Imaging Supramolecular Aggregates in Bile Models and Human Bile

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ABSTRACT Investigation of cholesterol crystallization is essential for the understanding of gallstone formation. Previous work has revealed a variety of aggregates of different sizes and shapes prior to the appearance of "classical" plate—like cholesterol monohydrate crystals both in native biles and model systems. In this article, we review existing data based on various microscopic techniques and present data on microstructural pathways leading to cholesterol crystal formation in two different bile models and in native bile.

In continuation of our recent investigation of the microstructures in nucleating human bile, we now present data suggesting that polymorphism is not limited to complex native bile, but also appears in two, simplified model systems. These studies employed cryo-transmission electron microscopy (cryo-TEM) and video-enhanced light microscopy, using Nomarski optics (VELM). Only the combined use of these two complementary, non-perturbing direct methods can cover the whole range of microstructures ranging from a few nanometers to several microns.

Concentrated isotropic solutions of bile models, composed of cholesterol, lecithin and taurocholate, were diluted to induce cholesterol supersaturation and start an evolution of microstructures, leading to cholesterol crystallization. Initially, small spheroidal micelles were observed by cryo-TEM. Subsequently, uni-, oligo- and multilamellar vesicles, compatible with structures seen at the same time by VELM, appeared in coexistence with micelles. Thereafter, during a dynamic phase of cholesterol crystallization, filaments, tubular and helical microstructures, as well as classical plate-like cholesterol monohydrate crystals were noted by light microscopy. Eventually, large plate-like crystals were observed by VELM, while cryo-TEM revealed only small spheroidal micelles.

The crystallization process in native human bile during ex vivo incubation was found to bear close resemblance to the findings in the model systems, further supporting the applicability of these systems to the exploration of microstructural aspects of nucleating human bile. *Microsc. Res. Tech., 39:85–96, 1997.* © 1997 Wiley-Liss, Inc.

INTRODUCTION

Cholesterol monohydrate crystals comprise the microscopic building blocks of cholesterol gallstones (Womack et al., 1963). Thus, understanding the process of cholesterol crystallization is essential for the understanding of gallstone formation. Cholesterol crystals form in supersaturated bile when cholesterol, which is excreted from the body via the liver into bile, precipitates out of solution.

Biliary cholesterol was initially suggested to be solubilized only in bile salt-rich micelles (Admirand and Small, 1968). Accordingly, cholesterol would precipitate out of solution and crystallize once the micellar solubilizing capacity was exceeded. This capacity was measured in model bile solutions and denoted by a cholesterol saturation index (CSI), equal to one at full saturation (Carey, 1978; Carey and Small, 1978). However, supersaturated human bile, with a CSI in excess of one, was found not only in gallstone patients but also in normal individuals (Holzbach et al., 1973). Cholesterol was later shown to be contained in phospholipid vesicles (Holzbach, 1990; Mazer and Carey, 1983; Pattinson, 1985; Somjen and Gilat, 1983; Somjen et al., 1986) in addition to bile salt or mixed bile salt/phospholipid micelles. In fact, there is strong evidence that cholesterol is secreted into bile within phospholipid vesicles (Cohen et al., 1990). Moreover, vesicles following aggregation and possible fusion are thought to be the metastable aggregates from which cholesterol precipitates and crystallizes in bile (Halpern et al., 1986a, 1986b; Peled et al., 1988, 1989).

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Direct evidence for the existence of vesicles in model (Halpern et al., 1986b) and native biles (Halpern et al., 1986a) was obtained by electron microscopy of specimens prepared by staining and drying. This method of specimen preparation, however, has long been shown to produce artifacts (Talmon, 1983). Transmission electron microscopy of vitrified thin liquid films have revealed spherical and thread-like micellar structures in model systems (Vinson et al., 1989; Walter et al., 1991). In bile, however, micelles have not been directly observed, and their exact shape, as well as the shape of the vesicles, is still under investigation. Lately, some additional intermediate lamellar structures have also been suggested to occur in model and native biles (Somjen et al., 1990), although their existence has been challenged as artifactual caused by negative stain electron microscopy (Cohen et al., 1993).

The process of cholesterol crystallization in bile can be divided into three steps: nucleation, crystallization and crystal growth. These steps are difficult to observe separately. The assay of "nucleation time" (Holan et al., 1979), commonly used in cholesterol crystallization studies, measures the time required for the appearance of cholesterol crystals in crystal-free bile samples incubated ex vivo. Since this assay is based on detection of crystals by light microscopy, it actually represents a combination of the three steps. Notably, the nucleation time of bile according to this assay has been shown to be significantly shorter in gallstone patients compared to normal individuals, despite a comparable cholesterol saturation (Holzbach et al., 1973). This assay is, however, crude and does not address the issue of crystal structure or growth. Recently, it has been shown that biliary cholesterol can crystallize as filaments that transform through intermediate microcrystalline structures before becoming classical plate-like cholesterol monohydrate crystals (Konikoff et al., 1992). Thus, the process of cholesterol crystallization in bile is a complex, dynamic process involving a multitude of microaggregates, structures and stages, that eventually lead to the formation of the building blocks of cholesterol gallstones.

In the present study we have employed a combination of cryo-transmission electron microscopy and light microscopy to characterize the complex structural path leading to cholesterol crystallization, with emphasis on the early stages of the process. We present the various microstructures appearing in two model systems and in native human bile, before the eventual formation of classical cholesterol monohydrate crystals.

MATERIALS AND METHODS

Chemicals

Cholesterol (Ch) and sodium taurocholate (TC) were purchased from Sigma Chemical Co. (St. Louis, MO), and egg-yolk lecithin (EYL) from Avanti Polar Lipids, Inc. (Birmingham, AL). Purity of the lipids (>98%) was checked by thin layer chromatography. All other chemicals were ACS or reagent grade. Glassware was acid washed and rinsed thoroughly in distilled water prior to use.

Bile Models

Cholesterol, EYL and TC were coprecipitated from chloroform-methanol (2:1 vol/vol) and dried under nitrogen at reduced pressure. The dried lipid film was dissolved in an aqueous solution (0.15 M NaCl, pH 6–7), containing 3 mM NaN_3 as an antimicrobial agent. Two different models were designed to have relative lipid compositions within the micellar phase limits of the phase diagram prior to dilution, but in the multiphase zone after dilution (Carey and Small, 1978; Konikoff et al., 1992). To ensure complete micellization prior to dilution, the systems were incubated for 1 hour at 60°C. Supersaturation of the models was induced by dilution with 0.15 M NaCl, resulting in lipid compositions beyond the micellar phase limit. The solutions were maintained at 37°C throughout the crystallization process.

Native Bile

Native bile samples were obtained from cholesterol gallstone patients during (gallbladder bile) or after (hepatic bile) cholecystectomy. Gallbladder bile samples were aspirated by puncture prior to ligation or manipulation of the gallbladder. Hepatic bile was collected from an indwelling T-tube catheter after cholecystectomy. All samples were obtained following informed consent of the patients.

