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EFFECTS OF TEMPERATURE AND LIPID COMPOSITION ON THE SERUM ALBUMIN-INDUCED AGGREGATION AND FUSION OF SMALL UNILAMELLAR VESICLES

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Small unilamellar vesicles of egg phosphatidylcholine (PC) or dimyristoylphosphatidylcholine, mixed with small unilamellar vesicles labelled with 2-(10-(1-pyrene)decanoyl)phosphatidylcholine, exhibit a constant average size and excimer to monomer (E/M) ratio for several hours when incubated at pH 3.6 at a temperature higher than the phase transition temperature (T_c) of the lipids. Addition of bovine serum albumin to this system produces a transient turbidity increase, a fast decrease in the E/M ratio, a partial loss of vesicle-entrapped [^{14}C]sucrose and a measurable leak-in of externally added sucrose. Sepharose 4B filtration of the system demonstrates that the E/M ratio decrease is strictly paralleled by the formation of liposomes which exhibit a low E/M ratio and a hydrodynamic radius larger than that of small unilamellar vesicles. These data demonstrate that the E/M ratio decrease can be unequivocally ascribed to a vesicle-vesicle fusion process induced by serum albumin. The rate of serum-albumin induced fusion of small unilamellar vesicles is: (a) maximal at a stoichiometric ratio of approx. 2 albumins per vesicle; (b) sensitive to the nature of the lipid and; (c) not altered when human serum albumin replaces bovine serum albumin. The rate of albumin-induced fusion of dimyristoylphosphatidylcholine small unilamellar vesicles is higher below the T_c of the lipid and increases with temperature above the T_c. The formation of protein-bound aggregates with defined stoichiometries and a high local vesicle concentration, as well as changes in the local degree of hydration, are proposed to be the driving forces for the protein-induced vesicle fusion in this system.

Introduction

Proteins have been demonstrated to be involved in virus-cell fusion [1], virus-mediated cell-cell fusion [1–3], and egg fertilization [4] and have been postulated to play a role in other fusion processes [5,6]. Several model systems have been developed for investigating the mechanism of protein-induced membrane fusion at a molecular level. Direct evidence for protein-mediated membrane fusion has been recently obtained for vesicle-planar membrane [7] and vesicle-vesicle fusion [8,9].

The sequence of events leading to fusion is necessarily preceeded by intermembrane approximation. Indeed, vesicle aggregation promoted by proteins is a well-established phenomenon [10–12], aggregation in itself does not necessarily lead to fusion [13]. Moreover, since discrimination between aggregation and fusion is not straightforward, certain rigorous criteria must be met to prove the occurrence of the latter event [14].

We have recently demonstrated that, under certain conditions, the addition of bovine serum albumin to small unilamellar vesicles prepared from egg phospha-

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Abbreviations: PyPC, 2-(10-(1-pyrene)decanoyl)phosphatidylcholine; egg PC, egg phosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-3-glycerophosphocholine; E/M, eximer to monomer ratio.

tidylcholine (egg PC) results in vesicle fusion [9]. In that work, a series of techniques, including time-dependent turbidity changes, filtration, centrifugation, Sepharose 4B chromatography and electron microscopy were employed to follow both the aggregation and fusion of small unilamellar vesicles. It was demonstrated that the first observable step is the formation of large aggregates, which are retained by 0.45 μm filters and give rise to a visible turbidity. Subsequently, these aggregates, composed of small unilamellar vesicles interconnected by serum albumin, undergo spontaneous collapse. During this process the size of the vesicle population increases and, after 14 h of incubation, electron microscopy reveals a homogeneous population of 50 ± 10 nm diameter liposomes [9].

In the present work, we report an investigation of the kinetics of the serum albumin-induced fusion of small unilamellar vesicles using a fluorescent lipid probe [15]. In addition, we examined the rates of leak-in and leak-out of a water-soluble marker and the effects of temperature and lipid composition on the fusion process. The data are analyzed in terms of the molecular events leading to protein-induced vesicle fusion.

