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The effect of albumin on the state of aggregation and phase transformations in phosphatidylcholine-sodium cholate mixtures

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Abstract

Dilution of phospholipid-detergent mixed micellar systems results in an increase of the fraction of monomeric detergent and, consequently, in a decrease of the effective ratio R_e between non-monomeric detergent and phospholipid. The value of R_e has been previously shown to be the main determinant of the state of aggregation in such mixtures: at R_e values below a critical value R_e^{SAT}, the mixture is vesicular; at R_e values higher than R_e^{SOL}, the mixture is micellar, whereas within the range of $R_e^{SAT} - R_e^{SOL}$, vesicles and micelles co-exist. Albumin binds bile salts. Therefore, in PC-cholate mixtures, R_e is reduced by the presence of albumin in the system. Within the range of PC concentrations of 2–23 mM, cholate concentrations of 2–15 mM and BSA concentrations of 0–100 mg/ml, binding of cholate to BSA results in reduction of the effective cholate concentration to the extent of 0.11 mM cholate per 1 mg/ml BSA, namely up to 7 cholate molecules bind to each BSA molecule. Yet, the values of R_e^{SAT} and R_e^{SOL} are essentially independent of BSA. In addition, at any given R_e value, the size of vesicles made by dilution of mixed micelles is a complex function of albumin and PC concentrations. Possible mechanistic details which may cause this effect are discussed. These effects of albumin on the state of aggregation of PC-cholate mixtures must be taken into account in studies of such mixtures in the presence of albumin when other effects of albumin (e.g., on phospholipolysis) are investigated. Practical conclusions are reached with respect to the procedures that can be used to prepare vesicles of identical composition and size in the presence of different concentrations of albumin.

Keywords: Albumin; Phospholipid; Detergent; Mixed micelle; Phospholipid; Lipid solubility; Aggregation

1. Introductions

The solubility of lipids in aqueous media is extremely low. Yet, biological fluids must contain relatively high concentrations of lipids, for the sake of lipid transport and metabolism. Dispersion of lipids in aqueous media is possible through their solubilization by surfactants such as bile salts in the biliary system [1] or through emulsification by surface active compounds, including phospholipids and amphiphilic proteins (e.g., apolipoproteins in the blood) [2]. In these mixed aggregates the hydrophobic moieties of the lipids and amphiphiles are shielded from water by the polar groups of the amphiphiles. The exact structural details of these aggregates are of great physiological and pathological importance, since both the stability and the metabolism of the individual components are critically dependent on the packing of lipids and amphiphiles within the aggregates. Accordingly, much research has been devoted to the structure of various such aggregates and a wealth of data exists on the dependence of structure on the composition of these lipid 'carriers' [1,2].

Body fluids include high concentrations of water-soluble proteins. Some of these proteins are known to interact with lipid aggregates by either coating them, absorbing lipidic components or inducing aggregation and/or fusion of the lipid aggregates [3–6]. Such interactions may markedly affect physiologically-important processes. Yet,

Abbreviations: BSA, bovine serum albumin; PC, phosphatidylcholine (from egg yolk); PE, phosphatidylethanolamine; DOPC, dioleylphosphatidylcholine; DOPE, dioleylphosphatidylethanolamine; OA, oleic acid; POPE, palmitoyloleylphosphatidylethanolamine; CF, 5(6)-carboxyfluorescein; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; BS, bile salts; PCS, photon correlation spectroscopy (quasi-elastic light scattering); NMR, nuclear magnetic resonance; cmc, critical micellar concentration; D_t, the total detergent concentration; D_w, the concentration of monomeric detergent in the solution; R_e, effective ratio, the ratio of non-monomeric detergent to lipids ($R_e = (D_t - D_w)/[\text{Lipid}]$); R_e^{SAT}, the value of R_e at the onset of solubilization; R_e^{SOL}, the value of R_e required for complete solubilization.

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understanding of these interactions is incomplete. Many data are available on the binding of free fatty acids and several other lipidic compound to albumin [7] and on the interactions of albumin and several other water-soluble proteins with phospholipid model-membranes (liposomes) [3]. The interactions of the latter lipid aggregates with serum proteins (including albumin) depend on the liposome composition [8] as well as on the acidity of the medium. In acidic solutions, albumin causes increased turbidity of liposomal dispersions, probably due to albumin-induced aggregation or/and fusion of the liposomes, whereas at neutral pH there is no evidence for interaction of phospholipid vesicles with albumin [8–10].

By contrast, when aqueous media contain physiological concentrations of free fatty acids and albumin, the tight binding of free fatty acids to albumin [4,7,11] markedly affects the state of lipid aggregation in the system. As an example, when albumin is added to vesicles composed of DOPE and oleic acid (OA), the binding of OA to albumin results in transformation of the PE from a bilayer into an hexagonal (HII) phase [12]. Similar (although different) phase transformations may occur when lipid hydrolysis is followed by binding of the resultant free fatty acids to albumin [13]. Such phase transformations may affect the propagation of hydrolysis, in addition to the effect of the mere extraction of reaction products, which are known to have dual (accelerating and/or inhibitory) effect on the rate of enzymatic hydrolysis [14–16].

Of special interest are systems composed of bile salts and phospholipids. As for any other phospholipid-detergent mixture, both the aggregation state of the lipids and the structure (and size) of the mixed aggregates are governed by the effective ratio of (non-monomeric) detergents to phospholipids ($R_e = (D_t - D_w)/L$, where L denotes the lipid concentration, D_t denotes the total detergent concentration and D_w is the concentration of detergent monomer [1,17]). At R_e values lower than a critical ratio (R_e^{SAT}) the system is essentially vesicular; at much higher R_e values, above another critical ratio (R_e^{SOL}), all the lipid is contained in mixed micelles whereas at intermediary R_e values (between R_e^{SAT} and R_e^{SOL}), vesicles and micelles co-exist.

These phase transformations are the basis of detergent-induced solubilization and of membrane-reconstitution procedures based on detergent-removal [1,18]. Moreover, systems containing cholesterol, in addition to phospholipids and bile salts, can be regarded as simplified but valid models of bile [19,20]. These systems (with and without cholesterol) are likely to be affected by binding of bile salts to water soluble proteins, particularly albumin. This binding has been studied by various methods [21–25], yielding significantly different values for the number of binding sites and respective binding constants (possibly due to large experimental errors in the measurement of free versus bound bile salts). Furthermore, interaction of albumin with other components of these systems is also possible [26,27]. The effects of albumin on the state of aggregation in lipid-detergent mixed systems and consequently on the lipid metabolism in these systems may result from such interactions. More specifically, the hydrolysis of aggregated phospholipids by water soluble phospholipases is governed by the state of aggregation of the phospholipid substrate (type, composition and size of the mixed aggregates). These reactions are often studied in phospholipid-bile salt mixtures in the presence of albumin (in most cases bovine serum albumin, BSA; [28]) used to absorb the resultant fatty acids. The possible alteration of the state of aggregation of the substrate by BSA is rarely considered and has never been addressed systemically, in spite of its obvious significance. This communication presents such a systemic study of PC-cholate mixtures.

