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CHEMICALLY INDUCED LIPID PHASE SEPARATION IN MODEL MEMBRANES CONTAINING CHARGED LIPIDS: A SPIN LABEL STUDY*

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Summary

The lipid distribution in binary mixed membranes containing charged and uncharged lipids and the effect of Ca^{2+} and polylysine on the lipid organization was studied by the spin label technique. Dipalmitoyl phosphatidic acid was the charged, and spin labelled dipalmitoyl lecithin was the uncharged (zwitterionic) component. The ESR spectra were analyzed in terms of the spin exchange frequency, W_{ex}. By measuring W_{ex} as a function of the molar percentage of labelled lecithin a distinction between a random and a heterogeneous lipid distribution could be made. It is established that mixed lecithin-phosphatidic acid membranes exhibit lipid segregation (or a miscibility gap) in the fluid state. Comparative experiments with bilayer and monolayer membranes strongly suggest a lateral lipid segregation. At low lecithin concentration, aggregates containing between 25 % and 40 % lecithin are formed in the fluid phosphatidic acid membrane. This phase separation in membranes containing charged lipids is understandable on the basis of the Gouy-Chapman theory of electric double layers.

In dipalmitoyl lecithin and in dimyristoyl phosphatidylethanolamine membranes the labelled lecithin is randomly distributed above the phase transition and has a coefficient of lateral diffusion of $D = 2.8 \cdot 10^{-8}$ cm^2/s at 59 °C.

Addition of Ca^{2+} dramatically increases the extent of phase separation in lecithin-phosphatidic acid membranes. This chemically (and isothermally) induced phase separation is caused by the formation of crystalline patches of the Ca^{2+}-bound phosphatidic acid. Lecithin is squeezed out from these patches of rigid lipid. The observed dependence of W_{ex} on the Ca^{2+} concentration could be interpreted quantitatively on the basis of a two-cluster model. At low lecithin and Ca^{2+} concentration clusters containing about 30 mol % lecithin are formed. At high lecithin or Ca^{2+} concentrations a second type of precipitation containing 100 % lecithin starts to form in addition. A one-to-one binding of divalent ions and phosphatidic acid at pH 9 was assumed. Such a one-to-

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one binding at pH 9 was established for the case of Mn^{2+} using ESR spectroscopy.

Polylysine leads to the same strong increase in the lecithin segregation as Ca^{2+}. The transition of the phosphatidic acid bound by the polypeptide is shifted from $T_t = 47.5°$ to $T_t = 62°C$. This finding suggests the possibility of cooperative conformational changes in the lipid matrix and in the surface proteins in biological membranes.

Introduction

Recent results in membrane research suggest that lipid bilayers and membrane bound (intrinsic) proteins form a functional entity. This postulate is sustained by the finding that removal of the natural lipid environment inactivates intrinsic proteins (cf. ref. 1). Examples of intrinsic proteins are: Cytochrome oxidase, the terminal member of the mitochondrial electron transport chain [2, 3] or the cytochrome *P*-450/450 reductase complex, the terminal member of the hydroxylation system in liver microsomal membranes [4].

It has been established by two recent spin label studies [3, 4] that intrinsic proteins may be embedded in a cluster of rigid lipid bilayers, while this protein-lipid entity swims in the fluid (or liquid crystalline) bulk membrane. The lipid structure surrounding the *P*-450/450 reductase system undergoes a conformational change from a rigid to a fluid state at a "transition" temperature $T_t = 32°C$ for rat liver microsomes [4]. This finding provides strong evidence that the lipid adjacent to the proteins differs in its molecular structure from the phospholipid forming the bulk membrane. Obviously the incorporation of intrinsic proteins in biological membranes may involve a heterogeneous lipid distribution (or lipid phase separation).

The class of the so-called "extrinsic" proteins that are associated with the membrane surface are generally thought to exhibit only a very weak and negligible lipid-protein interaction. However, experiments by Kimelberg and Papahadjopoulos [5] showed that positively charged proteins such as lysozyme and cytochromes increase the Na^+ permeability of phosphatidylserine vesicles considerably. Small changes in the lipid conformation induced by the adsorption of proteins to membrane surfaces have also been observed recently by the spin label technique [6]. A dramatic effect on the lipid distribution in binary mixed lipid bilayers containing phosphatidylcholine and phosphatidic acid is caused by adsorption of polylysine, provided the polypeptide is in its charged and therefore random coiled configuration [7]. The polylysine binds to the negatively charged phosphatidic acid which then forms rigid (crystalline) precipitations within the lipid bilayer. These experiments suggest the possibility of simultaneously and cooperatively induced conformational changes in the surface proteins and in the lipid matrix. In summarizing, the above mentioned experiments indicate that both, intrinsic and extrinsic proteins, may involve a heterogeneous lipid distribution in biological membranes. From a physical point of view two classes of conformational changes have to be considered in membranes composed of lipid mixtures:

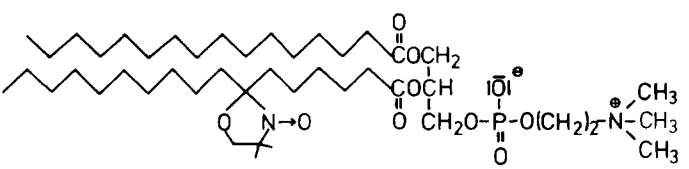
(1) The crystalline-to-liquid crystalline phase transition transforming the lipid structure from a crystalline to a highly fluid state, and (2) phase separation leading to a heterogeneous lipid distribution or domain formation.

Both types of conformational changes may be induced either thermally or chemically, for instance by altering the composition of the aqueous phase. Thermally induced phase separation in model membranes has been observed by several groups using calorimetry [8] or the spin label technique [9, 10, 11]. In model membranes containing charged lipids, such as phosphatidic acid, the lipid phase transition may be triggered chemically for instance by changes in the pH of the aqueous phase [12, 13]. The possibility of a chemically (and isothermally) induced phase separation has been demonstrated recently in model membranes of lecithin containing phosphatidic acid as charged lipid constituent [7]. In these experiments the conformational change has been triggered by the addition of Ca^{2+}.

In the present paper we performed a spin label study of these Ca^{2+} induced alterations in the lipid organization of model membranes composed of synthetic dipalmitoyl phosphatidic acid and dipalmitoyl phosphatidylcholine (lecithin). The lecithin was marked by a spin label group. Addition of Ca^{2+} causes a strong increase in the broadening of the ESR-spectra. This Ca^{2+}-induced broadening has been reported previously by Ito and Ohnishi [14, 16]. The broadened ESR-lines were analyzed in terms of the concentration dependence of the dipolar and the spin exchange interaction. It was thus possible to distinguish whether the radical interaction is caused primarily by an increase in the lipid lateral diffusion or by lipid phase separation. In addition quantitative information on the extent of phase separation could be obtained on the basis of such an analysis.

Materials and Methods

Lipids. Dipalmitoyl lecithin from Fluka and dipalmitoyl phosphatidic acid from Serdary (London, Ontario, Canada) were used without further purification. The dimyristoyl phosphatidylethanolamine was a gift of Dr Eibl of the Max-Planck-Institut in Göttingen.



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Spin-labelled lecithin (I) was used as one lipid constituent in the mixed membranes. The label was synthesized by acylation of 1-palmitoyl lysolecithin (from Serdary) according to the method described by Hubbell and Mc Connell [15]. The acylation was performed with the anhydride of stearic acid (Syva Associates, Palo Alto, Calif.) carrying a labelled oxazolidine ring at carbon atom number seven. The reaction product was purified by gel filtration on Sephadex LH-20 using an 8 : 2 chloroform/ethanol solvent.

Polylysine used in our experiments (from Miles Biochemicals) had a molecular weight of about 30 000.

The following buffers as described in ref. 16 were used:

(1) pH 9.0: 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 0.05 M NaCl.

(2) pH 5.0: acetate buffer: 70.5 ml of 1 M NaOH and 100 ml of 1 M acetic acid was diluted with water to a final volume of 500 ml.

