

FIGURE 2: X-ray diffraction from lipid samples recorded on X-ray-sensitive film. Details of sample preparation and composition are described under Experimental Procedures. (A) Diffraction patterns obtained at 22 °C from bulk mixtures of DPPC and POPC: (1) 100, (2) 50, (3) 20, (4) 8, (5 and 6) 4, and (7) 0 mol % DPPC in POPC. (5) and (6) represent, respectively, the first and second films of a multiple film package used to record the diffraction pattern. (B) Diffraction patterns observed at 23 °C from (1) egg PC with  $\text{Cd}^{2+}$ , (2, 3, and 4) egg PA/egg PC (0.4/0.6) with  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Ca}^{2+}$  plus  $\text{Mg}^{2+}$ , respectively, and (5 and 6) egg PA with  $\text{Mg}^{2+}$  and  $\text{Li}^{+}$ , respectively. (C) Diffraction patterns observed in the absence of exogenous metal ions (1) egg PC at 23 °C, (2) egg PA/egg PC (0.1/0.9) at 23 °C, (3) egg PA at 23 °C, (4) egg PA/DMPC (0.3/0.7) at 20 °C, and (5) egg PA/DMPC (0.3/0.7) at 37 °C. (D) Diffraction patterns observed in the presence of  $\text{Cd}^{2+}$  from (1) egg PC at 40 °C, (2) egg PA/egg PC (0.05/0.95) at 22 °C, and (3) egg PA/egg PC (0.2/0.8) at 22 °C, from (4) egg PA with uranyl acetate at 23 °C, and from (5) egg PA/egg PC (0.4/0.6) with  $\text{Ba}^{2+}$  at 23 °C.

line] phase appear as the mole fraction of the low melting lipid is raised.

To determine the sensitivity of this method for phase boundary identification, the diffraction patterns of mixtures of a gel phase lipid (DPPC, transition temperature 42 °C) and a liquid-crystal phase lipid [POPC, transition temperature -5 °C (Santaren et al., 1982)] were recorded at 23 °C (Figure 2). From a visual inspection of the wide-angle region of these diffraction patterns, the presence of gel and liquid-crystal phase was apparent at  $\geq 2$  and  $\geq 30$  mol %, respectively. Accordingly, the compositional uncertainty of the solidus boundary is much greater than that of the liquidus line. Microdensitometric tracings of the wide-angle reflections in this standard series are shown in Figure 3. Clearly the visual inspection method is more sensitive to small amounts of either gel or liquid-crystal phase. As an example, gel phase is discernible by eye in the original negatives when present at 2 mol %. In contrast, a (4.2

Å)<sup>-1</sup> line is not apparent in the microdensitometric tracings until  $\geq 4$  mol % of the total lipid is in the gel phase. Therefore, in constructing the phase diagrams the visual inspection method was used to identify phase boundaries.

A more precise location of the solidus line is found from EPR measurements of TEMPO partition between the aqueous phase and the lipid liquid-crystal phase (Hubbell & McConnell, 1968). Following Gaffney & Chen (1977) we designate the signal from TEMPO in the fluid lipid phase as  $h$  and the signal from TEMPO in the aqueous phase as  $p$  (Figure 5). The relative intensities of  $h$  and  $p$  depend upon the concentration of fluid lipid in the sample and on the partition coefficient of TEMPO between the lipid and aqueous phases. The appearance of a net  $h$  signal is taken to indicate the presence of liquid-crystal phase.

*Phase Behavior of Egg PA/Egg PC with and without  $\text{Cd}^{2+}$ .* By use of the above methods for identifying phase boundaries,

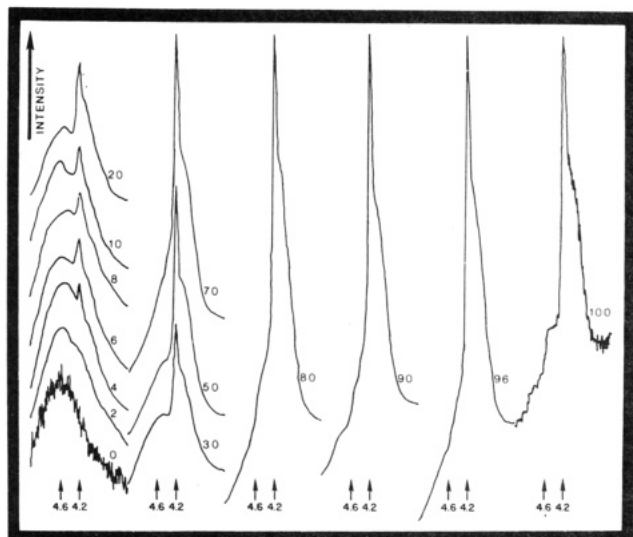


FIGURE 3: Microdensitometric tracing of the wide-angle region of the X-ray diffraction pattern obtained with bulk mixtures of gel (DPPC) and liquid-crystal (POPC) phase lipid. The mole percent DPPC in POPC is indicated along with the position of maximum scattering intensity from gel ( $4.2 \text{ \AA}^{-1}$ ) and liquid-crystal ( $4.6 \text{ \AA}^{-1}$ ) phase lipid. Details of the methods involved are described under Experimental Procedures. Exact copies of the tracings were made in the case of 0 and 100 mol % DPPC to show the noise level associated with the actual microdensitometric tracing.