2. Materials and methods

Egg PC, sodium cholate, bovine serum albumin (BSA fraction V, essentially fatty acid free) and Dextran M_r 2 000 000 were purchased from Sigma (St. Louis, MO.). NaCl, EDTA and $CaCl_2$ were analytical grade (Merck). Tris buffer was purchased from Fluka. Sepharose 4B and Sephadex G-25 were purchased from Pharmacia. Carboxyfluorecein (CF) was a product of Molecular Probes. Praseodymium chloride ($PrCl_3 \cdot 8H_2O$) was purchased from Aldrich.

2.1. Preparation of multilamellar vesicles (MLV)

PC dissolved in $CHCl_3$ was evaporated to dryness under a stream of nitrogen. The PC film was suspended in buffer A (140 mM NaCl, 0.5 mM EDTA, 0.02% NaN_3, 10 mM Tris, pH 7.4) to form multilamellar vesicles [29].

2.2. Preparation of large unilamellar vesicles (LUV)

A suspension of MLV was extruded four times through 0.2 μm filters (Schleicher and Schnell), and subsequently ten times through 0.1 μm filters. This results in the formation of large unilamellar vesicles (LUV) as previously described by Olson et al. [30]. PC concentration was determined chemically [31].

The vesicle diameters were characterized by PCS measurements (see below).

2.3. Preparation of small unilamellar vesicles (SUV)

A suspension of MLV was sonicated for 20 min, using an XL-2020 sonicator (Heat System Incorporated) as previously described [32].

2.4. Gel chromatography

Mixtures of egg PC LUV (20 mM) and BSA (50 mg/ml or 70 mg/ml) were incubated for 1 week. Follow-

ing equilibration, the mixtures (1 ml) were passed through Sepharose 4B columns (20 × 1.2 cm) and eluted by buffer A. Fractions of 1 ml were collected for analysis.

The concentration of the eluted PC and BSA, were determined chemically by established methods ([31] and [33], respectively).

2.5. Turibidity measurements

Turbidity was measured at 360 nm using a Shimadzu UV-VIS (UV-160) spectrophotometer.

2.6. Photon correlation spectroscopy (PCS) measurements

The particle size was measured on a Malvern photon correlation spectrometer Model 4700, equipped with an Argon laser of a wavelength of 488 nm, at 25° C, as previously described [17]. Vesicle dispersions were measured after (3–30-fold) dilution, to avoid multiple-scattering. Such dilutions have been previously shown to have only a slight effect (if any) on the size determination [1]. The viscosity of all measured samples differed from that of the medium by no more than 1%.

2.7. Preparation of mixed micelles and vesicles

Mixed micellar dispersions of PC and cholate were prepared from mixed solutions of the appropriate amounts of each lipid in a chloroform-methanol mixture (1:1). The solutions were dried under a stream of nitrogen. The lipid films were hydrated in buffer A. PC-cholate mixed vesicular dispersions of different compositions were prepared by dilution of the mixed micellar dispersion with buffer A containing different amounts of sodium cholate as previously described by Almog et al. [34]. The cholate concentration was determined chemically by established method [35].

2.8. Carboxyfluorescein (CF) entrapment in LUV

LUV were made as described above in buffer A containing 20 mM CF. Non entrapped CF was removed by passing the vesicle dispersion through Sephadex G-25 column as previously described [36].

2.9. ^{31}P-NMR measurements

10 mM SUV were mixed with 14.9 mM cholate and 60 mg/ml BSA. The size of the resultant vesicles as measured by PCS was 250 nm. Cholate was removed by dialysis against 4 × 200 ml of buffer A for 24 h. Praseodymium chloride ($PrCl_3 \cdot 8H_2O$) was added to the solution to a final concentration of 10 mM and the NMR measurements carried out at 202.46 MHz using Bruker ARX-500 NMR spectrometer. The relaxation delay time (D_1) was 2 s and acquisition time was 0.1966 s.

2.10. Microscopic observations

The sample made for NMR studies was also observed by light microscopy using a Nikon (Model Optiphot) microscope and interference (Nomarski) optics.

3. Results

In a control experiment, we have studied the interaction of albumin with egg PC large unilamellar vesicles (LUV, mean diameter = 145 nm). Addition of BSA (10–70 mg/ml) to dispersions of these vesicles (0.6 mM PC) at pH 7.4, did not affect the turbidity of the dispersions. Furthermore, when such mixtures were passed through sepharose 4B columns, each of the two components was eluted at the same volume as in the absence of the other, with no co-elution of PC and albumin (Fig. 1). These results indicate that the two components do not interact, although some 'loose association' can not be ruled out.

This conclusion is also consistent with the result of an experiment in which albumin addition to LUV containing partially self quenched CF did not affect the fluorescence intensity of the entrapped CF for 3 days (not shown). Addition of cholate to the same preparation resulted in an immediate 2-fold increase in the fluorescence intensity due to release of the entrapped CF. The lack of any such effect after addition of albumin to the LUV is consistent with the conclusion that at pH 7.4 albumin does not interact with the vesicles, as previously noted [3–6].

In mixtures of PC and cholate, the aggregation state of the lipid is governed by R_e, the effective ratio of non-monomeric cholate to PC [1,34]. Dilution of PC-cholate mixed-micellar solutions results in decreasing R_e due to increasing fraction of monomeric cholate. When the dilu-

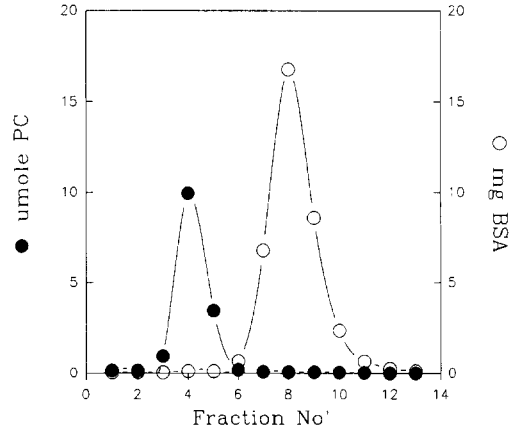

Fig. 1. Sepharose 4B elution profile of an equilibrated mixture of egg PC large unilamellar vesicles (LUV) and BSA. Large unilamellar vesicles (20 mM PC) were incubated with BSA (70 mg/ml) for 1 week at room temperature and pH 7.4 in buffer A (see methods). 1 ml of the mixture was applied to the column and eluted by buffer A. Fractions of 1 ml were collected for chemical analysis (see Materials and methods).