(3) pH 3.0: to a solution of 0.1 M KCl in water, 0.1 M HCl was added until the required pH value was reached. The pH values were controlled with a Beckman pH-meter to an accuracy of 0.001 pH units.

Preparation of the vesicles

Bilayer vesicles were prepared as described earlier: the wall of an 8 ml pear-shaped flask was coated with a thin film of a mixture of phosphatidic acid and labelled lecithin in appropriate amounts. The total amount of lipid in the film was $0.9 \cdot 10^{-5}$ mol. Then 5 ml of buffer solution was added and the sample was sonicated for about 10 min at a temperature well above the lipid phase transition (about 60°C). After addition of Ca^{2+} or polylysine in appropriate amounts the vesicle dispersion was again sonicated for about 3 min. Very clear solutions were obtained showing that small vesicles were formed also in the presence of Ca^{2+} or polylysine.

Monolayer vesicles: prepared as described in refs 17 and 18. Ca^{2+} was added after the preparation.

ESR measurements

ESR spectra were taken between 20°C and 65°C using a Varian E-line spectrometer equipped with an AEG-gaussmeter. The samples were sealed in capillaries with an inner diameter of 1.1 mm. Spectra were recorded at increasing and decreasing temperatures. While recording the spectra, the temperature was monitored to an accuracy of 0.2°C.

Spectra were recorded by varying the molar ratio, r, of lecithin-to-phosphatidic acid between $r = 0$ and $r = 0.25$. The molar ratio of Ca^{2+}-to-phosphatidic acid was varied between $R = 0$ and $R = 1$.

Computer simulation of ESR-spectra

An approximate procedure for the computer simulation of nitroxide radical spectra modified by radical interaction is described in ref. 18. This procedure is based on the solution of the Bloch equations containing additional terms that account for the spin exchange. Such a simplified method can be applied only in those cases where the anisotropies of the hyperfine interaction and the electronic g-factor are largely averaged out by the rapid rotational motion of the spin label in the membrane. Such quasi-isotropic spectra may be simulated to a good approximation by the superposition of three Lorentzian lines $V_{+1}(H, \Delta H^0_{+1})$, $V_0(H, \Delta H^0_0)$; $V_{-1}(H, \Delta H^0_{-1})$, centered at the magnetic field positions $H_0 - a_H$; H_0; $H_0 + a_H$:

$$V(H) = V_{-1}(H, \Delta H^0_{-1}) + V_0(H, \Delta H^0_0) + V_{+1}(H, \Delta H^0_{+1})$$

a_H is the isotropic hyperfine splitting constant which depends somewhat on the polarity of the solvent. For our computer simulation procedure we used a value of a_H = 15 G. Fig. 1 exhibits the first derivative ESR spectrum of the lecithin label (I) in vesicles of phosphatidic acid at pH 9 and at a temperature (T = 59°C) above the phase transition of the lipid lamellae. The transition temperature of phosphatidic acid at pH 9 is T_t = 47.5°C [7].

The line broadening at high label concentration (cf. Fig. 1b) is the result of two interaction mechanisms: the spin exchange interaction and the magnetic dipole-dipole interaction. Consequently the spectra are characterized by two independent interaction parameters, the exchange frequency W_{ex} (in MHz) and the dipolar broadening ΔH_d (in Gauss).

The dipolar interaction may be taken into account by replacing the line widths ΔH_j^0 by

$$\Delta H_j = \Delta H_j^0 + \Delta H_d$$

In order to analyze the spectra in terms of both the exchange and the dipolar interaction, the modified Bloch equation has to be solved.

Three cases had to be considered in the present work:

(1) *Weak radical interaction.* The three ESR lines are well resolved and slightly broadened. The exchange frequency may be determined from the linewidth of the central line according to $\Delta H_0 = \Delta H_0^0 + \Delta H_d + \Delta H_{ex}$. The two interaction mechanisms affect the linewidth in the same way.

(2) *Medium interaction.* The three ESR lines start to merge. An example is given in Fig. 1b. The two interaction mechanisms affect the spectra in a different way. The exchange interaction tends to shift the two outer lines towards the center of the spectrum. The dipolar broadening shifts the side bands

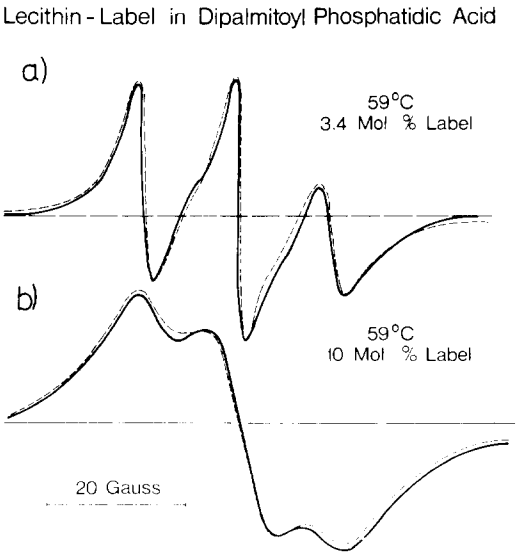

Fig. 1. (a) First derivative ESR spectrum of the lecithin label (I) in vesicles of dipalmitoyl phosphatidic acid at 59°C with negligible interaction. Molar ratio label-to-lipid r = 0.035 corresponding to 3.4 mol% label. —, measured spectra; - - - -, calculated spectra with ΔH_{+1}^0 = 1.5; ΔH_0^0 = 1.0 and ΔH_{-1}^0 = 2.5 G. (b) Spectrum at high label concentration. Best fit between calculated (- - - -) and experimental spectrum (—) was obtained for a dipolar broadening ΔH_d = 0.7 G and an exchange frequency W_{ex} = 6.5 MHz.

away from the center. Accordingly the two interaction parameters W_{ex} and ΔH_d may be determined by a trial and error method as described in ref. 18. The spectra calculation was performed with a PDP-8I computer equipped with a Tektronix display (model 4002 A). The latter allowed a rapid comparison of calculated and observed spectra.

(3) *Strong interaction.* The three hyperfine lines have fused to a single line of Lorentzian shape.

In the limiting cases of weak and strong exchange interaction, ΔH_d and W_{ex} may not be determined independently. In these cases the values of ΔH_d have been estimated by extrapolating the values obtained for the cases of medium radical interaction. The extrapolation was based on the assumption that ΔH_d is proportional to the radical concentration. Some values of ΔH_d used in the present work are summarized in Table I.

Experimental results

Binding of divalent ions to vesicles of phosphatidic acid

Organic phosphoric acids such as phosphatidic acids may form strong complexes with divalent ions. It is therefore expected that such ions bind strongly to vesicles of phosphatidic acid. For paramagnetic ions the complex formation may be directly observed via the broadening of the ESR spectra. Consider the case of Mn^{2+}: The ESR spectrum of this ion in aqueous solutions consists of six sharp lines. Upon binding to phosphatidic acid that is either in solution or incorporated into lipid lamellae this hyperfine structure is completely smeared out and a broad one line spectrum is observed. It is thus possible to distinguish between free ions (in solution) and bound ions. As an example the ESR spectrum of a phosphatidic acid vesicle preparation containing Mn^{2+} is shown in Fig. 2a. The molar ratio, R, of Mn^{2+}-to-lipid is $R = 1.0$. Obviously the spectrum consists of a superposition of a sharp sextet spectrum and a broad one line spectrum. At pH 9 the sharp sextet spectrum just starts to appear at an equimolar ratio of Mn^{2+} and phosphatidic acid. By plotting the intensity I of the sharp spectrum as a function of the molar ratio, R, of Mn^{2+}-to-lipid, the limiting value of R, at which the membrane is saturated with ions, may be determined. Using this procedure the maximum binding of Mn^{2+} to phosphatidic acid vesicles was measured as a function of pH. The result is given in Fig. 2b.

