STABILITY OF MIXED MICELLAR BILE MODELS SUPERSATURATED WITH CHOLESTEROL

D. LICHTENBERG,* S. RAGIMOVA,* A. BOR,* S. ALMOG,* C. VINKLER,* M. KALINA,‡ Y. PELED,§ AND Z. HALPERN§

Departments of Physiology and Pharmacology, Histology,‡ and Gastroenterology,§ Sackler Faculty of Medicine, Tel Aviv University Ramat Aviv 69978, Israel*

ABSTRACT The maximal equilibrium solubility of cholesterol in mixtures of phosphatidylcholine (PC)[1] and bile salts depends on the cholesterol/PC ratio (R_c) and on the effective ratio (R_e) between nonmonomeric bile salts and the sum (C_T) of PC and cholesterol concentrations (Carey and Small, 1978; Lichtenberg et al., 1984). By contrast, the concentration of bile salts required for solubilization of liposomes made of PC and cholesterol does not depend on R_c (Lichtenberg et al., 1984 and 1988). Thus, for $R_c > 0.4$, solubilization of the PC-cholesterol liposomes yields PC-cholesterol-bile salts mixed micellar systems which are supersaturated with cholesterol.

In these metastable systems, the mixed micelles sponstaneously undergo partial revesiculation followed by crystalization of cholesterol. The rate of the latter processes depends upon R_c, R_e, and C_T. For any given R_c and R_e, the rate of revesiculation increases dramatically with increasing the lipid concentration C_T, reflecting the involvement of many mixed micelles in the formation of each vesicle. The rate also increases, for any given C_T and R_e, upon increasing the cholesterol to PC ratio, R_c, probably due to the increasing degree of supersaturation. Increasing the cholate to lipid effective ratio, R_e, by elevation of cholate concentration at constant R_c and C_T has a complex effect on the rate of the revesiculation process. As expected, cholate concentration higher than that required for complete solubilization at equilibrium yields stable mixed micellar systems which do not undergo revesiculation, but for lower cholate concentrations decreasing the degree of supersaturation (by increasing [cholate]) results in faster revesiculation. We interpret these results in terms of the structure of the mixed micelles; micelles with two or more PC molecules per one molecule of cholesterol are relatively stable but increasing the bile salt concentration may cause dissociation of such 1:2 cholesterol:PC complexes, hence reducing the stability of the mixed micellar dispersions.

The instability of PC-cholesterol-cholate mixed systems with intermediary range of cholate to lipids ratio may be significant to gallbladder stone formation as: (*a*) billiary bile contains PC-cholesterol vesicles which may be, at least partially, solubilized by bile salts during the process of bile concentration in the gallbladder, resulting in mixtures similar to our model systems; and (*b*) the bile composition of cholesterol gallstone patients is within an intermediary range of bile salts to lipids ratio.

INTRODUCTION

Under physiological conditions biliary cholesterol is efficiently solubilized by bile salt-phospholipid mixtures either in the form of mixed micelles (1) or vesicles (2). However, quite frequently gallbladder stasis develops, due to cholesterol crystallizing in the gallbladder in the form of cholesterol gallstones. It is generally believed that cholesterol precipitates from bile only when the bile is supersaturated with cholesterol. Nevertheless, the absolute degree of supersaturation is not the sole parameter which governs the precipitation. Various promoters of crystallization, (heterogeneous nucleation seeds) as well as a variety of crystallization-inhibitors, may also play an important role (3). This, however, does not diminish the possible importance

Please address all correspondence to Dr. Lichtenberg.

Part of this work constitutes a part of a thesis to be submitted by C. Vinkler as partial fulfillment of the requirement of Tel-Aviv University toward an M.D. degree.

[1]Abbreviations used in this paper: PC, Phosphatidylcholine (from egg yolk). $[BS]^a$, Total bile salt concentration required for complete solubilization (micellization) of a vesicular lipid preparation. C_T, The sum of PC and cholesterol concentrations C_T = [PC] + [cholesterol] CMC, critical micellar concentration IMC, Intermicellar concentration; the concentration of surfactants present in a surfactant-containing dispersion (or solution) as monomers. Apparent cmc, The minimal concentration of surfactant required for solubilization of any lipid. R_e, (Effective ratio) Ratio of nonmonomeric bile salt to other lipids in a given dispersion (for micellar solutions: R_e = ([BS]-cmc)/C_T). R_e^a, The value of R_e at which solubilization is complete (critical solubilizing effective ratio). R_c, The ratio of cholesterol to PC in a mixed dispersion (or solution) containing these two components. $R_e^{a,eq}$, The critical effective ratio of bile salt to lipids (R_e) above which a solubilized (mixed micellar) lipid-bile salt system is in a state of equilibrium. R_e^{met}, The critical effective ratio of bile salts to lipids below which a metastable mixed micellar system is stable on a time scale of up to 24 h (see text for details; note: $R_e^a < R_e^{met} < E_e^{a,eq}$). OD, optical density.

of the degree of cholesterol supersaturation. Accordingly, much work has been devoted to investigating the limit of cholesterol solubility in equilibrated bile models which yielded detailed phase diagrams for bile salt-cholesterol-lecithin mixtures (1). We have previously suggested that the phase diagrams be analyzed in terms of the effective ration, R_e, between the concentration of nonmonomeric bile salt (which is about equal to the difference between the total concentration of bile salt and its critical micellar concentrations, [cmc]) and the sum of concentrations of the other biliary lipids (here denoted C_T; C_T = ([Phosphatidylcholine] + [cholesterol]) (4,5). For complete solubilization to occur, the ratio R_e should exceed a critical value, (R_e^s), which is independent of total lipid concentration. Yet, this value of R_e depends on the cholesterol to PC ratio (here denoted R_c). At low values of R_c (up to $R_c \simeq 0.2$), R_e^s is low ($R_e^s < 1$) and is only slightly dependent on R_c, whereas at higher cholesterol to PC ratios R_e^s depends strongly on R_c (for $R_c = 0.5$, R_e should be higher than 7).

A cholesterol to PC ratio (R_c) of 0.5 is quite frequent in human bile [6-9, Z. Halpern and Y. Peled, unpublished results]. For cholesterol and PC concentrations of 10 and 20 mM, respectively, complete solubilization (at an equilibrium state) therefore requires BS concentration in excess of 210 mM, which is not very frequent in human bile (6-9, Z. Halpern and Y. Peled, unpublished results). On the other hand, we have previously shown that complete solubilization of a vesicle dispersion made of 10 mM cholesterol and 20 mM PC requires only 30 mM bile salt (4). The resultant mixed micelles therefore constitute a metastable state of aggregation of the cholesterol-PC-BS mixture. Consequently, the initial solubilization of the vesicles is followed by a subsequent spontaneous phase transformation, which may be relevant to the processes leading to cholesterol precipitation in vivo.

In view of the possible effect of the degree of supersaturation on the rate of cholesterol precipitation, we found it of interest to investigate the stability of model systems with varying concentrations of cholesterol, PC, and cholate made by cholate-solubilization of PC-cholesterol vesicles. The results of this study are reported in this communication.

MATERIALS AND METHODS

Cholesterol (Sigma Chemical Co., St. Louis, MO) was crystalized from ethanol. Sodium cholat (Sigma Chemical Co.) was used without further purification. PC was prepared from egg yolks, according to Singleton et al. (10). Its purity was confirmed by thin layer chromatography. Bile salt concentrations were assayed by the enzymatic method of Turnberg and Anthony-Mote (11), using α-hydroxysteroid dehydrogenase and β-NAD^+ (Sigma Chemical Co.). Phosphatidylcholine concentration was measured by the method of Stewart (12), and cholesterol concentration as described by Zlatkis and Zak (13).