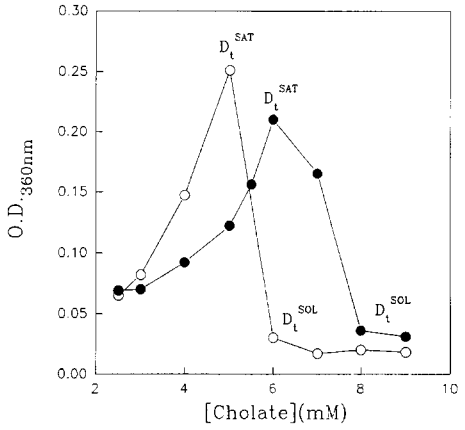

Fig. 2. The dependence of the steady-state turbidity of PC-cholate mixed aggregates on the cholate concentration, in the absence and presence of BSA. Dispersions were made by a 50-fold dilution of a mixed-micellar system of 100 mM PC and 100 mM cholate in buffer A containing varying cholate concentrations (○), or in buffer A containing 25 mg/ml BSA and varying cholate concentrations (●). The final PC concentration in all the dispersions was 2 mM.

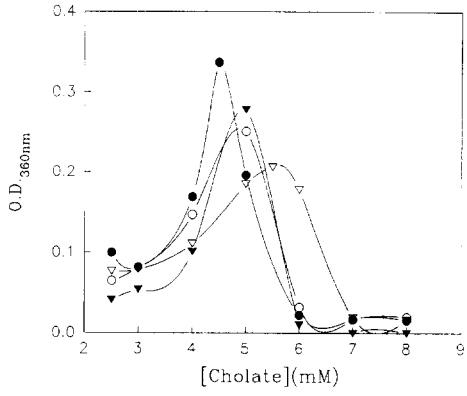

Fig. 3. The dependence of the steady-state turbidity of PC-cholate mixed-aggregates on the cholate concentration, in the absence and presence of BSA, and in the absence and presence of Ca^{2+}. Dispersions of 2 mM PC were made by diluting a mixed-micelle system of 100 mM PC and 100 mM cholate in buffered media (pH 7.4) containing, Tris-HCl (10 mM), NaN_3 (0.02%, w/v) and varying cholate concentrations. The diluting media were as follows: (○) buffer A (140 mM NaCl + 0.5 mM EDTA). (●) buffer B (125 mM NaCl + 10 mM $CaCl_2$). (▽) buffer A + BSA (6 mg/ml). (▼) buffer B + BSA (6 mg/ml).

tion is such that R_e decreases to values below R_e^{SAT}, all the PC is transformed into vesicles. However, if the diluting medium contains cholate, the mixed system will either be vesicular (for $R_e < R_e^{SAT}$), micellar (for $R_e > R_e^{SOL}$) or a mixture of vesicles and mixed micelles (for R_e within the range of $R_e^{SAT} - R_e^{SOL}$) [34].

An experiment carried out by a 50-fold dilution of a mixed micellar solution of 100 mM PC and 100 mM cholate in media containing varying cholate concentrations is described by the empty symbols in Fig. 2, in terms of the dependence of the optical density of the resultant mixtures on the final cholate concentration. This figure shows that at sub-solubilizing cholate concentrations the turbidity (which relates to the vesicle size) is an increasing function of cholate concentration whereas in the micellar range (at $R_e > R_e^{SOL}$) increasing R_e results in smaller mixed micelles, hence in reduced turbidity [34].

The solid symbols in Fig. 2 present the results of a similar experiment done in the presence of BSA (25 mg/ml) in the diluting media. As evident from this figure, the presence of albumin in the system causes a 'right shift' of the 'bell shaped' dependence of turbidity on cholate concentration. This shift could have been expected in light of the qualitatively well established binding of cholate to albumin. This binding implies that a higher total cholate concentration is required for the effective ratio of cholate/PC in mixed aggregates (R_e) to approach any given value. In this respect, the effect of albumin is opposite to that of Ca^{2+} ions, which reduce the solubility of cholate monomers (reduced cmc), thus causing a 'left shift' of the 'bell shaped' dependence of turbidity on cholate concentration [37]. Fig. 3 presents the latter dependence. It also shows that when the medium contains both Ca^{2+} (10 mM) and albumin (6 mg/ml), the effects of these two additives cancel each other and the O.D. versus cholate concentrations curve is similar to that obtained in media containing neither Ca^{2+} nor albumin.

The 'right shift' caused by albumin in the cholate concentration required for the onset (D_t^{SAT}) and completion (D_t^{SOL}) of solubilization can be described in terms of ΔD_t^{SAT} and ΔD_t^{SOL}. These factors are the differences between the two critical cholate concentration (for any given PC and BSA concentrations) and the corresponding concentrations in the absence of BSA. Fig. 4 presents ΔD_t^{SAT} as a function of the concentration of BSA at a constant concentration of PC (2 mM). The observed linear

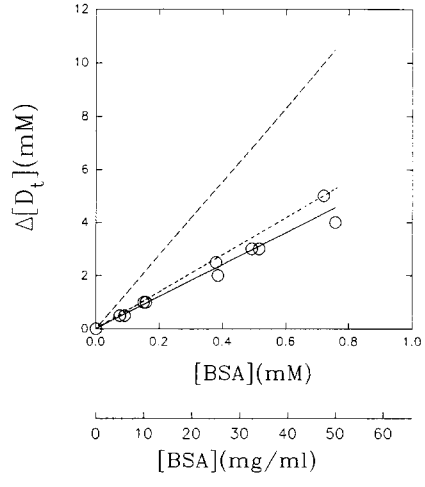

Fig. 4. The dependence of the ΔD_t^{SAT} on BSA concentration. The 'right shift' (e.g., Fig. 2) in cholate concentration required for the onset of solubilization of 2 mM PC (ΔD_t^{SAT}) is presented as a function of BSA concentration (in mM and mg/ml). The solid line was obtained by linear regression of the experimental data. The other lines are theoretical: the broken line is based on published binding constants [22] whereas the dotted line was obtained as described in Discussion.

dependence (Fig. 4; $r^2 = 0.968$) indicates that over the studied range of concentrations of BSA and cholate, each BSA molecule binds a constant number of cholate molecules.

