crystallization is primarily derived from studies in model bile systems and in native bile samples incubated *ex vivo*

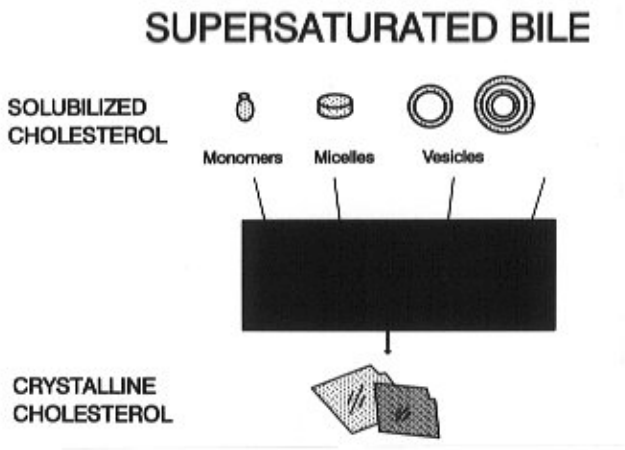


Figure 1. Schematic presentation of cholesterol solubilization and crystallization in bile. The crystallization process is still a "black box."

[28]. The investigation of biliary cholesterol crystallization is, however, particularly complex due to limitations derived from the special nature of bile, as well as the stringent limitations involved in the handling and preparation of bile samples for examination [77]. For example, the sizes of bile salt micelles, which are the smallest lipid aggregates that solubilize cholesterol in bile are far below the limits of resolution of light microscopy, and close to that of electron microscopy. Furthermore, conventional transmission electron microscopic techniques are prone to possible artifactual reorganization of the lipid structures embedded in the aqueous biliary environment [27, 79]. Thus, the visualization of micelles is still rudimentary [51, 58, 82], and their exact shape, and structure are still controversial [25, 53, 58]. Multilamellar vesicles have been observed in specimens prepared by staining and drying, but this procedure is known to produce artifacts in aqueous systems [39, 79]. Moreover, the presence of biliary pigments, mucin and some other proteins in gallbladder bile, and the relatively high concentrations and variability of lipid aggregates pose significant limitations to the use of many indirect techniques, such as spectroscopy, quasielastic light scattering (QLS), or nuclear magnetic resonance (NMR) [12, 15, 19, 27]. On the other hand, some of the techniques (e.g., QLS and photon correlation spectroscopy) have a preferential sensitivity for larger particles, whereas others are either insensitive to (e.g., NMR) or exclude (e.g., electron microscopy) them in the process of sample preparation [15, 19, 36]. Therefore, an integrated approach of combining a variety of physical-chemical techniques and appropriate model systems has to be employed in order to gain insight into the process of cholesterol crystallization in bile.

During the past few years, we have adopted such an integrated approach, and the purpose of the present paper is to review the data obtained from these studies.

Model bile systems

Bile models simulating native bile, based on the three major biliary components (cholesterol, lecithin and bile salts) in an aqueous solution, have been used extensively by several investigators [8]. Early work done by Small and Carey introduced the relevance of phase equilibria considerations and established the importance of micelles as cholesterol solubilizers and cholesterol supersaturation as a driving force for crystallization [8, 9, 54]. The existence of liquid crystals and additional lipid aggregates was, however, suggested by several authors [29, 30, 32, 60, 74]. Subsequently, it was shown by both indirect and direct methods that supersaturated bile contains lipid vesicles [49, 52, 61, 70, 71, 72], which were later described as being the cholesterol "carriers" responsible for cholesterol nucleation and crystallization [1, 20, 21, 23, 38, 64, 66]. More recently, the existence of intermediate crystal forms during biliary cholesterol crystallization has been demonstrated by several investigators [15, 34, 75, 83, 84]. The basic physical-chemical findings have been similar, irrespective of the methods used for the preparation of the supersaturated models, i.e., whether by cooling an initially isotropic lipid solution [38], mixing supersaturated vesicles with bile salt micelles [15, 50], or diluting a concentrated mixed micellar solution [42]. We have primarily used the dilution method (Fig. 2), in which an initially isotropic concentrated micellar solution is rapidly diluted to yield the final, pre-determined lipid composition. Dilution causes bile salts to be dissolved in the expanding aqueous medium, thereby inducing supersaturation of the cholesterol-solubilizing lipid aggregates (micelles and vesicles) at a clearly defined time point. This initiates a process of nucleation and growth of cholesterol crystals, which can then be studied by time-lapse analysis.

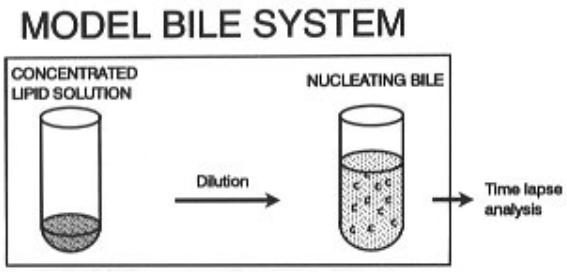
Experiments in dilute model bile

In a simple, quite dilute model system composed of cholesterol, egg yolk lecithin and taurocholate, cholesterol crystallization can to a large extent be followed by light microscopy. In particular, employing a model with low total lipid concentration (1.2 g/dl) but high proportion of bile salts (97.5 moles%), and sufficient cholesterol supersaturation (saturation index 208% [9]) to guarantee rapid cholesterol crystallization, enables us to investigate the crystallization process by a variety of physical-chemical modalities [42] (Fig. 2, model A).