PC-cholesterol vesicles were prepared by sonication of a lyophilized mixture of these two components in saline solutions (150 mM NaCl), adjusted to pH 7.5 ± 0.1 at PC concentration of 40 mM and varying cholesterol concentrations. Sonication was done by a Heat Systems 350 V sonicator, at 4°C under a stream of nitrogen (14). After sonication and low speed centrifugation (to remove metallic particles), the dispersions were analyzed for PC and cholesterol.

In most experiments, the vesicular dispersions were solubilized by mixing them with equal volumes of cholate solutions. The turbidity of the mixture was then followed continuously by a probe colorimeter (Brinkman Instruments Co., Westbury, NY) immersed in the stirred solution and connected to a strip chart recorder. In several experiments, the turbidity was not followed continuously, but measured at various times using a spectrophotometer (Shimadzu Scientific Instruments Inc., Kyoto, Japan (at 570 nm). Experiments were carried out in saline solutions (150 mM NaCl) at pH 7.5 ± 0.1 and room temperature, unless otherwise stated. Nuclear magnetic resonance measurements were made as described by Almog et al. (15).

For microscopic observations, a drop of a mixed dispersion was pippeted on a glass slide and covered with a number 1 glass coverslip. Birefringence was detected using a WL Zeiss microscope equipped with a polarizer and an analyzer. Specimens were also observed with a Leitz Ortholus epifluorescence microscope with differential interference phase contrast (DIC) equipped with a Leitz Vario-Orthomat Camera using a Leitz 100/1.32 oil immersion objective. Kodak Tri-X film was developed to 1,600 ASA with Diafine developer.

For electron microscopic examinations, mixed dispersions were placed on gold planchets and rapidly frozen by plunging into liquid Freon. Fracturing, etching, and shadowing were performed in a Balzers BAF 300 Freeze-etch apparatus under a vacuum of 2×10^{-6} m bar. Fracturing was done at −100°C followed by etching by sublimation for 30 s. Platinum was shadowed at a 45° angle to a depth of 20 nm. A 250 Å carbon film was then applied to the replica. The original sample was cleaned from the replicas by floating on Clorox bleach for 24 h. The replicas were examined in a Philips 300 electron microscope operating at 60 kV and 4,000-25,000 magnifications. The same microscope was used to observe the dispersion after negative staining by 2% amonium molibdate using the same procedure as in (15).

RESULTS

As stated above, a cholesterol-phospholipid mixture in which $R_c = 0.5$ is expected to be solely micellar at equilibrium only when $R_e > 7$ (1,4). In contrast, complete solubilization of PC-cholesterol liposomes with $R_c = 0.5$ (33 mol % cholesterol) occurs at $R_e^s \simeq 0.6$ [4, Lichtenberg, D., N. Younis, and T. Kushnir, manuscript submitted for publication]. For mixed micellar systems of PC, cholesterol and cholate the value of R_e is $\simeq R_e = ([\text{cholate}]-9)/C_T$, where concentrations are expressed in millimolar units (the IMC of cholate is approximately equal to the apparent cmc of 9 mM). Accordingly, when cholate is added to a liposomal preparation made of 20 mM PC and 10 mM cholesterol, the resultant mixed micellar system can be expected to be at equilibrium only if the final cholate concentration exceeds 219 mM, although a (metastable) mixed micellar state is obtained for any cholate concentration higher than 27 mM.

Fig. 1 presents the results of experiments in which PC-cholesterol liposomal dispersions (containing 20 mM cholesterol and 40 mM PC) were mixed with equal volumes of cholate solutions of various concentrations. When the final cholate concentration was 250 mM, the solubilization resulted in an immediate decrease of turbidity OD = 5% of the turbidity of the liposomal dispersion of 20 mM PC and 10 mM cholesterol) and the turbidity remained unaltered for at least 8d (Fig 1, curve A). In

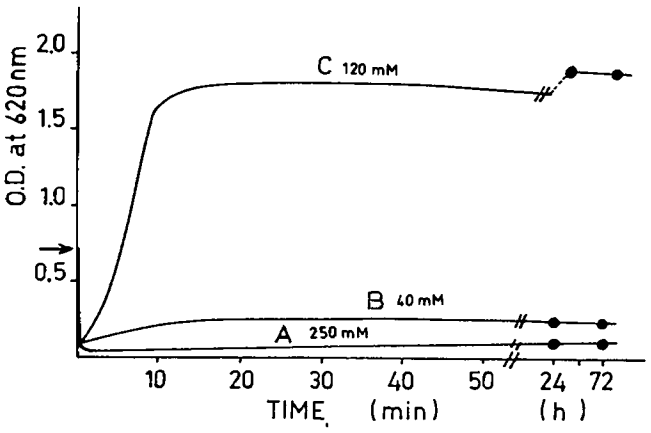


FIGURE 1 Time dependencies of the turbidity of PC-cholesterol-cholate mixed systems. Sonicated vesicle dispersions (1 ml) containing 40 mM PC and 20 mM cholesterol (with a turbidity indicated by the arrow at OD 0.7) were mixed at time zero with 1 ml cholate solutions so that the final concentrations of PC and cholesterol were 20 and 10 mM respectively, and the final cholate concentrations were as indicated in the figure. During the first 50 min after preparation, the turbidity of each of the (*stirred*) systems was measured continuously. The turbidity measurements performed after longer periods of time were also carried out on stirred dispersions. Without stirring, precipitation occured in the dispersion with 120 mM cholate (not shown). Note the change in time scale after 50 min of incubation.

contrast, when the solubilization was carried out such that the final cholate concentration was 40–200 mM, the very rapid decrease of turbidity was followed by a relatively slow increase of OD. The rate of the turbidity increase depended on the cholate concentration, as described for several cholate concentrations in Fig. 1. As seen in this figure, when the final cholate concentration was 40 mM the turbidity increased only slightly (*curve B*) and the ^{31}P-NMR spectra (not shown) were quite typical for micellar PC (16, 17).

In contrast, when the final cholate concentration was 120 mM, the turbidity increased markedly and quite rapidly, and an apparently constant turbidity was observed after several minutes (*curve C*). In the first several hours after mixing of the cholate solution with the vesicle dispersions, the measured turbidity and the rate of turbidity increase depended only slightly on whether or not the dispersions were mixed while being measured. However, after prolonged periods of time (many hours to days), precipitates were observed in the mixture which obviously did not contribute to the turbidity of the dispersions unless stirred.

Immediately after mixing, a sample examined by light microscopy did not contain sufficient large particles to be observed but such particles were formed in 15 min (Fig. 2 *A*). Subsequently, their number increased (Fig. 2 *B*) and they began to aggregate (Fig. 2 *C*). Electron microscopy revealed a few vesicles of diameters of ~30 nm immediately after mixing and many more were present 10 min later (not shown). Ninety minutes after preparation the dispersion already contained much birefringent material (Fig. 2*D*) and one day after preparation, the suspension contained lipid "blubs" with much birefringent material (Fig. 2*E*). A day later, electron microscopy revealed heterogeneous population of vesicles with 1–10 lamellae and diameters of 50 nm – 1.5 μm (Fig. 2*F*). At this point, electron microscopy of the negatively-stained dispersion revealed crystals of 1–10 μm (Fig. 2*G*) and five days after mixing, larger crystals were observed under polarized light (Fig. 2*H*).

We interpret these kinetic observations as follows:

(*a*) The initial drop of turbidity is due to solubilization of the PC-cholesterol vesicles, i.e., immediately after cholate addition the dispersion contained mostly if not exclusively PC-cholesterol-cholate mixed micelles.

