Vesicle aggregation in model systems of supersaturated bile: relation to crystal nucleation and lipid composition of the vesicular phase

Zamir Halpern,[1] Mary A. Dudley, Marc P. Lynn, Janet M. Nader, Anthony C. Breuer, and R. Thomas Holzbach[2]

Gastrointestinal Research Unit, Department of Gastroenterology and Department of Neurology and Cardiovascular Research, Cleveland Clinic Foundation, Cleveland, OH 44106

Abstract The presence of small vesicles composed of phospholipid and cholesterol has recently been demonstrated in supersaturated model and in dilute native human biles by several groups using differing methods. Among compositional factors shown to favor spontaneous vesicle formation and prolong the cholesterol monohydrate nucleation time in model bile systems are dilution, a raised cholesterol saturation index (CSI), and a low bile salt/phospholipid ratio. Time-lapse video-enhanced microscopy of a series of model bile systems representing systematically designed variations in the above factors revealed strong evidence for an essential linkage between antecedent vesicle aggregation and subsequent crystal nucleation. Stability of vesicles was inversely related to their degree of cholesterol saturation, i.e., the greater the degree of vesicular cholesterol saturation, the less their stability (metastability). Instability of vesicles was reflected by their early aggregation followed by rapid cholesterol crystal nucleation. The lowest degree of vesicular cholesterol saturation was found in dilute systems which also exhibited the greatest metastability despite a high degree of cholesterol solubility (raised CSI). Conversely, the more concentrated and least metastable systems exhibited both rapid vesicle aggregation and rapid onset of crystal nucleation. These systems, while influenced by the other compositional factors, were found to have a high degree of vesicular cholesterol saturation, i.e., cholesterol/phospholipid molar ratio = 2.0. An additional finding was the extreme variability in the proportionate distribution of total solution cholesterol distributed to the vesicular phase, i.e., from zero to as high as 37%. Higher solute concentration, raised bile salt/lecithin ratio, and raised CSI were interactive and almost equally capable of increasing the proportionate amount of cholesterol in the vesicular phase. In conclusion, lipid compositional differences in model bile systems drastically affect the cholesterol saturation of spontaneously formed phospholipid-cholesterol vesicles. This effect, in turn, exerts a potent influence upon the metastability of vesicles, subsequently affecting the cholesterol crystal nucleation time. —**Halpern, Z., M. A. Dudley, M. P. Lynn, J. M. Nader, A. C. Breuer, and R. T. Holzbach.** Vesicle aggregation in model systems of supersaturated bile: relation to crystal nucleation and lipid composition of the vesicular phase. *J. Lipid Res.* 1986. **27:** 295–306.

Supplementary key words vesicles • bile • aggregation • nucleation

Vesicles have been demonstrated in normal and abnormal human gallbladder bile, in the more dilute human T-tube and hepatic bile, and in a variety of model (artificial) biles (1–4). Recent studies of cholesterol monohydrate crystal nucleation in native bile have demonstrated that while nucleation is much slower in normal gallbladder, hepatic, or T-tube bile, the process itself is the same in all supersaturated specimens: first, de novo formation of small unilamellar vesicles; second, formation of small (1–5 μm) vesicular aggregates; third, enlargement of small aggregates to large, fused, often multivesicular and multilamellar forms closely followed by the appearance of cholesterol monohydrate crystals (5). The order of events and the association of crystals with fields of clustered vesicles suggests that crystal nucleation results from vesicular aggregation. It may be that aggregated vesicles serve both as a surface for the nucleation process and as a possible source of cholesterol needed for crystal formation (6, 7). A similar concept has recently been proposed by Collins and Phillips (8) as a result of their study of cholesterol-rich aqueous codispersions of cholesterol and phosphatidylcholine. Under their conditions, dispersions comprising vesicles with up to 4 moles of cholesterol/mole of phosphatidylcholine were metastable, i.e., upon incubation at 4°C and at 20°C, the vesicles aggregated and precipitated. There was, in addition, a

Abbreviations: HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; NaTC, sodium taurocholate; CSI, cholesterol saturation index; BS/PL ratio, bile salt/phospholipid ratio; CHOL/PL ratio, cholesterol/phospholipid ratio; VEM, video-enhanced microscopy, video-enhanced contrast-differential interference miscroscopy.

[1] Present address: Department of Gastroenterology and Internal Medicine "T", Ichilov Hospital, Sackler School of Medicine. Tel Aviv University, Tel Aviv, Israel.

[2] To whom correspondence should be addressed.

decrease in the cholesterol/phosphatidylcholine molar ratio in the particles and formation of cholesterol monohydrate crystals which appeared to "grow" from multilamellar vesicles.

While it is generally agreed that the predominant driving force in the nucleation process is the absolute degree of cholesterol supersaturation, a number of other phenomena are known to be involved. Dilution markedly prolongs nucleation time in supersaturated human and model bile (2, 9). The bile salt/lecithin ratio and the calcium ion concentration alter both the nucleation time in model bile and the number of vesicles present in these solutions (10). Crude preparations of biliary proteins are capable of inhibiting cholesterol crystal nucleation in human gallbladder bile (11). Furthermore, apolipoproteins A-I and A-II present in bile, are closely associated with the most potent nucleation-inhibiting crude protein fraction, and themselves prolong cholesterol crystal nucleation (12). In contrast, evidence for the presence of nucleation-promoting factors in the gallbladder bile of patients with cholesterol gallstones has been recently demonstrated (13). In addition, some evidence, albeit controversial, has recently been provided for a putative role of mucin glycoproteins in cholesterol crystal nucleation (14, 15). Although some of the factors altering cholesterol monohydrate nucleation are understood, little is known as to which step in the nucleation process is affected by inhibiting and promoting factors. If vesicles are important to nucleation, it may be that factors that affect nucleation time express this functional effect by means of their influence on vesicle stability.

The intent of the present study was threefold: first, to examine the role of vesicles in the nucleation process in defined model systems using time-lapse video-enhanced microscopy and transmission electron microscopy; second, to determine the effect of dilution, CSI, and BS/PL ratio on the concentration of cholesterol and phospholipid in vesicles; and third, to study the distribution of these lipids in the micellar versus the vesicular phase.

EXPERIMENTAL PROCEDURES

Preparation of model bile solutions

Cholesterol was purchased from Eastman Kodak Co. (Rochester, NY) and stored under nitrogen at 4°C. Upon recrystallization, its purity was assessed at 99.5% by melting point and gas-liquid chromatography. Egg lecithin was obtained from Lipid Products (Surry, U.K.) and stored at −80°C. Sodium taurocholate (NaTC) (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) was twice recrystallized by the method of Pope (16). The procedures for model bile preparation were slight modifications of those previously described (10, 11). Briefly, stock model biles were prepared as follows. Aliquots of NaTC in methanol, lecithin in chloroform–methanol 2:1 (v/v), and cholesterol in methanol were added to a vial equipped with a Teflon-faced septum and cap to yield the desired amount of these three lipids (**Table 1**). After flushing with nitrogen, the mixture was shaken at 37°C for 2 hr and the organic solvent was evaporated under a stream of nitrogen until the mixture had condensed to a viscous paste. To achieve complete solvent removal, the mixture was then lyophilized. Lastly, the required volume of HEPES-saline (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid–NaCl 10 mM:140 mM, pH 7.5) was added to the preheated lyophilized lipids. For several studies, dilution of the model biles in Table 1 was required and was performed by adding sufficient additional HEPES-saline to lyophilized lipids to reach the desired concentration (g/dl). The pH of each solution was then adjusted to 7.5 and the mixture was flushed with nitrogen. The resultant suspension (stock model bile or a dilution of the stock) was shaken at a constant sped of 100 rpm in a model G-24 Environmental Incubation Shaker (New Brunswick Scientific, New Brunswick, NJ) at 55°C until microscopically isotropic (2, 17).