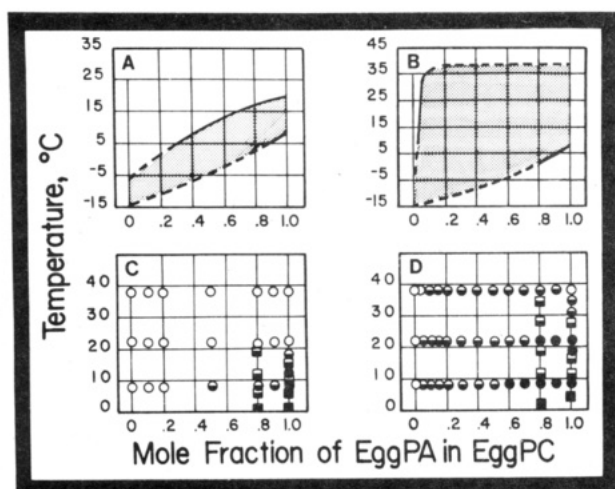


FIGURE 4: Phase properties of egg PA in combination with egg PC in the presence, (B, D) and absence (A, C) of  $\text{Cd}^{2+}$ . (A) and (B) are diagrammatic interpretations of the phase data presented in (C) and (D), respectively, and incorporate a literature value of  $-7$  to  $-15^\circ\text{C}$  as the transition temperature range for egg PC (Chapman, 1975). Methods used in sample preparation and in phase identification by wide-angle X-ray diffraction and EPR are described under Experimental Procedures. Lipid phase types identified by X-ray diffraction are denoted as follows: gel ( $\bullet$ ), liquid crystal ( $\circ$ ), and gel/liquid-crystal phase coexistence ( $\circ$ ). Phases identified by EPR are denoted as follows: ( $\blacksquare$ ) denotes gel phase while half-filled squares denote the presence of a liquid-crystal phase with or without coexisting gel phase. Phase coexistence regions are indicated by shadowing. Dashed lines in (A) and (B) indicate that phase boundaries in these regions are inferred and not determined experimentally as described in the text.

the phase behavior of egg PA/egg PC with and without  $\text{Cd}^{2+}$  was determined (Figure 4). Liquidus lines were drawn on the basis of the X-ray diffraction results while solidus line construction relied heavily on the detection of fluid lipid as revealed by TEMPO partitioning (Figure 5). To facilitate discussion, some examples of the X-ray diffraction patterns recorded on film from representative regions of the phase diagrams are presented in Figure 2.

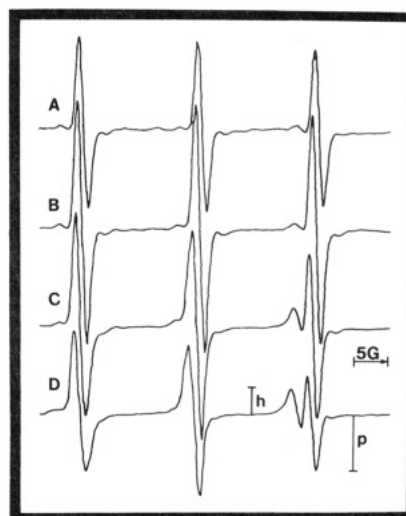


FIGURE 5: EPR spectra of TEMPO in phospholipid dispersions. (A) TEMPO in egg PA- $\text{Cd}^{2+}$  at  $4^\circ\text{C}$ ; (B) TEMPO in egg PA/egg PC (0.8/0.2)- $\text{Cd}^{2+}$  at  $1.2^\circ\text{C}$ ; (C) TEMPO in egg PA/egg PC (0.8/0.2)- $\text{Cd}^{2+}$  at  $35^\circ\text{C}$ ; (D) TEMPO in egg PA/egg PC (0.8/0.2)- $\text{Cd}^{2+}$  at  $44^\circ\text{C}$ . Details of sample composition and preparation are described under Experimental Procedures.  $h$  and  $p$  denote, respectively, TEMPO signal in a hydrophobic and a polar environment.