(*b*) Solubilization is followed by a subsequent partial reformation of vesicles. At this stage the dispersion was already turbid, it contained some large (and partially aggregated) vesicles but it gave rise to a ^{31}P-NMR signal of a halfwidth of ~50 Hz, similar to that of other micellar solutions [16,17]. At this stage (40 min after mixing) the dispersion (containing 20 mM PC, 10 mM cholesterol, and 120 mM cholate) was centrifuged at 160,000 *g* for 3 h., to yield a transparent supernatant, which contained only 80–85% of the total PC, 50–60% of the cholesterol and a much higher fraction (97–99%) of the cholate. These results show that the ratio of cholesterol to PC in the precipitate was ~1.0–1.3, while in the supernatant the cholesterol to PC ratio was ~0.3. The cholate to lipid ratio in the precipitate was lower than 0.35. (*c*) The further increase of turbidity, along with precipitation and appearance of birefringent material are probably due to vesicle aggregation followed by formation of cholesterol crystals. This process is similar to that described by Kibe et al. [18], Halpern et al. [19], and Mazer and Carey [20] for model systems of supersaturated bile prepared by heating hydrated coprecipitated mixtures of the three components.

The stability of mixed micellar supersaturated dispersions depends upon the total lipid concentration (C_T), the cholesterol, and <70 mM cholate are more stable than lipid ratio (R_c). Since these three factors are interdependent, we had to ensure that the effect of each of them be studied under conditions where the other two were kept constant. Fig. 3 describes the effect of R_c on the turbidity of PC-cholesterol-cholate mixed micellar systems, as measured 40 min after preparation. It is quite clear from this figure that dispersions made of 20 mM PC, 10 mM cholesterol, and $\lesssim$70 mM cholate are more stable than systems with the same concentrations of PC and cholesterol (same R_c and C_T), but with higher cholate concentrations. However, when the cholate concentration is raised above 220 mM the micellar system becomes stable and no revesiculation occurs for at least 8d.

Of special interest is the effect of temperature on the stability of the solubilized lipid dispersions. At room

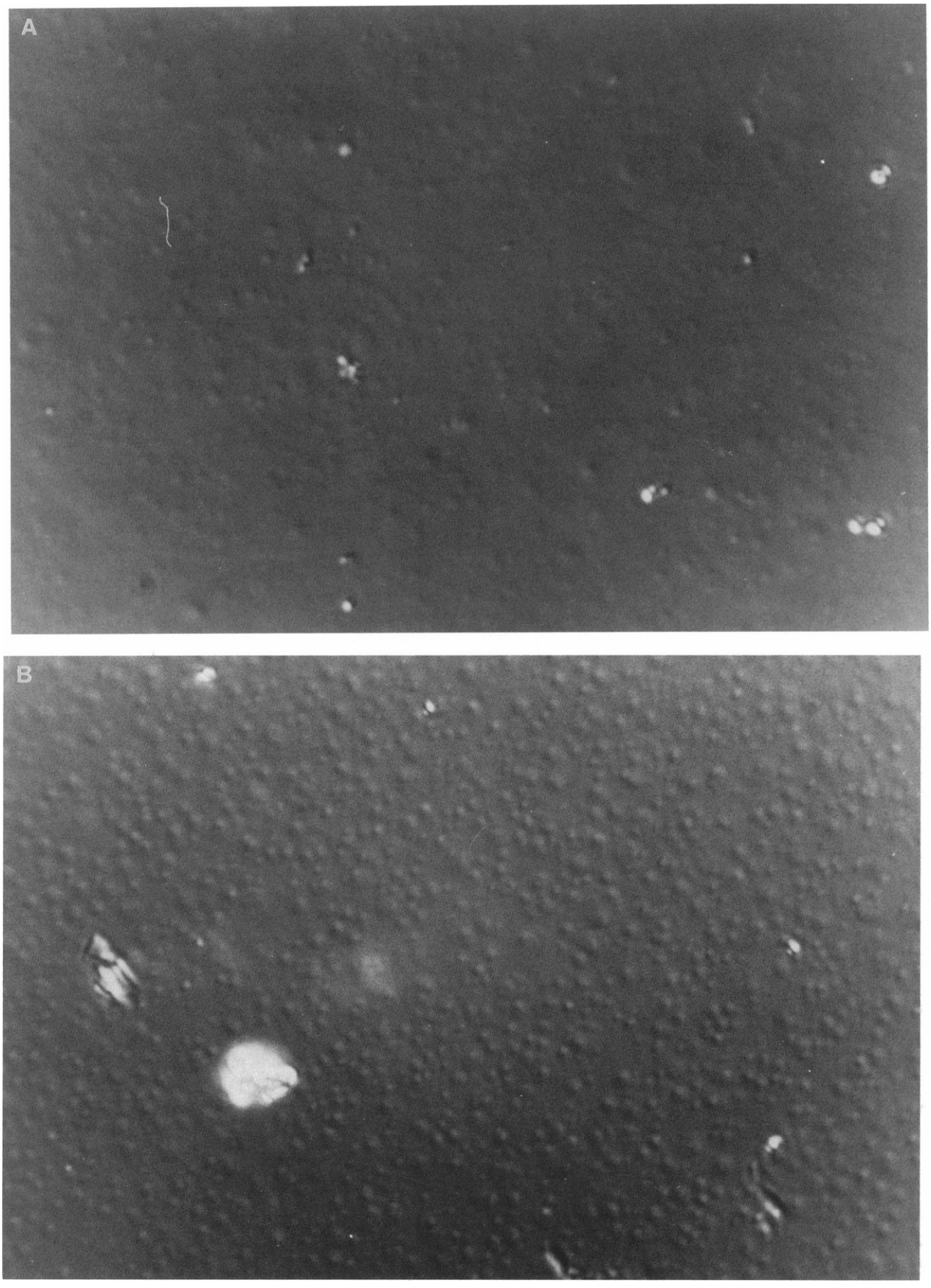
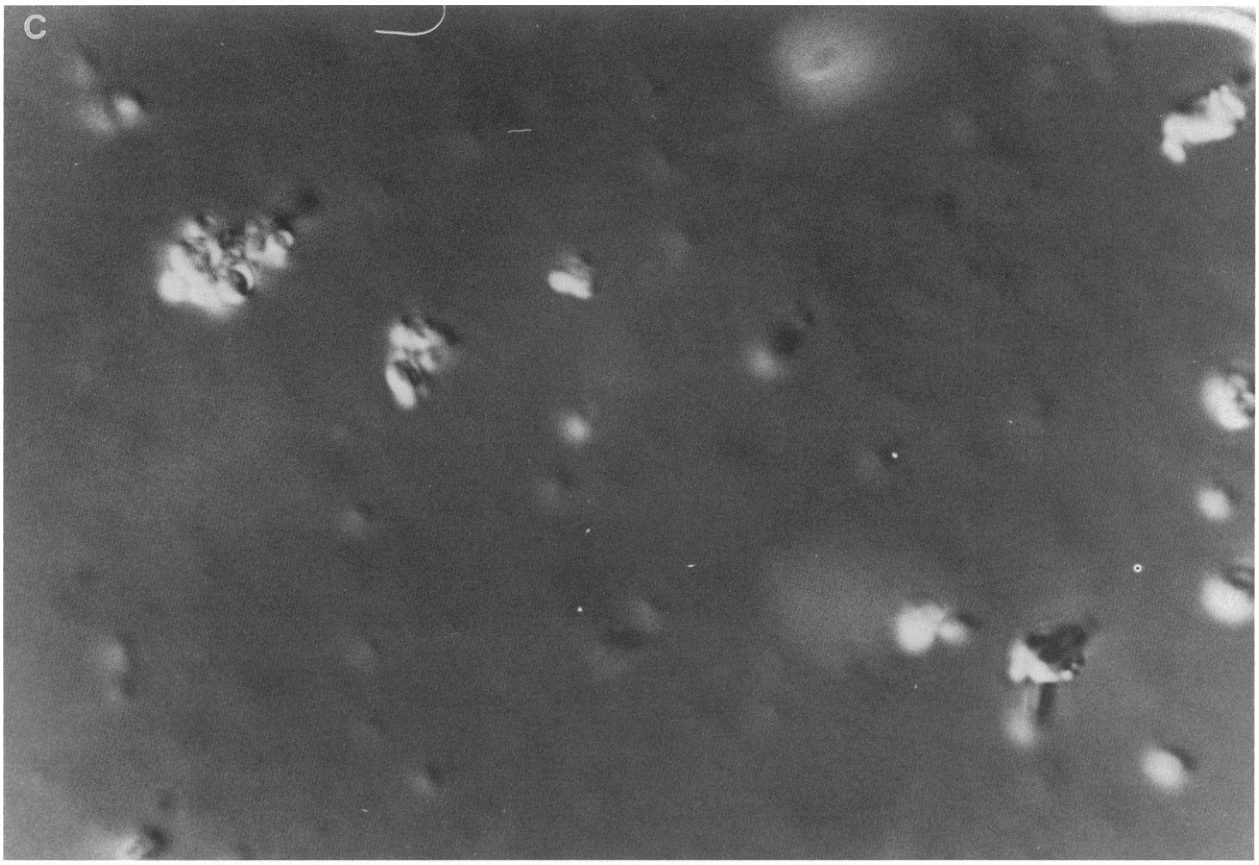
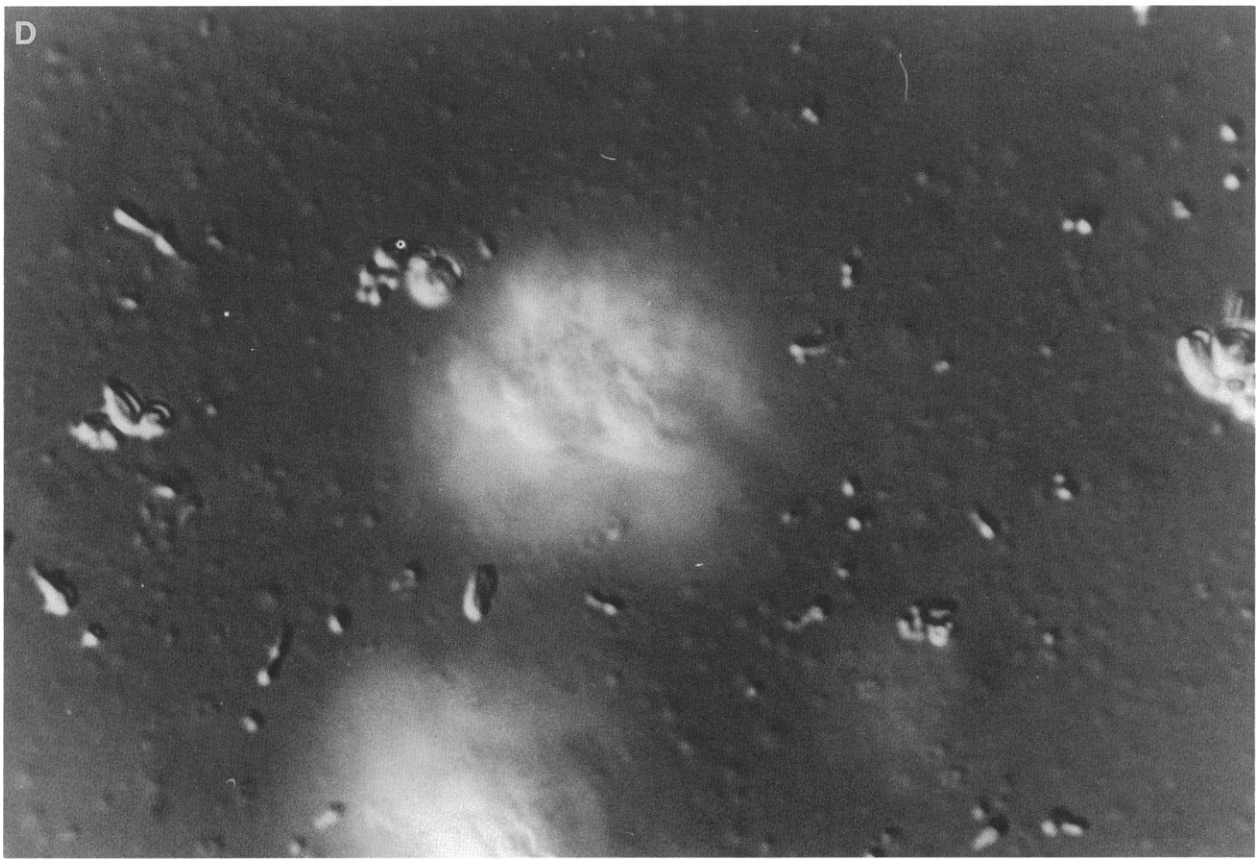


