cellar structural dimorphism with sphere to disk transition compatible with the results of this and the preceding paper (Müller, 1981) is applicable to native human bile.

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Carbon-13 and Phosphorus-31 Nuclear Magnetic Resonance Studies on Interaction of Calcium with Phosphatidylserine†

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ABSTRACT: The interaction between Ca^{2+} and phosphatidylserine was studied by ^{13}C and ^{31}P NMR spectroscopy, by IR analysis, by binding constant measurements, and through use of space-filling molecular models. NMR measurements of various salt forms of the lipid were made in two types of organic solvents that allowed sufficient averaging of chemical shift anisotropy and dipolar couplings to yield high resolution spectra. ^{13}C resonances of the polar head-group carbons were broadened relative to those of the acyl chains. This was especially true in samples prepared at neutral pH where ionic interactions appeared to restrict molecular motion. In $CDCl_3$ the marked line broadening of the resonances of the polar head-group atoms in the Ca^{2+} form indicated the formation of large, slow tumbling micelles. In the amphipathic solvent the large reduction in line broadening indicated the presence of freely tumbling Ca-(phosphatidylserine)$_2$ dimeric complexes. The 2:1 binding stoichiometry and the low chemical activity of the Ca-phosphatidylserine complex support this view. Analysis of the chemical shifts of the various lipid atoms under the differing ionic environments indicates that Ca^{2+} enhanced the deprotonation of both the carboxyl and amino groups and stabilized the entire polar head group against the effects of changing pH. The marked upfield shift of the ^{31}P phosphate resonance in the Ca^{2+} form and its insensitivity to changing pH indicate strong coordination binding. IR data indicate direct involvement of the carboxyl group in Ca^{2+} binding, as evidenced by the appearance of a C=O stretching mode. Binding studies indicated that the phosphate group was the primary binding force but that the carboxyl group also contributes positively. The amino group appears to exert a repulsive effect, which is supported by the chemical shift data which indicate that Ca^{2+} enhances the deprotonation of the amino group. Molecular models indicate direct involvement of the carboxyl and phosphate oxygens and that the amino group must be deprotonated to participate.

Calcium has long been known to bind with phosphatidylserine (Nash & Tobias, 1964; Abramson et al., 1964; Hendrickson & Fullington, 1965), and such interaction is considered to be of physiological significance in a variety of biological processes. In addition to the well-known function of Ca^{2+} binding to phospholipids in blood clotting (Davie & Fujikawa, 1975) and in various soft-tissue membrane-associated processes, Ca^{2+} has been shown to be complexed with phosphatidylserine and other acidic phospholipids in mineralizing tissues [e.g., Wuthier (1968, 1971), Eisenberg et al. (1970), and Boskey & Posner (1976)]. Inorganic phosphate has been shown to enhance quantitatively the binding of Ca^{2+} to phosphatidylserine (Cotmore et al., 1971) leading to the formation of stable noncrystalline lipid-calcium-phosphate complexes. Subsequently, both Anghileri & Dermietzel (1973) and Boskey & Posner (1976) found significant amounts of such complexes in mineralizing tissues. Most recently, phosphatidylserine and complexes of it with calcium phosphate have been discovered in membrane-enclosed matrix vesicles associated with the initiation phase of mineral deposition in calcifying cartilage (Wuthier & Gore, 1977; Majeska et al., 1979). However, the characteristics of the affinity of phosphatidylserine for Ca^{2+} which permit concomitant binding of inorganic phosphate are not well understood.

While numerous studies have been conducted on the binding of Ca^{2+} with phosphatidylserine by using a wide variety of techniques, the details of the specific interactions between Ca^{2+} and the various atoms of the polar head group remain unclear. Recently, increased attention has been focused on the use of NMR spectroscopy in determining the nature of metal binding sites (Behr & Lehn, 1973; Hauser et al., 1976; Czarniecki & Thornton, 1977) and of solution structural features such as

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hydrogen bonding (Czarniecki & Thornton, 1976) and segmental mobility (Gent & Prestegard, 1977). For example, numerous studies have now been conducted by using ^{31}P NMR for studying the binding of Ca^{2+} and other cations to phospholipids and to phosphatidylserine in particular (Hauser et al., 1975, 1977; Cullis & Verkleij, 1979; Kurland et al., 1979; Hope & Cullis, 1980; Browning & Seelig, 1980). These studies have shown that Ca^{2+} causes major broadening of the ^{31}P NMR signal in unsonicated aqueous dispersions of phosphatidylserine (Cullis & deKruijff, 1976), even at concentrations below that required to cause coacervation (Kurland et al., 1979). This cation, as opposed to Mg^{2+}, also causes a large increase in the ^{31}P relaxation time (Kurland et al., 1979; Hope & Cullis, 1980). These findings indicate that Ca^{2+} is directly bound to the phosphate group and causes immobilization of the polar head-group region. Hauser et al. (1977) used ^{2}H NMR spectroscopy of solvent water to investigate the nature of the complex formed between Ca^{2+} and phosphatidylserine and observed that Ca^{2+} binding was accompanied by release of D_2O from the lipid polar head group with resultant tighter packing of the head-group region. ^{2}H NMR studies of phosphatidylserine labeled in both the fatty acyl chains, the C-3 of glycerol, and the phosphate carbon of serine have provided evidence that the acyl and glycerol portions of phosphatidylserine are similar to those of other phospholipids but that the phosphoserine head-group is more rigid (Browning & Seelig, 1980).

Nevertheless, the problem of coagulation of aqueous dispersions of phosphatidylserine upon exposure to Ca^{2+} and the marked line broadening associated therewith have limited the amount of information obtainable concerning the involvement of specific atoms of the serine head group in the lipid–Ca^{2+} complex. In order for NMR spectroscopy to permit direct observation of such interactions in phosphatidylserine, we had to overcome two problems. One was to devise a solvent system in which the anisotropic nature of the aggregated form of the lipid could be overcome. The other was to obtain sufficient pure phosphatidylserine so that natural-abundance ^{13}C NMR spectra could be obtained within a reasonable time frame. Chloroform and an amphipathic organic lower phase composed of chloroform, methanol, and water were considered to be suitable solvents for dispersing and averaging the anisotropic nature of the polar head-group atoms, because of the solubility of the acyl portion of the molecule in these solvents. Our findings indicate that such solvents did provide high-resolution ^{13}C and ^{31}P NMR spectra such that analysis of both the line widths and chemical shifts of the various atoms of phosphatidylserine in varying ionic forms was possible. Further, the recently described biosynthetic technique for obtaining large amounts of phosphatidylserine described by Comfurius & Zwaal (1977) enabled us to obtain adequate amounts of lipid for natural-abundance ^{13}C NMR analysis.

In the present report we describe the interaction between phosphatidylserine and various metal ions, as measured by both natural-abundance ^{13}C and ^{31}P NMR spectroscopy. Interpretation of the NMR data was augmented by concomitant ^{13}C NMR analysis of model compounds with both high and low affinity for Ca^{2+}, as well as by infrared analsis of phosphatidylserine, by measurement of binding constants of Ca^{2+} with phosphatidylserine and two closely related phospholipids, and through study of space-filling molecular models of the lipid.

Materials and Methods

Preparation of Phospholipids. A relatively large amount of lipid (400–500 mg) was required for adequate sensitivity with natural-abundance ^{13}C NMR, especially of the polar head-group atoms. Accordingly, phosphatidylserine was prepared from egg yolk phosphatidylcholine by a one-step transphosphatidylation reaction catalyzed by phospholipase D in the presence of L-serine (Comfurius & Zwaal, 1977). The resulting product was a mixture of phosphatidylserine and phosphatidic acid which were separated by CM[1]-cellulose chromatography. Yield of phosphatidylserine from phosphatidylcholine was about 40–50%, the lipid being pure as judged by two-dimensional paper chromatography (Wuthier, 1976). Phosphatidylhydroxypropionate was also synthesized by transphosphatidylation of egg yolk phosphatidylcholine with hydroxypropionic acid (Aldrich Chemicals). Phosphatidylcholine and phosphatidylethanolamine were obtained from egg yolk by using the extraction method of Folch et al. (1957), followed by the purification procedure of Comfurius and Zwaal mentioned above.

Preparation of Ionic Species of Phosphatidylserine. The lipid was prepared in the desired ionic form (H^+, Na^+, Ca^{2+}, and Mg^{2+}) and at the desired pH by dissolving the lipid in an organic lower phase followed by equilibration with an aqueous upper phase which was prepared at the desired pH and cation species (1 M) in the chloride form. The solvent composition of the upper phase was $CHCl_3$–CH_3OH–H_2O, 3:48:47 v/v/v, the organic lower phase being composed of $CHCl_3$–CH_3OH–H_2O, 86:14:1 v/v/v (Folch et al., 1957). The pH of the upper phase was adjusted by titration with acidic or basic upper phase, followed by thorough mixing of the phases and separation by centrifugation. Partitioning with fresh upper phase was repeated until the desired pH was obtained. The amount of cation bound per mole of lipid was analyzed by either flame emission or atomic absorption spectrometry. The lipid salts were taken by dryness under N_2 and, for NMR studies, dissolved in either deuterated chloroform ($CDCl_3$) or the lower phase in which $CDCl_3$ was used in place of $CHCl_3$. EDTA and succinic acid were adjusted in aqueous solutions to the desired pH by the addition of sodium hydroxide (Na^+ form) and to the Ca^{2+} form by further addition of excess $CaCl_2$. Metallic cations and paramagnetic ion impurities were removed from the lipid preparatons by partitioning the lipid dissolved in lower phase with an acidic upper phase prepared from 1 N HCl.

