Effect of Calcium on Kinetic and Structural Aspects of Dilution-Induced Micellar to Lamellar Phase Transformation in Phosphatidylcholine–Cholate Mixtures†

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ABSTRACT: Previously, we have shown [Almog, S., Kushnir, T., Nir, S., & Lichtenberg, D. (1986) *Biochemistry 25*, 2597–2605] that the distribution of cholate between phosphatidylcholine (PC) vesicles and aqueous media apparently obeys a single distribution coefficient, K. In PC–cholate mixed micellar systems, the monomer concentration does not rise much above the cholate's critical micelle concentration (cmc). Consequently, for vesicular systems, the cholate:PC molar ratio in the mixed aggregates (R_e) is given by R_e = [cholate]/([PC] + $1/K$) whereas for mixed micellar systems R_e = ([cholate] − cmc)/[PC]. Dilution of mixed micellar systems results in a decrease of R_e, due to an increase in the fraction of monomeric PC. If the decrease in R_e is to values lower than 0.3, micellar to lamellar transformation occurs. This process involves a sequence of three steps, namely, micellar equilibration followed by vesiculation and subsequent vesicle size growth via a lipid transfer mechanism. The ultimate size of the resultant vesicles is an increasing function of R_e. This work is devoted to the effect of calcium on the dilution-induced vesicle formation. Its major findings and conclusions are as follows: (i) Calcium reduces the cmc of the detergent and raises its distribution coefficient between PC vesicles and the aqueous medium. Thus, for any given cholate and PC concentrations, calcium causes an increase of R_e. (ii) The rate of all the steps which ultimately lead to an apparent equilibrium vesicle size distribution increases dramatically with increasing calcium concentration. Thus, equilibration is attained in seconds to minutes rather than many hours required in the absence of calcium. (iii) The rate of the postvesiculation size growth is an increasing function of lipid concentration, indicating the probable involvement of a fusion mechanism in the vesicle size equilibration. This conclusion is strengthened by retention of dextran, entrapped in unilamellar vesicles during cholate-induced size growth. (iv) Calcium has no effect on the dependence of vesicle size on R_e. Calcium, indeed, affects the aggregation state of the lipid by increasing the value of R_e, but for any given R_e, the state of aggregation is independent of calcium. This means that, for mixtures with low values of R_e, addition of calcium causes an increase of the mean diameter of the vesicles, whereas for higher R_e values, it may lead to micellization and a subsequent decrease of micelle sizes. The lack of a direct effect of calcium on the state of aggregation of PC–cholate mixtures of any effective ratio, obtained in spite of the calcium-induced change in the mechanism of vesicle size growth, supports the conclusion of Schurtenberger et al. [Schurtenberger, P., Mazer, N. A., & Kanzig, W. (1985) *J. Phys. Chem. 89*, 1042–1049] that the ultimate size distribution obtained for PC–cholate vesicles represents a true thermodynamic equilibrium of the diluted systems.

The lipid aggregational state in mixed dispersions of lipids and surfactants is an issue of much importance from several points of view: First, the chemical characterization of biological membranes requires their solubilization, in the form of mixed micelles, for isolation of membrane proteins (Helenius & Simons, 1975). Purified membrane proteins will then have to be reconstituted into model membranes (proteoliposomes) for analysis of their function (Racker, 1979). This strategy is responsible for much of our current understanding of model membranes. Nonetheless, the present approach to these procedures of solubilization (by surfactants) and reconstitution (by surfactant removal) is completely empirical. A deeper understanding of these processes may contribute much to the development of a more rational general approach to solubilization and reconstitution experiments. The phase behavior in lipid–surfactant mixtures is also of importance in enzymatic lipolysis reactions, which can, in many cases, be carried out only if the lipid is solubilized by surfactants [e.g., see Dennis (1984)]. Finally, in several parts of the gastrointestinal tract, lipids are solubilized in the aqueous medium by bile salts. The lipid state of aggregation in these mixtures is important because it may affect the lipid digestion by various enzymes in the liver, gallbladder, and intestine. Furthermore, precipitation of cholesterol from a bile salt–phospholipid–cholesterol mixed aggregate, which is the major cause of bile stone formation, is very likely to depend upon the aggregational state of these mixtures in the gallbladder bile (Somjen & Gilat, 1983; Kibe et al., 1985). Therefore, much effort has been devoted to studies of the state of aggregation in bile salt–lipid mixtures, which resulted in detailed phase diagrams for many such mixtures (Carey & Small, 1978).

In a recent work (Almog et al., 1986), we have shown that the state of aggregation in egg phosphatidylcholine–cholate mixed dispersions is a function of the effective cholate to phosphatidylcholine molar ratio (R_e),[1] namely, the ratio *in the*

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mixed aggregates (micelles or vesicles); the state of aggregation is essentially lamellar (vesicular) if $R_e \leq 0.3$ and micellar if $R_e \geq 0.4$, whereas in the range of R_e values between 0.3 and 0.4, micelles and vesicles coexist. The size of the mixed vesicles increases with increasing R_e and reaches a maximal radius of 55 nm. Similar phase behavior has been previously described for other phospholipid–bile salt mixtures (Schurtenberger et al., 1985).

The present work is devoted to the effect of Ca^{2+} on the phase behavior of PC–cholate mixtures. As calcium concentrations in gallbladder bile are high (5–20 mM; Lentner & Wink, 1981), it may greatly affect the aggregational state of the lipid. Furthermore, enzymatic hydrolysis of phospholipids may be dependent upon calcium concentration, and to study this dependence, information on the possible indirect effect of calcium, through its effect on the aggregational state of the lipid substrate, is required. The reported results clearly support this approach and provide data of much relevance to the various types of studies of calcium-containing lipid–bile salt mixed dispersions.

Materials and Methods

Materials. Phosphatidylcholine (PC) from fresh egg yolk was prepared according to Singelton (1965). Its purity was confirmed by thin-layer chromatography. Sodium cholate and fluorescein isothiocyanate (FITC)–dextran (M_r 70 000) were purchased from Sigma (St. Louis, MO) and were used without further purification. Porcine pancreatic phospholipase A_2 (Sigma) was dialyzed against saline for 6 h prior to being used. Concanavalin A (Con A)–Sepharose was a product of Pharmacia (Sweden).