FIGURE 2 Micrographs of the mixture of Fig. 2 C composed of 20 mM PC, 10 mM cholesterol, and 120 mM cholate, at different times after preparation. Magnifications are as indicated on the photographs. *A–E* are DIC Micrographs (see Methods) taken 20 min (*A*), 40 min (*B*), 60 min (*C*), 90 min (*D*), and 20 h (*E*) after mixing the vesicle dispersion with the bile salt solution. *F* is an electron micrograph of a freeze-etched dispersion and *G*, of a negatively-stained dispersion, both made 45 h after preparation. *H* is a micrograph of the dispersion 5 d after preparation, taken with cross-polarization, using the WL Zeiss microscope.





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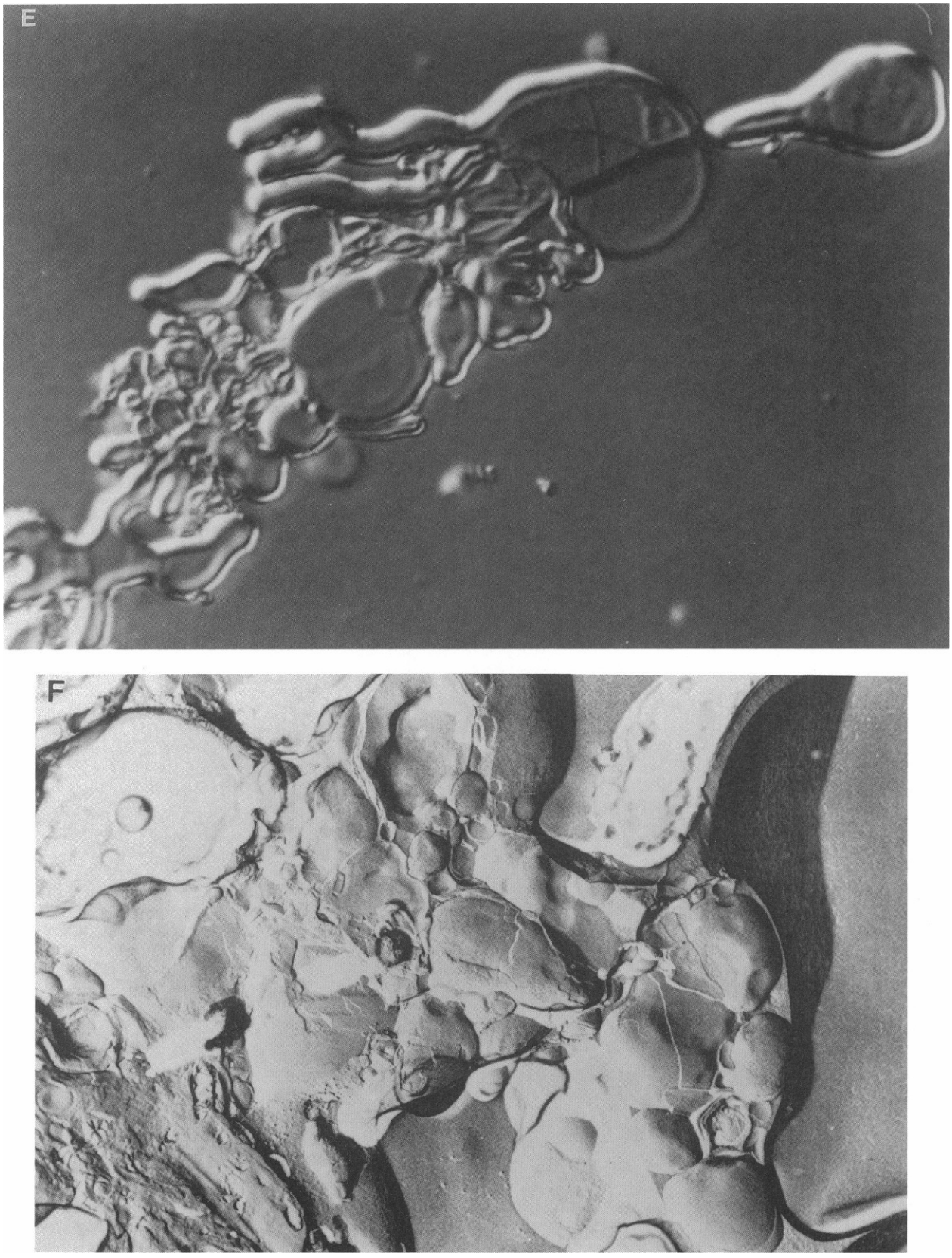