TABLE I

VALUES OF DIPOLAR BROADENING ΔH_d IN GAUSS OF LECITHIN LABEL (I) IN VESICLES OF DIPALMITOYL PHOSPHATIDIC ACID FOR $T = 59°C$; AS A FUNCTION OF THE MOLAR FRACTION OF LABEL

r is the molar ratio of lecithin-to-phosphatidic acid. (a) values obtained by simulation of the spectra at medium radical interaction, (b) values extrapolated.

	Molar fraction $r/(1+r)$	0.01	0.034	0.05	0.1	0.12	0.16	0.20
ΔH_d values	Absence of Ca^{2+}	0.0	0.0	0.2 [b]	0.7 [a]	1.0 [a]	1.3 [a]	1.5 [a]
	Molar fraction of Ca^{2+} $R = 0.5$	0.0	0.7 [b]	1.1 [a]	3.5 [a]	4.3 [a]	5.0 [b]	5.5 [b]

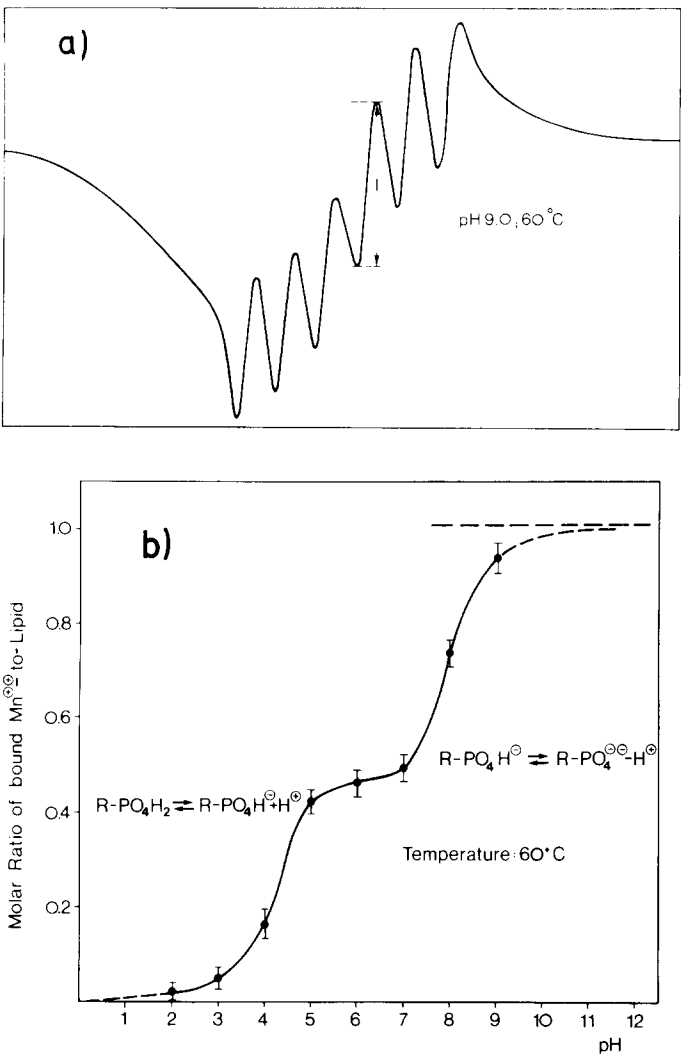

Fig. 2. (a) ESR spectrum of Mn^{2+} in an aqueous dispersion of phosphatidic acid vesicles at pH 9. The molar ratio of Mn^{2+}-to-phosphatidic acid was $R = 1.0$. The sharp 6-line spectrum is due to free ions in the bulk solution. The height I of the line near the centre of the spectrum is a measure of the concentration of free Mn^{2+}. (b) pH dependence of the binding of Mn^{2+} to the surface of phosphatidic acid vesicles at 60°C. The ordinate gives the molar ratio, R^*, of bound Mn^{2+}-to-lipid. A value of 1 corresponds to a one-to-one binding of Mn^{2+} to the lipid. The binding curve does not change appreciably with temperatures between 20°C and 60°C.

The complex formation of Mn^{2+} with the lipid increases sharply at pH 4 and at pH 8. Obviously the first and the second hydroxyl bond of the phosphate group dissociate at these pH values (cf. Fig. 2b). The corresponding pK values of the free phosphatidic acid are p$K \approx 3.0$ and p$K \approx 8.0$. Now it is well known that the pK values of fatty acids and phospholipids may change by 1—2 pH units if these molecules are associated in a monolayer (cf. ref. 19). Fig. 2b shows that two singly charged phosphatidic acids (between pH 5 and pH 7.5) are needed to bind one Mn^{2+} ion whereas one doubly charged lipid

forms a one-to-one complex with Mn^{2+}. It is reasonable to assume that the same is valid for Ca^{2+}. Very strong evidence for a one-to-one binding of Ca^{2+} to phosphatidic acid has been obtained in our previous optical study [7].

Vesicles of pure lecithin label

The ESR-spectrum of vesicles from pure lecithin label (I) consists of a single exchange narrowed line. This line exhibits Lorentzian shape at all temperatures. A sharp decrease in the line width at $T_t = 44°C$ indicates a crystalline-to-liquid crystalline phase transition of the labelled lecithin at this temperature. This value compares well with the transition temperature of dipalmitoyl lecithin vesicles ($T_t = 41$ °C).

Effect of Ca^{2+} on the radical interaction in mixed lecithin-phosphatidic acid vesicles

Typical ESR spectra of mixed vesicles composed of lecithin label (I) and dipalmitoyl phosphatidic acid are given in Fig. 3. The spectra were taken at 59°C, that is at a temperature well above the lipid phase transition of dipalmitoyl phosphatidic acid at pH 9. Spectra are presented as a function of the lecithin content. Moreover, Fig. 3 shows how addition of increasing amounts of Ca^{2+} affects the line shape. It is found that addition of Ca^{2+} has a dramatic effect on the line shape. Consider the spectra at 10 % lecithin. Upon addition of increasing amounts of Ca^{2+} the partially resolved triplet spectrum is transformed to an unstructured one line spectrum which exhibits to a good approximation Lorentzian shape. Obviously the binding of Ca^{2+} to the membrane leads to a strong increase in the radical interaction.

Fig. 4 shows the effect of temperature. Spectra are presented at a temperature below (31°C) and at two temperatures above (51°C and 59°C) the phase transition of phosphatidic acid ($T_t = 47.5°C$ at pH 9). At molar ratios of Ca^{2+}-to-phosphatidic acid of $R = 0.50$ and 1.0 Lorentzian lines are obtained at $T > T_t$. Their line widths decrease with increasing temperatures. Analysis of the spectra show that the exchange interaction increases with increasing temperature.

By computer simulation of the spectra presented in Fig. 3 values of the spin exchange frequency W_{ex} have been determined both as a function of lecithin concentration and of the Ca^{2+} concentration. The results are summarized in Fig. 5. In Fig. 5a, W_{ex} is plotted as a function of the molar fraction $r/(1+r)$, of labelled lecithin. In Fig. 5b W_{ex} is plotted as a function of $\sqrt{(1+r)}/r$.

Effects of pH changes

Fig. 6 shows how changes in the pH of the aqueous phase affect the ESR spectra of mixed vesicles containing 16 mol % lecithin. It is seen that a decrease in the pH value leads to a significant decrease in the radical interaction. This effect is most pronounced in the presence of Ca^{2+}. Fig. 7 summarizes the effect of pH changes on the spin exchange frequency W_{ex}. Between pH 7 and pH 5, where the phosphatidic acid molecules carry a single charge, Ca^{2+} addition has a much smaller effect on the label interaction than at high pH values. At pH 3, where part of the phosphatidic acid is uncharged, the influence of divalent ions on W_{ex} is very small. Measurements could not be performed at pH < 3 since the vesicles coagulated.