Micelle and Vesicle Preparation. PC and cholate were codissolved in methanol/chloroform (1:1). The solvents were removed completely under reduced pressure at room temperature, and the dry lipid–detergent film was hydrated in saline to yield a clear concentrated micellar dispersion. Vesicles of different compositions were prepared by dilution of the mixed micellar dispersion with saline solution (135 mM NaCl) containing different amounts of $CaCl_2$ and sodium cholate. The pHs of both the micellar system and the diluting media were preadjusted to 7.4.

Turbidity Measurements. Turbidity was measured usually at 450 nm, using a light path of 2 cm, on a Brinkmann PC-801 probe calorimeter equipped with Servograph Pen Drive recorder (READ 310, Radiometer, Copenhagen). The molar absorbance of vesicular dispersions (turbidity/M), here denoted e, increases linearly with the hydrodynamic radius (R_h) of the vesicles, as described in detail in our previous work (Almog et al., 1986), which can yield a rough estimate of the mean vesicle size.

Electron microscopy and nuclear magnetic resonance (NMR) measurements were performed as described in our previous work (Almog et al., 1986).

Phospholipase A_2 Activity. The time course of the enzymatic hydrolysis by pancreatic PLA_2 was monitored with a pH stat according to Nieuwenhuizen et al. (1973).

Determination of Cholate to PC Ratio in Lipid Bilayers. Ultracentrifugation of PC–cholate mixtures at 200000g for 8 h causes precipitation of all the vesicles but not of the monomeric or the micellar bile salt (Almog et al., 1986). Following the addition of "sub-solubilizing" cholate concentrations to PC vesicular dispersions and centrifugation, no PC was present in the supernatant, as detected by the colorimetric method of Stewart (1980). The bile salt concentration in the supernatant (D_w), detected enzymatically according to Turnberg and Anthony-Mote (1969), was smaller than the total cholate concentration (D_T) detected prior to centrifugation. The partition coefficient K is defined as

$$K = D_B/LD_W \quad (1)$$

where D_B is the cholate concentration associated with the lipid bilayers, D_W is the cholate level in the aqueous phase, and L is the lipid concentration. The cholate to PC ratio in cholate-containing vesicles is given by

$$R_e = D_T/(L + 1/K) \quad (2)$$

In micellar mixtures, the aqueous intermicellar cholate concentration (IMC) may increase (Mazer et al., 1980). However, for total cholate concentrations below 20 mM, the IMC increases only slightly above the cmc, and, therefore, R_e is approximately equal to

$$R_e = (D_T - \text{cmc})/L \quad (3)$$

Measurements of L, D_T, K, and the cmc therefore provide an approximate value of R_e for any given system.

Entrapment and Retention of Dextran in Unilamellar Vesicles. Multilamellar vesicles were made by hydrating dry egg PC in an aqueous medium containing 135 mM NaCl, 10 mM $CaCl_2$, and 5 mg/mL FITC–dextran. Following vortex mixing of the suspension (15 min), it was sequentially extruded through polycarbonate filters (Nucleopore) of diameter pore sizes of 0.2, 0.1, and then 0.03 μm, using "The Extruder" (Lipex Biomembranes Inc., Vancouver, B.C.). This procedure resulted in the formation of vesicles with a mean hydrodynamic radius of 30.7 nm, as detected by a laser scattering spectrometer (Nicomp, Model HN-5/90; equipped with a computer autocorrelator, Model 6894). Nonentrapped FITC–dextran was removed by passing the dispersion through a 15 × 50 mm Con A–Sepharose column with 135 mM NaCl and 10 mM $CaCl_2$ as eluant. Vesicles emerged in the void volume, whereas the nonentrapped dye was retained. The fraction of dye entrapped in the vesicles was about 0.6%, as detected from a calibration graph. This level of entrapment corresponds to an entrapment efficiency of 1.2 L of aqueous medium per mole of lipid. This level of entrapment efficiency is expected for unilamellar vesicles of a mean radius of 30 nm (Lichtenberg et al., 1981), indicating that the vesicle preparation was essentially unilamellar.

Retention of FITC–dextran in the vesicles, following the addition of cholate, was studied as follows: Prior to and at varying times after cholate addition, aliquots of the vesicle dispersion were passed through small (8 × 8 mm) Con A–Sepharose columns. The vesicles, emerged in the void volume, were solubilized by excess cholate, and the probe retained in them was determined by fluorescence intensity measurements (excitation 490 nm; emission 520 nm). As a control, the solubilized dispersions were then passed through the columns. Upon this second passage, all the probe was retained by the

[1] Abbreviations: PC, phosphatidylcholine; cmc, critical micellar concentration; OD, optical density; ϵ, molar optical density (OD/M); R_e, effective ratio, molar ratio of cholate to PC *in mixed aggregates* (micelles and vesicles); $[\text{cholate}]^{crit}$, cholate concentration at which the turbidity of the PC–cholate system containing vesicles and micelles is halfway between its maximal and minimal values; R_e^{crit}, slope of the line which describes $[\text{cholate}]^{crit}$ as a function of [PC]; K, distribution coefficient of cholate between PC vesicles and the aqueous medium; QLS, quasi-electric light scattering; NMR, nuclear magnetic resonance; $\bar{R}_h$, mean hydrodynamic radius of micelles or vesicles; FITC, fluorescein isothiocyanate; Con A, concanavalin A; PLA_2, phospholipase A_2; IMC, intermicellar cholate concentration; EDTA, ethylenediaminetetraacetic acid; SUV, small unilamellar vesicle.

