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Synthesis of a New Phosphatidylserine Spin-Label and Calcium-Induced Lateral Phase Separation in Phosphatidylserine–Phosphatidylcholine Membranes†

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ABSTRACT: A new phosphatidylserine spin label with nitroxide stearate attached at the 2 position has been synthesized by the reaction of spin-labeled CDP-diglyceride with L-serine under the catalytic action of phosphatidylserine synthetase. Some structural properties of pure phosphatidylserine (PS) and binary PS–phosphatidylcholine (PC) membranes were studied with the spin label. PS membrane became solidified on lowering solution pH, 50% solidification being attained at pH 3.5. The membrane was also solidified by addition of Ca^{2+}. The effect of Ba^{2+}, Sr^{2+}, and Mg^{2+} was smaller than that of Ca^{2+}. The calcium-induced lateral phase separation in the binary membrane was studied from the side of the calcium-receiving lipid. The results confirmed and extended our previous conclusion drawn with PC spin label. The phase diagram of the binary membrane in the presence of Ca^{2+} was determined. Not all PS molecules were aggregated to form the solid patches but some remained dissolved in the fluid PC matrix. The fluid PS fraction was larger for the membranes containing more PC. The membrane with 10% PS still had a significant fraction of solid phase. The rate of calcium-induced aggregation was greatly dependent on the PS content. The aggregation was almost complete within 5 min in the membrane containing 67% PS, while it was still proceeding after several hours in the membrane with 20% PS. The rate-limiting step was suggested to be in the formation of "stable" nuclei consisting of larger aggregates. The possible biological significance of the ionotropic phase separation was discussed whereby a transient density fluctuation was emphasized.

It has been generally accepted that phospholipid molecules arranged in the bilayer structure act as a two-dimensional fluid matrix for the proteins in biological membranes (see, for example, Singer and Nicolson, 1972). The physical properties of the phospholipid bilayer are not only related to the membrane phenomena taking place in that part of the membrane but also have a deep influence on the functional properties of the membrane proteins (Esfahani et al., 1971; Linden et al., 1973; Overath et al., 1971). Biological membranes contain a variety of classes of lipids and each of them appears to play its own characteristic role. Some provide the two-dimensional matrix while the others are involved more directly in the functions. It has been shown that phosphatidylserine (PS[1]) and phosphatidic acid (PA) responded to Ca^{2+} characteristically (Ohnishi and Ito, 1973, 1974; Ito and Ohnishi, 1974). These phospholipid molecules were aggregated by calcium through intermolecular chelation and segregated from the remaining fluid ma-

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[1] Abbreviations used are: PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid; PS*, PC*, and PA*, spin-labeled PS, PC, and PA, respectively, where acyl chains at the 2 position were replaced with 12-nitroxide stearic acid (4′,4′-dimethyloxazolidine-*N*-oxyl derivative of 12-keto stearic acid).

trix in binary PS–PC and PA–PC membranes. The lateral phase separations were directly demonstrated by calcium-induced exchange broadening in electron spin resonance (ESR) spectra of PS–PC*, PA–PC*, and PA*–PC membranes. The ionotropic phase separations would probably be involved in the calcium-requiring membrane phenomena such as nerve excitation, cell adhesion, cell fusion, hormone release, etc. PS and PA may thus be functional lipids whereas PC acts as fluid matrix.

For the study of the individual roles of lipids, we have synthesized a variety of new phospholipid spin labels: phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylserine spin labels. In the present paper, we report the synthesis of the PS spin label (see Figure 1) and its use in the study of calcium-induced phase separation in binary PS–PC membrane. This is to study the phase separation from the side of phospholipid molecules to be aggregated by calcium and complements our previous study that used PC spin label. The present results have confirmed and gave further details on the phase separation.