When observed by phase-contrast light microscopy, the crystallization process in this model starts by the formation of elongated filamentous cholesterol crystals. The filaments are gradually replaced by intermediate spiral, helical, and tube-like microstructures, which undergo a morphological transformation to become "classic" plate-like cholesterol monohydrate crystals [10, 42, 43].

By breaking down the whole process to sequential stages and employing a series of physical-chemical techniques, some significant additional information about the microstructures involved in this process can be attained. For example, by QLS, the appearance of vesicles in an initially isotropic micellar solution can be demonstrated rapidly after dilution, by an abrupt jump of the mean hydrodynamic radius from about 2 to 20 nm [42, 43].

Furthermore, the process can be visualized directly by two complementary, non-perturbing microscopic tools [36]. The first one, video-enhanced light microscopy (VELM) operated with Nomarski optics, provides direct images of structures in a range from 100 nm to a millimeter without sample processing. Bile specimens can be observed in sealed micro-cavity slides, allowing visualization of the whole bulk medium. The second method, cryo-transmission electron microscopy (cryo-TEM), provides direct images of microstructures ranging from 4 to 300 nm, and has been used in the study of many surfactant systems, without introducing staining, drying or freezing artifacts [4, 11, 69,



Lipid concentration (mM) after dilution

	model A	model B
Cholesterol	0.4	18
Lechitin	0.2	36
Taurocholate	21.2	120

Figure 2. The model system used in investigating biliary cholesterol crystallization. Dilution of an isotropic, concentrated micellar lipid solution (cholesterol, lecithin and taurocholate in 0.15 M NaCl, pH 7), induces cholesterol supersaturation and initiates the nucleation process, which is studied by time-lapse analysis of bile specimens.

80, 82]. In this technique, samples are cooled extremely rapidly by plunging them into liquid ethane at its freezing point, thereby causing vitrification of the specimens without crystallization or artifact formation. The samples are then observed, without staining, in the electron microscope at -170°C. Thus, the combined, simultaneous use of these two partly overlapping methods allows direct imaging of microstructures within the aqueous environment of bile, in a size range from 4 nm to a millimeter. In the dilute model described above, at the earliest stage of the process (1-4 hours after dilution) before any filaments or other microstructures can be seen by light microscopy, cryo-TEM reveals spheroidal micelles, approximately 6 nm in diameter, co-existing with unilamellar vesicles, with a diameter ranging from 50 to 250 nm [36]. When crystallization becomes apparent by light microscopy, cryo-TEM can detect elongated crystallites, compatible with the filaments seen by light microscopy.

Gel filtration chromatography provides quantitative data on the processes leading to cholesterol crystallization. This technique reveals that the vesicular fraction at the time of estimated nucleation (between 15 minutes and 2 hours from dilution) contains less than 0.1% of the cholesterol in the system [42]. At this time, the cholesterol to phospholipid (c/p) ratio of both the vesicles and the micelles is higher than 1. Thus, both fractions are supersaturated with cholesterol.

In the dilute system, the earliest crystallites are filamentous, between 100-500 nm wide, and several microns long, as disclosed by negative staining and scanning EM, as well as cryo-TEM of samples vitrified during the first few hours of nucleation and crystallization [36, 42]. Cryo-TEM has also suggested a helical internal pattern [45].

That the filamentous structures are indeed crystalline, has been confirmed by X-ray diffraction [42]. Conventional powder diffraction of filaments harvested from the crystallizing model bile display the characteristic diffraction pattern of cholesterol monohydrate. However, rapid synchrotron X-ray diffraction studies of the earliest separable filaments have revealed an additional wide-angle diffraction peak at 4.9 Å, suggesting that the filaments contain some remnants of anhydrous cholesterol, although an unidentified polymorph of cholesterol monohydrate can not be excluded. In addition, the filaments have been shown to be covered by a layer of lecithin molecules, possibly contributing to the growth pattern of these crystals [10, 42, 44].

Density gradient ultracentrifugation separates the filaments from the other crystals, disclosing a mass density of 1.029 g/ml, which is also consistent with anhydrous cholesterol [67]. Based on this feature, we have developed a rapid sucrose density gradient ultracentrifugation method, by which the time-dependent process of cholesterol crystallization can be sequentially monitored [41]. By this method, the density gradient profiles of cholesterol precipitates are recorded at various time points throughout the crystallization process [46]. Initially (at 18 hours), the precipitable cholesterol is confined to low-density crystals (1.029 g/ml), shown to be filaments by microscopy. These are then gradually replaced by high-density (1.045 g/ml) plate-like crystals, compatible with cholesterol monohydrate [13]. When a steady state is reached at the end of the crystallization process, close to apparent thermodynamic equilibrium (> 15 days in this system), a clear phase separation has occurred, revealing a precipitate of classic plate-like cholesterol monohydrate crystals within a clear micellar solution.

Experiments in concentrated model bile

To acquire additional insight into biliary cholesterol crystallization leading to gallstone formation, we have also studied a concentrated, physiologically more relevant model by the above mentioned techniques. The model, with a total lipid concentration of 10 g/dl (after dilution), has a cholesterol, lecithin and taurocholate concentration of 18, 37, and 120 mM, respectively (Fig. 2, model B), based on the mean composition of gallbladder biles from ten cholesterol gallstone patients [46].