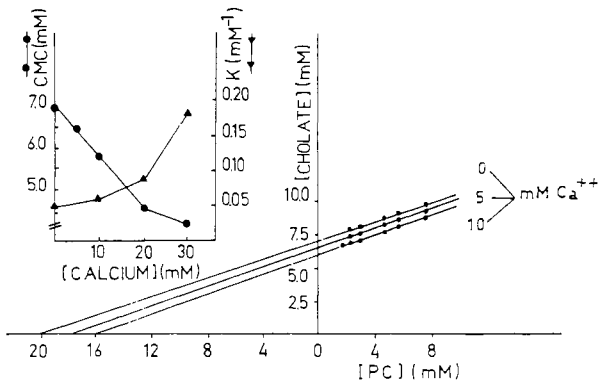

FIGURE 1: [Cholate]^{crit} (the cholate concentration at which the OD of a system of a given PC concentration is halfway between the maximal and minimal turbidity) as a function of the PC concentration at the three different concentrations of $CaCl_2$ indicated on the figure. Dispersions were made by diluting a mixed micellar system of 100 mM PC and 75 mM cholate in saline solutions containing $CaCl_2$ and varying cholate concentrations. For any given final PC concentration (attained by the appropriate dilutions), the turbidity depended in a "bell-shaped" fashion on the final cholate concentrations. The values of [cholate]^{crit}, extracted from these dependencies as described in the text, are plotted in this figure as a function of [PC] at the different calcium concentrations. The inset describes the dependence of the apparent cmc and the distribution coefficient (K) on the calcium concentration (see text for details).

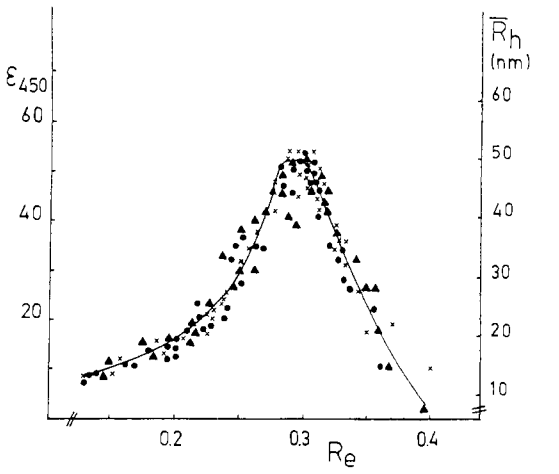

FIGURE 2: Dependence of the molar absorbance (ϵ_{450}) and the mean hydrodynamic radius ($\bar{R}_h$) of equilibrated PC–cholate dispersions on R_e, the cholate:PC molar ratio in mixed aggregates. The figure presents the data of many series of experiments in which R_e was varied either by dilution of PC–cholate mixed micelles in Ca^{2+}-containing media or by cholate addition to preequilibrated vesicles. The closed circles represent results of experiments done in the absence of Ca^{2+}, whereas the data represented by the times signs and the closed triangles are from experiments carried out in the presence of 5 and 10 mM $CaCl_2$, respectively. The solid line is hand-drawn through the data points.[2] $\bar{R}_h$ was calculated by use of eq 4 [$\bar{R}_h = (0.83\epsilon_{450} + 6.4)$ nm].

columns, indicating the potency of the Con A–Sepharose to maintain all the nonentrapped probe in the first passage.

Results and Discussion

Structural Studies of Equilibrated Dispersions

Effect of Calcium on the Cholate:PC Ratio in Vesicles. Previously, we have shown (Almog et al., 1986) that dilution of mixed micellar dispersions of PC–cholate in cholate-containing media (in the absence of calcium) results in a "bell-shaped" dependence of the turbidity of the diluted and equilibrated dispersions on the cholate concentrations. This dependence has been interpreted as follows: (1) At low cholate concentrations, the turbidity increases with increasing cholate concentrations due to an increase in the size of the resultant vesicles. (2) At a critical effective ratio of cholate to PC, solubilization of vesicles begins. Further addition of cholate yields larger fractions of micellar PC, thus, a consequent decrease of turbidity. (3) When complete solubilization occurs, further addition of cholate affects the turbidity only slightly. (4) The critical detergent concentration, [cholate]^{crit}, defined as the cholate concentration at which the turbidity is halfway between the maximal turbidity and the turbidity of the micellar dispersion, depends linearly on [PC]. The line describing this dependence intersects with the cholate axis at the cmc of the bile salt and with the PC axis at $1/K$. Its slope is the critical value of R_e (R_e^{crit}) at which micellar $\rightleftharpoons$ lamellar phase transformation occurs (Lichtenberg, 1985).

In the presence of calcium, the "bell-shaped" character of the dependence of turbidity of equilibrated dispersions on cholate concentration (not shown) was qualitatively similar to that observed in Ca^{2+}-free media (Almog et al., 1986). For 8 mM PC and 10 mM Ca^{2+}, solubilization began at 7.2 mM and was completed at 11.5 mM cholate. The [cholate]^{crit} value was 8.9 mM. Similar experiments were performed with different PC concentrations, and the [cholate]^{crit} values were plotted against [PC]. A straight line was obtained ($r^2 = 0.99$) which intersected with the lipid axis at −16.00 mM ($1/K$ = 16 mM) and with the detergent axis at an apparent cmc of 5.8 mM as compared to −20 mM and 6.8 mM, respectively, in the absence of calcium (Figure 1). The value of K in the Ca^{2+}-containing media was confirmed by direct determination as described under Materials and Methods. For vesicular mixtures containing 10 mM $CaCl_2$, a plot of the cholate concentration associated with the vesicles ($D_B = D_T - D_W$) against LD_W yielded a straight line ($r^2 = 0.98$) which passed through the origin of the axes and had a slope of 0.063 mM^{-1}. This value of K is higher than that measured in a calcium-free system (K = 0.050 mM^{-1}; Almog et al., 1986).

Additional series of dilution experiments were carried out at 5, 20, and 30 mM calcium concentrations. The values of K and the apparent cmc values derived from these experiments are presented as a function of [Ca^{2+}] in the inset of Figure 1. As obvious from this figure, increasing the Ca^{2+} concentration results in a decrease in the apparent cmc while increasing the affinity of cholate to lecithin vesicles (increasing the partition coefficient K). This implies that for any given concentration of cholate and PC, calcium reduces the fraction of monomeric cholate, thus increasing the value of R_e.

