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Chemically Induced Phase Separation in Mixed Vesicles Containing Phosphatidic Acid. An Optical Study

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Abstract: The possibility of chemically induced phase separation in lipid lamellae containing charged lipids is demonstrated. In mixed membranes of synthetic dipalmitoyllecithin (DPL) and dipalmitoylphosphatidic acid (DPA), a lipid domain structure may be triggered both by the addition of Ca^{2+} and of polylysine in random coil configuration. A new optical (excimer forming) probe, pyrenedecanoic acid, is introduced. The rate of excited complex formation is the crucial physical parameter yielding quantitative information on both the coefficient of lateral diffusion and on the lipid segregation. At all pH values bivalent ions bind strongly to DPA or mixed DPA–DPL lamellae. The number of ions bound per lipid molecule is one at pH 9 and about one-half at pH 5–7. The Ca^{2+} bound phosphatidic acid segregates into regions characterized by a rigid (or crystalline) structure. A quantitative analysis of the experiments shows that at pH 9 the number of DPA molecules segregated is about equal to the number of Ca^{2+} ions. At pH 9 polylysine attaches strongly to DPA membranes and triggers the formation of lipid domains that differ in their microviscosity from the rest of the membrane. At this pH the transition temperature T_t of the lysine-bound DPA is shifted from $T_t = 47°$ to $T_t = 61°$, corresponding to a shift from pH 9 to 2. Strong evidence is provided that the polylysine assumes such a (random coil) configuration that the charged lysine groups point toward one direction and that one lysine group may bind one DPA molecule. The coefficient of lateral diffusion of pyrenedecanoic acid in DPA at pH 9 and at 60° has been determined as $D_L = 1.7 \times 10^{-7}$ cm²/sec. The corresponding value for DPL at 60° is $D_L = 0.8 \times 10^{-7}$ cm²/sec.

Many properties of biological membranes may be understood on the basis of a two-dimensional fluid model. Direct evidence for this model was provided by the detection of the rapid lateral mobility of lipoids[2a] and phospholipids[2b] in lipid lamellae and of surface antigens in cells or cell hybrids.[3] The fluid membrane model also accounts for the observation of a rapid rotational mobility of membrane-bound proteins.[4] Both the rotational and lateral mobility of membrane-bound macromolecules may be directly related to the lipid lateral diffusion coefficients.[5,6] Ample evidence has

been provided that a fast mobility of the membrane constituents is essential for biological growth and transport processes (cf. ref 5, 7, and 8).

At first sight the fluid membrane model involves a complete and rapid randomization of the lipid and membrane-bound proteins. However, one expects that as in three-dimensional multicomponent systems the complete randomization of the lipids and protein molecules may be overcome by the tendency for phase separation. A lateral phase separation (that is a domain formation in multicomponent lipid lamellae) is expected at low temperatures where one lipid component undergoes a phase transition from the liquid crystalline to the crystalline state. In fact, a thermally induced lipid segregation in binary phospholipid lamellae had been detected several years ago by calorimetry.[9] More recently McConnell and coworkers reported ample evidence for thermally induced phase separation in both artificial and biological membranes.[10,11] Previously, a thermally induced domain structure was also observed in lipid lamellae containing steroids or other lipoids.[12] In the rigid state the lipoids form clusters of closely packed molecules that are embedded in the lipid matrix. More recently several groups[13-15] reported a lateral phase separation (or lipid segregation) in the neighborhood of membrane-bound proteins (e.g., cytochrome P 450 reductase,[13] cytochrome oxidase[14]) in biological systems well above the lipid phase transition.

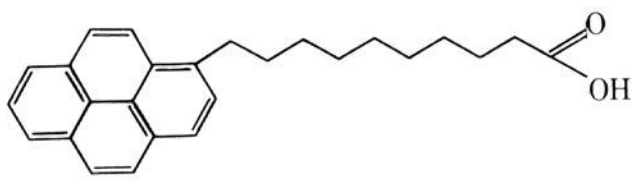
The primary purpose of the present work is the demonstration of chemically (that is, isothermically) induced lipid segregation in vesicles containing charged phospholipids. Mixed model membranes (vesicles) of dipalmitoylphosphatidic acid (DPA) and dipalmitoyllecithin (DPL) were studied. It is shown that domain formation may be triggered both by the addition of Ca^{2+} and polylysine (in random coil configuration). In fact, Ca^{2+} induced phase separation in lipid lamellae containing natural phosphatidic acid that were absorbed to millipore filters was reported very recently by Ito and Ohnishi (cf ref 16).

A pyrene-substituted fatty acid was used as optical probe for the detection of the lipid segregation. The rate of formation of optically excited complexes is used as a structural sensitive probe. A simple model allows an estimation of the relative amount of segregated lipid from the rate of excimer formation. Our spin label study of the DPA–DPL system will be presented in a forthcoming paper.

Experimental Section

Materials. Synthetic dipalmitoylphosphatidic acid (DPA) from Serdary (London, Ontario, Canada) and synthetic DL-dipalmitoylphosphatidylcholine from Fluka were used without further purification. Polylysine that was purchased from Miles Biochemicals had a molecular weight of about 33,000.

The optical label, a pyrene-substituted fatty acid [pyrenedecanoic acid, (I)], was a gift from H.-G. Scholz and Dr. W. Kühnle of the Max-Planck-Institute in Göttingen.



