Quantitation of cholesterol crystallization from supersaturated model bile[1]

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Abstract Cholesterol crystallization is an essential step in gallstone formation. Although spectrophotometry and nephelometry have been used for quantitation of crystallization, potential effects of crystal size and shape have not been evaluated. We determined crystallization in model biles [total lipid concentration 7.3 g/dl, egg yolk Phosphatidylcholine (EYPC)/(EYPC+taurocholate) molar ratio = 0.05, 0.15, or 0.30; cholesterol saturation index (CSI) = 1.2, 1.7, or 2.0; 37°C] plotting in the central three-phase (micelles, vesicles, and crystals containing) zone or in the left two-phase (micelles and crystals containing) zone of the equilibrium ternary phase diagram. Extent of crystallization estimated by spectrophotometry and nephelometry was related to chemical determination of crystal mass and to crystal size or shape (by microscopy). With all methods, crystallization was less extensive when vesicles were present (central three-phase zone) and at lower CSIs. In the left two-phase zone, particularly at EYPC/(EYPC+taurocholate), ratio of 0.15, there were strong increases in spectrophotometric and nephelometric readings during the first days of incubation, but decreases at later stages, despite progressive increases in crystal mass by chemical measurement. Initially, there were large numbers of very small crystals (<10 μm) in these biles, which were subsequently replaced by large cholesterol monohydrate crystals. Decreasing sizes of harvested cholesterol monohydrate crystals by sonication increased spectrophotometric and nephelometric values despite identical crystal mass. When cholesterol crystal mass is assayed by indirect methods such as spectrophotometry or nephelometry, results are strongly influenced by crystal size.—Portincasa, P., N. G. Venneman, A. Moschetta, A. van den Berg, G. Palasciano, G. P. vanBerge-Henegouwen, and K. J. van Erpecum. **Quantitation of cholesterol crystallization from supersaturated model bile.** ***J. Lipid Res.*** **2002.** 43: **604–610.**

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Precipitation of cholesterol crystals from supersaturated bile is a prerequisite for the formation of cholesterol gallstones (1). Normally, cholesterol in bile is solubilized in mixed micelles by bile salts (BS) and phospholipids (PL). Phosphatidylcholine is the major (>95% of total) phospholipid in bile. In case of cholesterol supersaturation, the excess sterol may be contained in vesicles together with phospholipids (2, 3) or precipitated as solid crystals. Wang and Carey (4) have pointed to the importance of the relative amounts of bile salts versus phospholipids in the system for crystallization behavior. In case of excess bile salts [i.e., PL/(BS+PL) molar ratios ≈≤0.2], crystals precipitate at fast rates, and both various intermediate anhydrous cholesterol crystals (needles, arcs, tubules, spirals) and mature rhomboid cholesterol monohydrate crystals can be detected by microscopy. In case of higher amounts of phospholipids, crystal precipitation proceeds at slower rates (with predominant formation of mature cholesterol monohydrate crystals), and large amounts of cholesterol are solubilized in vesicles together with phospholipids. In case of excess phospholipids [high PL/(BS+PL) molar ratios], solid cholesterol crystals do not occur, and cholesterol is mainly solubilized in vesicular phases. Based on these data, the equilibrium cholesterol-bile salt-phospholipid ternary phase diagram (**Fig. 1**) (4) is assumed to contain a one-phase zone (only micelles), a left two-phase (micelles- and cholesterol crystals-containing) zone, a central three-phase (micelles-, vesicles-, and cholesterol crystals-containing) zone and a right two-phase (micelles and vesicles-containing) zone.

Since crystallization is an essential step in cholesterol gallstone formation, accurate quantitation of the amounts

Abbreviations: CSI, cholesterol saturation index; DC, deoxycholate; EYPC, egg-yolk phosphatidylcholine; TC, taurocholate.

