

Studies of Naturally Occurring Acidic Lipids: Ionic, Surface and Membrane Properties

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UNITED STATES DEPARTMENT OF THE INTERIOR

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FOREWORD

This is the one hundred and seventy-eighth of a series of reports designed to present accounts of progress in saline water conversion with the expectation that the exchange of such data will contribute to the long-range development of economical processes applicable to large-scale, low-cost demineralization of sea or other saline water.

Except for minor editing, the data herein are as contained in the reports submitted by Yeshiva University, Albert Einstein College of Medicine, under Grant No. 14-01-0001-342, covering research carried out through June 30, 1965. The data and conclusions given in this report are essentially those of the Grantee and are not necessarily endorsed by the Department of the Interior.

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As the Nation's principal conservation agency, the Department of the Interior works to assure that nonrenewable resources are developed and used wisely, that park and recreational resources are conserved for the future, and that renewable resources make their full contribution to the progress, prosperity, and security of the United States--now and in the future.

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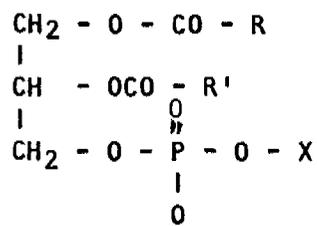
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INTRODUCTION

This research is directed toward a study of the properties of acidic lipids as they may be related to the role of these lipids in membrane processes occurring in living cells; in particular, those processes concerned with the movements of salts through these membranes. Across these membranes, active cellular processes 'pump' out Na with great energetic efficiency resulting in the accumulation of K as the chief intracellular cation. In excitable tissues, such as muscle and nerve, the membrane shows further ionic specificity; in the resting state, the K conductance is much greater than the Na conductance, whereas during the excited state this is reversed.

Acidic phospho- and glycolipids are important constituents of membranes. In brain tissue where there is the greatest concentration of excitable membrane, these chemicals account for about one-fourth of the anionic sites. More important, several of these lipids have been implicated in active transport of cations. The phospholipids studied are of the form



Where R and R' are the aliphatic chains of C₁₄ - C₂₂ fatty acids. In the simplest of these phospholipids, phosphatidic acid, X is a H; in lecithin, X is a choline moiety; in phosphatidylserine, X is a serine (in ester linkage through its -C-OH group).

It has been our purpose to define the physico-chemical properties of isolated phospho- and glycolipids as they relate to their ionic properties in aqueous dispersion. Thus, titration curves, ion exchange data, electrophoresis mobility are studied. A most important question is whether these lipids show any ionic specificity -- whether they combine more readily with Na or with K, whether they react differently to Ca or Mg. Therefore, measurements of the binding constants of these cations to the lipids are being made. Another significant aspect of the behavior of lipids in cell membranes is their cohesive ability, leading to the maintenance of an oriented bimolecular lipid array as the backbone of the cell membrane even when this membrane is undergoing the stress of movement or pressure. Related 'cohesive' properties of our acidic lipids

and the way these properties are affected by salt concentration are studied by measurement of the turbidity, light scattering, and viscosity properties of the dispersed micelles; by studies of lipid monolayers; and by electron microscopy.

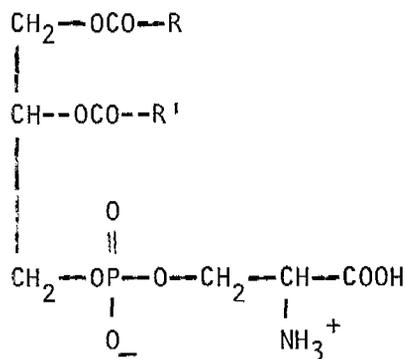
An additional problem we have considered is whether the information gathered on these lipids could be applied toward the construction of a model lipid membrane with properties similar to those of a cell membrane. This approach has been modified by the elegant development of just such a model system by Mueller et al¹. This membrane constructed out of an extract of whole brain lipids is endowed with electrical properties akin to those of the cell membrane by the addition of another biological extract, "excitatory inducing material". We have confirmed this work. The problems then arise: What is the exact chemical nature of the excitatory inducing material; how does it interact with the acidic and other lipids in the membrane; what is the effect of altering the concentrations of individual acidic lipids in the membrane; how does this membrane react to specific cations in the surrounding media. These problems, however, cannot be considered properly until this excitatory inducing material is isolated, purified and identified.

RESULTS AND DISCUSSION

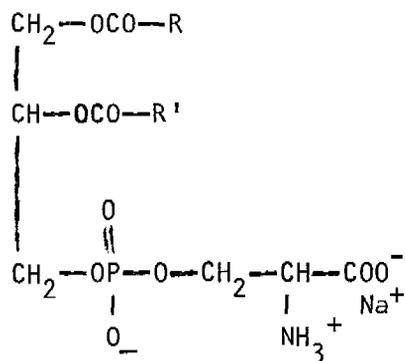
General properties of ultrasonicated sols.

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^{2,3}. Such lipids were formerly studied in crude aqueous suspensions by Christensen and Hastings⁴, or in mixed solvent systems⁵. The latter system permitted reasonable extrapolations of pK values, and hence a correct deduction of the ionic form in aqueous suspensions.

Our study of phosphatidylserine⁶ shows that ultrasonicated dispersions of this acidic lipid contain micelles with an average molecular weight of 4×10^6 . 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.



Isoelectric or acidic (HPS)



Monosodium salt (NaPS)

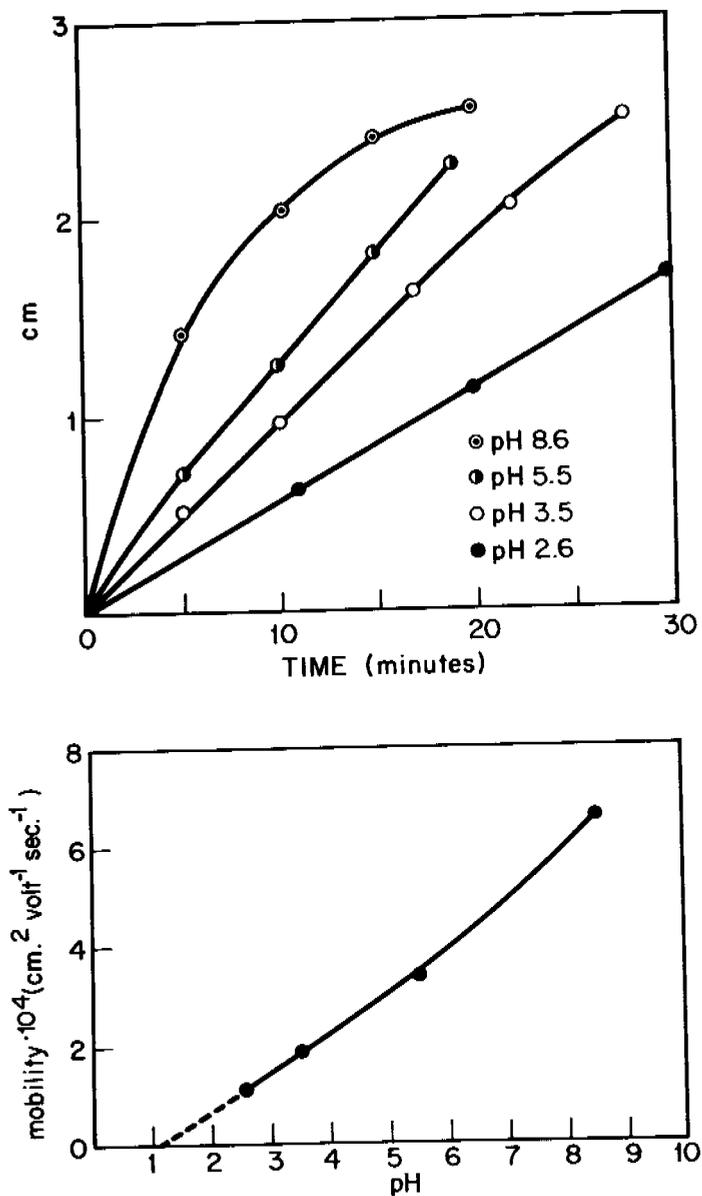


Fig. 1. Electrophoresis of ultrasonicated dispersions in 0.1 N NaCl. Upper graph shows movement of boundary as a function of time at various pH levels. Electrophoresis was performed in a Tiselius apparatus at 0° at 17.4 ma. Lower graph shows calculated mobilities as a function of pH. Extrapolation to 0 mobility gives an isoelectric point at 1.2.

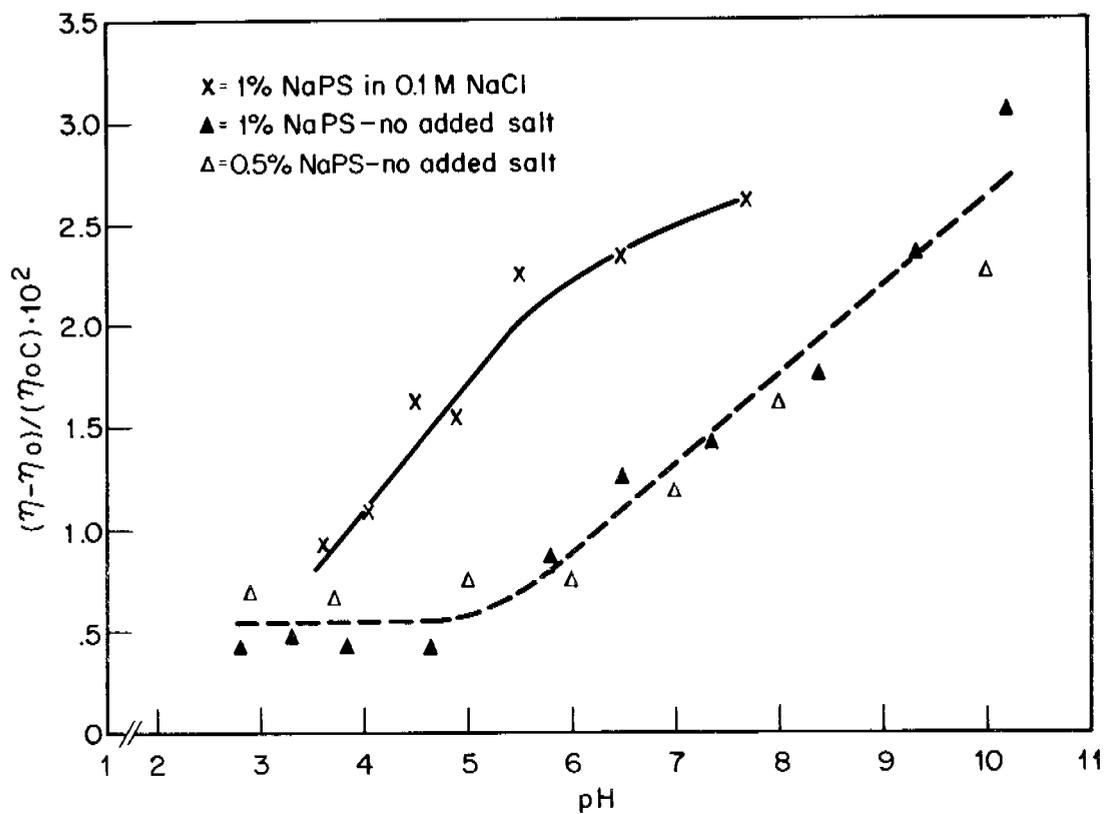


Fig. 2. Viscosity of ultrasonicated dispersions. Variation of reduced viscosity as measured by $(\eta - \eta_0) / (\eta_0 C)$ with pH. Viscosity increases at higher pH, particularly in the presence of added salt.

