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Calcium-Induced Phase Separations in Phosphatidylserine–Phosphatidylcholine Membranes†

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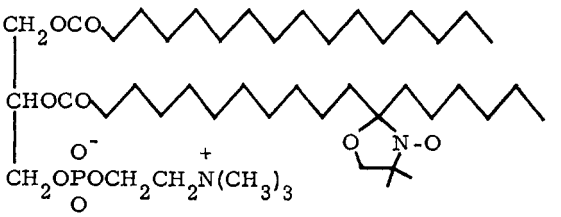
ABSTRACT: The effect of calcium ion on phosphatidylserine (PS)–phosphatidylcholine (PC) membranes has been studied using PC spin labels (PC*). From the exchange broadening in the electron spin resonance spectrum, it is directly demonstrated that calcium ion induces phase separation of PS–PC* bilayer membranes into a solid phase of PS aggregates bridged by calcium chelation and a fluid phase of PC* molecules. The phase separation is rapid and reversible. In the calcium-chelated PS aggregates, the lipid motions are considerably frozen and the lipid molecules are more closely packed. The surface of the aggregates is hydrophobic. The fluid PC* molecules form patches and their sizes make a distribution depending on the PC* contents. Barium and strontium ions induce similar phase separation, whereas the magnesium ion is completely ineffective and only causes slight motional freezing of lipid alkyl chains. Magnesium retards PS aggregation by calcium. A local anesthetic, tetracaine, also shows this type of antagonistic effect. Tetracaine replaces calcium ions bound to PS aggregates, leading to their disaggregation, whereas magnesium shows only slight replacements. Conditions for the phase separation are discussed from the present results. The phase separation will also occur in lipid portions of biological membranes containing PS and would affect lipids as well as proteins in the membrane. Possible involvements of this phenomenon in nerve excitation and cell adhesion are discussed.

The important roles of calcium ion in cellular functions involving membranes have long been recognized and well described. Among others, the essential role of calcium in nerve excitability was reported by Locke as early as 1894. Later works emphasized the effect and detailed analysis has been done with squid giant axons (Frankenhaeuser and Hodgkin, 1957; Tasaki, 1968). Calcium requirement in cell adhesion is also well recognized and its function in adhesiveness between the cells of amphibian gastrulae has been analyzed (Steinberg, 1962). However, the molecular basis underlying these and other calcium-requiring physiological phenomena is not yet clear and efforts for claifying molecular mechanism are being accumulated.

We have carried out investigations along this line and found calcium-induced phase separation in phospholipid membranes such as phosphatidylserine (PS)–phosphatidylcholine (PC) and phosphatidic acid (PA)–PC membranes using phospholipid spin labels PC* and PA*.[1] In the present paper, we present results on the PS–PC membrane using mostly 12PC* (see formula). A brief account of this finding has already been



12PC*

published (Ohnishi and Ito, 1973). The phase separation is directly indicated by calcium-induced exchange broadening in electron spin resonance (esr) spectra of PS–PC* membranes. This is a rapid and reversible two-dimensional phase separation into solid PS aggregates bridged by calcium chelation and a fluid phase consisting of PC*. Barium and strontium ions showed similar effects, whereas magnesium ion was completely ineffective for the phase separation. Antagonistic effects of magnesium and of a local anesthetic tetracaine on the phase separation were shown. These molecular events must be deeply connected with physiological phenomena and its biological significance is discussed from the fluid nature of biological membranes recently developed (Frye and Edidin, 1970; Scandella *et al.*, 1972; Singer and Nicolson, 1972). The interactions of divalent cations with phospholipids have

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[1] Abbreviations used are: PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid; 12SAL and 5SAL, 4′,4′-dimethyloxazolidine-*N*-oxyl derivatives of 12- and 5-keto stearic acids, respectively; 12PC* and 5PC*, spin-labeled phosphatidylcholines where the β-fatty acid chains were replaced with 12SAL and 5SAL, respectively; PA*, phosphatidic acid spin labels.

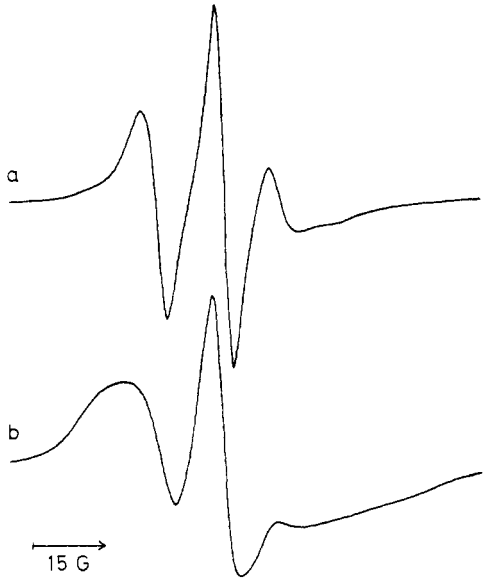

FIGURE 1: Effect of calcium ion on the esr spectrum of the PS–12PC* (50:1) membrane: (a) the spectrum in the presence of 10 mM EDTA and 100 mM KCl; (b) the spectrum measured after 10 min of soaking in 10 mM $CaCl_2$ and 100 mM KCl. The salt solutions are buffered with 50 mM Tris-HCl (pH 7.1).

been studied by measuring surface properties of monomolecular films and by measuring various properties of phospholipid vesicles (for example, Dawson and Hauser, 1970). Some of these results are discussed in relation to the present finding.

