Aqueous Dispersions of Phosphatidylserine*

IONIC PROPERTIES

MORRIS B. ABRAMSON,† ROBERT KATZMAN,‡ AND HARRY P. GREGOR

From the Department of Neurology, Albert Einstein College of Medicine, New York 61, New York, and the Department of Chemistry, Polytechnic Institute of Brooklyn, Brooklyn 1, New York

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The importance of acidic lipids as tissue ion exchange agents has been recently emphasized by a number of investigators (1-4). In addition, evidence is accumulating that these lipids may play a role in transmembrane cation transport (3, 5, 6). Consequently, it was felt desirable to study the ionic structure and ion exchange capabilities of the purified acidic lipids. Phosphatidylserine was selected for study.

Ultrasonicated sols provide the means for reproducible, quantitative measurement of the ionic properties of the acidic lipids. The ultrasonication produces visually clear, stable dispersions of lipids in water (7, 8). Such lipids were formerly studied in crude aqueous suspensions (9), or in mixed solvent systems (10). The latter system permitted reasonable extrapolations of pK values, and hence a correct deduction of the ionic form in aqueous suspensions.

Our study of phosphatidylserine shows that ultrasonicated dispersions of this acidic lipid contain micelles with an average molecular weight of $4 \times 10^6$. Essentially, all of the ionogenic polar groups are readily available for titration, and are therefore probably present on the micellar surface. The micelles are strongly acidic, and their isoelectric point is at pH 1.2. Structural evidence for the existence of the following three forms will be presented.

\[
\begin{align*}
\text{HPS} & : \text{CH}_{2}-\text{OCO}-R \\
\text{NaPS} & : \text{CH}_{2}-\text{OCO}-R' \text{Na}^+ \\
\text{Na}_2\text{PS} & : \text{CH}_{2}-\text{OCO}-R'' \text{Na}^+ \text{Na}^+
\end{align*}
\]

EXPERIMENTAL PROCEDURE

PS Preparation—Two preparations were used: Preparation I, the best of a number of commercial preparations (Nutritional Biochemicals Corporation) tested; and Preparation II, prepared by extraction of ox brain according to the method of Maltaner (11), followed by silicic acid column fractionation, and explained in detail elsewhere. From 60 to 80 µg of the lipid preparations were chromatographed by the thin layer technique on a basic plate prepared from a silica gel G made into a slurry in 0.01 M Na$_2$CO$_3$ and developed with a solution of CHC$_2$H$_5$OH-H$_2$O, 70:30:4 by volume, and both preparations ran as single spots. The lipid spots were identified by staining with 2,7-dichlorofluorescein and ninhydrin, and by charring with sulfuric acid.

HCl hydrolysates of the preparation, analyzed by paper chromatography, contained serine but were free of other amino acids, ethanolamine, galactose, glucose, and inositol. Chemical analysis by methods previously described (3) gave the following results: P, 3.5% in Preparation I; 3.40% in Preparation II; in the latter, the ratio of esters to α-aminonitrogen to phosphorus was 1.94:0.97:1.0 M. Preparation II contained less than 1% plasma-rogen and lipid galactose.

Preparation of HPS, NaPS, and Na$_2$PS—HPS was prepared by washing the extracted lipid with 0.1 N HCl and dialysing it for 24 hours against distilled H$_2$O. NaPS was prepared according to the method of Folch, Lees, and Sloane-Stanley (1) by dissolving the extracted lipid in CHCl$_3$:CH$_3$OH:H$_2$O, 86:14:1 by volume ("lower phase"), and washing with CHCl$_3$:CH$_3$OH:H$_2$O, 3:48:47 by volume, containing 0.14% NaCl. Na$_2$PS was prepared by washing the lipid in the "lower phase" with the NaCl of the "upper phase" (1) maintained at pH 10.0 by the addition of NaOH during the washing. Inorganic salts were analyzed in nitric acid digests of the lipids: Na and K by internal standard flame photometry; Ca and Mg as the sum of the di-

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1 The abbreviation used is: PS, phosphatidylserine.

2 R. Katzman and C. E. Wilson, to be published.

3 See the structural formulas for explanation of the abbreviations.
The resulting suspensions contained 0.3 to 1.2% lipid and were quite clear. The optical densities of the 47 NaPS and HPS dispersions studied varied between 0.04 to 0.06 at 546 m. The suspensions remained stable for several weeks when stored at 4°. When sonicated in dilute acid or in salt solutions, the suspensions were turbid and in some instances coagulated.