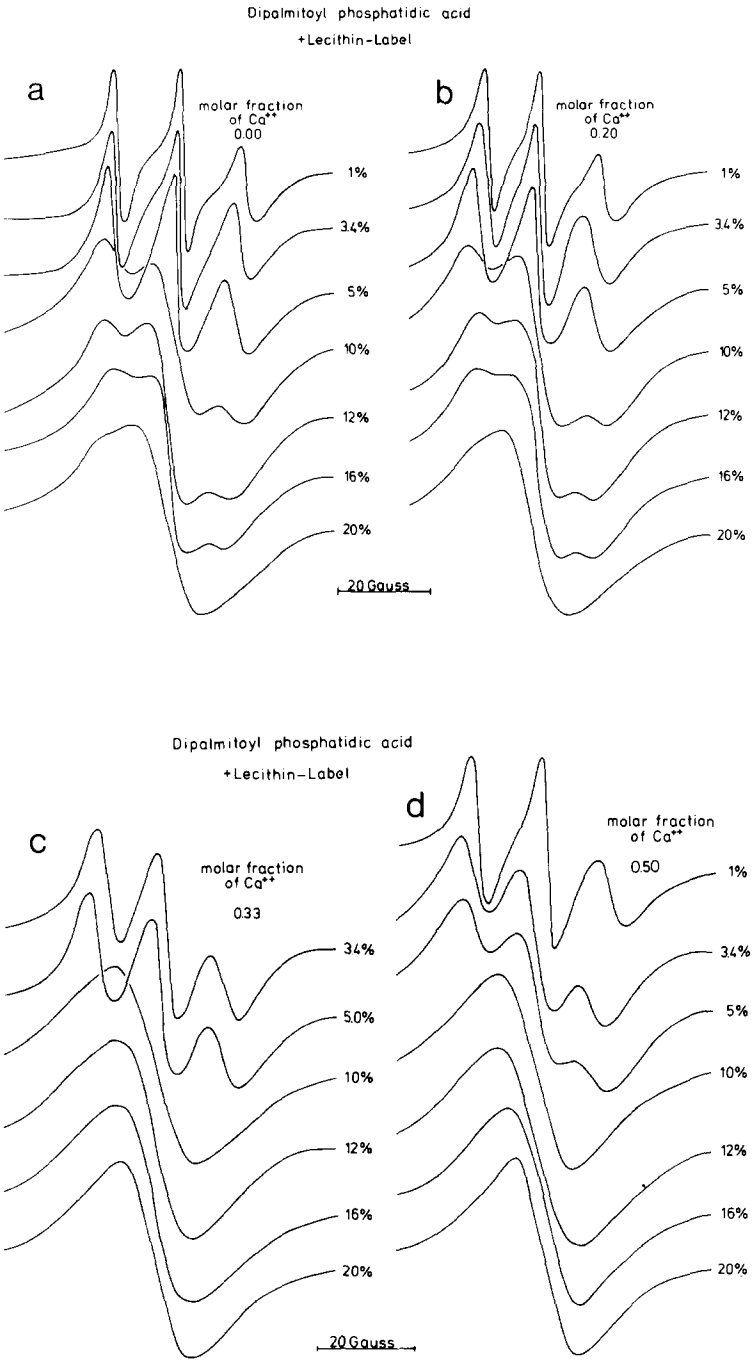

Fig. 3. First derivative ESR spectra of lecithin label (I) in phosphatidic acid vesicles taken at $T = 59^{\circ}C$ and pH 9. The numbers at the right side of the spectra give the molar percentage of labelled lecithin with respect to the total lipid content. (a) spectra taken in the absence of Ca^{2+}; (b), (c) and (d) spectra taken in the presence of different amounts of Ca^{2+}. The molar fractions of Ca^{2+} with respect to phosphatidic acid are in (b) 0.2; in (c) 0.33 and in (d) 0.5. Note: The values of the molar fraction are given by $R/(1+R)$ where R is the molar ratio of Ca^{2+}-to-phosphatidic acid.

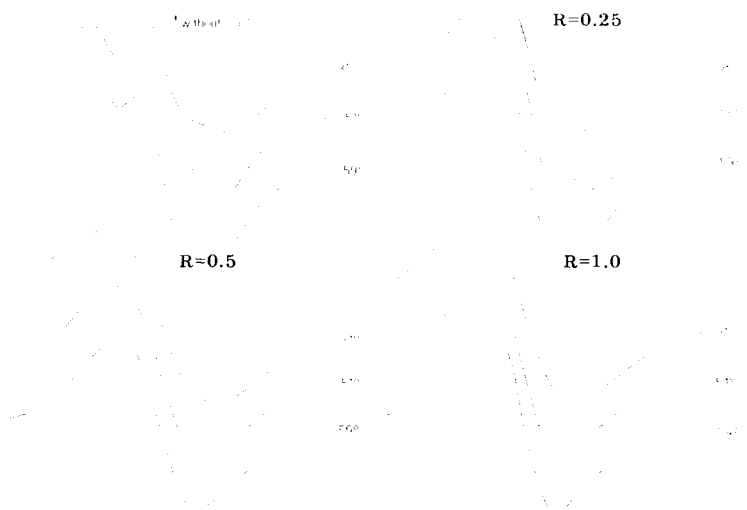

Fig. 4. Effect of temperature on the ESR line shape of mixed lecithin-phosphatidic acid vesicles in the absence and in the presence of different amounts of Ca^{2+}. The spectra were taken at pH 9. At this pH the transition temperature of dipalmitoyl phosphatidic acid membranes is $T_t = 47.5°C$ [7]. The linewidths of the Lorentzian lines at molar ratios of Ca^{2+}-to-phosphatidic acid ($R = 0.5$ and $R = 1$) decrease with increasing temperature.

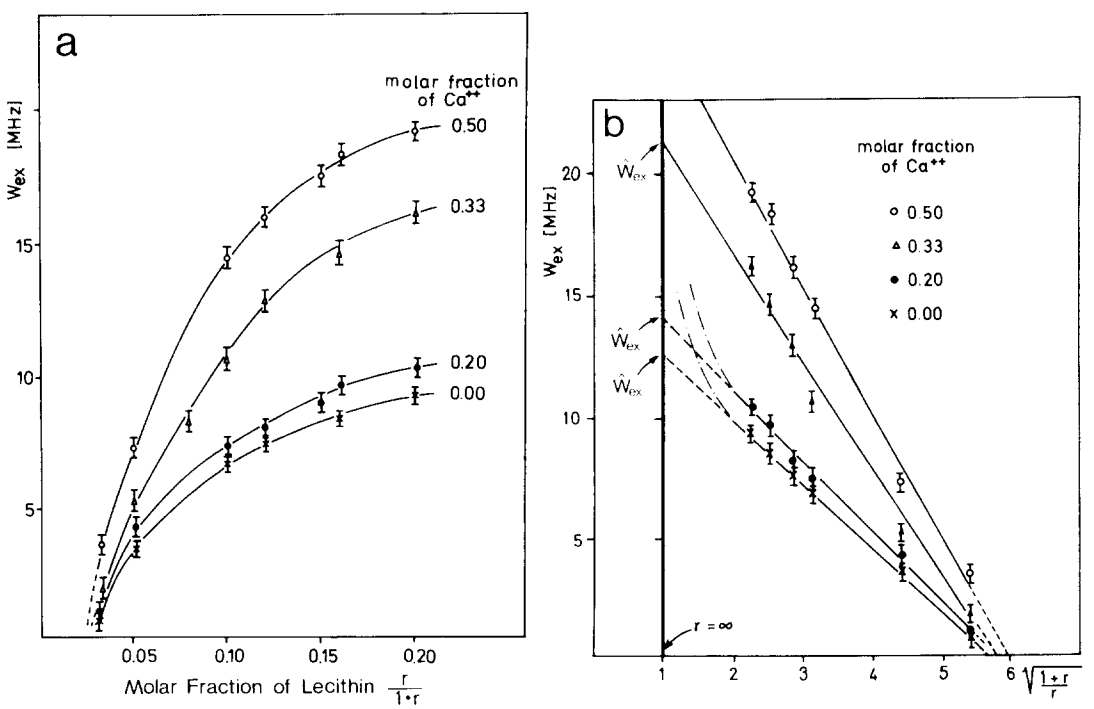

Fig. 5. Concentration dependence of the exchange frequency, W_{ex} at pH 9 and at 59° C, both in the absence and in the presence of different amounts of Ca^{2+}. (a) W_{ex} is plotted as a function of the molar fraction $(r/1+r)$ of lecithin. r is the molar ratio of lecithin-to-phosphatidic acid. (b) Plot of W_{ex} versus $\sqrt{(1+r)/r}$. Straight lines are obtained both in the absence and in the presence of Ca^{2+} up to lecithin concentrations of 20 mol %. At larger lecithin concentrations the curves are expected to exhibit an upward deflection indicated by the curve (-.-). At a Ca^{2+} molar fraction of $\frac{R}{1+R} \approx 0.5$ it is $W_{ex} \approx 27$ MHz.

