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Lipid miscibility and size increase of vesicles composed of two phosphatidylcholines

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The size increase of small unilamellar vesicles composed of binary mixtures either of saturated fatty acid phosphatidylcholines with different chain lengths or of saturated and unsaturated phosphatidylcholines was found to depend on the miscibility properties of the lipid components. No size increase was detected in vesicles formed by two miscible phosphatidylcholines. In vesicles composed of two lipids which are partially immiscible in the gel state, a size increase was observed at temperatures which mainly overlapped the range of temperatures of the lipid phase transition. The rate of size increase of vesicles composed of two lipids which are immiscible in the gel state was faster than that of vesicles composed of two partially immiscible phosphatidylcholines, and the process occurred not only at the temperature ranges of the lipid phase transition, but also when both lipids were in the gel state. The vesicle size increase process occurred without the mixing of the internal content of the vesicles. A model is proposed in which the presence of 'fractures' between membrane regions of different fluidity and/or lipid composition controls the rate of this process.

Introduction

Small unilamellar vesicles (SUV), composed of one saturated fatty acid phosphatidylcholine, free from contaminants, are slowly transformed into larger species at temperatures below the gel to liquid-crystalline lipid transition temperature [1–10]. This process has been extensively investigated using several techniques, including NMR, gel chromatography, electron microscopy, differential scanning calorimetry, photon correlation spectroscopy, and trapped volume, turbidity and ultracentrifugal studies. In spite of the numerous experimental data reported in the literature, the following questions are yet to be clarified: (1) whether the size increase process is due to vesicle fusion, in which case the internal contents of the vesicles mix without release into the suspension

Abbreviations: DLPC, dilauroylphosphatidylcholine (12 : 0/12 : 0-glycerophosphocholine); DMPC, dimyristoylphosphatidylcholine (14 : 0/14 : 0-glycerophosphocholine); DPPC, dipalmitoylphosphatidylcholine (16 : 0/16 : 0-glycerophosphocholine); DSPC, distearoylphosphatidylcholine (18 : 0/18 : 0-glycerophosphocholine); DAPC, diarachidoylphosphatidylcholine (20 : 0/20 : 0-glycerophosphocholine); DOPC, dioleoylphosphatidylcholine ($18:1_c/18:1_c$-glycerophosphocholine); DEPC, dielaidoylphosphatidylcholine ($18:1_t/18:1_t$-glycerophosphocholine); DLnPC, dilinoleoylphosphatidylcholine ($18:2_c/18:2_c$-glycerophosphocholine); PC, phosphatidylcholine; Tris, trishydroxymethylaminomethane; SUV, small unilamellar vesicle(s); N-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dioleoylphosphatidylethanolamine; N-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)dioleoylphosphatidylethanolamine.

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medium, or simply to the recombination of bilayer fragments produced by broken membranes, and (2) whether the nucleation center for the size increase process originates from packing disorders of the membrane lipids.

In order to clarify these problems, we report a systematic study of the size increase capacity of SUV composed either of two saturated phosphatidylcholines with different chain lengths, or of one saturated and one unsaturated phosphatidylcholine. The unsaturated phosphatidylcholines contain either mono-unsaturated hydrocarbon chains in the *cis* or in the *trans* conformation, or di-unsaturated chains in the *cis* conformation or one saturated and one *cis*-unsaturated chain. In these mixtures, the vesicle size increase is shown to occur through the rupture of the membrane. The rate of the process, which is strictly correlated with the miscibility properties of the lipids, depends on the formation of lipid domains with different fluidities and/or composition. The membrane destabilization was suggested to be due to the formation of 'fractures', or regions of mismatch in molecular packing at the interface of the domains. These phenomena are particularly important, because many natural membranes have been shown to possess macro or micro-regions with different lipid mobilities, composition and spatial arrangements. The presence of fractures between these regions may affect not only the cell fusion process, but also some membrane phenomena, such as thermal hysteresis [11], annealing [12], permeability [13–15], protein-lipid interactions [16,17] and susceptibility to enzymes [18].

Experimental procedures

Vesicle preparation

Saturated phosphatidylcholines, supplied by Sigma, were precipitated from acetone according to procedures described by us elsewhere [19]. Unsaturated phosphatidylcholines (Sigma and Supelco) were used without further treatment. Small single bilayer vesicles (SUV) were prepared by dissolving the lipids in chloroform. The solvent was evaporated under a nitrogen stream and completely removed by drying the sample under high vacuum for 20 h. A solution containing 0.1 M KCl and 10 mM Tris-HCl (pH 7) was added at a temperature well above the phase transition temperature of the lipids and the suspension was shaken for 10 min. The sample was exposed to ultrasonic pulsed radiation under a nitrogen atmosphere for a total of 15 min with a Branson sonifier model B-30. Undispersed lipids and titanium particles were removed by centrifugation at $40\,000 \times g$ for 20 min. For each lipid mixture, the temperature during centrifugation was selected to minimize the rate of formation of large particles.

Unilamellar vesicles of large diameter were prepared by the French press method according to Barenholtz et al. [20]. The vesicle dispersion, after four treatments by the French pressure cell operating at maximal pressure of 20000 p.s.i., was centrifuged for 4 h at $160\,000 \times g$. The inorganic phosphate was determined according to the method described by Bartlett [21].

Optical methods

Turbidity changes measurements. The turbidity changes of a SUV suspension were measured as absorbance changes, ΔA, at 334 nm in an Eppendorf photometer connected to a recorder. The temperature was regulated and kept constant by a circulating thermostated bath, and the solution in the sample cuvette was magnetically stirred. The SUV suspension was maintained at temperatures at which the bilayer was in the liquid-crystalline state. Aliquots of this suspension were cooled for 1 min at the experimental temperature, and the kinetics of the absorbance increase was then measured.