Methods

Egg PC was prepared, purified, and stored following established procedures[16,17] as previously described [9]. 1,2-Dimyristoyl-*sn*-3-glycerophosphocholine (DMPC) was prepared according to Robles and Van den Berg [18]. Lysophosphatidylcholine was prepared from egg PC by hydrolysis with *Crotalus adamanteus* phospholipase A_2 (generously donated by Dr. Schreier of the Instituto de Química da Universidade de São Paulo). 2-(10-(1-Pyrene)-decanoyl)-phosphatidylcholine (PyPC) was synthesized by the general procedure of Gupta et al. [19] by mixing purified lysophosphatidylcholine with the anhydride of 10-(1-pyrene)decanoic acid [20] and purified by column chromatography on silica gel (chloroform/methanol) [17]. The product migrated as a single fluorescent spot at the R_f of egg PC upon TLC on silica gel (chloroform/methanol/water, 65 : 35 : 4, v/v). In addition, the excitation and emission spectra of both the monomer and excimer of PyPC (incorporated in small unilamellar vesicles) were wavelength-independent, indicating the absence of extraneous fluorescent impurities. There was no evidence of chemical or photochemical degradation of PyPC under any of the conditions employed. Less than 0.5 mol% of free fatty acid was present in the vesicle preparations, as estimated by gas-liquid chromatography [21].

Bovine serum albumin (Sigma Chem. Co. Fraction V) was purified [22,23] as described previously [9]; human serum albumin (Sigma Chem. Co. fatty acid-free) was used without further purification.

Small unilamellar vesicles were prepared according to Barenholz et al. [24] as previously described [9] in 10 mM formate (K^+) buffer, pH 3.6, containing 0.16 M KCl (Buffer 1). Egg PC small unilamellar vesicles were prepared at 2°C and DMPC small unilamellar vesicles at 30°C. PyPC-containing small unilamellar vesicles were prepared analogously. [^{14}C]Sucrose (480 mCi/mmol, Schwarz, Mann) was incorporated into small unilamellar vesicles by carrying out the above preparation in Buffer 1 containing 10 μCi of [^{14}C]sucrose. After the ultracentrifugation step [24], the label in the bulk aqueous phase was separated by filtration (4.0 ml aliquot) through a Sephadex G-25 column (2.5 × 20 cm) equilibrated in Buffer 1.

The kinetic experiments were performed in Buffer 1 using mixtures of PyPC-labelled small unilamellar vesicles with either unlabelled small unilamellar vesicles or [^{14}C]sucrose-labelled small unilamellar vesicles. The reaction was initiated by addition of an aliquot of a stock solution of serum albumin. Turbidity changes were followed at 450 nm on a Beckman M-25 spectrophotometer in 0.2 cm pathlength cells. For the fluorescence measurements, aliquots (10 or 20 μl) of the reaction mixtures were diluted to 2 ml with 10 mM maleate (K^+) buffer, pH 6.5, containing 0.16 M KCl, in 1 cm pathlength quartz fluorescence cells (Hellma). This dilution at pH 6.5, which served both to stop the reaction [9] and to eliminate possible scattering artifacts, does not alter the excimer/monomer intensity ratios. Fluorescence emission spectra were recorded at 30.0 ± 0.1°C on a Hitachi-Perkin Elmer MPF-4 spectrometer operated in the ratio mode. [^{14}C]Sucrose leakage was followed by removing aliquots (0.21 ml) of the reaction mixtures at convenient times, adjusting the pH to 6.5 with 0.1 M imidazole buffer (pH 9) and applying 0.2 ml of this solution to a Sephadex G-25 column (0.6 × 12 cm) equilibrated with 10 mM maleate (K^+) buffer, pH 6.5 containing 0.16 M KCl. Gel filtration on

Sepharose 4B (0.9 X 40 cm) columns was performed in a entirely analogous manner. For egg PC preparations the column was operated at room temperature (22 ± 2°C), whereas for DMPC preparations the column was run at 32°C. Total phosphate [25] and albumin concentration [26] were determined by established procedures. Radioactivity was determined with a Beckman LS-250 liquid scintillation system in a Triton X-100-toluene scintillation fluid.

Results

Fluorescence spectra of PyPC-labelled egg PC-small unilamellar vesicles exhibit both the characteristic 1-alkylpyrene monomer emission and the long-wavelength structureless excimer emission (Fig. 1A). The excimer/monomer (E/M) ratio, measured as the ratio of uncorrected fluorescence intensities at 470 and 395 nm, is a linear function of the mol% of PyPC in small unilamellar vesicles up to 7% PyPC; analogous results were obtained when the phospholipid was DMPC.