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.

Of the several preparations of phosphatidic acid used, all those that had been washed with HCl and dialyzed extensively with water and HCl formed stable dispersions on sonic treatment in water. In some instances, when the dispersion appeared turbid, additional treatment for 2 minutes reduced the turbidity. The optical densities measured at 546 $m\mu$ ranged from 0.07 to 0.10. The suspensions were relatively clear but not as clear as comparable suspensions of phosphatidylserine with an optical density of 0.04 to 0.06.

The pH and cation content of these dispersed preparations are shown in Table 1⁹. It should be noted that the pH values for the systems varied within a narrow range for dispersions made from the same preparation of lipid. Preparations C3 and C4 obtained from the acid-washed preparation C2 by repartitioning with NaOH and KOH at pH 8.5 and 9.5, respectively, were found to disperse well. The significance of their pH values will be discussed.

Titration.

These aqueous dispersions gave reliable reproducible titration curves. With phosphatidylserine, 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. 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. 3. Again, $CaCl_2$ is more effective than NaCl or KCl (Table 2). 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.

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.

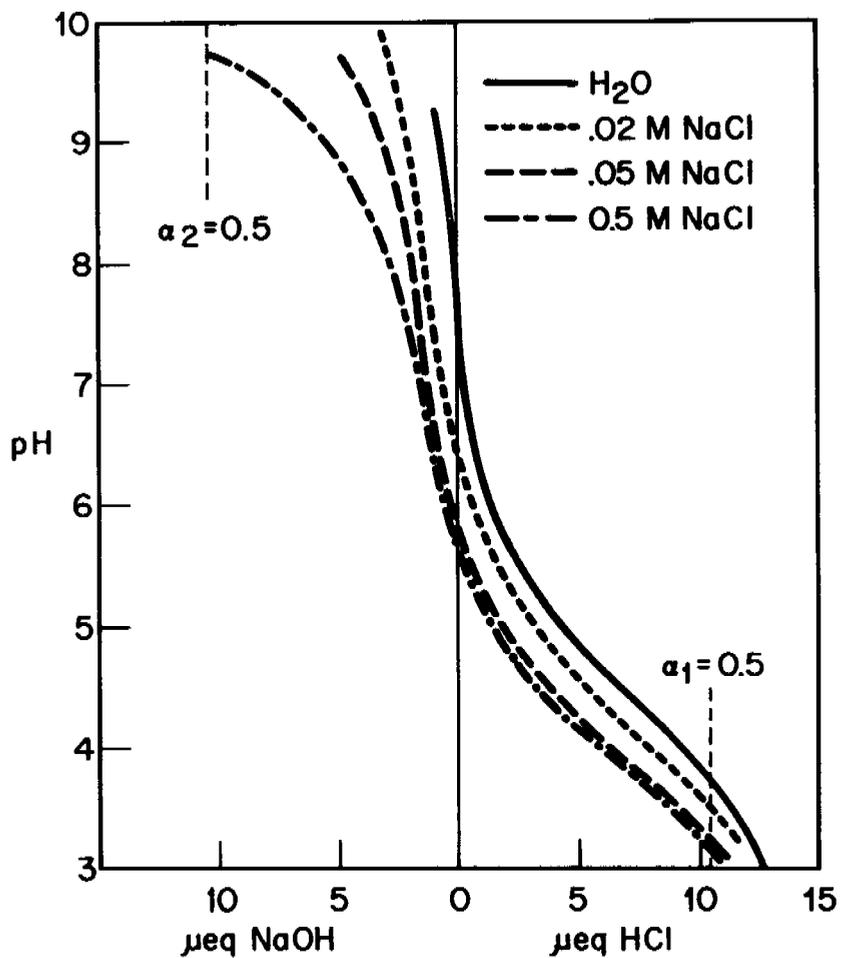


Fig. 3. Titration curves in water and NaCl solutions. The ultrasonicated dispersions contained 21 μ moles of NaPS dispersed in 8 ml of aqueous media. α_1 and α_2 refer to points of half-neutralization as described in text.

Cation analysis and pH of dispersions

TABLE I

Titration data of dispersions*

Phosphatidic acid preparation†	Dispersions	Cation to phosphorus ratio		Initial pH (no added salt)	Added salt	pH at first equivalence point (± 0.05)	pK ₁	pK ₂	No. of Titrations
		Sodium plus potassium	Calcium						
		$\mu\text{mole}/\mu\text{mole}$							
A1	1	0.56		4.90					
A2	7	0.31	<0.03	3.05-3.10		6.20	3.80	8.60	1
					0.1 N KCl	5.78	3.45	8.03	2‡
						5.40	3.18	8.15	2
B1	1	<0.3	0.23	4.85					
B2	1	<0.3	0.02	3.25		6.23	3.87	8.78	3
					0.1 N KCl	5.38	3.05	8.13	2
C2	6	0.42	<0.03	3.12-3.29	0.1 N NaCl	5.25	2.68	7.93	2
C3	1	1.19	<0.03	7.60		5.50	3.18	8.50	2
C4	1	1.32	<0.03	8.10		5.50	3.10	8.30	1

*For comparison, titrations of the soluble sodium β -glycerophosphate yielded a first equivalence point of 4.30, a second equivalence point of 8.00, and a pK₂ of 6.55 in low salt solutions and a first equivalence point of 4.25, a second equivalence point of 8.50, and a pK₂ of 6.45 in 0.10 N NaCl.

†A1 and B1, not acid-dialyzed, contained calcium; A2 and B2, acid-dialyzed; C2, acid-dialyzed; C3, acid-dialyzed, partitioned with KOH; C4, acid-dialyzed, partitioned with NaOH.

‡Titration over an 18-hour period.

TABLE 2

Medium	Concentration M	pH of NaPS	pK ₂	pK ₃
NaCl	0.0025	6.95	3.81	
	0.02	6.29	3.52	
	0.05	5.67	3.28	
	0.5	5.51	3.20	9.70
TMACl	0.1	5.93	3.33	9.65
KCl	0.02	6.26	3.50	
	0.2	5.86	3.00	< 10.00
CaCl ₂	2.5 X 10 ⁻⁴	6.82	3.50	
	5.0 X 10 ⁻⁴	6.53	3.00	

The pK₂ and the pK₃ values of PS are sensitive to salt concentrations, and can be calculated from their titration curves. In assembling the curves for Fig. 3, 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 pK₂ is equal to the pH at 50% neutralization (10.5 μeq of added acid); similarly pK₃ (the ionization constant for NH₃⁺) is the pH when 10.5 μeq of base was added (Table 2). Only in the most concentrated NaCl solutions was there sufficient increase in the acid strength of the NH₃⁺ groups to attain a pH corresponding to α₂ = 0.5 without saponification of the ester.

Our titration results are in general agreement with those of Garvin and Karnovsky⁵. 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 pK₂ value (4.6) is somewhat higher than ours (3.81), and their pK₃ value (for the NH₃⁺) 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.

With phosphatidic acid sols, titrations of preparations A2, B2, and C2 gave results which lead to a satisfactory interpretation of the titration characteristics. All titration curves showed two inflection points; Fig. 4 shows a typical curve. The first inflection point (where ΔpH/Δμeq is a maximum) corresponds to the conversion of dibasic acid to the monobasic acid (Table 3). The scale of the abscissa is based upon this inflection point. Taking 720 as the average molecular weight of phosphatidic acid containing approximately 0.4 equivalent of sodium plus

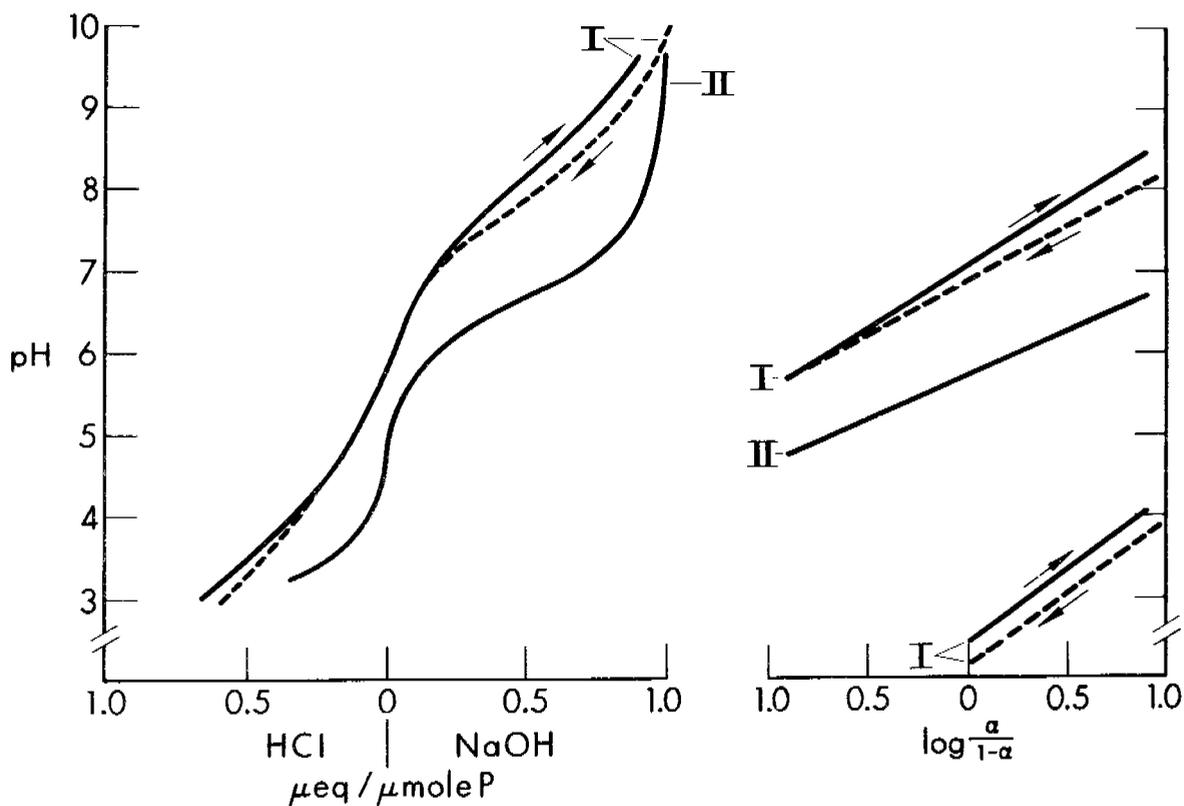


Fig. 4. I, 18-hour titration of an acid-dialyzed dispersion of phosphatidic acid. The titration curve was continuously monitored during this period. Dispersion contained 13.8 μ moles phosphatidic acid in 5 ml of water and had an initial pH of 3.10. II, titration of 13.5 μ moles of sodium β -glycerophosphate in 5 ml of H₂O. Panel at right shows $\log \frac{\alpha}{1-\alpha}$ against pH for first and second acid groups of I and second acid group of II.