Nuclear Magnetic Resonance Spectroscopy. Since direct comparison of spectra from the various ionic forms of the lipids was essential, standardized instrumental conditions were used throughout to minimize systematic errors. Spectra of the lipids were run as solutions in either $CDCl_3$ (nonpolar solvent) or deuterated lower phase (amphipathic solvent). EDTA and succinate were analyzed as solutions in deionized water, with an inserted capillary of deuterated water. For ^{13}C NMR spectroscopy, chemical shifts were measured relative to the principal peak of the acyl chain methylene carbons of the lipid. Concentrations of phospholipid were ~450 mg/mL. Solution viscosity was increased, but with insignificant increase in line broadening. Concentrations of EDTA and succinate were 1 M. Spectra were taken with a Varian CFT-20 spectrometer at ambient temperature (~30 °C) by using a sweep width of 5 kHz. Spectral data were accumulated by using a 90° pulse with an acquisition time of 0.819 s under conditions of proton noise decoupling. The field was stabilized by locking on solvent deuterium resonance. The sample tubes (8 mm) were spun and contained ~1 mL of solution. Accumulation times of

[1] Abbreviations used: CM, carboxymethyl; EDTA, ethylenediaminetetraacetate; CPK, Corey–Pauling–Koltun.

Table I: Chemical Shifts and Assignments of the Principal Downfield ^{13}C Resonances of Various Ionic Forms of Phosphatidylserine (PS)

		chemical shifts (ppm)[a]									
		PS, H^+ form		PS, Na^+ form				PS, Ca^{2+} form			
		pH 1		pH 7		pH 10		pH 7		pH 10	
functional group		$CDCl_3$	D-LP[b]	$CDCl_3$	D-LP	$CDCl_3$	D-LP	$CDCl_3$	D-LP	$CDCl_3$	D-LP
-*C*=O, acyl ester (carbonyl)	(1)[c]	192.4	192.5	192.3	192.6	192.7	192.6	192.5[e]	192.6	192.2[e]	192.7
	(2)	192.1	192.2	192.1	192.2	192.5	192.3		192.3		192.4
-*C*=O, serine (carboxyl)		188.5	188.1	189.1[d]	188.7	193.2	192.8	*g*	189.5	*g*	191.1
-*C*H=CHCH$_2$, acyl chain (methenyl)		148.8	148.7	148.8	148.7	148.8	148.7	148.8	148.7	148.8	148.1
-CH=*C*HCH$_2$CH=CH, acyl chain (methenyl)		146.9	146.8	147.0	146.8	146.8	146.6	146.8	146.8	146.8	146.8
H*C*O, glycerol	(2)	88.9	89.0	88.9	88.8	89.5	89.1	89.3[d]	89.1	89.4[d]	89.3
H$_2$*C*OP, glycerol and serine	(3)	83.4	83.0	83.3	82.8	82.0[e]	81.8	81.8[e]	82.7	81.8[e]	82.8
H$_2$*C*O, glycerol	(1)	81.4	81.3	81.4	81.3	82.0[e]	81.6	81.8[e]	81.6	81.8[e]	81.8
H*C*NH$_2$, serine		72.8	72.7	73.3[d]	72.9[d]	74.2[d]	74.5[d]	74.8[f]	73.6[d]	75.9[f]	74.4[f]

[a] Chemical shifts were measured relative to the principal peak of the acyl-chain methylene carbons. Values are for the italicized carbon atom in each functional group. [b] D-LP = lower-phase solvent composed of $CDCl_3$–methanol–water, 86:14:1 v/v/v. [c] Numbers in parentheses are those of the glycerol carbon to which the functional group is attached or from which it arises. [d] Resonance showed moderate line broadening ($V_{1/2}$ = 25–35 Hz). [e] Resonance showed both line broadening and overlap with adjacent lines ($V_{1/2}$ = 35–60 Hz). [f] Resonance showed major line broadening, but was still detectable above noise levels ($V_{1/2}$ = 60–75 Hz). [g] Resonance showed such line broadening that it was not detectable above noise levels ($V_{1/2}$ = >75 Hz).

8–10 h (~30000 transients) were necessary to yield adequate signal to noise ratios for the phospholipids, with EDTA, and succinate requiring only 15 min (~1000 transients). A pulse delay (0.2 s) was added to minimize saturation of slowly relaxing nonprotonated carboxyl carbons. In ^{31}P NMR spectroscopy chemical shifts were assigned relative to 85% phosphoric acid with 10% added D_2O as an external standard. ^{31}P NMR spectra were recorded at 40.5 MHz on a highly modified Varian XL-100-15 spectrometer (Byrd & Ellis, 1977) at ambient temperature (~40 °C). Concentration of lipid was ~30 mM, the 18-mm sample tubes containing ~5 mL of solution. Sample tubes were not spun, and proton noise decoupling did not affect line widths. Spectral data were accumulated with an acquisition time of 0.8 s which required ~15 min to generate adequate signal to noise ratios.

Infrared Spectroscopy. Vibrational spectra of phosphatidylserine in the H^+ form (pH 1), Na^+ form (pH 7 and 10), and Ca^{2+} form (pH 7 and 10) were obtained on a Digilab FTS-15B spectrophotometer. Dry phosphatidylserine (5 mg) was mixed with Nujol to make a mull and placed on a 2 × 4 cm KBr plate. All spectra were taken at room temperature, and the resolution of bands was 2.0 cm^{-1}. A range of 2000–800 cm^{-1} was studied in all cases.

Binding Constant Studies. Binding studies were conducted with phosphatidylserine, phosphatidylethanolamine, and phosphatidylhydroxypropionate in the organic lower phase partitioned against the aqueous upper phase which contained ^{45}Ca and varying concentrations (10^{-2}–10^{-7} M) of $CaCl_2$ at pH 7. The lipid in the Na^+ form (0.3 μmol) and ^{45}Ca (~6 μCi) were added to each biphasic incubation tube. The two phases were stirred vigorously for 90 min and allowed to stand undisturbed overnight. ^{45}Ca was determined by liquid scintillation spectrometry using a toluene–Triton N-101 (4:1 v/v) cocktail containing 0.4% w/v of Omnifluor (New England Nuclear). Aliquots of the upper phase and lower phase were assayed for radioactivity, counting efficiency being ~64% due to quenching by the small amounts of $CHCl_3$.

pH Measurements. For the purposes of this paper, the apparent pH of the upper phase was taken to be that measured with a standard glass electrode (Fisher 13-639-104) after standardization in aqueous buffer. It is known from the work of Harned & Owen (1958) that methanol reduces somewhat the H^+ activity coefficient (~15% for the mole fraction of methanol present in upper phase at pH 2, lesser percentages at higher pH). To determine the effect of the nonaqueous solvents on the apparent pH of the upper phase as measured by the glass electrode, we carefully adjusted aqueous solutions to known pH values ranging from 2 to 11 by using the glass electrode described above. These solutions were then used to prepare synthetic upper phases (chloroform–methanol–buffer, 3:48:47 v/v/v), and the apparent pH of the upper phase so prepared was measured by using the same glass electrode. The pH values obtained were only slightly different (0.01–0.19 pH units) from those predicted for the effects of dilution and of methanol on the aqueous system. These small discrepancies did not materially influence the interpretation of the data but have been corrected for in the pH values given in the text.

Results

^{13}C NMR Analysis. The assignments of the principal resonance lines of the various phosphatidylserine carbon atoms and the change in chemical shift under a variety of experimental conditions are given in Table I. Most assignments were made by comparison with those observed for phosphatidylcholine by Stoffel et al. (1972). However, the assignments of the resonances of the serine methylene carbon, the carbon adjacent to the amino group, and the C-1 methylene carbon of the glycerol moiety were made by comparison with the spectra of phosphoserine, phosphatidic acid, diethyl phosphate, and trioleoylglycerol. Note that the resonances of the methylene carbon to the phosphate group of both glycerol and serine appear to have the same chemical shift, within the limits of resolution.