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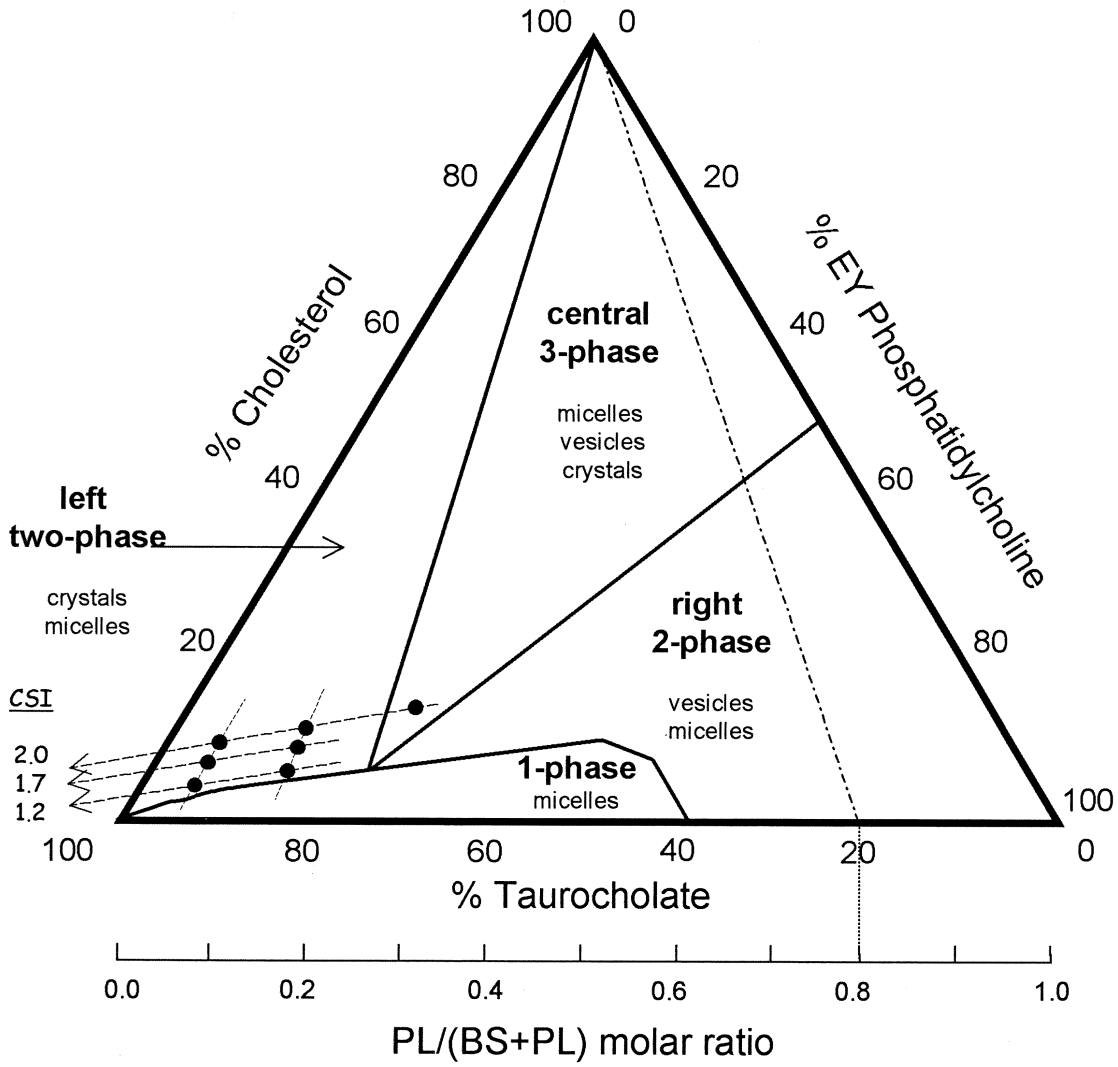

Fig. 1. The ternary equilibrium cholesterol-taurocholate-egg yolk phosphatidylcholine phase diagram (4). Components are expressed in mole percent. Depicted are a one-phase (micellar) zone at the bottom, a left two-phase zone (containing micelles and crystals), a central three-phase zone (containing micelles, vesicles, and crystals), and a right two-phase zone (containing micelles and vesicles). At the bottom, PL/(BS+PL) molar ratios are also given. Interrupted lines indicate identical PL/(BS+PL) molar ratios for all model biles plotting on the line (in this case ratio of 0.8). The positions of all model biles used in this study are also plotted.