This model produces immediately upon dilution a suspension, with a “milky” appearance to the naked eye,

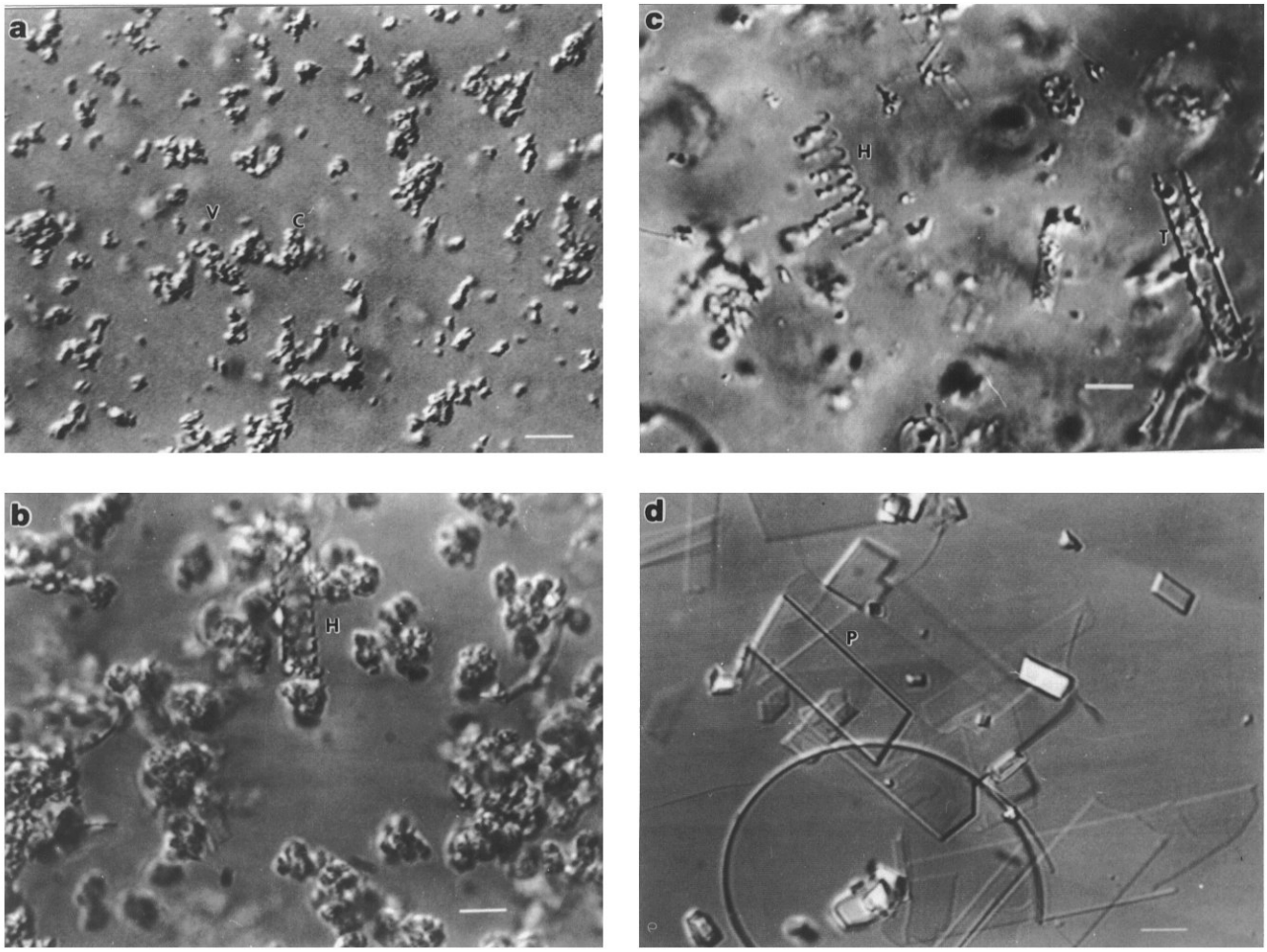


Figure 3. The evolution of structures in a pathophysiologically relevant bile model as observed by video-enhanced light microscopy (VELM): (**a**) 1 hour after dilution (Bar = 20 μm), (**b**) 48 hours after dilution (Bar = 10 μm), (**c**) 4 days after dilution (Bar = 20 μm), and (**d**) 19 days after dilution (Bar = 10 μm). The micrographs demonstrate vesicular aggregates (**V**), and clusters of them (**C**), helical structures (**H**), tubes (**T**), and plate-like crystals (**P**). Reproduced with permission from reference [36].

making certain techniques, such as QLS unsuitable. However, by video-enhanced light microscopy the process can be readily followed, revealing a more complex sequence of events than in the dilute system [36, 45]. As shown in Figure 3, during the first few hours after dilution, spherical particles with a diameter of about 1-2 μm, are observed separately as well as in clusters. By 48 hours, some filaments, and helical structures similar to those observed in the dilute system are noted, co-existing with growing clusters of spherules. Subsequently, less spherules and more helical structures are noted, and only 3 to 5 days after dilution, the first plate-like cholesterol monohydrate crystals are observed representing the "nucleation time," as originally defined by Holzbach and coworkers [26], and commonly used as an indicator of bile lithogenicity. Hence, this time point, more appropriately defined as the crystal observation time [6, 76], is in fact preceded by a series of crystallization processes leading to the formation of the "classic" plate-like cholesterol monohydrate crystals. Finally, close to an apparent equilibrium, at about three weeks of incubation, plate-like crystals are the dominant structures, although some long filaments still persist.