For nucleation studies, the model biles were filtered through a preheated Swinney filter assembly containing a 0.22 μm filter (Millipore Continental Water Systems, Bedford, MA), then flushed with nitrogen and incubated at 37°C without shaking. Nucleation studies were conducted as previously described (11, 18). Initial (zero) time for each study was 30 min after cooling to 37°C. The model bile specimens were examined by video-enhanced microscopy at zero time, and thereafter were observed at

TABLE 1. Lipid composition of model biles

Study	Model Bile	Total Lipids	Moles % Cholesterol	BS/PL	CHOL	PL	BS	CSI
		g/dl				*mM*		
Time-lapse	a	20	8.5	4.0	30.0	64.5	258.0	1.2
	b	8	8.5	4.0	12.0	25.8	91.2	1.36
	c	8	10.8	2.3	14.9	37.2	85.7	1.4
Dilution	d	20	10.8	2.3	37.4	93.0	214.3	1.2
BS/PL	e	8	11.3	1.9	15.3	41.4	78.8	1.4
	f	8	11.1	2.1	15.1	39.2	82.3	1.4
	g	8	10.8	2.3	14.9	37.2	85.7	1.4
	h	8	9.9	3.0	13.9	31.3	94.0	1.4
	i	8	7.3	5.7	10.3	19.6	111.9	1.4
CSI	j	8	7.8	2.3	10.5	37.8	86.8	1.0
	k	8	8.5	2.3	11.6	37.7	86.7	1.1
	l	8	9.4	2.3	12.9	37.2	86.6	1.2
	m	8	10.0	2.3	13.8	37.3	85.7	1.3
	n	8	10.8	2.3	14.9	37.2	85.7	1.4
	o	8	11.7	2.3	16.0	36.8	84.6	1.5

BS, bile salt; PL, phospholipid; CHOL, cholesterol; CSI, cholesterol saturation index.

2-hr intervals for 12 hr. They were then examined daily until they had nucleated. If vesicles were detected by video-enhanced microscopy, specimens were examined by electron microscopy (EM).

For studies involving the formation and characterization of vesicles formed in various model biles (Table 1, Solutions No. d–o), vesicles were allowed to form spontaneously in a supersaturated isotropic model bile by cooling from 55°C to 37°C for 6 hr (2, 17). With time, the solution became visibly turbid and vesicle formation was monitored by absorbance at 300 nm. The model biles were then centrifuged at 90,000 *g* for 18 hr at 37°C using a Beckman model L-50 centrifuge and a 50.3 Ti rotor (Beckman Instruments, Palo Alto, CA). The contents of the centrifuge tube (6 ml) were divided into ten 0.6-ml fractions and assayed for lipids (11, 19–23). Fractions were obtained by puncturing the bottom of the centrifuge tube and collecting aliquots by gravity (Model FSA101, Gradient Tube Fractionator, Hoefer Scientific Instruments, San Francisco, CA). Reproducibility of measurements was determined in replicate experiments. The lipid concentration of the vesicles was estimated as previously described (10). Briefly, the lipid distribution of a supersaturated model bile and an undersaturated model bile having a comparable total lipid concentration and bile salt/phospholipid (BS/PL) ratio were compared. The lipid profiles obtained from fractionation of the models showed that the cholesterol and phospholipid concentrations were raised only in the vesicle-containing fractions in the supersaturated model biles. The lipid concentration of the micelles within the system was estimated by superimposing the baseline from the undersaturated model bile on that of the saturated. The area above baseline was considered representative of vesicles and the area below baseline was considered representative of micelles. An approximate composition for the vesicle fraction based upon this assumption could then be determined using the lipid concentration of the fraction containing the largest number of vesicles and subtracting the lipid concentrations for lecithin and cholesterol below baseline from the respective total lipid concentration for each. Furthermore, using area triangulation, the size of the vesicle fraction under these conditions was estimated.

Video-enhanced microscopy studies

A Zeiss Axiomat microscope in the inverted configuration equipped with Nomarski differential interference contrast optics was used. Model bile stock solutions (Table 1) or dilutions thereof containing spontaneously formed vesicles were mounted between two No. 0 coverglasses (Gold Seal, Clay Adams Division, Becton, Dickinson, and Co., Parsippany, NJ) and observed at 37°C using a 50-watt DC-powered mercury arc and Zeiss heat reflection and green interference filters. All specimens were viewed using a high numerical aperture (NA) Achromatic-Aplanatic oil-immersed differential interference contrast condenser (NA 1.4) and 100 × planapochromat (NA 1.3) POL oil-immersed objective. Images were recorded using a Hamamatsu C-1000 Chalnicon head video camera on a ¾" Panasonic NV-9240-XD videotape unit using a previously described video-enhanced contrast method (24–26). The gain and offset features of the Hamamatsu Camera Control Unit were adjusted to optimize contrast and detection of small structures in the video-enhanced image. The illustrations presented in this paper were obtained by photographing still frames from a video monitor of images recorded on videotape following background mottle subtraction by digital image processing. Video raster lines were blurred by diffraction using a 50-lines ronchi grating (Rolyn Optics Co., Covina, CA) attached in front of the camera lens.

Electron microscopy studies

Transmission electron microscopy. Model solutions containing spontaneously formed vesicles were studied using Formvar-coated grids. Equal volumes of 1% phosphotungstic acid and the specimen were mixed on the grid. After eliminating excess liquid, the grid was allowed to dry and was examined with a Phillips 400 microscope (27).