Carboxyfluorescein release measurements. Carboxyfluorescein was supplied by Eastman Kodak and purified according to the method described by Ralston et al. [22]. Carboxyfluorescein-loaded vesicles were prepared by sonicating 2.5 ml of lipids (10 mM P_i) in 0.1 M KCl and 25 mM Tris at pH 9 for 15 min. Then 2.5 ml of a solution containing 66 mM dye neutralized with 0.1 M KOH, and 60 mM Tris at pH 9 was added and the final solution was sonicated for further 5 min. The vesicle suspension was maintained for 1 h at a temperature higher than the lipid transition temperature, and separated from the excess dye by eluting through a Sephadex G-75 column in 0.1 M KCl and 25 mM Tris. The column (1.6×30 cm)

was kept at a temperature of 4°C, where the rate of dye release from the vesicles was negligible. Negligible external concentration of free dye was found in the suspension of carboxyfluorescein-containing SUV after their elution through the column. Thus the difference between the inner dye concentration of the vesicle and the external medium dye concentration does not change when different lipid concentrations are used in the sample. The carboxyfluorescein release from the vesicles was measured as the dye fluorescence increase in a Perkin Elmer spectrofluorimeter model 650-40. The excitation and emission wavelengths were 492 and 523 nm, respectively. The complete release of the dye was achieved by the addition of Triton X-100, at a final concentration of 0.75% (v/v).

Measurements of fluorescence anisotropy. In the measurements of the fluorescence anisotropy of the probe diphenylhexatriene, the sample consisted of a suspension of vesicles previously incubated with diphenylhexatriene dispersed in the same buffer (0.5 mol diphenylhexatriene/100 mol lipids), maintained for 30 min at temperatures higher than the gel to liquid-crystalline phase transition temperature of the lipids. Fluorescence was measured at excitation and emission wavelengths of 360 and 430 nm, respectively. Fluorescence anisotropy was measured with the spectrofluorimeter equipped with plastic polarizers in the excitation and emission beams. Anisotropy values were calculated according to the formula

$$r = (I_{\parallel} - CI_{\perp})/(I_{\parallel} + 2CI_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are the emission intensities detected through a polarizer oriented parallel ($I_{\parallel}$) or perpendicular ($I_{\perp}$) to the direction of polarization of the excitation light. C is the instrumental correction factor. The phase diagrams of the binary mixtures were obtained by the temperature-dependent fluorescence anisotropy measurements of the probe diphenylhexatriene according to the graphical method previously described [23]. The temperature-dependent fluorescence anisotropy was recorded in cooling scans at an average scan rate of 25 K/h.

Lipid mixing measurements. The lipid mixing was monitored by the assay of energy transfer between two fluorescent phospholipids, as described by Struck et al. [24]. The two fluorescent probes *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dioleoylphosphatidylethanolamine (N-NBD-PE) and *N*-(lisssamine Rhodamine B sulfonyl)dioleoylphosphatidylethanolamine (N-Rh-PE) were cosonicated with the lipids (0.6 mol% each). Lipid mixing was directly initiated in the cuvette of the spectrofluorimeter by adding vesicles devoid of fluorescent lipids to vesicles containing the fluorescent lipids. The N-NBD-PE fluorescence increase was measured at 465 and 530 nm excitation and emission wavelengths respectively. The fluorescence intensity at infinite probe dilution was measured by adding Triton X-100 (0.75% v/v). The quenching by Triton X-100 on the fluorescence intensity of N-NBD-PE incorporated in lecithin vesicles at very low concentrations, 0.1%, was measured and a correction factor was calculated and applied to the lipid mixing experiments. N-NBD-PE and N-Rh-PE were synthetized in the Dr. C. Montecucco laboratory (this Institute).

Measurements of the mixing of the internal content of the vesicles. The assay of vesicle fusion was based on the mixing of the vesicle contents according to Wilschut and Papahadjopoulos [25]. The assay involves the encapsulation of terbium chloride ($TbCl_3$) in one population of vesicles and of dipicolinic acid in another. The enhancement of the terbium fluorescence consequent to the formation of the Tb-dipicolinic acid complex during the mixing of internal content of the two vesicle populations permits to follow the kinetics of vesicle fusion.

Electron microscopy

The electron micrographs were obtained from a Philips 300 electron microscope operating at 80 kV. A drop of lipid suspension (0.5 mM lipids at 10°C) was applied to a 300 mesh copper grid coated with collodion. After 30 s, the liquid was drawn off with a blotter and a drop of 1% molybdic acid solution (pH 7.4) was applied. The excess liquid was removed and the grid allowed to dry. Approximately 1000 vesicles were sized for each experiment.

Results

Size increase of vesicles composed of two miscible phosphatidylcholines

Saturated phosphatidylcholines having a difference of two carbon atoms in the acyl chain length mix ideally both in the liquid-crystalline and in the gel state [26–31]. The rates of vesicle size increase of DMPC/DPPC, DPPC/DSPC and DSPC/DAPC SUV suspensions, measured as turbidity increase at various temperatures and lipid ratios, were not appreciably different from those obtained with SUV composed of the pure lipid component ($\Delta A/10$ min < 0.02). The only exception was represented by the DLPC/DMPC system, in which $\Delta A/10$ min was found to be higher than 0.05 when the amount of DLPC was higher than 10% of the total lipids. The rate of absorbance increase showed a maximum in the temperature range of the gel to liquid-crystalline phase transition temperature of the lipids (not reported). Hauser and Barratt [32] concluded that short chain phosphatidylcholines, such as DLPC, form short-lived SUV which have unstable bilayers and which are not effective cation barrier. In our case, the different behaviour between the DLPC/DMPC and the other mixtures is probably due to a membrane instability of the former mixture.