TABLE 3

Preparation	Added base to first equivalence point*	Initial salt (sodium plus potassium)	Sum	2 (μeq at pK_2 - μeq at first equivalence point)†
A2	0.58	0.31	0.89	0.97
C2	0.49	0.42	0.91	1.17
C3	-0.24	1.19	0.95	1.0
C4	-0.43	1.32	0.89	1.02

*Equivalence point for the formation of the monobasic acid.

†This calculation is based upon the relationship that for neutralizing the monobasic acid, microequivalents at pK_2 - microequivalents at first equivalence point = one-half the number of microequivalents required.

potassium, the amount of lipid present ranged from 11.5 to 14.0 μ moles. If all the ionogenic groups were exposed to the aqueous medium and were available for reaction, and if the titration curve with the position of the equivalence point and the number of moles present (based on phosphorus) were known, one could read values of pK_1 and pK_2 from the curve. The values for pK_2 found this way agreed within 0.05 unit with those obtained independently from the curvature ($d^2\text{pH}/d(\mu\text{eq})^2$) of the titration curve at higher pH levels. Coagulation at low pH levels precluded a similar calculation for pK_1 .

Most of the titrations with base, when carried out to pH 10.25 to 10.5, showed a change in slope indicating an approach to the second equivalence point. The microequivalent of base used to bring the pH from the first equivalence point to this maximum pH was consistently from 0.9 to 1.0 per μ mole of lipid being titrated. This supported the view that essentially all the acid groups were available for titration.

Preparation C2 contained 0.34 μ mole of sodium and 0.08 μ mole of potassium per μ mole of phosphorus, and no detectable calcium; hence, it represented a concentration of cations somewhat below that corresponding to 50% neutralization of one acid group. The pH of the dispersions in water are shown in Table 1 to be somewhat lower than the pK_1 in a water medium, in agreement with the amount of cation contained.

To confirm this interpretation of the titrations, two samples of phosphatidic acid were prepared containing more than 1 μ eq of cation per μ mole of phosphorus. These were dispersed in the manner described. The aqueous suspension of a sample (C3) containing 1.04 μ eq of potassium and 0.15 μ eq of sodium per μ mole was at a pH of 7.60 (Table 1). A second

preparation (C4) containing 1.32 μ eq of sodium and potassium produced a suspension with a pH of 8.10 (Table 1). The pH observed for each dispersion was consistent with the pH predicted by the titration curve, assuming that all the acid groups are exposed to the aqueous medium and are titrable. When the equivalence point as determined from the titration curves is used, and when the total equivalents of the phosphatidic acid present is known (assuming the complete availability of the H^+ ions), α was calculated at different pH levels. Plotting pH against $\log \alpha/(1 - \alpha)$ (Henderson-Hasselbach equation) gave linear graphs (Fig. 4). The slopes of these plots are typical of those for polyelectrolytes, n having values of 1.6 to 1.8.

Titration in these systems without added salt showed significant differences in the pK values in upscale and downscale titrations (Fig. 4), even in titrations conducted slowly over an 18-hour period. Since these differences were absent in titrations carried out in the presence of salt (0.1 M), it was concluded that the effect was probably due to changes in the ionic strength. However, one cannot exclude the possibility that at higher pH levels the micelles are somewhat dispersed as a consequence of increased ionization and repulsion of the acid groups; adding salt to such dispersions would give lower values of pK_2 as in Table 1.

Large differences between the titrations with acid and base also appeared at the lower pH ranges of the titration (less than 4), probably because of coagulation and subsequent loss of capacity. Titrations carried out over 90 minutes showed more hysteresis than the 18-hour titrations; this suggests that, in the slow titration, redispersion may have occurred at high pH levels.

Titration in 10^{-3} M $CaCl_2$ consumed much less base than the micromoles of lipid present and were not useful for the determination of pK values. The presence of this cation thus appeared to reduce the availability of the acidic groups. Further evidence of this was obtained from the behavior of preparation B1 which did not disperse completely with sonic treatment in water and which, despite a low content of sodium and potassium, gave a pH of 4.85. Its calcium content (presumably bound to phosphatidic acid) was found to be 0.23 μ mole of calcium per μ mole of phosphorus. Titration of this dispersion above the first equivalence point showed a titer approximately 30% of that expected from the number of micromoles of phosphatidic acid present (Fig. 5). This reduction in titer is greater than can be accounted for by strong binding of calcium to a phosphate group and may indicate a geometric rearrangement of the calcium-bound lipid, such that the shape of the titration curve was similar to those obtained normally, and also that both types of acid groups were reacting where they were not bound to calcium. This preparation was then washed with 0.1 N HCl, dialyzed against 0.005 N HCl, and finally partitioned between chloroform-methanol, 2:1, and water. It (B2) now contained 0.02 μ mole of calcium per μ mole of phosphorus and gave dispersions in water with a pH of 3.25 which titrated in the same manner as preparations A2 and C2 (Fig. 5).

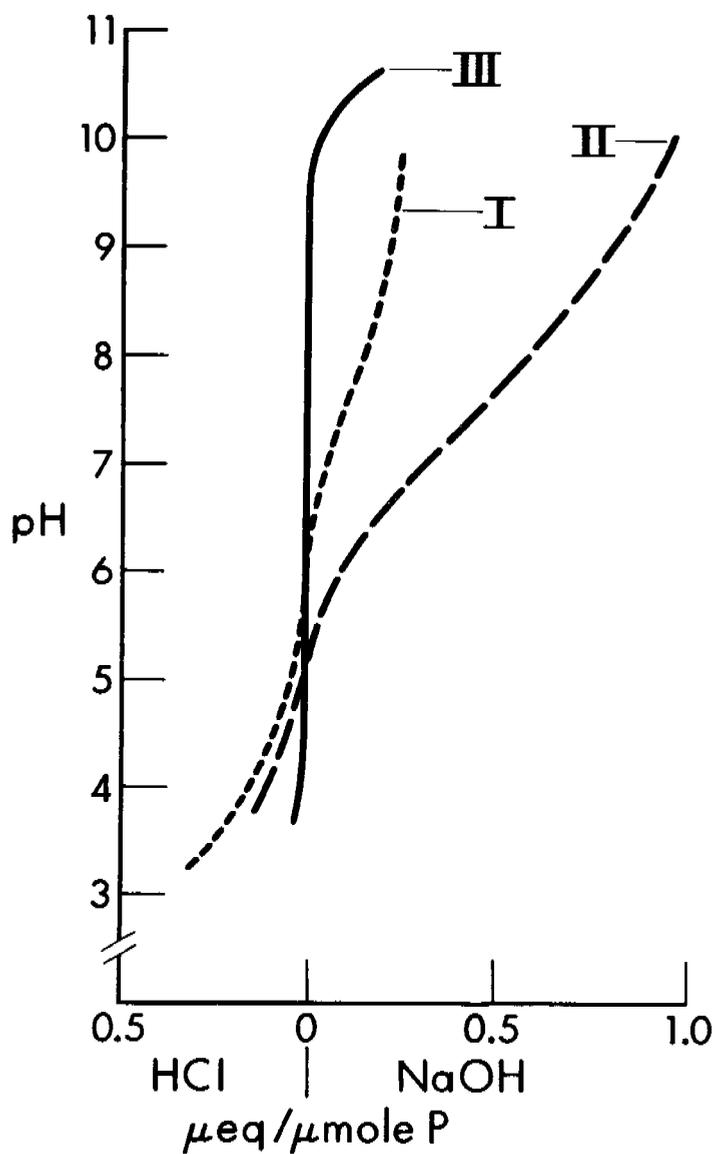


Fig. 5. Comparison of titration of calcium containing phosphatidic acid dispersion (I) with same preparation after removal of calcium by acid dialysis (II) and aqueous dispersion of egg lecithin (III).

Titration performed in 0.1 N KCl or 0.1 N NaCl showed complete availability of the acid groups for titration.

The values obtained for pK_1 and pK_2 in aqueous dispersions are somewhat lower than those calculated by Garvin and Karnovsky⁵ ($pK_1 = 4.1$; $pK_2 = 10.0$) from their titrations performed in nonaqueous media. This is as expected because of differences in activity coefficients of solutes in the two solvents of different dielectric constants and the inherent liquid junction of potential differences in the measurements themselves.

The lipid coagulated on standing after titration in salt systems at low pH. The infrared absorption spectrum of the dried lipid showed no noticeable difference from that of the original material. Thin layer chromatography also showed no decomposition of the lipid resulting from sonic treatment and exposure to varied media and brief exposure to a higher pH during titration.

Titration of the soluble disodium β -glycerophosphate were performed in the same manner. The pK_2 was at 6.55, comparable to the reported value of 6.650 at 25^o¹⁰. The major difference observed between the titration of suspensions of phosphatidic acid and of soluble glycerophosphoric acid may be ascribed to the surface reaction of the acid groups of the phosphatidic acid micelles. The values for pK_1 and pK_2 and the pH at the equivalence points of the glycerophosphoric acid are approximately 2 units lower than the corresponding values for the dispersed phosphatidic acid (Table I). The ionic reactions at the surface of the micelles can be compared with reactions of similar ionogenic groups on a monolayer exposed to an aqueous medium^{11,12}. Studies of monolayers of carboxylic acids have shown that the apparent pK (the point of half neutralization of the acid film) is approximately 2 to 3 units higher than for carboxylic acids in solution.

The addition of NaCl to a solution of glycerophosphates produced a small lowering of the pH of the system in contrast to the marked effect produced on phosphatidic acid. The release of H^+ on the addition of salts to these dispersions involves the exchange of ions in the double layer surrounding the micelles and profound changes in the charge on the micelle.