**Ion Exchange and Titration**—To study the reaction of PS with salts and in its titration with acid, base, or both, the 5-mL sonicated suspensions were brought to 8 mL by adding water or salt solutions. The pH was determined with a Radiometer titrator with glass and calomel electrodes. The instrument was calibrated before each use by appropriate buffer solutions. Titrations were performed at 24°C.

To measure the release of H⁺ by salts, small volumes of the appropriate chloride were added by pipettes or a microsyringe. For more concentrated solutions, weighed amounts of salt were added. In each case, the solution was stirred, and the pH was recorded when it reached a stable value, usually within 5 minutes.

In the titration of NaPS, the pH of the suspension was measured, the desired amount of salt added, the total volume brought to 8 mL, and the pH measured again. All titrations were performed in an atmosphere of nitrogen. The acid or base was added by a microsyringe. Recordings of pH were made automatically and manually. The suspension was first titrated with base to a pH of 10 to 10.5; in this titration the concentration of cation increased.

Infrared studies and analyses showed that hydrolysis of the ester groups occurred in the region of pH 11; titrations were not carried beyond pH 10.5. For all systems, a blank was titrated under comparable conditions without any lipid present. The volume of acid or base used by the blank was subtracted from that required by the lipid. In the neutral region, this volume was very small and increased to one-fifth to one-quarter of the volume required for the lipid titrations at the highest and lowest pH.

**Coagulation**—The effect of pH and concentration of cations on the coagulation of dispersions of PS was followed by measuring the optical densities at 546 m with a Beckman spectrophotometer. The desired concentration of salt was added to the sonicated dispersion at various pH levels. Optical density and pH were measured 5 minutes after the addition of salt. On prolonged standing, the coagulation of suspensions with high salt or hydrogen ion concentrations increased.

**Infrared Analysis**—Absorption spectra of HPS, NaPS, and Na₂PS were obtained by means of a Perkin-Elmer model 257 grating spectrophotometer. Samples were prepared by slow dropping of a solution containing 2 mg of the lipid in chloroform onto the salt plate and evaporating the chloroform. Gentle heating removed all traces of chloroform. In addition, the spectra of this lipid dissolved in tetrachloroethylene were obtained. The range from 4000 cm⁻¹ (2.5 μ) to 625 cm⁻¹ (16.0 μ) was studied in all cases.

**Light Scattering**—For turbidity measurement, a series of dilutions of a suspension of NaPS was prepared, ranging from 0.48% to 0.037% solids in water, filtered through a 0.4-μ Millipore filter. Light scattering was measured by a Brice-Phoenix photometer at 90°, 45°, 135°, and 0°. In addition, the refractive index was measured for several concentrations by a Zeiss dipping refractometer.

**Electrophoresis**—Mobilities of sonicated suspensions of NaPS were measured by electrophoresis with the Perkin-Elmer model 38A apparatus. Suspensions containing 7 mg of the lipid in 2 mL of water were dialyzed in cellulose bags for at least 24 hours. One liter of 0.1 NaCl brought to the desired pH by the addition of HCl was used for pH values of 5.5, 3.5, and 2.55. For pH 8.0, 0.1 NaCl and Veronal buffer were used.

The electrophoresis was performed at 0°. The current flow was 17 to 17.4 milliamperes. Mobility was calculated from the equation

\[ \mu = dK/A \]

in which \( d \), the distance in centimeters, was determined from the movement of the boundary during time (seconds); the constant values were: the cell constant, \( K = 0.978 \) cm; area \( A = 0.3 \) cm²; magnification, \( m = 1.06 \); current, \( I = 17-17.4 \) milliamperes. \( R \) was determined for each solution by measuring the conductivity at 0°.

**Viscosity**—The viscosity of the sonicated suspensions at 25° was measured by a 2-mL capillary viscosimeter. Aliquot portions of the suspension were brought to the desired pH by addition of HCl or NaOH. The average of five measurements was used. A water blank was measured with each series of determinations. One series was performed with 0.5% PS, another with 1% PS. With the latter series, the effect of NaCl was also studied by addition of 0.1 m NaCl before adjusting the pH; the blank was 0.1 m NaCl solution.