Experimental Procedure

In the present investigation we prepared phospholipid membranes impregnated in Millipore filter SMWP 02500 with an average pore diameter of 5 μ (Millipore Filter Corporation). This system was first used by Tobias *et al.* (1962) for electrical measurements. Chloroform solutions of lipid and lipid labels were pipetted into a small glass tube with tapered joint. The solvent was evaporated to dryness first by nitrogen stream and then by evacuation. Benzene was added to the dried lipid mixture and the filter, cut in pieces of ~0.5 cm × 0.4 cm, was soaked. After 10 min of soaking, the filters were taken from the solution and dried for 2 hr. Typically, 10 pieces of filters were soaked in 0.2 ml of benzene solution of lipids at a concentration of 50 mg/ml. The average amount of impregnated lipids was 0.33 mg/piece. The dried filters were then soaked in 100 mM KCl solution for at least 2 hr for conditioning. The conditioned filters were transferred to 5 ml of desired salt solutions and their esr spectra were measured. All the solutions contained 100 mM KCl in addition to the desired salts. The pH of salt solutions in distilled water ranged from 5 to 6. For experiments at neutral pH, salts were dissolved in 100 mM KCl–50 mM Tris-HCl buffer. $CaCl_2$, $MgCl_2 \cdot 6H_2O$, $SrCl_2 \cdot 6H_2O$, and EDTA were Guaranteed Reagent obtained from Nakarai Chemical Co. Ltd. $BaCl_2$ was Suprapur reagent from Merck.

Spin-labeled phosphatidylcholines, 12PC* and 5PC*, were prepared by acylation of egg lysolecithin with the anhydride of spin-labeled stearic acids, 12SAL and 5SAL,[1] respectively, according to the procedure described by Hubbell and McConnell (1971). 12SAL and 5SAL were synthesized by the method of Waggoner *et al.* (1969) and Jost *et al.* (1971), respectively. PC from egg yolk and PS from beef brain white matter were prepared following the procedure of Singleton *et al.* (1965) and Sanders (1967), respectively. The purity of these lipid samples was checked with thin layer chromatography. Phosphatidylethanolamine (bacterial), phosphatidylinositol (plant), and cardiolipin (bovine) were purchased from Applied Science Laboratories and used without purification.

The lipid-impregnated filter was put in a tissue cell with the remaining mother liquor and the esr spectra were measured with a commercial X-band spectrometer equipped with variable temperature accessories (JEOLCO Model ME-2X). The mother liquor did not show esr signals. Unless otherwise stated, soaking of the filters and esr measurements were made at 25°.

Results and Discussion

Phospholipid Membranes Impregnated in Millipore Filter. The lipid-impregnated filter, after conditioning in 100 mM KCl, gave essentially the same esr spectra as those of sonicated lipid vesicles. The spectra are well described by axially symmetric spin Hamiltonian (Hubbell and McConnell, 1971). As the temperature rises, the parallel principal value $T_{\parallel}$ decreases and the perpendicular value $T_{\perp}$ increases. In a plot of the order parameter against temperature, a certain critical temperature was often clearly observed: 40° for PC–5SAL, 53° for PC:cholesterol (2:1)–5SAL, 50° for PC–5PC*, and about 40° for PS–5SAL membranes. Some phospholipid membranes in the filter showed anisotropic spectra. For example, when the magnetic field was applied perpendicularly to the filter impregnated with PC:5PC* (200:1), the peak heights at $T_{\parallel}$ positions were larger and those at $T_{\perp}$ positions were smaller than those measured with the field applied in the plane. This anisotropy indicates preferred orientation of the alkyl chains of PC molecules perpendicular to the filter plane. These results are taken to suggest that phospholipids impregnated in the Millipore filter are in the lamellar bilayer structure.

The electric resistance of PS-impregnated filter was several ohms per square centimeter, far smaller than that of the lipid black membrane, 10^7 ohm cm^{-2} (Papahadjopoulos and Ohki, 1969). The difference may suggest that the ions do not move across the bilayers in the filter but move along water channels between the hydrophilic surfaces of the bilayer.

The lipid-impregnated filters, before conditioning in 100 mM KCl, gave broader esr spectra. Soaking in 100 mM KCl rapidly changed the spectra to sharper ones and, after 30 min, the spectra became indistinguishable from those conditioned for a longer time.

