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Fluorescence Method for Measuring the Kinetics of Ca^{2+}-Induced Phase Separations in Phosphatidylserine-Containing Lipid Vesicles†

Dick Hoekstra

ABSTRACT: The effects of Ca^{2+} and Mg^{2+} on the fluorescence behavior of the phospholipid analogues 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine in small unilamellar vesicles consisting of phosphatidylserine, mixtures of phosphatidylserine/phosphatidylcholine, and mixtures of phosphatidylserine/cholesterol were studied. Fluorescence quenching was observed when Ca^{2+}, but not Mg^{2+}, was added to phosphatidylserine vesicles containing 5 mol % fluorescent lipid. The quenching process, which could be monitored continuously, was virtually complete within 5–6 min at Ca^{2+} concentrations $\geq$ 1.5 mM and resulted in a decrease of fluorescence intensity of approximately 60%. Fluorescence quenching did not occur in the presence of 0.5 mM Ca^{2+}; however, simultaneous addition of 6 mM Mg^{2+} initiated a quenching process similar in rate and extent to that observed at higher concentrations of Ca^{2+} alone. This quenching of 4-nitro-2,1,3-benzoxadiazole (NBD) fluorescence is best explained in terms of Ca^{2+}-induced separation of lipid phases that leads to an increase in local concentration of NBD–lipid in the bilayer and hence to self-quenching of NBD fluorescence. The kinetics of Ca^{2+}-induced phase separation were also studied in several mixed lipid systems containing phosphatidylserine. In the case of mixtures of phosphatidylserine/cholesterol, the results indicate the presence of phase-separated regions as an intrinsic property of the vesicles in the absence of Ca^{2+}. Finally, results are presented indicating that the kinetics of phase separation is slow compared to vesicle–vesicle fusion.

The importance of divalent cations in promoting the fusion of artificial lipid vesicles composed of acidic phospholipids is well established (Papahadjopoulos et al., 1979). In particular, the involvement of Ca^{2+} in this process has attracted considerable attention because of its presumed role in potentiating various biological fusion events [see Poste & Nicolson (1978)]. Although the molecular basis for Ca^{2+} induction of membrane fusion is not known, several mechanisms have been proposed in which phase transitions (Papahadjopoulos et al., 1973, 1979) and/or formation of nonlamellar lipid particles (Cullis & De Kruijff, 1979) in the fusing membranes play a role in the fusion process. In addition, it has also been suggested that the ability of Ca^{2+} to promote membrane fusion may be related to its potential to induce phase separations of membrane lipids (Papahadjopoulos et al., 1974, 1977). However, because of limitations in current techniques for examining phase separations, such structural changes have only been studied under equilibrium conditions (Ito et al., 1975; Papahadjopoulos et al., 1977; Van Dijck et al., 1978), and potentially important membrane changes during the initial stages of the fusion process may not have been detected.

In this paper a method is presented for continuous monitoring of lipid phase separations during the time course of vesicle–vesicle fusion. The method is based on the use of small amounts of fluorescent phospholipid analogues incorporated into the vesicle bilayer, which, during phase separation, are

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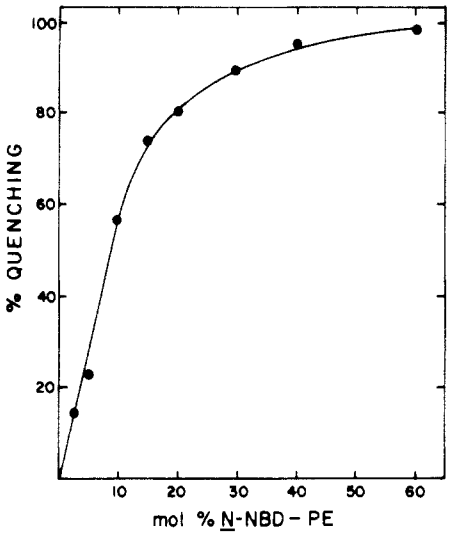

FIGURE 1: Self-quenching of *N*-NBD-PE fluorescence in PS vesicles. Small unilamellar vesicles were prepared containing PS and varying amounts of *N*-NBD-PE. At each NBD concentration aliquots were removed, and the fluorescence was measured (λ_{ex} = 475 nm, λ_{em} = 530 nm) before and after the addition of Triton X-100. The percentage of self-quenching was determined as described under Materials and Methods.

concentrated in restricted domains of the membrane and thus become self-quenched. This method is applied to study Ca^{2+}-induced phase separations in PS[1]-containing vesicles, and it is suggested that the kinetics of phase separation are slow compared to the process of vesicle–vesicle fusion.

Materials and Methods

Lipids. DOPC, bovine brain PS, C_6-NBD-PC, and *N*-NBD-PE were purchased from Avanti Biochemical Corp. (Birmingham, AL). Cholesterol was obtained from Sigma Chemical Co. All lipids were periodically monitored for purity by thin-layer chromatography and purified as required.

Preparation of Lipid Vesicles. Small unilamellar vesicles of various lipid compositions were generated in 0.1 M NaCl/0.01 M Hepes, pH 7.4, by ultrasonication (Branson sonifier, Model W-185) under an argon atmosphere at 20 °C. Lipid vesicles were more than 95% unilamellar as judged by their chromatographic behavior on Sepharose 4B.