## RESULTS

We prepared 15 dispersions of NaPS from Preparation I and 20 from Preparation II. In addition, 12 dispersions of HPS and 8 of Na₂PS were prepared. The pH values of these dispersions of HPS were 4.0 to 4.2; for NaPS, 6.70 to 6.95; and for Na₂PS, 7.9.

**Ion Exchange**—Adding NaCl to make the solution 0.1 m lowered the pH of the HPS dispersion by 0.75 unit and the NaPS by 0.80 unit. In 0.1 m KCl, the pH of the HPS dispersion was lowered 0.65 unit. However, making the medium only 0.001 m in CaCl₂ produced a lowering 1.1 pH units in NaPS dispersions.
With NaPS, which has an appreciable ionic strength contribution of its own, this hysteresis was much less pronounced. With increasing ionic strength, added salts produced more displacement of the titration curves, as shown in Fig. 4. Again, CaCl₂ is more effective than NaCl or KCl (Table II). Considerable hysteresis was observed on titration with tetramethylammonium hydroxide in the presence of the corresponding chloride, 0.1 M. The explanation for this is not clear.

Electrophoresis—Electrophoretic mobilities were measured in 0.1 n NaCl at pH 2.55, 3.5, 5.5, and 8.6. Well defined boundaries were obtained in all cases. Some change in the velocity of the boundary was observed as a function of time, particularly at the highest pH (Fig. 5). The plot of mobilities measured during the initial 5-minute period against pH gave a linear slope in the range from pH 2.6 to 5.5, permitting a satisfactory extrapolation

Thus, small amounts of salts added to suspensions of HPS and NaPS caused a sharp decrease in the pH of the medium, and when more salts were added, the pH changes grew progressively smaller (Figs. 1 and 2). The amount of NaCl and KCl needed to reduce the pH of the dispersion of NaPS in distilled H₂O by 1 pH unit was 100-fold that of the amount of CaCl₂ needed for the same change. Another measure of the ability of salts to displace H⁺ from these dispersions is the amount of base needed to maintain a constant pH with increasing salt concentration (Fig. 3). The curves for the addition of NaCl or KCl show a plateau at 0.5 M concentrations. With CaCl₂ at 3 X 10⁻³ M, the release of H⁺ is similar to that produced by 0.7 M NaCl.

Titrations—The titrations in water and in several of the salt systems were repeated twice. There was good agreement in the general form of the curves and little variation in the volumes of acid or base used except at the highest and lowest pH. Titrations performed without added salt showed a hysteresis effect when base was added to HPS, followed by back-titration with acid. The ascending and descending curves did not coincide, because the ionic strength changed during the titration period.

Fig. 1 (upper). The effect of added NaCl and KCl on pH of ultrasonicated dispersions of HPS plotted as in Fig. 2. The dispersion contained 21 μmoles of HPS in 8 ml of distilled H₂O.

Fig. 2 (lower). Effect of added salts on pH of ultrasonicated dispersions of NaPS. The pH is plotted against the square root of the final molar salt concentration. The initial material contained 21 μmoles of NaPS in 8 ml of distilled H₂O. The difference in effect between additions of CaCl₂ and the univalent salts may be noted.

Fig. 3. Base required to maintain pH of dispersions constant at 7.0 during additions of neutral salt. The ultrasonicated dispersions of 21 μmoles of HPS were initially brought to pH 7 with NaOH or KOH. The microequivalents of base are plotted against the square root of the molar concentration. CaCl₂ shown on upper scale.

Fig. 4. Titration curves in water and NaCl solutions. The ultrasonicated dispersions contained 21 μmoles of NaPS dispersed in 8 ml of aqueous media. α₁ and α₂ refer to points of half-neutralization as described in text.
to 0 mobility at pH 1.2, the isoelectric point of the lipid in 0.1 N NaCl.

**Infrared Spectra**—The main differences in the infrared spectra of HPS, NaPS, and NazPS appear in the regions of 6.1 μ (1640 cm⁻¹) and 5.75 μ (1740 cm⁻¹). The absorption at 5.75 μ is normally attributed to C=O in the ester groups and in the carboxy acid group. The absorption at 6.1 μ appears to be that of the COO⁻, which normally occurs at 6.25 to 6.41 μ in the ionized forms of amino acids and fatty acids. The absorption at 3.5 μ is due to C—H. Table III gives the absorbance at 5.75 and 6.1 μ referred to that at 3.5 μ.