Calcium-Induced Phase Separation in PS–PC Membrane as Evidenced by Exchange Broadening in Esr Spectra.* When the conditioned PS–PC* membrane was soaked in aqueous salt solution containing 10 mM $CaCl_2$, its esr spectrum immediately became exchange broadened. Figures 1 and 2 illustrate the dramatic change in esr spectra of PS–12PC* membranes (50:1 and 9:1, respectively), upon transferring to the calcium solution. The spectra were measured after 10 min of soaking and did not change appreciably on further soaking. The calcium-induced change was independent of pH in the range of 5–8. Calcium did not affect PC–PC* membranes at all. The exchange-broadened spectra were rapidly and completely reversed when the membranes were soaked in 10 mM EDTA solution. The change was completed in less than 1 min. The reverse change was very slow in aqueous KCl solution and the spectrum measured after 1 day was still an exchange-broadened one superimposed with sharper components.

Evidence that the observed changes are mostly due to ex-

change broadening and are not due to motional freezing was presented previously (Ohnishi and Ito, 1973). For example, a general feature of the calcium spectrum of the PS–PC* (9:1) membrane (Figure 2b) is quite similar to the model spectrum of PC–PC* (1:1) or PS–PC* (1:1) in the absence of calcium (dotted spectrum in Figure 2b). The broadening is dependent on the PC* contents in PS–PC–PC* membranes where the PS:(PC + PC*) ratio was kept constant. For example, the 100:0:2 PS–PC–12PC* membrane showed a broader spectrum than the 100:1:1 membrane, demonstrating that the broadening arises from intermolecular effects. Esr spectra of concentrated lipid labels in phospholipid membranes have been studied by Sackman and Träuble (1972a–c) and by Devaux *et al.* (1973). The intermolecular dipolar effects may also contribute to some extent to the observed broadening.

The exchange broadening arises when the labels come close together and the intermolecular spin–spin exchange interaction J becomes larger. Therefore, the observed broadening directly indicates calcium-induced clustering of PC* molecules in the PS–PC* membrane. The PS molecules, on the other hand, form solid aggregates bridged by intermolecular calcium chelation. The rapid PS aggregation or disaggregation and concomitant PC* clustering and spreading are made possible by the fast lateral diffusion of lipid molecules in the bilayer (Kornberg and McConnell, 1971; Devaux and McConnell, 1972).

The exchange broadening was dependent on the PS:PC* ratio, being larger for membranes with higher PC* contents. An example of such differences is seen by comparing the spectrum of the 9:1 PS–PC* (Figure 2b) with that of the 50:1 membrane (Figure 1b). The membrane with still lower PC* contents (100:1) showed smaller exchange-broadened background and higher central peaks. These spectra looked different from those obtained with the model homogeneous membranes in their higher central peaks. The calcium spectra can be interpreted as a superposition of variously exchange-broadened spectra with J values ranging from ~ 0 to a maximum. The distribution shifts to smaller J values for membranes with lower PC* contents since these membranes had higher central peaks. Such a difference in J values must result from formation of PC* patches and their size distribution. The distribution is probably a function of the fluid fraction, and, therefore, of the PC* contents and membranes with lower PC* contents would have smaller patches. The effective J becomes smaller in the smaller patches since the interaction between PC* and the surrounding nonparamagnetic PS molecules becomes more frequent. In the membranes with lower PS contents, the calcium-chelated PS aggregates would be distributed in patches in fluid PC medium.

In the calcium-chelated PS aggregates, the lipid motions are frozen to a considerable extent. For example, the stearic labels in PS membranes were markedly immobilized by the addition of calcium. The increase in the $2T_{\parallel}$ value and decrease in the $2T_{\perp}$ value of the PS–5SAL (100:1) membrane were from 46.1 to 54.6 G and from 20.4 to 18.7 G at 30°, respectively. The order parameter was therefore increased from 0.51 to 0.70 (37.6%) by calcium. In this case, the major change in the esr spectrum was due to motional freezing of the labels and exchange broadening was not appreicable. As will be reported in a forthcoming paper (T. Ito and S. Ohnishi, submitted, 1974[2]), PA behaved quite similarly to PS and the spectra of PA–12PC* (9:1) and 12PA*–PC (1:9) membranes became exchange broadened upon the addition of

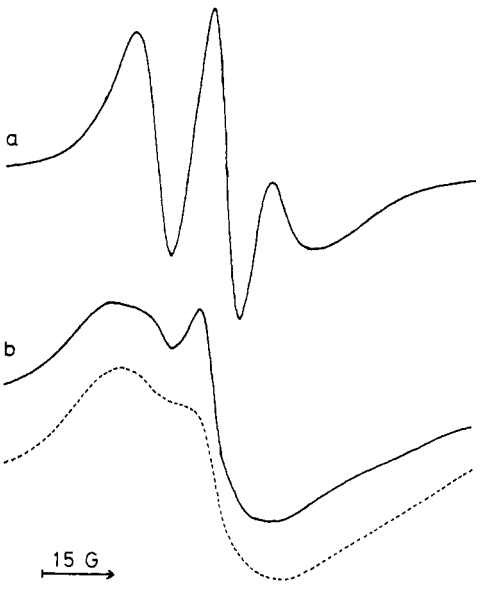

FIGURE 2: Effect of calcium ion on the esr spectrum of the PS–12PC* (9:1) membrane: (a) the spectrum in the presence of 10 mM EDTA and 100 mM KCl; (b) the spectrum measured after 10 min of soaking in 10 mM $CaCl_2$ and 100 mM KCl buffered with 50 mM Tris-HCl (pH 7.1). The dotted spectrum in b is for the PC–12PC* (1:1) membrane in 100 mM KCl–50 mM Tris-HCl buffer.