Cryo-transmission Electron Microscopy (Cryo-TEM)

Vitrified specimens for cryo-TEM were prepared in a flow-through controlled environmental vitrification system (FT-CEVS) as described elsewhere (Bellare et al., 1988; Fink and Talmon, 1994). Specimens were prepared at sequential time intervals following dilution of bile models or after withdrawal of native bile from patients. In brief, each bile sample was equilibrated in the FT-CEVS chamber at 25°C or 37°C and 100% relative humidity. A 5 µl drop was applied onto a holey carbon film supported on an electron microscopy copper grid, held by tweezers. The drop was blotted and immediately plunged into liquid ethane at its freezing point (90K). Specimens were stored under liquid nitrogen (77K) and later loaded into a cooling-holder (model 626, Gatan Inc., Warrendale, PA) and transferred to the electron microscope (JEOL JEM 2000FX). Specimens were equilibrated in the microscope at about 100K. The TEM was operated at 100 kV accelerating voltage, in a low-dose mode to minimize electron beam radiation damage. Images were recorded at a nominal underfocus of 4 µm on Kodak SO-163 film and developed in full-strength D-19 developer (Eastman Kodak Company, Rochester, NY).

Video-enhanced Light-microscopy

Specimens were observed at 25°C by an Olympus BH-2 light microscope at the time of cryo-TEM specimen preparation. Samples were put on a micro concavity slide (Clay-Adams) and sealed with a cover glass to prevent evaporation. The light microscope operated with Nomarsky differential interference contrast (DIC) optics. Images were recorded by a Sony CUE CCD video camera hooked up to a Galai Cue-4 system. The files were then converted to Macintosh files using the Adobe Photoshop software package. Contrast was further enhanced when needed. Images from a Macintosh 16-inch monitor screen were recorded with a Nikon F-3 camera on a Kodak TMX402 film and developed with

1:4 diluted T-MAX developer (Eastman Kodak Company, Rochester, NY).

RESULTS

A supersaturated dilute bile model, composed of 0.36 mM Ch, 0.18 mM EYL, and 21.2 mM TC (1.2 g/dl total lipid concentration after dilution), was previously studied by light and scanning electron microscopy as well as by several indirect methods (Konikoff et al., 1992). In the present study, a typical structural pathway was confirmed by VELM: filamentous structures were the first to appear (after 3–8 hours), individually or in bundles, followed by ribbons, helices and tubes, and finally by plate-like crystals in a clear supernatant (at equilibrium, after 2 to 3 weeks).

Cryo-TEM micrographs of a sample vitrified 4 hours after dilution revealed coexistence of micelles and vesicles (Fig. 1a). Micelles were the dominant structures. These were spherical, and approximately 6 nm in diameter. The vesicles were unilamellar and spherical with a 50–250 nm diameter range. A large tube-like structure was noted in a single micrograph (Fig. 1b). This tube had a 400 nm diameter and exceeded 4 μm in length. It seemed to be made by helical folding of a wide ribbon. Layers are denoted by arrowheads in the micrograph. The corresponding VELM images have been published previously (Konikoff et al., 1992). Although a filament of the size seen in Figure 1b is generally unlikely to be caught on cryo-TEM specimens (see Discussion for more details), the specific findings in the present work are in close agreement with the previous data obtained by direct and indirect methods.

Next, a more concentrated physiologically relevant bile model, simulating gallbladder bile of cholesterol gallstone patients (Konikoff et al., 1993), was studied. The model was composed of 18 mM Ch, 36 mM EYL, and 120 mM TC (10 g/dl total lipids, after dilution). Samples were studied by VELM and cryo-TEM from 1 hour to a few weeks after dilution. The time-lapse data of a typical structural pathway as observed by VELM are presented in Figure 2. One hour after dilution spherical aggregates were detected (Fig. 2a). These aggregates were observed separately as well as in clusters of several aggregates. The diameter of a single aggregate was on the order of 1.5–2.5 μm. Helical structures were initially noted from 48 hours after dilution (Fig. 2b). The helical structures coexisted with the clusters of the spherical aggregates described above. Subsequently, fewer clusters and more helical structures were noted. Plate-like cholesterol monohydrate crystals were initially observed 3 to 5 days after dilution (representing "nucleation time"). Light micrographs recorded on day 4 (Fig. 2c) demonstrated coexistence of helical and tube-like structures with cholesterol monohydrate crystals. In addition, long straight or curved filaments were observed. Figure 2d shows cholesterol monohydrate crystals 19 days after dilution. The plate-like crystals were the dominant structure observed at that time, with some long filaments still existing.

Cryo-TEM specimens vitrified 1 minute to 1 hour after dilution (Fig. 3a) were all similar, and revealed coexistence of small spheroidal micelles and unilamellar vesicles. The former, 6 nm in diameter, were the dominant structures. The diameters of the vesicles ranged between 50 and 100 nm. Subsequently, coexisting spheroidal micelles, unilamellar vesicles and multilamellar vesicles were observed, with the latter gradually becoming the predominant structure. Multilamellar vesicles were seen in specimens vitrified 48 to 93 hours after dilution (Fig. 3b,c). The multilamellar vesicles differed in the number and density of their bilayers, as demonstrated in micrographs 3b and 3c. In many of these vesicles the bilayers are either densly packed (arrows in Fig. 3c) or not fully resolved around the circumference of the multilamellar vesicles (arrowheads in Figs. 3b and 3c). In some of these there is dense material superimposed on the projection of bilayers (asterisk in Fig. 3b) suggesting the existence of a precipitate encapsulated within these aggregates. As cholesterol precipitated out of solution the supernatant became clear and was found to contain small spheroidal micelles (Fig. 3d). These spheroidal micelles were of the same size as those shown in the very early stages after dilution. Of note, in some micrographs within the first minutes after dilution, low contrast planar structures, possibly intermediate structures in a micelle-vesicle transition (Walter et al., 1991), were noted (Fig. 3e).

We have also studied the microstructures that form during cholesterol crystallization in native human bile employing the same combination of visualization techniques. These results are described in detail elsewhere (Kaplun et al., 1994). The two images presented in Figure 4 demonstrate the feasibility of the two direct methods applied to native bile. Helical and spiral microcrystalline structures were revealed by VELM in gallbladder bile from gallstone patients (Fig. 4a), while unilamellar vesicles were visualized by cryo-TEM in hepatic bile (Fig. 4b).

DISCUSSION

Characterization of the dynamic microstructural evolution of nucleating bile is important for understanding the process of cholesterol crystal formation. In this study we have employed a combination of two complementary direct methods to investigate the early stages of the process. The ability to observe structures ranging from sizes on the order of nanometers to tens of microns is essential in this study. Cryo-TEM provides direct images of microstructures larger than 4 nm and has been widely used in the study of many surfactant systems, including systems of biological relevance, without introducing artifacts typical of drying of specimens, as with standard TEM techniques (Talmon, 1983). Nevertheless, as will be discussed below, the specimen preparation method for cryo-TEM in the controlled environmental vitrification system (CEVS) is limited to structures smaller than about 300 nm. Therefore, light microscopy in the differential interference contrast (DIC) mode was employed as a complementary direct method for larger structures. The latter method is suitable for analysis of microstructures in the size range of 500 nm to 500 μm, and allows visualization of the bulk medium.