Fluorescence Measurements. Continuous monitoring of NBD fluorescence was carried out with an American Instrument Co. spectrophotofluorometer equipped with a chart recorder. The fluorescent sample was excited at 475 nm and monitored at 530 nm with narrow band-pass slits and crossed polarizers to minimize light scattering. Incubations were carried out in 1-cm quartz cuvettes at 23 °C (except where noted). $CaCl_2$, $MgCl_2$, or EDTA was added as a concentrated solution.

Other Methods. The concentration dependence of NBD-fluorescence self-quenching was determined by measurement of the fluorescence before and after addition of Triton X-100 (1% final concentration) as previously described (Nichols & Pagano, 1981). Light scattering (at 90°) was measured with excitation and emission wavelengths set at 400 nm. Lipid concentrations were determined by analysis of lipid phosphorus (Ames & Dubin, 1960).

[1] Abbreviations: PC, phosphatidylcholine; Chol, cholesterol; DOPC, dioleoylphosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; C_6-NBD-PC, 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PS, phosphatidylserine; EDTA, ethylenediaminetetraacetic acid; NBD, 4-nitro-2,1,3-benzoxadiazole; ESR, electron spin resonance; DSC, differential scanning calorimetry.

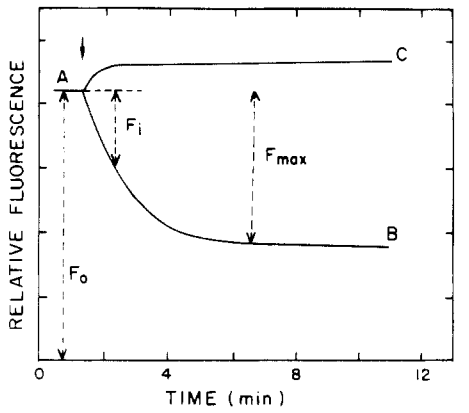

FIGURE 2: Fluorescence recordings from PS/*N*-NBD-PE (95:5) vesicles upon addition of Ca^{2+} or Mg^{2+}. Section A shows the initial fluorescence level. Sections B and C show the fluorescence development upon addition of Ca^{2+} or Mg^{2+} (arrow), respectively, as a function of time. F_0, F_i, and F_{max} represent the relative fluorescence level of the initial vesicle population, the decrease in fluorescence upon addition of Ca^{2+} after 1 min, and the maximal decrease of fluorescence, respectively. The fractional (Q_i) and maximal (Q_{max}) quenching were calculated as follows: $Q_i = F_i/F_0$; $Q_{max} = F_{max}/F_0$. See text for details.

Results

Figure 1 demonstrates that as the percentage of *N*-NBD-PE in PS vesicles was increased the fluorescence quantum yield decreased, presumably due to intermolecular quenching of the fluorescent probe. The degree of self-quenching increased linearly to approximately 75% when the *N*-NBD-PE content in the bilayer was increased from 2.5 to 15 mol %. Similar results to those shown in Figure 1 were obtained with the acyl chain labeled probe C_6-NBD-PC. Thus, with vesicles containing 5 mol % of either NBD–lipid, small increases in the surface density of NBD molecules resulted in a considerable quenching of the fluorescent signal.

A typical time course of fluorescence development recorded with small unilamellar PS vesicles containing 5 mol % *N*-NBD-PE upon addition of Ca^{2+} or Mg^{2+} is shown in Figure 2. Section A represents the initial fluorescence level of NBD-containing vesicles suspended in NaCl/Hepes buffer. Addition of Ca^{2+} (section B) resulted in a decrease of the fluorescence intensity with time. In general, the initial quenching rate was linear for several minutes, and for convenience the fractional quenching 1 min after the addition of Ca^{2+} (Q_i) was used to estimate the initial quenching rate. The maximal quenching, Q_{max}, was determined after completion of the reaction (5–6 min at $[Ca^{2+}] \geq 1.5$ mM; 1–2 h at $[Ca^{2+}] \leq 1$ mM) when curve B is parallel to A. Q_i and Q_{max} were calculated as described in the legend of Figure 2. Addition of Mg^{2+} (section C) resulted in a slight dequenching of fluorescence. The maximal extent of *de*quenching (D_{max}) obtained in the presence of Mg^{2+} was calculated in an analogous manner to the corresponding parameter for quenching in the presence of Ca^{2+}. Similar results to those shown in Figure 2 were obtained when C_6-NBD-PC was used in place of *N*-NBD-PE.

As shown in Figure 3, Q_i increased with increasing Ca^{2+} concentrations and became maximal at a concentration of approximately 5 mM, while at Ca^{2+} concentrations ≤ 1 mM, Q_i was negligible. Although the initial quenching rate was greater for C_6-NBD-PC than for *N*-NBD-PE, values of Q_{max} were found to be virtually identical (0.6) for both probes at