This constant number (α), can be expressed in terms of the molar ratio of cholate to BSA (α_2) or in terms of the reduction of cholate concentration (mM) per 1 mg BSA/ml (α_1). The value of α appeared to be independent of the PC concentration, as determined through experiments similar to the one presented in Fig. 4 using different PC concentrations of 1–4.75 mM. The experimental variation of α was quite large ($\alpha_2 = 5.9–8.6$) but independent of either PC concentration or on whether it was determined on the basis of D_t^{SAT} or D_t^{SOL} (Table 1). Furthermore, similar experiments in Ca^{2+}-containing systems gave identical results ($\alpha_2 = 7 \pm 1$; not shown). Thus, it appears that within the studied range of concentrations each molecule of BSA binds about 7 cholate molecules.

For any given albumin concentration, the dependence of D_t^{SAT} on the concentration of PC was linear (not shown), similar to the results obtained in the absence of BSA [17]. The slope of each such curve yields R_e^{SAT} and the intercept reflects the value of the detergent's cmc in the presence of lipid [1,38]. Similarly, the dependence of D_t^{SOL} on the concentration of PC yields R_e^{SOL} and an apparent cmc. These dependencies (D_t^{SAT} vic PC concentration and D_t^{SOL} vic PC concentration) in systems containing 25 mg BSA/ml yielded an R_e^{SAT} value of 0.31 and an R_e^{SOL} value of 0.70 (not shown), as compared to values of 0.3 and 0.7 obtained in the absence of BSA [34,37]. By contrast, the value of the apparent cmc, given by the intercepts of the dependencies of D_t^{SAT} and D_t^{SOL} on PC concentration, increased linearly ($r^2 = 0.99$) upon increasing the concentration of BSA (Fig. 5). Such dependencies were expected if the effect of BSA on the phase boundaries results merely from cholate binding to BSA. Furthermore, the dependence of the apparent cmc on the concentration of BSA had a slope of $\alpha_2 = 7.0$, in agreement with the values listed in Table 1. It thus appears that the presence of BSA affects the state of aggregation in PC-cholate mixtures only by binding cholate; the phase boundaries (i.e., the values of R_e^{SAT} and R_e^{SOL}) are not affected.

Table 1
BSA-related changes in critical detergent concentrations ($D_t = \alpha$[BSA]) in mixtures containing different PC concentrations

PC (mM)	D_t^{SAT}			D_t^{SOL}		
	α_1 [a]	α_2 [b]	r^2	α_1 [a]	α_2 [b]	r^2
1	0.117	7.722	0.983	0.1	6.6	0.85
2	0.09	5.94	0.968	0.112	7.392	0.97
2.67	0.095	6.27	0.983	0.114	7.524	0.96
4.25	0.101	6.66	0.964	0.1	6.6	0.98
4.75	0.1	6.6	0.98	0.13	8.58	0.97
Means	0.1006	6.638		0.1112	7.33	
S.D. (±)	0.009	0.599		0.011	0.73	

[a] α_1 (mM/mg per ml).
[b] α_2 (mol/mol).

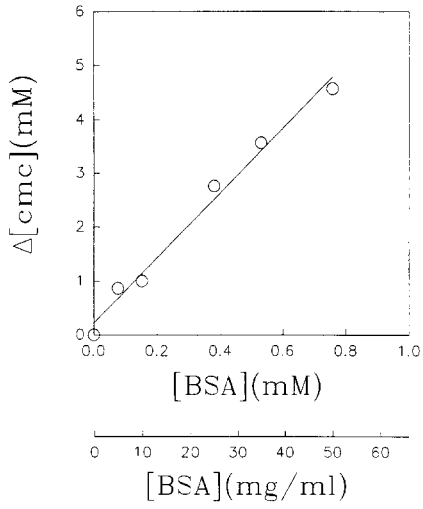

Fig. 5. The effect of BSA on the apparent cmc. Apparent Δcmc values were extracted from the dependencies of D_t^{SAT} and D_t^{SOL} on PC concentrations (1.0–4.75 mM), as measured at any given BSA concentration. The effect of BSA on the apparent cmc is given by the difference in cmc between BSA-containing systems and systems with no BSA (Δcmc).

However, in addition to the 'right shift' described above, the presence of BSA in the diluting medium also affected the turbidity of PC/cholate vesicles (e.g., Fig. 2). Thus, vesicle dispersions of any given R_e made in the presence of varying concentrations of BSA (0–100 mg/ml) differed in their turbidities. The effect of BSA on the turbidity depended in a complex fashion on the exact composition of the mixture: for any given concentration of PC, a maximal steady state turbidity was observed when $R_e = 0.3$, independent of the presence of BSA (see above). For 2 mM PC, the dependence of the maximal turbidity on BSA concentration was biphasic: the presence of 5–50 mg BSA/ml resulted in a decreased turbidity (e.g., Fig. 2) such that the maximal steady-state turbidity, obtained at $R_e = 0.3$ 16–24 h after preparation, was a decreasing function of the BSA concentration (Fig. 6). The close correlation between these measurements and the size of the vesicles, as studied by PCS after a 20-fold dilution of the mixture (Fig. 6), indicates that the variation of turbidity was due to BSA-induced changes in the size of the vesicles.

This effect is not a consequence of the increased viscosity of the medium, since in the presence of equi-viscous (1.13 cP), solutions of high molecular weight dextran, no such effect was observed (Fig. 7). In contrast to these results, when the diluting medium contained 100 mg BSA/ml, the resultant mixtures were very turbid and contained larger vesicles (see below).

Similar behavior was observed when cholate-containing vesicles of an $R_e = 0.3$ were formed by dilution of preformed PC vesicles (diameter = 40–50 nm) in media containing BSA (0–100 mg/ml) and cholate (4.5–14.5 mm).

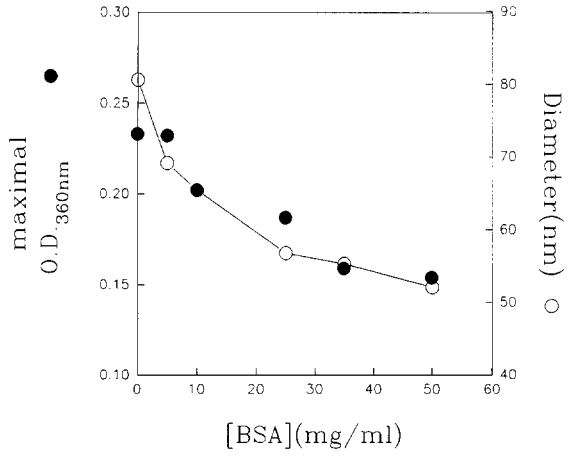

Fig. 6. The dependence of the maximal steady-state turbidity and vesicle size of cholate-containing vesicles ($R_e = 0.3$) on BSA concentration. The maximal turbidity (measured at 360 nm) and the corresponding diameters (as measured by PCS after 20-fold dilution) are given as functions of BSA concentration.