Effect of Calcium on Vesicle Size. In spite of affecting both the value of K and the cmc, calcium had no effect on the slope of the dependencies of Figure 1. In terms of our previously published interpretation (Lichtenberg, 1985), this result means that the critical effective ratio at which micellar $\rightleftharpoons$ lamellar phase transformation occurs (R_e^{crit}) is ca. 0.35, independent of [Ca^{2+}]. Furthermore, calcium has only a slight direct effect (if any) on the dependence of vesicle size upon R_e. This conclusion is based on a large number of experiments in which we prepared vesicles of varying R_e values in media containing 0, 5, and 10 mM Ca^{2+} and studied the vesicle sizes by turbidity measurements, NMR spectroscopy, and electron microscopy. At any given [Ca^{2+}], variation of R_e was achieved by two different protocols: (a) dilution of PC–cholate mixed micelles in aqueous media containing different cholate concentrations; (b) addition of cholate to preequilibrated vesicle dispersions prepared by procedure (a). Figure 2 presents the molar turbidities (ϵ) of all the dispersions, made by both procedures at all three Ca^{2+} concentrations as a function of the effective ratio R_e. Statistical analysis[2] shows that there is no significant

difference between the dependencies of ϵ on $\bar{R}_h$ for the systems with various Ca^{2+} concentrations.

In our previous work (Almog et al., 1976), we have shown that in the absence of calcium the molar turbidity is a linear function of the vesicle hydrodynamic radius $\bar{R}_h$ (in nanometers):

$$\epsilon_{450} = 1.2(\bar{R}_h - 6.4) \quad (4)$$

The following findings suggest that this correlation is also valid in the presence of Ca^{2+}: (i) Dilution of preequilibrated vesicular dispersions in calcium-containing media had no effect on their molar turbidities, indicating the aggregation contributes very little, if at all, to the turbidity. This conclusion is considerably strengthened by the finding that addition of equimolar concentrations of EDTA to calcium-containing vesicular dispersions did not alter their molar turbidities. In addition, electron microscopy of negatively stained vesicles, prepared by dilution of micelles in Ca^{2+}-containing media, gave no indication for vesicle aggregation. (ii) Vesicular dispersions containing different Ca^{2+} concentrations but equal molar turbidities gave rise to almost identical ^1H NMR spectra (not shown). This finding is of special importance in view of the sensitivity of ^1H NMR spectroscopy to small vesicles (Schmidt et al., 1981), as oppposed to light scattering, to which large vesicles make the major contribution. (iii) Vesicles with an R_e value of 0.28 made by dilution of micelles in the absence as well as in the presence of $CaCl_2$ (10 mM) had the same molar turbidities ($\epsilon = 41$). The electron micrographs of these two dispersions were indistinguishable. Both show a rather homogeneous population of vesicles of radii 40 nm, as could have been predicted on the basis of the value of ϵ_{450} and eq 4.

Given the validity of this equation and the data of Figure 2, we conclude that R_e is the predominant, if not the sole, determinant of vesicle size. In view of the scattering of the data points in this figure around the solid line, it is impossible to rule out a possible direct effect of Ca^{2+} on $\bar{R}_h$, but if such an effect exists, it must be minor.[2] The indirect effect of calcium on vesicle size, through the Ca^{2+}-induced increase of R_e, is much more significant. It is best demonstrated by the addition of Ca^{2+} to preequilibrated vesicular dispersions: When the addition of calcium causes R_e to increase to levels still lower than 0.30, it is accompanied by an increase of turbidity. In contrast, a calcium-induced increase in R_e to levels higher than 0.30 caused a decrease of the molar turbidity, which can be attributed to (partial) solubilization. This is evident from the effect of calcium addition on the ^{31}P NMR spectra of a cholate–PC vesicular dispersion containing 2.5 mM PC and 5.7 mM cholate ($R_e = 0.25$). Addition of 10 mM calcium to this dispersion resulted in an increase of R_e to a value of 0.3 and caused narrowing of the ^{31}P signal which may be attributed either to a cholate-induced decrease in the motional restriction present in vesicular PC (Brouillette et al., 1982) and/or to partial solubilization (London & Feigenson, 1979; Dennis & Pluckthan, 1984). Addition of calcium to a final concentration of 30 mM, which led to $R_e = 0.72$, resulted in a sharp ^{31}P NMR resonance typical for micellar dispersion, indicating complete solubilization of the vesicles.

In those experiments where calcium addition to equilibrated vesicles led to an increase of turbidity, a new equilibrium was established when the turbidity was similar to that obtained in the dilution experiments at the same R_e. Thus, the mean size of vesicles is dependent only on their composition (R_e) and is independent of either the calcium concentration or the preparation procedure. This is not the case for vesicles made by further dilution of equilibrated vesicular dispersions. Although the dilution resulted in a decrease of R_e, due to extraction of cholate from the vesicles into the aqueous medium, the vesicle size remained unaltered. This "kinetic trap" phenomenon is similar to that obtained with vesicles made in the absence of Ca^{2+} (Almog et al., 1986).

Kinetic Studies

Effect of Calcium on the Rate of Vesicle Formation and Equilibration. A 12.5-fold dilution of a mixed micellar dispersion containing 100 mM PC and 75 mM cholate, in the presence of 10 mM $CaCl_2$, led to a rapid enhancement of turbidity (not shown). The turbidity reached an apparent equilibrium level (Figure 2) in 2 min and then remained unaltered for the following 24 h. At any given time after equilibration, the dependence of log OD on the logarithm of the wavelength (log λ) was linear ($r^2 \geq 0.99$), with a slope of −4.00, as expected for Rayleigh scattering of light. Electron micrographs of the dispersion, examined both 15 min and 24 h after dilution, appeared to contain identical vesicular structures.

The characteristics of the hydrolysis of the PC in the dispersion, by porcine pancreatic phospholipase A_2, suggest that even 2 min after dilution all the PC was in fact contained in vesicles. Specifically, the enzymatic hydrolysis of liposomal PC by this enzyme is characterized by an initial phase of slow hydrolysis (latency period) followed by an abrupt increase of the rate. The latency observed for vesicular PC is extremely sensitive to the vesicle size while hydrolysis of micellar PC or mixtures of micellar and vesicular PC has never been preceded by such a latency (Gheriani-Gruszka Almog, Biltonen, and Lichtenberg, submitted for publication). The dispersion made by the procedure described above demonstrated a latency of 16.3 ± 0.6 min when the enzyme was applied to the system either 2 min, 25 min, 160 min, or 24 h after the dilution, suggesting that 2 min after dilution very little PC (if any) was contained in micelles. Furthermore, in view of the sensitivity of the latency to vesicle size, we conclude that the equilibration of turbidity indeed reflects equilibration of vesicle sizes in less than 2 min.