Control experiments demonstrated that the E/M ratio of egg PC small unilamellar vesicles containing 5 mol% PyPC is unaffected by dilution and remains virtually unchanged, after incubation for 2 h, at pH 3.6, in the presence of a 60-fold excess of unlabelled egg PC small unilamellar vesicles. Addition of bovine serum albumin to a mixture of 5 mol% PyPC-labelled and unlabelled small unilamellar vesicles (hereafter referred to as egg PC(PyPC) small unilamellar vesicles results in a decrease in the E/M ratio from 0.33 to 0.08 (Fig. 1B). The time dependent E/M ratio changes produced upon addition of serum albumin to egg PC(PyPC) small unilamellar vesicles are shown in Fig. 2, along with data for the concomitant turbidity changes and for release of vesicle-entrapped [^{14}C]-sucrose.

As shown previously [9], the turbidity of the reaction mixture exhibits a transient increase. When the turbidity reaches its maximum value, the E/M ratio is typically about one half of its initial value (Fig. 2). The loss of vesicle-entrapped [^{14}C]sucrose is apparently first order during 30 min after addition of serum albumin to egg PC(PyPC) small unilamellar vesicles (Fig. 2, insert) and accompanies the decrease in the E/M ratio. The uptake of externally added [^{14}C]sucrose by the vesicles is presented in Fig. 3.

The vesicle size distribution and the corresponding E/M ratios following addition of serum albumin to egg PC(PyPC) small unilamellar vesicles at pH 3.6 were analyzed by Sepharose 4B gel filtration. Egg

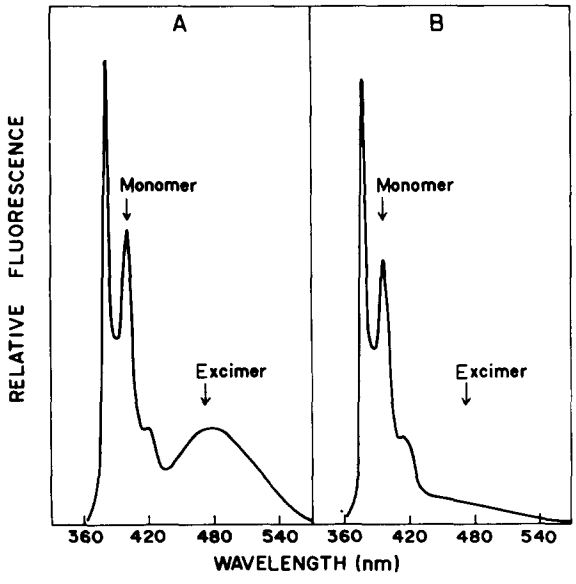

Fig. 1. Effect of bovine serum albumin on the fluorescence emission spectra of egg PC(PyPC) small unilamellar vesicles. Vesicles (10 mM egg PC; labelled : unlabelled = 1 : 60) incubated at 30°C in Buffer 1: (A) for 2 h; (B) for 30 min with bovine serum albumin (0.21 mg/ml).

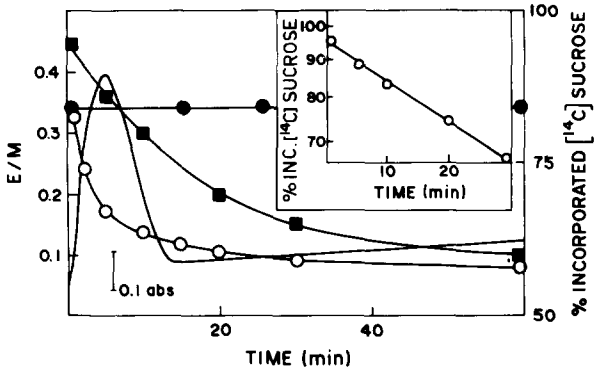

Fig. 2. Temporal variation of the E/M ratio, turbidity and leakage of [^{14}C]sucrose following the addition of bovine serum albumin (0.21 mg/ml) to egg PC(PyPC) small unilamellar vesicles (10.2 mM egg PC; labelled : unlabelled = 1 : 50) at 30°C in Buffer 1. Absorbance at 450 nm (———); E/M ratio with (○) and without (●) albumin, percent of [^{14}C]sucrose retained (■). Inset shows a log/linear plot of the [^{14}C]sucrose leakage data.