In the specimen preparation process for cryo-TEM a drop of solution is blotted to form a thin aqueous film of 250–300 nm. The thickness of the film determines the size range of structures that are maintained on the grid prior to plunging and vitrification. Structures larger than 300 nm are likely to be excluded from the grid and

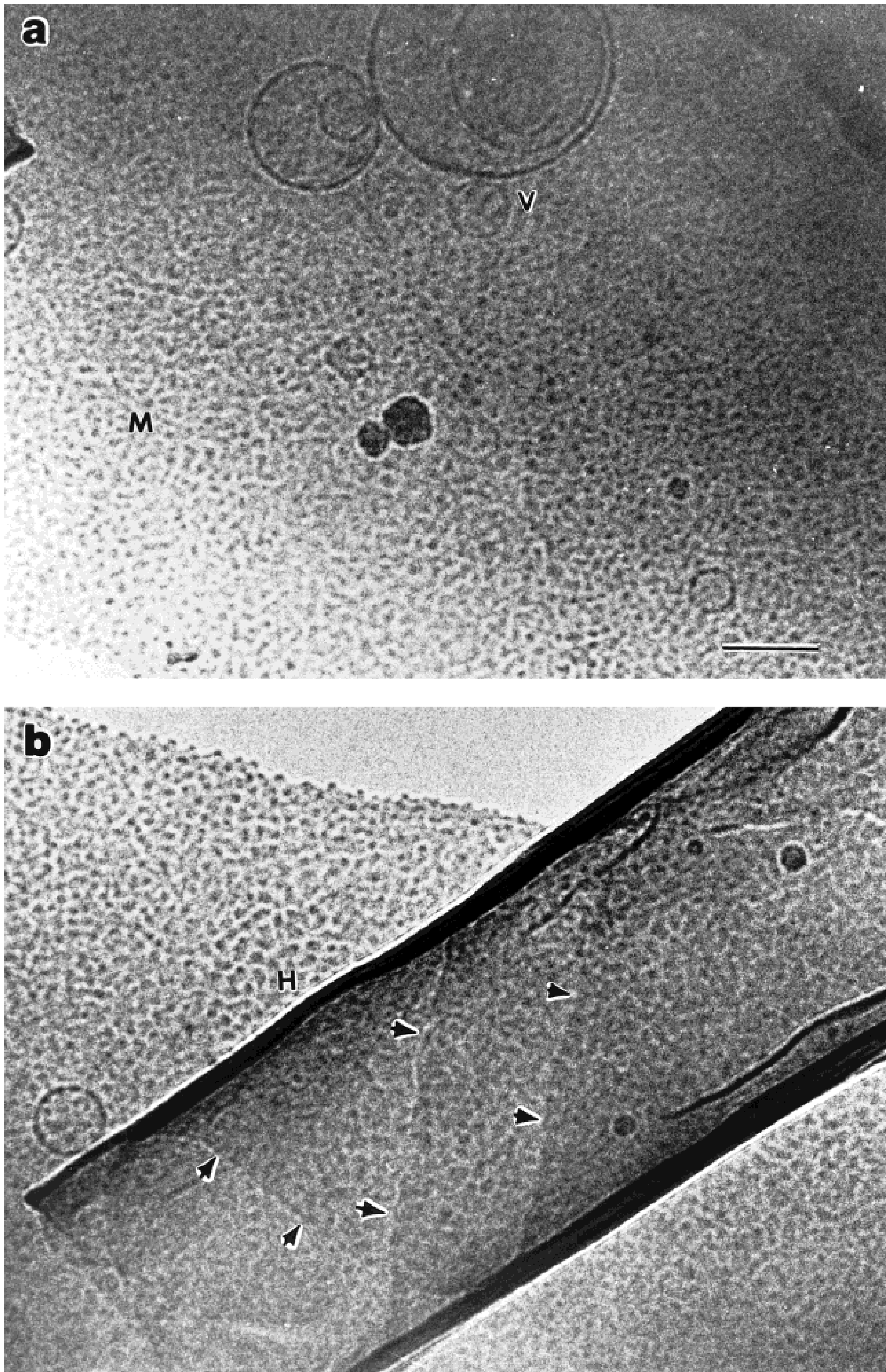

Fig. 1. **a:** Coexistence of spherical micelles (M) and unilamellar vesicles (V) observed by cryo-TEM of a specimen of a dilute bile model vitrified 4 hours after dilution. **b:** Coexistence of the same two structures and a tube-like structure with a helical backbone (H) observed in a single micrograph of the same specimen. The helical layers are denoted by arrowheads. Bar = 100 nm.