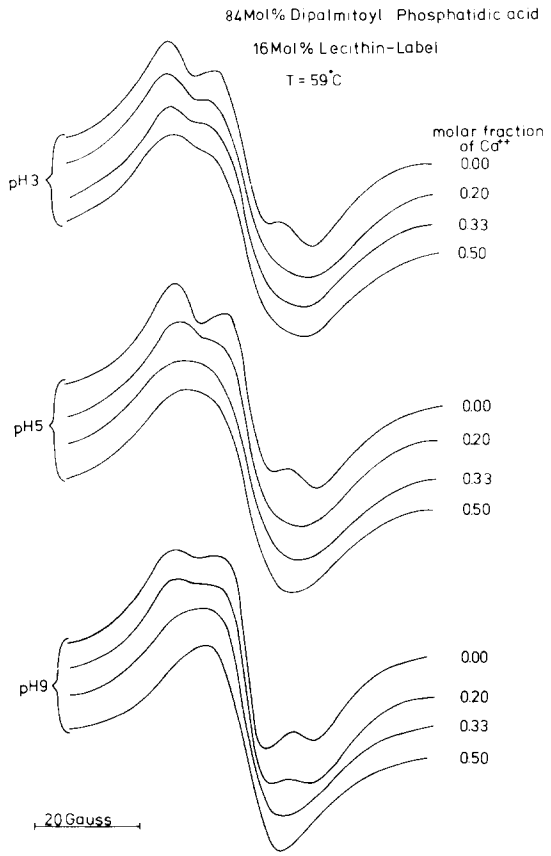

Fig. 6. Effect of pH changes on the ESR spectra of vesicles containing 16 mol% lecithin label. The numbers given at the right side of each spectrum are the molar fraction, $R/(1+R)$, of Ca^{2+} with respect to phosphatidic acid.

Experiments with monolayer membranes

For comparison, some experiments were also performed with vesicles formed by lipid monolayers. The interior of the vesicles contained a chloroform dibutylether mixture [17, 18]. A spectrum analysis in terms of the exchange frequency was only performed in the absence of Ca^{2+}. The results are given in Fig. 8. The exchange frequencies observed in the lipid monolayers agree well with the values of W_{ex} characterizing the corresponding bilayer system.

Comparison with uncharged vesicles

One aim of the present work was to compare the molecular organization of lipid lamellae composed of a mixture of charged and uncharged lipids with lipid bilayers containing only uncharged lipid mixtures. For this purpose we studied mixed vesicles of (1) lecithin label (I) in dipalmitoyl phosphatidylcholine and of (2) lecithin label in dimyristoyl phosphatidylethanolamine. Values of the exchange frequencies W_{ex} were again determined as a function of the label concentration up to label-to-lipid molar ratios of $r = 0.25$. The re-

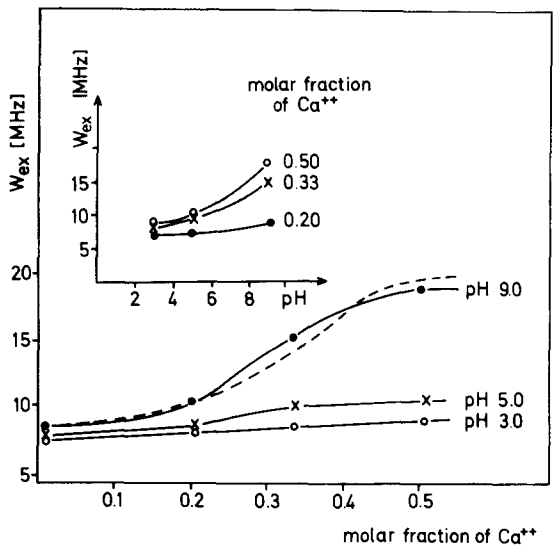

Fig. 7. Plot of W_{ex} as a function of molar fraction of Ca^{2+}, $R/(1+R)$, (as measured with respect to phosphatidic acid), for different pH. The effect of Ca^{2+} on the exchange frequency is greatly reduced at lower pH. This is most clearly seen in the insert of the figure. Broken curve: values of W_{ex} calculated with equation A2 of the Appendix.

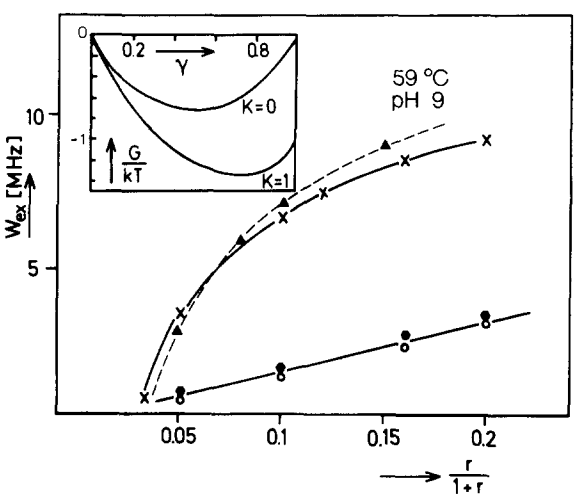

Fig. 8. Comparison of exchange frequencies, W_{ex}, of labelled lecithin (I) in membranes containing charged (dipalmitoyl phosphatidic acid) (X) and uncharged (dipalmitoyl lecithin) (○) and dimyristoyl phosphatidylethanolamine (●) lipids. ▲, values of W_{ex} obtained for phosphatidic acid monolayer vesicles. r is the molar ratio of labelled lecithin-to-unlabelled lipid. Insert: Normalized free energy $G/k\ T$ of a binary mixed monolayer containing both uncharged lipid and lipid carrying one net charge. γ molar fraction of charged lipid. Definition of K cf. Eqn 8.

sult is summarized in Fig. 8, together with the values of W_{ex} obtained for the lecithin-phosphatidic acid system. A pronounced difference in the radical interaction in charged and uncharged lipid lamellae is observed. Obviously W_{ex} is strictly proportional to the molar fraction $(r/1+r)$ of labelled lecithin in uncharged lipid mixtures. In charged lipid lamellae, a much stronger radical inter-

action is observed. In addition W_{ex} is no longer proportional to $r/1+r$. As will be shown below, this finding can be explained by assuming a heterogeneous distribution of the charged and uncharged lipids within the membrane.

Effects of polylysine

Fig. 9 shows the effect of polylysine on the lecithin interaction in phosphatidic acid vesicles. At pH 9 the lysine groups are positively charged and the polylysine exhibits a random coil configuration. Obviously addition of polylysine induces a significant increase in the radical interaction.

The temperature dependence of the ESR spectra at pH 9 and at low label concentration ($r \leqslant 0.01$) exhibits two well separated lipid phase transitions: a transition at 47.5°C is characteristic for phosphatidic acid membranes at pH 9. The second transition at 62°C is caused by regions of lipid layers to which polylysine is adsorbed. This effect of polylysine has been described in more detail in our optical study with excimer probes [7].