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Buffers as described in ref 17 were used: (a) pH 10.5, mixture of 47.2 ml of 0.1 *M* NaOH, 52.8 ml of 0.2 *M* Na_3BO_3; ionic strength 0.153; (b) pH 9, mixture of 81.6 ml of 0.05 *M* $Na_2B_4O_7$ and of 18.4 ml of 0.2 *M* H_3BO_3, containing also 0.05 *M* NaCl; (c) pH 7.8, same as in (b) but only 20.6 ml of 0.05 *M* $Na_2B_4O_7$ in 100 ml of buffer; (d) pH 5, acetate buffer (70.5 ml of 1 *M* NaOH, 100 ml of 1 *M* acetic acid, and 329.5 ml of H_2O) with ionic strength 0.14 *M*; (e) pH 2 and pH 3, mixture of KCl and HCl of ionic strength 0.1 *M*.

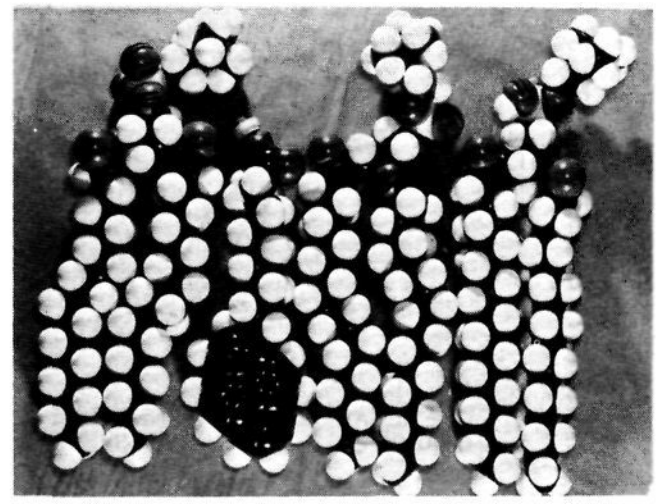


Figure 1. Pauling–Corey atomic model of pyrenedecanoic acid (I) incorporated between lipid molecules.

Vesicle Preparation. All experiments were performed with vesicles prepared by cosonication of the lipid components (DPA and DPL) together with the label in the presence of either polylysine or Ca^{2+}. The label concentration was varied between 1 and 4 mol % with respect to the lipid content. The vesicles were prepared as follows. First a thin film of DPA and label was deposited on the wall of a 20-ml glass flask under an atmosphere of purified, oxygen-free nitrogen. After addition of an appropriate amount of (oxygen-free) buffer containing either the Ca^{2+} ions (in the form of $CaCl_2$) or the polylysine, the dispersion was rinsed for about 10 min with oxygen-free nitrogen at about 50°. Thereafter, the sample was sonicated under a nitrogen atmosphere for about 10 min at a temperature above the DPA phase transition until a clear vesicle preparation was obtained. The final lipid concentration was 1.33×10^{-3} *M*.

Addition of Ca^{2+} to pure DPA vesicles caused coagulation if the Ca^{2+} concentration was about equal to the DPA concentration. Vesicles containing more than 5 mol % DPL were considerably more stable even at a high Ca^{2+} concentration. The preparations were more turbid in the presence of polylysine than in the absence of the polypeptide. However, the addition of polylysine did not lead to coagulation within several hours after preparation.

The fluorescence spectra were taken with a Baird atomic fluorescence spectrometer as described previously. The fluorescence lifetime was measured with a laser flash spectrometer.

The Excimer Probe

Aromatic molecules that form complexes in the excited state are very sensitive both against changes in the microviscosity and against cluster formation. Figure 1 shows that the pyrenedecanoic acid fits nicely in a monolayer of lipids containing palmitin chains.

The crucial physical parameter in the application of excimer probes is the rate of formation of (short-lived) excited sandwich complexes according to

$$P^* + P \overset{k_a \cdot c}{\rightleftharpoons} (PP)^*$$

The formation of excimers (PP)* is indicated by the appearance of a broad fluorescence band (maximum intensity I' at wavelength 470 nm) which is well separated from the wavelength region where the monomer P emits (maximum intensity I at wavelength 385 nm).

The principles of application of excimer-forming molecules as structural sensitive probes may be summarized as follows (cf. ref 18).

1. The ratio of maximum fluorescence intensities I'/I is related to the second-order rate constant k_a of complex formation according to[19]

$$I'/I = \kappa \frac{k_f}{k_f'} \tau_0' k_a \cdot c \qquad (1)$$

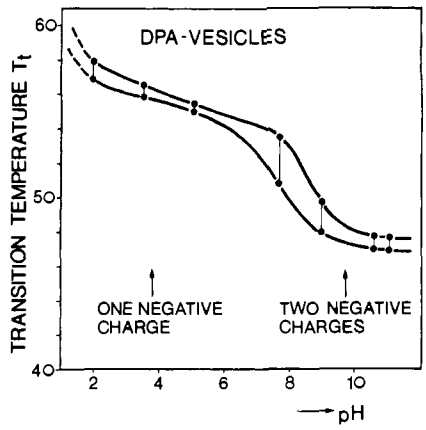


Figure 2. pH dependence of the transition temperature for DPA vesicles. The upper and the lower points (●) denote the transition temperature obtained upon increasing and decreasing the temperature.