In parallel, cryo-TEM of specimens vitrified immediately after dilution show coexistence of small spheroidal micelles and unilamellar vesicles (Fig. 4a). Initially the micelles, about 6 nm in diameter, dominate but gradually multilamellar vesicles emerge, and become predominant, so that in specimens vitrified 2 to 4 days after dilution, clusters of multilamellar vesicles, with diameters between 50-200 nm,

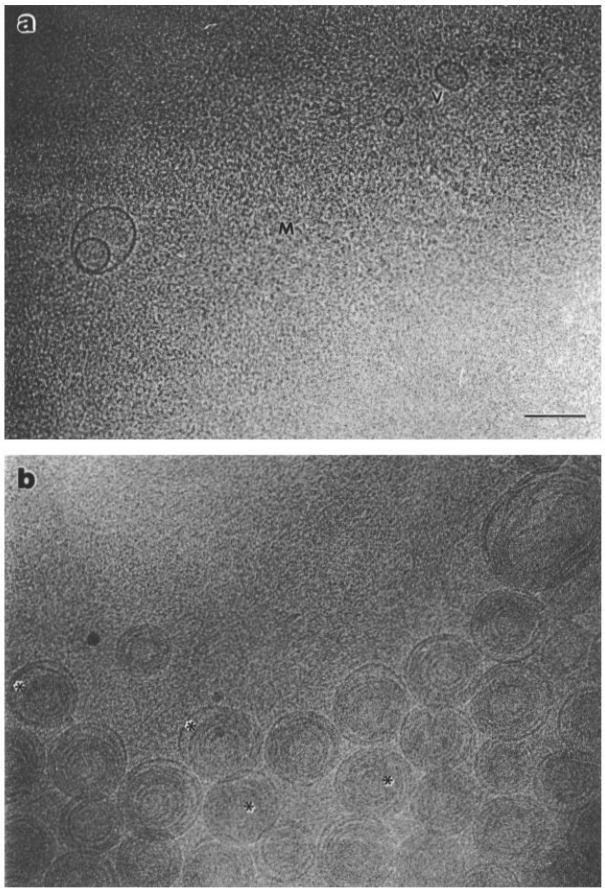


Figure 4. Cryo-TEM micrographs of nucleating model bile. Spherical micelles (**M**) and unilamellar vesicles (**V**) in a specimen (**a**) vitrified 1 hour after dilution, and multilamellar vesicles (**b**) 48 hours after dilution. Some of the multilamellar vesicles display electron-dense areas, marked by asterisks (*). Bar = 100 nm; a and b are at the same magnification. Reproduced by permission from reference [36].

are noted. Clusters of these multilamellar structures are compatible with the spherules seen by light microscopy (Fig. 5). Some of the vesicles have variable numbers of bilayers, whereas some exhibit electron-dense areas within their bilayers, possibly representing cholesterol-rich domains (Fig. 4b).

In the concentrated bile model, sucrose density gradient ultracentrifugation of precipitable cholesterol, separates into four bands of structures, representing cholesterol-containing aggregates of different densities [36, 46]. At 162 hours of incubation (which is about 2 days after nucleation has occurred), all four bands may be seen simultaneously. The upper band, (between 1.01-1.02 g/ml), consists of the spherules as seen before by light microscopy, and shown to be multilamellar vesicles by

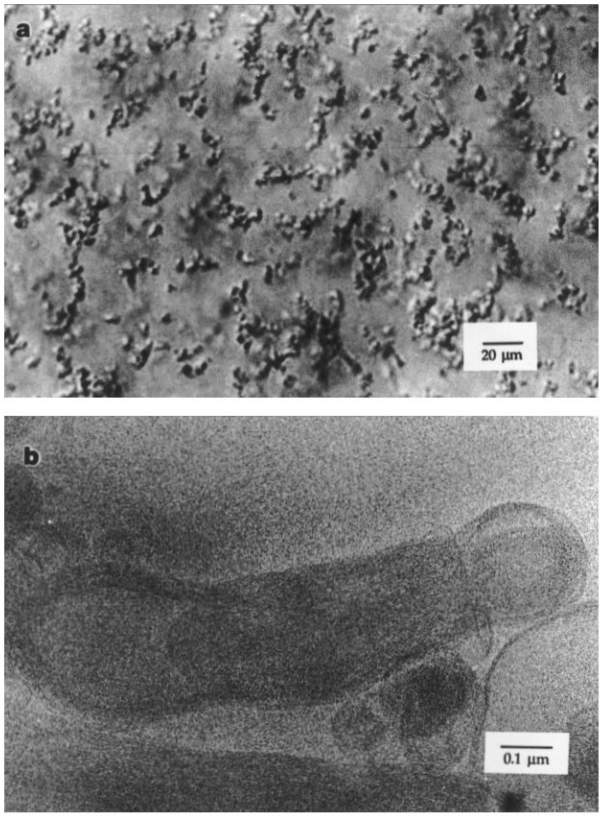


Figure 5. Demonstration of the size correlation between a single aggregate observed by VELM (**a**) and a large multilamellar vesicle observed by cryo-TEM (**b**) in model bile.

electron microscopy. The next band, (between 1.02-1.03 g/ml), contains thin filaments by light microscopy, similar to those seen in the dilute model. The third band, (between 1.03-1.04 g/ml), reveals the whole spectrum of intermediate crystal structures, and the lowest band, (density above 1.045 g/ml), contains classic plate-like cholesterol monohydrate crystals.

Like in the dilute model, the time-dependent process of cholesterol crystallization can be monitored by sequential density gradient separation of cholesterol precipitates [46]. Initially (at 18 hours), all the precipitable cholesterol is contained in low-density multilamellar vesicles. These vesicles are supersaturated (c/p > 1), and contain about 25% of the total cholesterol in the system. Thereafter (between 60-400 hours), a continuous transition of cholesterol to higher density crystal structures is observed. And, at an apparent equilibrium (about 600 hours) practically all precipitable cholesterol is in high-density monohydrate crystals. During cholesterol crystal formation, the c/p ratio of the vesicles gradually decreases and the vesicles become unsaturated.