Effect of Ionic Form and Solvent on ^{13}C and ^{31}P NMR Line Widths of Phosphatidylserine. Line widths of the polar head-group atoms of phosphatidylserine were sensitive to both the solvent and the ionic form of the lipid, whereas the acyl portions of the molecule were much less so. Figure 1A shows the ^{13}C NMR spectrum in $CDCl_3$ of phosphatidylserine prepared in the H^+ form by prior equilibration at pH 1 (see Materials and Methods). Note that between 60 and 90 ppm the resonances of the glycerol and the serine methylene and α-amino carbon atoms were broadened compared with those of the acyl chain (32–53 ppm) or solvent ($CDCl_3$ triplet, 94.3–97.5 ppm) carbon atoms. Notice, however, that the resonances of the fatty acid ester carbonyl (192.1–192.4 ppm) and the serine carboxyl (188.5 ppm) carbon atoms were narrow. Spectra in the amphipathic deuterated lower phase (D-LP) of phosphatidylserine also prepared at pH 1 (Figure 1B) showed increased resolution of the resonances of the

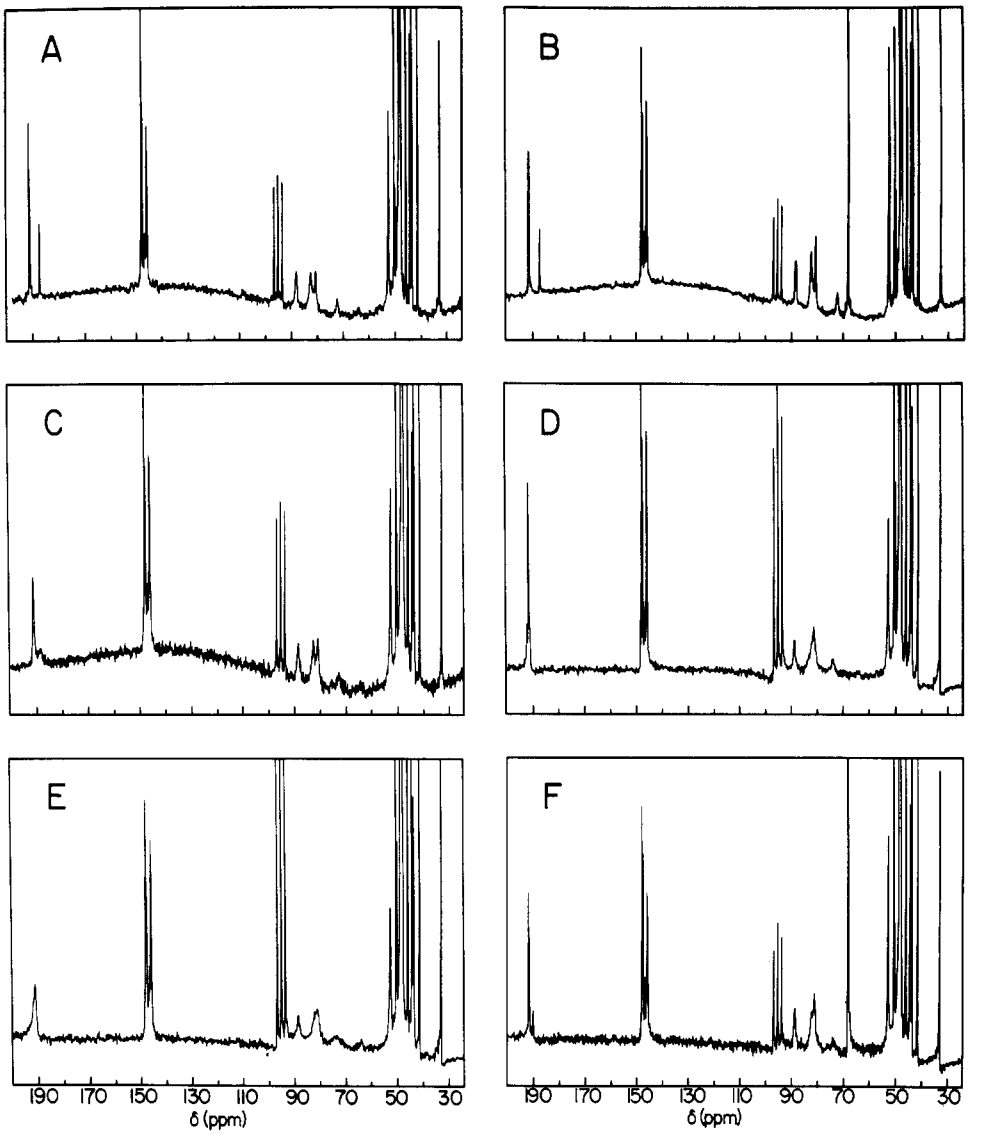

FIGURE 1: Proton noise-decoupled ^{13}C NMR spectra of 0.5 M phosphatidylserine (PS) in $CDCl_3$ and $CDCl_3$-containing lower phase solvent. Each spectrum represents data obtained from ~30 000 transients by using a 90° pulse with an acquisition time of 0.819 s. (A) Spectrum of the H^+ form (pH 1) in $CDCl_3$; (B) as in (A), except in $CDCl_3$-containing lower phase; (C) spectrum of Na^+ form (pH 7) in $CDCl_3$; (D) spectrum of Na^+ form (pH 10) in $CDCl_3$; (E) spectrum of Ca^{2+} form (pH 10) in $CDCl_3$; (F) as in (E), except in $CDCl_3$-containing lower phase. For assignments of carbon resonances, see Table I. Note in $CDCl_3$ (E) that the NMR signals from the polar head-group carbons of the Ca^{2+} form are greatly reduced in height, relative to the $CDCl_3$ carbon peak, and are broadened. In contrast, note the increased resolution and enhanced NMR signals of the polar head group when the Ca^{2+} form is present in the $CDCl_3$-containing lower phase (F).

glycerol and serine carbon atoms, the remaining portions of the spectrum being unaffected. With phosphatidylserine in $CDCl_3$ prepared in the Na^+ form by prior equilibration at pH 7 (Figure 1C), marked line broadening of all polar head-group carbon atoms was seen. Especially note the greatly increased line widths of the serine carboxyl and fatty acid ester carbonyl carbon resonances, compared to those of the H^+ form. Phosphatidylserine prepared in the Na^+ form at pH 10 and examined in $CDCl_3$ (Figure 1D) showed a marked reduction in line broadening of the carbonyl carbons and, to a lesser degree, in the polar head-group carbons, although this was partially obscured by the upfield shift of the methylene carbon to the phosphate which overlapped with the glycerol 1 carbon. In the amphipathic D-LP solvent phosphatidylserine prepared at both pH 7 and 10 (not shown) exhibited higher resolution of the resonances of the polar head-group atoms compared to that obtained in the nonpolar $CDCl_3$ solvent. Spectra in $CDCl_3$ of phosphatidylserine prepared in the Ca^{2+} form at pH 7 (Figure 1E) and at pH 10 (not shown) were similar, both showing marked line broadening in the entire polar head-group region, as well as in the unsaturated acyl-group carbon atoms (Figure 2). There was a large decrease in the signal to noise ratios of the phosphatidylserine carbon atoms relative to that of the solvent $CDCl_3$ resonance. In contrast, when the Ca^{2+} form was dissolved in amphipathic solvent (Figure 1F), resolution of the entire molecule was greatly enhanced, especially in the carbonyl and unsaturated carbon regions. We also examined the spectrum of phosphatidylserine in $CDCl_3$ after equilibration of the Na^+ form with 1 M $MgCl_2$ at pH 7. The spectrum (not shown) revealed only slight line broadening in the carbonyl region, the resonances of the amino-group carbon being unaffected. Chemical analysis revealed, however, that Mg^{2+} did not fully replace the Na^+. At pH 10 in $CDCl_3$ (not shown) Mg^{2+} was more tightly bound to phosphatidylserine and caused similar line broadening in the polar head-group region to that observed with Ca^{2+}. In D-LP phosphatidylserine prepared in the Mg^{2+} form at pH 10 showed increased resolution of all polar head-group carbon atoms as was seen previously with the Ca^{2+} form.

^{31}P NMR line widths of phosphatidylserine were similarly affected by ionic form and solvent (Table II). In $CDCl_3$ phosphatidylserine prepared in the H^+ form at pH 1 had a

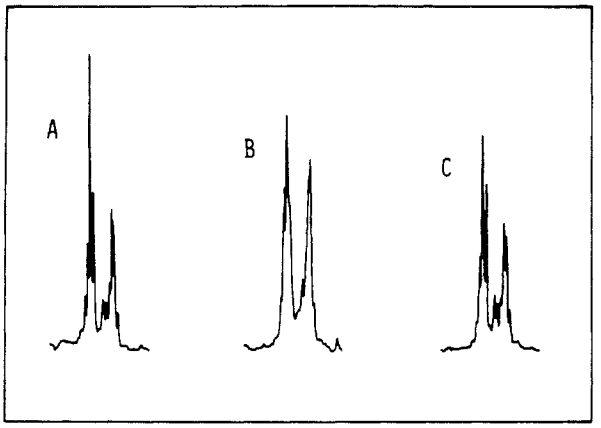

FIGURE 2: Effect of Ca^{2+} on the ^{13}C NMR spectrum of the acyl-chain double-bond carbons of phosphatidylserine. (A) H^+ form (pH 1) in $CDCl_3$; (B) Ca^{2+} form (pH 7) in $CDCl_3$; (C) Ca^{2+} form (pH 7) in $CDCl_3$-containing lower phase. Note the distinct line broadening and lack of resolution of the Ca^{2+} form in $CDCl_3$ (B), which is restored when the complex is present in the amphipathic lower-phase solvent (C). Spectral regions shown for (A), (B), and (C) are from 142 to 152 ppm (compare Figure 1).