In our previous work (Almog et al., 1986), the time-dependent increase of turbidity, observed upon dilution of a PC–cholate mixed micellar systems (in the absence of Ca^{2+}), was sigmoidal; initially, the turbidity increased relatively slowly, and only after a lag time of length τ, a somewhat abrupt turbidity increase was observed. During the time τ, the ^1H NMR signals broadened (e.g., the choline head group signal broadened from ca. 4 to ca. 10 Hz) while later on, no further broadening occurred. Our interpretation of these results was that vesicle formation occurred during the time τ, yielding broadening of NMR signals without causing marked increase of the turbidity. The second phase of a more pronounced increase of turbidity was attributed to a slow postvesiculation size growth. The resultant, larger, cholate-

[2] For the purpose of statistical comparison of molar absorbance versus R_e curves, we have separated each of the curves (0, 5, and 10 mM Ca^{2+}) into two portions: an ascending nonlinear phase up to the inflection zone ($R_e = 0.13$–0.3) and a linear descending phase beyond the inflection ($R_e = 0.3$–0.4). The ascending phase was fitted [according to Batschelet (1971)] to the equation $\epsilon_{450} - 15 = \epsilon_{max}/[1 + K - e^{-\gamma\epsilon_{max}(R_e - 0.13)}]$ whereas the descending phase was fitted to the equation $\epsilon_{450} - 15 = B - A(R_e - 0.13)$. Postfitting procedures were done on an HP-85 microcomputer using the iterative least-squares nonlinear regression program of Nichols and Peck (1971). The fitted regression parameters (K, γ, B, and A) were compared by examination of the mean values, standard errors, and percentage coefficient variation. There were not significant differences between the fitted regression parameters for the three curves.

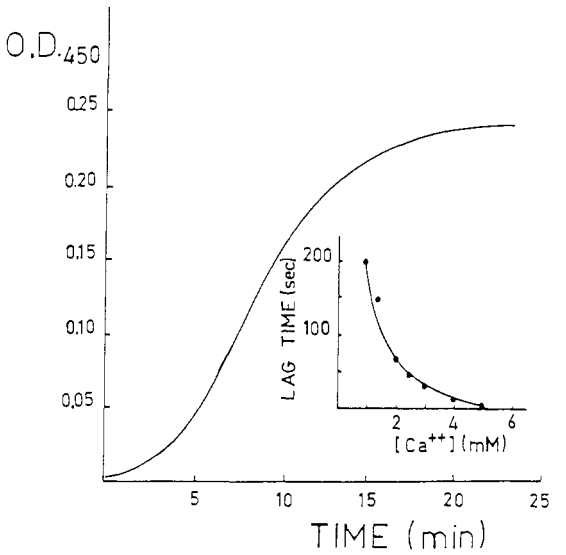

FIGURE 3: Time course of vesicle formation and equilibration upon dilution of a PC–cholate mixed micellar system. At time zero, a micellar solution containing 100 mM PC and 75 mM cholate was added to a 19-fold larger volume of a saline solution containing 1.0 mM $CaCl_2$. The length of the initial phase of slow increase of turbidity (lag time) observed in dilution experiments is presented in the inset as a function of $[Ca^{2+}]$. The diluting media contained cholate at concentrations chosen to maintain a constant R_e value of 0.25, as calculated according to eq 2 and the values of K appropriate for the respective $[Ca^{2+}]$ (Figure 1).

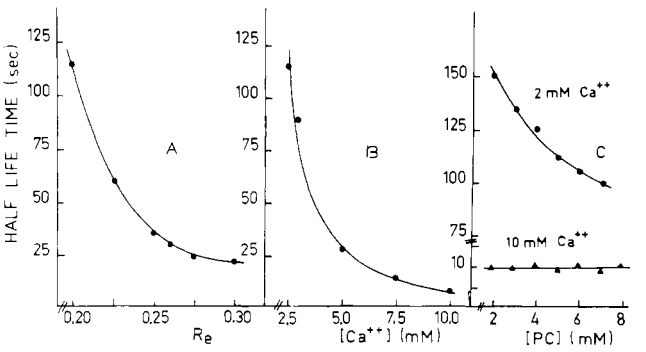

FIGURE 4: Dependence of the half-life ($t_{1/2}$) of vesicle size growth, induced by addition of sodium cholate to preequilibrated vesicles, on R_e (A), $[Ca^{2+}]$ (B), and [PC] (C). To study the dependence of $t_{1/2}$ on R_e, vesicles with an R_e of 0.163 were first made by 20-fold dilution of PC–cholate mixed micelles (100:75 mM) in saline containing 5 mM Ca^{2+} ($1/K = 18$). Cholate was then added to raise the R_e value of the vesicles from 0.163 to the varying final levels: $[\text{cholate}]_{\text{added}} = [(R_e - 0.163)(5 + 18)]$ mM. To evaluate the dependence of $t_{1/2}$ on $[Ca^{2+}]$, vesicles were first prepared by a 20-fold dilution of a mixed micellar solution of 100 mM PC and 75 mM cholate in media containing varying calcium and cholate concentrations, chosen such that the R_e value for all the vesicular dispersions was 0.25 [$0.25 = [\text{cholate}]_{\text{total}}/(5 + 1/K)$]. The K value for each calcium concentration was extracted from the inset to Figure 1. Following equilibration, an appropriate amount of cholate was added to each vesicular dispersion to raise the value of R_e from 0.25 to 0.30: $[\text{cholate}]_{\text{added}} = [(0.30 - 0.25)(5 - 1/K)]$ mM. To investigate the dependence of $t_{1/2}$ on [PC], vesicles were first made by 15–50-fold dilutions (and equilibration) of the PC–cholate (100:75 mM) mixed micelles in media containing 2 or 10 mM $CaCl_2$ (as indicated in the figure) and varying cholate concentrations, chosen to maintain an R_e value of 0.25. Subsequently, appropriate amounts of cholate were added to raise the R_e value from 0.25 to 0.30.

containing vesicles scatter much more light, but their ^1H NMR signals are not broader than those of the initially formed vesicles, probably because of the narrowing effect of cholate (Almog et al., 1986).