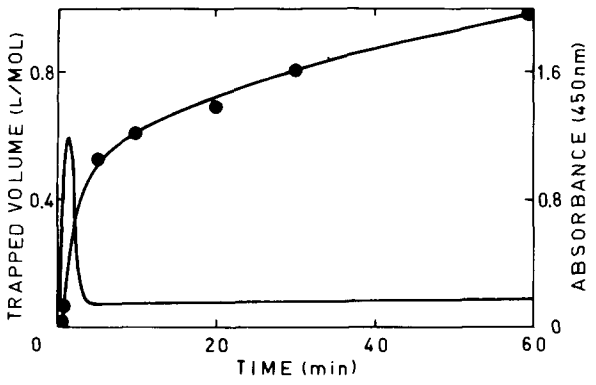

Fig. 3. [^{14}C]Sucrose incorporation into egg PC small unilamellar vesicles (17 mM egg PC) after the addition of bovine serum albumin (10,2 mg/ml), in Buffer 1, at 30°C (•). Absorbance at 450 nm (———).

PC(PyPC) small unilamellar vesicles elute as a single peak upon gel filtration with an average E/M ratio of 0.32 (Fig. 4A). When egg PC(PyPC) small unilamellar vesicles are incubated with serum albumin for 1.5 min, the elution profile reveals components with significant differences in the E/M ratio. Thus, the average E/M ratio of the phospholipid eluting at the void volume (V_0) is 0.05 while that of the phospholipids eluting in the region of the original small unilamellar vesicles is 0.30. After 24 h of incubation, filtration of the reaction mixture gives a phospholipid peak at V_0 with an average E/M ratio of 0.04 (Fig. 4C).

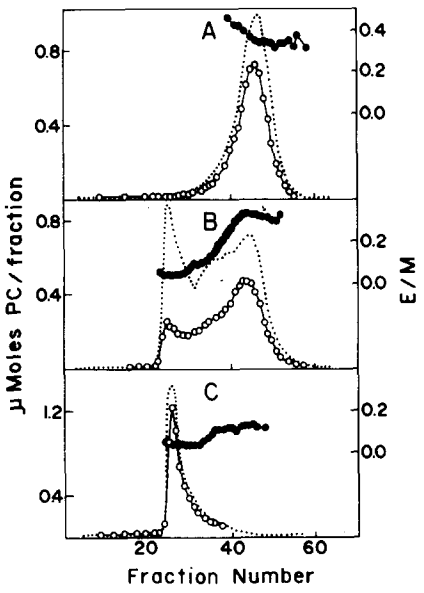

Fig. 4. Sepharose 4B elution profiles of egg PC(PyPC) small unilamellar vesicles (21 mM egg PC; labelled : unlabelled = 1 : 60) incubated in Buffer 1, at 30°C. (A) 2 h; (B) 90 s with bovine serum albumin (0.2 mg/ml); (C) 24 h with albumin (0.2 mg/ml). The fractions (0.41 ml) were assayed for total lipid (○), relative fluorescence intensity at 385 nm (· · · · ·) and E/M ratio (•).

The ability of two other soluble proteins, ovoalbumin and cytochrome *c*, to cause changes in the E/M ratio and Sepharose 4B elution pattern of egg PC(PyPC) small unilamellar vesicles was examined. Although small decreases (approx. 10%) in the E/M ratio were observed when egg PC(PyPC) small unilamellar vesicles were incubated with these proteins for 2 h at pH 3.6, no changes in the elution profile of the vesicles were detected upon Sepharose 4B filtration. Although limited in scope, these data clearly imply that the serum albumin induced changes in E/M ratio and Sepharose 4B elution pattern are not due to an unspecific protein-vesicle interaction at this pH.

As observed with egg PC(PyPC) small unilamellar vesicles, a mixture of PyPC-labelled DMPC small unilamellar vesicles with an excess of unlabelled DMPC small unilamellar vesicles (hereafter referred to as DMPC(PyPC) small unilamellar vesicles) can be incubated above the phase transition temperature (T_c) of DMPC, for 2 h at pH 3.6, with no observable change in the E/M ratio. Addition of bovine serum albumin to DMPC(PyPC) small unilamellar vesicles at 30°C results in turbidity changes which are comparable to, but smaller than, those observed for egg PC(PyPC) small unilamellar vesicles. At both 30 and 42°C, data for the vesicle size distribution from Sepharose 4B filtration and the corresponding E/M ratios reveal an inverse correlation between the decrease in the E/M ratio and the size increase (Fig. 5). More than 70% of the DMPC appears as Sepharose 4B-excluded lipid with low E/M, after incubation of DMPC(PyPC) small unilamellar vesicles with bovine serum albumin for 30 min at 42°C (Fig. 5). When the incubation temperature is lowered to 30°C the column profile again shows the inverse correspondence between the E/M ratio and size, but the fraction of lipid eluting in the V_0 is significantly lower.