of precipitated cholesterol is important. Although crystal mass has previously been estimated with the aid of radiochemical (5) or ultracentrifugal (6, 7) assays, these methods are rather tedious and time consuming. Therefore, most previous studies have quantified crystal mass indirectly with the aid of nephelometric light scattering (8–14) or spectrophotometric absorbance measurements (8, 15–26). Although some previous nephelometric (8–14) and spectrophotometric (15, 21) studies have quantitated crystal mass with the aid of standard curves of cholesterol crystals, potential effects of crystal sizes or shapes on nephelometric or spectrophotometric values have not been evaluated.

In the present study we have compared chemical measurement of crystal mass with nephelometric, spectrophotometric, and microscopic quantification in model biles plotting in various zones of the ternary phase diagram. We also examined potential effects of decreasing crystal sizes by means of sonication on nephelometric and spectrophotometric values.

MATERIALS AND METHODS

Materials

Taurocholate (TC) and deoxycholate (DC) were obtained from Sigma Chemical Co. (St. Louis, MO) and yielded a single spot on thin-layer chromatography (butanol-acetic acid-water, 10:1:1, v/v/v, application of 200 μg bile salt). Cholesterol (Sigma) was ⩾98% pure by reverse-phase HPLC (isopropanol-acetonitrile, 1:1, v/v, detection at 210 nm). Egg-yolk phosphatidylcholine (EYPC) (Sigma) yielded a single spot upon thin-layer chromatography (chloroform-methanol-water, 65:25:4, v/v/v, application of 200 μg lipid). As shown by reverse-phase HPLC, EYPC contained mainly 16:0 acyl chains at the *sn*-1 position and

mainly unsaturated (18:1 > 18:2 > 20:4) acyl chains at the *sn*-2 position, similar to PC in human bile (27). All other chemicals and solvents were of American Chemical Society or reagent grade quality. The enzymatic cholesterol assay kit was obtained from Boehringer (Mannheim, Germany) and the enzymatic phospholipid kit from Sopar Biochem (Brussels, Belgium). 3α-hydroxysteroid dehydrogenase for the enzymatic measurement of bile salt concentrations (28) was purchased from Sigma.

Preparation of model biles

Lipid mixtures containing variable proportions of cholesterol, phospholipids (both from stock solution in chloroform), and bile salts (from stock solutions in methanol) were vortex-mixed and dried at 45°C under a mild stream of nitrogen, and subsequently lyophilized for 24 h before being dissolved in aqueous 150 mM NaCl plus 3 mM NaN_3. Tubes were sealed with Teflon-lined screw caps under a blanket of nitrogen to prevent lipid oxidation and vortex-mixed for 5 min followed by incubation at 37°C in the dark. The final molar percentages of cholesterol, phospholipids, and bile salts did not differ more than 1% from the intended molar percentages. Also, model systems always plotted in the intended zones of the appropriate phase diagram (4), as inferred from microscopic examination.

Lipid measurement. Cholesterol and EYPC concentrations were determined by enzymatic assays (29, 30). Bile salt concentrations were measured with the 3α-hydroxysteroid dehydrogenase method (28). Cholesterol saturation index (CSI) was calculated according to Carey's critical tables (31).