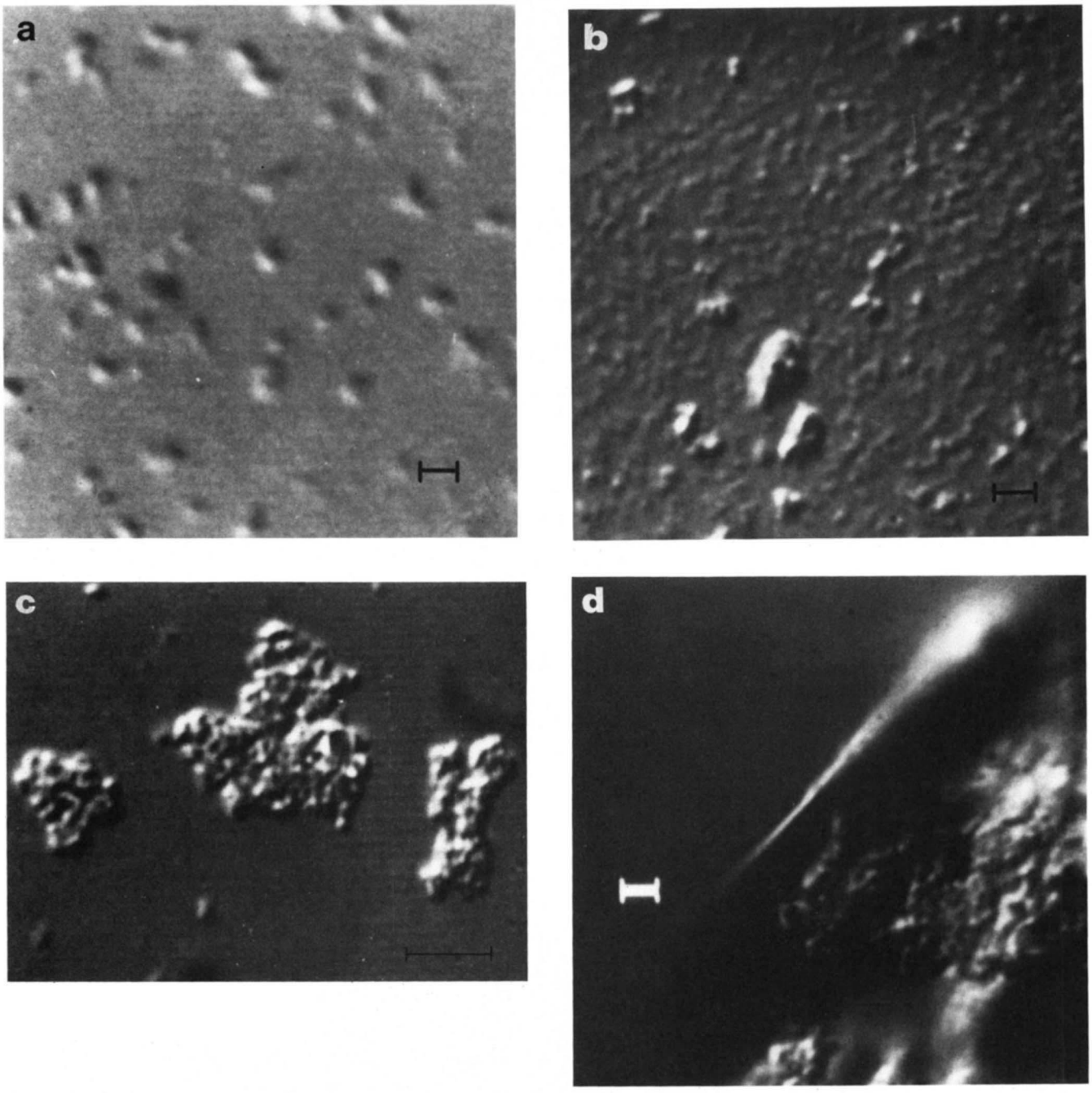
Statistical methods

The Kruskal-Wallis Test (a non-parametric analysis of variance procedure) was employed to assess possible comparative differences in the number of unilamellar vesicles and their aggregates in the three model bile solutions with respect to time (28).

RESULTS

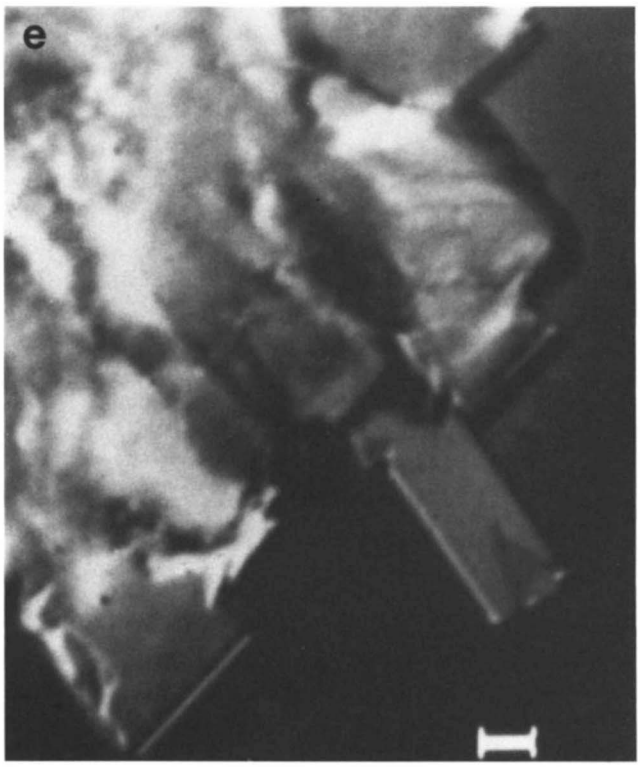
Time-lapse studies

Three model biles were employed for the time-lapse studies; one (Table 1, a) was comparable to the model bile system we have previously used for standardization of the nucleation assay; the remaining two (Table 1, b and c) were more dilute and thus comparable to native gallbladder bile. At zero time (at 30 min incubation at 37°C) two of the solutions (Table 1, a and b) appeared clear whereas the third (Table 1, c) was visibly turbid. When examined by video-enhanced microscopy (VEM), all three solutions regardless of their lipid concentration were found to contain vesicles and occasional small aggregates, 1 to 5 μm in size, but no cholesterol monohydrate crystals (**Fig. 1, a**). In model bile (a) (the most concentrated system) vesicles were both few and widely scattered, never constituting a major portion of the microscopic field. In model (b) the vesicles were much more numerous. As expected from the turbidity of the solution, vesicles in



model (c) were present in vast numbers. In all three solutions, the vesicles were monodisperse and demonstrated rapid Brownian movement.

At the 2-hr examination time-point, the initially clear dilute model bile (Table 1, b) had become turbid. Video-enhanced microscopy indicated that de novo formation of vesicles appeared to have taken place in all three model solutions. Both models (b) and (c) became a mass of seemingly monodisperse, closely packed particles showing no appreciable increase in the number of small vesicular aggregates (Fig. 1, b). In contrast, model bile (a) remained visually clear. In this latter, more concentrated system, an increased number of vesicles was observed, but these were fewer than those observed in the more dilute specimens. Aggregate formation had noticeably increased in this sample, however, and small clusters of vesicles were frequently seen floating in the solution or adhering to the surface of the lower coverslip. Electron microscopy demonstrated that in addition to the small unilamellar vesicles, 50 to 100 nm in diameter, the solutions contained small multilamellar vesicles with a diameter of 100–200 nm (**Fig. 2, a, b, c**). Clustering of the smaller aggregates



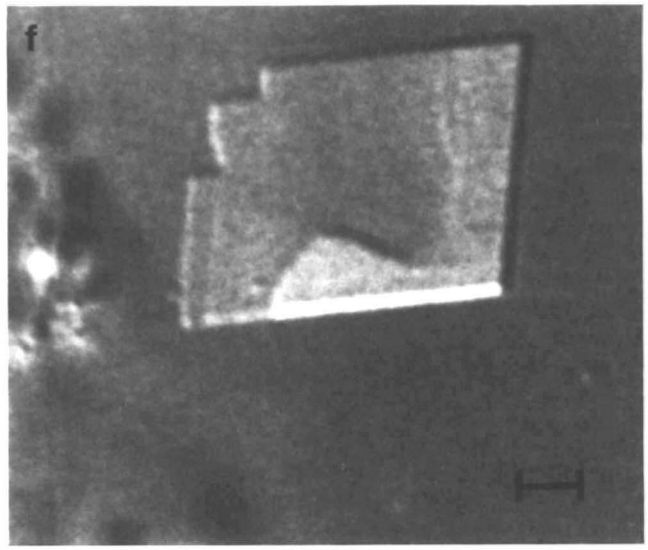


Fig. 1. Model bile (Table 1-a) observed by video-enhanced contrast-differential interference contrast microscopy—a time-lapse study. A) Zero time. Modest number of unaggregated unit-size particles (bar =100 nm). B) Two hours; larger field showing primarily unaggregated unit-size particles and some small vesicle aggregates (bar = 250 nm). C) Ten hours; slightly larger and structurally more complex (i.e., multivesicular and multilamellar) vesicular aggregates (bar = 1000 nm). D) and E) Twenty-four hours; large number of aggregated vesicles in the almost total absence of unaggregated ones; occasional typical cholesterol monohydrate crystals with associated apparently adherent huge vesicular aggregates. Crystals were also often seen in isolation unassociated with adjacent vesicles (bar = 250 nm). F) Twenty-four hours; phenomenon of twinning as a manifestation of rapid crystal growth (bar = 250 nm).

appeared to produce a multilamellar particle (Fig. 2, d).