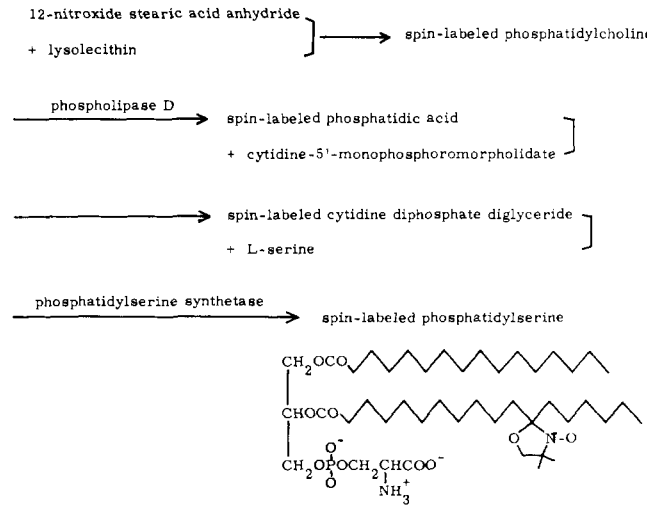

FIGURE 1: Reaction scheme for synthesis of spin-labeled phosphatidylserine.

Materials and Methods

Synthesis of Phosphatidylserine Spin Label. PS* was synthesized by the reaction of spin-labeled CDP-diglyceride with L-serine under the catalytic action of PS synthetase which has been recently isolated in soluble form from *Escherichia coli* B and purified free from PS decarboxylase (Ishinaga and Kito, 1974). The overall reaction scheme is outlined in Figure 1 and detailed procedures for some of these preparations are described below.

(a) 12-Nitroxide stearic acid was prepared by the method of Waggoner et al. (1969) with the following modification in purification and hydrolysis of methyl 12-nitroxide stearate. The crude product (30 g) was applied to a column containing 1.5 kg of activated silicic acid (100 mesh, Mallinckrodt) and eluted with hexane–ether (7:3, v/v). After taking off the early eluate which contained methyl 12-keto stearate, the residual material was rechromatographed over silicic acid with hexane–ether (8:2, v/v) as eluent. The purified product (10 g) gave a single spot with an R_f value of 0.4 on a silica gel H thin-layer chromatogram developed by hexane–ether (7:3, v/v). The purified ester was dissolved in 1 l. of methanol and hydrolyzed by adding 3 g of NaOH dissolved in 20 ml of water and refluxing overnight. The reaction mixture was added with 0.5 *M* HCl until the solution pH was 3.0 and extracted with chloroform. The hydrolysis was complete.

(b) PC* was synthesized by the reaction of egg lysolecithin with the anhydride of 12-nitroxide stearic acid following the procedure described by Hubbell and McConnell (1971).

(c) PA* was prepared by the action of phospholipase D on PC* in a similar manner to that described previously (Ito and Ohnishi, 1974). The method was essentially the same as that of Kornberg and McConnell (1971) used to convert egg PC into PA. A mixture of 0.5 g of PC*, 0.5 g of phospholipase D (obtained from savoy cabbage by the method of Yang (1969)) in 250 ml of 0.1 *M* acetate buffer (pH 5.6), 25 ml of 0.1 *M* $CaCl_2$, and 25 ml of diethyl ether was shaken overnight at 25°. After the reaction, the mixture was added to 8.75 g of citric acid monohydrate, shaken, and centrifuged. The upper ethereal phase was removed and the aqueous phase was extracted three times with 125-ml portions of chloroform. The combined ethereal phase and chloroform extracts were evaporated under reduced pressure. The residue was dissolved in absolute alcohol and the solution was evaporated to remove traces of water. The residue was dissolved in 3.75 ml of chloroform and added dropwise to a solution of 5 g of barium acetate monohydrate in 100 ml of water–methanol (1:1, v/v). The precipitate was collected by centrifugation, dissolved in 5 ml of ether, and reprecipitated by adding 20 ml of acetone. The purified precipitate was dissolved in 60 ml of chloroform, shaken for 10 min at room temperature with 60 ml of 0.5 *N* H_2SO_4, and centrifuged. The lower phase was extracted once more with 60 ml of 0.5 *N* H_2SO_4 and once with 125 ml of methanol–water (1:1, v/v). The upper phase was extracted with 125 ml of chloroform–methanol (1:1, v/v), and the resulting lower phase was extracted with 60 ml of 0.5 *N* H_2SO_4 and washed with water. The lower phases were combined and evaporated under reduced pressure. A trace of water was removed from the residue by adding absolute ethanol and evaporation, and a trace of ethanol was removed by evaporation of toluene, yielding 242 mg of PA*. The product gave one spot on the silica gel H thin-layer chromatogram developed by chloroform–methanol–17 *N* NH_4OH (65:25:4, v/v). The R_f value was almost the same as that of unlabeled PA derived from egg PC.