Quantitation of cholesterol crystal growth

Chemical determination of cholesterol crystal mass. Cholesterol crystal mass was determined daily during 21 days as described (32, 33). In case of coexistent cholesterol crystals and aggregated vesicles (i.e., central three-phase-micelles-, vesicles-, and crystals-containing zone) (Fig. 1), deoxycholate was first added in quantities sufficient to desaturate the model system (i.e., final CSI <1) in order to dissolve small unilamellar vesicles and vesicular aggregates. After 15 min incubation, both light microscopy and stability of turbidity measurements (absorbance at 405 nm) (24) revealed that all vesicular aggregates had been completely micellized. Cholesterol crystals were subsequently precipitated by ultracentrifugation during 30 min at 50,000 *g* and at 37°C in a TLS 55 rotor (Beckman, Palo Alto, CA) (34), and cholesterol content was determined after dissolving the pellet in isopropanol. We did not find remaining cholesterol crystals with microscopical examination of the supernatant. Experiments with isolated cholesterol monohydrate crystals showed that dissolution of the cholesterol crystals did not occur during the short incubation with deoxycholate. Also, addition experiments with cholesterol monohydrate crystals indicated full recovery. As model systems plotting in the left two-phase zone (Fig. 1) contain only micelles and cholesterol crystals at thermodynamic equilibrium, cholesterol crystals were precipitated in this case by ultracentrifugation, without added deoxycholate. Although studies by quasielastic light scattering spectroscopy (4) have suggested that small unilamellar vesicles may occur transiently in supersaturated model systems in the left two-phase zone before equilibrium is reached, amounts of cholesterol in these vesicles are expected to be minor. Indeed, amounts of micellar cholesterol [determined by highly selective 300 kDaA filter (32, 33)] and cholesterol crystal mass, determined as described above accounted for virtually all cholesterol in left two-phase model systems.

Nephelometry. Cholesterol crystal mass was measured daily for 21 days through light scattering (8–14). To avoid light scattering induced by cholesterol-phospholipid vesicles in case of model biles plotting in the central three-phase zone, the samples were 20-fold diluted with sodium deoxycholate (50 mmol/l) with subsequent incubation for 6 min at room temperature before measurement (final CSI always <1). In case of model biles plotting in the left two-phase zone, samples were diluted with equal volumes of aqueous 150 mM NaCl. Light scattering by cholesterol crystals was measured at 840 nm at an angle of 13–24° with a nephelometer (BN 100, Behring, Marburg, Germany). The apparent cholesterol crystal mass was calculated with the aid of a standard curve of cholesterol monohydrate crystals (8–14). The coefficient of variation of this method was <5%.

Spectrophotometry. The method of Sömjen et al. (24) was employed for spectrophotometric measurement of crystallization. In case of model biles plotting in the central three-phase zone, 50 μl biles were mixed in microplate wells with 150 μl NaCl 0.9% containing DC in quantities sufficient to desaturate the model system (final CSI always <1). After 15 min incubation and repeated shaking, stable turbidity values were reached and absorbance was measured at a wavelength of 405 nm. In case of model biles plotting in the left two-phase zone, absorbance was read after mixing 50 μl biles with 150 μl aqueous 150 mM NaCl.

Microscopy. Numbers and sizes of various cholesterol crystal shapes were determined by daily examination of 7 μl model biles during 21 days with the aid of a polarizing microscope and a KOVA Glasstic™ slide (Hycor Biomedical Inc., Garden Grove, CA) with 10 standardized examination chambers. Each chamber contains one large grid (3 × 3 mm), divided into 81 small grids (size 0.33 mm × 0.33 mm). Seven microliters from a model bile sample that was 10× diluted (because of abundant crystals in the left two-phase zone) was placed on a KOVA slide, crystal sizes and shapes were noted, and numbers were counted in nine consecutive small grids at a magnification of 100×. We found excellent reproducibility of this method with intra- and inter-assay variation of less than 10% and 15%, respectively.

Sonication and cholesterol crystals. Mature cholesterol monohydrate crystals (initial sizes 50–100 μm) were obtained by precipitation from 95% ethanol (35) or harvested from supersaturated model biles plotting in the left two-phase zone after 40 days incubation. Crystal sizes were subsequently progressively decreased by sonication of crystals dispersed in TC 150 mM (cholesterol crystal content concentration in all cases: 6 mM) at 100 W for 0, 5, 10, 20, 30, and 40 s using a Labsonic-L sonicator (B. Braun, USA). After each sonication step, numbers and sizes of crystals were assessed by microscopy and absorbance as well light scattering values were determined.