calcium. The motional freezing in calcium-chelated PA aggregates was directly indicated from the calcium spectrum of the PA–12PA* (50:1) membrane. The motional freezing must result from closer packing of lipid molecules in the bilayer structure. This is consistent with the observation by Papahadjopoulos (1968) that calcium ion decreased the surface pressure of the monomolecular film of PS and that of PA at pH 7.9. He proposed a molecular model for PS–Ca^{2+} and PA–Ca^{2+} complexes consisting of a linear polymeric arrangement. The enthalpy decrease due to the aggregate formation such as intermolecular calcium chelation and the increase in van der Waals interaction among closely packed alkyl chains would surmount the entropy decrease resulting from phase separation. The motive force for the phase separation is, therefore, formation of energy-gaining Ca–PS aggregates in the two-dimensional bilayer. Phospholipids must contain at least two chelating sites in the head group to develop the chelated structure. Calcium ion induced phase separation in the PA–PC membrane, whereas the ion caused only slight motional freezing of lipid alkyl chains and failed to induce phase separation in phosphatidylinositol–PC, phosphatidylethanolamine–PC, and cardiolipin–PC membranes. Phosphatidylinositol with only one anionic site cannot make the aggregates in the bilayer. Cardiolipin contains two anionic sites but they may be unable to develop the aggregate structure for steric reasons since the lipid has two anionic sites per four alkyl chains.

The calcium effect was dependent on the concentration. Figure 3 shows esr spectra of the PS–12PC* (50:1) membrane measured after 10 min of soaking in a 100 mM KCl solution containing 0.1–10 mM $CaCl_2$. At higher calcium concentrations (≥ 2 mM) the membranes showed almost the same exchange broadening, whereas at lower concentrations the broadening was smaller and increased with concentration. The broadening at lower concentrations, however, markedly progressed with time and the spectra measured after 18 hr of soaking approached those at higher concentrations. As a convenient measure for the broadening, we took the width at

[2] Submitted for publication to *Biochim. Biophys. Acta.*

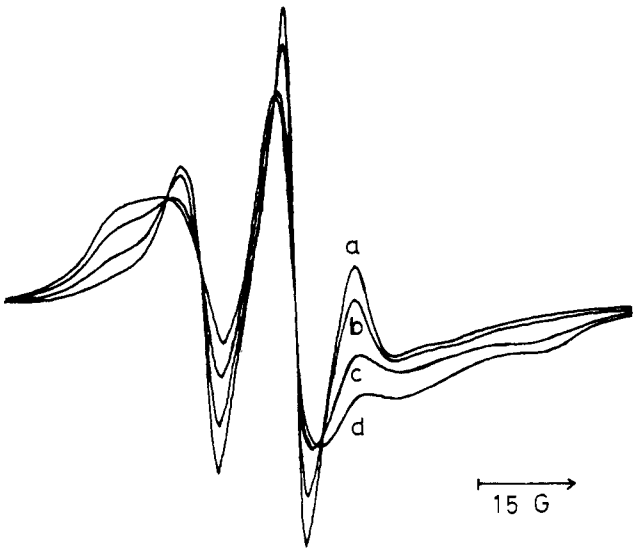

FIGURE 3: Esr spectra of the PS–12PC* (50:1) membrane soaked for 10 min in 100 mM KCl and $CaCl_2$ of various concentrations: (a) 10 mM EDTA; (b) 0.5 mM $CaCl_2$; (c) 1 mM $CaCl_2$; (d) 10 mM $CaCl_2$. The solution pH is 5–6.