(d) Spin-labeled CDP-diglyceride was synthesized by the reaction of PA* with cytidine 5′-monophosphoromorpholidate following the procedure described by Aganoff and Suomi (1963) for unlabeled CDP-diglyceride. PA* (220 mg) was dissolved in 10 ml of benzene and frozen in a Dry Ice–acetone bath. Cytidine monophosphoromorpholidate (212 mg) (prepared from cytidine 5′-monophosphate (Kojin), morpholine (Nakarai Chemicals), and dicyclohexylcarbodiimide by the method of Aganoff and Suomi (1963)) was suspended in 10 ml of benzene, added to the frozen mixture, and similarly frozen. The frozen mixture was then lyophilized and, when the benzene was completely removed, 40 ml of anhydrous pyridine was added. The flask was sealed and kept for 65 hr at room temperature. The reaction mixture was evaporated to dryness in vacuo at 30° and 20 ml of ice-cold water was added to the residue. The mixture was brought to pH 4 with 1 *N* formic acid, and 40 ml of methanol and 30 ml of chloroform were added. The upper phase was removed and extracted with 30 ml of chloroform. The extract was combined with the lower phase and 38 ml of methanol–water (2:1, v/v) and then 2 *N* NH_4OH

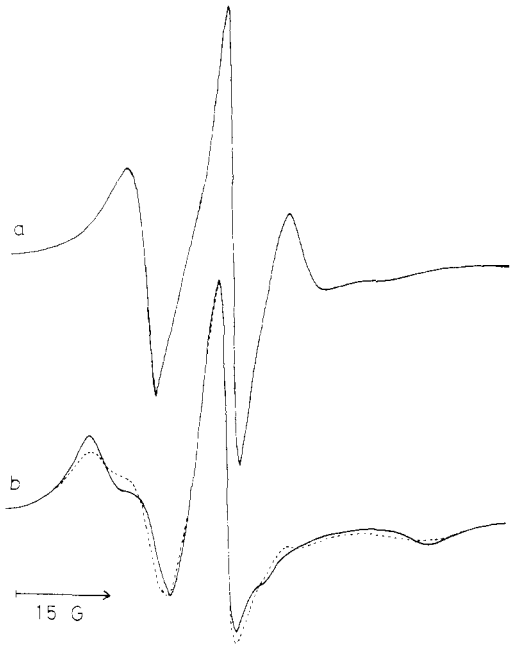

FIGURE 2: ESR spectrum of PS–PS* (100:1) membrane in (a) neutral salt solution (pH 7.2) and (b) acidic solutions (pH 2.1 for the full-line spectrum and pH 3.1 for the dotted-line spectrum). The solution contained 100 m*M* KCl, 10 m*M* EDTA, and 50 m*M* Tris-Cl in a and 100 m*M* KCl and 25 m*M* citrate–KOH in b. Spectra were measured at 23°. Those given in b were measured after soaking the membrane overnight in the acidic solutions.