RESULTS

In **Fig. 2** cholesterol crystal mass is given as measured in the same biles by chemical determination in the pellet, by nephelometry, and by spectrophotometry. Results are shown for model biles with low, intermediate, or high cholesterol saturation (i.e., CSI 1.2, 1.7, 2.0) in the left two-phase zone with PL/(BS+PL) molar ratio of 0.05, in the left two-phase zone with PL/(BS+PL) molar ratio of 0.15 as well as in the central three-phase zone [PL/(BS+PL) molar ratio of 0.30, CSI = 2.0]. In **Fig. 3**, numbers of various cholesterol crystals are shown as function of incubation time.

With all methods, crystal mass is higher in case of more pronounced cholesterol supersaturation (in rank order: CSI 1.2 < 1.7 < 2.0). In the left two-phase zone at low phospholipid content [PL/(BS+PL) molar ratio of 0.05],

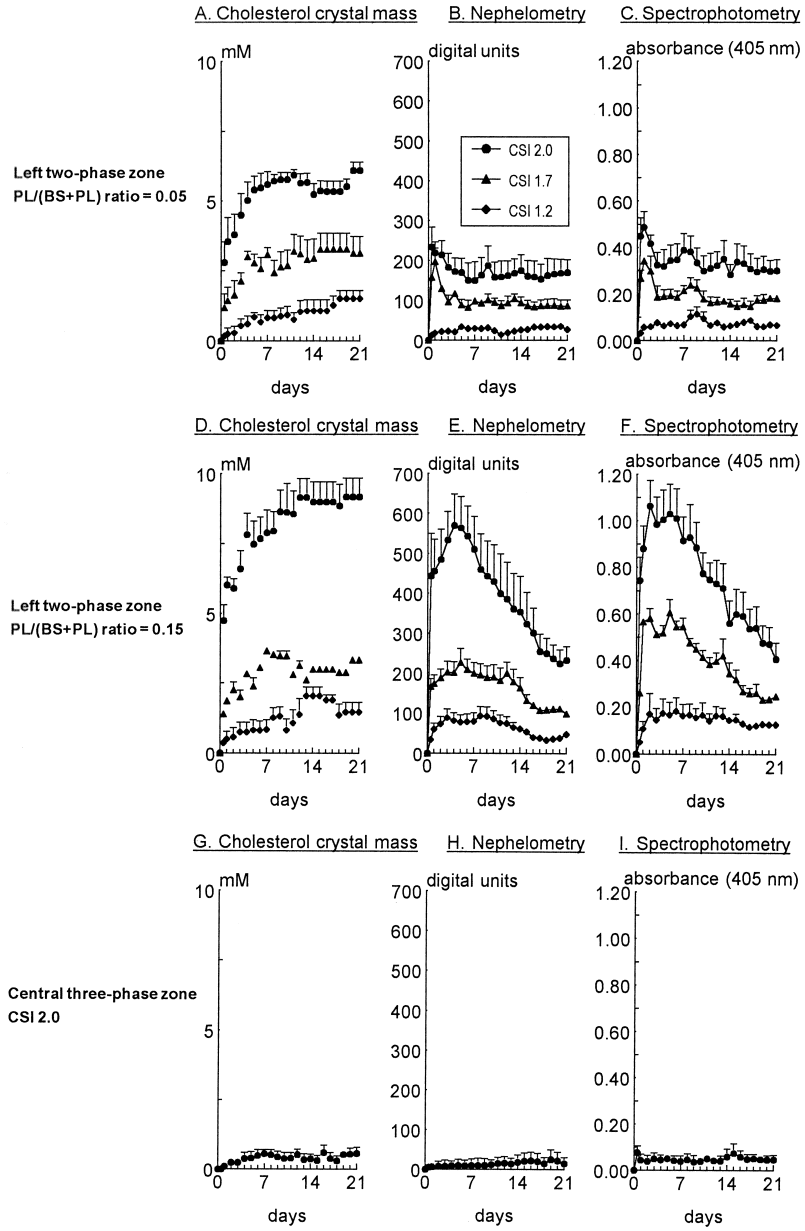

Fig. 2. Cholesterol crystallization as assessed by chemical determination (A, D, G), nephelometry (B, E, H), or spectrophotometry (C, F, I) in various model biles. In particular in model biles in the left two-phase zone with PL/(BS+PL) molar ratio of 0.15, there is a discrepancy between chemical determination and nephelometric or spectrophotometric readings (D vs. E and F). Mean ± SE.