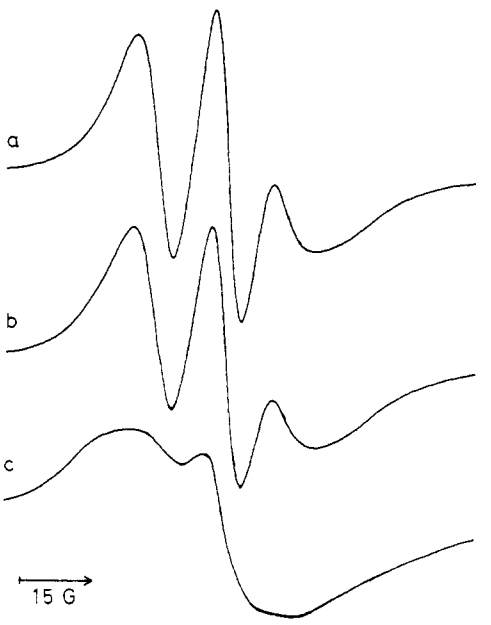

FIGURE 5: Time course of the esr spectrum of PS–12PC* (9:1) membrane after soaking in 0.5 mM $CaCl_2$ and 100 mM KCl buffered with Tris-HCl (pH 7.1): time (a) 0; (b) 10 min; (c) 4.5 hr.

half-height for the positive part of the low-field derivative line and plotted it as a function of calcium concentration in Figure 4. It is seen that ~1.2 mM $CaCl_2$ gave half of the effect after 10 min of soaking and the half-concentration shifted to smaller values with time. The same concentration dependence and time course were also observed with the PS–12PC* (9:1) membrane. Figure 5 clearly demonstrates development of exchange broadening with time at a low concentration of 0.5 mM. After 10 min of soaking, the broadening progressed to a similar extent as that of the model membrane PC–12PC* (4:1) and reached saturation after 4.5 hr. The broadening developed faster in 1 mM $CaCl_2$.

These results indicate that the rate of PS aggregation depends on the calcium concentration; it is slower with a lower concentration. The relevant processes are calcium binding to the head group of PS molecules and aggregation of calcium-bound PS molecules. The observed dependence is perhaps due to a difference in the concentration of Ca–PS molecules in the filter. Of course, the equilibrium concentration should be determined by the association constant which was estimated to be as large as 10^4 (Barton, 1968) and the spectra approached those at higher calcium concentrations with time. While the aggregation does not develop well, PC* molecules are concentrated in a fluid medium consisting of unaggregated Ca–PS and PS molecules. Such concentrating stages are indicated by the spectra measured after 10 min of soaking in 0.5 mM $CaCl_2$ (Figure 5b). Figure 6 schematically summarizes the calcium-induced phase separation as deduced from the present esr results.

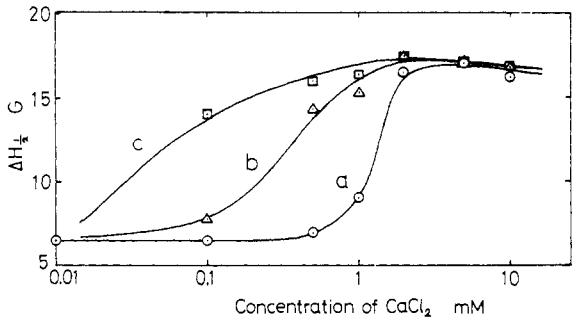

FIGURE 4: Concentration dependence of calcium-induced broadening in the esr spectrum of the PS–12PC* (50:1) membrane. The membranes are soaked for (a) 10 min, (b) 4 hr, and (c) 18 hr in 100 mM KCl and $CaCl_2$ of various concentrations buffered with 50 mM Tris-HCl (pH 7.1); $\Delta H_{1/2}$, width at half-height for the positive part of the low-field derivative peak.

The conditioned PS–PC* filters are hydrophilic so that when a small drop of mother liquor was put on the filter the drop was immediately flattened. However, the filter after soaking in $CaCl_2$ solution became repulsive to water drop. This change is correlated to the formation of calcium-chelated PS aggregates in the membrane. The hydrophilic to hydrophobic change has been observed by Tobias *et al.* (1962) with Millipore filters impregnated with animal cephalin.

The sonicated PS–PC* vesicles showed essentially the same change in their esr spectra as those of lipid-impregnated filters. The spectrum of PS–12PC* (9:1) vesicles rapidly changed to the exchange-broadened one upon addition of 6 mM $CaCl_2$. In this case, the clear dispersion became immediately turbid and precipitation occurred. Such coagulation of PS vesicles has been noted by Abramson *et al.* (1964) and by Papahadjopoulos and Miller (1967). The exchange-broadened signal was found in the washed precipitate. On addition of 10 mM EDTA, the precipitate became rapidly solubilized and the solution appeared as a clear dispersion. The esr spectrum was also reversed in less than 1 min. The precipitation is probably caused by intervesicular adhesion at the surface of the calcium-chelated aggregates in PS–PC* membrane. The hydrophobic interaction would also contribute to the adhesion.