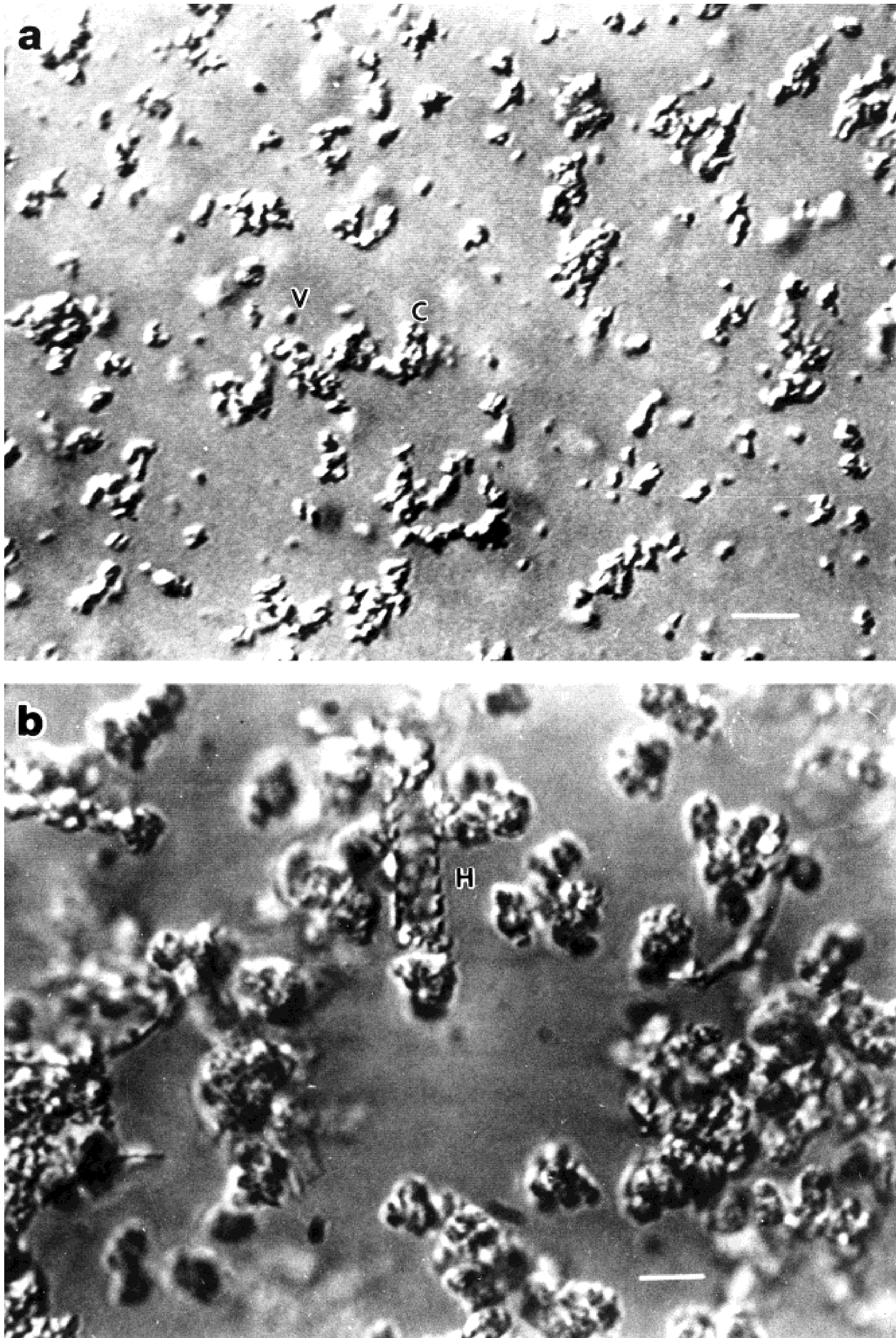

Fig. 2. The evolution of structures in a pathophysiologically relevant bile model as observed by VELM (**a**) 1 hour after dilution (bar = 20 μm), (**b**) 48 hours after dilution (bar = 10 μm), (**c**, overleaf) 4 days after dilution (bar = 20 μm), (**d**, overleaf) 19 days after dilution (bar = 10 μm). The images demonstrate single vesicular aggregates (V) and clusters of them (C), helical structures (H), tubes (T) and plate-like crystals (P).

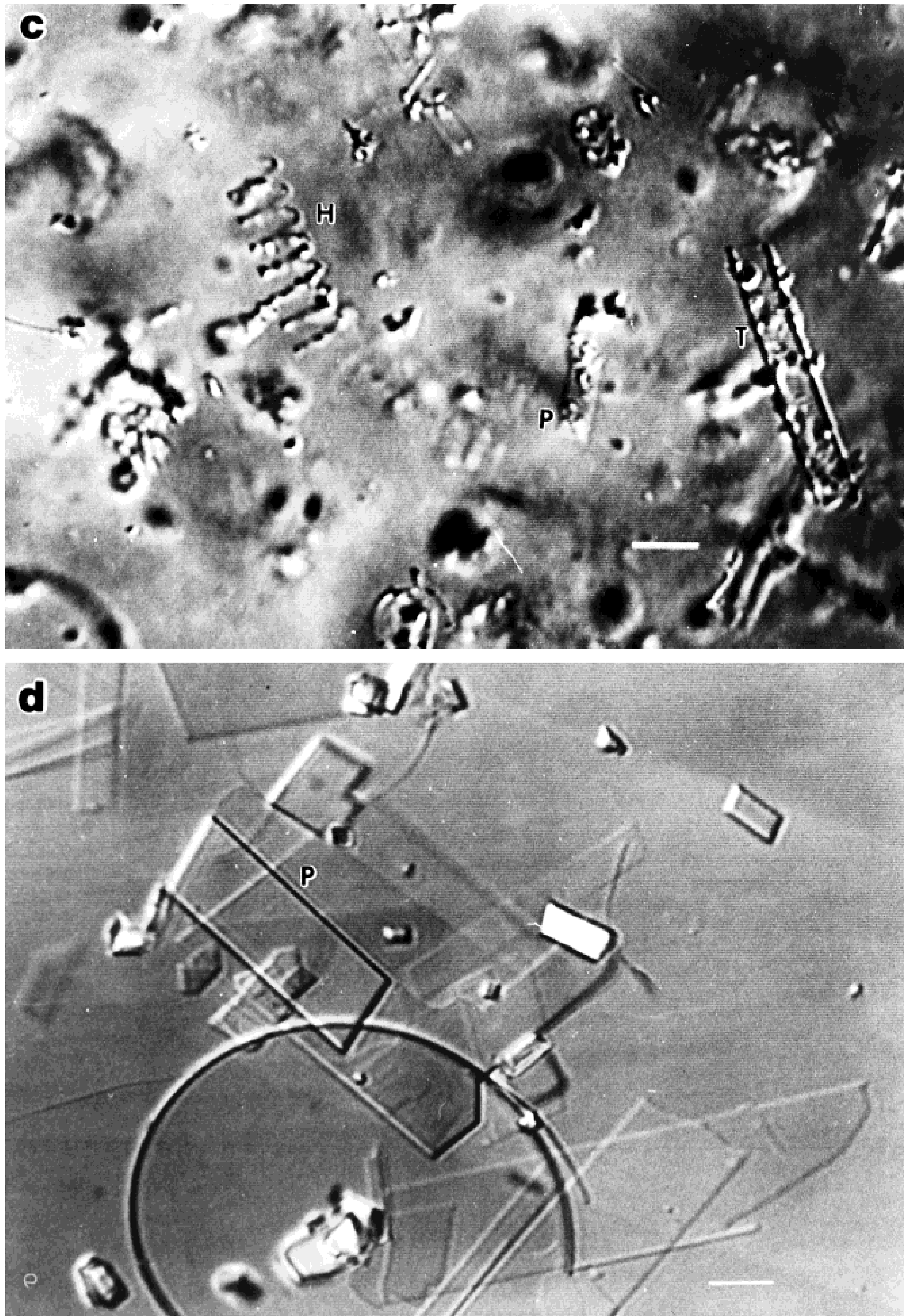

Fig. 2. (Continued.)

are not observed by the electron microscope. In some cases, vesicles with diameters exceeding 300 nm may remain on the grid but undergo shape deformation. Large structures with one dimension smaller than 250 nm remain on the grid if they are oriented with the large dimensions parallel to the plane of the grid. This