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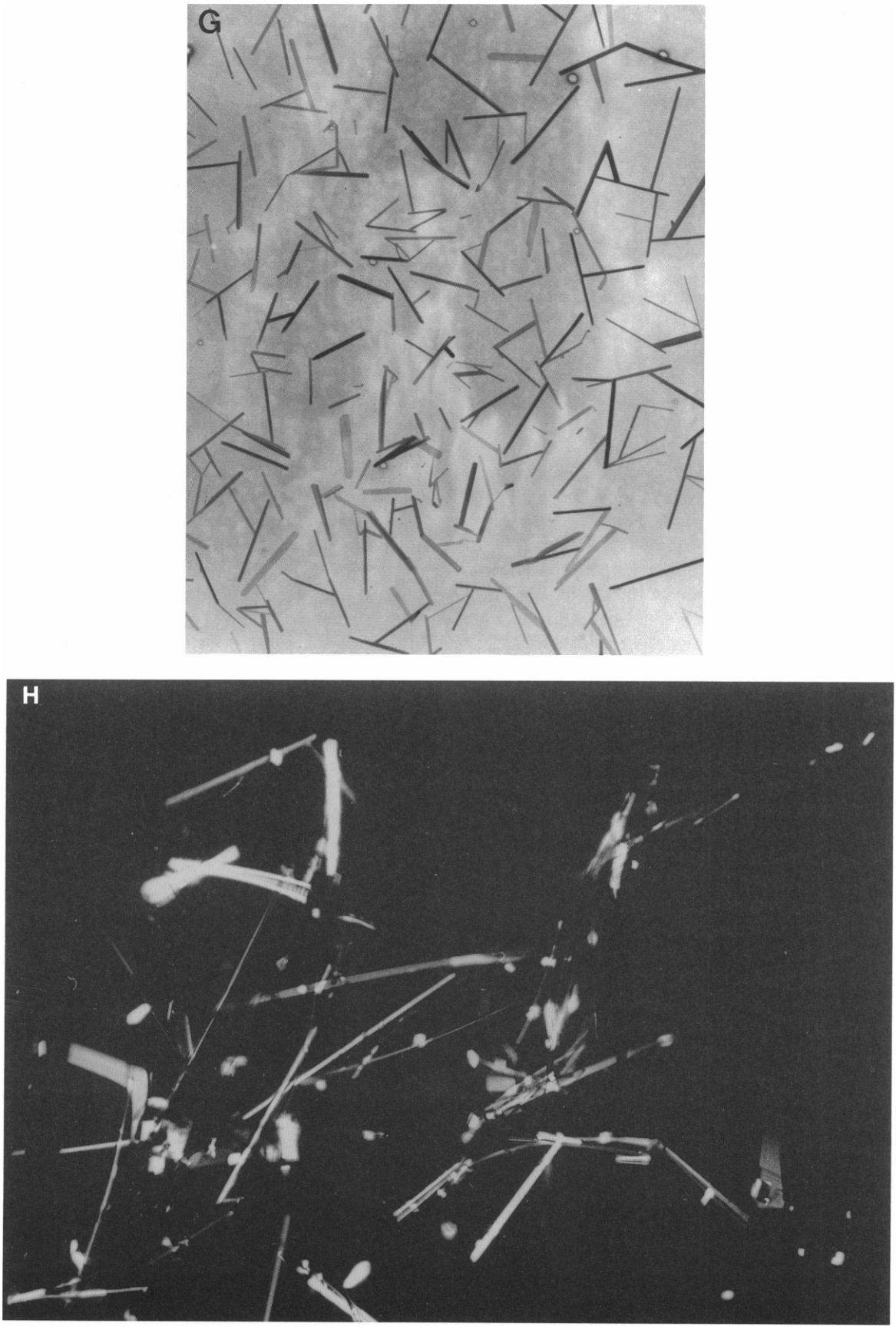

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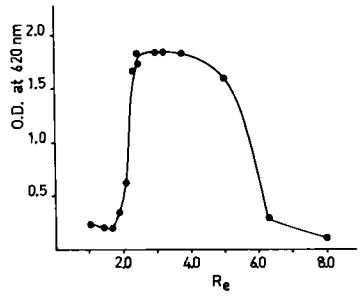

FIGURE 3 Dependence of the turbidity of PC-cholesterol-cholate systems (made as in Fig. 1) on the effective ratio R_e. All the dispersions contained final concentrations of 20 mM PC, 10 mM cholesterol, and varying cholate concentrations (expressed in terms of R_e). The turbidity described in this figure is as measured 40 min after mixing the PC-cholesterol vesicles with the respective cholate solutions, when an apparent equilibrium was established. The increase of turbidity, observed in systems with $2.25 < R_e < 5.00$, begins only after a latency of ~2 min and is characterized by a half life of 3–5 min (not shown).

temperature these dispersions are relatively stable up to an R_e value of ~2.8 (80 mM cholate in mixtures with 16.8 mM PC and 8.4 mM cholesterol). Mixtures with higher R_e values become turbid at a rate which increases with increasing the cholate content of the system. This effect is exemplified by the large increase of the rate of turbidity-increase observed when the cholate concentration in a mixture with 16.8 mM PC and 8.4 mM cholesterol is raised from 85 mM (R_e = 2.7; *curve C* in Fig. 4) to 90 mM (R_e = 3.2; *curve B* in Fig. 4). Increasing the temperature results in an increase of the limit of metastability. Thus, at 40°C, a solubilized dispersion containing 16.8 mM PC and 8.4 mM cholesterol becomes unstable only when it contains more than 110 mM cholate (R_e = 4.0; Fig. 4 *curve E*). This marked effect of temperature is in fact very informative as will be shown in the Discussion.

For dispersions made of lower PC and cholesterol concentrations at the same molar ratio (R_c = 0.5), we studied the rate of revesiculation after solubilizing the vesicles with various cholate concentrations at the same R_e of 3.7 ([BS] = 3.7 · C_T + 9). From the data presented in Fig. 5, it is very clear that the rate of revesiculation is markedly dependent on C_T.