Table II: Effect of pH and Ionic Forms on Chemical Shift and Line Widths of ^{31}P NMR Spectra of Phosphatidylserine

phosphatidylserine (ionic form)	line width (Hz)[a]		chemical shift (ppm)[c]	
	$CDCl_3$	D-LP[b]	$CDCl_3$	D-LP[b]
H^+ form, pH 1	50	55	(–) 0.86	(–) 0.23
Na^+ form, pH 7	160	60	(–) 0.51	(–) 0.04
Na^+ form, pH 10	40	38	(+) 0.90	(+) 0.60
Ca^{2+} form, pH 7	225	50	(–) 1.86	(–) 3.91
Ca^{2+} form, pH 10	225	38	(–) 1.74	(–) 3.85

[a] Width at half-height. [b] D-LP = lower-phase solvent composed of $CDCl_3$-methanol-water, 86:14:1 v/v/v. [c] Chemical shifts were assigned relative to 85% phosphoric acid (plus 10% D_2O) as an external standard; negative shifts are upfield from the standard. Addition of 10% D_2O caused a 0.12 ppm downfield shift compared to pure 85% phosphoric acid.

^{31}P NMR line width of ~50 Hz. In the zwitterionic Na^+ form prepared at pH 7, ^{31}P line widths were greatly broadened (160 Hz) but narrowed again (40 Hz) in the Na^+ form prepared at pH 10. In $CDCl_3$ phosphatidylserine in the Ca^{2+} form showed major line broadening of the ^{31}P signal (225 Hz), whether prepared at pH 7 or 10. However, in the amphipathic solvent ^{31}P line widths of all forms of phosphatidylserine were narrow (38–60 Hz), the narrowing effect on the Ca^{2+} form being particularly striking.

Effect of Ionic Form on ^{13}C and ^{31}P NMR Chemical Shifts of Phosphatidylserine and Phosphatidylethanolamine. The profile of the ^{13}C NMR chemical shifts of the fatty acid ester carbonyl, serine carboxyl, fatty acid double-bond, and serine methylene carbon to the phosphate of phosphatidylserine equilibrated at varying pH values from 1 to 12 and examined in amphipathic solvent are shown in Figure 3. Note first the almost total lack of effect of the pH of the equilibrating buffer on the chemical shifts of the fatty acid ester carbonyl and double-bond carbons, as well as those of the solvent molecules, $CDCl_3$ and methanol. In contrast, note the large chemical shifts of the serine carboxyl carbon and, to a lesser extent, of the serine methylene carbon to the phosphate. In the Na^+ form the chemical shift of the serine carboxyl carbon was relatively stable up to pH 7, whereupon a major downfield shift (4.4 ppm) occurred with a small inflection between pH 8.2 and 8.7. In the Ca^{2+} form the chemical shift pattern was markedly different. There was a sizeable downfield shift (2.1

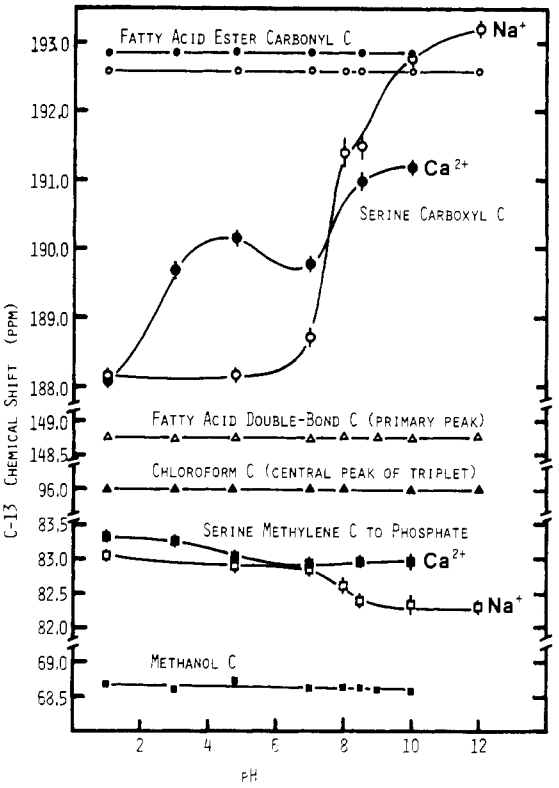

FIGURE 3: Effect of Na^+ and Ca^{2+} on the chemical shift–pH profile of the polar head-group (serine carboxyl and methylene phosphate) and fatty acid (ester carbonyl and double-bond) carbons of phosphatidylserine. Each point represents the ^{13}C NMR chemical shift of the specific carbon of phosphatidylserine in either the Na^+ or Ca^{2+} form at the indicated pH. Chemical shifts of the double-bond carbons of the Na^+ and Ca^{2+} forms were not significantly different, even though the line widths were broadened by Ca^{2+}. Spectra of the lipid were taken in the amphipathic $CDCl_3$-containing lower phase which had been equilibrated with an upper phase adjusted to the indicated pH. Note that the chemical shifts of the acyl carbons of the phospholipid, and of the solvent molecular carbons, were insensitive to changing pH, whereas those of the functional group carbons of the polar head group underwent sizable changes.

ppm) when phosphatidylserine was equilibrated at pH values increasing from 1 to 4.4, then a small upfield shift (0.4 ppm) between pH 4.4 and 6.6, and finally a further downfield shift (1.5 ppm) which occurred between pH 6.6 and 10.2. Two features stand out with the Ca^{2+} form: the downfield shift of the serine carboxyl carbon at much lower pH than with the Na^+ form and the smaller overall magnitude of the chemical shift with increasing pH of the equilibrating buffer (3.1 vs. 4.6 ppm, respectively). The pH values at which maximum change in chemical shift occurred were 2.9 and 8.2 for the Ca^{2+} form and pH 7.8 and 9.5 for the Na^+ form. Effects of equilibrating pH and cation form of the phosphatidylserine on the chemical shifts of the methylene carbon to the phosphate were much more muted than that seen with the carboxyl carbon but were nonetheless of interest. In the Na^+ form little change was seen between pH 1 and 7; however, a significant *upfield* shift (~0.5 ppm) occurred between pH 7 and 9. In the Ca^{2+} form the changes in the chemical shift with varying pH were very small: only a small transient downfield deflection (0.3 ppm) between pH 2.9 and 6.6.

In Figure 4 are shown the effects of equilibrating pH on the ^{13}C NMR chemical shift of the carbon to the amino group of both phsophatidylserine and phosphatidylethanolamine. For phosphatidylserine the pattern was remarkably similar to that seen for the carboxyl group: in the Na^+ form there was a single downfield shift (2.4 ppm) occurring between pH 6 and 10 (maximum shift at pH 8.2); in the Ca^{2+} form there was

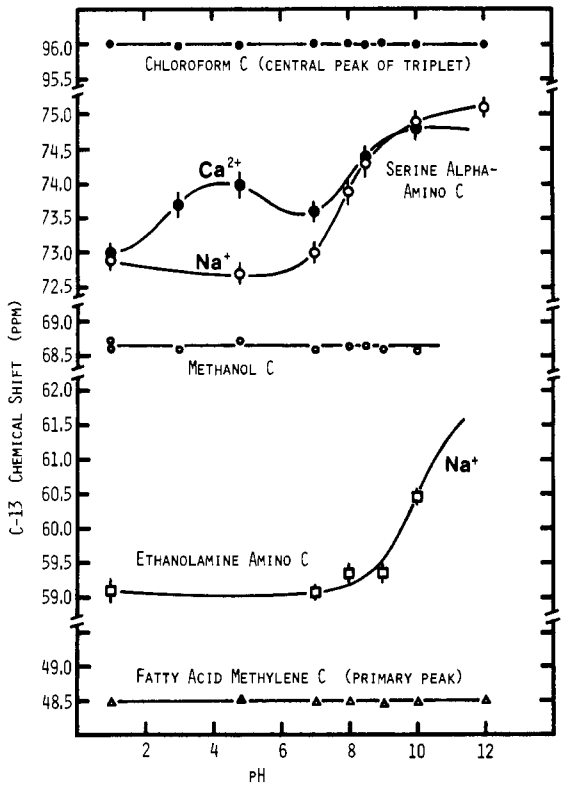

FIGURE 4: Effect of Na^+ and Ca^{2+} on the chemical shift–pH profile of the polar head-group (α-amino) and fatty acid (principal methylene) carbons of phosphatidylserine and phosphatidylethanolamine. Each point represents the ^{13}C NMR chemical shifts of the indicated carbon atoms of phosphatidylserine and phosphatidylethanolamine in either the Na^+ or Ca^{2+} forms. Note, as in Figure 3, that the chemical shifts of only the polar head-group carbons were effected by changes in pH. There were no significant differences between the Na^+ and Ca^{2+} forms of phosphatidylserine in the chemical shifts of the principal fatty acid acyl methylene carbons.