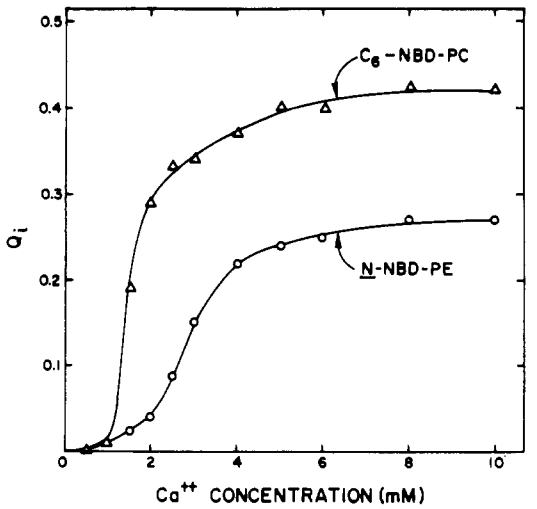

FIGURE 3: Initial rates of NBD fluorescence quenching in PS vesicles as a function of Ca^{2+} concentration. Approximately 60 nmol of PS/*N*-NBD-PE (95:5) or PS/C_6-NBD-PC (95:5) vesicles was suspended in 2 mL of 0.1 M NaCl/0.01 M Hepes, pH 7.4, at 25 °C. Ca^{2+} was added to the final concentrations indicated, and the fluorescence development was monitored continuously. The initial quenching rate, Q_i, was determined as described in the text and in the legend of Figure 2. (○) PS/*N*-NBD-PE; (△) PS/C_6-NBD-PC.

$[Ca^{2+}] \geq 2$ mM. An almost instantaneous recovery (5–10 s) of fluorescence to its original level was obtained when a 2-fold molar excess of EDTA (relative to Ca^{2+}) was added to the incubation mixture. Addition of EDTA prior to the addition of Ca^{2+} prevented the Ca^{2+}-induced quenching.

In contrast to Ca^{2+}, addition of Mg^{2+} to NBD–lipid containing PS vesicles induced a small but significant *de*quenching of fluorescence (Figure 2). The dequenching effect was dependent on the Mg^{2+} concentration and was maximal ($D_{max} \simeq 0.12$) at concentrations ≥ 6 mM. This maximum was reached within 2 min and remained constant over a period of hours. When a 2-fold excess of EDTA (relative to Mg^{2+}) was added, the original level of fluorescence was reestablished.

At a low Ca^{2+} concentration (0.5 mM) no detectable quenching of NBD fluorescence in PS vesicles was observed. However, subsequent addition of Mg^{2+} caused a combined Ca^{2+}/Mg^{2+} concentration-dependent quenching to become apparent (Figure 4). For each of the traces shown in Figure 4A, increasing concentrations of Mg^{2+} were added to the PS vesicles prior to the addition of 0.5 mM Ca^{2+}. A maximum value of Q_{max} of 0.6 was found at Mg^{2+} concentrations ≥ 6 mM. As shown in Figure 4B, Q_{max} did not appear to depend on the sequence of cation addition. It is also apparent that prior binding of Ca^{2+} to vesicles (curve f) did not have a significant effect on the *initial* (Mg^{2+} induced) dequenching rate when compared to the rate observed in the absence of Ca^{2+} (curve e). Rather, after reaching approximately half of the maximal Mg^{2+}-induced dequenching level, the Ca^{2+} action became effective as indicated by the initiation of fluorescence quenching.

The effect of the physical state of the bilayer lipid on NBD fluorescence is shown in Figure 5 in which the fluorescence of *N*-NBD-PE in PS vesicles was measured as a function of temperature in the presence or absence of 5 mM Mg^{2+}. No significant change in fluorescence at −3 (upper curve) or +19 °C (lower curve), the respective gel–liquid crystalline phase transition temperatures of bovine brain PS in the absence or presence of Mg^{2+} (Portis et al., 1979), was seen. Similar results to those shown in Figure 5 were obtained with DOPC/*N*-NBD-PE (95:5) or PS/C_6-NBD-PC (95:5) vesicles. Thus, it is concluded that the observed Ca^{2+}-induced quenching

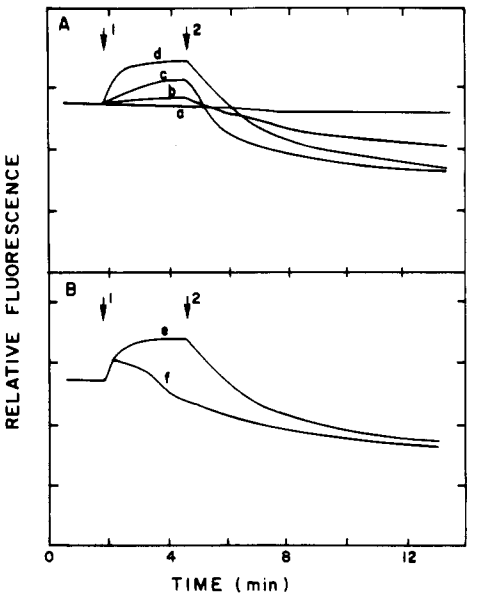

FIGURE 4: Ability of Mg^{2+} to facilitate Ca^{2+}-induced NBD fluorescence quenching in PS/*N*-NBD-PE (95:5) vesicles. (A) Vesicles were treated with various concentrations of Mg^{2+} (arrow 1). Then, 0.5 mM Ca^{2+} (final concentration) was added (arrow 2). Mg^{2+} concentrations were (a) 0, (b) 3, (c) 6, and (d) 10 mM. (B) In (e), vesicles were incubated with 10 mM Mg^{2+} (arrow 1) for approximately 2 min, followed by the addition of 0.5 mM Ca^{2+} (arrow 2); in (f), vesicles were preincubated with 0.5 mM Ca^{2+} for 2 min, followed by the addition of 10 mM Mg^{2+} (arrow 1). Fluorescence intensity was continuously monitored at 530 nm (λ_{ex} = 475 nm).