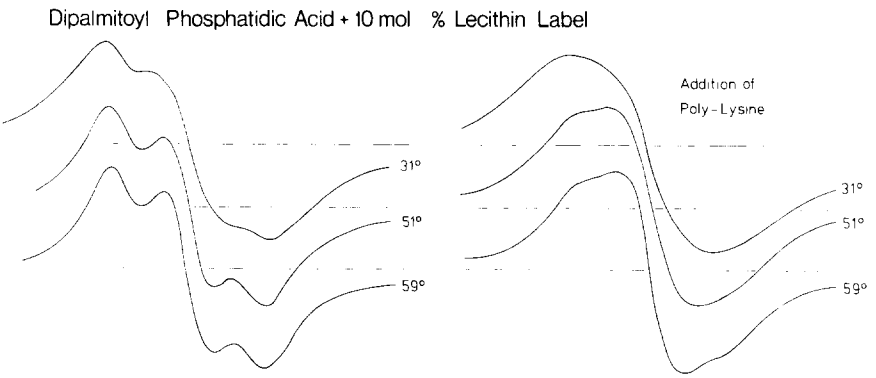

Fig. 9. Effect of polylysine on ESR spectra of mixed lecithin-phosphatidic acid membranes at pH 9. Spectra at left side: without polypeptide. Spectra at right side: after addition of polylysine. Number of positive lysine groups equal to number of phosphatidic acid molecules. At 59°C, W_{ex} increases from W_{ex} = 6.7 MHz to W_{ex} = 9.5 MHz upon addition of polylysine.

Discussion

According to ref. 9, the radical interaction in lipid lamellae containing a labelled lipid as one constituent may be determined by two factors:

(1) By the rate of lateral diffusion D of the labelled lipid molecules

(2) By the distribution of the lipids within the membrane, a factor which determines the average radical distance.

These two mechanisms lead to completely different concentration dependencies of the exchange frequency W_{ex}.

Provided the radical interaction is determined by the lipid lateral diffusion, W_{ex} is proportional to the molar fraction $(r/1+r)$ of labelled lipid:

$$W_{ex} = \frac{4\, d_c D}{3\, \lambda F} \frac{r}{1+r} \tag{5}$$

In this equation, d_c, λ and F are the critical radical interaction distance, the length of one diffusional jump and the area per lipid molecule, respectively.

D is the coefficient of the label lateral diffusion. This model is based on the assumption of a random lipid distribution within the lipid bilayers.

If W_{ex} is determined by the lipid distribution, that is by the formation of clusters of labelled lipid, one expects a functional dependence according to

$$W_{ex} = \hat{W}_{ex}\left(1 - \sqrt{\pi \cdot d_c^2 \cdot n}\sqrt{\frac{1+r}{r}}\right) \tag{6}$$

where n is the number of clusters per cm^2 and where $\hat{W}_{ex}$ is the maximum exchange interaction in the interior of the cluster.

By plotting W_{ex} both as a function of $r/1+r$ and as a function of $\sqrt{\frac{1+r}{r}}$, respectively, one should be able to distinguish between the above two cases.

Lipid segregation in the absence of Ca^{2+}

In Fig. 8, the exchange frequencies of labelled lecithin (I) in dipalmitoyl phosphatidylcholine, in dimyristoyl phosphatidylethanolamine and in dipalmitoyl phosphatidic acid are compared at a temperature (T = 59°C) well above the respective transition temperatures. W_{ex} is plotted as a function of the label molar fraction ($r/1+r$). Straight lines are obtained for the ethanolamine and the lecithin membranes, clearly demonstrating that the labelled lecithin is randomly distributed and is rapidly diffusing in these lipid lamellae. From the slopes of the straight lines the lateral diffusion coefficient D may be determined according to Eqn 5. Using values of $d_c \approx 8$ Å and $\lambda \approx 8$ Å one obtains $D = 2.8 \cdot 10^{-8}$ cm^2/s for both the ethanolamine and the lecithin membranes at 59°C. This value of D agrees well with the diffusion coefficient for androstane in lecithin membranes ($D = 3 \cdot 10^{-8}$ cm^2/s at 50°C). It is smaller by a factor of about 3 than the value of D found in the optical experiments [7] using Pyrene decanoic-acid labels.

A completely different behaviour is observed for mixed lecithin-phosphatidic acid vesicles. For this system W_{ex} is not proportional to $r/1+r$ even in the liquid crystalline state of the membrane ($T > T_t$). Accordingly the two phospholipids segregate in these mixed systems above T_t. Two types of lipid phase separation should be considered:

(1) A heterogeneity in the lipid distribution within each monolayer of the membranes (lateral phase separation)

(2) A difference in the lipid composition between the inner and the outer monolayer of the vesicles (transversal phase separation cf. refs 20 and 21).

In order to distinguish between these two possibilities we performed the experiments with mixed lecithin-phosphatidic acid monolayer vesicles. The concentration dependence of W_{ex} for this system is also plotted in Fig. 8. Good agreement between the values of the exchange frequency in the bilayer and in the monolayer membranes is observed. This finding provides strong evidence for the occurrence of lateral phase separation in mixed lecithin-phosphatidic acid membranes.

The question arises whether phase separation in fluid mixed membranes containing a component with a net electric charge is physically reasonable. Considering only the repulsion forces between the charged lipids one would

rather expect a randomization in the mixed system. However, at the surface of membranes composed of charged lipids an electric double layer forms by the absorption of counter ions. It has been emphasized by Overbeek [22] that a very characteristic feature of diffuse electric double layers of the Gouy-Chapman type is the negative sign of its Gibbs free energy.

At high surface potential the free energy is proportional to the surface charge:

$$G_{dl} = -2\,\Gamma \cdot kT \tag{7}$$

where Γ denotes the number of charges per cm^2. Consider a mixed monolayer composed of charged and uncharged lipids containing a molar fraction γ of charged lipids. Taking into account the mixing entropy, the total free energy of such a mixed lipid layer is given by (cf ref. 23, p. 237):

$$G = R\Gamma(\gamma \ln \gamma + (1-\gamma)\ln(1-\gamma) - K\gamma) \tag{8}$$

where $K\gamma = 2\Gamma$ is positive. In the insert of Fig. 8, G/kT is plotted as a function of the fraction, γ, of charged lipid for $K = 0$ and $K = 1$. These diagrams predict a complete randomization of the lipid at $K = 0$ (minimum of G at $\gamma = 1/2$). For $K > 0$ the minimum free energy is decreased considerably and in addition is shifted to higher fractions of charged lipids (minimum of G at $\gamma > 1/2$). In view of this result, lateral phase separation in membranes composed of charged and uncharged lipids seems physically reasonable.

Assume a value of $F = 60$ Å^2 for the area occupied by one phosphatidic acid molecule. This would correspond to a lipid density of $2.8 \cdot 10^{-10}$ mol/cm^2. According to Eqn 7 the free energy of the electric double layer of a membrane composed of single charged phosphatidic acid molecules would thus be $G_{dl} \approx -1.8\,R$. For this value of G_{dl} the free energy has a minimum for $\gamma \approx 0.8$. Accordingly, the formation of regions enriched and depleted in the charged lipid would lead to a lower free energy of the mixed membrane than a random mixture.

Enhancement of phase separation by Ca^{2+}

Fig. 5 shows the concentration dependence of W_{ex} for different molar fractions, $R/1+R$, of Ca^{2+} as measured with respect to the phosphatidic acid content. In Fig. 5a, W_{ex} is plotted as a function of $r/1+r$. No straight lines are obtained between $R = 0$ and $R = 1$. Obviously the radical interaction is not a diffusion controlled process. In Fig. 5b the exchange frequently is plotted as a function of $\sqrt{1+r/r}$. Straight lines are obtained both in the presence and in the absence of Ca^{2+}. Their slopes increase with increasing Ca^{2+} concentration. This finding demonstrates that addition of Ca^{2+} strongly enhances the lipid segregation in mixed lecithin-phosphatidic acid membranes. From the slopes of the straight lines, values of the density, n, of lecithin clusters may be estimated according to Eqn 6. The result, summarized in Table II, shows that the cluster density, n, does not depend on the Ca^{2+} concentration. However, since W_{ex} increases with increasing Ca^{2+} concentration, the size of the lecithin clusters must increase upon addition of Ca^{2+}.