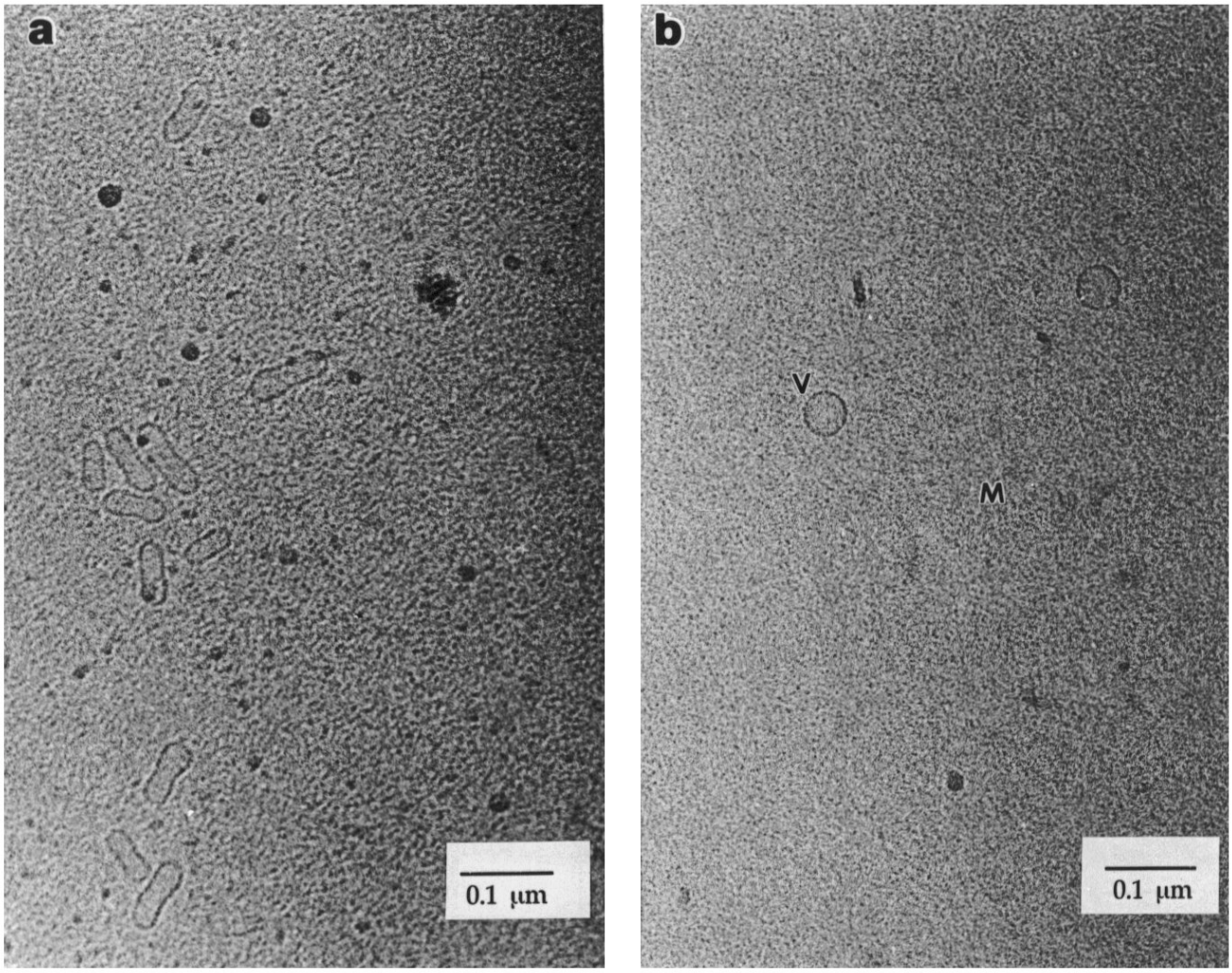


Figure 6. Electron micrographs (cryo-TEM) of native bile samples displaying spheroidal micelles (**M**) and unilamellar vesicles (**V**), some of which are bean-shaped. Specimens were prepared on the day of withdrawal of bile from two gallstone patients.

During the crystallization process, filamentous and intermediate crystal structures are consistently seen by microscopy. However, when quantitated in the density gradient profiles, the two intermediate bands comprise at all times only a small fraction of the precipitable cholesterol in the system. This may reflect a shorter half life of the intermediate structures, or suggest that not all the cholesterol crystallizes via the filamentous pathway, and that other crystallization pathways can occur in parallel [46].

Experiments in native bile

The composition of human bile is considerably more complex than that of the models [40]. Although the three major groups of lipids are the same as those used in the preparation of the model systems, it should be noted that both bile salts and phospholipids exist as a mixture of molecular species in native bile. Recently, it has been shown that cholesterol crystallization in bile depends on both the molecular species of lecithin [22, 44, 81], as well as bile salts [33, 34, 75], and humans prone to gallstone formation have increased concentrations of deoxycholic acid in their bile when compared to controls [68]. Moreover, bile pigments [37], elements such as calcium [56], and a wide array of proteins [2, 5, 7, 16, 18, 31, 55, 57] may have profound effects on cholesterol precipitation and crystallization in bile. Nevertheless, the microstructures observed in native bile are quite similar to those seen in the models [35], and the crystallization process in human bile is found to bear close resemblance to the findings seen in the model systems [35, 36].