Size increase of vesicles composed of two phosphatidylcholines partially miscible in the gel state

(a) Temperature-dependent rate of absorbance increase. Saturated phosphatidylcholines having a difference of four carbon atoms in the acyl chain length mix non-ideally in gel state membranes [26–31,33,34]. Also the mixture composed of DPPC and the *trans*-unsaturated DEPC mix non-ideally in the gel state (see Fig. 2B and Refs. 29 and 35). The rate of absorbance increase of DMPC/DSPC and DPPC/DEPC SUV suspension was measured at various temperatures and lipid ratios. Fig. 1 shows that the rate of absorbance increase of the various mixtures is high in a temperature range which mainly overlaps, as will be shown in the Fig. 2, the range of temperatures of the lipid phase transition.

Figs. 2A and B show the DMPC/DSPC and DPPC/DEPC SUV phase diagrams and the range of temperatures at which the vesicles size increase process takes place (vertical solidus bars). The open circles indicate the temperature at which the maximum rate of absorbance increase occurs.

(b) Lipid mixing and average diameter increase. In order to ascertain if the absorbance changes detected a vesicle size increase and not an aggregation process, we studied the lipid mixing and analyzed the electron micrographs of negatively

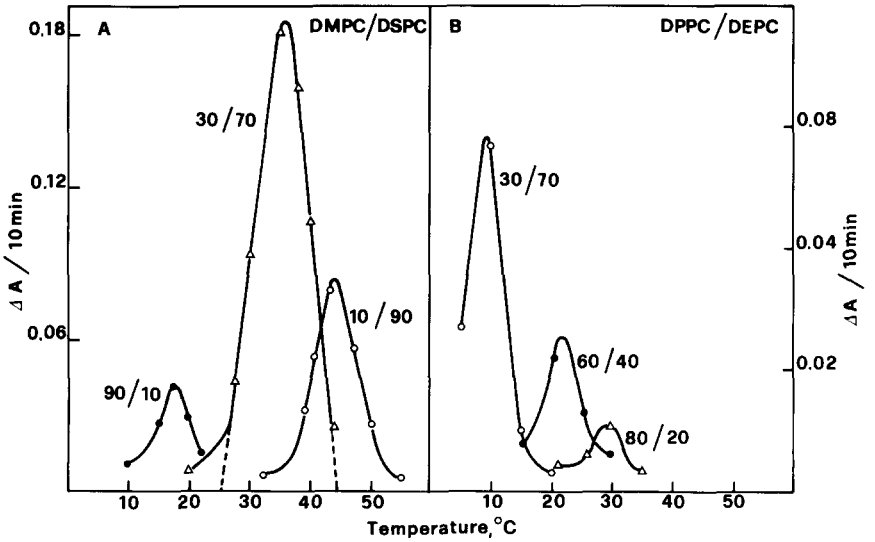

Fig. 1. Rate of absorbance increase of a suspension of SUV composed of DMPC/DSPC, or DPPC/DEPC. The lipid concentration was 1 mM (in A), and 0.5 mM (in B). The ratio of the two lipid components is indicated.

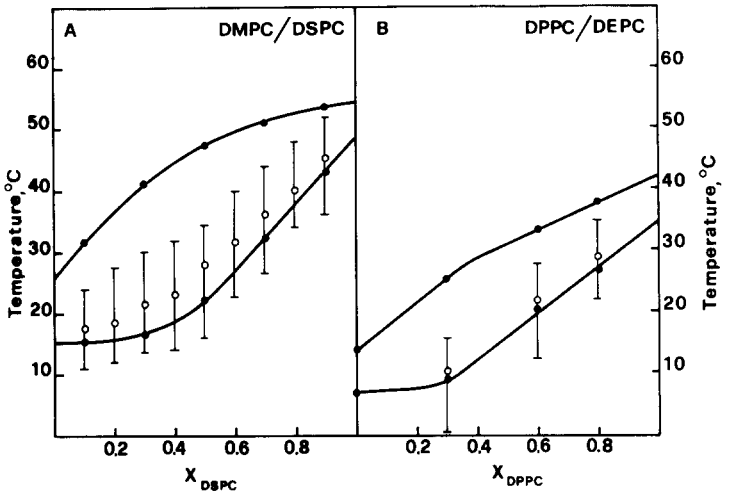

Fig. 2. Phase diagrams of DMPC/DSPC and DPPC/DEPC SUV and temperature ranges in which the vesicle size increase process takes place. The phase diagram was obtained from measurements of fluorescence anisotropy of the probe diphenylhexatriene, as described in Methods. The vertical bars represent the range of temperatures at which the vesicle size increase process takes place. The range of temperatures was estimated from the intercepts on the abscissa of the tangents to the curve of the rate of absorbance increase, dashes of Fig. 1. The open circles indicate the temperatures at which the maximum rate of absorbance increase occurs.

stained vesicles. Fig. 3A shows that DMPC/DSPC SUV in the 50/50 ratio exhibit a temperature-dependent lipid mixing which is parallel to the absorbance increase. The lipid mixing was monitored by the fluorescence intensity increase of the N-NBD-PE probe, which is due to the decrease in energy transfer that is produced by the dilution of N-NBD-PE and N-Rh-PE when membranes containing the two probes fuse with membranes devoid of them.

The average diameter, $\bar{d}$, of freshly sonicated DMPC/DSPC SUV in the 50/50 ratio, measured by electron micrographs was 25 nm. When incubated 24 h at 28°C, $\bar{d}$ increases to 125 nm; after 8 days $\bar{d}$ was approx. 450 nm. Fig. 3B shows the $\bar{d}$ value for this mixture incubated for 2 h at various temperatures: the $\bar{d}$ maximum occurs at the same temperature at which the rate of absorbance increase is maximum. As the interior of the vesicles is not usually stained, it was not possible to reliably ascertain the number of lamellae. However, unilamellar vesicles were sometimes unequivocally observed, and neither oligo- nor multilamellar structures were seen. Also unilamellar vesicles having a diameter larger than that of SUV initially show a significant size increase. Fig. 4 shows the relative frequency, measured by electron micrographs, of the diameters of DMPC/DSPC vesicles in the 50/50 ratio, prepared by passing the multilayered vesicle suspension through a French pressure cell. The vesicle size distribution shows a narrow peak centered at about 35 nm. When the vesicles were incubated for 24 h at 28°C, the peak shifted to about 120 nm. This value is approximately the same as that obtained with sonicated vesicles having the same composition after 24 h of incubation at the same temperature.