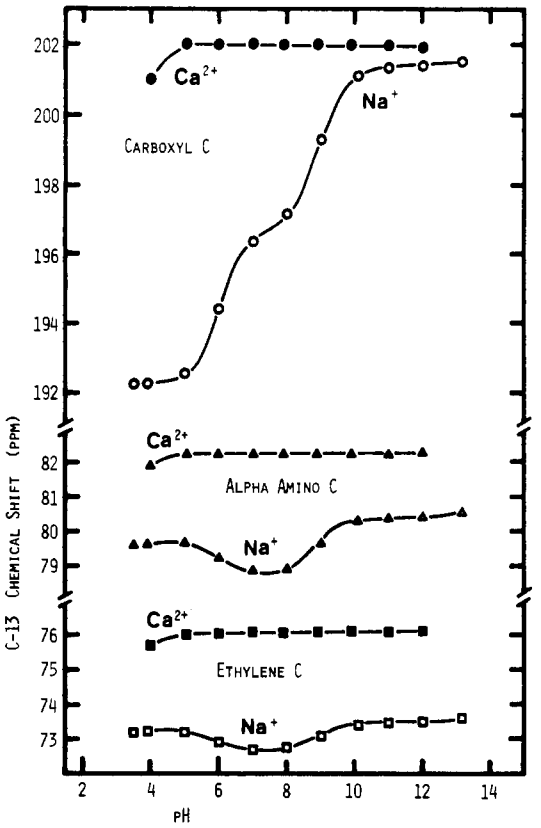

FIGURE 5: Effect of Na^+ and Ca^{2+} on the chemical shift–pH profile of the three types of carbons of ethylenediamine tetraacetate (EDTA). Each point represents the ^{13}C NMR chemical shift of the respective carbons of EDTA in either the Na^+ or Ca^{2+} forms at the indicated pH. Spectra were taken of EDTA dissolved in deionized water. Note the remarkable stabilization of the EDTA molecular structure in the presence of Ca^{2+}, in contrast to the major changes seen in the Na^+ form.

again the complex pattern with the low-pH downfield shift (1.0 ppm) between pH 1 and 3.9 and the upfield deflection and final downfield shift between pH 7 and 10. For phosphatidylethanolamine in the Na^+ form the pattern was similar to that seen for phosphatidylserine except that the change in chemical shift occurred at higher pH (maximum change at pH 9.9). The Ca^{2+} form of phosphatidylethanolamine was not studied.

Significant changes in the chemical shift of the ^{31}P NMR resonances of the phosphatidylserine phosphate group also occurred with change in cation form and the pH of the equilibrating buffer (Table II). Only small downfield shifts of 0.19 and 0.35 ppm for amphipathic solvent and $CDCl_3$, respectively, were observed in the Na^+ form in changing from pH 1 to 7. Increasing the pH of the equilibrating buffer to 10 caused further downfield shifts of 0.83 and 1.76 ppm, respectively, in the two different solvents. The chemical shifts of phosphatidylserine in the Ca^{2+} form were distinctly different. In $CDCl_3$ there was a 1.35 ppm *upfield* shift from that seen in the Na^+ form prepared at pH 7 and a 2.64 ppm upfield shift from that seen at pH 10. In amphipathic solvent the differences between Ca^{2+} and Na^+ forms were even greater (3.87 ppm upfield, pH 7; 4.45 ppm upfield, pH 10).

Effect of pH on ^{13}C NMR Chemical Shifts of EDTA and Succinic Acid. In an attempt to relate changes in chemical shift with changing pH to binding affinity for metal ions, two molecular species with high and low affinity for Ca^{2+} were studied: EDTA ($\log K_f = 10.6$) and succinic acid ($\log K_f = 1.2$) (Sillén & Martel, 1964). In Figure 5 the effect of cation form on the titration of EDTA is evident. In the Na^+ form the carboxyl carbons showed a very large downfield chemical shift (~9.5 ppm) with apparent pK_as at pH 6.0 and 8.9. In contrast, in the Ca^{2+} form, note the large downfield shift, relative to Na^+, at low pH, and the almost total insensitivity to changing pH thereafter. Similar but more muted perturbations were seen in the central carbon atoms of the molecule. In the Na^+ form the carbons to the amino group and the central ethylene showed a small transient upfield shift between pH 5 and 7, followed by a further downfield shift at higher pH. Note again the downfield shift and lack of sensitivity to pH in the Ca^{2+} form.

In contrast, with succinic acid (Figure 6), Ca^{2+} exerted only a slight effect when compared to that of Na^+. In both cation forms a major downfield shift (~5.5 ppm) occurred during the titration of the carboxyl groups, with Ca^{2+} causing only a small apparent acidic shift of the pK_as but with no reduction in the overall magnitude of the size of the chemical shift change. Note that the central methylene carbons almost totally mimicked the pattern seen with the peripheral carboxyl carbons, both in size of chemical shift and pH range.

Infrared Analysis of Phosphatidylserine. Several differences appeared in the infrared spectrum of the various forms of phosphatidylserine in the regions of 1745–1645 cm^{-1} (Figure 7). With phosphatidylserine in the H^+ form, a strong band was seen at 1745 cm^{-1} (ester carbonyl region; Abramson et al., 1964) with a slight shoulder at ~1720 cm^{-1}. In the Na^+ form prepared at pH 7, a broad weak band appeared in the 1650-cm^{-1} region (asymmetric C–O stretching mode) with a corresponding reduction in the intensity of the 1745-cm^{-1} vibration. At pH 10 the 1650-cm^{-1} peak was considerably

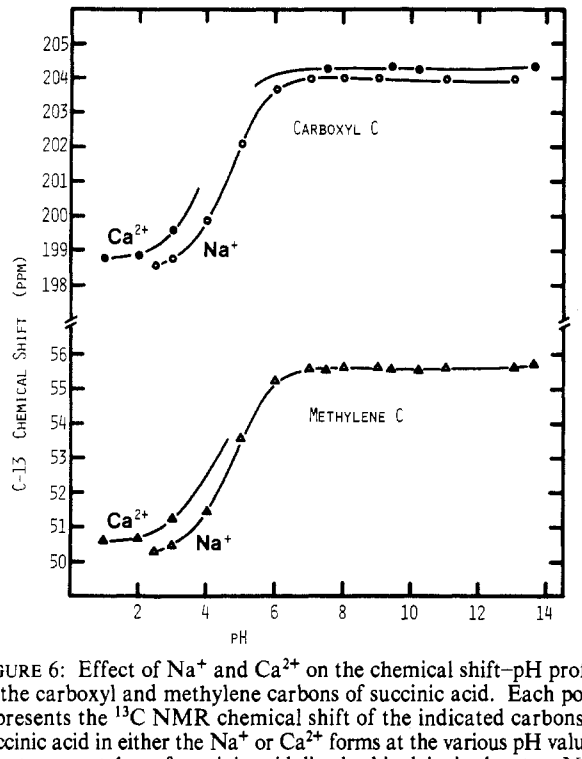

FIGURE 6: Effect of Na^+ and Ca^{2+} on the chemical shift–pH profile of the carboxyl and methylene carbons of succinic acid. Each point represents the ^{13}C NMR chemical shift of the indicated carbons of succinic acid in either the Na^+ or Ca^{2+} forms at the various pH values. Spectra were taken of succinic acid dissolved in deionized water. Note the lack of stabilization by Ca^{2+} of the molecular structure with changing pH, in contrast to that seen with EDTA (Figure 5).

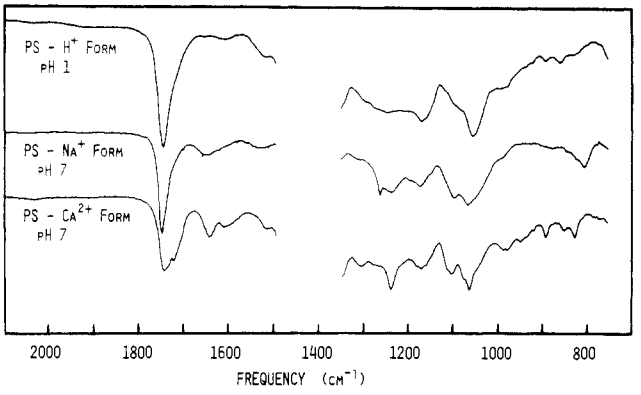

FIGURE 7: Infrared spectra of different ionic forms of phosphatidylserine. The different ion forms of phosphatidylserine were made into a Nujol mull and placed on 2 × 4 cm KBr plates. Spectra were taken at room temperature in the range from 2000 to 800 cm^{-1}. The band resolution was 2.0 cm^{-1}. Note the generally increased resolution of the spectrum of the Ca^{2+}, as opposed to the Na^+, form. In particular, notice the appearance of the new peaks at 1720 and 1610 cm^{-1}. PS = phosphatidylserine.

stronger (data not shown). In the Ca^{2+} form prepared at pH 7, note the broadening of the resonance in the ester carbonyl (1740-cm^{-1}) region with the appearance of a separate peak at 1720 cm^{-1}. Also note the increased intensity of the 1645-cm^{-1} band and the appearance of a second band at 1610 cm^{-1}. Examination of entire fingerprint region reveals a noticeable increase in the resolution of the spectrum of the Ca^{2+} form.