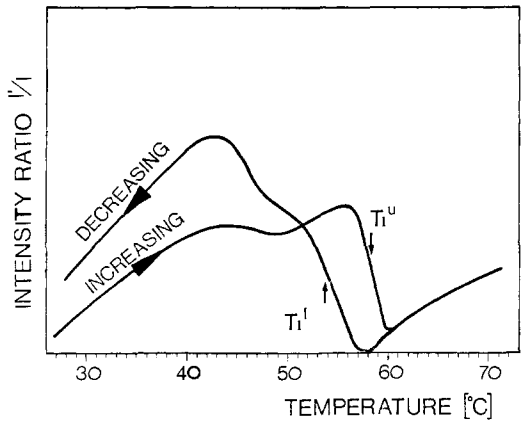


Figure 3. Transition curve of DPA vesicles at pH 7.8 observed at increasing and decreasing temperatures.

κ is a correction factor (cf. ref 18) and has a value[20] of κ = 0.62 for the pyrenedecanoic acid (I). The ratio of the transition probabilities, k_f/k_f', for the radiative decay is k_f/k_f' = 0.14.[20] This ratio is a characteristic constant of the label and is not influenced appreciably by the nature of the solvent. For membranes, the concentration of the label is measured in molecules/cm^2. The dimension of k_a is cm^2 molecules^{-1} sec^{-1}. τ_0' is the total lifetime of the excimer.

2. In fluid membranes the complex formation is a diffusion-controlled process. The rate of complex formation $k_a \cdot c$ is equal to the number, ν_c, of label collisions per second.[22] Therefore, the lateral diffusion coefficient, D_L, of the label may be determined from the intensity ratio I'/I according to[18]

$$D_L = \frac{\lambda k_a}{4d_c} = \frac{I'}{\kappa I} \cdot \frac{k_f}{k_f'} \cdot \frac{\lambda}{4d_c \cdot \tau_0' \cdot c} \qquad (2)$$

d_c is the critical interaction distance and λ is the length of one diffusional jump.

3. The solubility of the eximer probe in crystalline lipid lamellae is of the order of 0.1 mol % (cf. ref 18). If the label concentration exceeds this limiting value clusters of closely packed label molecules are formed that are embedded in the rigid lipid matrix.

4. A distinction may be made whether the excimer formation is determined by the lateral diffusion or by the cluster formation. In the first case, the intensity ratio I'/I increases linearly with the label concentration c, while in the second case a more complicated nonlinear concentration dependence is observed.

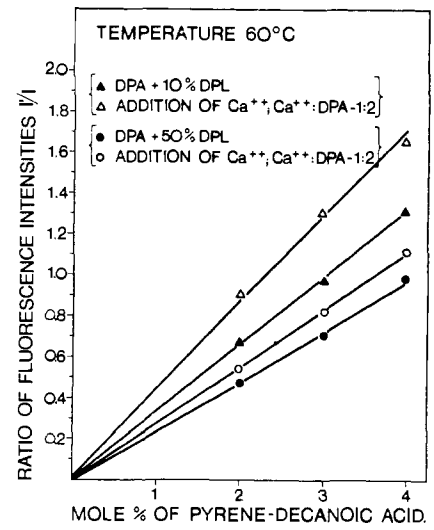


Figure 4. Concentration dependence of the fluorescence intensity ratio I'/I for two mixed DPA-DPL model membranes at 60°. △ and ▲ denote measured values for vesicles containing 10 mol % DPL and ○ and ● denote vesicles with 50 mol % DPL. Both in the absence and in the presence of Ca^{2+} the excimer formation is diffusion controlled (cf. eq 1). The coefficients of lateral diffusion obtained from the slopes of the straight lines are $D_L = 1.52 \times 10^{-7}$ cm^2/sec and $D_L = 0.99 \times 10^{-7}$ cm^2/sec for the Ca^{2+} free membrane containing 10 and 50% DPL, respectively.

5. Assume a membrane undergoes a transformation to a heterogenous structure of alternating rigid and fluid domains. It is then expected from the above principles (cf. item 4) that the label molecules will be squeezed out of the rigid domains into the fluid patches. Accordingly, the effective label concentration in the fluid membrane increases leading to a corresponding increase in I'/I. This demonstrates the applicability of excimer-forming molecules as optical probes for the investigation of domain formation in lipid lamellae.

Experimental Results

Mixed DPA-DPL vesicles were studied between 25 and 70°. Vesicles containing 5, 10, 25, 50, 75, and 100 mol % DPL were prepared. Ca^{2+} (or polylysine) was added (prior to sonication) up to concentrations corresponding to an equimolar ratio of DPA and Ca^{2+} (or DPA and lysine groups) of 1:1. The number of lysine groups was estimated from the molecular weight of polylysine used.

Transition Temperatures and pH Dependence. As shown recently by Eibl and Träuble[23] and by Verkleij et al.,[24] it is characteristic for charged phospholipids that the transition temperature depends strongly on the pH of the aqueous phase. The pH dependence of the transition temperature T_t of DPA is shown in Figure 2. The values of T_t have been determined from the temperature dependence of the fluorescence intensity ratio (cf. Figure 3). According to Figure 2, the transition temperature decreases strongly with increasing pH. A sharp decrease in T_t is observed at pH 7-8 where the second OH group in the phosphoric acid residue starts to dissociate. The sharp drop in the transition temperature at pH 7-8 is most probably caused by a decrease in the packing density of the twofold charged DPA molecules.[23]

In the pH region where single and double charged lipids are present simultaneously, the lipid phase transition is distinguished by a very strong hysteresis (cf. Figure 3). The thermally induced phase transitions observed at increasing and decreasing temperatures are separated by about 5°.