(c) Mixing of the internal aqueous compartments and carboxyfluorescein release. If the size increase process originates from the fusion between the vesicles, their internal content should mix. The Tb^{3+}-dipicolinic acid assay, performed on the DMPC/DSPC mixture, showed however no mixing at the temperature where the maximum of absorbance increase occurred. Both Tb^{3+} and dipicolinic acid were released into the suspension medium. To further analyze if the release of the markers contained in the internal aqueous space of the vesicles was due to a high membrane permeability, or to a break of the vesicles during the size increase process, we performed experiments

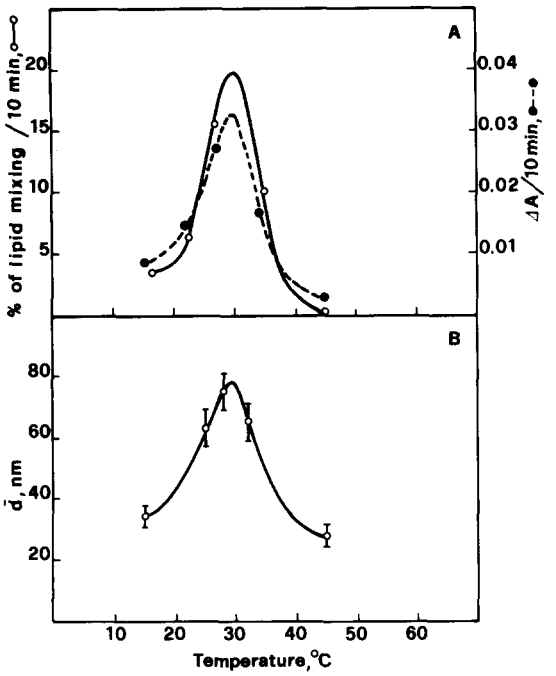

Fig. 3. Temperature-dependent absorbance increase, lipid mixing, and increase of the average diameter of DMPC/DSPC SUV in the 50/50 ratio. (A) The percentage of lipid mixing was obtained by mixing 40 μM of the SUV containing N-NBD-PE and N-Rh-PE with 160 μM of SUV devoid of the probes, and by measuring the fluorescence intensity increase as described in Methods. In the experiments of absorbance increase, the suspension consisted of 50 μM of the SUV containing the probes and 0.2 mM of the SUV devoided of the probes. (B) In the average diameter experiments, 0.5 mM SUV were incubated at various temperatures for 2 h. The bars represent the maximum error in the diameter measurements from the electron micrographs.

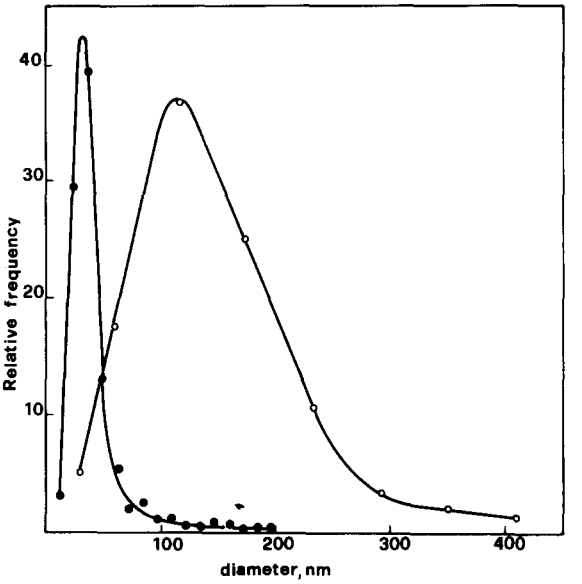

Fig. 4. Vesicle size distribution from micrographs of negatively stained DMPC/DSPC vesicles in the 50/50 ratio, prepared by passage through a French pressure cell. ●, 1 mM of lipids. ○, the vesicles were incubated for 24 h at 28°C.

carboxyfluorescein release from the vesicles. Fig. 5 shows experiments on the time dependent release of carboxyfluorescein entrapped in the DMPC/DSPC SUV in the 40/60 ratio, and the corresponding light scattering change. The experiments were performed at alkaline pH, where the permeability of the membrane to the dye is diminished consequent to the charging of the dye molecule [36]. The figure shows that the curve of the percentage of carboxyfluorescein release has two maxima, one at 32°C (temperature at which there is the maximal rate of light scattering increase), and the other at 37°C. The percentage of the dye release, at 37°C, is independent of the lipid concentration, and seems to be a diffusion process. In fact, when the dye release occurs by a diffusion process, the rate depends linearly on the lipid

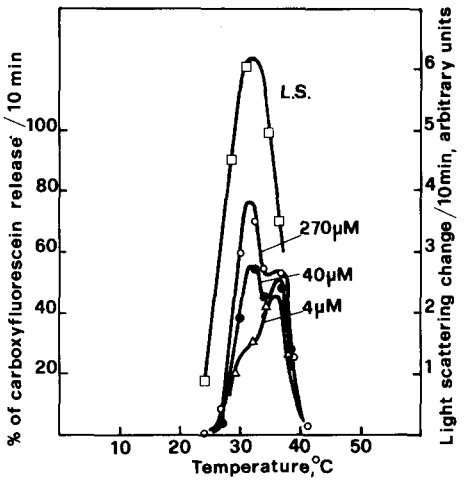

Fig. 5. Carboxyfluorescein release and light scattering change of DMPC/DSPC SUV in the 40/60 ratio. The pH of the sample was 9. The lipid concentration is indicated. The light scattering measurements (L.S.) were performed in the Perkin-Elmer spectrofluorimeter with excitation and emission wavelengths at 580 nm. The sample contained 0.27 mM of lipids.

concentration, and the percentage of release is independent of it. The percentage of the dye release, at 32°C, on the contrary, is strongly dependent on the lipid concentration. At this temperature therefore the release process depends on vesicle-vesicle contact. In conclusion it seems that, after aggregation or collision or immediately after fusion, the vesicle membrane breaks.