By the time of the 6 hr examination, model (a) had changed noticeably. Single vesicles were still present in large numbers, but they had become more polydisperse. Many aggregates were now seen in the solution. Most were 1–5 μm in diameter, but others had enlarged.

During the subsequent 6 hr (8, 10, 12 hr examinations), the only apparent change in the dilute systems was a notable increase in the number of vesicular aggregates. These remained small (1–5 μm) and the vesicles remained monodisperse. The aggregates in model bile (a), however, increased in size. Some had become so large as to occupy the entire microscopic field ($>$ 30 μm). Upward and downward adjustment of the focal plane permitted remarkably clear delineation of the internal structural features of individual aggregates as multilamellar and multivesicular (cochlear) forms (Fig. 1, c). Electron microscopy confirmed these structural observations.

At the time of the 22 hr examination, crystal nucleation had occurred in model (a). Multiple cholesterol monohydrate crystals were seen either in association with the large aggregates or floating freely in solution. These were typical birefringent plate-like crystals showing step and screw dislocations (29, 30). Only scattered vesicles were observed, and the most remarkable feature of the specimen was the occasional presence of huge complex vesicular aggregates which frequently had cholesterol monohydrate crystals protruding from their surface (Fig. 1, d and e). At times, the crystals also showed typical twinning phenomena (Fig. 1, f) (30).

Vesicles in the more dilute systems, i.e., 8 g/dl (Table 1, b and c), were still stable and unaggregated after 22 hr. Although vesicular aggregates could be seen in both solutions, stable monodisperse vesicles were the predominate feature. A careful search revealed no cholesterol monohydrate crystals.

After 46 hr, crystal nucleation had occurred in the more dilute model biles (Table 1, b and c). Cholesterol crystals were seen either protruding from large vesicular aggregates or floating free in solution. In contrast to model (a), at the time of nucleation, vesicles still predominated in these solutions and vesicular aggregates varied in size from 1–5 μm. Observations of dilute systems, i.e., 4 and 2 g/dl, were also obtained by VEM at 48 hr and at 1 week. During these periods, vesicles in the dilute systems remained stable and neither aggregates nor crystals were observed.

The data in **Table 2** comprise a morphometric portrayal of the kinetics of vesicle aggregation and cholesterol

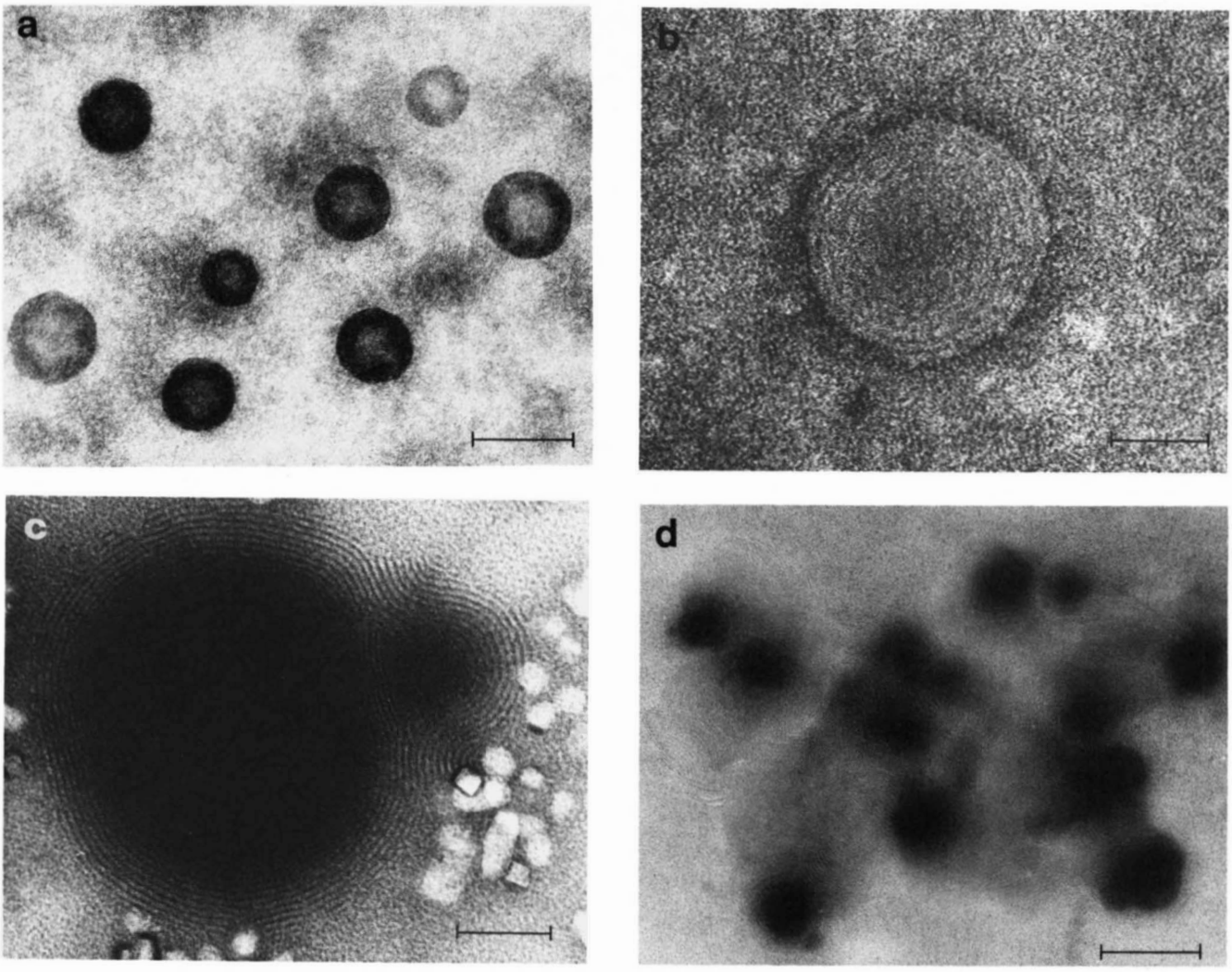


Fig. 2. Model bile (Table 1-a) observed by electron microscopy—a time-lapse study. A) Unilamellar vesicles (1 hr) (bar = 87 nm); B) multilamellar vesicles (4 hr) (bar = 50 nm); C) fusion of multilamellar vesicles (8 hr) (bar = 38 nm); D) clusters of vesicles (12 hr) (bar = 347 nm).

crystal nucleation in model biles (a), (b), and (c), respectively, as qualitatively described above. This was obtained by videotape replay and still frame counts (in triplicate) of the various particle species in typical frames (fields) from the three model bile systems. With time, the previously indicated pronounced trend favoring rapid aggregation in solution (c) is observable and in striking contrast to the comparative stability and lack of aggregation behavior observed in solution (a). The findings with regard to the aggregation behavior of solution (b) are intermediate between these extremes. Statistically significant time and model bile system differences in particle counts are indicated in Table 2. These data summarize and clarify the earlier narrative account of observations regarding model solution vesicle aggregation in our time-lapse studies.