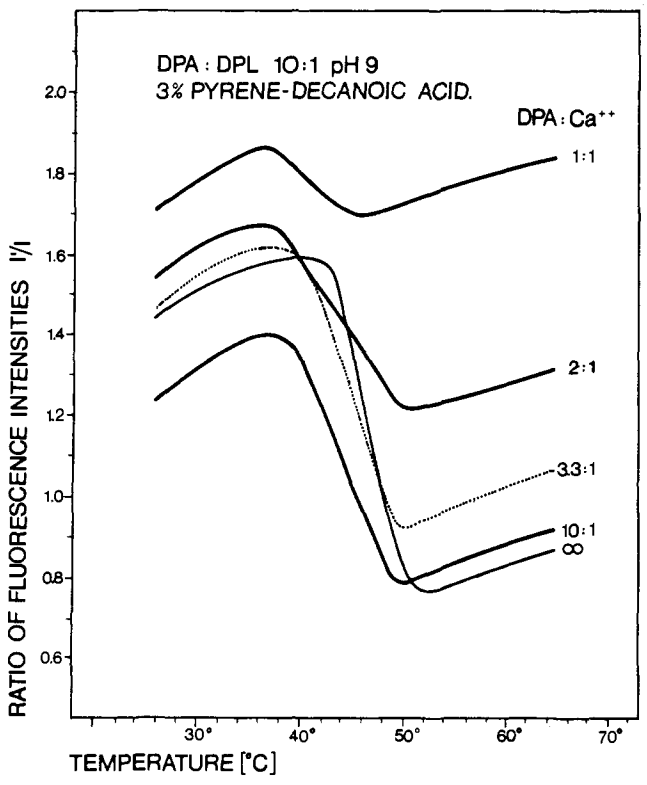


Figure 5. Effect of Ca^{2+} on the transition curves of the fluorescence intensity ratio for vesicles containing 90% DPA and 10% DPL. The curves measured at decreasing temperature are shown. At temperatures above 50° the excimer formation is diffusion controlled (see Figure 4). The transition temperatures are T_t = 42, 43, 45, 46, and 47° for DPA/Ca^{2+} molar ratios of 1:1, 2:1, 3.3:1, 10:1, and ∞.

Lateral Diffusion. First the lateral diffusion coefficient of the label (I) was determined in membranes containing different DPA/DPL molar ratios. For this purpose I'/I was measured for each membrane preparation as a function of the label concentration. Membrane preparations containing 2, 3, and 4 mol % label with respect to the total lipid were studied. Figure 4 shows two typical results at 60°, that is, at a temperature well above the phase transition of the lipid lamellae. In all cases studied I'/I increases linearly with the label concentration showing that the eximer formation in the absence of Ca^{2+} or polylysine is diffusion controlled.

In parallel experiments the excimer lifetime τ_0' was measured for each membrane preparation separately. The τ_0' value at 60° was $\tau_0' = 50 \pm 5$ nsec for all lipid compositions.

It is reasonable to assume that the values of the critical interaction distance d_c ($d_c \sim 8$ Å) and of the length of one diffusional jump, λ ($\lambda \sim 8$ Å), that have been used in the previous paper[18] for pyrene are also valid for pyrene attached to fatty acids. For the calculation of the label concentration c a value of $F \sim 60$ Å^2 for the area per lipid molecule has been assumed both for DPL and DPA. This value has been chosen since it is close to the experimental value of $F = 58$ Å^2 for DPL above the phase transition.[2a] The lateral diffusion coefficients obtained from the slopes of the straight lines using the above values of d_c, λ, and F are given in the text of Figure 4.

In Figure 4 plots of I'/I vs. label concentration are also given for the two membrane preparations after addition of Ca^{2+}. Obviously Ca^{2+} addition induces a considerable increase in the label collision rate ν_c. However, straight lines are obtained showing that even after the Ca^{2+} addition the excimer formation is still diffusion controlled.