In fresh gallbladder bile, before any microstructures can be seen by light microscopy, cryo-TEM discloses an

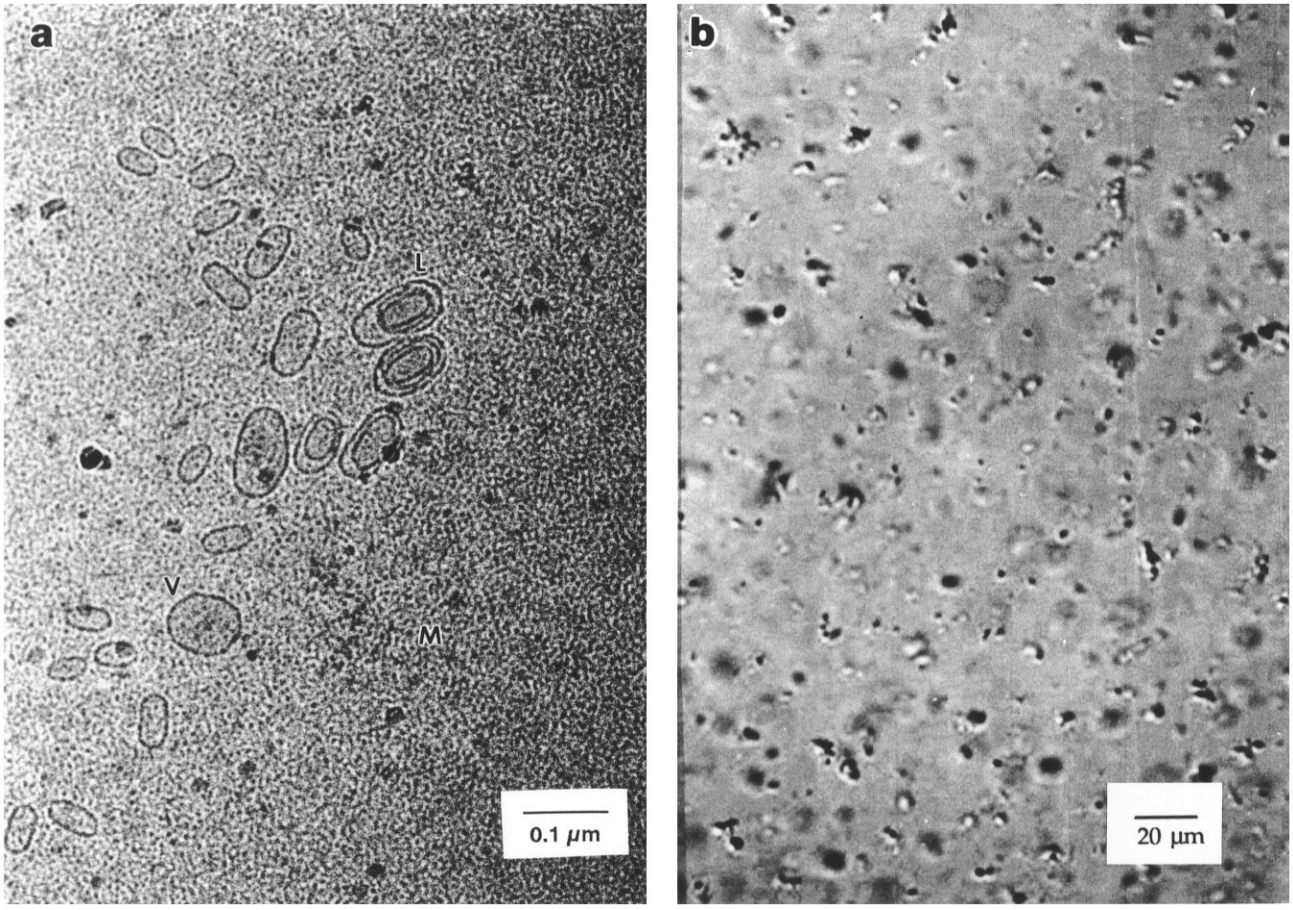


Figure 7. Gallbladder bile of a gallstone patient as observed one day after withdrawal by (**a**) cryo-TEM and (**b**) VELM. Micelles (**M**), unilamellar vesicles (**V**) and multilamellar vesicles (**L**) coexist in the electron micrograph, while spherical aggregates are seen in the light micrograph.

abundance of spheroidal micelles similar to the ones seen in the models, co-existing with small unilamellar vesicles (Fig. 6). Some of the vesicles are identical to those in the models, while some appear to be discoidal, or "bean-shaped." As aggregates appear by light microscopy, cryo-TEM shows the gradual emergence of oligo- and multilamellar vesicles, and these too are of heterogenic shapes (Fig. 7). Finally, during the dynamic phase of crystallization when morphological changes can be seen by light microscopy, vesicles become larger, more abundant and diverse, also disclosing uneven densities and numbers of their bilayers (Fig. 8).

Nucleating human bile also exhibits similar time-dependent density gradient profiles to those seen in the concentrated bile model. Initially, all precipitable cholesterol is in multilamellar vesicles, but as crystals form, the vesicles become desaturated and gradually disappear. In human gallbladder biles studied so far (unpublished observations), the quantity of precipitable cholesterol in the intermediate bands was variable, but less than that in the initial multilamellar vesicles or final monohydrate plates, therefore supporting the existence of additional crystallization pathways also in native bile.

Open Questions

The available data indicate that crystallization of biliary cholesterol monohydrate from metastable solubilized cholesterol is a multi-phase process, involving intermediate microcrystalline structures and possibly a number of structural pathways. Only an approach utilizing a combination of complementary physical-chemical techniques throughout the crystallization process can lead to a comprehensive understanding of the complex sequence of events during cholesterol crystallization in bile.