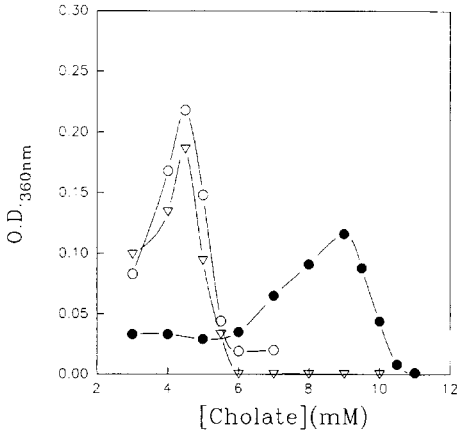

Fig. 7. The dependence of the apparent equilibrium turbidity (at 360 mM) of PC-cholate mixed aggregates on the cholate concentration, in the presence of BSA and Dextran. Dispersions of 2 mM PC were made by dilution of a mixed-micellar solution of 100 mM PC and 100 mM cholate in: (○) buffer A containing varying cholate concentrations. (▽) buffer A containing 0.275% (w/v) Dextran (M_r 2000000) and varying cholate concentrations. (●) buffer A containing 5% (w/v) BSA and varying cholate concentrations.

In the absence of BSA, the pre-formed SUV grew in size, due to cholate-induced size growth [34]. At low BSA concentrations smaller vesicles were formed, whereas at higher BSA concentrations, larger vesicles were formed, as indicated by the larger apparent mean size determined by PCS (Fig. 8) and by microscopic observation (Fig. 9). The mean size of these vesicles, as measured by PCS, was 250 nm. The individual size, as estimated from blown-up micrographs, was somewhat larger, indicating that the vesicles are heterogenous with respect to their sizes as the smaller vesicles can not be observed microscopically. Vesicle aggregation was rather rare (Fig. 9) and the vesicles were predominantly unilamellar, as evident from the out/in ratio (out/in = 0.84) in their ^{31}P-NMR spectrum measured in presence of $PrCl_3$ (not shown). Similar trend was observed at higher final concentrations of PC. However, as the PC concentration increased the 'turning' BSA concentration decreased, as exemplified in Fig. 8.

In an attempt to shed light on the factors which govern the steady state size of vesicles in the presence of albumin we have carried out several kinetic experiments. Previously we have shown [17] that the transformation of PC-cholate mixed micelles into cholate-containing vesicles occurs through a three-step mechanism: initially, the removal of cholate leads to micelle-micelle interactions and a consequent micelle size growth; subsequently the resultant large mixed micelles close into small vesicles, which then undergo a series of post-vesiculation size growth processes to yield larger vesicles whose size is an increasing function of R_e. It is the latter, relatively slow, processes which determine the apparent equilibrium size of the resultant vesicles [34]. This is further demonstrated by the results of the two-step dilution experiments in which a 50-fold dilution of PC-cholate mixed micelles in media containing the exact cholate concentration required to yield vesicles with $R_e = 0.3$, and the resultant solution was then diluted 2-fold in media containing the exact cholate concentration required to maintain this R_e value. The turbidity of the resultant mixture was a half of that obtained at equilibrium for the system that did not undergo a second step of dilution, regardless of the time of dilution (not shown). This is consistent with previous results [1] which showed that dilution of equilibrated cholate-containing vesicles has no effect on their size, in spite of the reduction of R_e due to dilution.

In contrast, when a 50-fold dilution of mixed micellar solutions was followed by a second step of (2-fold) dilution in media containing albumin (50 mg/ml) and the appropriate cholate concentration required for retaining R_e

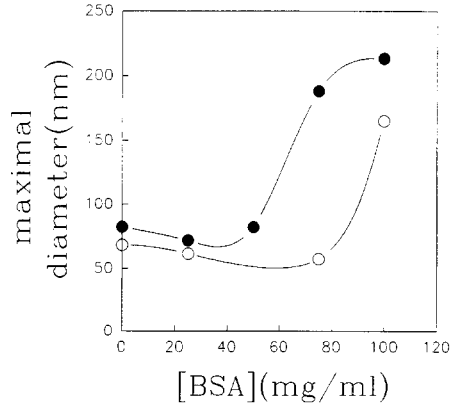

Fig. 8. The dependence of maximal diameter on BSA concentration. The maximal diameters of vesicles obtained at $R_e = 0.3$ at 2 mM PC (○) and 10 mM PC (●), were measured by PCS after a 20 fold dilution. (The viscosity of the diluted solutions differed from that of the diluting media by less than 1%.) The diameters (nm) are given as functions of BSA concentration.

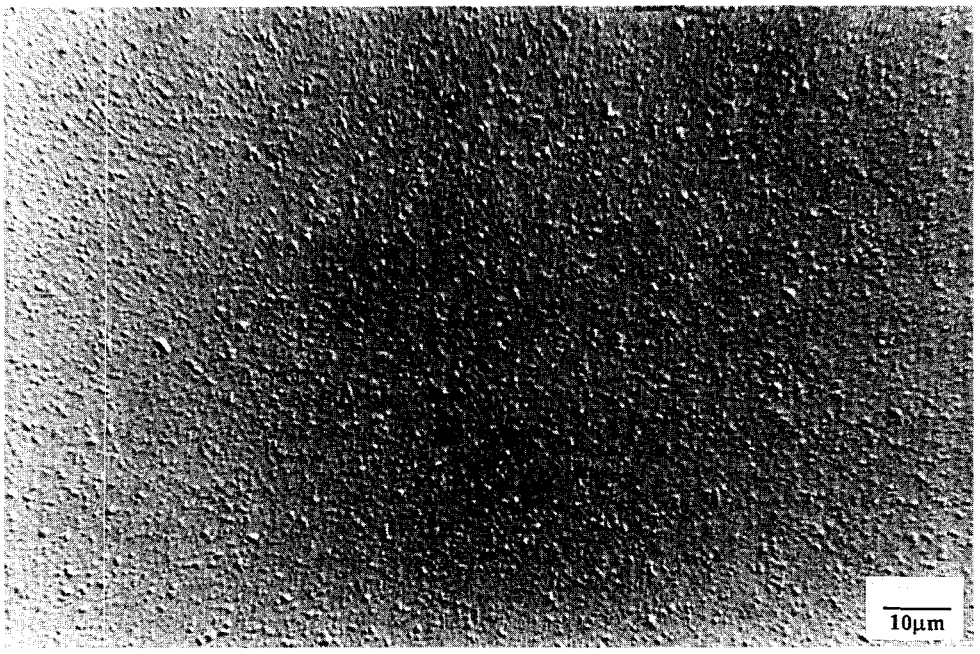

Fig. 9. Micrograph of PC vesicles. The vesicles were prepared by mixing PC SUV (10 mM) with cholate (14.9 mM) in the presence of BSA (60 mg/ml) and subsequently dialysing the cholate as described in Materials and methods. The bar represents 10 μm.