In the absence of  $\text{Cd}^{2+}$ , the gel-liquid-crystal phase transition temperature of fully hydrated egg PA was found to be broad ranging from  $8$  to  $20^\circ\text{C}$  (Figure 4C). In order to draw the complete egg PC/egg PA phase diagram as depicted in Figure 4A, a literature value of  $-7$  to  $-15^\circ\text{C}$  was used for the transition temperature range of egg PC (Chapman, 1975) since measurements below  $0^\circ\text{C}$  were not made in the present study.

The addition of  $\text{Cd}^{2+}$  raises the midpoint of the egg PA phase transition temperature range from  $14$  to  $26^\circ\text{C}$ . The transition in the presence of  $\text{Cd}^{2+}$  now ranges from  $10$  to  $38^\circ\text{C}$ .  $\text{Cd}^{2+}$  raises the position of the liquidus line in the phase diagram at each of the mole fractions of egg PA examined. The steepness of the liquidus line in the low egg PA concentration region suggests that the smallest addition ( $5 \text{ mol } \%$ ) of egg PA results in phase separation at all but the highest temperature examined. In the experiments shown in Figure 4D at  $38^\circ\text{C}$ , phase coexistence was detected for egg PA/egg PC from  $0.10/0.90$  to  $0.90/0.10$ , but not for pure egg PA. We infer in this composition range that the liquidus line is probably close to this temperature, as indicated by the dashed line in Figure 4B.

The results presented above were obtained with samples containing  $2 \text{ mM}$  total lipid with and without  $8 \text{ mM}$   $\text{CdCl}_2$ . In another experiment the ability of  $\text{Cd}^{2+}$  to induce gel formation in an egg PA/egg PC ( $0.4/0.6$ ) mixture was examined by preparing samples with  $2 \text{ mM}$  total lipid and increasing levels of  $\text{Cd}^{2+}$ . There was no evidence of gel phase at a  $\text{Cd}^{2+}$ /egg PA molar ratio  $< 1/20$ . However, at  $\text{Cd}^{2+}$ /egg PA  $= 1/10$  phase coexistence was apparent, and this continued up to the highest  $\text{Cd}^{2+}$ /egg PA molar ratio measured ( $10/1$ ) in agreement with the data in Figure 4. These results show that  $80 \mu\text{M}$   $\text{Cd}^{2+}$  in the suspending medium is sufficient to induce phase separation, suggesting relatively high-affinity binding of  $\text{Cd}^{2+}$  by egg PA in this egg PA/egg PC mixture.

**Phase Behavior of Egg PA/(7,6)PC with  $\text{Cd}^{2+}$ .** The phase properties of egg PA/(7,6)PC mixtures in the presence of  $\text{Cd}^{2+}$  are of interest since spin-labeled lipids are used in the membrane protein redistribution studies mentioned in the introduction. X-ray diffraction measurements were made at a total lipid concentration of  $18 \text{ mM}$  and  $20 \text{ mM}$   $\text{Cd}^{2+}$  in  $10 \text{ mM}$  HEPES and  $100 \text{ mM}$  KCl, pH  $7.0$ . The smallest addition of

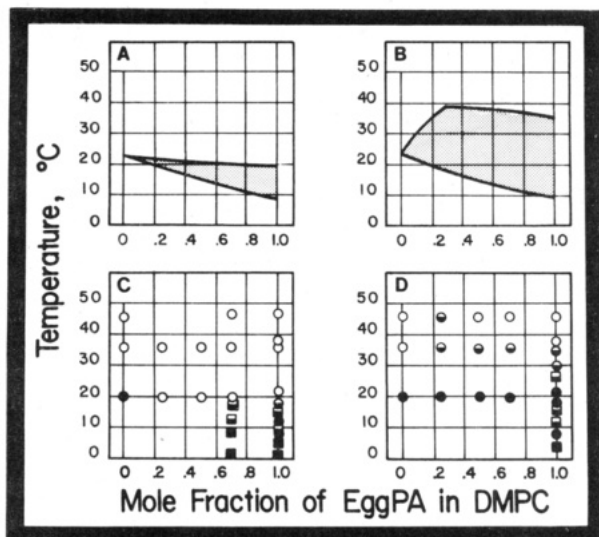


FIGURE 6: Phase properties of egg PA in combination with DMPC in the presence (B, D) and absence (A, C) of  $\text{Cd}^{2+}$ . Refer to the legend in Figure 4 and to the text for details and notation used.

egg PA (mole fraction 0.10) led to the appearance of gel phase as judged by the presence in the diffraction pattern of a sharp reflection at  $(4.2 \text{ \AA})^{-1}$ . Although sample temperature in these (7,6)PC experiments was not measured precisely, coexisting gel and liquid-crystal phases also were detected in the presence of  $\text{Cd}^{2+}$  for egg PA/(7,6)PC = 0.10/0.90 to 0.60/0.40 mixtures over a 20 °C range centered at room temperature (23 °C). Thus, although enough data to construct a phase diagram were not obtained, the phase behavior of egg PA/(7,6)PC is much like that of egg PA/egg PC.