Such a result is expected from the findings reported in our optical study [7]. There it was shown that the transition temperature of Ca^{2+}-bound

TABLE II

CHARACTERISTIC PARAMETERS OF LECITHIN PRECIPITATIONS IN MIXED LECITHIN-PHOSPHATIDIC ACID MEMBRANES

r and R molar ratios of lecithin-to-phosphatidic acid and of Ca^{2+}-to-phosphatidic acid, respectively. r_c: value of r in the lecithin containing clusters of type 1 (r_c^1) or type 2 (r_c^2). n: density of clusters (per cm^2), N: number of molecules per cluster, Ω radius of cluster.

Molar fraction of Ca^{2+} $R/(1+R)$		0.0	0.2	0.33	0.5
$n\left(\frac{\text{clusters}}{cm^2}\right)$		3.4×10^{11}	3.3×10^{11}	3.4×10^{11}	3.2×10^{11}
Values for $r = 0.25$	$\hat{W}_{ex}$ (MHz)	9	—	—	19
	Ω (Å)	$\Omega_1 = 80$ Å	—	—	$\Omega_2 = 45$ Å
	r_c	$r_c^1 \approx 0.5$	—	—	$r_c^2 \to \infty$

phosphatidic acid is shifted to very high temperatures. Consequently Ca^{2+}-bound phosphatidic acid forms rigid clusters which are embedded in liquid crystalline lipid regions composed of non-bound phosphatidic acid and lecithin. As shown above, lecithin-phosphatidic acid membranes most probably exhibit a mosaic-like structure of lecithin enriched and lecithin depleted regions. It is therefore reasonable to assume that Ca^{2+} attaches mainly to the lecithin depleted regions leading there to the formation of rigid phosphatidic acid clusters. Lecithin would be squeezed out from these crystalline regions. Judged from these considerations, the cluster density, n, is expected to remain unchanged upon addition of Ca^{2+}.

Clearly the maximum exchange rate, $\hat{W}_{ex}$, defined in Eqn 6 depends on the molar fraction, $r_c/(1+r_c)$, of lecithin within the clusters. Depending on the Ca^{2+} concentration, two limiting cases have to be considered:

(1) $R = 1$: Nearly all phosphatidic acid is bound by Ca^{2+} and is in a rigid state at pH 9. Clusters of fluid lecithin are formed which are embedded in the rigid matrix of the Ca^{2+}-bound lipid. Since $r_c/(1+r_c) \approx 1$, $\hat{W}_{ex}$ should be equal to the value $\hat{W}^0_{ex} = 30$ MHz characteristic for membranes of pure lecithin label. The value of $W_{ex} = 27$ MHz obtained from Fig. 9 is in rather good agreement with this expectation*.

For circular two-dimensional clusters the radius Ω_2, may be determined as a function of the lecithin concentration according to (cf. ref. 9, Eqn 28a):

$$\Omega_2 = \frac{1}{\sqrt{\pi \cdot n}} \sqrt{\frac{r}{1+r}} \tag{9}$$

As an example the value of Ω_2 for $r = 0.25$ is given in Table II.

(2) $R = 0$ (absence of Ca^{2+}): Two types of lecithin clusters have to be considered: At low label concentration a first type (1) of cluster containing both lecithin and phosphatidic acid will be formed. Its composition is determined by the minimum of the free energy, G_m. From our above estimation of G_m the clusters are expected to contain less than 50 mol % lecithin, that is $r_c^1/(1+r_c^1) < 0.5$. These clusters would grow in size with increasing lecithin concentration, $r/(1+r)$, up to a limiting value:

*The small discrepancy of about 10 % is most probably due to the fact that according to Fig. 2 the complex formation of Ca^{2+} with phosphatidic acid is not a perfect 1 : 1 binding at pH 9.

$$\frac{r_m}{1+r_m} = \frac{r_c^1}{1+r_c^1}$$

Up to this concentration W_{ex} is a linear function of $\sqrt{(1+r)/r}$ as found in Fig. 5b.

At higher lecithin concentrations, $r > r_m$, a second type (2) of cluster containing approx. 100 % lecithin ($r_c^2/r_c^2+1 \approx 1$) starts to form. Accordingly one expects that at $r \gg r_m$, W_{ex} should increase rapidly with increasing lecithin concentration, since at $r \to \infty$ one expects $W_{ex} = \hat{W}^0_{ex}$. This behaviour has been indicated in Fig. 5b by the broken curve (- - -). For circular clusters of type (1), the radius Ω_1 is given by

$$\Omega_1 = \frac{1}{\sqrt{n \cdot \pi}} \sqrt{\frac{r}{1+r} \frac{1+r_c^1}{r_c^1}} \tag{10}$$

This equation follows directly from Eqn 27 in ref. 9: The value of Ω_1 for the lecithin concentration $r = 0.25$ is given in Table II.

The rate of spin exchange $\hat{W}_{ex}$ within the two types of lecithin clusters is diffusion controlled (at $T = 59°C$) and one thus expects

$$\hat{W}_{ex} = \hat{W}^0_{ex} \frac{r_c}{1+r_c} \tag{11}$$

From this equation, the molar fraction of lecithin in the clusters of type (1) may be estimated. Consider the case $R = 0$: $\hat{W}_{ex}$ is determined by the value of the exchange frequency at the lecithin concentration, r_m, where the second type of cluster starts to form. In principle, r_m can be determined from that point in the plot of W_{ex} versus $\sqrt{(1+r)/r}$ where the straight line exhibits an upward deflection (curve -.- in Fig. 5b). An upper limit of $\hat{W}_{ex}$ is given by the intercept of the straight line with the ordinate ($\hat{W}_{ex} = 12.5$ MHz). A lower limit of $\hat{W}_{ex}$ is the exchange frequency at $r/1+r = 0.2$ ($\hat{W}_{ex} = 9$ MHz). The lecithin concentration in the first type of cluster thus has a value between $r_c^1/1+r_c^1 = 0.3$ and $r_c^1/1+r_c^1 = 0.4$ (cf. Eqn 11).

Intermediate case ($0 < R < 1$)

The above discussion of the experiments at the limiting cases $R = 0$ and $R = 1$ and at 59°C lead to the following conclusion: At lecithin concentrations $r < r_m$, patches (clusters of type (1)) of lipid monolayer containing 30—40 mol % lecithin ($r_c^1 = 0.43$—0.67) are formed in the fluid phosphatidic acid membrane. The size of these clusters increases with increasing lecithin concentration r (cf. Eqn 10) while the cluster density, n, remains constant. At $r > r_m$ ($r_m = r_c^1 = 0.43$—0.67) a second type (2) of cluster of pure lecithin ($r_c^2/1+r_c^2 = 1$) starts to form. At $R = 1$ clusters of type (2) are formed at all lecithin concentrations.