We examined the effect of temperature on the kinetics of the decrease of the E/M ratio following

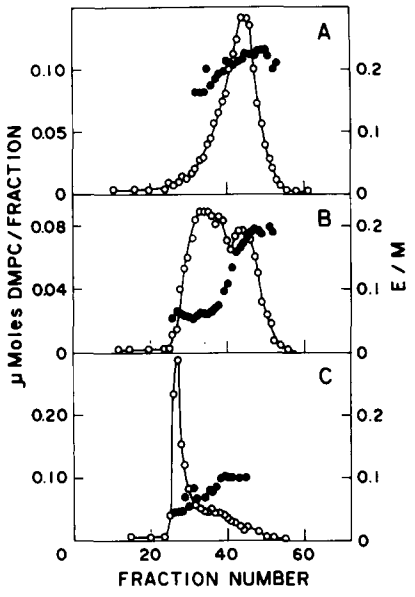

Fig. 5. Sepharose 4B elution profile of DMPC(PyPC) small unilamellar vesicles (4.3 mM DMPC; labelled : unlabelled - 1 : 60) incubated for 30 min in Buffer 1. Without albumin at 30°C. (A) With 0.2 mg/ml of bovine serum albumin at 30°C (B) and 42°C (C). The fractions (0.41 ml) were analyzed for total lipid (○) and E/M ratio (●).

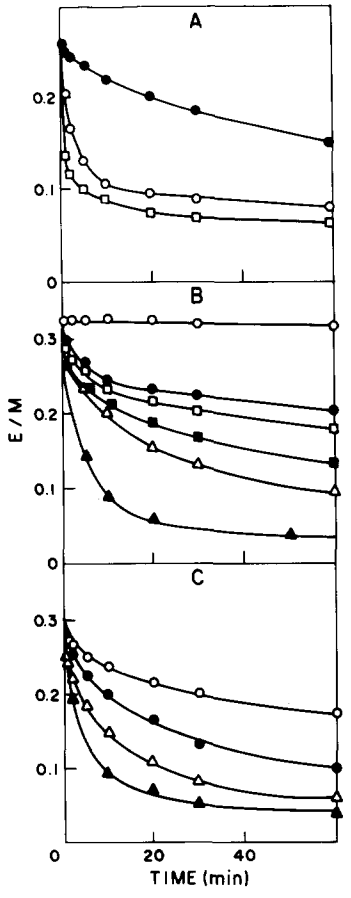

Fig. 6. Temperature effect on the E/M ratio of small unilamellar vesicles (labelled : unlabelled = 1 : 60), after addition of bovine of human serum albumin in Buffer 1. (A). Egg PC(PyPC) small unilamellar vesicles (17.5 mM egg PC) and bovine serum albumin (0.4 mg/ml) incubated at 2°C (●); 20°C (○) and 30°C (□). (B). DMPC(PyPC) small unilamellar vesicles (8 mM DMPC), incubated at 24°C without serum albumin (○) or with 0.2 mg/ml bovine serum albumin at 24°C (●), 27°C (□), 30°C (■), 35°C (△) and 45°C (▲). (C). Same as B with 0.2 mg/ml human serum albumin at 30°C (○), 35°C (●), 40°C (△) and 45°C (▲).

the addition of either bovine or human serum albumin to egg PC- and DMPC(PyPC) small unilamellar vesicles. In all cases, the rate of decrease of the E/M ratio increases with temperature above the T_c of the lipid (Fig. 6). The temperature dependence of the half-life for the decrease in the E/M ratio of DMPC-(PyPC) small unilamellar vesicles provoked by either human or bovine serum albumin yielded an activation energy of 140 kJ/mol (25–50°C) (Fig. 7). The corresponding activation energy for the bovine serum albumin-induced changes in the E/M ratio of egg PC-(PyPC) small unilamellar vesicles is 88 kJ/mol (2–30°C) (Fig. 7).

The rate of change of the E/M ratio induced by serum albumin is highly sensitive to protein and lipid concentration. 9 mM egg PC(PyPC) small unilamellar vesicles exhibit a constant rate of change of the E/M ratio between 0.2 and 0.4 mg/ml of bovine serum albumin, but this rate diminishes drastically, if the protein concentration is either increased or decreased, at the same lipid concentration (Fig. 8A). The lipid concentration dependence of the E/M ratio at constant serum albumin is shown in Fig. 8B. Analogous results were obtained for the lipid and serum albumin dependences of the E/M ratio with DMPC(PyPC) small unilamellar vesicles above the T_c.