in the vesicles, the apparent equilibrium turbidity depended on the point of time of the second step of dilution, as shown in Fig. 10: when the second step of dilution was done after apparent equilibration (at point III) the turbidity decreased to a half, similar to the result without BSA. In contrast, when the second dilution was done on non-equilibrated mixtures (at stages when the vesicles were still undergoing size growth), smaller vesicles were obtained upon equilibration, yielding lower turbidities (points I and II). It thus appears that the size of vesicles made by two step dilution procedures depends upon the detailed protocol and is not merely a function of the composition of the mixture.

Different results were obtained at high BSA concentrations at which BSA causes a size increase. Specifically, unlike in those cases where the presence of BSA resulted in a reduction of size, size growth was obtained at high BSA concentrations, regardless of the stage at which BSA was added. As an example, when a mixture containing 8 mM PC and 6.3 mM cholate ($R_e = 0.3$) was diluted (1:1) in a medium containing 200 mg BSA/ml and 24 mM cholate, to maintain R_e, vesicles of diameters of 150 ± 30 nm were obtained, regardless of the time of dilution (not shown). Furthermore, other procedures used for preparation of a given PC-cholate-BSA mixtures all yielded similar large vesicles, as given in Table 2. This table lists the size of vesicles made by four different procedures, based on mixing equal volumes of aggregated PC and aqueous media containing cholate or/and BSA. In two of these experiments, PC-SUV (without or with BSA) were mixed with solutions containing cholate (with or without BSA); In the third experiment, LUV were made by adding cholate to the SUV (to $R_e = 0.3$) and then mixed with a cholate-BSA solution whereas in the fourth experiment, a PC-cholate mixed micellar solution was mixed with a BSA solution. In all these experiments, the final composition was the same and the apparent sizes, as measured either 1 h or 24 h after preparation, were all within the same range

Table 2
Vesicle size in mixtures of PC (4.0 mM), cholate (15.15 mM) and BSA (100 mg/ml) made by several procedures

1:1 (v/v) Mixing of two solutions						Average diameter (nm) after			
Solution A				solution B		1 h	24 h	4 days [a]	
Aggregates	PC (mM)	cholate (mM)	BSA (mg/ml)	cholate (mM)	BSA (mg/ml)			as is	1:2
SUV	8.0	–	–	30.3	200	154	177	15.4	156
SUV	8.0	–	200	30.3	–	161	184	14.6	166
LUV	8.0	6.3	–	24.0	200	185	169	14.2	
Mixed micelles	8.0	30.3	–	–	200	173	193	14.8	161

[a] Vesicles were kept for 4 days at room temperature either at their original solutions (as is) or after being diluted (24 h after preparation) in equal volumes of buffer A solution (1:2).

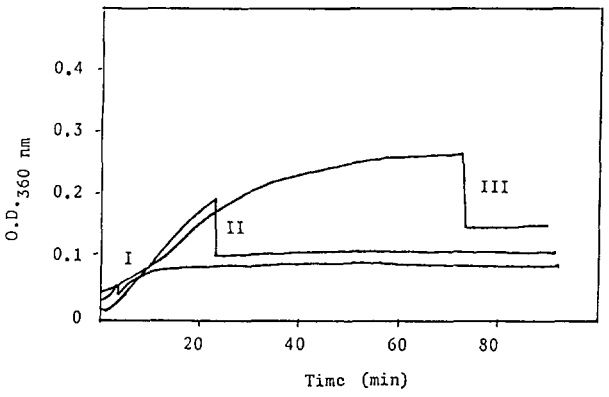

Fig. 10. Kinetic profiles of dilution-induced vesicle formation and size-growth, followed by a second step of dilution in cholate-containing solutions in the presence of BSA. PC-cholate mixed micelles (100 mM each) were first diluted 50-fold in buffer A containing 2.5 mM cholate ($R_e = 0.3$). A second step of a 2-fold dilution was carried out (at time points I, II or III) in buffer A containing 50 mg/ml BSA + 8.95 mM cholate (required for maintaining $R_e = 0.3$ since D_w in this mixture is 3.95 mM and 50 mg BSA/ml reduces the effective cholate concentration by 5 mM).

of 154–193 nm. This may indicate that the vesicles obtained at this composition represent a state of equilibrium.

This hypothesis, however, is not valid since with time all these mixtures became micellar, containing aggregates of merely 15 nm (smaller than SUV; see results after 4 days 'as is' in Table 2). This spontaneous vesicle → micelle transportation can be explained by a reduction of cholate-binding to BSA and a consequent increase of R_e from $R_e = 0.3$ to $R_e > 0.7$. Such a reduction of binding may be caused by a change in the conformation of albumin due to its long exposure to cholate, a possibility that will have to be further studied.

Interestingly, a 2-fold dilation of the mixtures described in Table 2 prevented the transformation of the vesicles into mixed micelles (Table 2; 4 days, 1:2). Apparently, the reduction of R_e due to the dilution (from $R_e = 0.3$ to $R_e = 0.17$) did not cause a decrease in vesicle size (similar to all other cases studied thus far). On the other hand, in the dilute solutions reduction of cholate binding either does not occur (possibly because a conformational change in BSA does not occur) or else, the reduction of binding is such that R_e increases to a value much lower than 0.7.

4. Discussion

4.1. The effect of BSA on the phase boundaries and aggregate composition in PC-cholate mixed systems

Based on our results, it appears that R_e^{SAT} and R_e^{SOL} are independent of the presence of albumin. In other words, the phase boundaries in any given mixture of PC and cholate apparently depend upon BSA concentration only through binding of cholate to the BSA.

Within the vesicular range, it has been shown [1] that R_e is a function of the total cholate and lipid concentrations (D_t and L, respectively) and the partition coefficient describing the distribution of cholate between the bilayer and the aqueous medium (K).