Determination of Binding Constants of Phosphatidylserine, Phosphatidylethanolamine, and Phosphatidylhydroxypropionate for Ca^{2+}. Binding constants were measured by analyzing the transfer of $^{45}Ca^{2+}$ at varying initial concentration from an aqueous upper phase at pH 7 to an organic lower phase containing a constant amount of phospholipid. A Scatchard plot (Scatchard, 1949) of Ca^{2+} binding to phosphatidylserine was constructed. Two apparent binding constants were evident: one at high affinity ($K_f = 2.4 \times 10^4$) with

Table III: Binding of Ca^{2+} to Phosphatidylethanolamine, Phosphatidylserine, and Phosphatidylhydroxypropionate

phospholipid	K_f[a]	log K_f
phosphatidyl-ethanolamine	$(6.00 \pm 0.96) \times 10^1$ (9)	1.78 ± 0.07
phosphatidylserine	$(2.96 \pm 0.43) \times 10^4$ (24)	4.47 ± 0.07
phosphatidyl-hydroxypropionate	$(8.22 \pm 2.01) \times 10^5$ (5)	5.91 ± 0.10

functional group	log K_f' [b]	K_f'
carboxyl	(+) 2.69	4.90×10^2
phosphate	(+) 3.22	3.66×10^3
amino	(–) 1.44	3.63×10^{-2}

[a] The apparent formation (binding) constants (K_f's) were calculated for each phospholipid by determining the amount of Ca^{2+} bound to the phospholipid in the lower phase [Ca-PL] relative to the concentration of Ca^{2+} remaining in the upper phase after equilibrium of the lipid [Ca^{2+}]. These were used to determine K_f values by using either the equilibrium constant equation (K_f = [Ca-PL]/([Ca^{2+}][PL]), where [PL] = concentration of free phospholipid in the lower phase), or a Scatchard plot (Scatchard, 1949), where the intercept of the $\bar{Y}$/[Ca^{2+}] axis = K_f and $\bar{Y}$ = moles of Ca^{2+} transferred to the lower phase per mole of phospholipid. $\bar{Y}$ intercepts of the Scatchard plots indicated Ca/PL binding ratios for phosphatidylserine and phosphatidylhydroxypropionate of 1:2 and for phosphatidylethanolamine of 1:1. Weaker K_f values at Ca/PL binding ratios of 1:1 were also observed for phosphatidylserine [$K_f = (2.2 \pm 0.1) \times 10^3$] and for phosphatidylhydroxypropionate [$K_f = (2.5 \pm 1.3) \times 10^2$]. [b] Log K_f' = log of partial formation constants for each functional group. These were calculated from the log K_f values of the three analogous phospholipids by using simultaneous equations as follows: (1) let P = log K_f' of the phosphatidyl moiety (phosphate), (2) let C = log K_f' of the carboxyl moiety, and (3) let A = log K_f' of the amino moiety. Thus, the log K_f of phosphatidylserine = $P + C + A$ = 4.47, the log K_f of phosphatidylethanolamine = $P + A$ = 1.78, and the log K_f of phosphatidylhydroxypropionate = $P + C$ = 5.91. Hence, the log K_f' of the carboxyl group (C) = log K_f of phosphatidylserine – the log K_f' of phosphatidylethanolamine, the log K_f' of the amino group (A) = the log K_f of phosphatidylserine – the log K_f of phosphatidylhydroxypropionate, and the log K_f' of the phosphate group (P) = the log K_f of phosphatidylhydroxypropionate – the log K_f' of the carboxyl group. Values in the table are the means ± SEM, the numbers in parentheses being the number of determinations made.

a binding ratio of ~0.5 mol of Ca^{2+}/mol of phospholipid; a second of low affinity ($K_f = 2.3 \times 10^3$) with a binding ratio of 1 Ca^{2+}/mol of lipid. The binding constants of phosphatidylethanolamine and phosphatidylhydroxypropionate were similarly determined and are shown with that of phosphatidylserine in Table III. It can be seen that phosphatidylethanolamine, which lacks a carboxyl group, has an affinity $^1/_{400}$ that of phosphatidylserine, whereas phosphatidylhydroxypropionate, which possesses a carboxyl group but lacks an amino group, has a high affinity for Ca^{2+}, almost 15 times higher than that of phosphatidylserine. From the K_f values of these analogous phospholipids, it was possible to calculate partial formation constants (K_f') for the three functional groups of phosphatidylserine, with the assumption that the observed K_f is the summation of the attractive and repulsive forces. These computed values, also shown in Table III, are as follows: K_f' of phosphatidyl phosphate = 1.66×10^3, K_f' of serine carboxyl = 4.90×10^2, and K_f' of serine amino = 3.63×10^{-2}.

Space-Filling Molecular Models. Molecular models of the complex between Ca^{2+} and two phosphatidylserine molecules were constructed in an attempt to gain further insight into the possible configuration of the ionic interaction. Two identical models of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine were constructed from CPK atomic models and arranged in a strain-free conformation which accommodated a sphere with dimensions (1.35-Å radius) approximating that of Ca^{2+} (0.9-Å

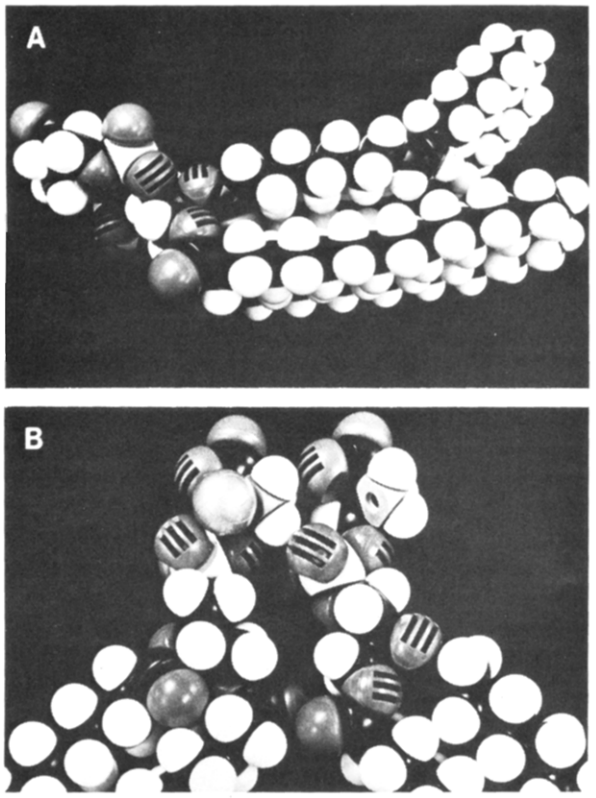

FIGURE 8: Space-filling molecular model of Ca^{2+}–phosphatidylserine complex. (A) Model showing bimolecular Ca^{2+}–phosphatidylserine complex; (B) model opened to show trimodal hemicoordination pocket. The Ca^{2+} ion (sphere) is shown on the left filling the hemicoordination pocket. The exposed pocket (right half) shows the carboxyl oxygen (top left of pocket), deprotonated amino group nitrogen (top right of pocket), and phosphate oxygens (bottom of pocket). Note that removal of the proton from the amino group is necessary to form the cleft in which the Ca^{2+} ion fits.

crystal lattice radius). A complex composed of two phosphatidylserine molecules enclosing one Ca^{2+} ion is shown in Figure 8A. In Figure 8B is shown the polar head-group region of the phosphatidylserine dimer, opened to expose the coordination pocket formed by the functional groups. The Ca^{2+} ion (sphere) is shown at the left filling the hemicoordination pocket. The unfilled pocket (right half) is bounded by a carboxyl oxygen (top left of pocket), the deprotonated amino nitrogen (top right of pocket), and a phosphate oxygen (bottom left of pocket). Note that removal of a proton from the amino nitrogen is necessary to form the cleft into which the sphere fits.