DMPC(PyPC) small unilamellar vesicles maintain a constant E/M ratio for at least 2 h at 10°C (i.e., below the T_c of DMPC). It is noteworthy that addi-

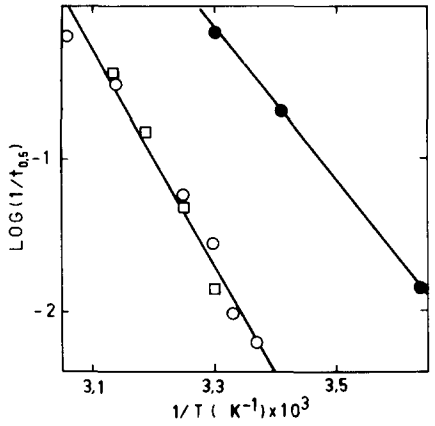

Fig. 7. Arrhenius plot for the rate of change of the E/M ratio of egg PC(PyPC) small unilamellar vesicles (closed symbols) or DMPC(PyPC) small unilamellar vesicles (open symbols) after addition of bovine (○) or human (□) serum albumin. Half lifes ($t_{0.5}$) were obtained from the data of Fig. 5 (see text).

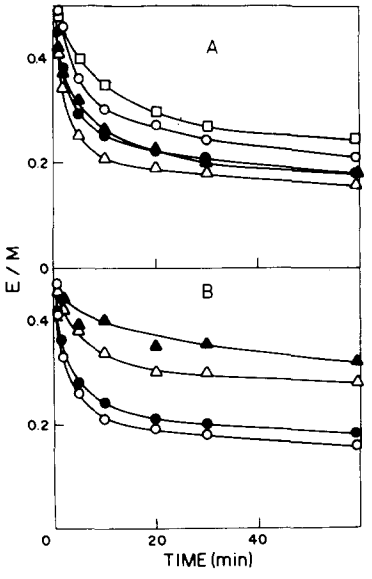

Fig. 8. (A). Effect of bovine serum albumin on the E/M ratio of egg PC(PyPC) small unilamellar vesicles (9 mM egg PC; labelled : unlabelled = 1 : 60) incubated at 20°C in Buffer 1. Serum albumin concentrations were 1.21 (○), 0.81 (●), 0.4 (△), 0.2 (△), 0.08 (▲) and 0.04 (□) mg/ml. (B). Effect of lipid concentration on the bovine serum albumin (0.4 mg/ml)-induced change in E/M ratio of egg PC(PyPC) small unilamellar vesicles incubated in Buffer 1 at 20°C at egg PC concentrations of 17.4 (○), 14 (○), 10.5 (○), 7 (●), 3.5 (△) and 1.7 (▲) mM. (labelled : unlabelled = 1 : 60).

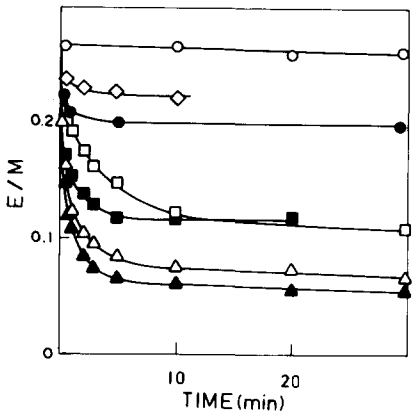

Fig. 9. Effect of bovine serum albumin in the E/M ratio of DMPC(PyPC) small unilamellar vesicles (9.14 mM DMPC; labelled: unlabelled = 1 : 60) incubated in Buffer 1 at 10°C without serum albumin (○) or with 2.8 (●); 1.7 (□), 1.1 (△), 0.7 (▲), 0.2 (□) and 0.02 (◇) mg/ml of bovine serum albumin.

tion of bovine serum albumin to DMPC(PyPC) small unilamellar vesicles at 10°C leads to a fast turbidity increase, which does not diminish upon further incubation and can be only partially decreased by increasing the pH to 6.5. Upon addition of serum albumin to DMPC(PyPC) small unilamellar vesicles at 10°C, the E/M ratio decreases rapidly and reaches a constant value which is minimum at a protein/lipid ratio of approx. 0.06 mg of albumin/μmol DMPC (Fig. 9). Higher or lower protein/lipid ratios produce smaller changes in the E/M ratio of DMPC(PyPC) small unilamellar vesicles.