The numerical values for the monosodium and disodium forms are essentially the same for all three ratios, evidence for their purity and for the validity of the procedure. The CO:CH ratio of the acid lipid HPS is larger than those of the other two forms, and the COO⁻:CH and COO⁻:CO ratios are considerably smaller. This is in good agreement with the analytical results given earlier, which show that about 10% of the HPS was in the NaPS form.

The absorption spectra of phosphatidylethanolamine of the form prepared with neutral solvents (pH 6) and after washing with acid and with base did not show any appreciable absorbance in the 6.1- to 6.5-μ region. Since the carboxyl group is not present in phosphatidylethanolamine, the absence of absorbance at 6.1 μ supports the identification of COO⁻ absorption in this region. These findings are similar to those obtained by Kimura and Nagai (13).

**Light Scattering**—For five dilutions of the same NaPS dispersion, light intensities at 0° and 90° gave the turbidity, τ, which increased linearly with concentration (Fig. 6). The change in refractive index with concentration (dn/dc) measurements permitted the calculation of the He/τ ratio; this parameter, plotted against c and extrapolated to 0 concentration, gives 1/M as an intercept. The average molecular weight was 4 × 10⁶.

Asymmetry ratios obtained from the scattering intensities at 45° and 135° varied from 2.27 to 2.38.

**Viscosity**—The specific viscosities (η - η₀)/(η₀c) of 0.5% and 1% NaPS, to which acid or base was added, and of 1% lipid suspensions measured similarly in 0.1 M NaCl are illustrated in Fig. 7. In the pH region more acid than the second equivalence point of the lipid (pH ~ 6), the viscosity was low and remained constant. The addition of NaCl produced a greater increase in viscosity at the same pH, the degree of ionization of the lipid in the presence of salt being different.

**Coagulation**—Coagulation of the ultrasonicated suspensions occurred at high salt and H⁺ concentrations. The coagulation measured as a change in optical density is shown in Fig. 8. A marked increase in optical density occurred when 0.4 M NaCl was added to suspensions of NaPS. KCl was less effective in the neutral range, since the equivalent amount of this salt did not produce an immediate increase in optical density, coagulation occurring only after standing overnight. CaCl₂ rapidly produced observable coagulation at 0.5 × 10⁻³ M, a concentration 1:100 of that required for NaCl. As more CaCl₂ was added, the optical density increases linearly with concentration. With

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**TABLE II**

<table>
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<tr>
<th>Medium</th>
<th>Concentration</th>
<th>pH of NaPS</th>
<th>pK₁</th>
<th>pK₂</th>
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<td>0.0025</td>
<td>5.95</td>
<td>3.81</td>
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</tr>
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<td></td>
<td>0.02</td>
<td>6.20</td>
<td>3.52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>5.67</td>
<td>3.28</td>
<td></td>
</tr>
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<td>3.20</td>
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<td>3.33</td>
<td>9.65</td>
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<td>0.2</td>
<td>5.86</td>
<td>3.00</td>
<td>&lt;10.00</td>
</tr>
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<td>CaCl₂</td>
<td>2.5 × 10⁻⁴</td>
<td>6.82</td>
<td>3.50</td>
<td></td>
</tr>
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<td></td>
<td>5.0 × 10⁻⁴</td>
<td>6.53</td>
<td>3.00</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE III**

<table>
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<th>Wave length</th>
<th>HPS</th>
<th>NaPS</th>
<th>NazPS</th>
</tr>
</thead>
<tbody>
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<td>CO:CH</td>
<td>0.75:3.5</td>
<td>0.79</td>
<td>0.68</td>
</tr>
<tr>
<td>COO⁻:CH</td>
<td>6.1:3.5</td>
<td>0.073</td>
<td>0.29</td>
</tr>
<tr>
<td>COO⁻:CO</td>
<td>6.1:5.75</td>
<td>0.093</td>
<td>0.43</td>
</tr>
</tbody>
</table>
FIG. 6. Light scattering of ultrasonicated dispersions at different concentrations. *Upper graph* shows $\tau$ (turbidity) as a function of milligrams per ml of NaPS. *In lower graph*, the extrapolation of $HC/\tau$ to 0 concentration gives the reciprocal of the molecular weight.