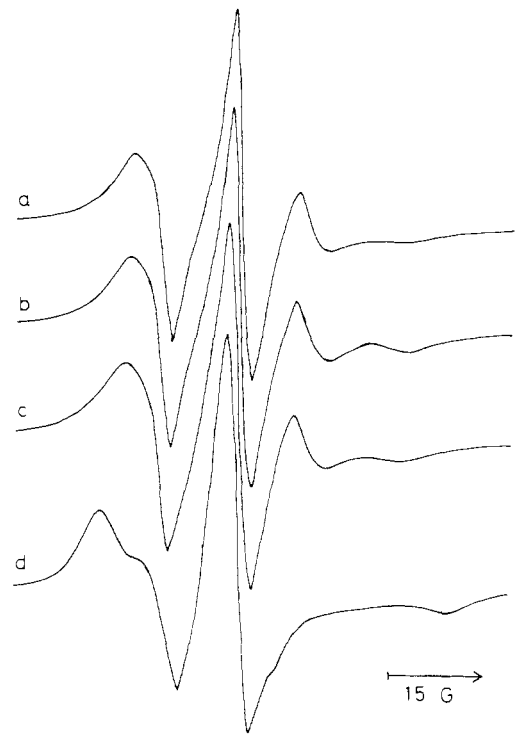

FIGURE 3: ESR spectrum of PS–PS* (100:1) membrane in the presence of (a) $MgCl_2$, (b) $SrCl_2$, (c) $BaCl_2$, and (d) $CaCl_2$. The solution contained 10–50 m*M* of the indicated divalent salt, 100 m*M* KCl, and 50 m*M* Tris-Cl (pH 7.2).

in methanol were added until the pH of the upper phase was 7.6. The lower phase was washed twice with methanol–water and the resulting upper phase was combined with the initial upper phase. The combined aqueous extracts were evaporated to dryness in vacuo and then lyophilized from 10 ml of benzene, yielding 120 mg of spin-labeled CDP-diglyceride. The product contained about 20% of PA* and 30% of water but this was used for the next reaction without further purification.

(e) Finally, PS* was synthesized by the reaction of the spin-labeled CDP-diglyceride with L-serine in the presence of PS synthetase. The reaction mixture contained 100 mg of the crude spin-labeled diglyceride, 5 m*M* L-serine, 0.1% of Cutscum (a polyoxyethylene-type detergent, Fisher Scientific Co.), 1800 units of PS synthetase, and 33 m*M* potassium phosphate buffer (pH 7.2) in a volume of 100 ml. The reaction was allowed to proceed for 40 min at 37° and terminated by the addition of 450 ml of chloroform–methanol (2:1, v/v). After being washed with 800 ml of 2 *M* KCl and 900 ml of water, the chloroform phase was dried in vacuo. Lipids were separated by thin-layer chromatography on silica gel plates with the solvent of chloroform–methanol–acetic acid (65:25:8, v/v). The area of PS was scraped and extracted with chloroform–methanol (2:1, v/v), yielding 21 mg of PS*. The product gave a single spot on a silica gel H thin-layer chromatogram with an R_f value slightly larger than unlabeled PS. The spin-labeled PS was converted to corresponding phosphatidylethanolamine by the action of PS decarboxylase.

Preparation of Phospholipid Membrane. PS was obtained from bovine brain white matter by the method of Sanders (1967) and PC from egg yolk according to the procedure described by Singleton et al. (1965). Phospholipid membrane was prepared on Millipore filter SMWP 02500 with an average pore diameter of 5 μm as described previously (Ohnishi and Ito, 1974). The lipid-impregnated filter, after conditioning, was soaked in aqueous salt solutions and its ESR spectrum was measured at 23° or at various temperatures with a commercial X-band spectrometer (JEOLCO Model ME). All the solutions contained 100 m*M* KCl in addition to the indicated salts. The pH was adjusted with 50 m*M* Tris-Cl buffer (pH 7.2) or with 25 m*M* citric acid–KOH buffer in acidic solution.

Results

ESR Spectrum of PS–PS Membrane and Effect of pH.* The ESR spectrum of PS–PS* membranes showed the characteristics of the labels undergoing axially symmetric rotational motion. The overall splitting, or twice the parallel principal value, was read as 42 G at 23° for the spectrum measured in neutral 100 m*M* KCl solution (Figure 2a). The PS membrane is therefore in a fluid state at that temperature. The overall splitting became markedly larger at temperatures lower than 10°. The change indicates immobilization of the alkyl chains caused by closer packing of lipid molecules and corresponds to transition from the liquid-crystalline to crystalline phase. Papahadjopoulos et al. (1973) measured the transition temperature with differential scanning calorimetry and obtained 13° for vesicles prepared from bovine brain PS.