there is a progressive increase of cholesterol crystal mass by chemical measurement as a function of incubation time (Fig. 2A). In contrast, in case of CSI 1.7 or 2.0, there is a minor increase in nephelometric or spectrophotometric readings during the first 24 h, but a decrease thereafter (Fig. 2B and C). By microscopy, there are progressive increases in numbers and sizes of cholesterol monohydrate crystals with time (Fig. 3A and B). Anhydrous crystals (arcs, needles, tubules) are abundant in the early stages in these model biles, but decrease strongly during longer incubation (Fig. 3C and D).

Discrepancies between cholesterol crystal content by chemical measurement and by nephelometry or spectrophotometry are more prominent in left two-phase model biles with higher phospholipid content [PL/(BS+PL) molar ratio of 0.15]. In these models there is a progressive increase of cholesterol crystal mass with time (Fig. 2D). In contrast, there are strong increases in both nephelometric and spectrophotometric readings during the first days of incubation, but progressive decreases at later stages (Fig. 2E and F). By microscopy, cholesterol monohydrate crystals increase progressively in numbers and sizes with time (Fig. 3E and F). It should be noted that large numbers of very small crystals (<10 μm) were seen during the early incubation days. At later stages these small crystals disappear, and there are increasing numbers of emerging mature

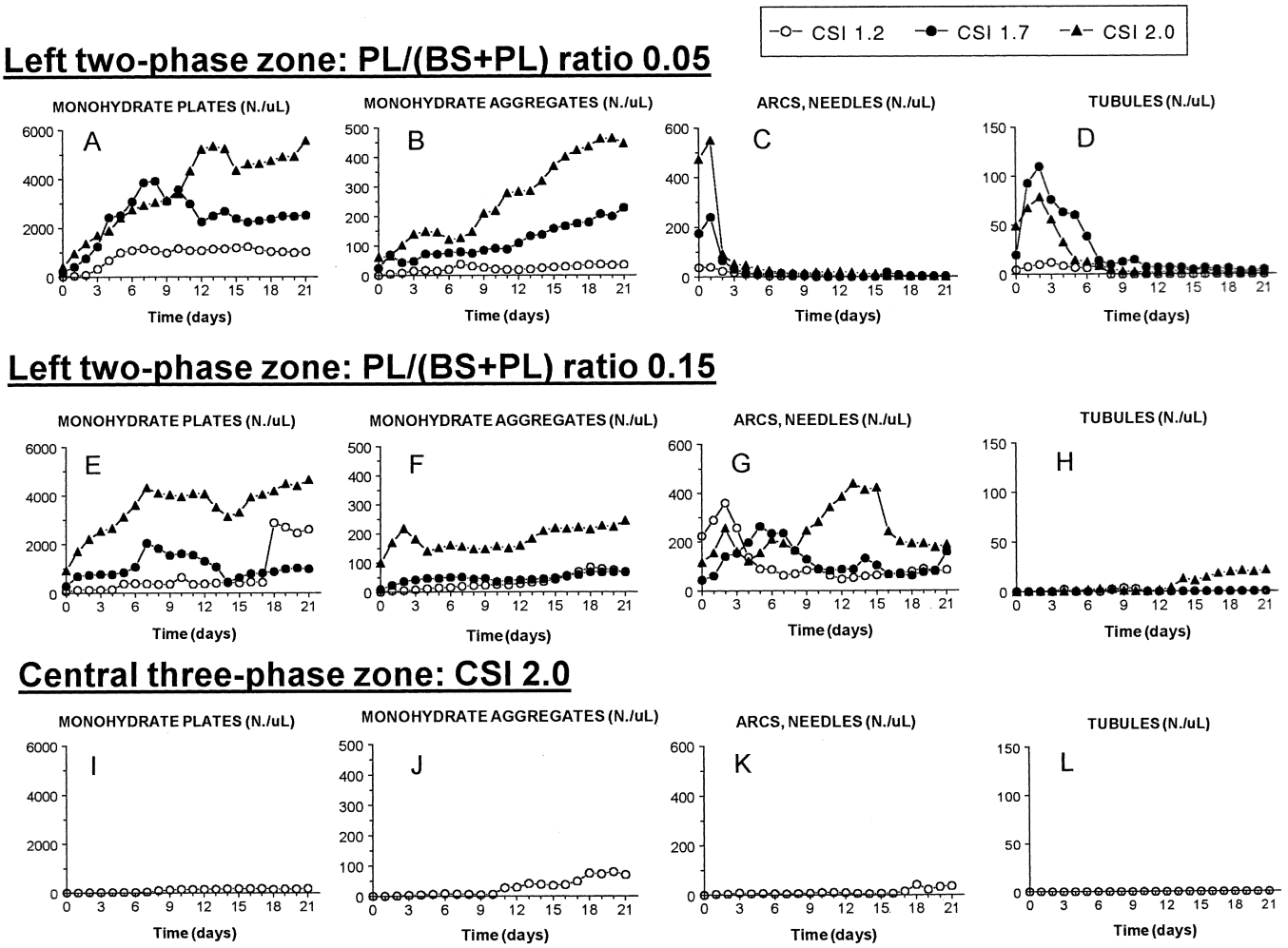

Fig. 3. Numbers of various cholesterol crystals (assessed by microscopy) as a function of incubation time in various model biles (see text).

cholesterol monohydrate crystals of progressively increasing sizes (maximal size at CSI = 2.0: ~150 μm at the end of the study). Arcs and needles are present at relatively low numbers throughout the study period and tubules appear only at the end of the incubation (Fig. 3G and H).