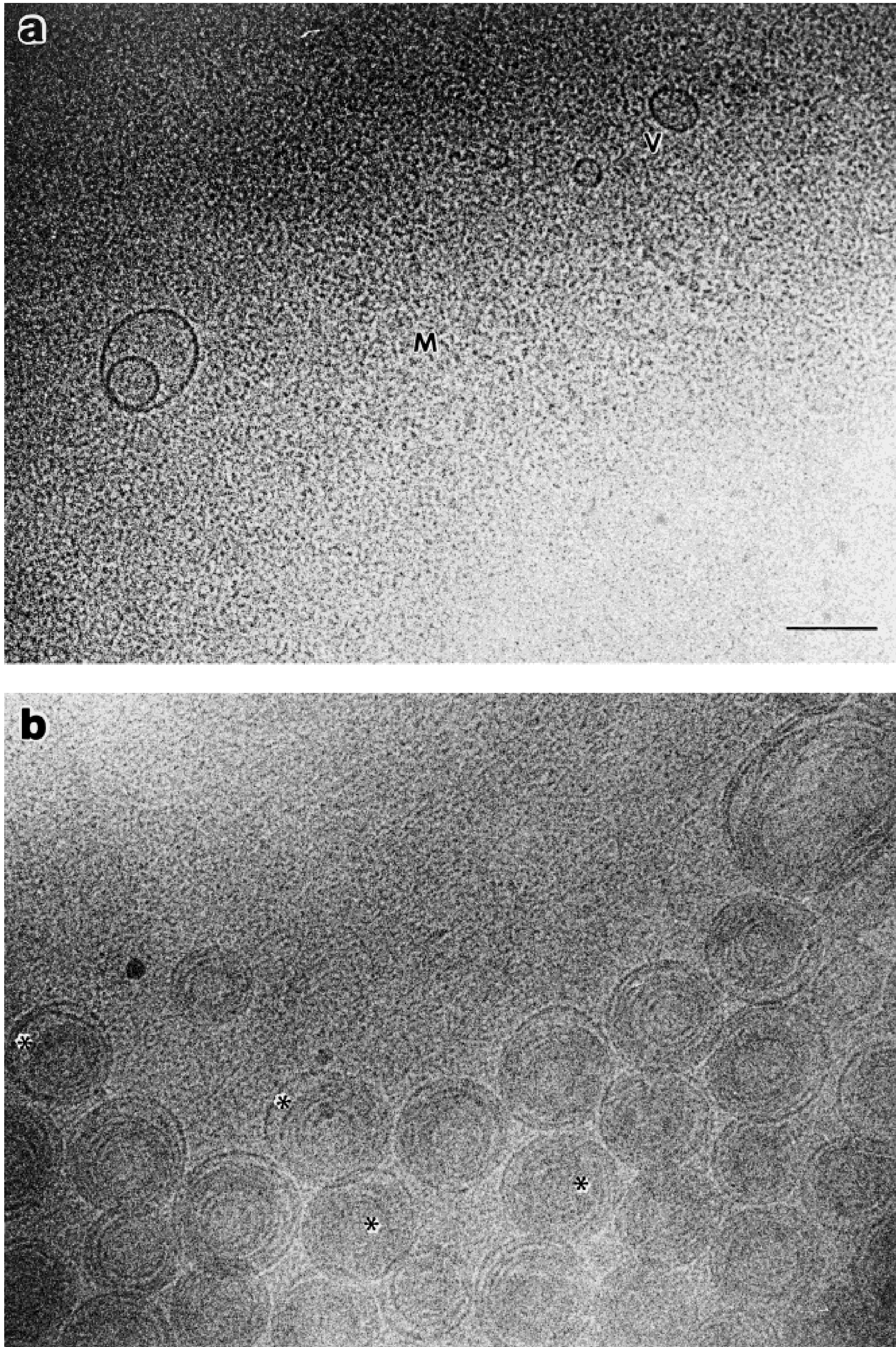

Fig. 3. Cryo-TEM micrographs of typical microstructures in a pathophysiologically relevant bile model: Spherical micelles (M) and unilamellar vesicles (V) in a specimen vitrified 1 hour after dilution (**a**), and multilamellar vesicles (**b**) 48 hours and (**c**, overleaf) 66 hours after dilution. The multilamellar vesicles are either densely packed (arrows) or not fully resolved around their circumference (arrowheads). There is also electron-dense material in some of the vesicles (asterisks). (**d**, overleaf) Spherical micelles at equilibrium (3 weeks) after cholesterol precipitation. (**e**, page 93a) A coexistence of spherical micelles and low contrast planar structures (arrows) was noted in some of the micrographs 1 minute after dilution. Bar = 100 nm.

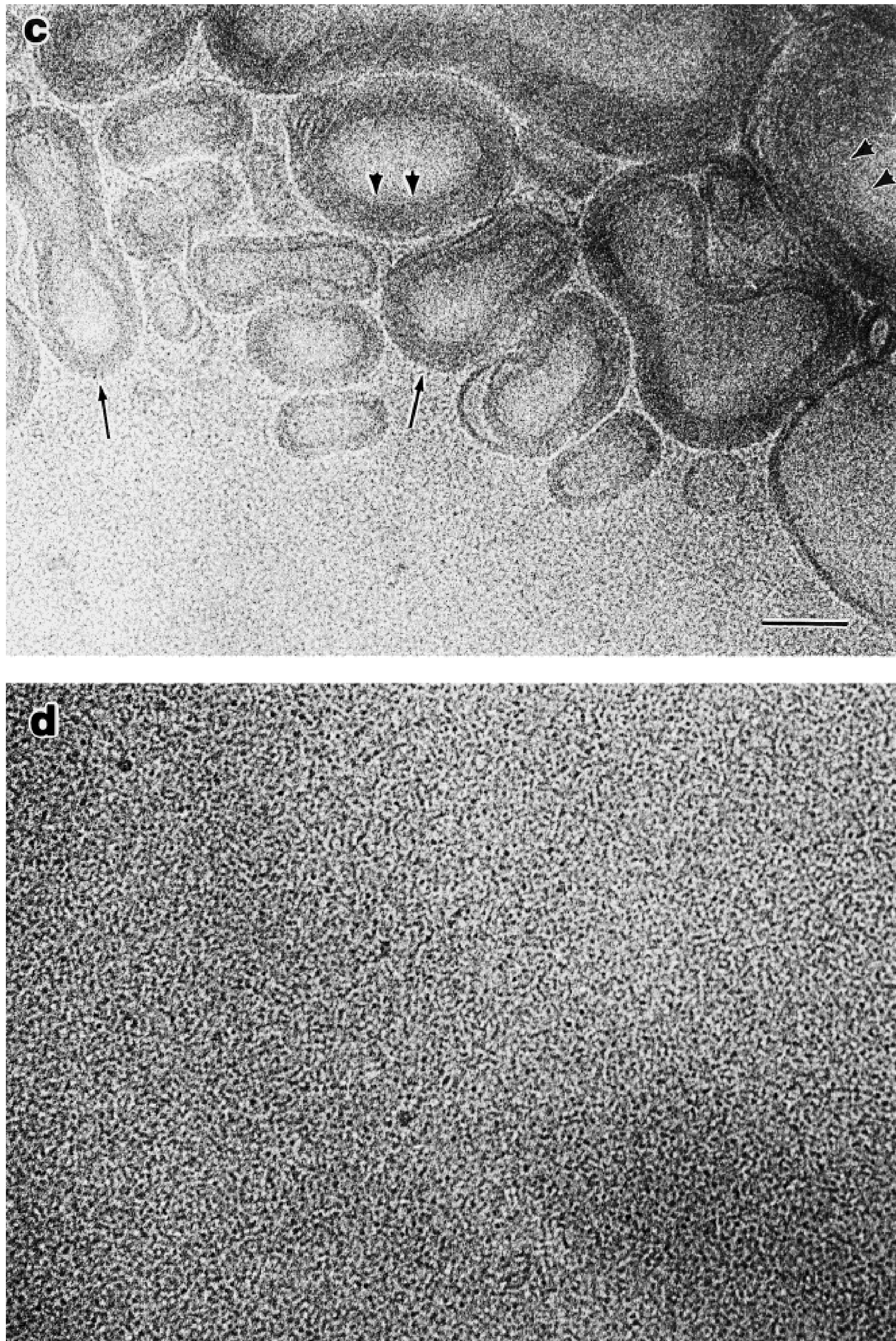

Fig. 3. (Continued.)

explains the visualization of only one tubelike structure in the dilute model bile. The tube with a diameter of 400 nm and a length exceeding 4 μm lies parallel to the plane of the film and is therefore contained in it, possibly after flattening. Generally, such tubelike structures are likely to be excluded from the film.