The ESR spectrum of the membranes was greatly affected by solution pH (see Figure 2b). The overall splitting was markedly increased at pH 2.1. The splitting value 54 G indicates strong immobilization of the lipid alkyl chains. The spectrum at pH 3.1 consisted of two components: one due to the immobilized labels and the other due to fluid phase labels. On increasing the pH further, the fluid component became larger at the expense of the rigid component. At pH 5.2 the spectrum was the same as that in neutral solutions, consisting only of the fluid component. The

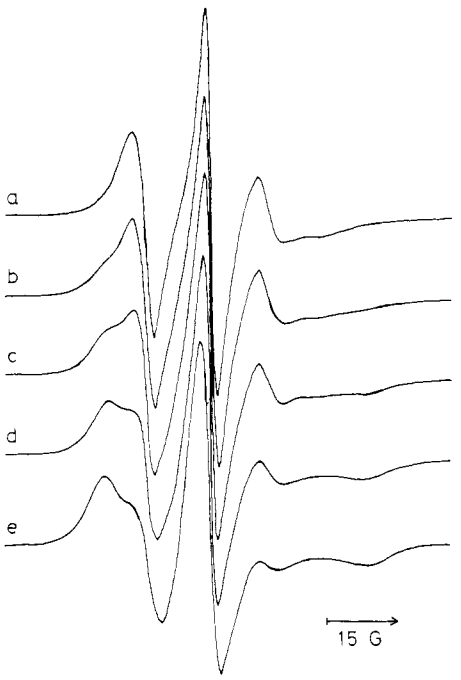

FIGURE 4: ESR spectrum of binary PS-PC membrane containing 0.5% PS* in the presence of Ca^{2+}. The mole fraction of PS in the binary membrane was (a) 0, (b) 0.2, (c) 0.33, (d) 0.5, and (e) 0.67. The spectra were measured at 23° after soaking the membranes for 1 day in 10 m*M* $CaCl_2$, 100 m*M* KCl, and 50 m*M* Tris-Cl (pH 7.2).

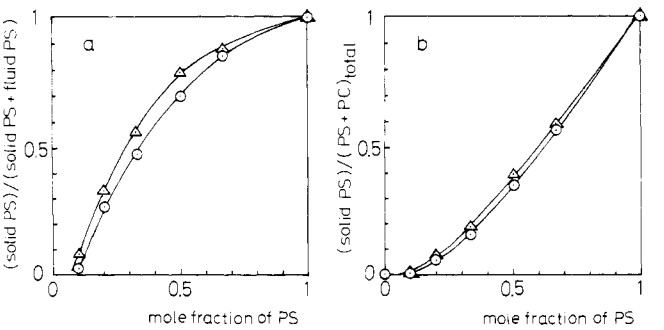

FIGURE 5: (a) Mole fraction of solid phase PS of total PS vs. mole fraction of total PS in binary PS-PC membrane in the presence of Ca^{2+}. The membrane was incubated for 1 day at 23° (○) and at 40° (△) and the ESR spectrum was measured at 23°. The mole fraction of solid phase PS was estimated by the fraction of the rigid component in the ESR spectrum. (b) Fraction of solid phase PS of total phospholipid in binary PS-PC membrane in the presence of Ca^{2+}. The solid fraction was calculated by the mole fraction of solid phase PS of total PS.

fraction of the rigid component in the measured spectra was estimated by comparison with computer-simulated spectra obtained by addition at various ratios of the rigid spectrum and the fluid spectrum. A plot of the rigid fraction vs. pH indicated that 50% of PS molecules were solidified at pH 3.5. The pH value is close to the reported p*K* for the carboxyl (4.0) and phosphate (3.7) groups of PS in monomolecular form (Seimiya and Ohki, 1973). The solidification is therefore brought about by increased interaction among the polar head groups through protonation (e.g., hydrogen bonding). The spectral changes occurred rather slowly. After several hours at 23°, the spectrum contained more fractions of the fluid component. It took about 1 day for equilibration of the pH-induced solidification.