**Effect of pH on the Phase Properties of Egg PA with and without  $\text{Cd}^{2+}$ .** Phosphatidic acid is doubly ionizable, with  $\text{pK}_a$ 's of <3 and about 8 (Abramson et al., 1966). It was of interest to determine the sensitivity of the phase behavior of egg PA with and without  $\text{Cd}^{2+}$  to bulk pH close to neutrality. As shown by the diffraction pattern in the wide-angle region, there was no discernible effect on the phase properties of egg PA in 100 mM HEPES at 23 °C from pH 6.2 to 8.2 in the absence of  $\text{Cd}^{2+}$ . In the presence of  $\text{Cd}^{2+}$  a similar result was obtained at pH 6.2 and 7.2. However, at pH 8.2,  $\text{Cd}^{2+}$  is not fully soluble at 8 mM, and the lipid diffraction pattern has superimposed sharp reflections from crystalline  $\text{Cd}^{2+}$  salts. As a result, a conclusion concerning the effect of alkaline pH on the phase behavior of egg PA- $\text{Cd}^{2+}$  cannot be made in this case.

**Phase Behavior of Egg PA/DMPC and Egg PA/DPPC with and without  $\text{Cd}^{2+}$ .** Phase diagrams for the egg PA/DMPC and egg PA/DPPC systems with and without  $\text{Cd}^{2+}$  are shown in Figures 6 and 7, respectively. Representative X-ray diffraction patterns upon which the diagrams are based are included in Figure 2.

In the absence of  $\text{Cd}^{2+}$  the phase behavior of egg PA in combination with DMPC and DPPC appears relatively simple. The data show that phase separation is minimal, suggesting a high degree of miscibility of egg PA with both of these fully saturated phosphatidylcholines over the entire composition range. Upon the addition of  $\text{Cd}^{2+}$ , the miscibility gap for both egg PA/DMPC and egg PA/DPPC widens.

**Phase Behavior of Egg PA and Egg PA/Egg PC with Ions Other Than  $\text{Cd}^{2+}$ .** Hauser & Shipley (1981) have demonstrated pronounced effects of  $\text{Li}^+$  on the phase properties of the synthetic lipid dimyristoyl-PS (DMPS). The addition of LiCl at 0.5 M induces "crystallization" of the acyl chains as

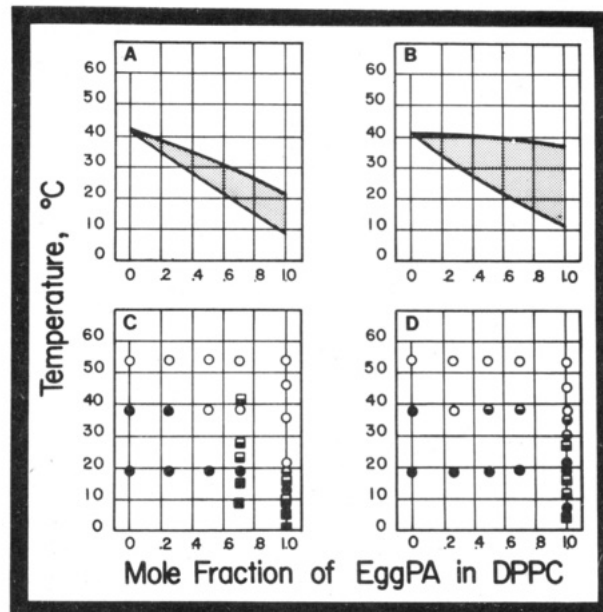


FIGURE 7: Phase properties of egg PA in combination with DPPC in the presence (B, D) and absence (A, C) of  $\text{Cd}^{2+}$ . Refer to the legend in Figure 4 and to the text for details and notation used.