The same pattern of time-dependent increase of turbidity was observed in the presence of 1 mM $CaCl_2$ (Figure 3). However, both the lag time (τ) and the time of complete equilibration were dramatically shortened by the presence of Ca^{2+}. The "lag" time observed in the presence of 1 mM $CaCl_2$ is merely 3 min, as compared to a lag of 20–30 min observed in the absence of Ca^{2+}. In the NMR spectrum measured 3 min after dilution, the choline head-group signal had a half-width of 11 Hz, indicating that the PC was already contained in vesicles (Brouillette et al., 1982). No further broadening was observed in this line width, similar to the results obtained in the absence of Ca^{2+} after the lag phase of turbidity increase (Almog et al., 1986).

These findings indicate, indirectly, that the series of processes which occur after diluting PC–cholate mixed micelles in Ca^{2+}-containing media are qualitatively similar to those obtained in the absence of calcium. Calcium, however, facilitates all the steps involved in postdilution equilibration: it accelerates vesicle closure (shortens τ, as evident from the inset of Figure 3) as well as postvesiculation size growth. In fact, when the media contained more than 5 mM $CaCl_2$, the formation of vesicles appeared to be instantaneous, as the turbidity increase was not preceded by any detectable latency ($\tau \rightarrow 0$). Complete equilibration under these conditions took less than 5 min, as compared to 12 h in Ca^{2+}-free media.

The assignment of the "lag phase" in dilution experiments done at lower $[Ca^{2+}]$ to vesicle formation is supported by the finding that if vesicles were formed and equilibrated prior to the addition of more cholate, the resultant further size growth, induced by cholate addition, never demonstrated a lag phase (not shown). Thus, vesicle size growth always causes increased turbidity; formation of small vesicles has smaller effects.

Cholate-Induced Size Growth of Vesicles. The slowest step in the equilibration of diluted micellar systems was always the postvesiculation size growth. To study this step, we have first prepared (and equilibrated) vesicular dispersions and subsequently increased the effective ratio (R_e) in them by adding either cholate or calcium. In all cases, the reequilibration yielded dispersions whose molar turbidity was as expected for their R_e values on the basis of Figure 2. A parameter P describing the extent of the reequilibration reaction was defined by $P = (A_\infty - A_t)/(A_\infty - A_0)$, where A_0, A_∞, and A_t are the turbidities as measured at time zero (prior to the addition of cholate or Ca^{2+}), after reequilibration, and at any time t, respectively. The logarithm of P decreased linearly with time, indicating that the reequilibration follows first-order kinetics (at least apparently).

The time at which P decreases to a value of $^1/_2$ (half-life of reequilibration, $t_{1/2}$) depended on the concentrations of PC, cholate, and calcium, as described in Figure 4. The dependence of $t_{1/2}$ on R_e is described in Figure 4A. The experiment described in this figure was conducted such that we first prepared vesicles of a mean radius of about 20 nm ($\epsilon_{450} = 20.6$ OD units/M) and an R_e value of 0.163, by a 20-fold dilution of a micellar system of 100 mM PC and 75 mM cholate in a solution of 5 mM $CaCl_2$ in saline. The vesicular system was then equilibrated and subsequently divided into six aliquots, and varying amounts of cholate were subsequently added to yield dispersions of different R_e values in the range of 0.20–0.30. The half-life of the turbidity increase (Figure 4A) was a decreasing function of R_e. The same trend was observed in experiments done with vesicles made (and equilibrated) at higher R_e values, indicating the generality of the finding that the rate of size growth is an increasing function of R_e.

Studying the effects of calcium and lipid concentration on the vesicle size growth is more complex since each of these

factors, in addition to affecting the size growth by itself, changes the effective ratio R_e. Therefore, we had to plan experimental protocols by which we can study the effects of both calcium and PC concentrations without varying R_e. This was done as follows: (i) To study the effect of $[Ca^{2+}]$ on the postvesiculation size growth, we first prepared vesicle dispersions with constant [PC] (5 mM) and R_e (0.25) by diluting mixed micelles in media containing different calcium and cholate concentrations. The cholate concentrations were chosen to yield an R_e value of 0.25 in all the dispersions, by the use of eq 2 and the values of K appropriate for the various $[Ca^{2+}]$ (inset to Figure 1). The equilibrated dispersions all had the same molar turbidity (ϵ_{450} = 25.0 ± 1.1 OD units/M), indicating that the vesicle size was about constant ($\bar{R}_h$ = 27 nm). Cholate was then added to the equilibrated dispersions at concentrations chosen to yield an R_e value of 0.30 in all of them. All the resultant equilibrated dispersions contained vesicles of the same size (ϵ_{450} = 55 ± 3.5 OD units/M; i.e., $\bar{R}_h$ = 54 nm), in agreement with Figure 2. The rate of the time-dependent increase of turbidity was an increasing function of calcium concentration (Figure 4B). This increase of rate is especially pronounced for low $[CaCl_2]$, in comparison with the 2 orders of magnitude longer equilibration time in the absence of calcium.

(ii) To test the effect of lipid concentration on the rate of vesicle size growth, we have first diluted PC–cholate mixed micelles (100 mM PC + 75 mM cholate) in media containing 2.0 mM calcium and varying cholate concentrations chosen such that R_e is maintained at 0.23 while the PC concentration varied from 2.0 to 7.0 mM. In agreement with other experiments, the ultimate size of the resultant vesicles was constant (ϵ_{450} = 20 ± 1 OD units/M). Cholate was then added to the equilibrated dispersions at concentrations calculated to yield an increase of the value of R_e from 0.23 to 0.29. This cholate addition resulted, in all cases, in an increase of the molar absorbance from its original value of 23 ± 1 OD units/M to a final constant value of 53 ± 1 OD units/M. As shown in Figure 4C, increasing the PC concentration resulted in an increase in the rate of equilibration (decrease of $t_{1/2}$).

This dependence of $t_{1/2}$ on [PC] is inconsistent with a lipid transfer mechanism (Lawaczeck, 1978). Calcium-induced aggregation and subsequent fusion of the cholate-containing negatively charged vesicles are more likely. In general, vesicle fusion processes occur via a two-step mechanism, namely, aggregation of the vesicles and subsequent fusion (Nir et al., 1980). While the former is second order in vesicle concentration, the fusion itself is believed to be first order (Nir et al., 1980). As a consequence, if the rate-limiting step is aggregation, $t_{1/2}$ can be expected to decrease linearly with [PC] whereas if the fusion is rate limiting, $t_{1/2}$ should be independent of the phospholipid concentration. At high calcium concentration (e.g., 10 mM), $t_{1/2}$ was in fact independent of [PC] (Figure 4C), as expected for a fusion-controlled mechanism. This is not unexpected when aggregation is very rapid, as in other cases of highly charged vesicles in the presence of high calcium concentrations (Nir et al., 1980). On the other hand, when the calcium concentration is lower ($[Ca^{2+}]$ = 2 mM), aggregation also affects the overall reaction, and $t_{1/2}$ decreases with [PC], although less than according to a linear relationship.