Effect of Various Divalent Cations on the PS–PC Membrane.* The effects of Mg^{2+}, Ba^{2+}, and Sr^{2+} ions were investigated for comparison with the effect induced by Ca^{2+}. Figure 7 shows the esr spectra of the PS–12PC* (9:1) membrane measured after 10 min of soaking in aqueous KCl solution containing 50 mM of divalent cations. It is clearly indicated that Ba^{2+} and Sr^{2+} caused exchange broadening. The broadening, however, was smaller than that induced by Ca^{2+},

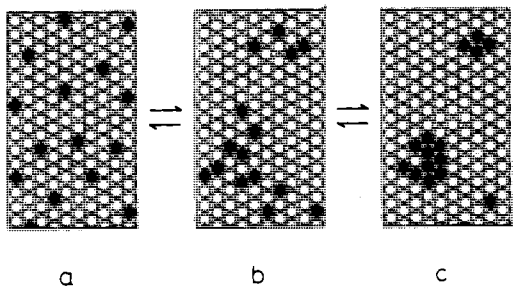

FIGURE 6: Schematic representation of calcium-induced phase separation in phospholipid membranes viewed from the top. The membrane rapidly changes to the right from a to c on addition of Ca^{2+} and returns to the left from c to a on removal of Ca^{2+} by EDTA; b is an intermediate stage. The ratio of the open circle (either PS or PC) to the filled circle (either PC or PS) is set to 9:1.

decreasing in the order $J_{Ca} > J_{Ba} > J_{Sr}$. Mg^{2+} showed a marked contrast to the other divalent cations in that the ion did not cause exchange broadening. The spectrum was almost superposable on the control spectrum in the absence of Mg^{2+} (Figure 7a). The magnesium ion caused only slight motional freezing of lipid alkyl chains. This type of effect is easier to observe with more dilute membranes and with labels having the nitroxide closer to the hydrophilic group. Figure 8 shows the result with the PS–5PC* (50:1) membrane. $2T_{\parallel}$ increased from 57 to 59 G and $2T_{\perp}$ decreased from 16.9 to 16.1 G at 22°. The increase in the order parameter was from 0.74 to 0.79. The anisotropy of the esr spectrum was also enhanced by the presence of Mg^{2+} (see Figure 8b). The magnesium effect occurred at a higher concentration than that of calcium, a half-increase in the $2T_{\parallel}$ value being observed at 6.5 mM $MgCl_2$ in the 100 mM KCl solution. The motional freezing and the increased anisotropy are related to the observation by Butler *et al.* (1970) that the ordering of alkyl chains of brain lipids increased on addition of mono- and divalent cations.

Magnesium ion retarded the calcium-induced PS aggregation. Figure 9 shows the spectrum of the PS–12PC* (50:1) membrane soaked in aqeous KCl solution containing both $MgCl_2$ (50 mM) and $CaCl_2$ (10 mM). After 10 min, the spectrum appeared to consist of two components, the exchange-broadened background superimposed with sharper components. This is to be compared with the spectrum in the presence of 10 mM $CaCl_2$, which consisted only of the broadened

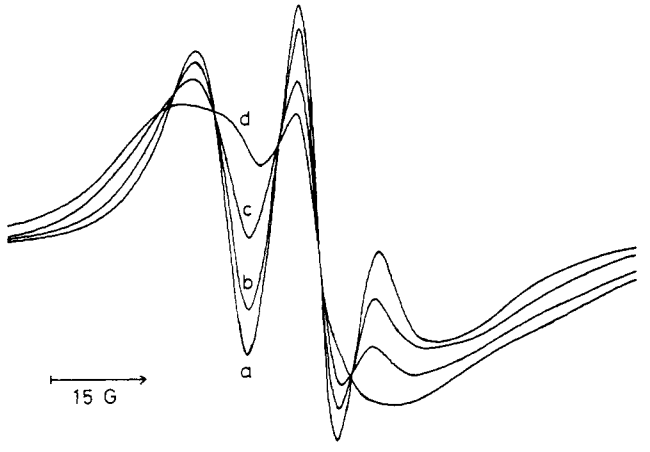

FIGURE 7: Effect of various divalent cations on the esr spectrum of the PS–12PC* (9:1) membrane. The spectra are measured after 10 min of soaking in (a) 10 mM EDTA; (b) 50 mM $SrCl_2$; (c) 50 mM $BaCl_2$; (d) 10 mM $CaCl_2$. The solutions contain additionally 100 mM KCl and 50 mM Tris-HCl (pH 7.2).

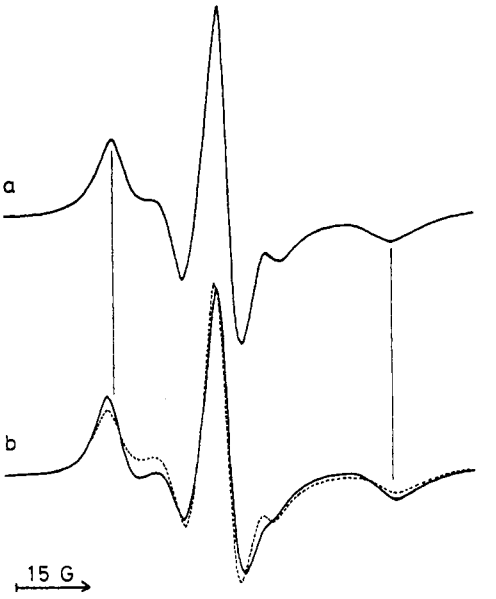

FIGURE 8: Effect of magnesium ion on the esr spectrum of the PS–5PC* (50:1) membrane: (a) in 100 mM KCl; (b) in 50 mM $MgCl_2$ and 100 mM KCl. The full line and the dotted line spectra in b are measured with the external field applied perpendicular to the lipid-impregnated filter plane and in the plane, respectively; pH 5.5; temperature, 22°.