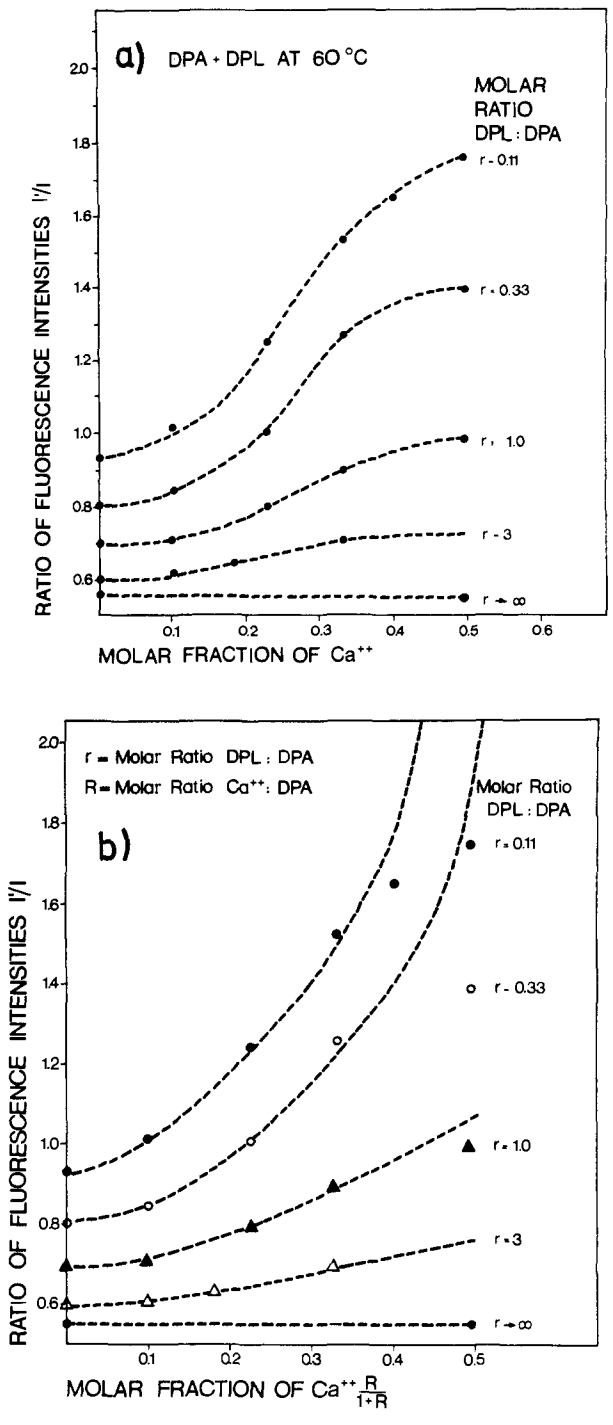


Figure 6. (a) Rate of excimer formation in mixed DPA-DPL vesicles as a function of Ca^{2+} content and of the DPA/DPL molar ratio. The ordinate gives the fluorescence intensity ratio at 60° and at pH 9. The dots (●) are the measured values. (b) Solid lines: intensity ratio calculated under the assumption that the Ca^{2+}-bound DPA forms rigid clusters in which the label solubility is negligibly small (cf. eq 5). Points (symbols ●, □, ○, and △) are experimental values taken from Figure 6a.

Ca^{2+} Induced Phase Separation. As a typical example, Figure 5 shows the effect of Ca^{2+} on the rate of eximer formation in lamellae containing 10 mol % DPL and 3 mol % label. The transition curves have been taken both at increasing and decreasing temperatures. The fluorescence spectra were recorded at temperature intervals of 2°. The abrupt change in the ratio I'/I is an indicator of the lipid-phase transition. The sharp increase in I'/I upon lowering the temperature below the lipid-phase transition is interpreted in

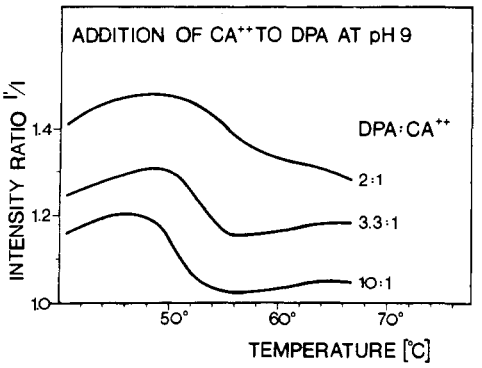


Figure 7. Effect of Ca^{2+} on transition curves of DPA vesicles. Above 50 mol % Ca^{2+} the vesicle system coagulates immediately after preparation.

terms of the formation of clusters of closely packed label molecules in the rigid state.[18] Addition of Ca^{2+} affects the transition curves in two ways. Firstly, the rate of excimer formation above the phase transition increases strongly with increasing Ca^{2+} concentration. Secondly, the lipid phase transition is shifted to lower temperatures with increasing Ca^{2+} concentration.

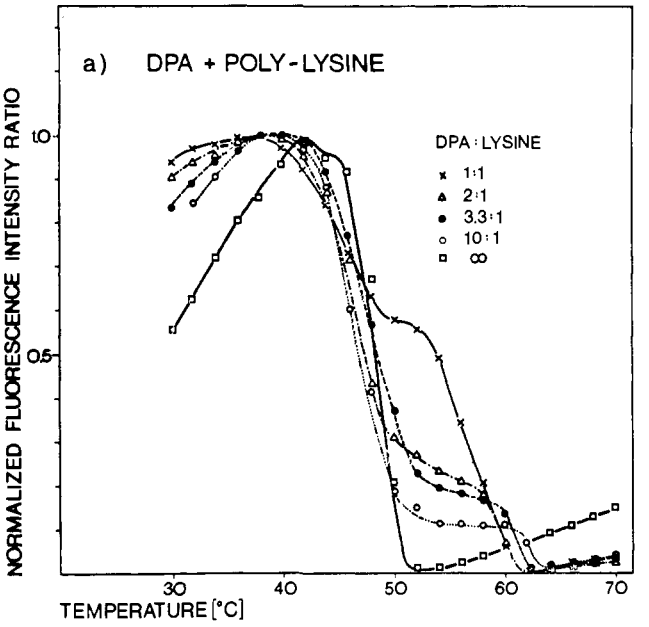
The above two Ca^{2+} induced effects have also been observed for membrane preparations containing higher DPL concentrations. By recording transition curves as shown in Figure 5, the fluorescence intensity ratio has been measured as a function of the DPL concentration of the Ca^{2+} concentration and of the label concentration. In Figure 6a the measured values of I'/I are plotted as a function of the Ca^{2+} concentration for different DPL concentrations and for a temperature (60°) well above the transition temperature of DPA ($T_t \sim 47°$) and at pH 9. In all cases studied I'/I was a linear function of the label concentration measured with respect to the total lipid content, showing that the excimer formation is diffusion controlled.