A Ca^{2+}-induced fusion mechanism of the cholate-containing negatively charged vesicles is also consistent with the dependence of the rate of vesicle size growth on R_e and $[Ca^{2+}]$. More specifically, on the basis of this mechanism, the rate of vesicle size growth can be expected to increase with increasing negative charge of the vesicles (increasing R_e), increasing

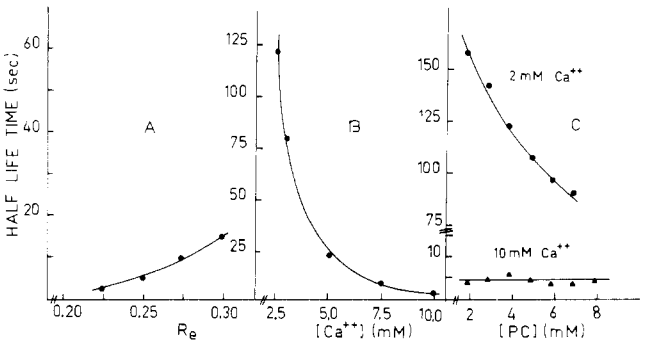

FIGURE 5: Dependence of the half-life ($t_{1/2}$) of *vesicle formation* (from mixed micelles) and equilibration on R_e (A), $[Ca^{2+}]$ (B), and [PC] (C). A PC–cholate (100:75 mM) mixed micellar solution was diluted in saline solutions containing cholate and Ca^{2+} at varying concentrations, and the postdilution turbidity increase was followed at 450 nm. In (A), the final [PC] and $[Ca^{2+}]$ both were 5 mM. The cholate concentration was varied from 5.17 to 6.90 mM to obtain different R_e values from 0.225 to 0.300. In (B), the final PC concentration was 5 mM. The cholate concentration was chosen such that the R_e value for all dispersions was 0.25 [0.25 = $[cholate]_{total}/(5 + 1/K)$]. The K value for each calcium concentration was extracted from the inset of Figure 1. In (C), $[Ca^{2+}]$ was constant for each series of experiments (2 and 10 mM). [Cholate] was varied such that R_e remained constant (0.25) at all [PC]. For 2 mM Ca^{2+}, $[cholate]_{total}$ = 4.8 + 0.25[PC], while for 10 mM Ca^{2+}, $[cholate]_{total}$ = 4.0 + 0.25[PC].

$[Ca^{2+}]$, and increasing vesicle concentration, as in fact is observed in this study.

More direct evidence for the involvement of a fusion mechanism was obtained by entraping fluorescently labeled dextran in PC vesicles in a Ca^{2+} (10 mM)-containing medium, inducing size growth by cholate addition, and determining the fraction of dextran retained in vesicles during the size growth. Cholate addition to R_e = 0.27 resulted in a rapid increase of the vesicle mean radius from 30.7 to 41.0 nm, with low polydispersities for both vesicle populations. For a lipid transfer mechanism to account for this size growth, about 45% of the vesicles would have had to disintegrate. Yet, much more than 55% of the probe was retained in the vesicles during the cholate-induced size growth (percent retention was 95.2 after 1 min, 85.6 after 4 min, 83.3 in 8 min, 80.9 in 12 min, and 78.6 after 1 h; that is much after the mean size approached an apparent equlibrium). This finding lends strong support to our conclusion that cholate induces vesicle size growth via a fusion mechanism.

Formation and Equilibration of Vesicles upon Dilution of Cholate–PC Mixed Micelles in Calcium-Containing Media. The equilibration of diluted mixed micellar solutions is a more complex series of processes. As the slowest of these processes is always the size growth occurring after the closure of micelles to small unilamellar vesicules, one could expect the rate of the overall equilibration to depend on [PC], $[Ca^{2+}]$, and R_e in a similar fashion to the size growth of premade vesicles, observed upon cholate addition. The dependence of $t_{1/2}$ of the overall process on $[Ca^{2+}]$ and [PC] (parts B and C of Figure 5, respectively) is consistent with this expectation. Similar to the vesicle size growth (Figure 4), the overall equilibration following dilution of the mixed micelles (Figure 5) was also facilitated by increasing the $[Ca^{2+}]$. The dependence of $t_{1/2}$ on [PC] again depended on $[Ca^{2+}]$. At low $[Ca^{2+}]$ (2 mM), the rate was an increasing function of [PC] while at high $[Ca^{2+}]$ (10 mM), the rate of equilibration was independent of [PC]. In contrast, increasing the value of R_e resulted in a decreased rate of equilibration (Figure 5A), unlike the cholate-induced size growth, which was an increasing function of R_e (Figure 4A).

Noteworthy is the finding that, at any given R_e, the rate of vesicle formation and equilibration in the dilution experiments of Figure 4B was faster than that of the corresponding size growth processes described in Figure 5B. To explain the latter observation, recall that in dilution experiments the initially formed vesicles have higher cholate content than the equilibrated ones. Simultaneously to being formed, the cholate to PC ratio in the vesicles keeps decreasing. At the same time, postvesiculation size growth is also under way. When the latter series of steps is rapid, it may therefore involve vesicles with actual cholate:PC ratios lower than the final R_e. By contrast, in cholate-induced size growth, the initial cholate:PC ratio in the vesicles is lower than R_e, and the initial steps of vesicle size growth probably involve vesicles whose cholate:PC ratio is lower than R_e. As the rate of size growth is an increasing function of R_e, it is therefore not surprising that at any given R_e the rate of postdilution equilibration (which involves vesicles with cholate:PC ratios higher than R_e) is always faster than that of the cholate-induced size growth. Thus, although in the "dilution" experiments small vesicles were formed and then grew into larger ones, the latter step was so much faster than in the corresponding size growth experiment (in spite of the final R_e being equal) that the overall equilibration in the latter experiments was always faster.