component. After 1 hr of soaking in the Mg^{2+}–Ca^{2+} solution, the sharper components decreased and the spectrum became similar to the calcium spectrum. The sharper components can be assigned to PC* molecules in Mg^{2+}-bound PS and in unaggregated Ca^{2+}-bound PS molecules. The coexisting magnesium thus retards the aggregation by competing in the binding to PS.

When the PS–PC* membrane was first soaked in 10 mM $CaCl_2$ and then transferred to 50 mM $MgCl_2$ solution, the exchange broadening was slightly reduced after 1 hr and the spectrum still remained exchange broadened after 1 day.

The marked ionic selectivity in the phase separation of the PS–PC membrane arises from the enthalpy requirement. The divalent cations must be able to form the chelated structure

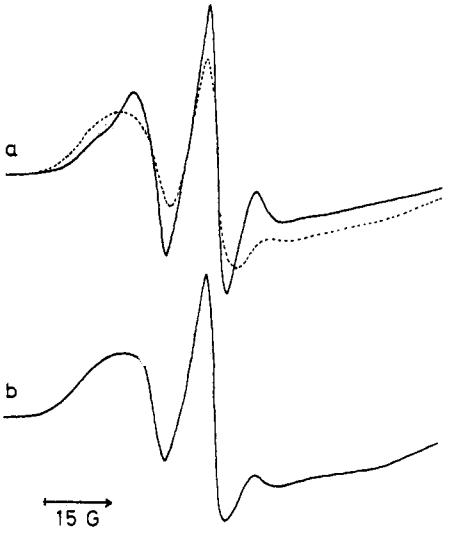

FIGURE 9: Esr spectrum of the PS–12PC* (50:1) membrane in the presence of both $CaCl_2$ and $MgCl_2$. The spectra are measured after soaking for (a) 10 min and (b) 1 hr in 10 mM $CaCl_2$, 50 mM $MgCl_2$, and 100 mM KCl buffered with 50 mM Tris-HCl (pH 7.3). The dotted spectrum is for the membrane soaked in 10 mM $CaCl_2$.

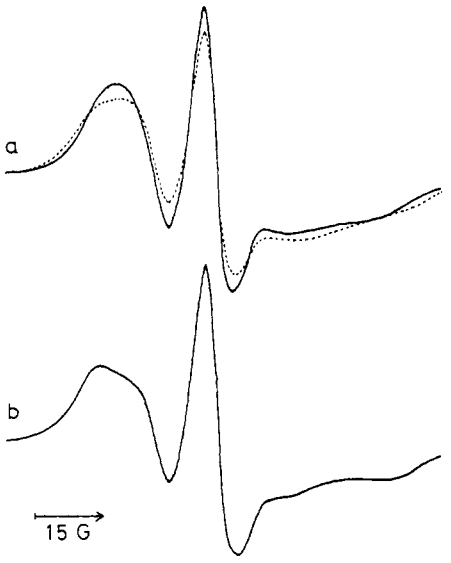

FIGURE 10: Esr spectrum of the PS-12PC* (50:1) membrane in the presence of both $CaCl_2$ and tetracaine. The spectra are measured after soaking for (a) 10 min and (b) 4.5 hr in 10 mM $CaCl_2$, 10 mM tetracaine, and 100 mM KCl buffered with 50 mM Tris-HCl (pH 7.8). The dotted spectrum is for the membrane soaked in 10 mM $CaCl_2$. Some precipitation of tetracaine was observed at this pH.

with the head groups of more closely packed PS molecules. Differences in the ionic radius of the cations may be responsible for the selectivity. Magnesium ion may be unable to form the aggregate structure with the anionic sites of the PS head group, phosphate, and carboxyl on the bulky tail. The phase separation of the PA–PC* membrane was induced by Mg^{2+} as well as Ca^{2+}, Ba^{2+}, and Sr^{2+}, although the exchange broadening caused by Mg^{2+} was smaller (T. Ito and S. Ohnishi, submitted, 1974[2]). Magnesium can therefore form the chelated structure with the head group of PA consisting only of phosphate with two chelating sites and having no bulky tail. Differences in the exchange broadening of the PS–PC* membrane induced by Ca^{2+}, Ba^{2+}, and Sr^{2+} should be sought to differences in the phase diagram, not only in the fluid composition but also in the fluid patch sizes.