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

R_e can also be described as a function of cholate concentration in the aqueous media (D_w).

$$R_e = KD_w/(1 - KD_w) \quad (2)$$

Since at a constant concentration of PC and varying concentrations of BSA the same R_e was obtained at the same D_w (but different D_t), it follows that K is not affected by BSA.

Binding of cholate to albumin has been previously analysed in terms of n_1 'strong binding sites' ($K_a = K_1$) and n_2 'weak binding sites' ($K_a = K_2$). The values of n_1, n_2, K_1 and K_2 depended upon the albumin used (HSA versus BSA) and the technique by which the binding was assayed [21–25]. For BSA, the published results, based on fluorescence measurements, were interpreted in terms of 5 strong binding sites ($K_1 = 6.8 \cdot 10^4$ M^{-1}) and 17 weak binding sites ($K_2 = 0.028 \cdot 10^4$ M^{-1}) [22].

In our studies, cholate partitions between the aqueous media, PC-containing aggregates and albumin binding sites. At the onset of solubilization $R_e = R_e^{SAT} = 0.3$ and the concentration of cholate contained in vesicles is $D_b = 0.3L$. The concentration of cholate that is bound to albumin (D_A) is a function of D_w and albumin concentration (A_T):

$$D_A = n_1 A_T(K_1 D_w/(1 + K_1 D_w)) + n_2 A_T(K_2 D_w/(1 + K_2 D_w)) \quad (3)$$

The concentration of cholate at the onset of solubilization should therefore be higher in the presence of albumin than in its absence by $\Delta D_t^{SAT} = D_A$. Similar results should be obtained for ΔD_t^{SOL} (where $D_b = 0.7L$ but D_w is the same [1]).

Using Eq. (3) and the values of n_1, n_2, K_1 and K_2 given by Pico and Houssier [22], the expected dependence of ΔD_t^{SAT} and ΔD_t^{SOL} is that given by the broken line in Fig. 4. To explain the large discrepancy between this expected dependence and the experimental curve, we have re-evaluated the results given by Pico and Houssier. We noticed that within the given experimental errors several series of values of n_1, n_2, K_1 and K_2 are consistent with these data. In fact, the most straightforward interpretation of the Scatchard plot given in Fig. 3 of this paper [22] is that the total number of binding sites is approximately 7. Deviation of the Scatchard plot from linearity (with one type of 7 binding sites of $K_a = 5.3 \cdot 10^4$ M^{-1}) is quite questionable. This deviation can be an outcome of the experimental error in computing 'site occupation' at high cholate concentrations and/or due to non-linear dependence of the quenching of fluorescence intensity at 'high occupation'. Alternatively, two types of binding sites exist,

5 binding sites of $K_a = 7.5 \cdot 10^4$ M^{-1} and 2 binding sites of $K_a = 2.7 \cdot 10^4$ M^{-1} (which is very consistent with their data).

The dotted line in Fig. 4 is the theoretical dependence of ΔD_t^{SAT} on BSA concentration based on the latter assumption ($n_1 = 5$; $n_2 = 2$). The line computed for 7 equal binding sites ($K_a = 5.3 \cdot 10^4$ M^{-1}) is indistinguishable from the latter line. We can therefore conclude only that 7 relatively strong cholate binding sites are involved. Whether or not this interpretation is generally valid, for the range of cholate, albumin and PC concentrations studied here, 7 mol of cholate have to be added to a cholate-PC mixture per mole of albumin to obtain aggregates of the same composition as in the absence of albumin.

These results are very reproducible and as such are valuable in that they allow the design of experiments with varying concentrations of added albumin which are identical in terms of the concentration, composition and size of cholate-PC mixed aggregates (see below).

4.2. The effect of albumin on the size of PC-cholate mixed aggregates

Apart from the effect of albumin on the composition of PC-cholate mixed aggregates, which determine their state of aggregation, it often affects their size at a given R_e value. This is evident from the data presented in Figs. 6 and 8, where the maximal turbidity and vesicle size are described as functions of the concentration of BSA at $R_e = 0.3$. Fig. 8 describes the size of these vesicles as a function of the BSA concentration at different PC concentrations. At 2 mM PC and BSA concentrations below 80 mg/ml the hydrodynamic diameter was a decreasing function of concentration of BSA whereas at higher BSA concentrations the vesicles present in the equilibrated mixtures were much larger than in the absence of BSA. Qualitatively similar results were obtained at different PC concentrations. However, at higher PC concentration the size-increase was observed at lower BSA concentration. Being measured after a 20–40-fold dilution, the much larger apparent sizes observed at high concentrations of BSA are probably due to vesicle size growth and not aggregation. Microscopic examination (Fig. 9) strengthen this conclusion. The ratio between the number of PC head groups that are exposed to the external medium and the number of inwards facing PC head groups (out/in ratio), as determined from ^{31}P-NMR measurements (out/in = 0.84) indicate, that the formed large vesicles are essentially unilamellar [1].

The inequality of the apparent equilibrium size of the three dispersions in Fig. 10, which had the same composition and differed only in the sequence of dilution steps leading to their formation, clearly indicate that the steady-state size does not always represent a state of equilibrium: when vesicles were first made and equilibrated in the absence of albumin, their dilution in albumin-containing media did not affect their size. This means either that after the second step of dilution they were kinetically trapped in the larger size, due to the lack of efficient size-decreasing mechanisms, or else, that in the presence of albumin the size growth is inhibited by (a) not yet understood kinetic factor(s).

The mechanism responsible for this effect of albumin on vesicle size is not clear. The results of Fig. 7 indicate that it is not caused by the effect of albumin on the viscosity of the medium. Other factors may include the effect of albumin on the stationary (very low) PC concentration in non-lamellar structures (probably in the form of albumin-bound PC), which may affect the rate of post-vesiculation size growth through lipid transfer mechanisms. Alternatively, BSA, at sufficiently high concentration, may induce aggregation and fusion of cholate-containing vesicles. The rate (and possibly the extent) of such processes may explain the dependence of the maximal size of vesicles on PC concentration (Fig. 8).

The mechanism responsible for aggregation and size growth of the vesicles at high BSA concentrations may be similar to that obtained in the presence of high concentrations of water soluble polymers [39]. Specifically, dehydration of the vesicle surface due to exclusion of BSA from the vesicle hydration sphere may reduce the energetic barrier for vesicle aggregation [40].

However, understanding of the mechanism(s) responsible for the effect of BSA on the size of reconstituted vesicles will require more experiments. Yet, the mere occurrence of this effect must be taken into account in any further experiments aimed at understanding the effect of albumin on reactions in which PC is involved in the presence of cholate (see below).