Effect of Divalent Cations on PS Membrane. The effect of divalent cations was investigated using the PS-PS* (100:1) membrane. Figure 3 shows the ESR spectrum of the membrane in aqueous salt solution containing Mg^{2+}, Sr^{2+}, Ba^{2+}, or Ca^{2+}. It is visually clear that Ca^{2+} has a predominantly large effect on the PS membrane. The overall splitting was increased to 54.2 G at 23°. The immobilization of lipid alkyl chains indicates closer packing or solidification of PS molecules by intermolecular chelation of Ca^{2+}. The spectrum showed relatively small dependence on temperature; the overall splitting was 53.5 G at 40° and 50.3 G at 60°.

The other divalent cations also affected the PS membrane but the effect was markedly smaller. The overall splittings at 23° were 42.2, 42.9, and 43.1 G for Mg^{2+}, Sr^{2+}, and Ba^{2+}, respectively. These cations therefore freeze the motion of lipid alkyl chains in that order.

Calcium-Induced Lateral Phase Separation in PS-PC Membrane. The effect of Ca^{2+} on the binary membrane was investigated using both PS*-PC membranes and PS-PC membranes containing very small amounts of PS*. The PS*-PC (4:1) membrane showed an exchange-broadened ESR spectrum due to a rather high concentration of PS*. The spectrum was further broadened slightly but definitely upon the addition of Ca^{2+}, indicating calcium-induced aggregation of PS* molecules in the binary membrane.

In the PS-PC membranes containing 0.5% PS*, Ca^{2+} caused the rigid component to appear in the ESR spectrum. Figure 4 shows spectra of the binary membranes having various mole fractions of PS, f_{PS}. It is seen that the rigid component increases with f_{PS}. The spectrum at $f_{PS} = 0.67$ approached closely that of the PS-PS* (100:1) membrane in the presence of Ca^{2+} (compare with Figure 3d). The observed spectra at various f_{PS} can be simulated as a sum of the fluid spectrum (e.g., Figure 4a) and the rigid spectrum (Figure 3d). The addition of the spectra at various ratios was carried out with the aid of a computer. Owing to broadness of the rigid component, its presence by small fractions did not largely modify overall appearance of the spectra. The high-field line in the composite spectra was mainly due to the fluid component and the central line was contributed by both components. The peak-height ratio of the high-field line to the central line was plotted against the fraction of the rigid component, *f*, in the simulated spectra. The plot gave a monotonously decreasing curve starting with 0.17 at $f = 0$ and tending to 0 as *f* approached 1. The *f* value of the observed spectra was obtained by comparison with the plot and the result is given in Figure 5a. The *f* represents mole fraction of solid phase PS of total PS in the binary membrane; f = (moles of solid phase PS)/[(moles of solid phase PS) + (moles of fluid phase PS)]. It is seen that the mole fraction of solid phase PS is greatly dependent on f_{PS}. It is only as low as 0.03 at $f_{PS} = 0.1$ and increases to 0.86 at $f_{PS} = 0.67$. The mole fraction of solid phase PS was rather insensitive to temperature. The *f* values for the membranes incubated at a higher temperature of 40° were slightly larger (see Figure 5a). The fraction of solid phase PS of total phospholipid in the binary membrane, i.e. (solid phase PS)/$(PS + PC)_{total}$, was calculated using the *f* value and plotted against f_{PS} in Figure 5b.

The time course of the calcium-induced aggregation of PS was followed by the growth of the rigid peak. The rate of aggregation was greatly dependent on f_{PS} as shown in Figure 6. The aggregation was complete within 5 min at $f_{PS} = 0.67$, whereas it was still proceeding after several hours at $f_{PS} = 0.2$. At lower f_{PS} values the aggregation approached equilibrium after 1 day. The data given in Figure 5 repre-