These studies clearly indicate that, in model bile systems of comparable solute concentration, a comparatively high degree of cholesterol supersaturation combined with a low bile salt/phospholipid (BS/PL) ratio, rapid vesicular aggregation is favored (solution c). In contrast, even in a more concentrated system, but one in which the degree of cholesterol supersaturation is much lower together with a considerably higher BS/PL ratio, there is both a great reduction in the amount of vesicles formed ab initio as well as a marked reduction in their aggregation behavior (solution a).

Vesicle composition as a function of dilution

Dilution of a supersaturated model bile (Table 1, d) containing 20 g/dl total lipids to a final concentration of

TABLE 2. Number of particles (range) observed in different model solutions (a, b, and c) per VEC-DIC microscopic field as a function of time

					Aggregates											
	Unilamellar Vesicles				3 μm			3-30 μm			30 μm			Solid Crystals		
Time	a	b	c	*P*	a	b	c	a	b	c	a	b	c	a	b	c
hr		*100 nm*														
0	10	228	753	0.03	1	0	0	0	0	0	0	0	0	0	0	0
					0	0	0	0	0	0	0	0	0	0	0	0
					1	0	0	0	0	0	0	0	0	0	0	0
2.5	13	250	876	0.03	1	0	0	0	0	0	0	0	0	0	0	0
					1	0	0	0	0	0	0	0	0	0	0	0
					1	0	0	0	0	0	0	0	0	0	0	0
4	21	251	877	0.03	0	0	0	0	0	0	0	0	0	0	0	0
					0	0	1	0	0	0	0	0	0	0	0	0
					0	0	0	0	0	0	0	0	0	0	0	0
7	28	302	45	0.03	0	0	4	0	0	1	0	0	0	0	0	0
					0	1	5	0	0	2	0	0	1[a]	0	0	0
					0	0	3	0	0	1	0	0	1[a]	0	0	0
10	32	340	27	0.04	1	0	6	0	0	2	0	0	2[a]	0	0	0
					0	0	10	0	0	1	0	0	1[a]	0	0	0
					1	0	8	0	0	2	0	0	1[a]	0	0	0
24	3	76	3	0.06	4	2	3	0	0	3	0	0	3[a]	0	0	0
					2	3	6	0	0	2	0	0	2[a]	+ +	+	+
					2	2	4	0	0	2	0	0	1[a]	occas	rare	occas
P	0.01	0.01	0.01													

[a] Lower magnification was used with larger microscopic field sizes (3,000 μm^2) for estimates of numbers of those largest aggregate forms which did not occupy every field but, when present, occupied one or more than one *entire* field. Particle number estimates for other smaller particle forms were made with a high zoom application and consequently smaller microscopic field size (500 μm^2). Estimates for the smaller particle forms were in triplicate from separate still frames.

15, 12, 10, 8, 4, or 2 g/dl raised the cholesterol saturation index (CSI) from 1.2 to 1.8, but did not affect the relative mole % cholesterol or the bile salt/phospholipid ratio. Solutions containing 15, 12, 10, and 8 g/dl total lipids became microscopically isotropic at 55°C. In contrast, dilute solutions, i.e., 2 and 4 g/dl, remained turbid even when heated to 95°C for 1 hr.

Video-enhanced microscopic examination of the six model biles after incubation at 37°C revealed that all contained vesicles. The more dilute the model solution, the greater the apparent number of vesicles seen in a single microscopic field. Ultracentrifugation caused the vesicles to form discrete, visibly turbid bands, and comparative lipid profiles, obtained from fractionation of each model bile, showed that the phospholipid and cholesterol concentrations were elevated in the turbid fractions. From the lipid profiles, the amount of cholesterol and phospholipid in the vesicular fractions were calculated (10). As expected from the sparsity of microscopic vesicles, only 1.5% of the total cholesterol (0.4 mM) and 0.3% of total phospholipid (0.2 mM) was in vesicular form in solutions containing 15 g/dl total lipid (**Table 3, 1 to 6**). With dilution to 12, 10, and 8 g/dl total lipids, the amount of vesicular cholesterol increased 10-fold. Approximately 23% of the total cholesterol (3.4 mM) and 5% of the total phospholipid (2 mM) was distributed to the vesicle fraction. With further dilution to 4 and 2 g/dl, the amount of cholesterol in the vesicle fraction decreased to 1.4 mM, whereas the percentage of total cholesterol distributed in vesicles increased. In the most dilute solution, 37% of total solution cholesterol was incorporated in vesicles (Table 3, 5 and 6). In contrast, vesicular phospholipid increased slightly to 2.5–3.0 mM in these more dilute solutions, and 27% of the total solution phospholipid was in the vesicular fraction of the most dilute solution.

Dilution affected the cholesterol/phospholipid (CHOL/PL) ratio in vesicles only if the solution contained less than 8 g/dl total lipids. In solutions containing 15 and 12 g/dl, the cholesterol/phospholipid ratio remained relatively constant (CHOL/PL = 2.0). Below 10 g/dl, the ratio fell in a linear fashion until in the 2 g/dl solution, the cholesterol/phospholipid ratio was only 0.42.

Nucleation studies revealed a direct correlation between the time required for nucleation, the degree of dilution, and the cholesterol/phospholipid ratio in the vesicles (**Fig. 3**). Nucleation times increased from 10 hr in a

TABLE 3. Cholesterol and phospholipid of vesicular phase[a]

	No.	Total Lipids	BS/PL	CSI	mM of Lipid in Vesicles		% of Lipid in Vesicles		CHOL/PL Ratio in Peak Fraction
					CHOL	PL	CHOL	PL	
		g/dl							
Dilution	1	15	2.3	1.2	0.4	0.2	1.5	0.3	2.05
	2	12	2.3	1.3	2.1	1.1	9.1	1.9	2.03
	3	10	2.3	1.3	3.1	1.6	16.1	3.3	1.95
	4	8	2.3	1.4	3.4	1.9	22.9	5.1	1.85
	5	4	2.3	1.6	2.6	3.1	34.3	16.4	0.83
	6	2	2.3	1.8	1.4	2.5	37.1	27.1	0.42
BS/PL	7	8	1.9	1.4	4.4	3.5	30.4	8.4	1.06
	8	8	2.1	1.4	3.6	2.1	23.9	5.3	1.60
	9	8	2.3	1.4	3.4	1.9	22.9	5.1	1.85
	10	8	3.0	1.4	2.9	1.3	20.6	3.5	2.0
	11	8	5.7	1.4	0.24	0.12	2.3	0.6	2.0
CSI	12	8	2.3	1.0					
	13	8	2.3	1.1	0.9	1.4	8.0	3.5	0.62
	14	8	2.3	1.2	1.7	1.4	13.0	3.9	1.70
	15	8	2.3	1.3	2.2	1.5	15.9	4.1	1.75
	16	8	2.3	1.4	3.4	1.9	22.9	5.1	1.85
	17	8	2.3	1.5	4.5	2.3	28.4	6.2	2.00

CHOL, cholesterol; BS, bile salt; PL, phospholipid; CSI, cholesterol saturation index.
[a]All values listed represent an average derived from at least three determinations.