revealed by the appearance of multiple reflections in the wide-angle region of the X-ray diffraction pattern and an increase in both the temperature and the enthalpy of the phase transition. Since both PA and PS are lipids bearing a net negative charge at physiological pH, the possibility exists for strong interaction between PA and certain monovalent cations which might alter the phase behavior of PA in the present study. Accordingly, the diffraction pattern of fully hydrated egg PA at 23 °C in the presence of 0.5 M NaCl, LiCl, KCl, and CsCl was measured. A high buffer concentration of 50 or 100 mM HEPES, pH 7, was used such that the addition of monovalent ions at 0.5 M had no effect on the final pH of the lipid suspension. Regardless of the identity of the monovalent cation, egg PA was found to exist in the liquid-crystal phase. This was shown by the presence of a single diffuse  $(4.6 \text{ \AA})^{-1}$  reflection in the wide-angle region of the diffraction pattern. Thus, if any of these ions influence the short range organization of the fatty acyl chains of this natural phospholipid, the effect is not apparent at 23 °C. We note that in the case of  $\text{K}^+$  and  $\text{Cs}^+$ , the low-angle lamellar reflections were diffuse as observed in HEPES buffer alone. In distinct contrast, these lines were remarkably sharp in the presence of  $\text{Na}^+$  and  $\text{Li}^+$ . These differences in diffraction behavior correlate with differences in hydrated ionic radii and with the relative ion binding affinity of egg PA (Loosley-Millman et al., 1982), i.e.,  $\text{Li}^+$ ,  $\text{Na}^+$  >  $\text{K}^+$ ,  $\text{Cs}^+$ . It is possible that the more tightly bound  $\text{Li}^+$  and  $\text{Na}^+$  ions screen charges on the PA bilayer surface and more effectively collapse dispersed bilayers into an orderly array of multilayers.

For the sake of completeness, the effect of certain monovalent anions on the phase properties of fully hydrated egg PA at 23 °C in 100 mM HEPES, pH 7, was also examined. It was found that  $\text{F}^-$ ,  $\text{Cl}^-$ ,  $\text{Br}^-$ , or  $\text{I}^-$  at 0.25 M  $\text{K}^+$  salt had no discernible effect on the state of organization of the PA acyl chains since the diffraction pattern in the wide-angle region was unaffected by their presence. Regardless of the identity of the anion, the lamellar reflections were broad. However, with KI, there were two additional sharp reflections of unknown origin on the low-angle side of the first lamellar line.

In view of the demonstrated capacity of  $\text{Cd}^{2+}$  to induce an ionotropic isothermal phase transition in egg PA/egg PC

membranes, the ability of a number of other divalent cations to effect a similar transformation was examined. Samples were prepared containing 2 mM total lipid (egg PA/egg PC, 0.4/0.6) in 100 mM KCl, 10 mM HEPES, pH 7, and 8 mM in either  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{BaCl}_2$ , or  $\text{SrCl}_2$ . The diffraction patterns were recorded at 23 °C. All four divalent cations induced phase separation as judged by the diffraction behavior at wide angles. With  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  the amount of residual liquid-crystal phase was much greater than was observed with  $\text{Ba}^{2+}$ ,  $\text{Sr}^{2+}$ , or  $\text{Cd}^{2+}$ . With  $\text{Ba}^{2+}$  and  $\text{Ca}^{2+}$  additional sharp wide-angle reflections of unknown origin were observed (Figure 2B<sub>2</sub>, B<sub>4</sub>, D<sub>5</sub>). In the PA/PC- $\text{Ca}^{2+}$  system a sharp line at  $(4.0 \text{ \AA})^{-1}$  was seen along with the usual  $(4.2 \text{ \AA})^{-1}$  and  $(4.6 \text{ \AA})^{-1}$  reflections from coexisting gel and liquid-crystal phases, respectively. The  $(4.0 \text{ \AA})^{-1}$  reflection was present under a number of other conditions: (1) in the egg PA/egg PC (0.4/0.6) system prepared with 8 mM  $\text{CaCl}_2$  plus 8 mM  $\text{MgCl}_2$  and (2) in a pure egg PA system with 1 and 8 mM  $\text{CaCl}_2$  or with 8 mM  $\text{CaCl}_2$  plus 8 mM  $\text{MgCl}_2$ . We note, however, that with pure egg PA the two sharp wide-angle reflections could not be seen at  $<0.05 \text{ mM Ca}^{2+}$ . In the PA/PC- $\text{Ba}^{2+}$  system sharp wide-angle reflections were observed at  $(4.6 \text{ \AA})^{-1}$ ,  $(4.1 \text{ \AA})^{-1}$ ,  $(3.9 \text{ \AA})^{-1}$ ,  $(3.1 \text{ \AA})^{-1}$ , and  $(2.3 \text{ \AA})^{-1}$  in addition to the  $(4.2 \text{ \AA})^{-1}$  line characteristic of the gel phase. These results suggest a highly ordered packing of the acyl chains in the presence of either  $\text{Ca}^{2+}$  or  $\text{Ba}^{2+}$ .