Discussion

Analysis of the ^{13}C resonances of various atoms of different ionic species of phosphatidylserine dissolved in the two organic solvent environments showed significant line broadening of most of the polar head-group atoms of the lipid, compared to the acyl-chain carbon atoms. Similar line broadening of the phosphate P atom was also seen from the ^{31}P NMR studies. ^{31}P line widths observed in the organic solvents were significantly broader than those we and others (London & Feigenson, 1979) obtained with sonicated aqueous cholate dispersions of phosphatidylserine and other phospholipids. This increased line broadening does not appear to be caused by field inhomogeneity, since spinning of the samples did not alter line widths. Neither does the presence of trace amounts of paramagnetic metal ions appear to be responsible, since the repeated acid-washing procedure we routinely employed was able to quantitatively remove Mn^{2+}, a strong line-broadening agent, from phosphatidylserine–Mn^{2+} complexes in which the ^{31}P NMR signal was broadened into the base line. The line broadening rather appears to be caused by some restriction in the rotational freedom of the polar head group, as was concluded by Browning & Seelig (1980) from ^{2}H NMR studies. The fact that the resonance line of the serine carboxyl carbon was more highly resolved when prepared at pH 1 or 10 than at pH 7 indicates that charge (dipolar) interaction with the amino group is a probable factor in the restriction of molecular motion. At either pH extreme charge interaction would be obivated by either protonation of the carboxyl or deprotonation of the amino group. Ca^{2+} caused a marked increase in line width of the carboxyl carbon resonance when phosphatidylserine was dissolved in $CDCl_3$, a relatively nonpolar solvent, indicating further restriction in rotational freedom of the polar head group. We interpret these broadened lines in $CDCl_3$, especially in the zwitterion forms, as being indicative of *the formation of both intra- and intermolecular electrostatic bonds*. In this solvent these effects would lead not only to restricted intramolecular motion but also to the formation of large, slow tumbling micelles in this solvent. This latter mechanism seems to be particularly evident with the divalent Ca^{2+} form where ionic cross-bridging would be favored. Hope & Cullis (1980) and numerous others have shown that in aqueous dispersions of phosphatidylserine, Ca^{2+} also causes marked line broadening of the ^{31}P NMR signal producing an anisotropic powder pattern like that of the rigid anhydrous form. They further showed that the spin–lattice relaxation time (T_1) of the Ca^{2+} form was greatly *in*creased (>20-fold) over that of the Na^+ form, indicating the immobilization effect that Ca^{2+} has upon the phosphate group.[2] The distinct narrowing of the resonance lines of the polar head-group atoms (both carbon and phosphorus) which we observed when the Ca^{2+} and other ionic forms of phosphatidylserine were dissolved in the methanol- and water-containing amphipathic lower phase (chloroform–methanol–water, 86:14:1 v/v/v) points to a line-narrowing mechanism which involves increased mobility of the molecular complex relative to that seen in the more hydrophobic solvent. The apparent increase in rotational freedom suggests a significant reduction in the intermolecular interactions between lipid molecules. That is, in the more hydrophobic solvent, dipole–dipole interactions between the zwitterionic phosphatidylserine molecules would lead to clustering of lipid molecules and the formation of large, slow tumbling micelles. In the more hydrophilic solvent, dipole–dipole interaction between the polar head group and the polar solvent molecules would impede lipid–lipid interactions. The dramatic decrease in the line widths of the polar head-group atoms of the Ca^{2+} form of phosphatidylserine in the amphipathic solvent compared to that in the more nonpolar solvent indicates a marked reduction in the size of the lipid clusters in solution. On the basis of the 2:1 binding stoichiometry of phosphatidylserine with Ca^{2+} derived from Scatchard plots, it is probable that Ca^{2+}–(phosphatidylserine)$_2$ dimers were responsible for the increased mobility revealed by the marked narrowing of the resonance lines. Data from both Portis et al. (1979) and Hope & Cullis

[2] For these highly anisotropic systems, the effective correlation time (or times) may be on either side of that corresponding to a T_1 minimum, so that either a decrease or an increase in T_1 can correspond to decreased head-group mobility. Thus, the Ca–phosphatidylserine complexes discussed by Hope & Cullis (1980) are illustrative of systems *out* of the region of extreme narrowing. There, an *increase* in T_1 denotes a *decrease* in mobility. However, the Ca–phosphatidylserine complexes utilized by Kurland et al. (1979) correspond to systems *in* the region of extreme narrowing. Here, a *decrease* in T_1 corresponds to a *decrease* in mobility.

(1980) also indicate that Ca^{2+}–(phosphatidylserine)$_2$ complexes form upon binding of Ca^{2+} with the lipid. Our finding of broadened line widths of the acyl-chain double-bond carbons when the Ca^{2+} form is dissolved in $CDCl_3$ (Figure 2) also supports the concept that Ca^{2+} induces highly ordered structure in the acyl chains, as suggested by Portis et al. (1979).

In a separate set of experiments to study the binding of inorganic phosphate to phosphatidylserine in the formation of phospholipid–calcium–phosphate complexes (Cotmore et al., 1971), we used the previously mentioned biphasic system in which either Ca^{2+} or inorganic phosphate or both were added to the aqueous upper phase and the lipid to the organic lower phase. We found that when [^{32}P]orthophosphate alone was added to the aqueous upper phase, no sequestration of radioactivity into the phosphatidylserine-containing lower phase occurred. However, if Ca^{2+} (1 mM) were present in the upper phase concomitantly with the labeled phosphate (1 mM), large amounts of [^{32}P]phosphate were rapidly transferred by the lipid into the organic phase. Significantly, if the phosphatidylserine in the organic layer was in the Ca^{2+} form (Ca/lipid, 1:2, as determined by direct chemical analysis), again no radioactivity was transferred to the organic phase. These findings show that when Ca^{2+} is bound to phosphatidylserine in a stoichiometric ratio of 1 Ca^{2+} to 2 lipid molecules, it has markedly reduced chemical activity and is no longer able to bind to inorganic phosphate. Taken together with the finding of the notable line-narrowing effect of this amphipathic organic solvent mentioned above, these data further support that when Ca^{2+} alone is equilibrated with phosphatidylserine in this solvent, freely tumbling dimeric complexes of Ca–(phosphatidylserine)$_2$ are formed.

Further insight into the nature of the ionic interaction between phosphatidylserine and either Na^+ or Ca^{2+} was gained by studying the ^{13}C NMR chemical shifts of the polar head-group atoms. It is evident from the chemical shifts shown in Figures 3 and 4 that deprotonation of the serine carboxyl group of the Na^+ form of phosphatidylserine in lower phase does not begin until the pH of the aqueous phase almost reaches 7. Both Quirt et al. (1974) and Cozzone & Jardetzky (1976) have shown that a downfield chemical shift of the carboxyl carbon accompanies deprotonation. The large magnitude of the chemical shift in changing from pH 7 to 12 and the small inflection at pH 8 suggest that in the Na^+ form both the carboxyl and the amino groups are deprotonated almost concomitantly. The apparent pK_a of the carboxyl group in the Na^+ form at the interface between the aqueous and organic phases clearly appears to be anomalous ($pK_a' = 7.6$) compared to that seen with micelles in aqueous environment ($pK_a' \simeq 4$; Abramson et al., 1964; Seimija & Ohki, 1973). This suggests that the interactions between the negatively charged phosphate and positively charged Na^+ or amino group may interfere with deprotonation of the carboxyl group in this interfacial environment. Examination of the chemical shift–pH profile of the amino carbon (Figure 4) reveals a pattern very similar to that seen for the carboxyl carbon (Figure 3). This indicates that the chemical environments around both carbon atoms are closely interrelated and that the titration of each functional group is reflected in the chemical shift of the other. In the presence of Ca^{2+} two major effects on the chemical shift profile of the serine carbon atoms were observed: a decrease in the overall magnitude of the change in chemical shift with changing pH and, in the case of the carboxyl- and amino-group carbons, a significant downfield chemical shift at much lower ambient pH when compared to that of the Na^+ form. The first suggests that Ca^{2+} stabilizes the chemical environment around not only the carboxyl carbon (especially in the pH range from 3 to 10) but also the carbons to the amino and phosphate groups as well. The second effect will be discussed later.

The stabilizing effect of Ca^{2+} on a chemical environment is particularly evident in the pH–chemical shift profile of EDTA, a strong chelator of Ca^{2+} (Figure 5). The carboxyl carbons were almost totally insensitive to changing pH with EDTA in the Ca^{2+} form. However, in a situation where only weak binding of Ca^{2+} occurred, as with succinic acid (Figure 6), Ca^{2+} had only a minor effect on the chemical shift–pH profile. These findings indicate that the sensitivity of the chemical shift of the various ionic forms to changing ambient pH is a good indicator of the tightness of cation binding. Where very strong binding occurs, the chemical environment about the functional group is stabilized and is insensitive to changes in the surrounding pH; where weak binding occurs, changes in the ambient environment are readily sensed by the functional group and are expressed by changes in the chemical shift. Thus, we conclude that Ca^{2+} has fairly strong interaction with both the serine carboxyl and amino groups. The interaction with the carboxyl group is not as pronounced as with EDTA but is stronger than with succinate. An even greater stabilizing effect of Ca^{2+} was seen in the chemical shift of the ^{31}P NMR resonances of the phosphate group of phosphatidylserine (Table II) and in the carbon to the phosphate group (Figure 3). Ca^{2+} not only stabilized the ^{31}P resonance in the face of changing pH but also caused a marked ($\sim$4 ppm) upfield chemical shift relative to the Na^+ form of phosphatidylserine. Such an upfield shift is indicative of increased shielding of the P nucleus and is consistent with greater charge neutralization, as suggested by Kurland et al. (1979). On the basis of the greater chemical shift stabilization of the ^{31}P resonances, Ca^{2+} appears to possess a greater affinity for the phosphate than the carboxyl group. As will be discussed later concerning Scatchard plots of Ca^{2+} binding constants of different phospholipid analogues, these independent observations support the same conclusion.