The difference between the actual cholate:PC ratio in vesicles and the calculated final value of R_e may also explain the finding that, in dilution experiments performed in Ca^{2+}-containing media, the rate of equilibration is a decreasing function of R_e. As suggested above, at an R_e value, many "size growth steps" involve vesicles whose cholate:PC ratio is higher than the final R_e. Under these conditions, it takes longer to form larger vesicles. In contrast, in the size growth experiments, the increase in R_e accelerates the fusion process to the extent that the larger vesicles formed at higher R_e values faster than the smaller ones are formed at lower cholate to PC molar ratios.

This explanation of the difference between the results of dilution and size growth experiments is consistent with the results of the similar experiments carried out in the absence of Ca^{2+} (Almog et al., 1986). In both latter series of experiments, the rate was an increasing function of R_e, similar to size growth experiments described above. Since in the absence of Ca^{2+} vesicle formation is so much slower than in its presence, the initially formed vesicles may already be composed of the same cholate:PC ratio as the final vesicles, and the dependence of the rate of equilibration on R_e is qualitatively similar to that obtained in the vesicle size growth experiments carried out in calcium-containing media.

Concluding Remarks

Calcium has two major effects on the micelles $\rightleftarrows$ vesicles transformation obtained upon dilution of PC–cholate mixed micellar dispersions: (i) Both the formation of vesicles and the subsequent postvesiculation size growth are dramatically accelerated by the presence of calcium. The latter series of processes probably involves fusion of vesicles to larger ones, as opposed to the lipid transfer mechanism responsible for the size growth in the absence of Ca^{2+}. (ii) Calcium lowers the cmc of cholate while increasing the coefficient (K) which describes its partition between PC–cholate mixed aggregates and the aqueous medium. Thus, for any given PC and cholate concentrations, R_e is an increasing function of the calcium concentration in the medium.

Nonetheless, the ultimate size of the vesicles depends on R_e and is not directly affected by calcium. In other words, for any given R_e, the ultimate size of the vesicles is independent of $[Ca^{2+}]$, in spite of the large effect of calcium on both the rate and mechanism of vesicle size growth. This supports very strongly the conclusion of Schurtenberger et al. (1985) that the ultimate size of vesicles formed upon dilution of mixed micelles (both in the absence and in the presence of calcium) represents an equilibrium state of aggregation. This implies that the most stable state of aggregation of mixtures containing PC and 5–12 mol % cholate is that of small unilamellar vesicles (SUVs) of diameter about 30 nm. More work will be needed to establish the relevance of this finding to the unsettled question of the equilibrium state of aggregation of pure liquid-crystalline PC (in the absence of cholate), although our results appear to be consistent with the prediction of Israelachvili et al. (1977) that SUVs are the most stable aggregates of liquid-crystalline PC.

From a technical point of view, the calcium-induced acceleration of vesicle formation and equilibration may be useful in membrane reconstitution experiments because "speeding up" the process may limit the exposure of proteins to cholate, thus avoiding protein denaturation in the course of this process. It may also be used in liposome technologies. Furthermore, when solubilized lipids serve as substrates for lipolytic enzymes, the data of this paper may be of assistance in planning the appropriate protocols to answer specific questions regarding the possible effects of R_e, $\bar{R}_h$, and [PC], which can now be varied one at a time.

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Thermotropic Phase Behavior of Model Membranes Composed of Phosphatidylcholines Containing Cis-Monounsaturated Acyl Chain Homologues of Oleic Acid: Differential Scanning Calorimetric and ^{31}P NMR Spectroscopic Studies†

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ABSTRACT: The thermotropic phase behavior of dioleoylphosphatidylcholine and six of its longer chain homologues was studied by differential scanning calorimetry and ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Aqueous dispersions of these compounds all exhibit a single endotherm upon heating but upon cooling exhibit at least two exotherms, both of which occur at temperatures lower than those of their heating endotherm. The single transition observed upon heating was shown by ^{31}P NMR spectroscopy to be a net conversion from a condensed, subgel-like phase (L_c phase) to the liquid-crystalline state. Aqueous ethylene glycol dispersions of these compounds also exhibit single endotherms upon heating and cooling exotherms centered at temperatures lower than those of their corresponding heating endotherm. However, the behavior of the aqueous ethylene glycol dispersions differs with respect to their transition temperatures and enthalpies as well as the extent of "undercooling" observed, and there is some evidence of discontinuities in the cooling behavior of the odd- and even-numbered members of the homologous series. Like the aqueous dispersions, ^{31}P NMR spectroscopy also shows that the calorimetric events observed in aqueous ethylene glycol involve net interconversions between an L_c-like phase and the liquid-crystalline state. However, the L_c phase formed in aqueous ethylene glycol dispersions exhibits a considerably broader powder pattern than that observed in water. This, together with the fact that the transition enthalpies of the aqueous ethylene glycol dispersions are considerably higher than those of the aqueous dispersions, indicates that these lipids form more ordered L_c phases in aqueous ethylene glycol. These results demonstrate that although the presence of a cis double bond can perturb the solid-state packing of the acyl chains, its presence does not preclude the formation of highly ordered subgel-like phases in lipid bilayers. In the particular case of these unsaturated phosphatidylcholines, the formation of their subgel phases is more kinetically favorable than is the case with their saturated *n*-acyl counterparts.

Unsaturated fatty acids are common and widespread constituents of many biological membranes. Of these, the monounsaturated fatty acids are found as membrane lipid constituents of many species of eubacteria, while both the mono- and polyunsaturated fatty acids are major constituents of all eucaryotic cell membranes. The biological importance of unsaturated fatty acids is believed to be related to the fact that their melting points are much lower than those of their saturated counterparts, with the result that membrane lipids containing carbon-carbon double bonds tend to have lower gel/liquid-crystalline phase transition temperatures. A number of studies have shown that a predominance of gel-state lipid is not compatible with the maintenance of "normal" membrane function [for reviews, see McElhaney (1982, 1984)], and for most living organisms, the maintenance of a viable liquid-crystalline cell membrane is achieved primarily by the presence of double bonds in the acyl chains of a significant fraction of their membrane lipids. In addition, other studies have sug-

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