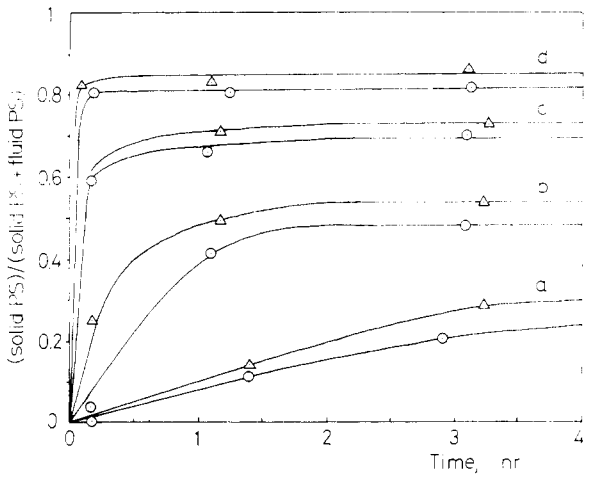

FIGURE 6: Growth of the rigid component in the ESR spectrum of the PS–PC membrane containing 0.5% PS* after addition of Ca^{2+}. The mole fraction of PS in the binary membrane was (a) 0.2, (b) 0.33, (c) 0.5, and (d) 0.67. The membrane was incubated in aqueous calcium solution at 23° (○) and at 40° (△) and the ESR spectrum was measured at 23°. The ordinate plotted the mole fraction of solid phase PS of total PS calculated from the fraction of the rigid component.

sent the values measured after 1 day. The dependence of the aggregation rate on f_{PS} can be roughly expressed in a form of f_{PS}^n where n was in the range of 3–5. The aggregation rate was dependent on temperature, being larger at a higher temperature of 40°.

Discussion

The present study has demonstrated that a specific phospholipid spin label provides a powerful tool for investigating phospholipid mixture membranes that contain the particular lipid. Use of such specific labels widely opens up the way of tracing behavior of functional lipid in biological membranes. Specific interaction with proteins and with phospholipids could be detected successfully.

The calcium-induced lateral phase separation in PS–PC binary membranes has been studied from the side of the calcium-receiving lipid. The results confirmed and extended our previous conclusion drawn with PC* (Ohnishi and Ito, 1973, 1974). The calcium-induced solidification of the PS membrane was directly shown. The phase diagram of the binary membrane in the presence of Ca^{2+} was determined. The rate of aggregate formation was obtained in a wider range of PS content.

Not all PS molecules were aggregated to form the solid patches but some remained dissolved in the fluid PC medium. The fraction of fluid phase PS was larger for membranes containing more PC, which is reasonable taking a solubility limit into account. The membranes containing larger fractions of PC still had significant fractions of solid phase PS. For example, the solid fraction was 2–9% in the binary membrane consisting of 90% PC and 10% PS.

Many biological membranes contain some fractions of PS. If we look at tables of phospholipid composition for a variety of mammalian tissues and subcellular fractions, compiled by White (1973) for example, PS is found to be a ubiquitous component. The content varies with species, tissues, and organella. The average content is 15.7% in erythrocyte, 14% in myellin, 11.2% in brain, 10% in peripheral nerve, 7.7% in spleen, etc. The organ containing the least is the heart (2.8%). These data therefore indicate possible involvement of the ionotropic aggregate formation of PS in some calcium-requiring membrane phenomena. Phospholipid composition may be different from the average in some membranes. PS may be concentrated in a certain membrane system of a cell or localized in some part of a membrane. Localization of PS in the inner layer of erythrocyte membrane was suggested by several investigators (for example, Bretscher, 1973).

The rate of aggregate formation was greatly dependent on PS content in the binary membranes. The two-dimensional crystallization occurred slowly in the membranes containing smaller amounts of molecules to be aggregated. The lateral diffusion of PS molecules in the membrane is not the rate-limiting step since such diffusion is known to be quite rapid. The diffusion constant is on the order of 10^{-8} cm^2/sec (Devaux and McConnell, 1972). The rate-limiting step is probably in the formation of "stable" nuclei. Larger aggregates would be "stable" so that these can bind fluid PS to grow the two-dimensional crystals. Smaller aggregates would be "unstable" so that these are readily disaggregated before growing. Fluctuation in density could be quite large in the transient aggregation–disaggregation stage. We believe that such transient instability of the membranes would play a crucial role in calcium-dependent membrane phenomena. Immediately after addition of calcium, the density fluctuation and therefore the lateral compressibility would become very large and could result in transient increase in permeability, transient modification of activity of proteins that are associated with PS, etc. The transient instability would occur likewise upon removal of calcium. For example, K^+ current removed the bound calcium and made the PS aggregates disaggregated. This was demonstrated by a change in the ESR spectrum of the PS–PC* (9:1) membrane when an electric current was applied through the lipid-impregnated Millipore filter in KCl solution (S. Tokutomi and S. Ohnishi, unpublished).