In the central three-phase zone, cholesterol crystal mass determinations by chemical analysis, by nephelometry, and by spectrophotometry all yield very low values throughout the 21-day study period (Fig. 2G, H, and I). In this zone, there are only small amounts of mature cholesterol monohydrate crystals (Fig. 3I and J) and anhydrous crystals (Fig. 2K and L, arcs, needles, tubules) are exceptional and small.

As shown in **Fig. 4A–C**, upon progressive sonication of harvested cholesterol monohydrate crystals, increasing absorbance measurements (by spectrophotometry) coincide with increasing crystal numbers and decreasing crystal sizes. Essentially the same results were obtained with nephelometry (Fig. 4D).

DISCUSSION

Since nucleation and growth of cholesterol crystals are important steps in cholesterol gallstone formation (1), accurate quantitation of crystallized cholesterol is essential in studies on gallstone pathogenesis. In previous publications, onset of crystallization and crystal growth have been quantitated indirectly by means of nephelometry (8–14) or spectrophotometry (8, 15–26). With these methods, amounts of scattered or absorbed light are assumed to relate directly to cholesterol crystal mass. Based on this assumption, standard curves of cholesterol monohydrate crystals were employed (8–15, 21), without consideration of the distinct possibility that in model biles of varying composition, variations in sizes, or shapes of the crystals might affect light scattering and absorbance.

We therefore related amounts of cholesterol crystals by nephelometry and spectrophotometry during 21-days incubation of various supersaturated model biles to chemical measurement of crystal mass and to crystal sizes and shapes by microscopy. With all methods, crystallization was much more extensive in model biles plotting in the left two-phase (micelles- and crystals-containing) zone than in the central three-phase (micelles-, vesicles-, and cholesterol crystals-containing) zone, despite identical cholesterol saturation (CSI = 2.0) (Fig. 2). This can easily be explained by the fact that in the central three-phase zone, considerable amounts of cholesterol are solubilized in vesicles (4).