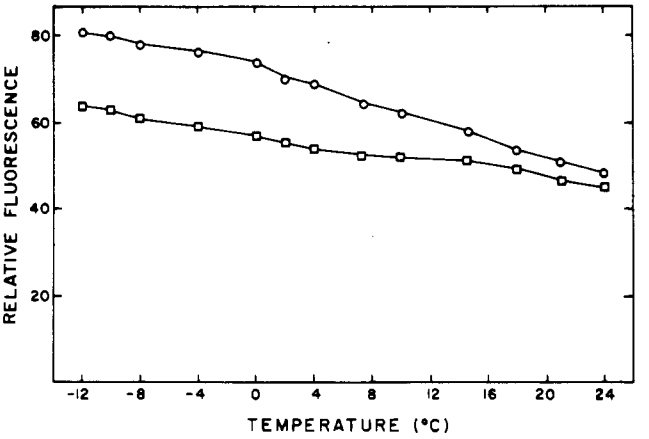

FIGURE 5: Effect of temperature and lipid phase transition on NBD fluorescence quantum yield. PS/*N*-NBD-PE (95:5) vesicles were suspended in NaCl/Hepes buffer and equilibrated at the indicated temperatures, and the fluorescence intensity was determined and plotted as a function of the temperature (○). Alternatively, the vesicles were incubated in the presence of 5 mM Mg^{2+} (10 min, ~23 °C) and subsequently equilibrated at the desired temperature before the fluorescence intensity was measured (□). The results were identical when C_6-NBD-PC was used in place of *N*-NBD-PE.

cannot be explained by a change in thermotropic properties of the PS vesicles.

Figure 6 shows the fluorescence behavior of *N*-NBD-PE in PS/PC and in PS/cholesterol bilayers upon addition of 5 mM Ca^{2+}. Since long incubation times (>6 h) were required to reach maximal quenching in the mixed PS/PC bilayers, the ordinate in Figure 6A is the extent of NBD quenching (Q) after an arbitrary (4 h) incubation period. As can be seen, Q decreased gradually and continuously with increasing mole fractions of PC. The initial rate, Q_i, dropped from 0.25 to 0.19 to 0.01 when the concentration of PC (relative to PS) in PS/PC/*N*-NBD-PE vesicles increased from 0 to 15 to 30 mol %, respectively. No appreciable *initial* quenching rates were

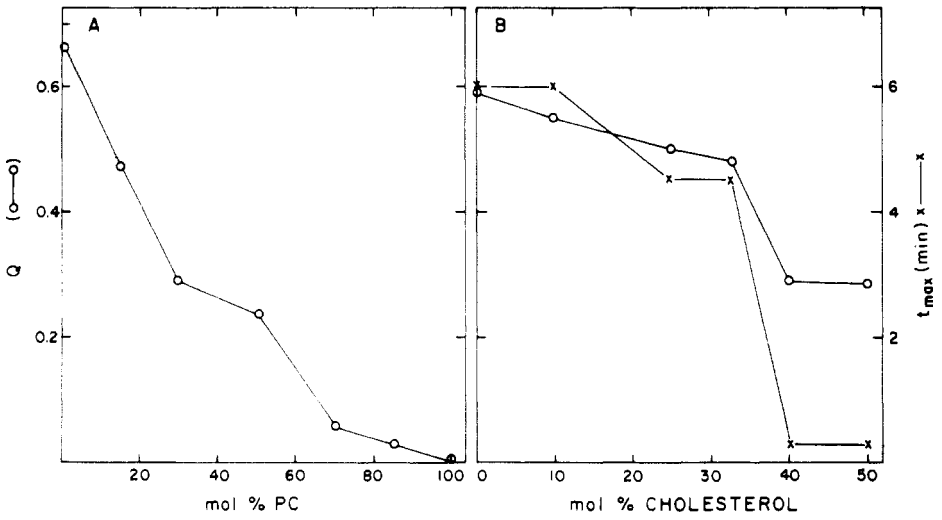

FIGURE 6: Calcium-induced fluorescence quenching in PS/PC and PS/cholesterol vesicles. (A) Vesicles consisting of PS and PC in varying molar ratios and containing 5 mol % *N*-NBD-PE were incubated in the presence of 5 mM Ca^{2+}. The extent of quenching was determined after 4 h. The mol % PC indicates the amount of PC relative to PS. See text for further details. (B) Vesicles, composed of PS and cholesterol in different molar ratios and containing 5 mol % *N*-NBD-PE, were incubated in the presence of 5 mM Ca^{2+}. The mol % cholesterol indicates the amount of cholesterol relative to PS. (O) Q_{max}; (×) t_{max}, i.e., the time required to attain Q_{max} (see text).