4.3. Design of enzymatic experiments in the presence of albumin

Enzymatic phospholipolysis is of course dependent on the concentration of the substrate and on the enzyme to substrate ratio. In addition, it is extremely sensitive to the state of aggregation of the phospholipid substrate both in terms of the type of aggregates and their composition and size [41–43]. To study the effect of any of these interdependent factors on the hydrolysis, experimental protocols must be designed individually for studying the effect of each possible factor while keeping the other factors constant [41].

In the presence of albumin, phospholipolysis is complex and may be affected by albumin interactions with either the enzyme, the substrate and/or with the reaction products. Only if the size and composition of the mixed aggregates remain unaltered, other effects of albumin on the hydrolysis can be revealed. In the following section, possible protocols are described by which the effects of various factors (including albumin) on the phospholipolysis can be studied.

Enzymatic studies on mixed micelles

In studying the hydrolysis of PC contained in PC-detergent mixed micelles studying separately the effects of micellar size and composition is not possible since the size appears to be always determined by the micellar composition. The (combined) effect of these two factors can be easily studied by using a constant lipid concentration with different concentrations of the detergent, as previously described [41]. Studying the effects of mixed micellar concentration and composition (and size) in the presence of albumin is only slightly complicated by detergent-binding to the albumin. If the studies are carried out under conditions that albumin is saturated (i.e., 7 cholate molecules are bound to it), cholate addition to the largest mixed micelles (with the smallest R_e value) can be used to study the effect of composition and size at any given PC concentration. Dilution of a concentrate PC-cholate mixed micellar solution in a medium containing cholate with a concentration = cmc can be used to study the lipid concentration-dependence at any given R_e (and size).

Similar protocols can be designed to study the effect of albumin on the rate of hydrolysis of solubilized phospholipids. Being aware of the 'indirect effects' of albumin, through its effect on the state of aggregation, experiments should be carried out such that the state of aggregation is retained in the presence of albumin by adding cholate to compensate for these effects.

Enzymatic studies on PC-cholate mixed vesicles

The hydrolysis of PC in cholate-containing vesicles is more complex as it depends on the PC concentration and on the composition and size of the vesicles. The effect of each of these interdependent factors can be studied separately by keeping the other two constant. As previously described [41], this can be done as follows: to study the effect of concentration and composition, relatively large vesicles can first be made at an R_e value close to R_e^{SAT} so that $D_w <$ cmc. Diluting these vesicles in equal volumes of media containing varying cholate concentrations lower than D_w, yields vesicles of constant size and PL concentration, but varying compositions (R_e) according to Eq. (1). On the other hand, diluting the vesicles in media containing D_w mM cholate results in vesicles of constant size and composition but varying phospholipid concentration, which is a simple function of the extent of dilution. To study the effect of vesicle size, vesicles with various sizes can be prepared by first diluting a mixed micellar solution in varying volumes of media with no cholate so that R_e (and therefore the size of the vesicles) is different in the various preparations. Following equilibration, the less diluted solutions can all be brought to the same volume so that the PC concentration and the R_e value are identical in all the solutions, which therefore differ only in size of the vesicles.

Based on our present work, similar (although more complex) protocols have to be designed in the presence of albumin:

(1) To study the effect of R_e in the presence of a given concentration of albumin, large vesicles with $R_e = 0.3$ can be prepared in the presence of albumin if

$$D_t = R_e(L + 1/K(1 + R_e)) + 7[\mathrm{BSA}] \quad (4)$$

Dilution of these vesicles in a constant volume of cholate solutions of concentrations lower than the cmc, results in vesicle dispersions of the same albumin and PC concentrations and the same size but varying R_e values.

(2) To study the effect of PC concentration, vesicle dispersions of constant BSA concentration, R_e value and size but varying PC concentration can be prepared by dilution of the vesicles described above ($R_e = 0.3$) in different volumes of media containing the same BSA and cholate concentrations (D; given by $D = (D_t - R_e L) + 7[\mathrm{BSA}]$, where D_t, L and R_e are the corresponding values prior to dilution and BSA concentration is given in mM).

(3) Studying the effect of size in the presence of albumin can be done by the two steps dilution protocol used in the absence of albumin, if albumin is contained in the diluting media in the second step of dilution. The resultant vesicles will have the same composition and concentration but different sizes. Using this protocol, the effect of size can only be studied at low phospholipid concentrations since at sufficiently high concentrations of PC, albumin-induced size growth is unavoidable (Table 2).

(4) To study the effect of albumin requires that at varying BSA concentrations the substrate vesicles have the same composition, size, and concentration. The detailed protocol depends on whether or not the presence of albumin may result in an increased size of the vesicles: when the composition of the system to be studied is such that albumin does not cause a size increase (Fig. 8), studying the effects of albumin at a constant vesicle concentration, composition and size is relatively simple and may be achieved by adding to pre-formed vesicles a fixed volume of media containing albumin and cholate at a concentration needed to maintain R_e (according to Eq. (4)). The resultant vesicles will all have the same composition and size and the only difference will be the presence of albumin. The effects of albumin at concentrations where the size of the vesicles is an increasing function of the concentration of BSA can only be studied if it is added to pre-formed unilamellar vesicles of a size equal to or larger than the largest size obtained in the presence of albumin (and cholate). When solutions containing cholate and albumin are added to such preformed vesicles the resultant desired vesicles (in terms of composition) can be obtained if the mixed solution contains the desired concentration of BSA and the concentration of cholate given by Eq. (4). The resultant vesicles will all have a constant concentration, composition and size but varying concentrations of albumin (always larger than zero).

5. Concluding remarks

The presence of albumin in cholate-PC mixed systems affects the state of lipid aggregation in these mixtures by (1) binding cholate very tightly such that as long as [cholate]/[BSA] > 7, each BSA molecule binds 7 molecules of cholate, regardless of PC concentration; and (2) altering the size of vesicles such that at low concentrations of BSA its presence causes a slight decrease of the size of the vesicles, whereas at much higher BSA concentrations, the vesicle size increases markedly.

The mechanism(s) responsible for these effects are not clear as yet. Nevertheless, the findings of this report make it possible to design protocols for investigating the effects of albumin on processes in which PC-cholate mixed aggregates are involved. In view of the effects of albumin on the state of aggregation, such special protocols are absolutely essential. Furthermore, these findings found the basis for studying the effects of lipid concentration and state of aggregation on various reactions in the presence of a fixed albumin concentration. Adhering to the protocols proposed above is important to avoid apparent irreproducibilities.

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