FIG. 7. Viscosity of ultrasonicated dispersions. Variation of reduced viscosity as measured by $(\eta - \eta_0)/(\eta_0C)$ with pH. Viscosity increases at higher pH, particularly in the presence of added salt.

FIG. 8. Coagulation of dispersions as shown by increase of O.D. measured at 546 nm. In *upper graph*, the pH was maintained constant by addition of base as salt was added. Note absence of effect on addition of KCl. The molarity of CaCl$_2$ shown on *upper scale* is 0.005 that of Na or KCl. *Lower graph* shows change in O.D. of HPS dispersions as salts were added. The pH was not maintained constant.

DISCUSSION

The sonication of PS in water produces micelles of the lipid with the hydrophilic groups of the molecules exposed to the aqueous medium. Presumably, the hydrophobic portions comprise an inner double layer, and therefore resemble fragments of myelin figures (14). The average molecular weight, $4 \times 10^6$ calculated from light scattering, is in the same range as that reported by Saunders et al. (8) for lecithin micelles produced by ultrasonication in water. If 840 is used as the molecular weight of the lipid, an average of 4800 molecules per micelle is indicated.

From available data for related materials which give a molecular area equal to 50 Å$^2$ (15), and postulating a micellar structure consisting of 2 disk-shaped lamellae (each containing on average 2400 molecules), it is possible to calculate an area of $12 \times 10^4$ Å$^2$ and a diameter of 400 Å for the micelle.

At a pH higher than the isoelectric point, ionization of acid groups imparts negative charges to the micelles with hydrogen or metallic cations, or both, as counter ions. These micelles are typical colloidal electrolytes or polyampholytes. The ionization of such packed and oriented molecular assemblies has been shown to be markedly influenced by the absorption of metallic cations into the region of the interface (16, 17).

With NaPS in water (Fig. 2), the addition of K, Na, and tetrathylammonium cations produced lowering of the pH. The effect of the three salts is the same. At this pH, the release of H$^+$ can only result from the conversion of the dipolar ion to the corresponding phosphate salt and free amine. Had some of these cations shown a marked preference for binding to the phosphate group, some differences in the pH decrease would have been observed. Since the quaternary ammonium ion is not bound to phosphate (18, 19), it is reasonable to expect that Na$^+$ or K$^+$ are also not bound by phosphate. Addition of CaCl$_2$ to NaPS produced a marked release of acid (Figs. 2 and 3), with about 0.35 mole H$^+$ freed per mole of CaCl$_2$, suggesting that Ca$^{2+}$ is bound strongly to the phosphate group.

On the other hand, at acid pH the addition of Na and K salts to aqueous suspensions of HPS produced a differential pH change, well beyond experimental error (Fig. 1). A reasonable assumption is that there is a preferential binding of Na over K;

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since in this case the source of H\(^+\) is the carboxylic acid group, this is the binding site.

For 21 \(\mu\)moles of HPS (of which 2 \(\mu\)eq are in the Na form), 19 \(\mu\)eq of NaOH or KOH were required to bring the system to pH 7 (Fig. 3). The pH of NaPS in water is 6.9, which is evidence that essentially all the acid groups of HPS are exposed to the aqueous medium and directly titrable to form NaPS.

**Titrations**—In 0.1 M NaCl, the isoelectric point is pH 1.2. Since the amino group is fully protonated (NH$_3^+$) at this low pH, the isoelectric lipid must contain an equal number of ionized acid groups. As the infrared absorption spectra showed, the carboxyl group was not ionized at low pH levels; ionized phosphate groups were therefore present at the isoelectric point. The titration from pH 1.2 to 5.80 (the pH of NaPS in 0.1 M NaCl) therefore involves neutralization of the carboxyl group.

The pK$_2$ and the pK$_3$ values of PS are sensitive to salt concentrations, and can be calculated from their titration curves. In assembling the curves for Fig. 4, the pH values for the NaPS in the respective salt systems before titration were placed along the same vertical axis, which corresponds to the equivalence points for NaPS in the different salt solutions. The ionization constant for the carboxyl group pK$_2$ is equal to the pH at 50% neutralization (10.5 \(\mu\)eq of added acid); similarly pK$_3$ (the ionization constant for NH$_3^+$) is the pH when 10.5 \(\mu\)eq of base was added (Table II). Only in the most concentrated NaCl solutions was there sufficient increase in the acid strength of the NH$_3^+$ groups to attain a pH corresponding to \(\alpha_2 = 0.5\) without saponification of the ester.