The effect of  $\text{MgSO}_4$  and  $\text{MnSO}_4$  on the phase properties of pure egg PA was also examined. Experiments were carried out as above at 23 °C with 2 mM egg PA, 10 mM  $\text{SO}_4^{2-}$  salt, and 100 mM HEPES, pH 7. In both cases phase separation was observed, with  $\text{MnSO}_4$  effecting the more complete gel formation. In the case of  $\text{MgSO}_4$  residual fluid phase was clearly evident as diffuse scatter centered at  $(4.6 \text{ \AA})^{-1}$  as was observed for both PA/PC (0.4/0.6)- $\text{MgCl}_2$  and PA- $\text{MgCl}_2$  systems. The diffraction pattern for the PA- $\text{MnSO}_4$  system at low angle had a lamellar periodicity out to 7 orders with a repeat distance of 53 Å. Odd orders were noticeably more intense than even orders. With PA- $\text{MgSO}_4$ , what appeared to be two sets of lamellar repeats [50 Å (6 orders) and 56 Å (7 orders)] were observed. In addition, a strong, sharp reflection at  $(37 \text{ \AA})^{-1}$  of unknown origin was observed. A similar result was obtained with pure egg PA dispersed in 10 mM HEPES and 100 mM KCl, pH 7, containing 8 mM  $\text{MgCl}_2$ .

Uranyl acetate has been shown to interact with membrane lipids (Furuya et al., 1976; Stamatoff et al., 1979, 1980; Parsegian et al., 1981; Huang et al., 1983), and its effect on the phase properties of egg PA was examined. It proved to be quite effective in inducing gel phase formation (Figure 2D<sub>4</sub>) presumably reflecting tight binding of the uranyl ( $\text{UO}_2^{2+}$ ) ion to the phosphate head group of egg PA. This ionotropic effect of uranyl acetate is not unique to PA and has been found with other model and biological membrane systems (unpublished observations).

## Discussion

**Utility and Interpretation of the Phase Diagrams.** The present study is one in a series designed to facilitate interpretation of ongoing fluorescence quenching measurements on proteins and other fluorophores reconstituted into lipid membranes (Caffrey & Feigenson, 1981a,b; London & Feigenson 1981a,b; Bayer, 1983; Caffrey, 1982; Feigenson, 1983). To obtain information regarding the partitioning of fluorophore between gel and liquid-crystal phases in these membranes, it is necessary to know (1) the composition and (2) the relative amounts of the two phases. This is precisely the information provided by a phase diagram. The lipids used

in the present series of experiments represent some of those employed in the fluorescence quenching studies, namely, egg PC, egg PA, DMPC, and DPPC. However, in the fluorescence quenching experiments a nitroxide-labeled derivative of egg PA or egg PC is also included in the membrane. Due to a scarcity of these lipids most of the experiments reported on here were carried out by using the corresponding nonlabeled precursors, egg PC and egg PA. Nonetheless, the measurements made with egg PA/(7,6)PC in the presence of  $\text{Cd}^{2+}$  reveal a phase behavior very similar to that of egg PA/egg PC.

In this study we find the wide-angle region of the X-ray diffraction pattern to be quite sensitive to the appearance of gel phase and hence to the location of the liquidus line. On the other hand, EPR measurements of TEMPO partitioning are sensitive to the appearance of fluid phase and hence to the location of the solidus line. The diffraction pattern at low angles was not used in this study as an indicator of phase coexistence because of the ambiguities introduced by coexisting phases displaying diffuse and/or poorly resolved low-angle scattering.

We point out that the wide region of phase coexistence shown in the phase diagram of Figure 4B is found for systems which are predominantly multilamellar. It could well be that the ion binding and phase behavior are strongly determined by bilayer apposition (Portis et al., 1979). Recently, Ekerdt & Papahadjopoulos (1982) reported that tight binding of  $\text{Ca}^{2+}$  to PS vesicles corresponded to aggregation of the vesicles, implying that close contact of bilayers is an important aspect of ion binding to membranes. Thus, we expect that single PA-containing bilayers not in contact will show a reduced ionotropic phase separation.

**Egg PA-Metal Ion Interactions.**  $\text{Cd}^{2+}$ -containing, egg PA-rich samples diffracted strongly, giving rise to sharp lamellar reflections with up to 9 orders discernible (Figure 2). Since  $\text{Cd}^{2+}$  is a high Z element, strong scattering is expected because coherent scattering intensity in the forward direction is proportional to  $Z^2$  (Cullity, 1967). The sharpness of the lamellar reflections suggests a high degree of order normal to the bilayer surface. It is also interesting to speculate on the in-plane, or lateral order in these structures. Liao & Prestegard (1981) have estimated that in-plane ordering in a PA- $\text{Cd}^{2+}$  system should give rise to reflections at ca.  $(4.6 \text{ \AA})^{-1}$  and  $(4.0 \text{ \AA})^{-1}$  due to in-plane  $\text{Cd}^{2+}$ - $\text{Cd}^{2+}$  spacings. In their experiments using DMPA and DPPA, reflections at these positions were not observed, leading to the conclusion that in-plane disorder prevails. They also point out that these lines are very close to the  $(4.2 \text{ \AA})^{-1}$  reflection from hexagonally packed acyl chains and the possibility of lateral ordering in the presence of  $\text{Cd}^{2+}$  was not fully excluded. However, sharp reflections at  $(4.0 \text{ \AA})^{-1}$ ,  $(4.2 \text{ \AA})^{-1}$ , and  $(4.6 \text{ \AA})^{-1}$  should be readily discernible if present.