The titration curves of lecithin dispersions showed very little buffering between pH 3.5 and 10.0 as previously reported⁴. However, mixed micellar dispersions of phosphatidic acid and lecithin provide an area for interesting investigations. These two closely related lipids (in this instance the phosphatidic acid was obtained from the lecithin) were mixed in solution, and the dissolved solids were deposited by evaporating the solvent. This deposit was dispersed in water. Presumably the micelles contained a mixture of both lipids. Two preparations of phosphatidic acid and lecithin were dispersed this way and titrated. In one of these, the phosphatidic acid was the major component; in the other, it was present as the minor component. The titration curves for the mixed

micellar systems were quite similar to those of phosphatidic acid alone, but, more significant, the number of equivalents of acids and base consumed were equal to the moles of phosphatidic acid present. Thus, the phosphatidic acid, even when present in small amounts, is oriented in the surface of the micelle with all of the acid groups directed toward the aqueous medium. This stoichiometry is shown in Table 4.

Optical properties of aqueous dispersions.

The addition of cations to the aqueous dispersions produced coagulation of the dispersions. It was found possible to utilize this to determine qualitatively and quantitatively the binding of cations to the micelles. With phosphatidylserine, 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. 6. 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. $CaCl_2$ rapidly produced observable coagulation at 0.5×10^{-3} M, a concentration 1:100 of that required for NaCl. As more $CaCl_2$ was added, the optical density increases linearly with concentration. With the more acidic HPS, KCl as well as NaCl and $CaCl_2$ readily coagulated the suspension (Fig. 6).

The coagulation 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 acids¹². As noted by Webb and Danielli¹³, 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.

The coagulation characteristics of dispersions of phosphatidic acid resembled those of phosphatidylserine systems. At pH 3, coagulation of salt-free dispersions took place after standing overnight. At pH levels greater than 7, these preparations did not show coagulation after standing several weeks. The addition of salts increased the extent of coagulation, with smaller concentrations of salt needed at lower pH levels (Fig. 7). At pH 3 to 5, the onset of coagulation could be detected when the system was 10^{-3} M in $CaCl_2$. At pH 4, the effect of NaCl and KCl were alike. At pH 5, however, the optical density increased from an initial value of 0.08 to 0.30 when the system was 0.5 M in NaCl, but to reach the same optical density with KCl required making the system 0.9 M. At pH 7, coagulation could be achieved with 0.5 M NaCl, but several systems prepared with as much as 0.9 M KCl remained stable for several days.

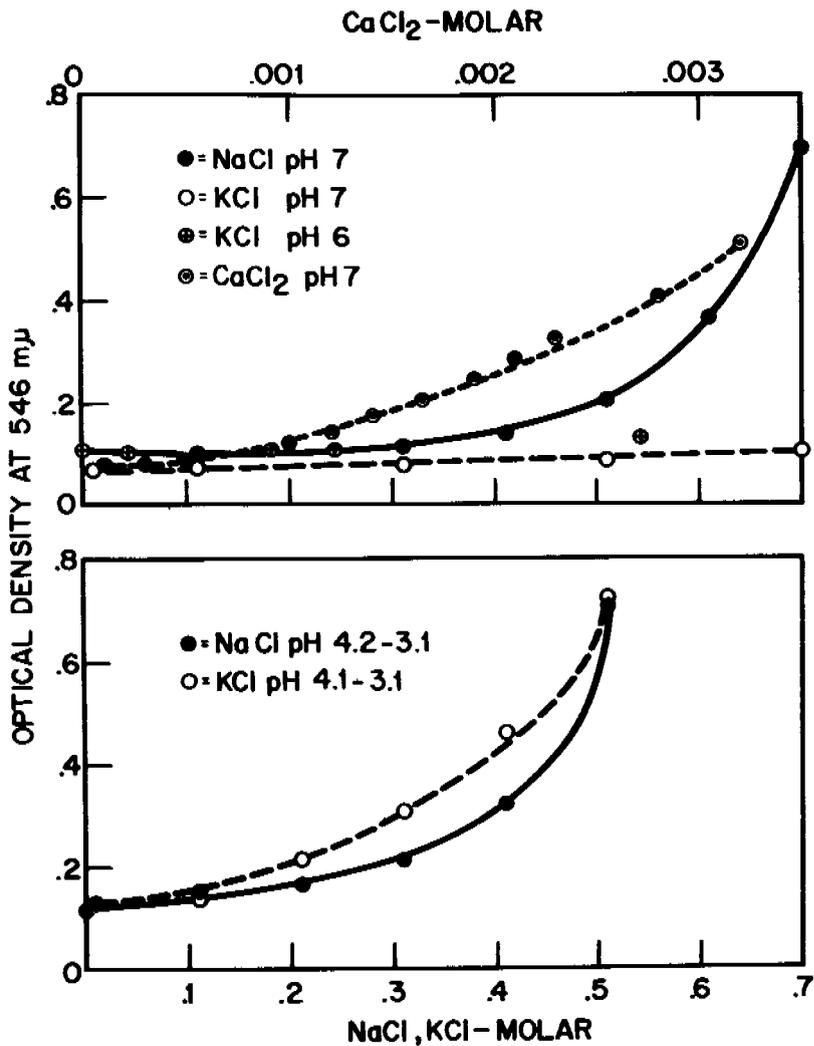


Fig. 6. Coagulation of dispersions as shown by increase of O.D. measured at 546 mμ. In upper graph, the pH was maintained constant by addition of base as salt was added. Note absence of effect on addition of KCl. The molarity of CaCl₂ shown on upper scale is 0.005 that of Na or KCl. Lower graph shows change in O.D. of HPS dispersions as salts were added. The pH was not maintained constant.

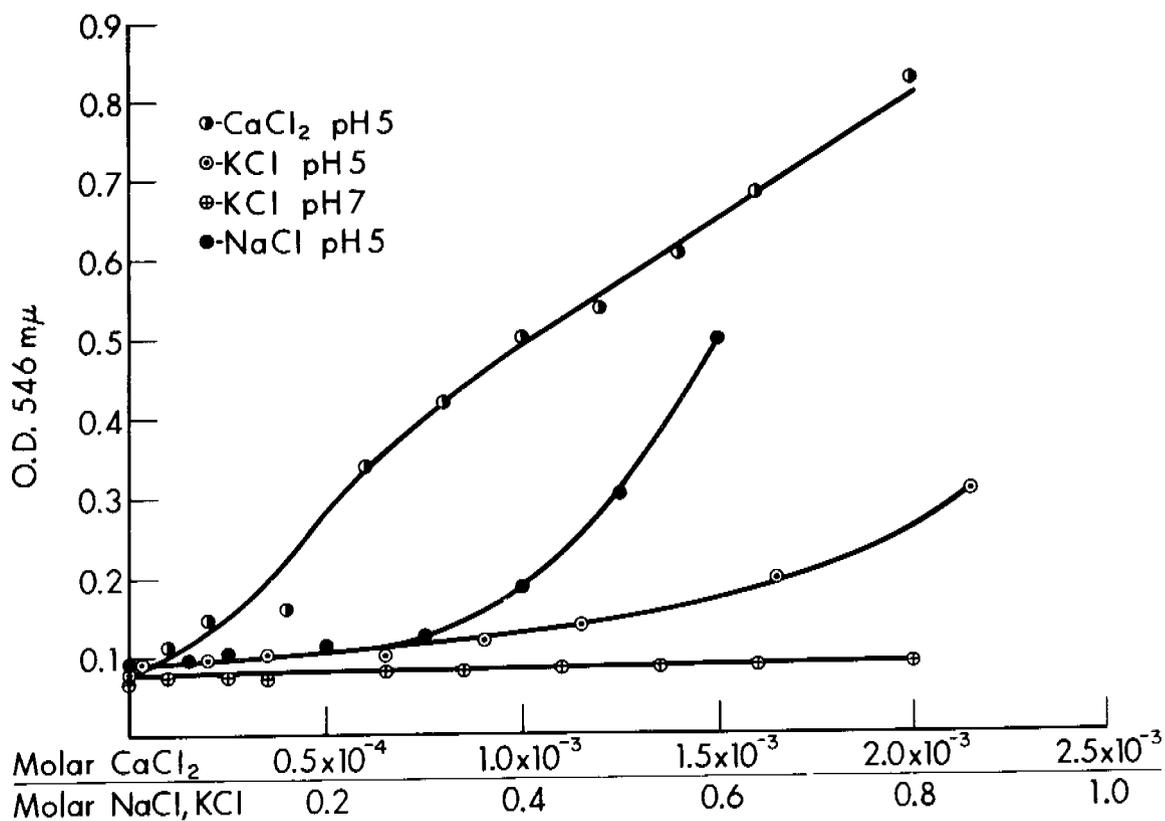


Fig. 7. Coagulation of dispersions as shown by the increase in optical density measured at 546 m μ . The pH was maintained constant by addition of base as salt was added. Note minimal effect on addition of KCl.

TABLE 4

Composition	Medium	pH at first equivalence point	pK ₁	pK ₂	Stoichiometry*
Phosphatidic acid, 13.9 μmoles	H ₂ O	6.30	3.70	8.70	0.92
Lecithin, 7.2 μmoles	0.005 N KCl	5.75		8.65	0.92
Phosphatidic acid, 5.3 μmoles	H ₂ O	5.85		8.75	1.02
Lecithin, 17.0 μmoles	0.005 N KCl	5.35		8.15	0.98

*Calculation:
$$\frac{2 (\mu\text{eq at pK}_2 - \mu\text{eq at first equivalence point})}{\mu\text{mole phosphatidic acid}}$$

This calculation is based upon the relationship that for neutralizing the monobasic acid, microequivalents at pK₂ - microequivalents at first equivalence point = one-half the number of microequivalents required.

The increased stability of the sols at higher pH levels can be ascribed to the increased negative charge of the particles. The binding of cations leads to a decreased charge and, accordingly, decreased stability. The pronounced differences in the effect of sodium and potassium are doubtless due to small differences in the charge of the micelle in the region of the critical charge for colloid stability.

Measuring the turbidity of dilute dispersions has proven to be an extremely sensitive method of detecting changes in the structure of aqueous micelles of lipids. Not only has it been possible to detect changes produced by the ionic content of the medium, but information can be obtained on the relative effects of different ions. Although the changes in the transmission of light (as measured by the optical density) are influenced by the ions present, the intensity of the light scattered at 90° is more sensitive to ionic changes, permitting differences between ions to be readily detected. Moreover, the apparent formation constants of Ca compounds of these lipids can be calculated by using such measurements made in the presence of a chelating agent of known binding constants.