(d) Effect of foreign lipid molecules. The incorporation of foreign lipid molecules into the DMPC/DSPC bilayer does not modify the shape of the curve of the rate of absorbance increase versus the temperature. However the maximum rate is dependent on the concentration of the lipid molecules, as shown in Fig. 6. The rate of vesicle size increase is inhibited by DPPC and by low concentrations of lysophosphatidylcholines, and it is stimulated by stearic acid and high concentrations of lysophosphatidylcholines. The carboxyfluorescein release, measured in DMPC/DSPC SUV in the 40/60 ratio containing 2% DPPC, at alkaline pH, was inhibited. The inhibition was about 25% when measured at 32°C, and about 15% when measured at 37°C.

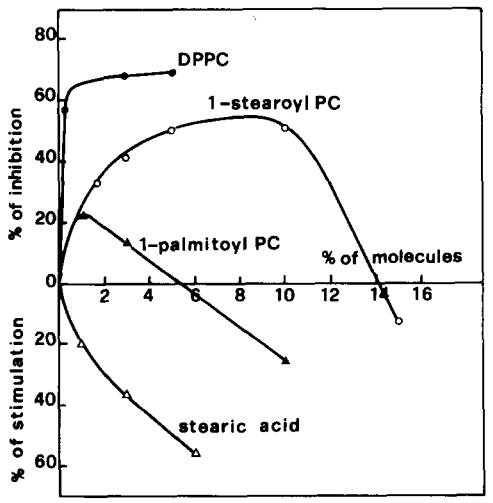

Fig. 6. Effect of foreign lipid molecules on the rate of size increase of DMPC/DSPC SUV in the 30/70 ratio. The percentage of inhibition or stimulation of the rate of absorbance increase was calculated at the temperature at which the maximum rate occurs. DPPC, lysophosphatidylcholines and stearic acid were incorporated in the membrane by sonicating them together with the phospholipids. When stearic acid was used, the sonication and the experiments were performed at pH 5, where the fatty acid in the membrane is not negatively charged.

Size increase of vesicles composed of two phosphatidylcholines immiscible in the gel state

(a) Temperature-dependent rate of absorbance increase. Saturated phosphatidylcholines having a difference of six carbon atoms in the acyl chain length are immiscible when the membrane is in the gel state (see Fig. 9 and Refs. 23, 27 and 33). The introduction of *cis*-double bonds into the fatty acid chains of saturated phosphatidylcholines produces immiscibility of the lipids in the gel state, even when the acyl chain length of the lipid components is comparable [28,37,38]. Also mixtures of DSPC and the *trans*-unsaturated DEPC are largely immiscible in the gel state (see Fig. 9). We measured the rate of absorbance increase of a SUV suspension composed of (1) two saturated phosphatidylcholines differing six carbon atoms in the acyl chain length (DLPC/DSPC and DMPC/DAPC), (2) one saturated (DPPC, DSPC and DAPC) and one *cis*-unsaturated phosphatidylcholine (DOPC, DLnPC and egg yolk PC) and (3) DSPC/DEPC. Figs. 7 and 8 show the temperature-dependent rate of absorbance increase of the SUV composed of DLPC/DSPC, DMPC/DAPC, DSPC/DEPC and of saturated and *cis*-unsaturated phosphatidylcholines: more than one maximum is observed in all mixtures. In mixtures containing egg yolk PC, the maximum rate occurs with very low amounts of egg yolk PC.

(b) Phase diagrams. Figs. 9A and B show the phase diagram of DMPC/DAPC and DSPC/DEPC mixtures. In the DMPC/DAPC mixture, two distinct phase transitions are evident. Immiscibility of the two lipids and large deviations from the miscibility are demonstrated for the first and the second mixture, respectively, in the gel state. In both diagrams, the vertical solidus bars represent the range of temperatures at which there is an increase in vesicle size, and the open circles indicate the temperatures at which there is a maximal rate of absorbance increase. The size increase begins at temperatures between the temperatures of the onset and the completion of the phase transition and extends widely to the gel phase.

The lipid mixtures composed of saturated and *cis*-unsaturated phosphatidylcholines, in the temperature interval between 10°C and 70°C, showed only one phase transition, probably due to the transition of the saturated phosphatidylcholine.

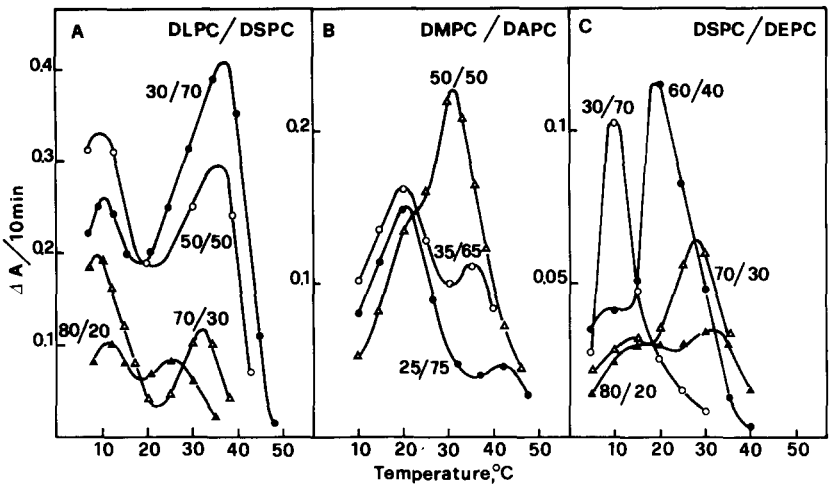

Fig. 7. Rate of absorbance increase of a suspension of SUV composed of DLPC/DSPC, or DMPC/DAPC or DSPC/DEPC. The lipid concentration was 1 mM (in A and B), and 0.5 mM (in C). The ratio of the two lipid components is indicated in the figure.