Discussion

The initial turbidity increase, observed after addition of bovine serum albumin to egg PC small unilamellar vesicles at pH 3.6, has been shown to be due to the formation of micron-size aggregates consisting of small unilamellar vesicles and serum albumin [9]. The metastable nature of these aggregates, evidenced by the subsequent decrease in turbidity, was proposed to arise from egg PC small unilamellar vesicles fusion. The use of a fluorescent phospholipid probe not only permits the investigation of the occurrence of vesicle fusion at this stage, but also provides a more detailed understanding of its mechanism.

For a mixture on unlabelled and PyPC-labelled

small unilamellar vesicles, the occurrence of protein-induced vesicle fusion during the transient turbidity stage [9] would imply a concomitant decrease in the E/M ratio due to redistribution (i.e., dilution) of the fluorescent probe over the available lipid. This behavior is evident in Figs. 1 and 2, the E/M ratio decreasing rapidly from 0.33 to 0.08, corresponding to an effective dilution of the probe by a factor of 4.

The decrease in the E/M ratio, observed upon addition of bovine serum albumin to mixtures of unlabelled and PyPC-labelled small unilamellar vesicles, does not unequivocally demonstrate the occurrence of fusion; an analogous decrease would be expected to occur as a result of label exchange among intact small unilamellar vesicles. In fact, energy transfer in mixtures of fluorescent-labelled small unilamellar vesicles and E/M ratio changes of PyPC have been utilized to investigate both fusion [27,28] and spontaneous phospholipid transfer [15] between small unilamellar vesicles in other model systems. However, as demonstrated by our controls in the absence of protein and by other work [15], the time scale of spontaneous label exchange in unaggregated small unilamellar vesicles is much too slow (of the order to hours) to explain the observed rate of decrease of the E/M ratio. Alternatively, it might be suggested that serum albumin increases the rate of spontaneous monomer exchange between small unilamellar vesicles. In this regard, the column profiles provide conclusive evidence for both the occurrence of vesicle fusion and the existence of a correlation between the E/M ratio and the apparent liposome size. Thus, the liposome population eluting in the V_0 of the Sepharose 4B column exhibits an E/M ratio of 0.04, corresponding to a approx. 6–8-fold dilution of PyPC. Both the observed dilution of the fluorescent probe and the 50 nm diameter exclusion limit of Sepharose 4B [29] imply that the liposomes appearing in V_0 must result from the fusion of (at least) five 25 nm diameter small unilamellar vesicles. These phenomena are already observed in the time when most of the lipid is in a highly aggregate stage (Fig. 2) [9].

The stoichiometric ratio of serum albumin to small unilamellar vesicles in the large aggregates [9] corresponds to that which produces a maximum in the fusion rates (Fig. 8). Moreover, within a large aggregate the local concentration of vesicles must be greater than that in bulk solution. Hence, both high local concentration and optimal protein/small unilamellar vesicle stoichiometry would be expected to facilitate fusion within the initial aggregate, leading to the formation of Sepharose 4B-excluded liposomes of low E/M ratio during the time of maximum turbidity. Taken together, these data conclusively rule out the possibility that monomer exchange is responsible for the decrease in the E/M ratio and point to vesicle fusion as the predominant mechanism for the decrease in the E/M ratio.

In related systems, it has been shown that small unilamellar vesicle fusion generally results in partial loss of the contents of the internal aqueous compartment to the surrounding medium [28,30–32]. The amount and velocity of loss of internal marker varies markedly with the chemical nature of the lipids [33–35]. In the bovine serum albumin-egg PC small unilamellar vesicle system, about 50% of the vesicle-entrapped [^{14}C]sucrose is released to the external medium during the time that corresponds to a 4-fold dilution of the fluorescent lipid marker. The existence of communication between the inner aqueous compartment and the external medium, evidenced by the partial leak out, implies that the reverse process, i.e. leak-in of external marker, should be measurable if the internal volume/mole of lipid increases during fusion [36]. This leak-in of external probes has in fact been observed in the spontaneous fusion of dipalmitoylphosphatidylcholine small unilamellar vesicles below the T_c [37]. Similarly, in our system, addition of bovine serum albumin to egg PC(PyPC) small unilamellar vesicles is accompanied by a measurable leak-in of externally added [^{14}C]sucrose. The maximum leak-in rate is observed during the high turbidity phase (Fig. 3), indicating the occurrence of a volume increase [36] concomitant with the fusion at the aggregate stage. At the point where the average E/M ratio corresponds to a 4-fold dilution of the fluorescent lipid probe over the liposome population, an effective internal volume of approx. 1 l/mol (1.3 ml/g) can be calculated [36] from the data in Fig. 3. This value is significantly higher than the values reported for small unilamellar vesicles [36], but is well within the range reported for the internal volume of lipid vesicles larger than 50 nm in diameter [37].