The addition of LiCl, NaCl, KCl, or tetramethylammonium chloride to buffered systems at pH 7.2 yielded interesting differences for these cations. The systems containing 2.6×10^{-4} M phosphatidic acid (preparation B) in 0.05 M tris had a very low turbidity in the absence of alkali cations (Fig. 8). A sharp increase in turbidity appeared with the addition of LiCl, beginning at a concentration of LiCl equal to 0.2 M. Similar effects were produced with NaCl, but beginning at 0.4 M NaCl. Neither KCl nor tetramethylammonium chloride produced increased turbidities up to concentrations of 0.75 M. The dissymmetry as measured by $I_{45^\circ}/I_{135^\circ}$ had values of 1.8 to 2.0 for the original dispersion and increased in a manner paralleling the change in turbidity on addition of LiCl and NaCl, but showed only small changes with KCl or tetramethylammonium chloride.

Similar dispersions of preparation A, which contained a small amount of Ca, had an initial turbidity higher than those of preparation B. The addition of KCl to these systems produced a small progressive decrease in turbidity with increasing K concentration. With NaCl, the turbidity decreased until a concentration of 0.25 M was reached, after which further additions of NaCl produced an increase. The effect of the addition of CaCl_2 was much more pronounced. Bringing the system to a concentration of CaCl_2 equal to 1×10^{-4} M doubled the turbidity and caused a sharp increase in the dissymmetry, showing the much stronger binding of this divalent cation to the phosphate groups of the micelles. Small additions of MgCl_2 also produced an increase in turbidity, although requiring somewhat higher concentrations than for CaCl_2 .

Since the effects of Ca^{+2} are different from those of the univalent cations, it is possible to detect competitive or antagonistic effects arising from the presence of both types of ions. An indication that reversible exchanges take place between univalent and divalent cations was shown by the following experiment. A buffered dispersion at pH 7.2 was brought to

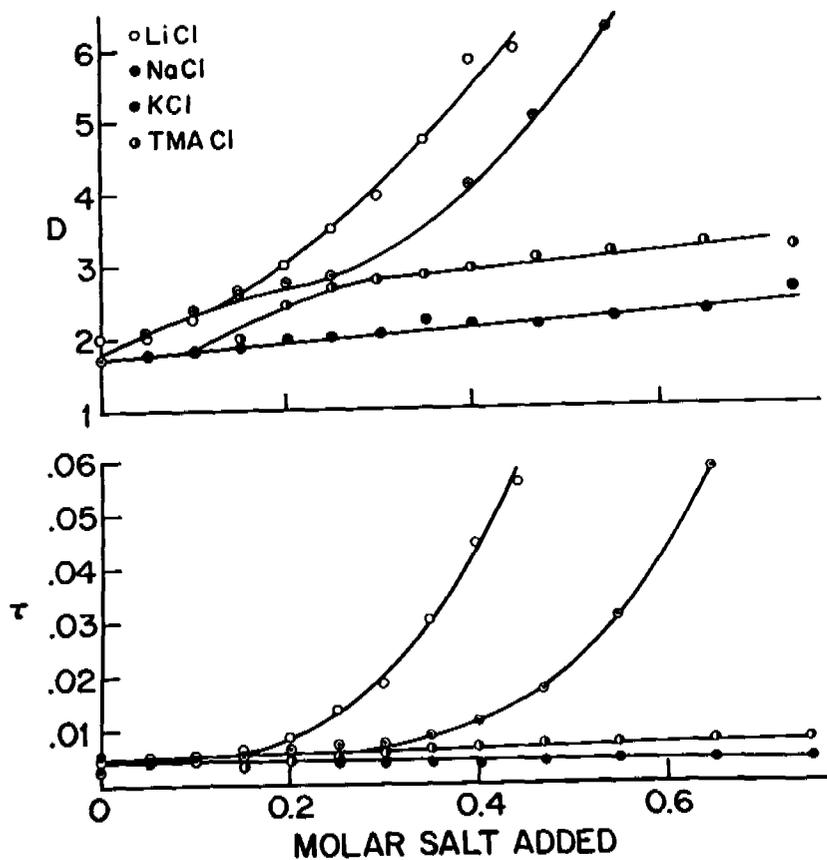


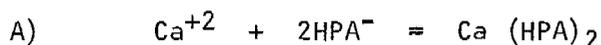
Fig. 8. The relative effects of LiCl, NaCl, KCl, and tetramethylammonium chloride on the light scattered by aqueous micelles of phosphatidic acid. Additions of the salts to equal aliquots of a 2×10^{-4} M dispersion buffered at pH 7.2 in 0.05 M tris show the greater effect of the smaller cations in increasing the turbidity (lower graph) and the dissymmetry measured by $I_{45^\circ}/I_{135^\circ}$ (upper graph) using 546 m μ light.

a concentration of CaCl_2 equal to 1×10^{-4} M, resulting in the anticipated sharp increase in turbidity, but without heavy coagulation. Addition of KCl to this dispersion decreased the turbidity, until at a concentration of KCl equal to 0.15 M the turbidity was reduced to half its value before the addition of KCl. The univalent cation when present in sufficient concentration can exchange for Ca that is bound to the surface with a resulting decrease in turbidity. Further studies of the effects of various ion systems are being made.

The increase in turbidity produced by Ca^{+2} contrasting with the absence of such effects in the presence of moderate concentrations of Na^+ led to a procedure for determining the apparent formation constants for the binding of Ca^{+2} by the anionic form of phosphatidic acid. The procedure was as follows: two equal aliquots of the same dispersion of phosphatidic acid were buffered with 0.05 M tris at the desired pH level of 7.2 or higher. One of these, system I, was made 0.03 M in NaCl, and then small additions of CaCl_2 were made, recording the turbidity at each concentration level of CaCl_2 . The second aliquot, system II, was made 0.01 M in sodium citrate, maintaining equal Na concentration. It showed the same turbidity as the system containing 0.03 M NaCl. For system III, larger additions of CaCl_2 were required to produce increases in turbidity equal to those produced in the absence of citrate ion in system I (Fig. 9).

The difference in the curves for the turbidity in the presence of citrate and in its absence is due to the complexing of Ca^{+2} with the citrate ion, since the citrate ion does not produce any change in the turbidity of the lipid suspension. Ancillary experiments performed with two different concentrations of the same dispersion at the same pH and CaCl_2 concentration showed parallel but different increases in turbidity which, however, were related to the concentration of lipid. From this, it is assumed that equal turbidities for two similar aliquots of lipid dispersion indicate equal concentrations of calcium bound phosphatidic acid, designated as (CaPA). Points of equal turbidity on the parallel curves in Fig. 9 then represent equal concentrations of CaPA. Using Ca_T for the total Ca added, Ca^{+2} for its equilibrium concentration, CaCit^- for the calcium bound to citrate, all in moles per liter, the difference in the values for Ca_T for the two systems gives the CaCit^- , that is, $\text{CaCit}^- = \text{Ca}_T^{\text{I}} - \text{Ca}_T^{\text{II}}$. In system II, Ca^{+2} is in equilibrium with both citric acid and phosphatidic acid anions. At the pH levels used, > 7 , citric acid may be considered to be completely ionized, and its reaction with Ca^{+2} is: $\text{Ca}^{+2} + \text{Cit}^{-3} = \text{CaCit}^-$.

Phosphatidic acid above its first equivalence point of pH 5.6 exists in the form of the anions HPA^- and $\text{PA}^{=9}$. Hence, in determining the apparent formation constant for the binding of Ca to phosphatidic acid, at pH levels above the first equivalence point, the selection of an appropriate reaction must be made from the following alternatives:



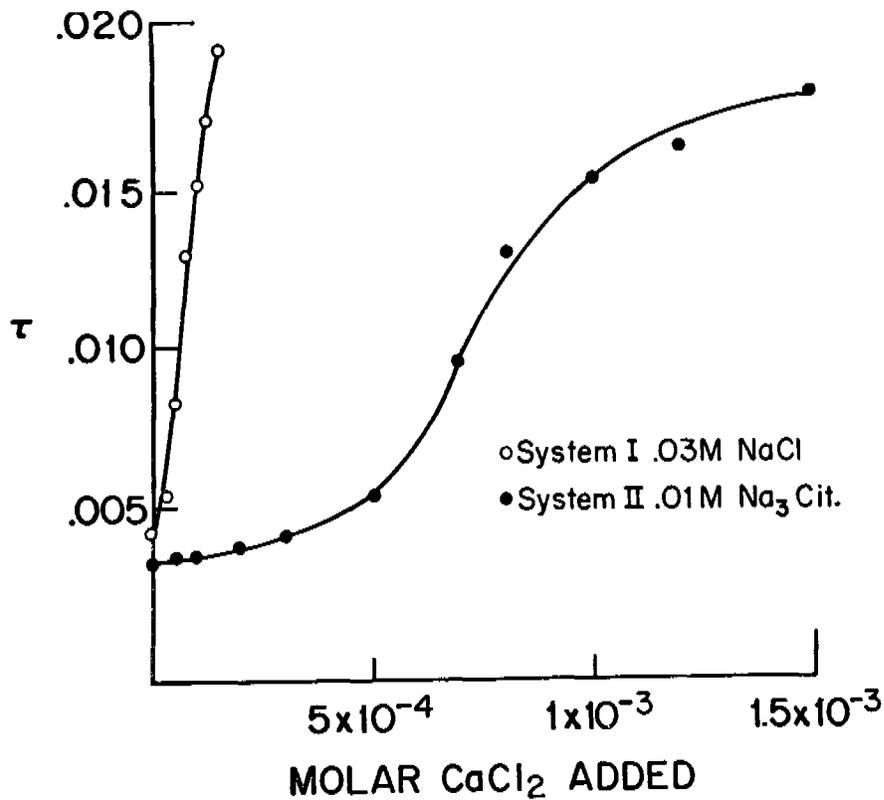
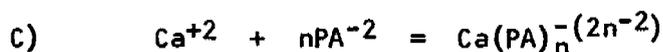


Fig. 9. Turbidities of micellar dispersions of phosphatidic acid with increasing concentrations of CaCl₂. Equal aliquots buffered at pH 7.23 in 0.05 M tris, System I containing 0.03 M NaCl, System II 0.01 M Na₃ citrate. For equal turbidities the difference in the concentrations of CaCl₂ gives the concentration of CaCit⁻ in System II. This permits the calculation of the apparent formation constant of CaPA as described in text.



The first equation is eliminated, because numerous experiments have shown that the addition of Ca to aqueous dispersions of acidic lipids produces an immediate release of H^+ which increases with further addition of Ca. The binding does not take place with HPA^- but with PA^{-2} . Removal of the latter ion leads to the further ionization of HPA^- releasing H^+ . Results obtained in this laboratory (to be published) show that formation constants for the binding of cations by phosphatidic acid can be calculated on the basis of this H^+ ion release. Unfortunately, the method of Bjerrum cannot be applied here to determine other possible values for n for higher orders of complexing¹⁴. The ratio of the total Ca to the total phosphatidic acid in these systems ranged from 1/5 to 1/1.3 and, therefore, did not permit investigation of higher values for n. The formation constants are therefore calculated on the basis of the second equation (B).