Calorimetric measurements of the DPPC/DOPC mixture, performed by Phillips et al. [37], showed two phase transitions, the first in the same temperature interval of our experiments, and the second at temperatures below 0°C. These two transitions have been attributed to the DPPC and DOPC phase transition, respectively [37].

(c) Average diameter increase, lipid mixing, and carboxyfluorescein release. The $\bar{d}$ value of freshly sonicated DMPC/DAPC SUV in the 36/65 ratio, measured by means of electron micrographs, was about 42 nm. Large sheets of a few μm in length, together with large vesicles, appeared in the electron micrographs when these SUV were incubated for 2 h at 20°C. The sheets appeared to have textured surfaces. The average diameter of freshly sonicated DSPC/DEPC SUV, in the 60/40 ratio, was 24 nm. After incubation of these vesicles for 24 h at various temperatures, $\bar{d}$ was maximum at 20°C (75 nm), the same temperature at which the absorbance increase is maximum. The average diameter of freshly sonicated DAPC/DOPC SUV, in the 80/20 ratio, was 38 nm. The temperature dependent increase of the average diameter of these SUV, incubated for one hour at the various temperatures, as well as the lipid mixing and the carboxyfluorescein release were measured (experiments not reported). The similar shape of the curves of the measured parameters confirmed that also in this system a vesicle size increase and a lipid mixing were associated to an absorbance increase. In DSPC/DEPC SUV in the 70/30 ratio, the temperature at which the release of carboxyfluorescein is maximum coincides with that at which the absorbance increase is maximum. On the contrary, carboxyfluorescein-loaded DMPC/DAPC vesicles, in the 30/70 ratio, lost their internal content during the elution through the Sephadex G-75 column at 4°C and pH 9. Their failure to maintain a solute gradient, even at temperatures at which the bilayer is in the gel state, may be explained by the permeability properties of binary lipid mixtures in the gel state. In our laboratory [39], it was indeed found that the permeability of the membrane in the gel state increases as the immiscibility of the two saturated phosphatidylcholine components is enhanced.

(d) Effect of foreign lipid molecules. Low amounts of DSPC, in DMPC/DAPC bilayers in the 35/65 ratio, inhibited the rate of absorbance increase at all temperatures. At 25°C, 2% DSPC reduced the rate of absorbance increase by 30%, and 4% DSPC reduced the rate by 60%. The insertion of low amounts of DMPC in DSPC/DEPC SUV in the 50/50 ratio inhibited the rate of absorbance increase at all temperatures. The inhibition was about 30% with 3% DMPC.

Peculiar behaviour is shown when low amounts

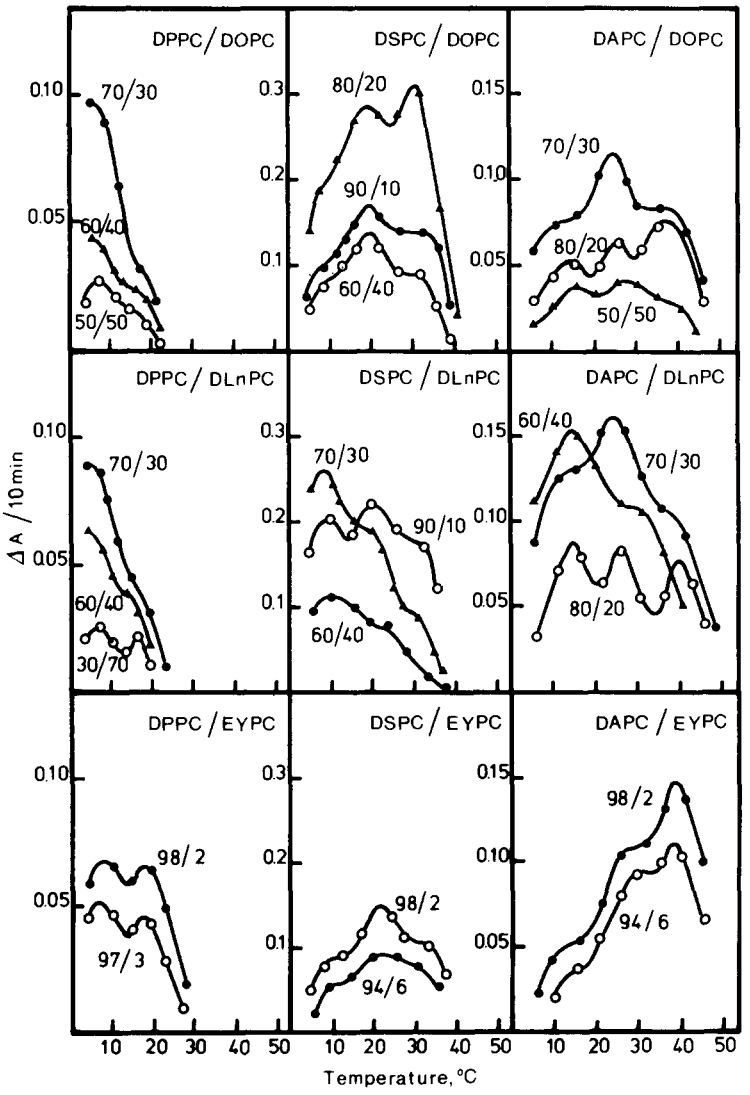

Fig. 8. Rate of absorbance increase of a suspension of SUV composed of saturated and *cis*-unsaturated phosphatidylcholines.. The lipid concentration was 0.5 mM for all lipid mixtures, except for the saturated/egg yolk PC mixture, which had a lipid concentration of 1 mM. The lipid composition and the lipid ratios are indicated in the figure. Each point of the experimental curves represents the average of two or three values obtained with different preparations.