With regard to lateral order it is worthwhile considering the effect of  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$  on the PA/PC system observed in the present study. In both cases, sharp wide-angle reflections in addition to the  $(4.2 \text{ \AA})^{-1}$  gel phase line were noted. It is possible that the multiple sharp wide-angle reflections indicate a partial crystallization of the acyl chains as noted for  $\text{Li}^+$ -DMPS (Hauser & Shipley, 1981) and for low-temperature DPPC (Füldner, 1981; Ruocco & Shipley, 1982a,b; Stumpel et al., 1983). It is also possible that whereas the  $(4.2 \text{ \AA})^{-1}$  line corresponds to hexagonally packed gel phase hydrocarbon chains, the additional sharp lines correspond to in-plane  $\text{M}^{2+}$ - $\text{M}^{2+}$  spacings from the orderly arrangement of metal ions at the bilayer surface.



In their study of metal ion binding to DMPA and DPPA, Liao & Prestegard (1981) report what appears to be isomorphous substitution of  $\text{Ca}^{2+}$  and  $\text{Cd}^{2+}$ . Identical lamellar repeats were observed by using a 1:1 molar complex of DPPA and either  $\text{Ca}^{2+}$  or  $\text{Cd}^{2+}$  at 25 °C and pH 6. Isomorphous substitution is reasonable because  $\text{Ca}^{2+}$  and  $\text{Cd}^{2+}$  have the same net charge and approximately the same ionic radius (0.95 and 0.92 Å, respectively) and are equally effective in inducing fusion of PA/PC vesicles (Liao & Prestegard, 1980). Still, the large differences in electronegativity and anion affinity between  $\text{Ca}^{2+}$  and  $\text{Cd}^{2+}$  (Chabareck & Martell, 1959; Sillen & Martell, 1971) suggest that isomorphous substitution might not be completely general. In the present study egg PA at pH 7 interacts somewhat differently with  $\text{Ca}^{2+}$  and  $\text{Cd}^{2+}$ . Whereas both cations give a lamellar  $d$  spacing of approximately 54 Å, for  $\text{Cd}^{2+}$  the low-angle reflections were slightly sharper than those for  $\text{Ca}^{2+}$ . Furthermore, 9 orders of the primary lamellar repeat were observed with  $\text{Cd}^{2+}$  while only  $n = 1, 2, 3, 5$ , and 7 were seen with  $\text{Ca}^{2+}$ . Differences are also seen in the wide-angle region. With  $\text{Cd}^{2+}$  a single sharp reflection at  $(4.2 \text{ Å})^{-1}$  was observed. With  $\text{Ca}^{2+}$ , two sharp reflections, one at  $(4.2 \text{ Å})^{-1}$  and one at  $(4.0 \text{ Å})^{-1}$ , were recorded. Thus, the diffraction pattern observed for egg PA- $\text{Cd}^{2+}$  in the present study is very similar to that of Liao & Prestegard (1981) for DPPA- $\text{Cd}^{2+}$ . When the diffraction patterns for the egg PA- $\text{Ca}^{2+}$  and DPPA- $\text{Ca}^{2+}$  are compared, however, peak intensity differences are apparent in both wide- and low-angle regions. It may well be that egg PA and DPPA interact differently with  $\text{Ca}^{2+}$ . However, the significance of these differences is not yet known. It should also be noted that the experiments of Liao & Prestegard (1981) were performed at pH 6.0 compared to pH 7.0 in the present study.

Ito & Ohnishi (1974) have examined metal ion binding to mixed PA/PC membranes in Millipore filters using an EPR technique. To this end, spin-labeled PA and PC were prepared from egg PC with the nitroxide spin-label on one of the hydrocarbon chains. The PA/PC membranes in 100 mM KCl and 50 mM Tris-HCl, pH 7.9, were shown to undergo lateral phase separation in the presence of  $\text{Ca}^{2+}$ . This effect was reversed on addition of EDTA. The behavior of a PS/PC system was similar to that observed for PA/PC. However, PS were found to be more selective than PA in its interaction with  $\text{Ca}^{2+}$  and a number of other metal ions. At pH 7.9, PS bound  $\text{Ca}^{2+} > \text{Ba}^{2+} > \text{Sr}^{2+} \gg \text{Mg}^{2+}$ , compared to PA which bound  $\text{Ca}^{2+} = \text{Ba}^{2+} = \text{Sr}^{2+} > \text{Mg}^{2+}$ . In the present study, the interaction of egg PA in an egg PA/egg PC system with  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Ba}^{2+}$ , and  $\text{Cd}^{2+}$  was examined at pH 7 (100 mM KCl and 10 mM HEPES). As described under Results a qualitative estimate of the effectiveness of these metal ions in inducing gel formation at pH 7.0 is as follows:  $\text{Cd}^{2+} = \text{Sr}^{2+} = \text{Ba}^{2+} > \text{Ca}^{2+} = \text{Mg}^{2+}$ .