Our titration results are in general agreement with those of Garvin and Karnovsky (10). In their titration of PS in mixed solvents, the equivalence point of NaPS was at an apparent pH of 7.5 and the isoelectric point at approximately pH 1.5. Their pK$_2$ value (4.6) is somewhat higher than ours (3.81), and their pK$_3$ value (for the NH$_3^+$) is given as 10.3. This appears to be in agreement with ours which is 9.70 in 0.5 M NaCl and probably close to 10 in more dilute salt systems.

**Electrophoresis**—The high electrophoretic mobilities of PS resembled that of many other micelles (20). The mobility (and the \(\zeta\) potential) increased with increasing pH of the medium, in keeping with the greater ionization of the acid groups of the lipid. From pH 1.2 to 5.5, the increased mobility results from ionization of the COOH groups. Above the latter equivalence point, an increase in the pH produces an increase in negative charge (PO$^-$) by removal of protons from the NH$_3^+$ group.

In their study of paper electrophoresis of lipids in mixed solvents, Wallach and Garvin (21) found the mobility of PS to be high. As anticipated, the mobility of PS was higher than that of phosphatidylethanolamine or lecithin.

**Coagulation**—The coagulating characteristics of the lipid micelles are typical of those of hydrophobic particles, their stability being sensitive to salt concentrations. The increased tendency to coagulate at low pH levels is related to the decreased charge density of the particle as it approaches the isoelectric pH. Absorption of cations at higher pH levels has a similar effect by reducing the negative charge of the particles. The coagulating effect of low concentrations of CaCl$_2$ indicates a strong binding of this cation. Similar effects have been reported for ionized monolayers of fatty acids (17). As noted by Webb and Danielli (22), equal amounts of Ca and Na are present in such monomolecular films when the concentration ratio in the same medium was Ca:Na = 1:100.

**Viscosity**—A pronounced increase in the viscosity of these suspensions occurred when the pH was increased above 5 to 6, the region in which deprotonization of the dipolar ion occurs. Repulsion between the two negatively charged acid groups could lead to lateral expansion of the micelle, or to an increase in the thickness of the double layer with a change in the micelle dimension, or to both. The increase in viscosity on addition of NaCl at constant pH can in part be attributed to the increased charge on the micelles as a result of increased ionization.

**SUMMARY**

Optically clear, aqueous dispersions of phosphatidylserine were prepared by ultrasonication. Dispersions of the mono- sodium form in distilled water consisted of micelles with an average molecular weight of 4 \(\times\) 10$^6$. The micelles were strongly acidic and had a high electrophoretic mobility; the isoelectric point was estimated at pH 1.2. All of the charged groups were readily accessible to titration.

The infrared and titration data indicate that at the isoelectric point the molecule is a dipolar ion of phosphate and amino groups. As the isoelectric point is approached, coagulation occurs even in the absence of added salts. The pK$_2$ in 0.1 M NaCl is 3.35, and is attributed to ionization of the carboxyl group. The pK$_3$ at 10.0 corresponds to deprotonization of the amino group; as this pK is approached, the viscosity of the dispersions increases.

The micellar dispersions showed ion exchange properties. Salts added to the dispersions displaced the titration curves, altering the pK. With the addition of enough NaCl to bring the salt concentration of a dispersion prepared in distilled H$_2$O to 0.1 M, the pH decreases by 1 unit. High salt concentrations produced coagulation even at neutral pH. CaCl$_2$ was 100-fold as effective as NaCl, on a molar basis, in producing such changes.

The data thus indicate that the micelles are highly charged with all of the ionized groups on the surface, despite the fact that there are nearly 5000 molecules per micelle. This suggests that phosphatidylserine has unusual colligative characteristics, forming oriented structures even when subjected to ultrasonication.

**Addendum**—Attention should be drawn to the work of DeVichian (23), who studied the titration properties and ion exchange properties of crude suspensions of phosphatidylserine.

**REFERENCES**

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