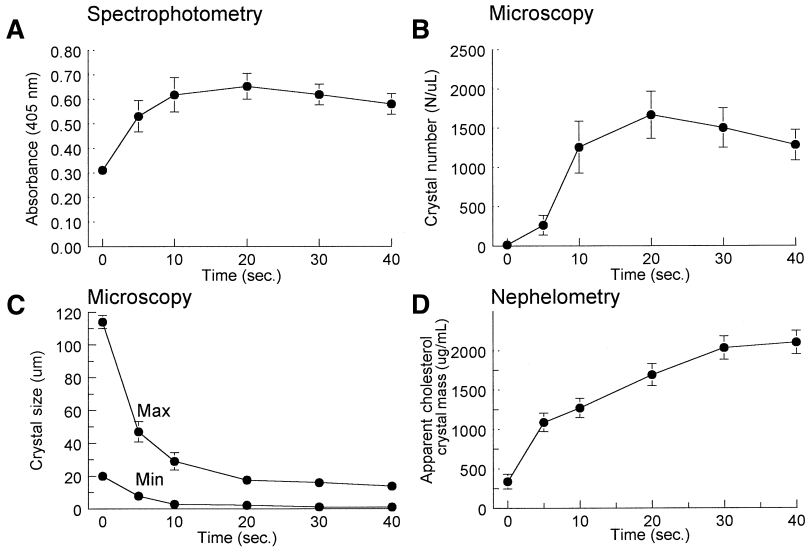

Fig. 4. Effect of sonication on spectrophotometric values (A), crystal numbers (B), and sizes (C). There is an increased absorbance despite identical crystal mass, coinciding with increased crystal numbers and sizes. D: Apparent crystal mass by nephelometry (with the aid of cholesterol crystal standard curve) increases progressively with longer sonication.

In the left two-phase (micelles + crystals-containing) zone, chemical measurement of cholesterol crystal mass yielded progressively increasing values throughout the 21-day study period, particularly at higher phospholipid contents [PL/(BS+PL) molar ratio = 0.15]. Light absorbance and scattering, however, showed an early increase during the first week followed by a late decrease (Fig. 2D–F).

Simultaneous microscopy indicated that these model biles initially contained a large number of very small crystals that were replaced after the first week by larger, but less numerous, crystals. We also performed sonication experiments with harvested cholesterol monohydrate crystals. During sonication, crystals progressively decreased in size, with increased spectrophotometric and nephelometric readings as a result, despite identical crystal mass (Fig. 4). Small crystals apparently absorb more light than large crystals. Since "ideal" absorbing or scattering spherical particles are lacking, the physics of light interaction with matter (36) might not totally apply in model biles and no theoretical deduction can be made from the relation between cholesterol crystal shape and crystal mass (15).

Immunoglobulins IgM and IgG (9, 10, 18, 21, 23, 37), α1-acid glycoprotein (9, 18, 20), aminopeptidase N (9, 38, 39), haptoglobin (23), α1-antichymotrypsin (12, 23), some glycoproteins (12, 15, 16, 19), and mucin (13, 40, 41) are regarded as pronucleating proteins. By contrast, human apolipoprotein A-I (15), some glycoproteins (17), and IgA (22, 25) have been postulated to exert antinucleating activity.

These conclusions were based on different methods, including polarizing microscopy (9, 37, 39, 40), nephelometry (9–13), or spectrophotometry (16–23, 25). Our present findings suggests that the in vitro studies with nephelometry or spectrophotometry should be reevaluated. In this respect it is also relevant that a growing number of publications have marshalled experimental evidence arguing against a role of biliary proteins in cholesterol gallstone formation. In a recent study on a large number of gallstone patients, the speed of cholesterol crystallization correlated in an univariate analysis with cholesterol saturation, protein, immunoglobulins, α1-acid glycoprotein, and aminopeptidase N, but stepwise logistic regression analysis revealed that only cholesterol saturation correlated independently with the speed of crystallization (42). Furthermore, Wang et al. (43) showed that after the extraction of biliary lipids from human bile and their reconstitution in buffer solution, the resulting model system displayed the same speed and pattern of crystallization as the original bile sample. We also recently found in the inbred mouse model that "pronucleating" proteins in bile generally decrease during the earliest stages of gallstone formation (44), arguing against an appreciable role of biliary proteins in gallstone pathogenesis.

In summary, morphology and size of cholesterol crystals strongly affect nephelometric and spectrophotometric estimates of crystal mass with lower readings for larger crystals. Chemical measurement of crystal mass should be considered the method of choice for quantitation of cholesterol crystallization.

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