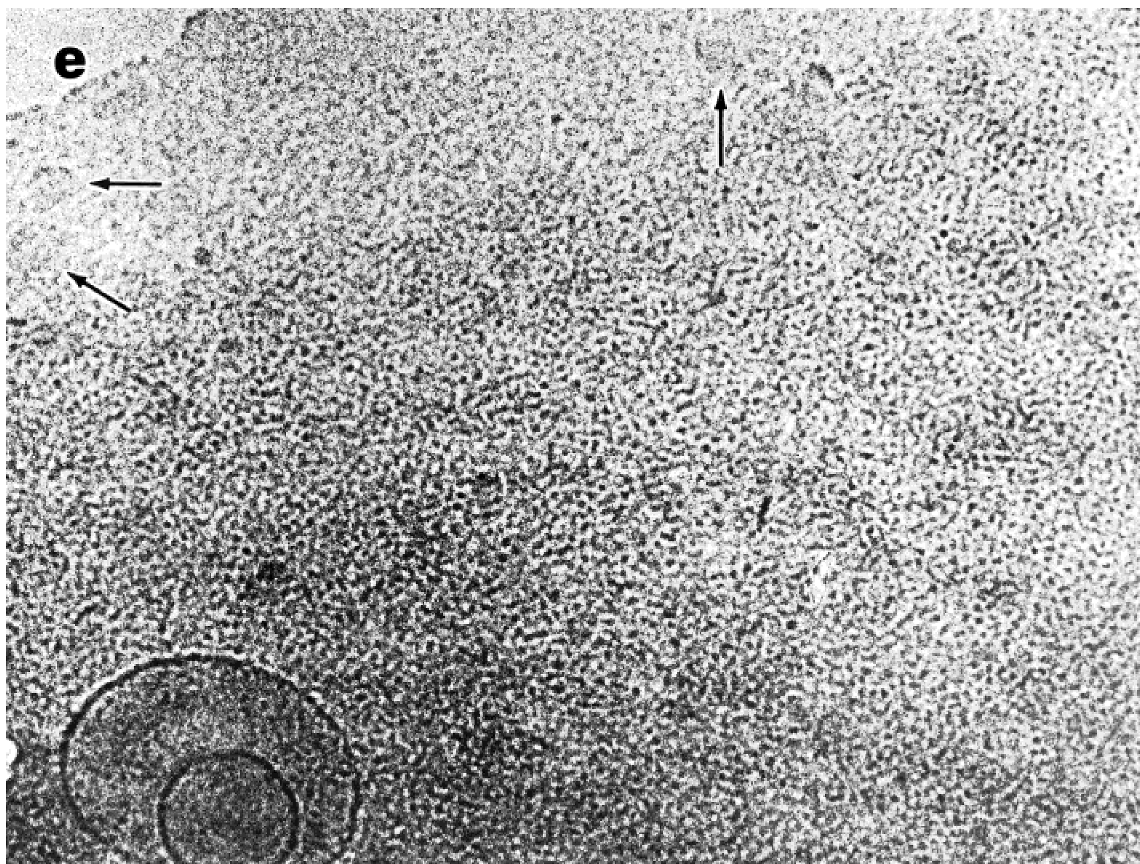

Fig. 3. (Continued.)

The probability of observing microstructures in TEM images depends on their concentration in the sample. At lower concentration the probability to observe structures decreases. Also, some suspended particles, especially larger ones, are excluded from the specimen during preparation. Therefore, cryo-TEM is only a qualitative tool, unable to provide exact size distribution data. The dilute model bile contains a total lipid concentration of 1.2 g/dl. A similar total lipid concentration was sufficient to allow the observation of vesicles and micelles in a bile salt/phospholipid system (Walter et al., 1991). In the present study, the vesicle-forming amphiphiles EYL and Ch totalled only 0.02 g/dl. Therefore, vesicular structures that exist in this particular system in small amounts are likely to be missed in the cryo-TEM images. In the physiologically relevant model, Ch and EYL are at a concentration of 3.5 g/dl and vesicular structures are easily observed by cryo-TEM.

Our light microscopy results of the dilute bile model were similar to those described and discussed elsewhere (Chung et al., 1993; Konikoff and Carey, 1994; Konikoff et al., 1992), and were consistent with findings observed in more concentrated systems made by solubilization of cholesterol/phospholipid vesicles (Fudim-Levin et al., 1995; Lichtenberg et al., 1988, 1990). In the present study we have focused on the early stages of this structural path. Most images of the early stages of this model disclosed micelles and only a small number of vesicles, as previously suggested by gel filtration chromatograpy and quasi-elastic light scattering measurements that require model assumption to interpret the data (Konikoff et al., 1992, 1994). In this study, cryo-TEM data provided the first images of micelles in the system, and confirmed the previously suggested existence of vesicles at the initial stage of crystallization. Moreover, the assumption that the vesicles in this system are unilamellar (Konikoff et al., 1992) was now confirmed by direct imaging. The vesicles and micelles resemble in shape and size the structures previously observed in systems composed of EYL and the non-ionic surfactant octylglucoside (Vinson et al., 1989) and EYL/sodium-cholate (Walter et al., 1991). The single image of a tube with a helical internal structure (Fig. 1b) does not provide additional insight as to the nature of this structure or possible relation to the filaments observed by VELM at the same time. Further studies are required. These should be combined with an additional direct method, such as freeze-fracture electron microscopy (Rigler et al., 1986), where particles are less likely to be excluded from the specimen.

The second model that we studied contained the same components, but at higher concentrations and different relative compositions. The composition is of pathophysiological relevance, and the higher concentrations increase the probability to observe the structures in the system. In this model a more complex and detailed structural path was observed. As in the dilute model system, the coexistence of micelles and vesicles immediately after dilution was in agreement with