To study the effect of the cholesterol:PC ratio, R_c, we changed both [PC] and [cholesterol] while keeping the sum of their concentrations (C_T) and [cholate], thus R_e, constant. Results obtained at C_T = 30 mM and R_e = 3.7, plotted as a function of R_c, demonstrate that increasing the cholesterol to PC ratio resulted in an increase of the rate of vesicle formation (Fig. 6).

In an attempt to evaluate the stability of supersaturated mixed-micellar systems to changes in the cholate to lipid effective ratio (R_e), we carried out the following experiments: First, we formed a micellar system with R_e = 3.7 by adding crystalline sodium cholate to a vesicular dispersion with 20 mM PC and 10 mM cholesterol. The resultant dispersion with 120 mM cholate was unstable and the time course of the increase of turbidity (Fig. 7, *curve A*) was essentially identical to that obtained by preparing a mixture of the same composition by mixing equal volumes of a vesicle dispersion and a cholate solution (Fig. 1 *C*). In contrast, a system made by addition of cholate to a concentration of 70 mM (R_e = 2.03) remained unaltered for at least 2 h after further addition (at point II) of cholate, to a final cholate concentration of 120 mM (Fig. 7, *curve C*). Thus, the dispersion made by this two step cholate addition to the PC-cholesterol vesicles was very different in terms of its turbidity from a dispersion of the same composition made by adding the same amount of cholate in only one step (Fig. 7, *curve A*).

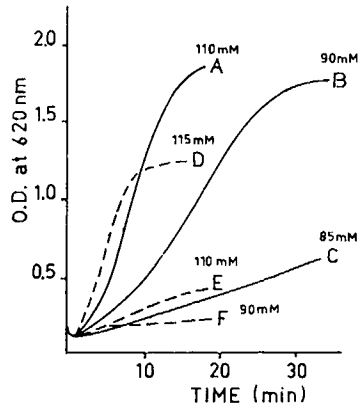

FIGURE 4 Time dependencies of turbidity of PC-cholesterol-cholate mixtures (made as in Fig. 1) on cholate concentration at room temperature (*solid lines*; *curves A, B, and C*) and at 40°C (*broken lines*; *curves D, E, and F*). The final concentrations of PC and cholesterol were 16.8 mM and 8.4 mM, respectively. Final cholate concentrations were as indicated in the figure.

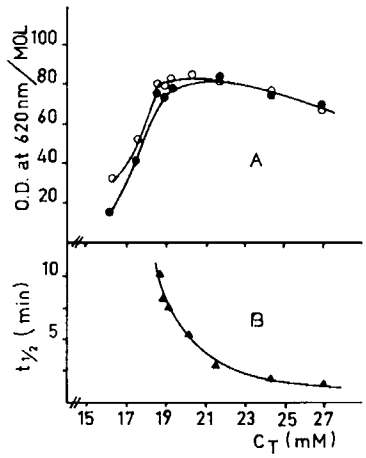

FIGURE 5 Effect of C_T (sum of PC and cholesterol final concentrations) on: (*A*) The molar turbidities of PC-cholesterol-cholate mixtures made as described in Fig. 1, as measured at 620 nm; 20 min (●—●) and 24 h (○—○) after mixing the PC-cholesterol vesicles with the respective cholate solutions. (*B*) The half lives ($t_{1/2}$) of the increase of turbidity during the first 1 h after mixing. R_e (as given by R_e = ([cholate]-9)/C_T) had a value of 3.7 and R_c (cholesterol/PC molar ratio) was 0.5 for all the mixtures.

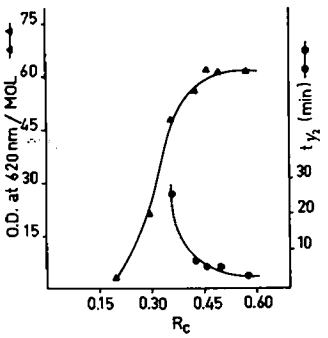

FIGURE 6 Effect of R_c (cholesterol/PC ratio) of mixtures made as in Fig. 1 on: (*a*) the molar absorbance at 620 nm, as measured 1 h after mixing of vesicles with cholate solutions (▲—▲) and (*b*) the half life ($t_{1/2}$) of the turbidity increase (●—●). Vesicles were made of 60-mM lipids (C_T = 60 mM) and mixed with equal volumes of 240 mM cholate, to yield mixtures containing 120 mM cholate (R_e = 3.7) and varying concentrations of PC and cholesterol chosen to give a constant total lipid concentration (C_T = PC + cholesterol = 30 mM) but varying ratios of cholesterol to PC (R_c values).

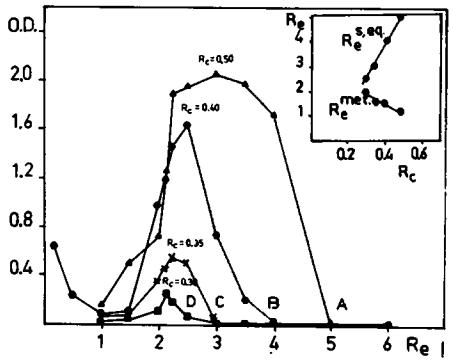

FIGURE 8 Stability of PC-cholesterol-cholate mixed micellar systems of various compositions. Mixed micellar systems were made by mixing cholate solutions with PC-cholesterol vesicles. They were all transparent immediately after being made provided that $R_e \geq 1.0$. Five days later, the turbidity of the dispersions (OD at 570 nm) was as described in this figure. The sum of PC + cholesterol concentrations in all the dispersions was 30 mM but the cholesterol/PC ratio (R_c) was as indicated in the fig. (0.50 in *A*, 0.40 in *B*, 0.35 in *C*, and 0.30 in *D*.) The cholate concentration in each solution is given here in terms of R_e (R_e = ([cholate]-9)/30). The inset describes R_e^{met} and R_e^s as a function of R_c (see text for details).

The most straightforward interpretation of this difference is that a relatively stable 1:2:6 (cholesterol:PC:cholate) complex had been formed after the addition of cholate to an R_e value of 2 (and R_c = 0.5). Further addition of cholate (to an R_e value of 3.7) did not affect this complex as the rate of exchange of both cholesterol and/or PC from the initial mixed micelles to the newly added pure cholate micelles is slow. In contrast, cholate contained in the stable mixed micelles of $R_e > 7$, made by addition cholate to a concentration of 240 mM at time zero in curve B, solubilized the PC-cholesterol vesicular dispersion added at point I. This can be concluded from the similar instability (similar rate of turbidity increase) of curve B, after addition of the vesicles (at point I), to that of the dispersion of curve A. Thus, cholate contained in mixed micelles exchanges rapidly between them.

The results described above on the relatively short-time stability of metastable systems made by vesicle solubilization also apply, qualitatively, to the stability of the same systems as measured after longer periods of time. Fig. 8 *A* presents the turbidity of the samples of Fig. 3, again as a function of R_e, as measured 5d after preparation. Clearly, the aggregational state in several samples changed with time (as reflected by their increased turbidity), resulting in broadening of the range of instability. Also shown in Fig. 8 are the turbidities of samples made by the same procedure with lower cholesterol/PC ratios. In all these curves it appears again that micellar systems are stable whenever R_e is either higher than a critical value $R_e^{s,eq}$ or lower than another critical value, here denoted R_e^{met}. These critical values of R_e, extracted from the curves of Fig. 8 are presented as a function of the cholesterol to PC ratio (R_c) in the inset to this figure. Both $R_e^{s,eq}$ and R_e^{met} are apparently linear functions of R_c (over the range of $0.3 \leq R_c \leq 0.5$): $R_e^{s,eq} = 3.3 - 4.6\,R_c$ and $R_e^{met} = 12.9\,R_c - 1.4$.