At this stage, however, we are still left with several unanswered questions, such as: what is the nature of the critical nucleus, and does it originate from the bilayers or

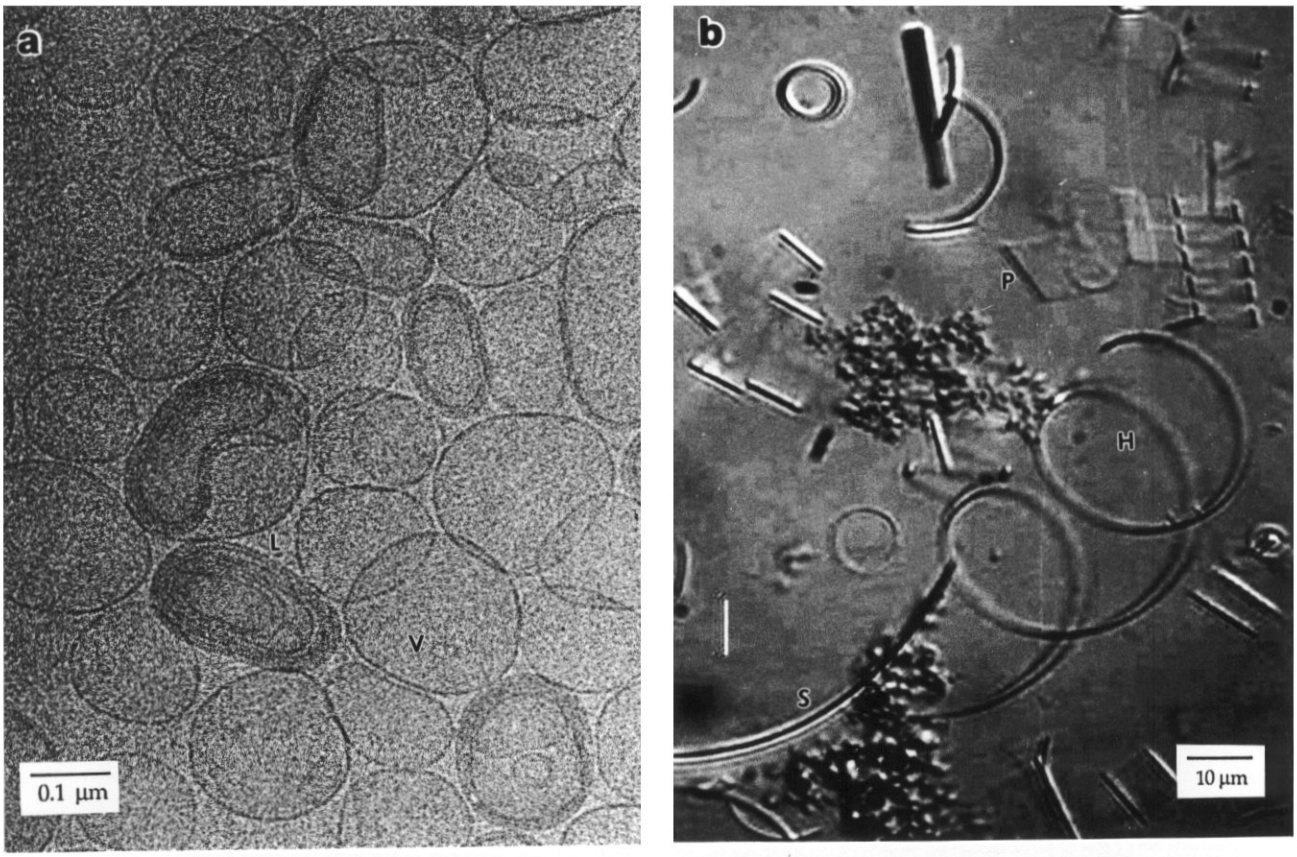


Figure 8. Cryo-TEM (**a**) and VELM (**b**) images of native bile obtained one week after withdrawal from a gallstone patient. Unilamellar (**V**) and multilamellar (**L**) vesicles coexist with spiral (**S**), helical (**H**) and plate-like (**P**) structures.

other structures? From what point is the crystallization process irreversible? Is initial anhydrous/polymorphic crystallization involved, and if so, what is the relevance of this feature? What other, non-filamentous pathways, are involved in native bile, and is there a major or critical pathway? What is the role of pro- and anti-nucleators, whether proteins, lipids or other compounds? What is the (patho) physiologically relevant time-frame of events, pertinent for crystallization *in vivo*? Moreover, while human data are obtained from crystallization studies *ex vivo*, this may not represent what really is going on *in vivo*, where fresh bile flows into the gallbladder changing the composition and cholesterol saturation continuously, making the process even more complex.

All these points will have to be systematically addressed in order to gain a more complete understanding of cholesterol crystallization in bile.

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Discussion with Reviewers

K.M. Kim: What evidence is there that cholesterol gallstones are held together by mucin? Mucin could have been simply trapped between crystals.

Authors: Mucin has been shown to be present in all types of gallstones [89]. This could indeed be interpreted as non-specific precipitation or entrapment between the crystals. However, more recent data have shown that mucin is unevenly distributed within the stones [63, 87]. Mucin is present particularly in cholesterol-rich areas, suggesting an active role in gallstone formation, and not a non-specific secondary process of entrapment [87].

K.M. Kim: During cholesterol crystal formation, the cholesterol/phospholipid ratio of the vesicles is said to be reduced. What is the evidence?