Ohnishi & Tokutomi (1980) discuss the  $\text{Ca}^{2+}$  binding properties of a PS/PC system in terms of an induced surface hydrophobicity. The idea is that upon binding of  $\text{Ca}^{2+}$  there is a loss of water of hydration of the PS head group. This dehydration of the lipid upon metal ion binding was also observed in the present study with egg PA-rich samples. For example, as noted under Experimental Procedures, egg PA-rich samples prepared with metal ions gave waxy water-repelling pellets. In addition, the lamellar repeat for egg PA- $\text{Cd}^{2+}$  of 54 Å is quite similar to the 53 Å spacing reported for bovine brain PS- $\text{Ca}^{2+}$  (Newton et al., 1978; Portis et al., 1979). Such short lamellar  $d$  spacings suggest low levels of hydration.

A question arises as to whether the metal ion induced phase separations reported here are lateral or if instead bulk phase

separation occurs. It is difficult to answer this question by using X-ray diffraction since it is impossible to discriminate between reflections that arise from phases which coexist within the same lamellae and phases which coexist but are separated, e.g., by aqueous medium (so-called bulk phase separation). Ohnishi & Tokutomi (1980) have addressed this issue in the case of the PS/PC- $\text{Ca}^{2+}$  system and consider that lateral rather than bulk phase separation obtains. They base this conclusion on the observation that thin-section electron micrographs reveal a uniform multilamellar structure for PS/spin-labeled PC (9/1) membranes on a Millipore filter support with and without  $\text{Ca}^{2+}$ . Additional evidence is the rapid restoration of a sharp electron spin resonance signal characteristic of homogeneously dispersed spin-labeled PC in egg PS upon removal of  $\text{Ca}^{2+}$  by EDTA both in the Millipore filter system and in small unilamellar vesicles.

## Conclusions

Synchrotron radiation has been employed as a very intense, naturally collimated source of X-rays for diffraction analysis of unoriented hydrated lipid samples. The nature of the diffraction at wide-angles was used to identify lipid that was in the gel and/or liquid-crystal phase. Thus, phase diagrams were constructed for egg PA in combination with (1) egg PC, (2) DMPC, and (3) DPPC in the temperature range 0–55 °C. The manner in which  $\text{Cd}^{2+}$  interacted with these systems was also examined. Since the X-ray diffraction method proved much more sensitive to gel ( $\geq 2\%$ ) than to liquid-crystal ( $\geq 30\%$ ) phase lipid, the position of the liquidus line in these diagrams is determined with more certainty by this method than is the position of the solidus line. An EPR method based on TEMPO partitioning between fluid lipid and the aqueous phase complemented the X-ray diffraction technique in phase diagram construction. TEMPO partitioning is sensitive to lipid in the liquid-crystal phase and so was used to locate the solidus boundary.

At 23 °C, where egg PA is in the liquid-crystal phase,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Cd}^{2+}$ , and  $\text{UO}_2^{2+}$  but not  $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cs}^+$ ,  $\text{F}^-$ ,  $\text{Cl}^-$ ,  $\text{Br}^-$ ,  $\text{I}^-$ , or  $\text{H}^+$  (from pH 6 to 8) brought about an isothermal phase transformation in a pure egg PA or mixed egg PA/egg PC system. Of the divalent metal ions examined  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$  were unusual in that they caused the appearance in the X-ray diffraction pattern of multiple sharp wide-angle reflections, suggesting a highly organized acyl chain packing. This represents the first detailed X-ray diffraction study of the phase properties of PA derived from a natural source, alone and in combination with synthetic and natural PC's and the effects thereon of a variety of ionic species, both anionic and cationic.

Synchrotron radiation proved particularly useful in this study primarily because it is an extremely bright X-ray source. Thus, compared to conventional laboratory X-ray sources, exposure times are markedly reduced—the principle advantage in phase diagram construction where large numbers of samples must be analyzed. In addition shorter exposures lessen the likelihood of time-dependent sample deterioration during data acquisition.

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**Registry No.** DMPC, 18194-24-6; DPPC, 63-89-8; Cd, 7440-43-9; Mg, 7439-95-4; Ca, 7440-70-2; Ba, 7440-39-3; Sr, 7440-24-6; Mn, 7439-96-5; uranyl, 16637-16-4.

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