of DMPC, DPPC or DSPC are incorporated into the bilayer of DAPC/DOPC SUV in the 80/20 ratio: the three maxima of the rate of absorbance increase are shifted towards lower temperatures. Fig. 10 shows that the temperature shift of the second maximum is larger both for increasing lipid concentrations and for the shorter chain length lipid. Furthermore, the rate of absorbance increase is enhanced at all temperatures when DSPC is added, but it is not influenced by the addition of DPPC or DMPC (see Fig. 8, where the rate of absorbance increase of the DSPC/DOPC mixture is faster than that of the DAPC/DOPC mixture, while the rate of the DPPC/DOPC and DAPC/DOPC mixtures are almost the same, when identical molar fractions of DOPC are compared). It seems therefore that the added lipid gradually transforms the behaviour of the DAPC/

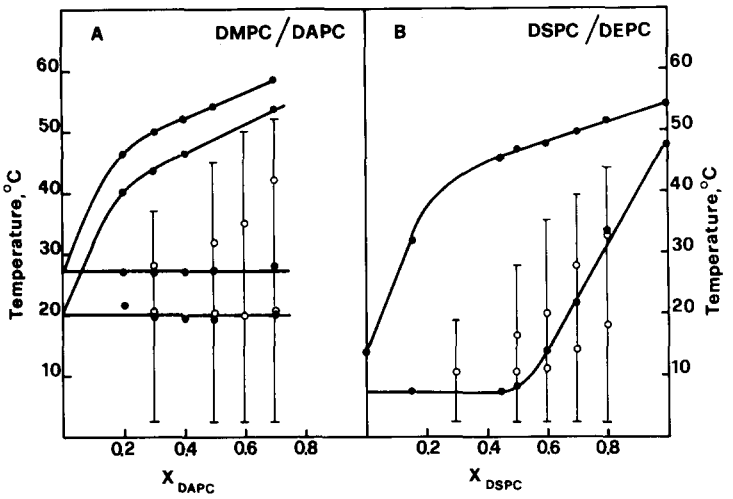

Fig. 9. Phase diagrams of DMPC/DAPC and DSPC/DEPC SUV and temperature ranges in which the vesicle size increase process takes place. The phase diagrams and the temperature intervals in which the vesicle size increase process takes place were obtained as described in the legend of Fig. 2.

DOPC mixture into that of a mixture where DAPC is extensively replaced by the lipid added.

Summary of the vesicle size increase process concerning binary lipid mixtures

The different behaviour of the various lipid mixtures may be analyzed on the basis of the differences between the phase transition temperatures of the pure lipid components, ΔT_c. As shown in Table I, SUV composed of two phosphatidylcholines, whose phase transition temperatures differ by more than 40 K, have a very poor

TABLE I

COMPARISON BETWEEN THE LIPID MISCIBILITY AND THE PARAMETERS CHARACTERIZING THE VESICLE SIZE INCREASE PROCESS FOR ALL THE MIXTURES TESTED (RATE, NUMBER OF MAXIMA AND EXTENSION TO THE GEL PHASE)

The rate of vesicle size increase is referred to as: fast when $\Delta A/10$ min is > 0.3, and slow when it is < 0.2. These values are taken at the temperature and at the lipid molar fraction at which the rate is maximal. ΔT_{gel}, the temperature interval at which the vesicle size increase process takes place when the membranes are in the gel state, was calculated from the phase diagrams as that part of the vertical line (at a molar fraction of 0.6) starting from the completion temperature of the phase transition and elongating to the gel phase. PC_{sat} and PC_{cis} indicate saturated and *cis*-unsaturated phosphatidylcholines, respectively.

	Lipid mixtures	Lipid miscibility in gel state bilayers	Rate of vesicle size increase	Number of maxima	ΔT_{gel} (K)
$\Delta T_c \geqslant 40$ K	PC_{sat}/PC_{cis}	Immiscible	fast-slow	more than one	–
	DMPC/DAPC	Immiscible	fast	more than one	18
	DLPC/DSPC	Immiscible	fast	more than one	–
	DSPC/DEPC	Immiscible (partial)	fast	more than one	24
$\Delta T_c \cong 30$ K	DMPC/DSPC	Partial miscible	slow	1	5
	DPPC/DEPC	Partial miscible	slow	1	8
$\Delta T_c \leqslant 20$ K	DMPC/DPPC	Miscible	zero	0	0
	DPPC/DSPC	Miscible	zero	0	0
	DSPC/DAPC	Miscible	zero	0	0

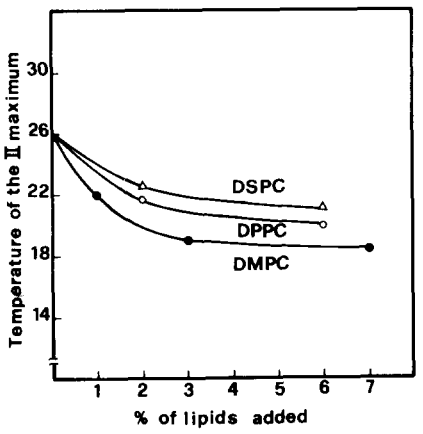

Fig. 10. Effect of foreign lipid molecules on the process of size increase of DAPC/DOPC SUV in the 80/20 ratio. The phospholipids were incorporated in the membrane by cosonication.

miscibility of the two lipids in the gel state; here the vesicle size increase process is very fast, it extends widely in the gel phase and its dependence on the temperature has more than one maximum. SUV composed of two phosphatidylcholines whose phase transition temperatures differ by approx. 30 K, show a partial miscibility of the two lipids in the gel statte; the vesicle size increase process is slower, with a reduced extension in the gel phase, and its dependence on the temperature has only one maximum. Finally, SUV composed of phosphatidylcholines whose phase transition temperature differs less than 20 K, show a complete miscibility of the two lipids; the vesicles do not increase in size.