Table 5 gives in columns 1 and 2 the values for Ca_T in the systems containing 0.03 N NaCl or 0.01 N Na_2Cit having equal turbidities. From these data, the concentrations for $CaCit^-$ are found. With the use of the value for the formation constant of $CaCit^-$ obtained by Hastings¹⁵ of $\log K = 3.21$ at pH 7.4 and $\mu = 0.15$ and the concentration of $CaCit^-$ (Table 5, column 3), the equilibrium concentration of Ca^{+2} (Table 5, column 4) in our systems can be calculated from the expression:

$$K = \frac{(CaCit^-)}{(Ca^{+2})(Cit^{-3})}$$

On the basis of the reaction of Ca^{+2} with the lipid at the pH levels studied ($Ca^{+2} + PA^{-2} = CaPA$), the mass balance gives

$$PA_T = HPA^- + PA^{-2} + CaPA \quad (1)$$

A curve made for the titration of phosphatidic acid with tetramethylammonium hydroxide in 0.03 N NaCl + 0.05 N TMACl gives values for at various pH levels where $\alpha = (PA^{-2}) / (HPA^-) + (PA^{-2})$ or

$$\alpha = \frac{(PA^{-2})}{PA_T - CaPA} \quad (2)$$

The apparent formation constant for CaPA is found by substituting (2) in the equilibrium equation.

$$K' = \frac{(CaPA)}{(Ca^{+2})(PA^{-2})} = \frac{(CaPA)}{\alpha(Ca^{+2})(PA_T - CaPA)}$$

Values for the apparent formation constant calculated this way at pH 7.2, are shown in Table 5.

TABLE 5

Molar concentrations

Ca_T in 0.03 M NaCl	Ca_T in 0.01 M Na_3Cit	CaCit^-	Cit^{-3}	Ca^{+2}	K'
5.91×10^{-5}	7.26×10^{-4}	6.67×10^{-4}	9.33×10^{-3}	4.28×10^{-5}	0.85×10^4
6.61×10^{-5}	7.70×10^{-4}	7.04×10^{-4}	9.30×10^{-3}	4.54×10^{-5}	1.04×10^4
7.54×10^{-5}	8.03×10^{-4}	7.28×10^{-4}	9.27×10^{-3}	4.72×10^{-5}	1.42×10^4
8.22×10^{-5}	8.37×10^{-4}	7.55×10^{-4}	9.25×10^{-3}	4.89×10^{-5}	1.67×10^4
9.02×10^{-5}	8.97×10^{-4}	8.07×10^{-4}	9.19×10^{-3}	5.27×10^{-5}	1.78×10^4
1.08×10^{-4}	1.10×10^{-3}	9.92×10^{-4}	9.01×10^{-3}	6.62×10^{-5}	1.60×10^4
1.27×10^{-4}	1.34×10^{-3}	1.21×10^{-3}	8.79×10^{-3}	8.27×10^{-5}	1.41×10^4
1.39×10^{-4}	1.50×10^{-3}	1.36×10^{-3}	8.64×10^{-3}	9.45×10^{-5}	1.33×10^4

$K' = 1.39 \times 10^4 \pm 0.11 \times 10^4$ (SEM).

For the three pH levels studied, the degree of ionization of HPA⁻ and the values for the formation constant for CaPA at 24° ± 1 are summarized below.

pH	α	K'
7.23	0.24	$1.39 \times 10^4 \pm 0.11 \times 10^4$ (SEM)
8.82	0.55	$1.21 \times 10^4 \pm 0.01 \times 10^4$
9.58	0.73	$0.92 \times 10^4 \pm 0.15 \times 10^4$

The success of this method for calculating the formation constant depends upon the determination of the Ca⁺² concentration using a complexing anion the formation constant of which at the desired ionic strength is known. Furthermore, the degree of binding of the cation with the complexing ion should not differ greatly from that of the lipid studied. In experiments performed with EDTA rather than sodium citrate, the addition of CaCl₂ did not increase the turbidity until the amount of added Ca was equivalent to the EDTA present. Further addition of CaCl₂ sharply raised the turbidity. The equilibrium Ca⁺² could not be determined in such instances.

The values for the apparent formation constant calculated for CaPA (Table 5) are reasonably constant through the pH interval 7.23-9.58 with a maximum range in values from 5.2×10^3 - 1.8×10^4 . Recalculating the constant for the possible formation of Ca (PA)₂⁻² gives values ranging from 3.2×10^7 to 7.5×10^8 . Although the greater constancy of the values obtained for the formation of CaPA is not a sufficient basis for eliminating higher values for n, this value obtained for the formation constant of CaPA agrees well with values measured at pH 6.0 and 7.0 in 0.1 M TMACl using the H⁺ ion released to calculate the constant (to be published). These were 2.5×10^4 at pH 6 and 1.5×10^4 at pH 7 for a concentration of phosphatidic acid of 1.9×10^{-3} M and CaCl₂ from 1×10^{-4} to 4×10^{-3} M.

The apparent constants reported here must be viewed with the uncertainties imposed by treating the phosphate groups on the surface of the micelle as independent units and applying the Law of Mass Action to their reaction with dissolved ions. There is no doubt that an inaccuracy is also introduced by using the concentration of the cation in the solution in place of the effective concentration at the surface of the micelle. This concentration, although proportional to the bulk concentration, is also a function of the surface potential which at a given pH will change with the extent of cation binding.

A comparison of the reactions of the highly charged phosphate groups on the surface of these micelles with some polyphosphates is of interest. Values reported for the binding of Ca by tetrametaphosphonic acid and trimetaphosphonic acid^{16,17} are in the same range with values for log K = 4.89 and 3.48, respectively. For ATP⁻⁴ the stability constant for the binding with Ca in 0.2 ionic strength is reported by Smith and Alberty¹⁸ as 1970. However, other values reported¹⁹ in 0.1 ionic

strength of 1.2×10^4 agree very well with our values for phosphatidic acid.

The addition of calcium ion had the most pronounced effect upon the micelles increasing turbidity and dissymmetry and producing coagulation. At pH levels above the first equivalence point phosphatidic acid micelles are composed of singly and doubly charged ions. The double layer may contain H^+ and other univalent cations. On addition of Ca or Mg these ions exchange for univalent cations in the double layer. The strong ionic binding between the phosphate ions and the divalent metals causes their incorporation into the surface of the micelle with a reduction in negative charge. The effect of this cation in its binding to ionized acid lipid groups is (1) to combine with the anion groups reducing the negative charge of the surface of the particle, (2) to expel water from the interlayer spaces by reducing the hydration of the phosphate groups, and (3) to bind together adjacent molecules on a plane and possibly bind together layers of lipid molecules.

The action of divalent cations can be partially antagonized by suitable concentrations of univalent cations. Preparation A containing a small amount of Ca bound to the phosphatidic acid gave dispersions which had a higher initial turbidity than that of the micelles produced from preparation B containing no Ca. However, the addition of alkali metal chlorides to the dispersions of preparation A gave a progressive decrease in turbidity. In the case of NaCl specific effects reversed this change at high NaCl concentrations. This action can be seen as the exchange of univalent ions for Ca when the relative concentration of the former is high. Despite the strong binding of Ca to the phosphate groups, it must be considered to be reversibly exchanged with alkali metal ions at high concentrations or H^+ (at low pH levels). An additional effect of salts of univalent ions is to increase the solubility of the lipid by the decrease in the activity coefficient of the lipid anion. Similar effects are well known, as in the case of the "salting-in" of proteins²⁰. Another effect of salts added to dispersions of acidic lipids is to increase the degree of ionization of the acid groups^{6,9}. Studies of the infrared spectra of phospholipids²¹ show that increased ionization of the phosphate groups is accompanied by increased hydration. The decrease in turbidity may then be associated with increased solvation of the micellar particles.

The univalent cations are not equally effective in their action on micellar turbidity. As we have seen, NaCl at concentrations greater than 0.30 M produces an increase in both the turbidity and dissymmetry. LiCl produces a like effect at 0.15 M concentration. For systems at pH 7 or higher, the addition of KCl to a concentration of 0.75 M does not produce an increase in turbidity nor any change in dissymmetry. At pH 5, KCl does produce an increase in turbidity but at greater concentrations than is needed for NaCl. At still lower pH levels the difference between

NaCl and KCl is minimal. Since at lower pH levels the number of ionized phosphate groups is less, the micelle has a smaller negative charge. The addition of neutral salts contracts the double layer permitting the aggregation of particles by the formation of bonds between P-OH groups of adjoining particles. The infrared studies made in this laboratory as well as those reported by Thomas, et al.²² attest to the strong tendency of these and related organophosphorus acids to form intermolecular hydrogen bonds. At higher pH levels where the surface charge is greatly increased by the formation of PO_4^{3-} groups, bonds between POH do not form. The difference between the effect of LiCl and NaCl contrasted with KCl can be attributed to a specific binding of Li and Na at moderate salt concentrations to the negative groups. The stability of similar dispersions in the presence of high K concentrations then indicates a much reduced binding of this cation to the ionized groups. The relative effectiveness of $K < Na < Li$ parallels the decreasing size of the crystallographic radius of the ion. From this it may be argued that the phosphate ion perturbs the hydration shell of the small Li and Na ions. The smaller univalent ions with more intense force fields are thereby more effective in binding to the phosphate anions. A similar trend was found by Strauss and Ross²³ for the binding of alkali metal cations to long-chain polyphosphates.

The effects of these ions upon the stability of phosphatidic acid micelles mirrors their action on other lipid systems. The antagonistic effect of Na and Ca has been observed in living membranes, where Ca increases rigidity in contrast to the dispersive effect of NaCl²⁴.

A dispersive effect of NaCl was noted by Schonhorn and Gregor²⁵ on their Ca "electrodes" composed of oriented multilayers of fatty acids. Presumably the presence of NaCl increased the movement of water into the lipid layer. Somewhat different results were obtained in x-ray diffraction studies of aqueous lipid emulsions; Palmer and Schmitt²⁶ found large spacings between lipid layers which increase with water content. They, however, found that KCl and NaCl in concentrations up to 0.4 M reduced the water content between lipid layers in the micelle to 20% of its value in the absence of salts; however, a much greater effect resulted from the addition of small concentrations of $CaCl_2$ which caused expulsion of water from the intramicellar spaces with Ca binding the layers of lipid.

Nash and Tobias²⁷ constructed a model membrane by impregnating a millipore filter with phosphatidylserine, a complex acidic lipid existing as a polyanion at pH 7. On addition of Ca the resistance of the membrane rose, whereas Na and K reduced it. They ascribed this to the dehydration of the phospholipid by $CaCl_2$ and rehydration in KCl or NaCl. These findings are consistent with our observations of the decrease in turbidity of micelles of phosphatidic acid in NaCl and KCl. In these salt systems the converse is observed on the addition of $CaCl_2$. The competitive behavior of mono- and divalent ions for the negative binding site of these lipids mentioned by Nash and Tobias²⁷ are clearly shown by our experiments.