In Figure 7 the transition curves of the excimer formation rate (or I'/I) are shown for pure Ca^{2+}-doped DPA vesicles. With increasing Ca^{2+} concentration the phase transition is shifted somewhat to higher temperatures. At molar ratio of DPA/Ca^{2+} = 2:1 the transition is very broad and nearly suppressed. The transition curve for an equimolar ratio of DPA and Ca^{2+} could not be measured accurately since at this Ca^{2+} concentration the vesicle preparation coagulated immediately after sonication.

Polylysine Induced Phase Separation. Figure 8a shows the temperature dependence of the excimer formation rate (or I'/I) for pure DPA vesicles after addition of polylysine. The curves were taken at pH 9. At this pH, polylysine is expected to be in a random coil configuration and the lysine groups are protonated (that is, positively charged). In the presence of polylysine the transition curves clearly exhibit two well-separated phase transitions. A lower transition is observed at a temperature T_t^l which decreases slightly from T_t^l = 48° (in the absence of polylysine) to T_t^l = 44° at an equimolar ratio of lysine groups and DPA.

The upper transition at T_t^u is slightly shifted from T_t^u = 57° at a molar ratio (DPA)/(lysine) = 1:1 to T_t^u = 62° at a molar ratio (DPA)/(lysine) = 10:1.

Figure 8 clearly shows that a phase separation has been induced by the addition of polylysine. The lower transition temperature agrees well with the corresponding value of DPA vesicles at pH 9. The upper transition temperature corresponds to DPA lamellae at a pH of ~2.



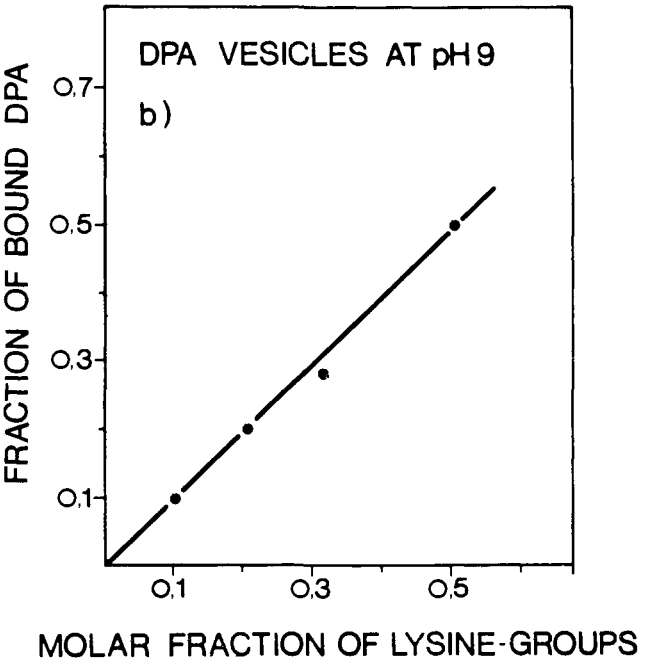


Figure 8. (a) Temperature dependence of fluorescence intensity ratio I'/I for sonicated DPA vesicles and the effect of polylysine at pH 9. The curves have been normalized in such a way that the maximum intensity ratio is I'/I = 1. In the presence of polylysine two clearly separated transitions are observed. (b) Fraction of bound phosphatidic acid plotted as a function of the molar fraction of lysine groups (with respect to the DPA). The experimental values (●) have been determined from the height of the second step in the transition curve.

Discussion

Effect of Ca^{2+}. According to Figures 4–6 addition of Ca^{2+} to mixed DPA/DPL lamellae has a dramatic effect on the rate of excimer formation, k_a, both above and below the phase transition. Figure 4 clearly shows that above the phase transition the excimer formation is diffusion controlled both in the absence and in the presence of Ca^{2+}. Obviously the optical probe is still in a fluid lipid environment after addition of Ca^{2+}. The strong increase in the excimer formation rate upon addition of Ca^{2+} must therefore be due to an increase in the effective label concentration. This finding may only be interpreted as follows. Ca^{2+} binds strongly to DPA leading to the formation of rigid clusters of the bound phosphatidic acid. The label is squeezed out from these rigid patches into the fluid lipid regions of the vesicles

and consequently the label concentration in the latter increases. This interpretation of our findings in terms of a lipid segregation is supported by the following additional observations. The transition temperature T_t is shifted from $T_t = 48°$ in the absence of Ca^{2+} to $T_t = 42°$ at an equimolar ratio of DPA and Ca^{2+}. The latter value of T_t agrees with the transition temperature of DPL. This shows that nearly all DPA is transformed into a rigid state at an equimolar ratio of DPA and Ca^{2+} and at a pH of 9. In the Appendix it is shown (at least for the case of Mn^{2+}) that at pH of 9 each bivalent ion may bind one phosphatidic acid molecule.