measurable when the content of PC in the bilayer was greater than 30 mol %. It should be noted that addition of 5 mM Ca^{2+} to PC/*N*-NBD-PE (95:5) vesicles did not cause any fluorescence change over a 4-h period, thus excluding a nonspecific effect of Ca^{2+} on the probe itself. A more complicated picture emerged when similar experiments were performed with PS/*N*-NBD-PE vesicles containing increasing amounts of cholesterol (Figure 6B). In the case of the cholesterol-containing vesicles both Q_{max} and the time required to reach Q_{max} (t_{max}) were measured. Both of these parameters decreased with increasing cholesterol concentrations, with a possible discontinuity in the data between 30 and 40 mol % cholesterol. Indeed, for cholesterol concentrations less than 30 mol %, the time required to reach maximal quenching (t_{max}) was ~4 min, while at 40 and 50 mol % cholesterol, t_{max} was approximately 30 s. Finally, it is interesting to note that in the case of PS/PC/*N*-NBD-PE vesicles the *initial* levels of fluorescence *prior to* the addition of Ca^{2+} were identical, regardless of the mole percent PC, while in the case of the PS/Chol/*N*-NBD-PE system, the *initial* fluorescence of the vesicle preparations decreased with increasing cholesterol concentrations. For example, the initial fluorescence level of vesicles containing 40–50 mol % cholesterol was approximately 65% of that measured for vesicles containing no cholesterol. These results suggest a preexisting heterogeneous distribution of fluorescent lipid in PS/Chol/*N*-NBD-PE bilayers (see Discussion).

The reversibility of Ca^{2+}-induced *N*-NBD-PE quenching in PS vesicles upon the addition of nonfluorescent vesicles was investigated and is shown in Figure 7. Addition of Ca^{2+} (see arrow 1) induced rapid quenching of NBD fluorescence that could be reversed to varying degrees by subsequent addition (see arrow 2) of increasing amounts of nonfluorescent PS (*but not PC*) vesicles. Indeed, addition of an 8-fold molar excess of PS vesicles resulted in complete recovery of the original *N*-NBD-PE fluorescence. These results are presumably due to the relief of self-quenching (cf. Figure 1) resulting from a decreased surface density of the NBD molecules. When the experiments were carried out with C_6-NBD-PC in place of *N*-NBD-PE, similar results to those shown in Figure 7 were seen when nonfluorescent PS vesicles were used. However, when nonfluorescent PC vesicles were used, a rapid dequenching of fluorescence was also seen. This latter result is consistent with the fact that intervesicular exchange of C_6-NBD-PC (Nichols & Pagano, 1981) but not *N*-NBD-PE (Struck et al., 1981) can occur.

Discussion

Ca^{2+}-Induced Quenching of NBD Fluorescence. In this paper it has been shown that addition of Ca^{2+} to PS vesicles containing NBD–lipids results in a rapid quenching of fluorescence that is reversible by EDTA. In principle this quenching could be due (i) to an altered environment of the probe molecules or to a direct effect of Ca^{2+} on the fluorescent probes, (ii) to an effect of Ca^{2+} on vesicle properties (e.g., flip-flop rates and/or vesicle aggregation), or (iii) to separation of the membrane lipids into discrete phases. The arguments presented below support the last possibility, namely, that the quenching of the NBD–lipids monitors the process of lipid phase separation.

The observed quenching of NBD fluorescence is probably not due to environmental effects on the probe molecules such as binding of Ca^{2+} to the probe and/or a Ca^{2+}-induced alteration in bilayer physical state. An effect of physical state was ruled out in the experiment shown in Figure 5 in which no effect on NBD fluorescence quantum yield was seen when the vesicles were taken through a phase transition. Addition of Ca^{2+} to DOPC/NBD–lipid bilayers did not affect fluorescence intensity, thus excluding a direct effect of Ca^{2+} on the probe molecules (Figure 6A). In addition, Ca^{2+} binding to PS bilayers may result in a change in the probe environment, presumably causing a difference in fluorescence properties (Lancet & Pecht, 1977). Such a possibility is ruled out, however, by the finding that when the probe apparently becomes diluted into nonfluorescent PS vesicles in the presence of Ca^{2+}, fluorescence quenching is relieved (see below).

An effect of Ca^{2+} on vesicle properties as a cause of NBD quenching can similarly be excluded. Ca^{2+}-induced aggregation of PS/*N*-NBD-PE (or PS/C_6-NBD-PC) vesicles does not result in intermolecular quenching due to close apposition of NBD molecules from adjacent bilayers. If so, Q_i would be expected to be faster (and Q_{max} greater) for *N*-NBD-PE than C_6-NBD-PC because of the different locations of the fluorophore on the molecule (and presumed different locations in the vesicle bilayer). Moreover, addition of Mg^{2+} similarly results in aggregation of the vesicles, but without quenching of fluorescence. Thus, these experiments clearly demonstrate

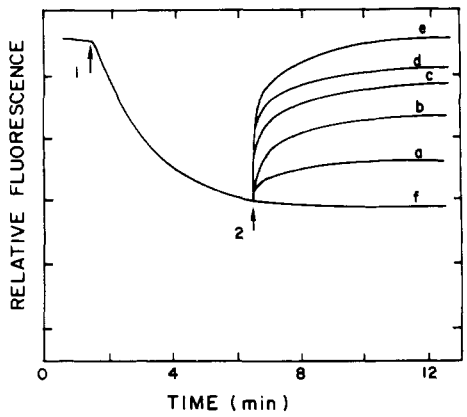

FIGURE 7: Dequenching of NBD fluorescence by fusion of PS/*N*-NBD-PE vesicles with nonlabeled PS vesicles. 25 nmol of PS/*N*-NBD-PE (95:5) vesicles was incubated in the presence of 5 mM Ca^{2+} (arrow 1) to promote maximal NBD fluorescence quenching. At arrow 2, various concentrations of nonlabeled PS vesicles (a–e) or a large excess of nonlabeled DOPC vesicles (f) was added. (a) 25, (b) 50, (c) 75, (d) 150, or (e) 200 nmol of PS or (f) 200 nmol of DOPC vesicles was used.