Effect of a Local Anesthetic Tetracaine on Calcium-Induced Phase Separation. Tetracaine acted antagonistically to the calcium-induced phase separation. It rapidly disaggregated the calcium-chelated PS aggregates. When the PS–PC* membrane soaked in 10 mM $CaCl_2$ was transferred to 10 mM tetracaine, the exchange broadening was rapidly reduced and the spectrum measured after 2 hr was almost reversed to that in the presence of 10 mM tetracaine only. This is replacement of the bound Ca^{2+} by tetracaine. In the absence of Ca^{2+}, tetracaine caused slight motional freezing of lipid alkyl chains, the $2T_{\perp}$ value of the PS-12PC* (50:1) membrane being decreased by 3.6%.

When the PS–PC* membrane was soaked in medium containing both $CaCl_2$ (10 mM) and tetracaine (10 mM), development of the exchange broadening was retarded at pH 7.8. This is shown in Figure 10a which compares the spectrum measured after 10 min with the calcium spectrum. After 4.5 hr, the spectrum was not yet as broad as that of 10 mM $CaCl_2$ only and, moreover, peaks due to immobilized labels became clearly noticeable (Figure 10b). The latter component may be due to PC* molecules trapped in the solid phase. The coexisting tetracaine thus retards the formation of Ca–PS aggregates and also apparently modifies the phase diagram.

A marked difference in the antagonistic actions of tetracaine and magnesium is that tetracaine strongly replaced Ca^{2+} bound to PS aggregates, leading to their disaggregation, whereas magnesium was very weak in the replacement. It is also noted that the esr spectrum in the presence of both tetracaine and Ca^{2+} was different from that in the presence of both Mg^{2+} and Ca^{2+} (compare Figure 9 with Figure 10).

Effects of local anesthetics on phospholipids and biological membranes have been studied with various spin labels (Hubbell and McConnell, 1968; Hubbell *et al.*, 1970; Trudell *et al.*, 1973; Butler *et al.* 1973). All these results indicated an increase in the fluidity of the membranes by anesthetics except for brain lipids with a reduced cholesterol content (Butler *et al.*, 1973). Our results indicated that, in the absence of Ca^{2+}, tetracaine decreased slightly but noticeably the fluidity of the PS–PC* membrane. This is probably reflecting the specific nature of PS in the interaction. Formation of a specific complex of PS–tetracaine (2:1) has been suggested (Feinstein, 1964). More interesting and perhaps more physiologically significant is the finding that tetracaine disaggregates the Ca–PS aggregates. The inhibitory effect of calcium for the blocking action of local anesthetics on nerve conduction has been established (for example, Blaustein and Goldman, 1966). Displacement of Ca^{2+} bound to PS by local anesthetics has also been demonstrated (Feinstein, 1964).

Biological Significance

The present results indicate that, when phospholipid bilayer membranes containing PS are exposed to aqueous calcium solution, rapid and reversible phase separations occur in the membranes into a solid phase of calcium-chelated PS aggregates and a fluid phase of neutral lipids such as PC. The phase separations will modify various properties of the lipid membranes. Obviously, the lateral diffusion in the aggregates is greatly reduced since the lipids are more closely packed and the motions of alkyl chains are considerably frozen. The transverse movement of molecules and ions across the aggregates will also be reduced. The electric resistances of PS black films (Papahadjopoulos and Ohki, 1969) and of Millipore filters impregnated with animal cephalin (Tobias *et al.*, 1962) were actually increased by the addition of calcium. It is also possible that calcium ions may create asymmetric distribution of lipid molecules in vesicular membranes.

The calcium-induced phase separations will also occur in the lipid bilayer portion of biological membranes which contain PS in most cases. Formation of the aggregates or patches will not only affect the lipid portions but also influence the lateral and transverse motions of proteins in the membranes. When some proteins are interacting with PS more preferentially, the effects on the protein activities would be more direct. The notion that the lateral phase separations may affect transport both parallel and perpendicular to membrane surfaces is supported and generalized by a recent observation by Shimshick and McConnell (1973). These authors have found phase separations in binary phospholipid mixtures in the absence of calcium.

Finally, we point out possible participation of this phenomenon in some specified biological events. Tasaki *et al.* (1967) showed that the external $CaCl_2$, $BaCl_2$, or $SrCl_2$ was essential for developing action potential in the squid giant axon, whereas $MgCl_2$ was completely ineffective. The ionic selectivity is remarkably parallel to that for inducing phase separations in membranes containing PS. Moreover, Cook *et al.* (1972) showed involvement of PS in nerve excitation by enzymatic conversion of PS and its reversal. The monovalent

cations diffused into the excitable membranes would disaggregate the calcium-chelated PS aggregates in a cooperative way and increase ionic permeability across the lipid bilayer portions or modify the ion-transporting proteins associated with the PS aggregates. The calcium-chelated ordered state and the disaggregated disordered state might be related to the two states in the theory of Tasaki (1968). In the cell adhesions, the hydrophobic interactions at the surfaces of Ca–PS aggregates would be important at least partly and location of the patches on the cell surfaces might be connected to the specificity in the intercellular interaction.

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