Discussion

Electron microscopy experiments demonstrate a time-dependent vesicle size increase parallel to the time-dependent absorbance increase. During this process the internal content of the vesicles is completely released into the external medium.

The time-dependent size increase of the SUV formed by PC mixtures is correlated with the miscibility properties of the lipid components. In fact: (1) when the two phosphatidylcholines are miscible in all ratios and at all temperatures, the vesicles are stable. (2) The rate of vesicle size increase is enhanced as the lipid immiscibility in the gel state increases. (3) The vesicle size increase process takes place in a temperature range which progressively extends to the gel state just as the immiscibility of the two lipids is progressively enhanced in this state.

Within the lipid phase transition temperature range, domains of lipids in the liquid-crystalline and gel state coexist. Distinct regions of mismatch in molecular packing at the interface of these domains has been postulated by many authors [13–15,40–43]. In binary lipid mixtures in which the lecithins are poorly miscible, the lipid composition of the liquid-crystalline and the gel domains become quite different, as a consequence of the non-ideal lipid miscibility. This may lead to a more pronounced disorganization of the interface region. We therefore propose that the vesicle size increase process is due to a membrane instability consequent to the formation in the membrane of strongly disorganized interface regions, or fractures, between domains whose lipid compositions and/or fluidities are different.

The presence of more than one maximum in the temperature-dependent absorbance curves of immiscible lecithins is a complex phenomenon, whose explanation is at the present difficult. With these mixtures, the formation of large vesicles in the temperature region in which the lipids are in the gel state indicates that the presence of lipid domains in the liquid-crystalline state is not a prerequisite for membrane fusion. When the two lipids are remarkably different in the structure, they crystallize in separate domains, leading to the formation of discontinuity regions between different crystalline domains.

The effect of lipid molecules on the vesicle size increase process deserves some comments. Low concentrations of fatty acids, and lysophosphatidylcholine above a threshold concentration (approx. 12%), have been shown to stimulate the rate of size increase of vesicles composed of one saturated phosphatidylcholine [44,45]. We obtained similar results with mixed vesicles composed of saturated phosphatidylcholines. However, at lower concentrations, an inhibition of the process was observed not only with lysophosphatidylcholines, but also with foreign phospholipid molecules. Larrabee [3] found that 1% of 1,3-DSPC, incorporated in 1,2-DSPC, reduced vesicle

fusion to 30% after 10 h. This great inhibitory effect has been interpreted as due to the incorporation of the molecule into the fractures. Similarly McConnell and Schullery [46] suggested that the inhibitory effect of cholesterol on the fusion rate of DPPC SUV is due to the incorporation in and the stabilization of the bilayer lattice defect sites. In our experiments, the inhibition of the vesicle size increase and of the carboxyfluorescein-release processes by low amounts of foreign phospholipid and lysophosphatidylcholine molecules may be due to the presence of a high concentration of these molecules in the fractures, which stabilizes the lattice defect sites. Facilitated incorporation of some molecules, such as N-BD-PE and 1-palmitoylphosphatidylcholine was similarly suggested to occur within boundaries of the bilayer lattice defect sites [45,47,48]. The addition of small amount of saturated phosphatidylcholines to the DAPC/DOPC membrane gradually transforms the temperature-dependent absorbance curve of this system into that of a mixture where DAPC seems to be extensively replaced by the saturated phosphatidylcholine added. These data may be explained by supposing that the added molecules gradually replace the molecules of the saturated phosphatidylcholines that constitute the domain boundaries.

When our results, obtained with binary lipid mixtures, are compared with those obtained with a single saturated phosphatidylcholine by other authors [1–10], three major differences emerge. (1) In SUV composed of a single commercial grade saturated phosphatidylcholine, the ultimate products of the vesicle size increase process are unilamellar vesicles with diameter of about 70 and 100 nm for DPPC and DSPC, respectively. However, as reported by Gaber and Sheridan [8] and confirmed by us [19], when the lipid is first precipitated from acetone, the SUV exhibit a higher stability toward fusion. SUV, composed of two acetone-precipitated phosphatidylcholines which do not mix ideally, have on the other hand a faster rate of size increase, the final products being vesicles with diameters of over 400 nm. (2) The dimensions of DPPC and DSPC SUV, in the gel state, increase spontaneously at a rate which progressively increases as the temperature is lowered. In SUV composed of two phosphatidylcholines, the kinetics of growth is not monotonically enhanced by lowering the temperature, but have one or more maxima in the range of temperatures of the lipid phase transition. (3) Vesicles composed of a single saturated phosphatidylcholine do not increase in size when their diameter is larger than 40 nm [5]. On the other hand, vesicles composed of two saturated phosphatidylcholines which do not mix ideally still increase in size even when the initial diameter is in the order of 40 nm. These considerations indicate that a different mechanism must be involved in the two systems. The size increase process of SUV composed of a single phosphatidylcholine presumably depends upon the packing strains and defects induced when the lipids of vesicles, having a very small radius of curvature, crystallize [6]. On the other hand, the size increase process of SUV composed of two non-ideally miscible phosphatidylcholines does not depend only by the small radius of curvature of the vesicles, since the process occurs with similar rate also for larger vesicles. The dependence of the rate of vesicle size increase on the formation of lipid domains in the membrane indicates that the presence of these domains are a prerequisite for the initiation of the process.

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