that NBD fluorescence quenching is not due to vesicle aggregation. In principle, it is also possible that NBD fluorescence quenching might result from a preferential flip-flop of the NBD–lipid to one leaflet of the vesicle bilayer induced by Ca^{2+}; such a phenomenon has been reported to occur for PC in PC/cardiolipin bilayers (Gerritsen et al., 1980). In this study this possibility is excluded since fluorescence quenching of C_6-NBD-PC (or *N*-NBD-PE) by Ca^{2+} can be fully recovered upon addition of EDTA, whereas had flip-flop occurred, only incomplete recovery would be expected.

Thus, it is concluded that the present results are best explained by a separation of lipid phases leading to an increase in local concentrations of the NBD–lipid in the bilayer, and hence to self-quenching of NBD fluorescence. Therefore, Q_{max}, which would measure phase separation under equilibrium conditions, can be compared with results obtained by others using different techniques. In this context it is interesting to note that Q_{max} attained a maximal value at Ca^{2+} concentrations $\geq$ 2 mM, a value similar to the optimum Ca^{2+} concentration required for phase separation in mixed PS systems as studied by ESR (Ohnishi & Ito, 1974) and DSC (Papahadjopoulos et al., 1979). In addition, the time required to attain Q_{max} is about 5–6 min (see Figure 2), in agreement with estimates of the time required for complete phase separation to occur, as reported by Ohnishi & Ito (1974). Finally, the observation that Mg^{2+} does not cause NBD quenching in PS bilayers is in agreement with its known inability to induce phase separations in such systems (Papahadjopoulos et al., 1979). Thus, it is concluded that the rate and extent of NBD quenching are measures of the rate and extent of phase separation in PS bilayers. The advantage of the technique presented here is not only that it can easily detect equilibrium phase separations but that this process can be conveniently and continuously monitored with time. It is important to keep in mind, however, that different probe molecules may give rise to slightly different rates of phase separation (see Figure 3) because of inherently different rates of lateral diffusion [see Wu et al. (1977)] and/or because of different interactions with neighboring nonfluorescent molecules (Klausner & Wolf, 1980).

Effect of Mg^{2+}. In contrast to Ca^{2+}, Mg^{2+} did not induce quenching of the NBD probes, although a small but significant *de*quenching of NBD fluorescence was seen. This dequenching of fluorescence cannot be due to direct interaction of Mg^{2+} with the probe since incubation of Mg^{2+} with DOPC vesicles containing 5 mol % *N*-NBD-PE (or C_6-NBD-PC) did not affect the fluorescence. Since 5 mol % NBD–lipids are partially quenched in PS vesicles (see Figure 1), binding of Mg^{2+} to the bilayer could result in a rearrangement of NBD molecules, which may subsequently lead to a decreased surface density of the molecules and thus a relief of self-quenching. Alternatively, binding of Mg^{2+} to the PS vesicles may involve a reorientation or environmental change of the probe, which could result in a small increase in fluorescence (Lancet & Pecht, 1977). At present these alternative explanations for the Mg^{2+} dequenching effect cannot be distinguished.

In Figure 4 it is seen that at low Ca^{2+} concentrations, which do not cause detectable NBD quenching in PS vesicles, addition of Mg^{2+} causes a combined Ca^{2+}/Mg^{2+} concentration-dependent quenching to become apparent. This enhancement of the Ca^{2+} induction of phase separation in PS bilayers by Mg^{2+} is probably best explained by a Mg^{2+}-facilitated enhancement of Ca^{2+} binding to PS vesicles as reported by Portis et al. (1979). The kinetics of phase separation seen in curve f (Figure 4B) suggest that in the presence of both Mg^{2+} and Ca^{2+} initially only a Mg^{2+} (but not Ca^{2+}) effect on fluorescence is seen and that the Ca^{2+} effect becomes dominant after a critical level of Mg^{2+} (and/or Ca^{2+}) has been bound to the vesicle surface. Finally, it is interesting to note that the kinetics and extent of phase separation induced by 0.5 mM Ca^{2+}/6 mM Mg^{2+} (Figure 4A) appear to be very similar to that seen with 2 mM Ca^{2+} alone (data not shown), implying that the amount of Ca^{2+} bound in both cases should be the same. Indeed, with the data of Portis et al. (1979), it is estimated that about 0.47 mol of Ca^{2+}/mol of PS is bound to the vesicle surface under both sets of experimental conditions.