15-g/dl solution to over 700 hr in a 2-g/dl model bile. Thus, under these conditions, the time required for cholesterol nucleation appears to be an inverse function of the amount of cholesterol in the vesicles; as the cholesterol content of the vesicles decreases, the time required for nucleation is prolonged.

Vesicle composition as a function of bile salt/phospholipid (BS/PL) ratio

When the CSI and the total lipid concentration for a series of model biles was held constant at CSI = 1.4 and 8 g/dl, vesicle formation could be studied as a function of the BS/PL ratio (Table 1, e, f, g, h, and i). Both the cholesterol and the phospholipid content of the vesicle fraction dropped precipitously as the BS/PL ratio of the solution rose (Table 3, 7 to 11). When the solution contained a BS/PL ratio of 1.9, the concentration of solution cholesterol distributed to the vesicular phase was 30.4% (4.4 mM); whereas when the solution composition reflected a BS/PL ratio of 5.7, the concentration of solution cholesterol distribution to the vesicular phase fell markedly to a level of 2.3% (0.24 mM). The percentage fall (86%) in vesicular phase distribution of cholesterol under these conditions was nearly identical to that of phospholipid (Table 3). As determined by the CHOL/PL ratio in the vesicle fraction, vesicles became fully saturated with cholesterol at a low BS/PL ratio, (e.g., BS/PL = 2.3). As the BS/PL molar ratio of these solutions fell from 5.7 to 2.3, their nucleation times increased slightly (**Fig. 4**), but the CHOL/PL ratio of the vesicles remained relatively constant (CHOL/PL = 2.0). When the BS/PL ratio fell to 1.9, the nucleation time became significantly prolonged and the CHOL/PL ratio fell to 1.0.

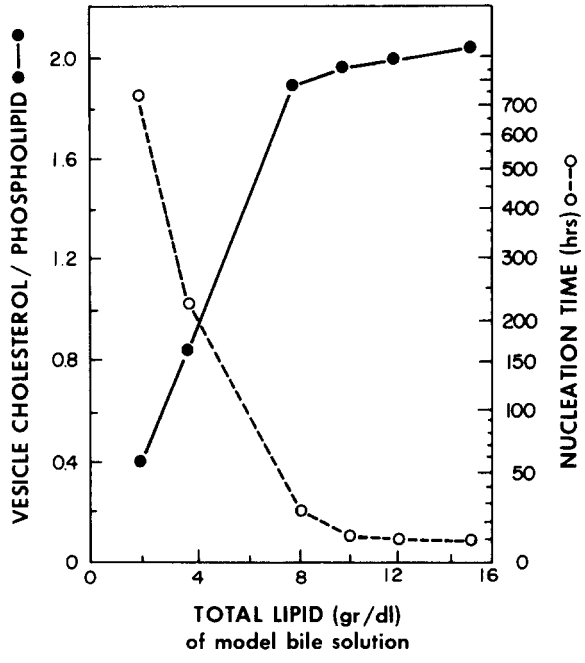


Fig. 3. Relation between nucleation time and cholesterol/phospholipid ratios in vesicles as a function of *dilution* from different model bile systems.

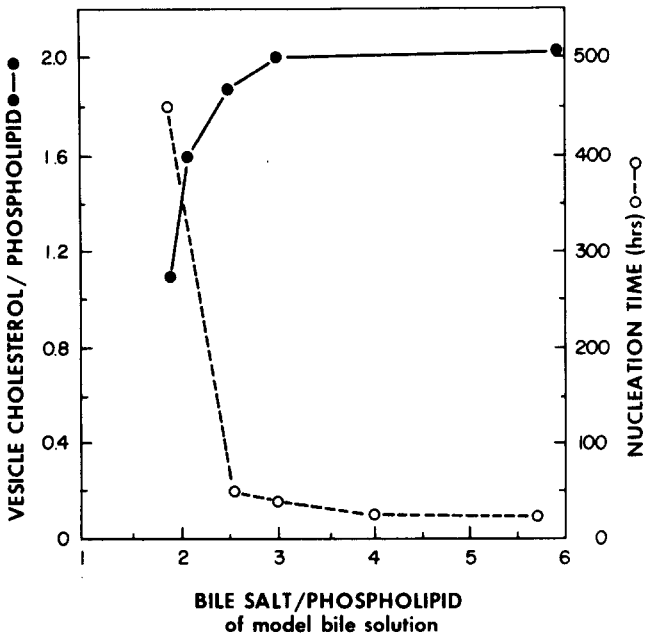


Fig. 4. Relation between nucleation time and cholesterol/phospholipid ratios in vesicles as function of *bile salt/phospholipid ratio* from different model bile systems.

Vesicle composition as a function of CSI

Using a series of model biles in which the total lipid concentration and the BS/PL ratio were held constant (8 g/dl, BS/PL = 2.3) while the CSI was increased from 1.1 to 1.5, it was possible to study the properties of the vesicles as a function of the cholesterol concentration in the solution (Table 1, j, k, l, m, n, and o). Under these conditions, the amount of phospholipid found in the isolated vesicle fraction remained relatively constant at 2 mM (Table 3, 12 to 17). The percentage of phospholipid in vesicles likewise remained stable between 4 and 6%. In contrast, as the CSI increased, the cholesterol content of the vesicle fraction likewise increased; rising nearly fourfold from 8.0% (CSI = 1.1) to 28.4% (CSI = 1.5). A simultaneous increase in the CHOL/PL ratio resulted in the vesicles being almost fully saturated by the time the CSI had reached 1.2 (Fig. 2, a). Nucleation time studies of these same solutions demonstrated a nearly linear correlation between the CSI and nucleation time, but an inverse relationship between the nucleation time and the CHOL/PL ratio (**Fig. 5**).

DISCUSSION

Cholesterol crystal nucleation is essential to gallstone formation and, as shown in the present study, depends upon prior extensive vesicle aggregation. Moreover, vesicle aggregation seems important to nucleation events in supersaturated native biles so that information on vesicle lipid composition is helpful in understanding vesicle aggregation (5). Evidence in the present study for linkage between vesicle aggregation and cholesterol monohydrate crystal nucleation is as follows. When nucleation is retarded as in dilute supersaturated systems, vesicle aggregation is likewise very slow in onset. When nucleation is rapid as in the more concentrated systems, i.e., 15–20 g/dl, vesicle aggregation is also rapid. Invariably, vesicle aggregation precedes crystal nucleation.

We often observed what appeared to be simple fusion of unit vesicles preceding their clustering or aggregation, but the extent to which the process takes place is not possible to quantify. Other workers have noted that agents capable of inducing vesicle fusion likewise promote aggregation but were unable to experimentally isolate the two processes (31–35). An indication that vesicle aggregation could be a precursor of crystal nucleation is to be found in the observations of Collins and Phillips (8) using stored supersaturated cholesterol-phospholipid co-dispersions. They showed by sequential electron microscopy that such systems were unstable (metastable); upon storage, vesicle aggregation and cholesterol crystal precipitation occurred. The events were time-dependent and were not observed when equilibrium saturation of vesicles was reached at a cholesterol/phospholipid molar ratio of < 2:1. Observations of Igimi, Nishijima, and Shimura (36) with supersaturated model systems have also led to the suggestion that vesicle aggregation and fusion could comprise an element in the pathway between unstable micelles and liquid crystal formation, another form of precipitate believed to often precede solid crystal nucleation. It is difficult to precisely correlate polarizing microscopic evidence of liquid crystals as solid crystal precursors with the present vesicle studies using higher resolution microscopy (37). Despite this difficulty, it is reasonable to believe that large multilamellar aggregates could contain liquid crystalline domains capable of