Infrared analysis.

The infrared absorption spectra of lipids have been studied by numerous investigators chiefly interested in using the spectra to identify the various substances studied or for quantitative estimations. However, the spectra can be further used to obtain an understanding of the ionic groups that are present in the molecule and to identify changes in ionic structures arising as the lipid reacts with an ionic medium.

Kimura and Nagai²⁸ in a study of the spectra of phosphatidylserine pointed out changes that were evident as this lipid was treated with acid or base, and attributed some of these to changes in the ionization of the carboxylic acid group. Abramson, Katzman, and Gregor⁶, in agreement with this work, found that the main differences in the infrared spectra of HPS, NaPS, and Na₂PS 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 6 gives the absorbance at 5.75 and 6.1 μ referred to that at 3.5 μ .

TABLE 6

Wave length μ	HPS	NaPS	Na ₂ PS
CO:CH, 5.75:3.5	0.79	0.68	0.68
COO ⁻ :CH, 6.1:3.5	0.073	0.29	0.30
COO ⁻ :CO, 6.1:5.75	0.0093	0.43	0.44

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.

In the following study the changes occurring in the ionization of the phosphate group of several phospholipids are described. It is shown that the infrared spectra can be used to study the changes in the ionization of these compounds and related esters of orthophosphoric acid. Although various authors have assigned absorption frequency ranges to the ionized and unionized phosphate groups²⁹⁻³², these ranges are broad and overlap. These authors also disagree in their assignment of specific absorption bands to definite chemical groups. An attempt has been made to identify these bands more closely for phospholipids and to use them for the recognition of structural characteristics of these compounds.

To aid in the understanding of the more complex phospholipid molecules a study was made of octadecyl dihydrogen phosphate, dioctadecyl hydrogen phosphate, and the sodium salts of both these esters. Phosphatidic acid and its salt resemble octadecyl dihydrogen phosphate; while phosphatidylserine, lecithin, sphingomyelin, and phosphatidylethanolamine, diesters of orthophosphoric acid, are similar in a limited way to dioctadecyl hydrogen phosphate.

The major absorption attributable to the phosphate groups is in the 1110 to 955 cm^{-1} region. The spectrum of dioctadecyl hydrogen phosphate (Fig. 10, Part 1) shows a very strong band or series of overlapping bands in this region with the maximum at 1020 cm^{-1} . The absorption in this region upon formation of the sodium salt (Fig. 10, Part 2) shifts to shorter wave lengths, and the maximum is found at 1095 cm^{-1} . The strong absorption in the acid form at 1055 to 1000 cm^{-1} is markedly reduced in the salt form.

The acid form of octadecyl dihydrogen phosphate in either CCl_4 or Nujol is similar to that of dioctadecyl hydrogen phosphate. A series of strong bands is present in the 1110 to 955 cm^{-1} region, with the maximum at 1030 cm^{-1} . Unfortunately, the direct comparison of sodium octadecyl hydrogen phosphate with the corresponding acid form or with the two forms of dioctadecyl hydrogen phosphate is made difficult by the fact that sodium octadecyl hydrogen phosphate is too polar to dissolve in CCl_4 . Spectra in Nujol mulls frequently differ considerably from those in solution because of the intermolecular association effects in the dispersed solid. The spectrum of sodium octadecyl hydrogen phosphate in Nujol however does show that the 1030 cm^{-1} band is absent; there is a marked reduction in absorbance in the 1055 to 955 cm^{-1} region, and the strongest band in the 1110 to 955 cm^{-1} region is at 1105 cm^{-1} .

In order to make these changes in the spectra studied more readily apparent, Table 7 gives the relative intensities of the major absorption bands associated with the phosphate group expressed as ratios of the intensity of that band divided by that of the CH absorption band at 1470 cm^{-1} . Because of the wide variation in the number of CH groups per phosphate among the compounds studied, these ratios can be used only for comparing the intensities within a spectrum or for qualitative

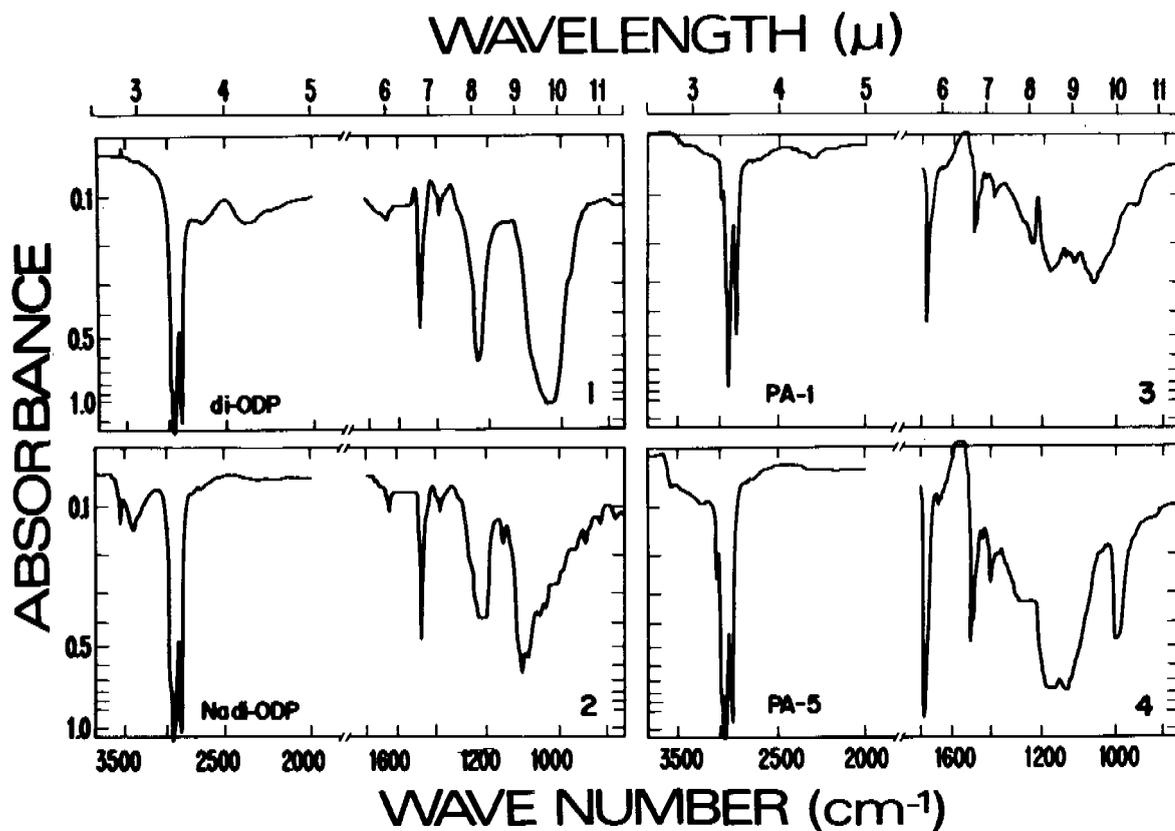


Fig. 10. Infrared absorption spectra of solutions in CCl_4 of dioctadecyl hydrogen phosphate (di-ODP) (Part 1), phosphatidic acid (Part 3), and their sodium salts (Parts 2 and 4). These serve as model compounds showing the differences in absorption bands for the acid and ionized forms. The major differences appear at 3430 to 3230 cm^{-1} showing increased OH (H_2O) for the ionized forms, while 2780 to 2630 cm^{-1} show increased associated P—OH bands for the acid forms. The P—OH of the acid forms is at 1040 to 1000 cm^{-1} while the P—O⁻ is at 1110 to 1090 cm^{-1} . Na di-ODP, sodium dioctadecyl phosphate.

TABLE 7

Absorption ratios* of selected regions of infrared spectra**.

Compound	Na + K eq/mole P	Wavelength									
		μ cm ⁻¹	2.9-3.0 3430-3330	3.7-3.8 2700-2630	8.1 1230	8.5-8.6 1175-1160	9.1 1100	9.3 1075	9.5 1050	9.6 1040	9.7 1030
ODP	0.0		0.06	0.48	0.99	0.29	0.30	0.55	1.20	1.48	2.38
di-ODP	0.0		0.02	0.31	1.40	0.25		1.35	2.0	2.5	
Na di-ODP	1.13		0.27	0.07	1.0	0.29	1.36	1.46	0.95	0.78	0.68
+ODP	0.0		0.03	0.52	0.38	0.20	0.42	0.55	0.72	0.79	0.86
+Na ODP	1.21		0.03	0.07	0.20	0.25	0.27	0.20	0.12	0.12	
Phosphatidic acid											
PA - 1	0.14		0.12	0.24	1.11	1.50	1.39		1.72		1.33
PA - 2	0.19		0.13	0.32	0.81	1.46	1.35		1.35		1.23
PA - 3	1.2		0.60	0.27	0.88	1.47	1.29		1.35		0.82
PA - 4	1.32		0.75	0.15	0.77	1.50	1.63		1.01		0.82
PA - 5	1.75		0.70	0.10	0.74	1.60	1.60		0.80		0.40
Phosphatidylserine											
PS - 1	0.0		0.19	0.40	0.73	1.3	1.2		1.8		
PS - 2	0.0		0.19	0.33	1.05	1.0	1.4		1.4		
PS - 3	0.05		0.16	0.36	1.01	1.0	1.3		1.5		
PS - 4	0.8		0.14	0.31	1.01	0.82	1.1		1.2		
PS - 5	1.4		0.34	0.25	1.0	0.76	1.0		1.2		
Lecithin	---		0.49	0.05	1.38	1.02	1.59		1.4		
Sphingomyelin	---		0.64	0.08	1.05	0.25	1.44		1.2		
Phosphatidylethanolamine	---		0.05	0.45	1.67	1.13	1.41	2.0		1.31	

*Ratios computed by dividing absorbance of peak by that of C-H at 1470 cm⁻¹ (6.8 μ) except for those indicated by+.**Compounds dissolved in CCl₄ except for those marked + which were in a Nujol mull.

evaluation of spectra of related compounds. This Table illustrates that the acid forms of octadecyl dihydrogen phosphate and dioctadecyl hydrogen phosphate have the most intense absorption at 1055 to 1000 cm^{-1} , and the salt forms at 1110 to 1070 cm^{-1} .