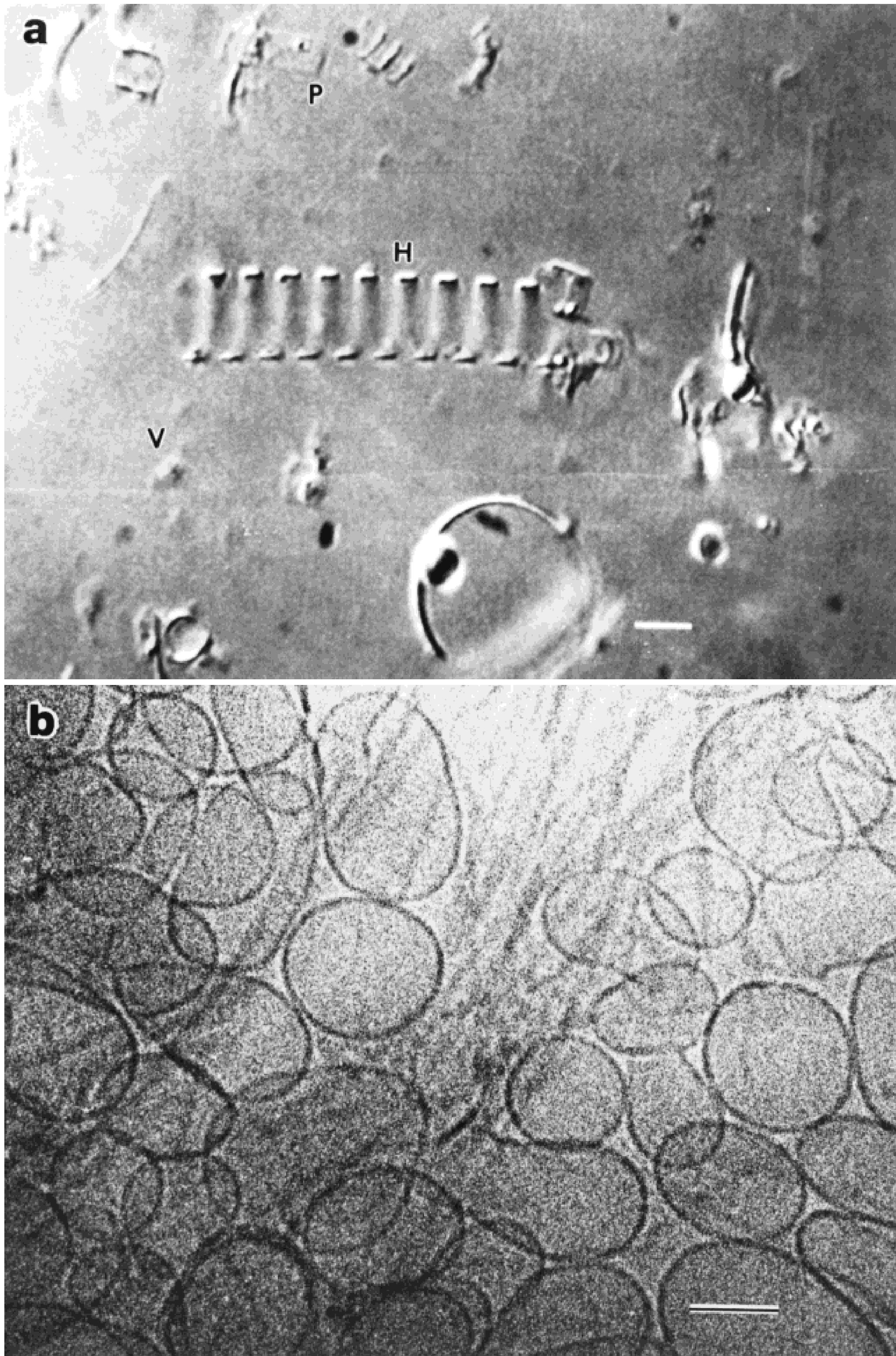

Fig. 4. **a:** Helical (H) and spiral structures coexisting with vesicular aggregates (V) and plates (P), observed by VELM in a gallbladder bile sample from a gallstone patient during cholesterol crystallization at 6 days of ex vivo incubation. Bar = 10 μm. **b:** Cryo-TEM micrograph demonstrating unilamellar vesicles in human hepatic bile. Bar = 100 nm.

previously suggested structures (Konikoff et al., 1992; Somjen and Gilat, 1985).

Of note is the lack of elongated worm-like micelles in any of the bile model systems investigated, as found by Fudim-Levin et al. (1995). Such structures have been previously observed in systems of EYL/sodium-cholate, devoid of cholesterol (Walter et al., 1991). It thus appears that the presence of cholesterol prohibits the lipid assembly in the form of worm-like micelles. Instead, the lipids assemble either in the form of vesicles or planar lamellar structures. The appearance of low contrast planar structures during the initial stages, although rare, might be related to the “lamellar cholesterol carriers” previously suggested (Somjen et al., 1990). This relationship, however, needs to be further investigated.

Spherical aggregates and clusters were detected by light microscopy at the time when multilamellar vesicles were observed by cryo-TEM. The diameter of a single aggregate corresponds to a cluster of several multilamellar vesicles, suggesting a process of multilamellar vesicle aggregation from a size range observed by cryo-TEM to that observed by light microscopy. Other investigators have studied vesicle aggregation and its relation to crystal nucleation in bile models (Halpern et al., 1986b) and native bile (Halpern et al., 1986a). However, those data are controversial due to the electron microscopy specimen preparation methods used. Electron microscopy of stained and dried specimens was demonstrated to produce artifacts like images of multilamellar vesicles in systems that were pure micellar before specimen preparation (Talmon, 1983). It may well be that the systems studied in the previous papers did contain multilamellar vesicles, yet the structures described in their images may, at the same time, be artifacts. Only a non-perturbing specimen preparation method of TEM specimens, like the one we have used, can confirm the existence of multilamellar vesicles in the solutions. Since multilamellar vesicles are believed to be the sites of nucleation, cryo-TEM may help to provide insight into the process by which the earliest critical nuclei form. This emphasizes the need to combine quantitative indirect methods to further investigate and characterize the nature of the multilamellar vesicles. It demonstrates the importance of cryo-TEM as a qualitative direct tool that allows one to detect and distinguish between microstructures, and provides a basis for model assumption in the interpretation of quantitative data obtained by indirect methods.

The structural paths of the two model systems revealed differences during the early stages leading to cholesterol crystallization. These differences may be due to different total and relative concentrations of lipids. The physiologically relevant model is about eightfold more concentrated (w/w), with a Ch/EYL molar ratio of 0.5 compared to 2 in the dilute model. There is also a difference in the TC to Ch and EYL molar ratio. Both models demonstrate a dominant population of small spheroidal micelles and a minor population of unilamellar vesicles, immediately after dilution. In the dilute model, no multilamellar vesicles were seen and no aggregates were observed by light microscopy. Unilamellar vesicles preceded and coexisted with filamentous structures in the dilute model, whereas multilamellar vesicles coexisted with helical structures in the second model. Thus, both models confirm the existence of micelles and vesicles in bile models immediately after supersaturation, but differ in the dynamic stages leading to formation of filamentous, helical and plate-like crystals.

The bile model systems have well-defined components and concentrations, and the sequence of structures is measured from the start of dilution. Dilution causes bile salt depletion of micelles and subsequent cholesterol supersaturation that induces cholesterol crystallization. This well defined zero time provides a basis for comparison between several solutions of the same model, as well as between different models. Native human bile is composed of about 10% solutes of which over 95% are biliary lipids: BS, PL and Ch (Konikoff, 1994). Unlike in model systems, the bile salts and phospholipids in native bile are composed of various subspecies and they also contain small amounts of pigment, electrolytes and proteins. These may act as nucleation promoters and inhibitors, and so their abscence in model bile systems could alter the time scale of cholesterol crystallization. The study of native bile was described in detail in a previous paper (Kaplun et al., 1994). In brief, micellar aggregates were directly visualized in human bile for the first time. The coexistence of unilamellar vesicles in human gallbladder bile at the early stages after withdrawal from patients was confirmed by this non-perturbing technique. Light microscopy indicated the appearance of helical structures prior to the formation of the classical plate-like monohydrate crystals. The mere observation of similar polymorphism in native and model systems lends further support to the validity of studies performed in model systems. A continued ongoing study of native bile focuses on the additional effects of the various non-lipid components in native bile.

In conclusion, this study demonstrates a large variety of structures present in model and native bile during the dynamic process of cholesterol crystallization. This structural and size variety could only be observed with a combination of two complementary methods like cryo-TEM and VELM.

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REFERENCES

Admirand, W.H., and Small, D.M. (1968) The physicochemical basis of cholesterol gallstone formation in man. J. Clin. Invest., 47:1043–1052.