Addition of Ca^{2+} leads to the formation of rigid phosphatidic acid regions from which lecithin is squeezed. Accordingly, the effective molar ratio, $r^\star$, of lecithin-to-free phosphatidic acid increases. At pH 9, where a 1 : 1 binding of Ca^{2+} and phosphatidic acid occurs, one obtains

$$r^\star = \frac{r}{1-R}$$

Depending upon whether $r^{\star} < r_m$ or $r^{\star} > r_m$, addition of Ca^{2+} leads to an increase in the size of the clusters of type (1) or type (2), respectively. In both cases Ca^{2+} addition causes an increase in the exchange frequency W_{ex}. In the appendix a model based on the above consideration is presented which allows to calculate W_{ex} as a function of the Ca^{2+} concentration, (cf. Eqn A2). As an example W_{ex} has been calculated for an initial molar ratio, of lecithin-to-phosphatidic acid of $r = 0.25$ and for a value $r_m = r_c^l = 0.33$. The calculated values of W_{ex} have been plotted in Fig. 7 as a function of the molar fraction, $R/(1+R)$, of Ca^{2+} (broken curve) good agreement between the calculated and the experimental plots of W_{ex} versus $R/(1+R)$ is obtained for pH 9. As reported by Schreier-Muccillo et al. [23] addition of Ca^{2+} increases the apparent order of films formed from a mixture of lecithin, cholesterol and phosphatidic acid. This finding may also be explained as an effect of phase separation. Upon addition of Ca^{2+} rigid patches of phosphatidic acid may form within the multilayers. As a consequence the spin probes would be restricted to the fluid membrane regions formed from lecithin and cholesterol and would reflect the order characteristic for this system.

pH dependence

The nearly negligible effect of Ca^{2+} on the exchange frequency at pH 3 (cf. Fig. 6) is expected from the finding that the binding of divalent ions to phosphatidic acid is negligibly small at this pH. An explanation for the small effect of Ca^{2+} at pH 5 cannot be given yet.

Effect of polylysine

As demonstrated in Fig. 9, positively charged polylysine has the same effect on mixed lecithin phosphatidic acid membranes as Ca^{2+}. Obviously adsorption of polylysine to the mixed lipid bilayer leads to the formation of clusters of phosphatidic acid held together by the polypeptide. This type of chemically induced phase separation has been studied in more detail in ref. 7. The clusters held together by the polylysine have a chain melting temperature of $T_t \approx 60°C$ which corresponds to an effective pH of about pH 2. A model of the polylysine induced lipid segregation is presented in Fig. 10. A much weaker, however, noticeable effect on the lipid distribution in mixed lecithin-phosphatidic acid membranes has been observed upon addition of natural proteins such as cytochrome. Smith and coworkers [25] observed an increase in the lipid order upon addition of polylysine (or lysine-leucine copolymers) to (1) brain-lipid bilayers and (2) to mixed egg lecithin-phosphatidic acid membranes. Their results (cf. Figs 2 and 3a of ref. 25) may well be interpreted in terms of the distribution of steroid labels in lysine bound rigid patches of charged lipid and in nonbound fluid regions.

These examples show that extrinsic proteins that are supposed to be only loosly connected with the membrane may have a remarkable effect on the lipid structure. This observation suggests that, in principle, conformational changes in surface proteins may trigger in a cooperative way (structural changes) in the local lipid composition.

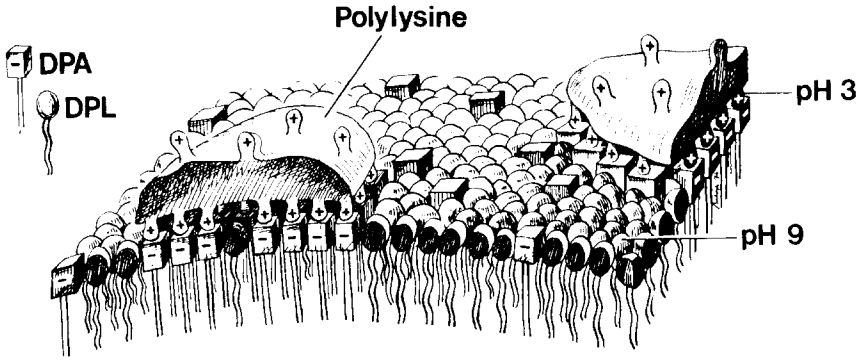

Fig. 10. Possible effect of randomly coiled polylysine (with positively charged N^+H_3) on a mixed membrane of dipalmitoyl phosphatidic acid (DPA) and dipalmitoyl lecithin (DPL). Hydrocarbon chains that are symbolized by straight lines are in a crystalline conformation.

Kinetics of lipid segregation

The fluid lecithin-phosphatidic acid lipid lamellae may be considered as a two dimensional alloy that exhibits a miscibility gap. The rate of formation of clusters of one lipid component by uphill diffusion [26] is determined by the rate of the lipid lateral diffusion, D. The times τ needed for the formation of a circular cluster of N lipid molecules may be estimated according to (cf. ref. 24) Eqn 13:

$$\tau = \frac{2NF}{\pi \cdot D}$$

F is the area per lipid molecule ($F \approx 60$ Å^2). With a lipid diffusion coefficient of $D \approx 10^{-7}$ cm^2/s one estimates $\tau \approx 4 \cdot 10^{-6}$ s for $N = 1000$. This shows that dynamic processes in biological membranes involving lipid phase separation may proceed very rapidly.

Appendix

Calculation of W_{ex} as a function of Ca^{2+} concentration

The following definitions are used:

r : molar ratio of lecithin to the total amount of phosphatidic acid
r_c : molar ratio lecithin to charged lipid in clusters
r_m: value of r at the minimum of the free energy, G_m (cf. Eqn 8)
$r^\star$: molar ratio of lecithin to non-bound phosphatidic acid
R : molar ratio Ca^{2+}-to-phosphatidic acid

Obviously $r^\star = r/(1 - R)$ for pH $\geqslant$ 9.

Assumption: two types of lecithin precipitations (clusters) may form: a first type of cluster (1) containing a lecithin concentration $r_c^1 = r_m$ is solely present up to a limit of $r = r_m$. At $r > r_m$ a second type (2) of cluster containing 100 % lecithin starts to form. Ω_1 and Ω_2 denote the radius of cluster of type (1) and (2), respectively, $\pi\Omega_1^2$ and $\pi\Omega_2^2$ are the corresponding areas of the clusters.

In order to allow for the fact that the cluster type (2) starts to form at $r > r_m$ while type (1) ceases growing at this limit, it is possible to introduce effective areas in the following way:

$$\pi(\Omega_1^\star)^2 = \pi\Omega_1{}^2 \frac{1}{1+\alpha^2}\,;\ \pi(\Omega_2^\star)^2 = \pi\Omega_2{}^2 \frac{\alpha^2}{1+\alpha^2} \tag{A1}$$

where $\alpha = r^\star/r_m$. Other functions with a sharper step at the critical value $r = r_m$ would in principle yield the same result. The average exchange frequency may be calculated in complete analogy to the method developed in ref. 9 (cf.Eqns 25, 26, 27 and 28a). The only difference is that in the present case two types

of clusters for which $\hat{W}_{ex} = \hat{W}^0_{ex}$ and $\hat{W}_{ex} = \hat{W}^0_{ex} \frac{r_c^1}{1+r_c^1}$ have to be considered.

The clusters are again divided in a core characterized by an exchange frequency $W_{ex} = \hat{W}_{ex}$ and a belt of width d_c where $W_{ex} = 1/2\ \hat{W}_{ex}$. Eqn 24 of ref. 9 is then valid for each type of cluster. By inserting the effective areas $\pi\Omega_1{}^{\star 2}$ and $\pi\Omega_2{}^{\star 2}$, in this equation the average exchange frequency is then given by

$$W_{ex}\{(\Omega_1^\star)^2\rho_m + (\Omega_2^{\star 2})\} = W_{ex}\Omega_2{}^2$$

$$= \Omega_2{}^2 \hat{W}^0_{ex} \left\{ \frac{\rho_m + \alpha^2}{1+\alpha^2} - \frac{d_c}{\Omega_2} \frac{\sqrt{\rho_m}^3 + \alpha}{\sqrt{1+\alpha^2}} \right\} \tag{A2}$$

In this equation the following abbreviations

$$\rho_m = \frac{r_m}{1+r_m}\ ;\quad \alpha = \frac{r^\star}{r_m} = \frac{r}{r_m(1-R)}$$

have been used. The concentration dependence of Ω_2 is given by Eqn 9. As an example W_{ex} has been calculated as a function of R for $r = 0.25$, $r_m = 0.33$ and for a value of the cluster density $n = 3.3 \cdot 10^{11}$ cm^{-2}. The curve has been plotted in Fig. 7 (broken line). Reasonable agreement between the calculated and the experimental plots of W_{ex} versus R is obtained.

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