Phase Separations in PS/Cholesterol Vesicles. Previous studies have demonstrated the presence of phase separations in mixed phospholipid/cholesterol vesicles (Gebhardt et al., 1977; Snyder & Freire, 1980). Such intrinsic phase separations can be considered as distinct cholesterol-rich and cholesterol-poor areas of the membrane that coexist in the plane of the bilayer (Snyder & Freire, 1980). The observation that the fluorescence level of 5 mol % *N*-NBD-PE in mixed PS/Chol/*N*-NBD-PE vesicles decreases with increasing cholesterol concentrations in the absence of Ca^{2+} is also consistent with the existence of phase separations in this system. Such a nonrandom partitioning of *N*-NBD-PE in the PS/Chol bilayer may be explained by the preferential association of cholesterol with PS (Van Dijck, 1979), which results in an increased concentration of the fluorophore in either cholesterol-rich or cholesterol-poor domains. Apparently this concentration is further enhanced upon the addition of Ca^{2+} to the system, which presumably could result in additional phase separation in the cholesterol-poor regions of the membrane. It is interesting to note that both the absolute value of Q_{max} and the time required to reach Q_{max} decreased with increasing cholesterol concentration (Figure 6B) and showed a discontinuity around 33 mol % cholesterol. This discontinuity may be related to the formation of a cholesterol–PS complex of specific stoichiometry (Snyder & Freire, 1980; Martin & Yeagle, 1978), thus leading to a further exclusion of *N*-NBD-PE. Whereas the absolute value of Q_{max} decreases with increasing cholesterol concentration, the *initial* fractional quenching (prior to addition of Ca^{2+}) increases. However, the *overall* quenching in mixed PS/Chol vesicles appears to be essentially the same as that seen with PS vesicles in the absence of cholesterol. For example, at 50 mol % cholesterol, Q_{max} is $\sim$0.3 and the initial fractional quenching (prior to addition of Ca^{2+}) is also $\sim$0.3.

Thus, an overall quenching of 0.6 is obtained, which is essentially identical with Q_{max} obtained for PS alone (Figure 6B). Therefore, it is concluded that the decreasing value of Q_{max} with increasing cholesterol content reflects the occurrence of a progressively increasing extent of intrinsic phase separation in the PS/Chol system prior to Ca^{2+} addition.

Dequenching of NBD Fluorescence by Fusion with Nonlabeled Vesicles. Addition of Ca^{2+} to PS-containing vesicles induces not only phase separations as discussed above but also concomitant vesicle–vesicle fusion (Papahadjopoulos et al., 1973; Hoekstra et al., 1979; Wilschut et al., 1980; Struck et al., 1981). However, during the fusion of PS/*N*-NBD-PE vesicles the surface density of the probe remains unchanged. Indeed, addition of EDTA after fusion results in a complete relief of quenched NBD fluorescence due to Ca^{2+}-induced phase separations, suggesting that the surface density of the *N*-NBD-PE molecules in the fused product remains unaltered. In the experiment shown in Figure 7, *nonfluorescent* PS vesicles were subsequently added to PS/*N*-NBD-PE vesicles in which Ca^{2+}-induced phase separation (and also fusion) had already taken place, resulting in an immediate dequenching of fluorescence (Figure 7, curves a–e). Since *N*-NBD-PE, in contrast to C_6-NBD-PC, is not readily transferred between vesicles via exchange (Struck et al., 1981; Pagano et al., 1981), its dilution must occur from vesicle–vesicle fusion, leading to a decrease in *N*-NBD-PE surface density and thus an increase in fluorescence. Dilution of the probe as a consequence of fusion is evidently a very rapid process and is presumably accomplished by rapid intermixing of the molecules in the "newly" formed membrane. This conclusion is also supported by an experiment in which the kinetics of vesicle aggregation (assessed by light scattering), which proceed on a similar time scale as fusion (Wilschut et al., 1980), were found to be much faster than the kinetics of Ca^{2+}-induced phase separation determined by NBD quenching.

Several parameters, i.e., vesicle concentration, Ca^{2+} concentration, and vesicle size, appear to determine the extent of the fusion-mediated dilution of the fluorescent probe. (i) The extent of dilution increases with increasing concentrations of unlabeled PS vesicles. (ii) In a previous study (Struck et al., 1981) it was shown that no significant fusion occurred when equimolar amounts of prefused and nonfused vesicles were mixed in the presence of 2 mM Ca^{2+}. However, in the presence of 5 mM Ca^{2+} as used in this study, some fusion (and consequent dilution of the fluorophore) may occur as shown in Figure 7, curve a. This would suggest that the prefused vesicle population does not readily undergo fusion at low Ca^{2+} concentrations (2 mM) but may do so at higher concentrations (5 mM). Such an interpretation is also consistent with observations made by Vanderwerf & Ullman (1980). (iii) Addition of unlabeled vesicles, which had been "prefused" by Ca^{2+}, did not result in significant dequenching but rather gave a result identical with that obtained upon addition of PC vesicles (Figure 7, curve f).

It is of interest that following the *rapid* dequenching of fluorescence by addition of nonfluorescent PS vesicles to PS/*N*-NBD-PE vesicles in which Ca^{2+}-induced phase separation (and fusion) had already taken place (see Figure 7), no subsequent phase separation occurs, as evidenced by a *slower* fluorescence quenching rate. This may be explained when it is considered that the *N*-NBD-PE becomes substantially diluted into the PS membrane following fusion and may now be present at too low a concentration to exhibit Ca^{2+}-induced fluorescence quenching (and phase separation). Indeed, no significant Ca^{2+}-induced NBD self-quenching can be observed in PS vesicles containing less than 1.0 mol % *N*-NBD-PE (unpublished data). This observation and the fact that the maximal extent of Ca^{2+}-induced NBD quenching seen with PS/*N*-NBD-PE (95:5) vesicles was always 60% suggest that not all the *N*-NBD-PE molecules participate in the phase separation process. Rather, they might remain completely intermixed in the PS matrix without exhibiting self-quenching. While it is tempting to speculate that the pool of NBD molecules (40% of the total) that do not participate in fluorescence self-quenching might represent those molecules restricted to the inner leaflet of the vesicle bilayer, preliminary experiments with PS vesicles containing an asymmetric distribution of C_6-NBD-PC (Pagano et al., 1981) showed no difference from control, uniformly labeled vesicles with respect to Ca^{2+}-induced self-quenching.