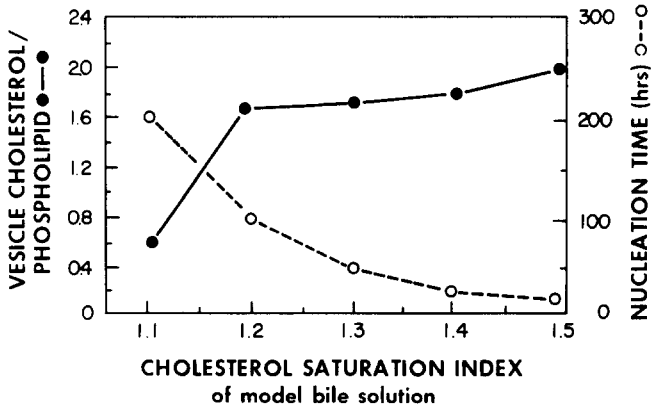


Fig. 5. Relation between nucleation time and cholesterol/phospholipid ratios in vesicles as function of *CSI* in different model bile systems.

producing the typical polarizing microscopy and X-ray diffraction pattern of smectic mesophase.

Among the various factors influencing the metastability of the different supersaturated model bile systems, the degree of cholesterol saturation of vesicles (cholesterol/phospholipid molar ratio below 2.0) emerged as the primary determinant. The relationship between degree of cholesterol saturation of the vesicles and nucleation was directly proportionate as indicated in Figs. 3–5. Both vesicle aggregation and crystal nucleation were observed to be more rapid in vesicles metastably supersaturated in cholesterol. Accordingly, a key finding in the present study was that the vesicular CHOL/PL ratio (i.e., degree of cholesterol saturation of vesicles) was variable with different solution conditions. Vesicular cholesterol saturation was lowest (1:2) in the more stable dilute systems, e.g., 2 g/dl, and although influenced by other variables, highest (2:1) in many of the more concentrated systems. By contrast, it should be emphasized that at the greatest degree of dilution employed, i.e., 2 g/dl, when such solutions were unsaturated, vesicles could not be observed. In principle, however, under conditions of dilution greater than that employed in the present study such as canalicular bile, vesicle formation could occur even in unsaturated solutions merely as a consequence of dilution alone (38–40). Moreover, under conditions of bile salt depletion, e.g., chronic biliary fistula, phospholipid-rich vesicle formation would also be expected due to the comparative inability of such solutions to form micelles. An extrapolation of the trend reflected in our solution No. 7 result (with the lowest BS/PL ratio studied) is also compatible with this view.

Recent evidence has indicated that, in supersaturated model and native biles, the presence of vesicles in addition to micelles adds a complementary mode of cholesterol transport and may help explain metastable supersaturation (1–4). The importance of this consideration lies in its conceptual application to dilute systems, e.g., hepatic bile, where the solution cholesterol saturation index may be markedly raised. In this situation, our studies indicate that a highly significant amount (about 37% of total) of the excess cholesterol is distributed to the vesicular phase (Table 3, solution No. 6), yet the vesicles themselves remain unsaturated with cholesterol and are therefore relatively stable. In more concentrated systems with elevated CSI's, the amount of excess cholesterol distributed to the vesicular phase is comparably raised (about 1/3 total), e.g., solution No. 17. The vesicular cholesterol content of these solutions, however was high (CHOL/PL molar ratio of 2.0) and nucleation was rapid. These observations underscore the view that the degree of vesicular saturation in cholesterol is of greater importance in influencing vesicle aggregation than the proportionate distribution of cholesterol to the vesicular phase. Our estimates of the amount of cholesterol in the vesicular phase varied from zero to as high as 37% of total soluble cholesterol. All of the variables studied, including dilution, BS/PL, and CSI, were shown almost equally capable of increasing vesicular cholesterol.

Using quasielastic light scattering, Mazer and Carey (2) have provided observations comparable to ours regarding the relative stability of dilute model bile systems, i.e., 3 g/dl, stable to 30 days. They also observed an instability in more concentrated systems, i.e., 10 g/dl and described the rapid onset of lipid precipitation which evolved into detectable liquid crystals (2). Their estimate of the degree of cholesterol saturation of vesicles in 3 g/dl dilute systems (i.e., CHOL/PL molar ratio) was markedly higher, however, than ours (i.e., CHOL/PL molar ratios = 1:2). Experimental agreement with our vesicle cholesterol saturation estimate in dilute, i.e., 2 g/dl, native bile has recently been observed by others (F. O. Nervi, personal communication). In addition, Mazer and Carey (2) failed to find compositional differences between vesicles derived from dilute systems and those from more concentrated systems. Instead, they reported a correlation in the more dilute and stable lipid precipitates with smaller particle size, i.e., 2–40 nm, and conversely, a correlation in the more concentrated unstable systems with larger particles. They speculated that the comparative stability of the dilute systems was a function of small vesicle size. Our observations clearly differ both in regard to vesicle composition and in our estimates of comparative vesicle size in dilute and more concentrated systems using electron microscopy as reported here and by means of a combination of electron microscopy and quasielastic light scattering in another recent study (10). In none of these structural studies, have we been unable to discern a systematic difference in particle size resulting from dilution. We cannot at present account for these apparent discrepancies.

The presently suggested key role for vesicle aggregation in cholesterol monohydrate crystal nucleation leads to a final consideration. Factors capable of both inhibiting and promoting nucleation have recently been described in native bile (9, 13–15). Among these factors are apolipoproteins A-I and A-II, crude bile proteins, and Ca^{2+}. It is not unreasonable to imagine that these counteracting factors could mediate their effects by means of an influence on vesicle aggregation (23–27). Viewed in this perspective, the necessary presence of cholesterol supersaturation leads to spontaneous vesicle formation (10). This, in turn, is affected by both the stability of the vesicles themselves as described in this report and ultimately by the outcome of a balance between the activity of naturally occurring nucleation inhibitors and promotors (41).

Manuscript received 18 June 1985.