The velocity of the bovine serum albumin-induced fusion increases with temperature above the T_c of the

phospholipid. This effect contrasts sharply with the temperature dependence observed in some model systems, such as the fatty acid-induced fusion of phosphatidylcholine vesicles [38] and the Ca^{2+}-induced fusion on phosphatidyl serine vesicles (Ref. 39, see however Ref. 35), where maximum fusion is observed near the T_c of the lipid. On the other hand, more complex systems, such as that derived from myoblasts [6] and virus-cell [40], exhibit an increase in fusion rate with temperature above the T_c. It can be postulated that the type of temperature effect we observe is prevalent in models of membrane fusion, which are most closely related with biological systems.

The apparent activation energy for the bovine and human serum albumin induced fusion of DMPC(PyPC) small unilamellar vesicles is significantly higher than that for the albumin-induced fusion of egg PC(PyPC) small unilamellar vesicles. This difference strongly suggests that an effect on the protein conformation [41,42] cannot be the only source of the temperature-dependent increase in fusion rate. Moreover, for egg PC(PyPC) small unilamellar vesicles, the magnitude of the activation energy for the fusion process is comparable to that for phospholipid flip-flop in the bilayer [43]. This similarity suggests that both phenomena might be closely related. Indeed, fusion must certainly be dependent on reorganization, of at least, part of the bilayer.

Over the temperature range investigated, the rate of bovine serum albumin-induced fusion of DMPC vesicles is faster below T_c of the lipid. Although spontaneous vesicle fusion below the T_c has been reported [37], the exact nature of this phenomenon has not been established; it has been suggested that fractures in the bilayer structure may be a possible cause of such spontaneous fusion [44]. Under our conditions, spontaneous fusion of DMPC(PyPC) small unilamellar vesicles was not detected at 10°C in the absence of protein. However, addition of bovine serum albumin to DMPC(PyPC) small unilamellar vesicles results in the formation of aggregates responsible for the stable high turbidity in the system. The kinetics of E/M ratio changes are indicative of a very fast initial rate of fusion which subsequently diminishes drastically (Fig. 9). This high fusion rate, below the T_c of DMPC, can be rationalized on the basis of the stability of the aggregates and the instability of bilayers with high curvature in the gel phase [45].

Below the T_c of DMPC, the optimal protein/lipid stoichiometry for fusion occurs at around 12.8 mol of lipid per mg of bovine serum albumin (Fig. 9). For egg PC, the optimal stoichiometry is within the same range (Fig. 8 A and B). The reported aggregation number of sonicated egg PC or DMPC small unilamellar vesicles is approx. 2500 lipid monomers per vesicle and is apparently temperature-independent, despite marked changes in shape and degree of hydration with temperature [46,47]. Consequently, the optimal molar stoichiometry for bovine serum albumin-induced fusion corresponds roughly to 2 albumin molecules per small unilamellar vesicle. This value is identical to that encountered in the initially formed large aggregates of bovine serum albumin and small unilamellar vesicles [9]. Hence both above and below the T_c, fusion occurs after formation of aggregates with a 2 : 1 serum albumin : small unilamellar vesicle ratio.

In general, the effect of temperature on the fusion of vesicles has been analyzed in terms of membrane lateral phase separation, generating unstable boundaries which can lead to fusion upon vesicle contact [48]. Thus, fusion has been observed when the vesicle undergo thermotropic [37] or ionotropic [49] phase transitions. In the present case, fusion occurs both in the gel and liquid crystalline state and is preceeded in both cases by an initial aggregation step. Changes in hydration have been reported to be a major contributor to the barrier for bilayer contact and fusion [50]. Thus, the higher fusion rate in the gel state may be a consequence of the lower degree of hydration of DMPC small unilamellar vesicles below the T_c.

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