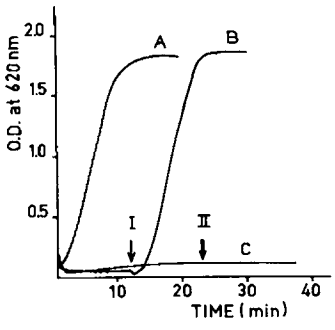

FIGURE 7 Time dependencies of the turbidity of PC-cholesterol-cholate mixtures. Crystalline sodium cholate was added at time zero to 2 ml of (sonicated) vesicular dispersions containing 20 mM PC and 10 mM cholesterol. The final cholate concentration was equal in all three experiments (120 mM) but in the dispersion of curve A the cholate was added to this concentration at time zero, in curve B cholate was first added to a concentration of 240 mM and then, at point I, the mixed micellar system was diluted with an equal volume of the sonicated dispersion, while in curve C cholate was first added to a concentration of 70 mM and at point II the cholate concentration was increased to 120 mM by adding more crystalline cholate.

DISCUSSION

Solubilization of PC-cholesterol vesicles by cholate occurs when $R_e > 0.6$ i.e., when the cholate concentration (in millimolars) exceeds a level of [cholate] $> 9 + 0.6 \cdot C_T$, where C_T = [PC] + [cholesterol]. At equilibrium, the cholate concentration required for complete solubilization is an increasing function of R_c, (the cholesterol to PC molar ratio). For $R_c > 0.25$, the cholate concentration required for complete solubilization of PC-cholesterol mixtures at equilibrium is higher than the cholate concentration needed for solubilization of the PC-cholesterol vesicles. Thus, solubilization of PC-cholesterol vesicles with more than 20 mol % cholesterol results in supersaturated essentially mixed micellar dispersions, which spontaneously undergo reorganization. The degree of supersaturation in these dispersions is a function of the cholate concentration, which can be experimentally varied over the range between the concentration required for solubilization of the liposomes and that needed for establishing equilibrium solubilization.

Here, we have studied the spontaneous reorganization of

the metastable mixed micelles, formed by solubilization of vesicles with cholate concentrations that are sufficient for solubilizing the vesicles but not necessarily sufficient for establishing, at equilibrium, systems containing only mixed micelles. These spontaneous processes were studied as functions of R_c, C_T, and the effective ratio R_e. The results of these studies are that: (*a*) The lipids in solubilized supersaturated micellar dispersions undergo partial revesiculation into cholesterol-rich lipid bilayers, from which cholesterol subsequently precipitates. (*b*) In mixtures with constant total lipid concentration (C_T) and effective cholate to lipid ratio (R_e), the rate of revesiculation is an increasing function of the cholesterol to PC ratio (R_c), i.e., of the degree of supersaturation. (*c*) In mixtures with constant ratios R_c and R_e, the rate of revesiculation is an increasing function of the total lipid concentration C_T, namely, the stability of the micellar dispersions decreases with increasing the total lipid concentration. (*d*) In mixtures with constant PC and cholesterol concentrations (thus constant R_c and C_T), and varying cholate concentration within the range equivalent to R_e of 2 to 6, the rate of revesiculation is an increasing function of R_e. In other words, (to our surprise) the stability of the micellar dispersions is an increasing function of the degree of supersaturation. (*e*) At 40°C, dispersions with R_e values of up to 4.0 are relatively stable, as compared with a limit of ~2.8 at room temperature. (*f*) The long-lived supersaturated mixed micellar dispersions exchange cholate relatively rapidly, unlike PC and cholesterol.

To interpret these results, it is important to note that inclusion of more than 37 mol % cholate in PC-cholesterol liposomes ($R_e = 0.6$) causes rupture of the lipid bilayers. This results in formation of mixed micelles in which R_c is the same as in the liposomes. For dispersion with $R_e > 0.6$, the actual molar ratio of cholate to lipids in lipid-containing mixed vesicles may be lower than R_e and as low as 0.6, since pure cholate micelles may coexist in the solution along with the PC-cholesterol-cholate mixed micelles. However, the turbidity of the micellar dispersions (which is a function of the cholate to lipid ratio in the mixed micelles [20]) decreases with R_e, indicating that for $R_e > 0.6$ the actual cholate to lipid ratio is higher than 0.6. Presumably, in the time frame of micelle formation, equilibration is rapid and, for mixtures with $R_e > 0.6$, the cholate to lipid ratio in the mixed micelles is approximately equal to R_e (21).

Mixed micellar systems with R_e values higher than 0.6 but lower than the value required for the micelles to be at equilibrium (here denoted $R_e^{s,eq}$) are metastable. Equilibration may subsequently occur through revesiculation of portion of the lipid in the form of cholesterol-rich, bile salt-poor vesicles. As a consequence, the cholesterol content (thus R_c) of the remaining mixed micelles decreases, the relative bile salt content (thus R_e) rises, to yield thermodynamically stable mixed micelles. As an example, for a mixed micellar dispersion with 20 mM PC, 10 mM cholesterol, and 120 mM cholate, $R_c = 0.5$ and $R_e = 3.7$. The latter value is considerably lower than $R_e^{s,eq}$, which for $R_c = 0.5$ is equal to 5.0. Due to partial revesiculation, the concentration of PC in the mixed micelles decreased to ~16 mM, the concentration of cholesterol decreased to ~5 mM and that of cholate decreased to 117 mM. Consequently, in mixed micelles the cholesterol to PC ratio (R_c) decreased to ~0.32 and the cholate to lipid ratio increased to a value of ~5, which is higher than $R_e^{s,eq}$ at $R_c = 0.35$.

Because the driving force for revesiculation is cholesterol-supersaturation i.e., the high cholesterol content of the micelles, it is not surprising that increasing R_c results in decreased stability of the micellar system (increasing rate of revesiculation).

On the other hand, the increase of the rate of the latter process with increasing the cholate concentration (increasing R_e at any given C_T and R_c) is much more difficult to understand. In fact, at any R_e value below a critical value (e.g., 2.8 at room temperature when $C_T = 30$ mM and $R_c = 0.5$), mixed micellar solutions appear to be relatively stable and only above this critical value (here denoted R_e^{met}) the micelles undergo rapid (partial) revesiculation. In other words, mixed micellar systems are stable when $R_e > R_e^{s,eq}$, they are long-lived when $R_e < R_e^{met}$ and least stable when R_e is lower than $R_e^{s,eq}$ but higher than R_e^{met}.

This complex dependence of stability on R_e can be rationalized on the basis of the structural model of Small and coauthors (22), which for the small lipid-cholate mixed micelles obtained at high R_e values is probably valid in its original formulation (modification of this model according to Mazer et al. (23) and/or Lichtenberg et al. (24) are of little significance, if any, for $R_e > 2$). According to this model, the radius of lipid-bile salt mixed micelles is inversely proportional to the effective ratio (i.e., to the bile salt to lipid ratio in the mixed micelles). For $R_c = 0.5$, each monolayer of cholesterol-containing discoidal mixed micelle contains, on the average, two PC molecules per one molecule of cholesterol in its central, hydrophobic, bilayer-like core. The number of cholate molecules required for covering the hydrophobic perimeter of one 1:2 cholesterol:PC complex, of course depends on the cross section of the complex and on the dimension of the hydrophobic face of the bile salt molecules. If the latter parameter has a value of 8Å (24) then six to seven bile salt molecules will be required if the cross section of the PC-cholesterol complex is 250 Å^2 ($R_e \leq 7/(2 + 1) = 2.33$). Increasing the surface area of the hypothetical cholesterol-PC (1:2) complex by elevating the temperature should therefore be expected to result in an increase of the value of R_e^{met}. This prediction is qualitatively consistent with the experimental data presented in Fig. 4, which supports our interpretation of the relative stability of mixed systems with $R_e < R_e^{met}$. More specifical-

ly, we propose that at R_e^{met} the mixture is essentially homogeneous, consisting of relatively stable micelles, each built of a 1:2 cholesterol:PC complex whose hydrophobic edges are covered by the amphiphilic cholate.