Provided the above interpretation is correct the flouresence intensity ratio $v = I'/I$ in the presence of Ca^{2+} may be calculated as follows. Denote by R the molar ratio of Ca^{2+} to DPA and by r the molar ratio of DPL to DPA. Then the concentration of free phosphatidic acid is $n_A{}^f = n_A{}^0(1 - R)$, where $n_A{}^0$ is the initial DPA concentration.

The experimental values of the intensity ratio $v_m{}^0 = I'/I$ for the mixed DPL/DPA vesicles in the absence of Ca^{2+} (cf. Figure 9) obey the following empirical rule

$$v_m{}^0 = (1 - x_L{}^0)v_A{}^0 + x_L{}^0 v_L{}^0 \qquad (3)$$

where $v_A{}^0$ and $v_L{}^0$ are the intensity ratios for pure DPA and DPL vesicles, respectively. According to Figure 9 $v_L{}^0 = 0.55$, $v_A{}^0 = 0.95$. $x_L{}^0$ is the molar fraction (or molar percentage) of DPL with respect to DPA in the absence of Ca^{2+} ($x_L{}^0 = r/1 + r$). Provided the free lipid is arranged in a continuous bilayer (or monolayer) the fluorescence intensity ratio, v_m, in the presence of Ca^{2+} may easily be expressed in terms of the molar ratios r and R and of the intensity ratios $v_A{}^0$ and $v_L{}^0$.

In the presence of Ca^{2+}, the molar fraction of DPL changes to $x_L = r/(1 - R + r)$. The molar ratio of optical label to free lipid, ρ, increases according to

$$\rho = \frac{1 + r}{1 - R + r}\rho_0{}^0 \qquad (4)$$

where the superscript 0 refers to the absence of Ca^{2+} and $\rho_0{}^0$ is the molar ratio of optical label to the total lipid DPL and DPA in the absence of Ca^{2+}. Equation 4 is valid for $\rho_0 \ll 1$. This condition is easily fulfilled for vesicle preparations containing at least 10 mol % DPL and not more than 3 mol % pyrenedecanoic acid. Upon addition of Ca^{2+} the intensity ratios $v_A{}^0$ and $v_L{}^0$ in eq 3 have to be replaced by $v_A = v_A{}^0\rho/\rho_0{}^0$ and $v_L = v_L{}^0\rho/\rho_0{}^0$, respectively. One therefore obtains from eq 3

$$v_m = \frac{1 + r}{(1 - R + r)^2}\{(1 - R)v_A{}^0 + rv_L{}^0\} \qquad (5)$$

The intensity ratios calculated according to eq 5 with $v_A{}^0 = 0.95$ and $v_L{}^0 = 0.55$ are plotted in Figure 6b as a function of the molar fraction, $R/1 + R$, of Ca^{2+} (with respect to the DPA content). Up to a molar fraction $R/1 + R \sim 0.33$ very good agreement between the calculated and the experimental intensity ratios is obtained. This finding supports our above conclusion (1) that the binding of Ca^{2+} to DPA induces the formation of domains or regions of rigid DPA lipid and (2) that the label is squeezed out from these patches.

The agreement between the calculated and the experimental intensity ratio becomes poor if the Ca^{2+} concentration approaches the DPA concentration. This discrepancy is most pronounced at very low DPL concentrations and is therefore most probably due to the finite label solubility in the rigid membrane regions. The exact concentration dependence of the excimer formation rate in rigid membranes is not known yet. It was therefore not possible to calculate the intensity ratios at large values of R.

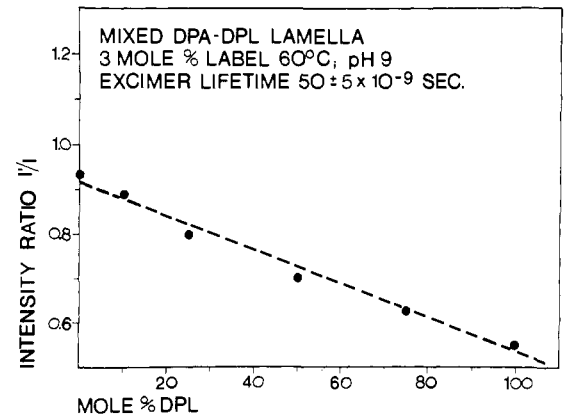


Figure 9, Intensity ratio I'/I as a function of DPL content in mixed DPA-DPL vesicles at a temperature of 60° and pH 9. The excimer lifetime τ_0' is 50 ± 5 nsec for all lipid compositions.