Bellare, J.R., Davis, H.T., Scriven, L.E., and Talmon, Y. (1988) Controlled environment vitrification system: An improved sample preparation technique. J. Electron Microsc. Tech., 10:87–111.

Carey, M.C. (1978) Critical tables for calculating the cholesterol saturation of native bile. J. Lipid Res., 19:945–956.

Carey, M.C., and Small, D.M. (1978) The physical chemistry of cholesterol solubility in bile. Relationship to gallstone formation and dissolution in man. J. Clin. Invest., 61:998–1026.

Chung, D.S., Benedek, G.B., Konikoff, F.M., and Donovan, J.M. (1993) Elastic free energy of anisotropic helical ribbons as metastable intermediates in the crystallization of cholesterol. Proc. Natl. Acad. Sci. U.S.A. 90:11341–11345.

Cohen, D.E., Angelico, M., and Carey, M.C. (1990) Quasielastic light scattering evidence for vesicular secretion of biliary lipids. Am. J. Physiol., 257:G1–G8.
Cohen, D.E., Kaler, E.W., and Carey, M.C. (1993) Cholesterol carriers in human bile—are lamellae involved? Hepatology, 18:1522–1531.
Fink, Y., and Talmon, Y. (1994) The flow-thru controlled environment vitrification system. 13th International Conference on Electron Microscopy, Paris, 17–22 July, pp. 37–38.
Fudim-Levin, E., Bor, A., Kaplun, A., Talmon, Y., and Lichtenberg, D. (1995) Cholesterol precipitation from cholesterol-supersaturated bile models. Biochim. Biophys. Acta, 1259:23–28.
Halpern Z., Dudley, M.A., Kibe, A., Lynn, M.P., Breuer, A.C., and Holzbach, R.T. (1986a) Rapid vesicle formation and aggregation in abnormal human biles. A time-lapse video-enhanced contrast microscopy study. Gastroenterology, 90:875–885.
Halpern, Z., Dudley, M.A., Lynn, M.P., Nader, J.M., Breuer, A.C., and Holzbach, R.T. (1986b) Vesicle aggregation in model systems of supersaturated bile: Relation to crystal nucleation and lipid composition of the vesicular phase. J. Lipid Res., 27:295–306.
Holan, K.R., Holzbach, R.T., Hermann, R.E., Cooperman, A.M., and Claffey, W.J. (1979) Nucleation time: A key factor in the pathogenesis of cholesterol gallstone disease. Gastroenterology 77:611–617.
Holzbach, R.T. (1990) Detection of vesicles in native and model biles by morphological and other structural techniques: Applications and limitations. Hepatology, 12:106S–112S.
Holzbach, R.T., Marsh, M., Olszewski, M.F., and Holan, K. (1973) Cholesterol solubility in bile. Evidence that supersaturated bile is frequent in healthy man. J. Clin. Invest., 52:1467–1479.
Kaplun, A., Talmon, Y., Konikoff, F.M., Rubin, M., Eitan, A., Tadmor, M., and Lichtenberg, D. (1994) Direct visualization of lipid aggregates in native human bile by light- and cryo-transmission electron microscopy. FEBS Lett. 340:78–82.
Konikoff, F. (1994) New mechanisms in cholesterol gallstone disease. Isr. J. Med. Sci., 30:168–174.
Konikoff, F.M., and Carey, M.C. (1994) Cholesterol crystallization from a dilute bile salt-rich model bile. J. Crystal Growth, 144:79–86.
Konikoff, F.M., Chung, D.S., Donovan, J.M., Small, D.M., and Carey, M.C. (1992) Filamentous, helical and tubular microstructures during cholesterol crystallization from bile: Evidence that biliary cholesterol does not nucleate classic monohydrate plates. J. Clin. Invest., 90:1155–1160.
Konikoff, F., Laufer, H., Messer, Y., and Gilat, T. (1993) Monitoring cholesterol nucleation and crystallization from physiologically relevant model bile by time-lapse density gradient ultracentrifugation. Hepatology 18:96A.
Konikoff, F.M., Cohen, D.E., and Carey, M.C. (1994) Filamentous crystallization of cholesterol and its dependence on lecithin species in bile. Mol. Cryst. Liq. Cryst., 248:291–296.
Lichtenberg, D., Ragimova, S., Bor, A., Almog, S., Vinkler, C., Kalina, M., Peled, Y., and Halpern, Z. (1988) Stability of mixed micellar bile models supersaturated with cholesterol. Biophys. J., 54:1013–1025.
Lichtenberg, D., Ragimova, S., Bor, A., Almog, S., Vinkler, C., Peled, Y., and Halpern, Z. (1990) Stability of mixed micellar systems made by solubilizing phosphatidylcholine-cholesterol vesicles by bile salts. Hepatology, 12:149S–154S.
Mazer, N.A., and Carey, M.C. (1983) Quasi-elastic light-scattering studies of aqueous biliary lipid systems. Cholesterol solubilization and precipitation in model bile solutions. Biochemistry, 22:426–442.
Pattinson, N.R. (1985) Solubilisation of cholesterol in human bile. FEBS Lett., 181:339–342.
Peled, Y., Halpern, Z., Baruch, R., Goldman, G., and Gilat, T. (1988) Cholesterol nucleation from its carriers in human bile. Hepatology, 8:914–918.
Peled, Y., Halpern, Z., Eitan, B., Goldman, G., Konikoff, F., and Gilat, T. (1989) Biliary micellar cholesterol nucleates via the vesicular pathway. Biochim. Biophys. Acta, 1003:246–249.
Rigler, M.W., Honkanen, R.E., and Patton, J.S. (1986) Visualization by freeze fracture, in vitro and in vivo, of the products of fat digestion. J. Lipid Res., 27:836–857.
Somjen, G.J., Marikovsky, Y., Lelkes, P., and Gilat, T. (1986) Cholesterol-phospholipid vesicles in human bile: An ultrastructural study. Biochim. Biophys. Acta, 879:14–21.
Somjen, G.J., Marikovsky, Y., Wachtel, E., Harvey, P.R.C., Rosenberg, R., Strasberg, S.M., and Gilat, T. (1990) Phospholipid lamellae are cholesterol carriers in human bile. Biochim. Biophys. Acta 1042:28–35.
Somjen, G.J., and Gilat, T. (1983) A non-micellar mode of cholesterol transport in human bile. FEBS Lett., 156:265–268.
Somjen, G.J., and Gilat, T. (1985) Contribution of vesicular and micellar carriers to cholesterol transport in human bile. J. Lipid Res., 26:699–704.
Talmon, Y. (1983) Staining and drying-induced artifacts in electron microscopy of surfactant dispersions. J. Colloid Interface Sci., 93:366–382.
Vinson, P.K., Talmon, Y., and Walter, A. (1989) Vesicle-micelle transition of phosphatidylcholine and octyl glucoside elucidated by cryo-transmission electron microscopy. Biophys. J., 56:669–681.
Walter, A., Vinson, P.K., Kaplun, A., and Talmon, Y. (1991) Intermediate structures in the cholate-phosphatidylcholine vesicle-micelle transition. Biophys. J., 60:1315–1325.
Womack, N.A., Zeppa, R., and Irvin, G.L. III (1963) The anatomy of gallstones. Ann. Surg., 157:670–686.