In conclusion, it has been shown that fluorescent NBD–phospholipid analogues can be used to study Ca^{2+}-induced phase separation in PS bilayers. The present method permits a continuous monitoring of the kinetics of this process immediately after addition of Ca^{2+}, an important advantage over previous techniques. Moreover, because the *N*-NBD-PE probe can also be used in resonance energy transfer studies of vesicle–vesicle and vesicle–cell fusion (Struck et al., 1981), it is possible to study the relationship of phase separations to the fusion process in these systems. This will be the subject of a forthcoming paper.

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Structure and Thermotropic Behavior of Phosphatidylserine Bilayer Membranes†

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ABSTRACT: The structure and thermotropic properties of a homologous series of diacylphosphatidylserines (PS) in the anhydrous and hydrated state have been examined with low-angle X-ray diffraction and differential scanning calorimetry. In the anhydrous state at low temperatures both acidic PS and its NH_4^+ salts exhibit lamellar bilayer crystal forms that transform to liquid-crystalline hexagonal (type II) structures at higher temperatures. The crystal → liquid-crystal transition temperature increases with increasing chain length, the transition temperature of an NH_4^+-PS being higher than that of its corresponding acidic form. In contrast, the transition enthalpies of the acidic PS are higher than those of the NH_4^+ salt forms. Hydrated acidic PS and NH_4^+-PS exhibit reversible lamellar gel → liquid-crystal transitions. In this case the acidic form undergoes this chain length dependent transition at a higher temperature, but with a lower enthalpy change, than the NH_4^+-PS. Both below and above the hydrocarbon chain melting transition, hydrated lamellar bilayer structures are present. The temperature–composition phase diagram of the NH_4^+-dimyristoyl-PS/H_2O system has been studied in detail. The chain melting transition decreases with increasing hydration, reaching a limiting value of 39 °C. X-ray diffraction shows that both the bilayer gel structure and the bilayer liquid-crystal form take up water continuously (i.e., no hydration limit), a characteristic of lipid bilayers with a net charge. Electron-density profiles of NH_4^+-dimyristoyl-PS at different hydration levels permit detailed analysis of the structural parameters of the PS bilayer.

The structure and properties of uncharged zwitterionic membrane phospholipids, notably phosphatidylcholine and phosphatidylethanolamine, have been studied with a wide range of physical–chemical techniques. This approach has provided detailed information on their molecular conformation, bilayer-forming properties, and order–disorder thermotropic transitions, which, in turn, has led to a better understanding of their structural role in model and natural cell membranes [for reviews, see Shipley (1973) and Hauser et al. (1981)]. This approach has benefited greatly by the study of synthetic phospholipids in which the fatty acyl chain length and/or degree of unsaturation are systematically varied. In contrast, anionic membrane lipids such as phosphatidylserine (PS),[1] phosphatidylglycerol, and cardiolipin have received much less attention, and precise details of their structure and properties are lacking.

Phosphatidylserine is found in significant amounts in a wide variety of animal and bacterial cell membranes. In red blood cells it appears to be located mainly on the cytoplasmic side of the membrane. Physical–chemical studies of PS have been concerned primarily with the behavior of natural, mixed fatty acid PS, isolated usually from bovine brain. Bovine brain PS has been shown by X-ray diffraction and electron microscopy to form bilayer structures (Atkinson et al., 1974), and its structure and hydration were shown to be clearly dependent on its ionic environment (Hauser & Phillips, 1979). The structure and thermotropic properties of bovine brain PS have been shown to be sensitive to the presence of divalent cations Ca^{2+} and Mg^{2+}, and these studies have suggested that cation–PS complexes may be involved in membrane fusion processes (Papahadjopoulos et al., 1977). More recently, studies of synthetic PS with controlled fatty acid chain length have been reported (MacDonald et al., 1976; Luna & McConnell, 1977; van Dijck et al., 1978; van Dijck, 1979; Browning & Seelig, 1980). For example, DPPS has been shown to undergo a thermotropic transition at ~53 °C, utilizing differential scanning calorimetry (MacDonald et al., 1976; van Dijck,

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[1] Abbreviations: PS, phosphatidylserine; DDPS, didecanoylphosphatidylserine; DLPS, dilauroylphosphatidylserine; DMPS, dimyristoylphosphatidylserine; DPPS, dipalmitoylphosphatidylserine; DSPS, distearoylphosphatidylserine; DL-DLPS, dilauroylphosphatidylserine with glycerol C-2 in the racemic form; DL-DPPS, dipalmitoylphosphatidylserine with glycerol C-2 in the racemic form; DSC, differential scanning calorimetry; TLC, thin-layer chromatography. Unless stated otherwise, the glycerol is in the native D configuration and the serine in the native L form.

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