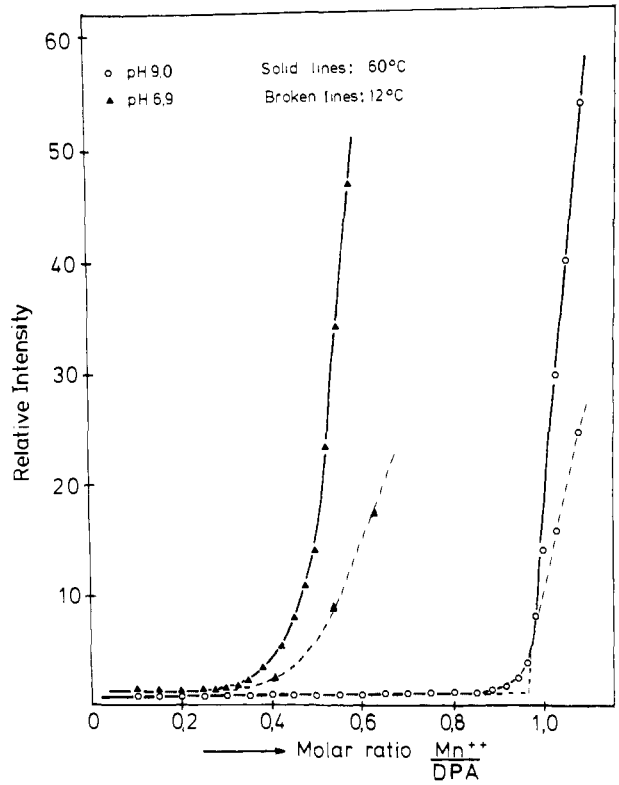


Figure 10, Binding of Mn^{2+} to DPA vesicles. In the ordinate the amplitude of the ESR signal of free Mn^{2+} (sextet spectrum) is plotted.

Effect of Polylysine. The appearance of a two-step transition curve upon addition of polylysine clearly shows that this polypeptide binds strongly to DPA lamellae. The transition temperature of DPA in contact with polylysine is shifted to $T_t{}^u \sim 60°$, corresponding to a pH of 2. Obviously the positively charged lysine groups decrease the local (effective) pH at the polar head group region of the membranes. The occurrence of a sharp phase transition at $T_t{}^u$ shows that the lipid bound to the protein forms a continuous domain of closely packed lipids. Judged from the Pauling-Corey model of polylysine, the protein may in principle assume a random coil configuration in such a way that most of the lysine groups point in one direction. In this configuration the average distance of the lysine groups is about 8 Å

and is thus close to the average distance of the lipid molecules.

The height of the high-temperature steps in the normalized transition curves of Figure 8a is an approximate measure of the relative amount of bound lipid. In Figure 8b this relative concentration is plotted as a function of the molar fraction (with respect to DPA) of lysine groups. Obviously half of the lipid is bound at an equimolar ratio of lysine groups and DPA.

Conclusions

The evaluation of the present experiments demonstrated the possibility of chemically induced lipid segregation or phase separation in membranes containing phosphatidic acid as charged lipids. Experiments by Ohnishi and Ito[25] and preliminary experiments in our laboratory show that model membranes containing neutral phosphatidylserine show similar phase separation effects upon addition of bivalent ions.

The data have been discussed on the basis of lateral phase separation alone, The vesicles have been prepared by cosonication of the lipid and Ca^{2+}. In small vesicles the possibility of unequal lipid distribution between the outer and the inner monolayer has to be considered. Some experiments were also performed with unsonicated, that is, large multilamellar, systems. Identical results were obtained. We therefore assume that lateral phase separation is the dominant process responsible for our observations in small vesicles.

Most interesting is the phase separation induced by polylysine. This shows that proteins adsorbed to the surface of lipid lamellae may have a dramatic effect on the lipid distribution. As shown recently, polypeptides with a net positive charge may affect the molecular alignment in the lipid matrix considerably.[26] From these findings it is expected that changes in the lipid matrix may trigger changes in the distribution and (or) in the conformation of the surface proteins of membranes. In fact, Kleemann and McConnell[11] provided evidence recently that the thermally induced phase separation in the lipid matrix of *Escherichia coli* auxotrophs may lead to a domain-like distribution of the proteins.

Such cooperative and simultaneous conformational changes of surface (or integral) protein and the lipid matrix may well play a role as a switching mechanism for short-term memories. The possible role of lateral phase separation to membrane permeability has been stressed recently.[27,28]

It should be emphasized that the ultimate cause for the domain formation is the shift in the transition temperature induced by the change in the charge of the polar head group or in the local pH at the lipid water interface.

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Appendix

Bivalent ions such as Ca^{2+} and Mn^{2+} bind strongly to membranes of DPA both at low and high pH. The strong binding of Mn^{2+} to DPA vesicles can easily be studied by EPR spectroscopy. If a Mn^{2+} ion is transferred from the bulk aqueous phase to the membrane surface, the symmetry of the Mn^{2+} solvation shell is distorted. Consequently, the ESR spectra become very broad. Since Mn^{2+} in the bulk phase exhibits a sharp sextet spectrum, the relative concentration of free Mn^{2+} may be easily determined. In Figure 10 the results of such an experiment are shown for DPA at pH 6.9 and 9. For pH 6.9 no ESR signal of free Mn^{2+} is observed up to a molar ratio of $R = 0.45$. At pH 9 the concentration of free Mn^{2+} is zero up to a molar ratio of $R = 0.9$. This shows that at pH 6.9 two DPA molecules can bind one Mn^{2+} while at pH 9 one Mn^{2+} ion may be bound by one lipid molecule. The very strong binding of Mn^{2+} to the lipid matrix is also shown by the finding that the Mn^{2+} concentration of bound ions is not affected considerably by temperature changes between 10 and 60°.

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