We have recently observed that Ca^{2+} immediately and greatly enhanced transfer of PS molecules between PS–PC binary membranes (Maeda and Ohnishi, 1974). The transfer reaction afterward was quite similar to that in the absence of Ca^{2+}. The transient instability seems to accelerate the transverse motion of lipid molecules in the membranes in this case. After reaching equilibrium in the phase separation, the fluctuations may not be so large. McConnell and his colleagues have emphasized the important role of the thermotropic phase separations in membrane activities (Linden et al., 1973). The onset of lateral phase separation on lowering temperature would lead to a sudden increase in the isothermal lateral compressibility which then facilitates the insertion of the transport protein. We emphasize that, in addition to the importance of such fluctuations in the *equilibrium* state of phase separation, the *transient* fluctuations would be even more important in the ionotropic lateral phase separations.

The ionotropic phase separations occur isothermally and therefore could be more significant in physiological phenomena of homeothermic animals. The importance of ionotropic effects has recently been pointed out by other investigators also. For example, Träuble and Eibl (1974) have emphasized electrostatic effects of mono- and divalent cations on lipid phase transition. Verkleij et al. (1974) have observed H^+- and Ca^{2+}-induced solidification of phosphatidylglycerol in a pure form and also in a mixture with PC.

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Circular Dichroism and Gel Filtration Behavior of Subtilisin Enzymes in Concentrated Solutions of Guanidine Hydrochloride†

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ABSTRACT: The circular dichroism of diisopropylphosphorylsubtilisins Novo and Carlsberg in both the near- and far-ultraviolet spectral regions is unaltered by concentrations of guanidine hydrochloride as high as 4 *M* at neutral pH. At concentrations of guanidine hydrochloride greater than 4 *M* slow irreversible time-dependent changes, apparently obeying second-order kinetics, are evident in both the near- and far-ultraviolet circular dichroism of these enzymes. Gel filtration studies of inactivated subtilisin enzymes reveal the circular dichroism changes to be accompanied by the appearance of aggregated protein material. The changes in circular dichroism and the production of associated subtilisin species are sensitive to protein concentration, denaturant concentrations, and pH. The circular dichroism of active subtilisins Novo and Carlsberg in guanidine hydrochloride exhibits irreversible changes similar to those observed for the inactivated subtilisins. Aggregated protein material is also formed initially in the presence of guanidine hydrochloride, but is rapidly autolyzed to low molecular weight fragments.

The diisopropyl fluorophosphate sensitive alkaline proteases elaborated by *Bacillus subtilis* and related species are noted for their unusual conformational stability in the presence of biopolymer denaturants. Previous work with the subtilisin enzymes has established that they retain considerable enzymatic activity in concentrated solutions of urea, guanidine hydrochloride (Gdn·HCl),[1] and ethanol. For example, subtilisin Novo[2] is enzymatically active in 6 *M* urea or 50% ethanol, although it is rapidly and irreversibly denatured below pH 5 (Gounaris and Otteson, 1965). Subtilisin Carlsberg is reported to be stable in 10 *M* urea or 6 *M* Gdn·HCl on the basis of optical rotatory dispersion and vis-

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[1] Abbreviations used are: Dip-F, diisopropyl fluorophosphate; Dip, diisopropylphosphoryl; $PhCH_2SO_2$, phenylmethanesulfonyl; CD, circular dichroism; uv, ultraviolet; Gdn·HCl, guanidine hydrochloride; Gdn·SCN, guanidine thiocyanate.

[2] Subtilisins Novo and BPN′ are identical enzymes (Olaitan et al., 1968; Robertus et al., 1971; Drenth et al., 1972). We use the name subtilisin Novo when referring to this enzyme.