At higher R_e values (higher cholate concentrations), there could have been a relatively stable state of aggregation if such mixed micelles would have coexisted in the solution with pure cholate micelles, similar to the mixture described in Fig. 7 *B*. However, addition of excess cholate to vesicles (in contrast to adding it to the relatively stable micelles at $R_e < R_e^{met}$) probably yields mixed micelles of varying compositions, some of which have no cholesterol and some of which have less than two PC molecules per one cholesterol molecule. The latter micelles are very likely to be unstable.

At R_e values below R_e^{met}, the mixed micelles are likely to contain in each of their monolayers more than one complex of 1 molecule of cholesterol and 2 molecules of PC. As an example, if each monolayer contains two such complexes, covering the perimeter of this hydrophobic core will require about eight bile salt molecules. Such mixed micelles are therefore likely to exist when $R_e = 8/[(2 + 1)2] = 1.3$. Similarly, it can be shown that when $R_e = 1{:}1$, the discoidal mixed micelles are likely to contain in each monolayer three 2:1 PC:cholesterol complexes. At R_e values lower than 1.1, the mixed micelles can further increase in size and probably contain some bile salt molecules in their hydrophobic cores (23).

Micelles similar to the unstable PC-cholesterol-cholate mixed micelles formed by cholate addition to PC-cholesterol liposomes at R_e above R_e^{met}, but below $R_e^{m,eq}$, are likely to be present in supersaturated gallbladder bile. This hypothesis is based on the finding that much of the lipids in hepatic bile are contained in vesicles (2) and on the reasonable assumption that the process of bile concentration in the gallbladder must be accompanied by a decrease in the amount of monomeric bile, thus by an increase in R_e and by a consequent solubilization of at least a part of the vesicles.

To evaluate the possible significance of our findings to gallbladder stone formation, we compared the composition of native gallbladder bile samples of cholesterol gallstone patients with that of normal subjects in terms of R_c and R_e and in relation to R_e^{met} and $R_e^{s,eq}$. Data for this comparison were collected from tables and figures reported in the literature (6–9, Z. Halpern and Y. Peled, unpublished

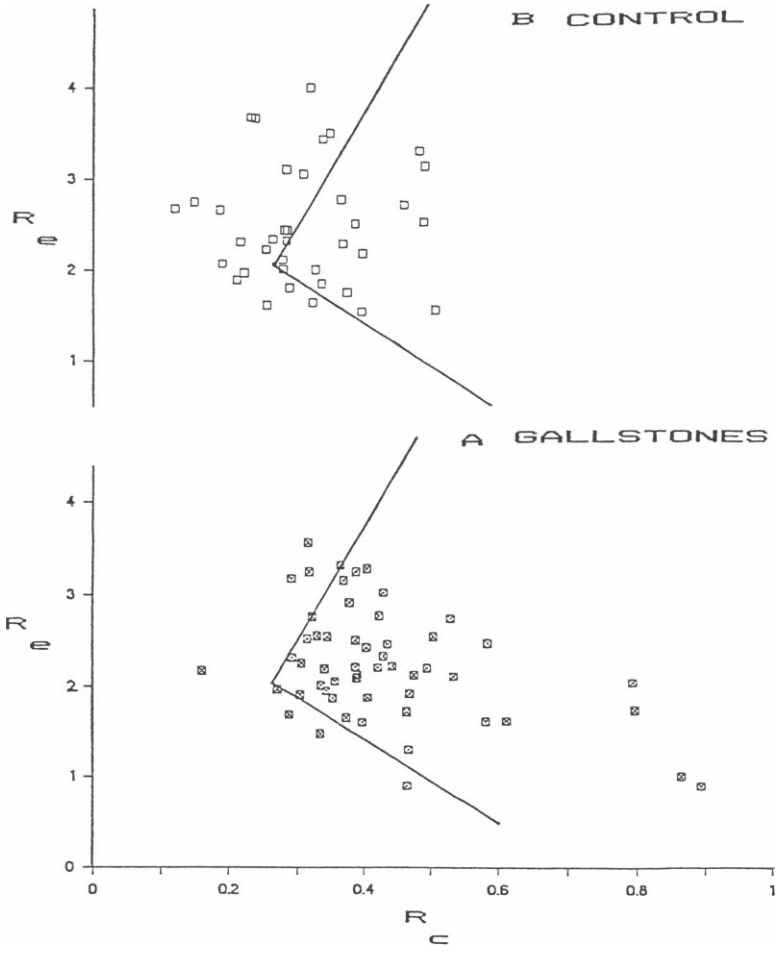

FIGURE 9 The composition of gallbladder bile of gallstone patients (*A*) and normal subjects (*B*, control) given in terms of R_e and R_c (see text for details). Data are from Ref. 2, 6, 20–23. The lines in both panels are those of the inset of Fig. 8.

results) in which sufficient data was given to enable us to compute R_c and R_e. Panel A in Fig. 9 presents the composition of 36 gallbladder biles of cholesterol gallbladder stone patients. Panel B presents bile compositions of 55 normal subjects. In both these groups, the composition is given in terms of the cholesterol to PC ratio (R_c) and the molar effective ratio (R_e) of bile salts to lipids (PC + cholesterol). The number of cases analyzed in Fig. 9 is limited because in most reports there is no indication of absolute concentrations and therefore we were unable to compute R_e. The solid lines in both panels of this figure are taken from the inset of Fig. 8. The instability region of $R_e^{met} < R_e < R_e^{s,eq}$ contains 22 out of the 36 data points of gallbladder stone patients but only 8 out of the 55 composition data points of bile samples of normal subjects. A chi square test of this distribution of data points suggests that this difference in distribution is statistically significant ($p < 10^{-5}$). Similar to the model systems, the range of instability broadens with increasing R_c.

A perfect fit between the lithogenicity of bile and the oversimplified model system presented in Fig. 8 could not have been expected and the values of R_e and R_c are certainly insufficient for prediction of stone formation. However, the general trend is that stone formation depends in many cases on whether or not the BS concentration is higher than the metastability limit and not on whether or not it is lower than the equilibrium solubilizing ratio. In other words, the degree of supersaturation is an important factor in determining the probability of stone formation but this probability is not a monotonic function of the cholesterol supersaturation index (CSI). In fact, in some bile samples a higher bile salt content would mean higher probability of cholesterol crystallization. Nucleating and/or anti-nucleating agents may of course play a predominant role in cholesterol precipitation (3), but spontaneous precipitation may also occur. For many biles the probability of such precipitation is likely to increase with increasing the bile salt concentration. This has to be taken into account in any further studies of the pathogenesis of gallstones.

We thank Margeretta Allietta of the Department of Pathology of the University of Virginia for her expert performance of the freeze-fracture electron micrographs, Dr. C. Little of the Department of Anatomy of the University of Virginia for his help with the light microscopy, and Dr. T. Kushnir of the Department of Chemistry at Tel Aviv University for her expert assistance with the nuclear magnetic resonance measurements. Thanks are also due to Dr. I. Friedberg of the Department of Microbiology at Tel Aviv University, Dr. H. Kutchai of the Department of Physiology at the University of Virginia, and Dr. Y. Barenholz of the Department of Biochemistry at the Hebrew University for several helpful discussions.

This study was partially supported by grants from the Israeli Academy of Sciences and the Chief Scientist of the Ministry of Health.

Received for publication 25 February 1988 and in final form 30 June 1988.

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