Authors: We and others have shown that prior to cholesterol crystallization the amount of cholesterol carried in vesicles increases, as does the C/P ratio of the vesicles [28, 65]. Fudim-Levin *et al*., and our group have demonstrated that the C/P ratio of the non-particulate phase (i.e., micelles and vesicles) decreases as crystals start to form [15, 65]. We have shown recently that multilamellar vesicles and cholesterol crystals can be isolated from nucleating bile and quantitated employing density gradient

ultracentrifugation (DGUC) during the dynamic process of cholesterol crystallization [46]. By sequential DGUC, the C/P ratio of the isolated vesicles can be seen to decrease concomitantly with the increase in crystal mass.

K.M. Kim: What is the relationship between the C/P ratio and the cholesterol saturation index (CSI)?
Authors: Halpern *et al*. have studied the relationship between the C/P ratio of vesicles and the CSI in different bile solutions [21]. They demonstrated a sharp increase of the C/P ratio at relatively low supersaturation (CSI 1.1 -> 1.2), whereafter additional increase of CSI resulted in only a minor increase in the C/P ratio.

K.M. Kim: How was crystallization induced in natural bile? Was it by dilution, as well?
Authors: Native bile was obtained from cholesterol gallstone patients. These biles are always supersaturated with cholesterol - an obligatory, although by itself an insufficient condition for bile lithogenicity. In these metastable biles cholesterol crystals will form *de novo* within a few days, even after removing pre-existing crystals by ultracentrifugation [26].

K.M. Kim: In addition to micelles and vesicles, cigar-shaped lamellar bodies are said to be a cholesterol carrier in bile. What is the significance? Did you observe any lamellar bodies in your study?
Authors: Besides micelles and vesicles, other particulates or aggregates have been noted or suggested to exist in native bile [32, 60, 73]. There is still significant controversy regarding the (patho)physiological relevance of many of these findings. Cigar-shaped lamellar bodies have been repeatedly detected in bile by electron microscopy [58, 73]. In general, they have been regarded as drying-induced artifacts [79, 86]. Recently, Somjen *et al*. have presented small-angle X-ray diffraction data suggesting that there might be multilamellar structures, other than vesicles in model as well as native biles [88]. In our models, we could not detect any multilamellar structures, other than vesicles during the process of cholesterol crystallization. We have, however, noted some lamellar fragments and electron dense elongated structures in human biles by cryo-TEM [35]. The chemical composition, molecular structure, and significance of these particulates remains to be determined.

K.M. Kim: How do bile salts solubilize cholesterol?
Authors: Bile salts are amphiphilic molecules that solubilize cholesterol primarily by incorporating them into micellar complexes [8, 85]. Although the exact structure of these micelles is unknown, the bile salt molecules are believed to be arranged so that their hydrophilic, hydroxyl-containing parts are facing the aqueous exterior, while their more hydrophobic part is facing the hydrophobic cholesterol molecules. This arrangement "solubilizes" cholesterol molecules within the micelles, thus preventing their precipitation. The cholesterol solubilizing capacity of the micelles is considerably increased by the incorporation of phospholipid molecules. In fact, while simple bile-salt micelles are capable of solubilizing only 1 cholesterol molecule per 20 bile salt molecules, the addition of a lecithin molecule increases this capacity to 1:3 [17]. When comparing this ratio to the up to 2:1 C/P ratio (with only minute amounts of bile salts) found in biliary vesicles, one can reach the conclusion that although the most abundant lipid molecules in bile, bile salts may not be the most efficient, and even most important cholesterol solubilizers in bile.

Reviewer IV: The "nucleation time," better named "crystal detection time" has been used as parameter of lithogenicity by many groups. Does this parameter still hold in the light of the new understanding about the pathways in evolution of lipid aggregates?
Authors: The "nucleation time" (better named "crystal observation time"), although crude, has been shown to reproducibly differentiate lithogenic from non-lithogenic human biles [26]. Based on our newer observations of a multi-step crystallization process and a multiplicity of crystallization pathways, newer and more elaborate tests are being proposed, and examined. At the present time, however, the "nucleation time" is still the best tested parameter to analyze the lithogenicity of human bile.

Reviewer IV: In contrast to model systems, native bile contains mixtures of various phospholipid and bile acid species as well as proteins promoting or inhibiting cholesterol crystallization. Can the authors provide data about how, e.g., proteins influence cholesterol crystallization by favoring specific intermediate pathways?
Authors: At present, the effect of biliary proteins on the cholesterol crystallization process is controversial, and in flux. Thus we find it premature to speculate on putative mechanisms.

Reviewer IV: Gallstone formation involves multiple steps beyond precipitation of crystals. Since cholesterol monohydrate crystals are the endpoints of crystallization, does it really matter which pathway is preferred in a given system determining different times to reach the final equilibrium? Is it not rather the amount of cholesterol that finally is found in the crystalline phase, that has an impact on stone formation?
Authors: The steps in gallstone formation, beyond crystal precipitation have been little studied. It is actually possible that, some crystallization occurs on the stone surface. We do not dispute the reviewer's opinion, that it is the total

amount of cholesterol in the crystalline phase that is important.

Reviewer IV: Compared to model bile systems, do native bile systems of equal total lipid composition form less amount of crystalline cholesterol at final equilibrium? Less amount of total crystalline cholesterol would imply less supply for cholesterol stone formation. Can the authors provide data on this topic?

Authors: We all know the classical experiments of Holzbach's group, showing that cholesterol crystallizes much faster in model solutions than in human bile having the same lipid composition [31]. This implies the presence of crystallization retarding compounds (proteins?) in normal human bile. Whether their effect is also quantitative has to our knowledge not been demonstrated.

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