REFERENCES

1. Sömjen, G. J., and T. Gilat. 1983. A non-micellar mode of cholesterol transport in human bile. *FEBS Lett.* **156:** 265–268.
2. Mazer, N. A., and M. C. Carey. 1983. Quasi-elastic light-scattering studies of aqueous biliary lipid systems. Cholesterol solubilization and precipitation in model bile solutions. *Biochemistry.* **22:** 426–442.
3. Kibe, A., A. C. Breuer, and R. T. Holzbach. 1984. Cholesterol nucleation in human bile by video-enhanced contrast-differential interference microscopy (VEM): the role of vesicles in metastable supersaturation. *Gastroenterology.* **85:** 1326 (Abstract).
4. Pattinson, N. R. 1985. Solubilization of cholesterol in human bile. *FEBS Lett.* **181:** 339–342.
5. Halpern, Z., M. A. Dudley, A. Kibe, M. P. Lynn, A. C. Breuer, and R. T. Holzbach. 1986. Rapid vesicle formation and aggregation in abnormal human biles: a time-lapse video-enhanced contrast microscopy study. *Gastroenterology.* **90:** In press.
6. McLean, L. R., and M. C. Phillips. 1984. Cholesterol transfer from small and large unilamellar vesicles. *Biochim. Biophys. Acta.* **776:** 21–26.
7. McLean, L. R., and M. C. Phillips. 1984. Kinetics of phosphatidylcholine and lysophosphatidylcholine exchange between unilamellar vesicles. *Biochemistry.* **23:** 4624–4630.
8. Collins, J. J., and M. C. Phillips. 1982. The stability and structure of cholesterol-rich codispersions of cholesterol and phosphatidylcholine. *J. Lipid Res.* **23:** 291–298.
9. Gollish, S. H., M. J. Burnstein, R. G. Ilson, C. N. Petrunka, and S. M. Strasberg. 1983. Nucleation of cholesterol monohydrate crystals from hepatic and gallbladder bile of patients with cholesterol gallstones. *Gut.* **24:** 836–844.
10. Kibe, A., M. A. Dudley, Z. Halpern, M. P. Lynn, A. C. Breuer, and R. T. Holzbach. 1985. Factors affecting cholesterol monohydrate crystal nucleation time in model systems of supersaturated bile. *J. Lipid Red.* **26:** 1102–1111.
11. Holzbach, R. T., A. Kibe, E. Thiel, J. H. Howell, M. Marsh, and R. E. Hermann. 1984. Biliary proteins: unique inhibitors of cholesterol crystal nucleation in human gallbladder bile. *J. Clin. Invest.* **73:** 35–45.
12. Kibe, A., R. T. Holzbach, N. F. LaRusso, and S. J. Mao. 1984. Inhibition of cholesterol crystal formation by apolipoproteins A-I and A-II in model systems of supersaturated bile: implications for gallstone pathogenesis in man. *Science.* **225:** 514–516.
13. Burnstein, M. J., R. G. Ilson, C. N. Petrunka, R. D. Taylor, and S. M. Strasberg. 1983. Evidence for a potent nucleating factor in the gallbladder bile of patients with cholesterol gallstones. *Gastroenterology.* **85:** 801–807.
14. Levy, P. F., B. F. Smith, and J. T. Lamont. 1984. Human gallbladder mucin accelerates nucleation of cholesterol in artificial bile. *Gastroenterology.* **87:** 270–275.
15. Gallinger, S., R. D. Taylor, P. R. C. Harvey, C. N. Petrunka, and S. M. Strasberg. 1985. Effect of mucous glycoprotein on nucleation time of human bile. *Gastroenterology.* **89:** 648–658.
16. Pope, J. L. 1967. Crystallization of sodium taurocholate. *J. Lipid Res.* **8:** 146–147.
17. Carey, M. C., and D. M. Small. 1978. The physical chemistry of cholesterol solubility in bile. Relationship to gallstone formation and dissolution in man. *J. Clin. Invest.* **61:** 988–1026.
18. Holan, K. R., R. T. Holzbach, R. E. Hermann, A. M. Cooperman, and W. J. Claffey. 1979. Nucleation time: a key factor in the pathogenesis of cholesterol gallstone disease. *Gastroenterology.* **77:** 611–617.
19. Allain, C. C., L. S. Poon, C. S. G. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total serum cholesterol. *J. Clin. Chem.* **20:** 470–475.
20. Cholesterol (Enzymatic Method). 3rd Edition. 1979. Boehringer Mannheim Corporation, Indianapolis, IN.
21. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66:** 375–400.
22. Bartlett, G. R. 1959. Phosphorus assay by column chromatography. *J. Biol. Chem.* **234:** 466–468.
23. Turley, S. D., and J. M. Dietschy. 1978. Re-evaluation of the 3α-hydroxysteroid dehydrogenase assay for total bile acids in bile. *J. Lipid Res.* **19:** 924–928.
24. Allen, R. D., N. S. Allen, and J. L. Travis. 1981. Video-enhanced, differential contrast (AVEC-DIC) microscopy: a new method capable of analyzing microtubule-related motility in the reticulopodial network of *Allogromia laticollaris*. *Cell Motil.* **1:** 291–302.
25. Allen, R. D., and N. S. Allen. 1983. Video-enhanced microscopy with a computer frame memory. *J. Microsc.* **129:** 3–17.
26. Kachar, B., D. F. Evans, and B. W. Ninham. 1984. Video-enhanced differential interference contrast microscopy: a new tool for the study of association colloids and prebiotic assemblies. *J. Colloid Interface Sci.* **100:** 287–301.
27. Forte, T. M., A. V. Nichols, E. L. Gong, S. Lux, and R. I. Levy. 1971. Electron microscopic study on reassembly of plasma high density apoprotein with various lipids. *Biochim. Biophys. Acta.* **248:** 381–386.
28. Hollander, M., and D. A. Wolf. 1973. Non-parametric statistical methods. John Wiley & Sons, Inc., New York. 115–119.
29. Nancollas, G. H. 1979. The growth of crystals in solution. *Adv. Colloid Interface Sci.* **10:** 215–252.
30. Toor, E. W., D. F. Evans, and E. L. Cussler. 1978. Cholesterol monohydrate growth in model bile solutions. *Proc. Natl. Acad. Sci. USA.* **75:** 6230–6234.
31. Duzgunes, N., J. Wilschut, R. Fraley, and D. Papahadjopoulos. 1981. Studies on the mechanism of membrane fusion: role of head-group composition in calcium- and magnesium-induced fusion of mixed phospholipid vesicles. *Biochim. Biophys. Acta.* **642:** 182–195.
32. Young, T. M., and J. D. Young. 1984. Protein-mediated intermembrane contact facilitates fusion of lipid vesicles with planar bilayers. *Biochim. Biophys. Acta.* **775:** 441–445.
33. Ohki, S., and K. Leonards. 1982. Effects of proteins on phospholipid vesicle aggregation and lipid vesicle-monolayer interactions. *Chem. Phys. Lipids.* **31:** 307–318.
34. Rossi, J. D., and B. A. Wallace. 1983. Binding of fibronectin to phospholipid vesicles. *J. Biol. Chem.* **258:** 3327–3331.
35. Garcia, L. A. M., P. S. Araujo, and H. Chaimovich. 1984. Fusion of small unilamellar vesicles induced by a serum albumin fragment of molecular weight 9000. *Biochim. Biophys. Acta.* **772:** 231–234.
36. Igimi, H., S. Nishijima, and H. Shimura. 1983. The development process from micelle to liquid crystal in the ursodeoxycholate, phosphatidylcholine and cholesterol solution. *J. Pharmacobio-Dyn.* **6:** 267–271.
37. Holzbach, R. T., and C. Corbusier. 1978. Liquid crystals and cholseterol nucleation during equilibration in supersaturated bile analogs. *Biochim. Biophys. Acta.* **528:** 436–444.
38. Schurtenberger, P., N. Mazer, and W. Känzig. 1983. Static

and dynamic light scattering studies of micellar growth and interactions in bile salt solutions. *J. Phys. Chem.* **87:** 308–315.

39. Mazer, N. A., P. Schurtenberger, M. C. Carey, R. Preisig, K. Weigand, and W. Känzig. 1984. Quasi-elastic light scattering studies of native hepatic bile from the dog: comparison with aggregative behavior of model biliary lipid systems. *Biochemistry.* **23:** 1994–2005.
40. Schurtenberger, P., N. Mazer, and W. Känzig. 1985. Micelle to vesicle transition in aqueous solutions of bile salt and lecithin. *J. Phys. Chem.* **89:** 1042–1049.
41. Holzbach, R. T., and A. Kibe. 1985. Chapter 4. Pathogenesis of cholesterol gallstones. *In* Contemporary Issues in Gastroenterology. Volume 4. Gallstones. S. Cohen and R. D. Soloway, editors. Churchill-Livingstone, New York. 73–100.