The effect of Ca^{2+} on both the carboxyl and amino groups, as judged by the downfield chemical shift at much lower pH than that seen with Na^+ (Figures 3 and 4), appears to be that of an enhancement of deprotonation. That is, Ca^{2+} appears to displace H^+ from both groups at ambient H^+ concentrations well above those seen with Na^+. However, some care must be exercised in the interpretation of these chemical shift perturbations. It is clearly evident, as stated before, that the chemical environments around the head-group atoms are closely interrelated and perturbations at one locus appear to be transmitted through at least two or more chemical bonds. They may reflect both conformational and electrostatic effects. This possibility is evident in the pH–chemical shift profiles of the internal carbon atoms of both EDTA and succinate. In the Na^+ form of EDTA the methylene carbons adjacent to the carboxy groups show a chemical shift pattern partially mirroring that of the peripheral carboxyl carbons. In the Na^+ form of succinate the chemical shift profile of the central methylene carbons almost perfectly parallels that of the peripheral carboxyl carbons. Thus, adjacent carbons may show either similar or opposite chemical shifts with changing pH, depending on the presence of other functional groups.

Infrared data (Figure 7) also support a direct interaction between the carboxyl group and Ca^{2+}. In the Na^+ form the broad, weak absorbance band in the asymmetric C–O stretching region (1650 cm^{-1}) at pH 7 (which was even stronger at pH 10) suggests the presence of a carboxylate

group in which C–O bonds possess some π-bonding character. The appearance in the Ca^{2+} form of a distinct C=O stretching mode at 1720 cm^{-1} suggests that Ca^{2+} must polarize the carboxylate group leading to the reappearance of the C=O form, and is indicated by the generally increased resolution in the whole fingerprint region to a more ordered structure.

The binding constant studies (Table III) indicate that phosphatidylserine has a moderate affinity for Ca^{2+}, intermediate between that of EDTA and that of succinate. The apparent formation constant we observed for phosphatidylserine at pH 7 [$K_f = (3.0 \pm 0.4) \times 10^4$ M^{-1}] is almost 1 order of magnitude greater than that measured by Portis et al. (1979; 3.9×10^3 M^{-1}) but is in closer agreement with that observed by Hendrickson & Fullington (1965; 1.35×10^4 M^{-1}), who used titration methods. On the other hand, our value is several orders of magnitude smaller than that measured by Hauser et al. (1969; 1.7×10^7 M^{-1}) and Seimiya & Ohki (1973; 6.4×10^5–2.9×10^6 M^{-1}), who studied phospholipid monolayers at an air–water interface. There are several possible explanations for the marked discrepancy between these measured values. We used a liquid–liquid biphasic system and measured the ability of phosphatidylserine to transfer Ca^{2+} from the aqueous phase into the organic layer. We used classic Scatchard techniques and obtained two affinity constants, one with a binding ratio of 2 lipids/Ca^{2+} (high affinity) and one with a ratio of 1 lipid/Ca^{2+} (low affinity). The data reported by Portis et al. (1979) also provide evidence for a second binding constant at high cation concentration, but their analysis involved equilibrium dialysis of sonicated lipid vesicles with an ionophore (X-537A) to permit Ca^{2+} penetration into the liposomes. Hauser et al. (1969) and Seimiya & Ohki (1973) used air–water monolayers of phosphatidylserine and measured surface binding of Ca^{2+}. In their studies they obtained binding ratios of only 0.25–0.46 Ca^{2+}/lipid, depending on the surface pressure. In general, it appears that methods which involve large dipolar differences between the two phases (e.g., air–water) give high apparent binding constants, and those using aqueous micelles give much lower values. Our studies using liquid–liquid interfaces provide intermediate values. Thus, with all of the widely varying experimental conditions, it is not surprising that the apparent binding constants obtained do not agree well. Further, it is not obvious which of these constants is most applicable to physiological conditions.

Our observation of the weak affinity of Mg^{2+} for phosphatidylserine, relative to either Na^+ or Ca^{2+}, is in agreement with several recent studies showing that Ca^{2+} has a much higher affinity for this lipid than does Mg^{2+} (Portis et al., 1979; Kurland et al., 1979; Newton et al., 1978). It is of interest that the affinity of Na^+ for phosphatidylserine was greater than that of Mg^{2+} in our liquid–liquid biphasic system. However, at elevated pH (10) where the amino group is deprotonated, Mg^{2+} binds more tightly and displaces Na^+.

Through the use of two phospholipid analogues of phosphatidylserine (phosphatidylethanolamine and phosphatidylhydroxypropionate) an attempt was made to quantitate the contribution of the individual functional groups of the polar head group to Ca^{2+} binding. The values obtained indicate that the phosphate group is the major contributor to Ca^{2+} binding but that the carboxyl group also contributes positively. Our findings regarding the relative affinities of Ca^{2+} for the carboxyl group and phosphate group are at odds with those reported by Seimiya & Ohki (1973) using titration of an air–water monolayer of phosphatidylserine. They report an apparent binding constant of Ca^{2+} for the carboxyl group of 2.9×10^6 M^{-1} and for phosphate of 6.4×10^5 M^{-1}. However, in their study they assumed that the affinity of Ca^{2+} for the phosphate group of phosphatidylethanolamine, which they measured, would be the same as that for phosphatidylserine, which they did not measure. Our findings, which are several orders of magnitude smaller and appear to be founded on solid thermodynamic principles, provide quantitative support to those of Dawson & Hauser (1970), who also concluded that the phosphate group was the main binding site. Our finding that the amino group is inhibitory to Ca^{2+} binding is not surprising since the positive charge of this group should be repulsive to Ca^{2+}. Also, the space-filling models indicate that the amino group must be deprotonated to participate in the formation of the coordination pocket (Figure 8B). Nevertheless, care must be exercised in extrapolating to other systems the affinity values we obtained for the various functional groups. The calculations of the partial formation constants are based on the assumption of an additive behavior of the log K_f's of the various charged groups in their interaction with Ca^{2+}. Since the Gibbs free energies are known to be additive properties and are proportional to the log of the K_f, this seems to be a logical approach. Nevertheless, it is evident from the widely varying values of binding constants obtained by the various experimental methods that a variety of factors are involved, including the probability that Ca^{2+} induces conformational changes in the polar head group. However, final determination of the bonding character of Ca^{2+} with phosphatidylserine will probably require single-crystal X-ray diffraction of a crystalline lipid–Ca^{2+} complex, but even here questions will still remain regarding its relation to the native state in biological membranes.

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Photochemical and Functional Properties of Bacteriorhodopsins Formed from 5,6-Dihydro- and 5,6-Dihydrodesmethylretinals†

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ABSTRACT: 5,6-Dihydroretinal and 5,6-dihydro-1,1,5,9,13-desmethylretinal are synthesized, and their all-trans isomers are shown to form pigment analogues (λ_{max} at 475 and 460 nm, respectively) of bacteriorhodopsin (purple membrane protein). The shift of the absorption maximum of the pigment from that of the protonated Schiff base of the chromophore for 5,6-dihydrobacteriorhodopsin is small compared to that of the native pigment, suggesting that negative charges similar to those controlling the λ_{max} of visual pigment rhodopsin exist near the cyclohexyl ring. Both pigment analogues undergo reversible light-induced spectral shifts reflecting cyclic photoreactions of the pigments. These results indicate that the absence of the C-5–C-6 double bond and of the five methyl groups of retinal does not abolish the photochemistry of these pigment analogues and strongly suggest that these structural features are not directly required for the photoreactions of native bacteriorhodopsin. The apparent rates of the photochemical transformations of these artificial pigments are quite different from those of bacteriorhodopsin. A *working hypothesis* is proposed for the photocycle of the pigment analogues, which includes a slower light-induced cycling rate (for the light-adapted pigments) than that of native bacteriorhodopsin and an increased rate of dark adaptation. When incorporated into egg lecithin vesicles both pigment analogues show proton pumping ability, again indicating that the missing double bond and the methyl groups are not structurally required for the function of the pigments.

The light-transducing pigment bacteriorhodopsin (bR) is the only protein in the purple membrane of the halophilic bacteria, *Halobacterium halobium*. Its chromophore, retinal, is linked to the apomembrane through a protonated Schiff base bond and can be extracted from the light-adapted form of the pigment as the all-trans isomer. Various synthetic bacteriorhodopsins have been prepared from retinal analogues in order to study the binding of retinal to the apoprotein, as well as the role of chromophore structure on the photochemistry and the function of the pigment (Oesterhelt & Christoffel, 1976; Tokunaga et al., 1977; Marcus et al., 1977; Tokunaga & Ebrey, 1978; Crouch et al., 1979). Chemical modifications of the retinal polyene side chain and/or the β-ionone ring are possible. We report here results of studies of pigments regenerated from bleached bacteriorhodopsin and two retinal

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