A series of spectra were made of phosphatidic acid with cation content varying from 0.14 eq of sodium plus potassium per mole of phosphorus to 1.75 eq of sodium plus potassium per mole of phosphorus. The first and last of the series are shown in Fig. 10, Parts 3 and 4. When arranged in order of increasing cation content the spectra show differences in the acid and salt forms in the 1110 to 955 cm^{-1} region similar to those in the model (octadecyl dihydrogen phosphate and sodium octadecyl hydrogen phosphate) compounds. The acid form of phosphatidic acid has a broad shoulder in the 1020 cm^{-1} region and a maximum at 1055 cm^{-1} . As the cation content increases, the absorption at 1020 cm^{-1} decreases, and the absorption at 1100 cm^{-1} becomes relatively stronger than that at 1055 cm^{-1} . In the preparation with the highest cation content the maximum is at 1100 cm^{-1} , and the region at 1055 to 955 cm^{-1} has a much lower intensity than that of the free acid form (Table 7).

Other regions of the spectra associated with the ionic state of the phosphate group are at 3430 to 3330 cm^{-1} , 2700 cm^{-1} , 1235 cm^{-1} , and 1175 cm^{-1} . The band in the 1235 cm^{-1} region is moderately strong in all cases. This band is stronger in the acid forms of octadecyl dihydrogen phosphate and phosphatidic acid than in the salt forms. The bands at 3430 to 3330 cm^{-1} and 2700 cm^{-1} appear to change in a reciprocal fashion as the ionic state is changed. The acid forms of octadecyl dihydrogen phosphate, dioctadecyl hydrogen phosphate, and phosphatidic acid show a broad band at 2700 cm^{-1} and no absorption or weak absorption at 3430 cm^{-1} . The salts of these compounds, however, show increased absorption at 3430 cm^{-1} and reduced intensity at 2700 cm^{-1} . This effect is more pronounced in phosphatidic acid with 1.75 μeq of sodium plus potassium per μmole of phosphorus than in preparations containing approximately 1 eq cation per phosphorus.

In Fig. 11 the intensities of some of the important absorption bands for phosphatidic acid, expressed as ratios of their intensities to that of the band at 1470 cm^{-1} are plotted against the cation content of the phosphatidic acid. The bands which show a significant increase in intensity are at 3430 to 3330 cm^{-1} and at 1100 cm^{-1} , while striking decreases occur at 1230 cm^{-1} and 1050 to 1030 cm^{-1} . Since increasing equivalence of cation is associated with increasing $\text{P}=\text{O}^-$ groups and decreasing $\text{P}=\text{OH}$ groups the changes in the aforementioned bands may be attributed to changes in these groups.

The series of spectra of phosphatidylserine prepared at increasing pH and with increasing cation content show regular changes. Phosphatidylserine prepared at pH 1 having no cation (Fig. 12, Part 1)

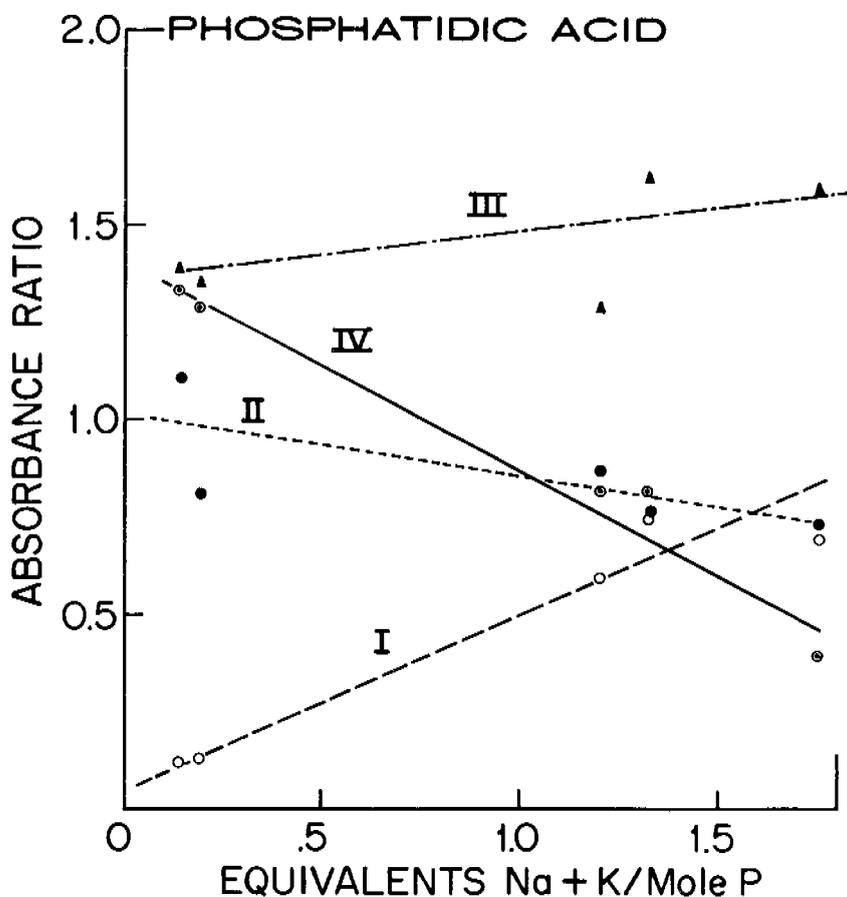


Fig. 11. Changes in intensities of the absorption of groups related to the POH and PO⁻ structures in phosphatidic acid. The ratio of the intensity of an absorption band to that of the C--H absorption at 1470 cm⁻¹ is plotted against the cation content (a measure of the ionization) of the phosphatidic acid preparation. The absorption bands are: I, 3430 to 3230 cm⁻¹ (0---0); II, 1250 to 1220 cm⁻¹ (●---●); III, 1110 to 1090 cm⁻¹ (▲---▲); and IV, 1040 to 1000 cm⁻¹ (●---●).

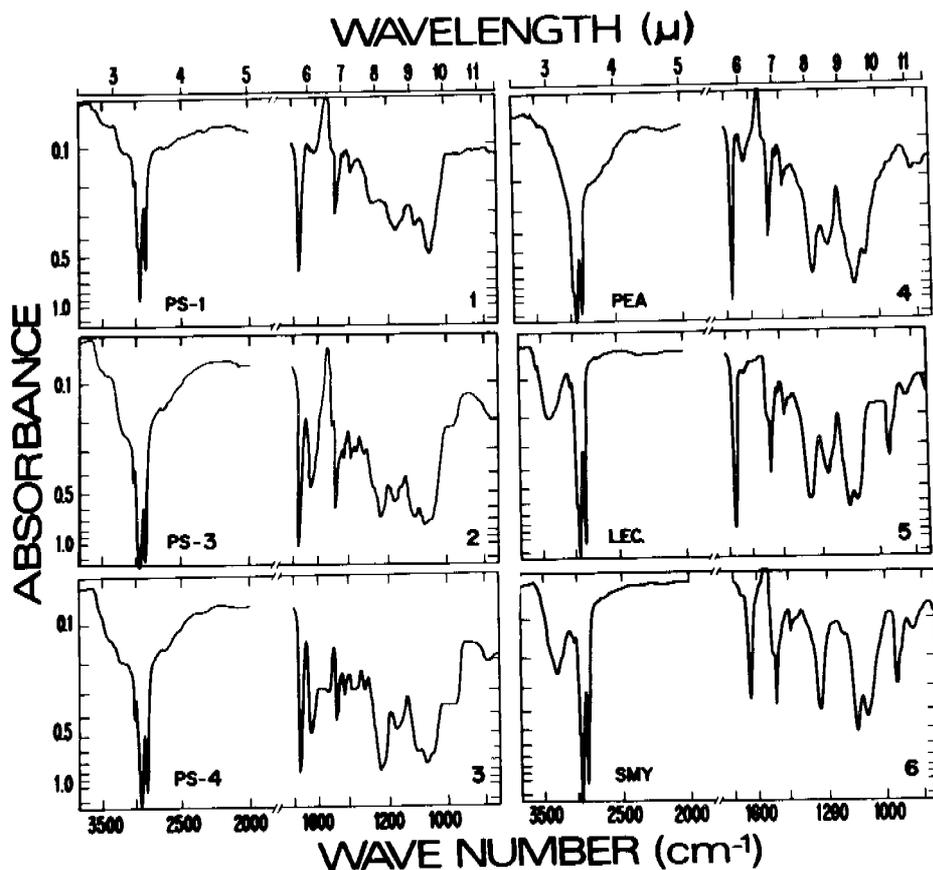


Fig. 12. Infrared absorption spectra of phospholipids in CCl_4 solution. Phosphatidylserine (PS) containing 0.0, 0.8, and 1.4 eq of sodium plus potassium per mole of phosphorus are shown in 1, 2, and 3. Phosphatidylethanolamine (PEA), lecithin (LEC), and sphingomyelin (SMY) spectra are shown in 4, 5, and 6. The ionic structures related to the POH and PO^- groups as interpreted from the spectra indicate the presence of ionized phosphate groups in 5 and 6.

has a maximum absorption at 1055 cm^{-1} and a shoulder at 2700 cm^{-1} . As the pH is increased and the cation content increases, the absorption at 1055 cm^{-1} diminishes; the 1100 cm^{-1} band remains prominent, and the band at 2700 cm^{-1} decreases in intensity. At the highest pH (Fig. 12, Part 3) the absorption at 3430 cm^{-1} increases noticeably. Table 7 gives the relative intensities for these absorption bands for four preparations of phosphatidylserine with increasing cation content.

The band at 1175 to 1160 cm^{-1} is moderately strong in those compounds having ester groups such as phosphatidic acid, phosphatidylserine, phosphatidylethanolamine, and lecithin. There is a weak band in this region in octadecyl dihydrogen phosphate but none in dioctadecyl hydrogen phosphate. The sodium salts of these two compounds, as well as sphingomyelin, have a weak band at 1145 to 1135 cm^{-1} .

Our spectra of phosphatidylethanolamine, lecithin, and sphingomyelin (Fig. 12, Parts 4, 5, and 6) are similar to those obtained by others³³⁻³⁵. Sphingomyelin and lecithin are similar in all of the regions of interest with the exception of the 1175 cm^{-1} band previously discussed. They both have strong bands at 3430 to 3230 cm^{-1} , 1250 to 1235 cm^{-1} , 1100 to 1090 cm^{-1} , and 1065 to 1055 cm^{-1} . They have no strong absorption at 2700 cm^{-1} or at 1030 to 1020 cm^{-1} . The spectrum of phosphatidylethanolamine is quite different from the two choline phospholipids. It has bands at 2700 cm^{-1} , 1235 cm^{-1} , 1070 cm^{-1} , and 1040 cm^{-1} .

In the assignment of infrared bands to specific groups we have been guided mainly by our observations of octadecyl dihydrogen phosphate, dioctadecyl hydrogen phosphate, phosphatidic acid, and their salts, compounds in which the ionic states of the phosphate are known. There is support for each of our assignments in previous studies of organophosphorus compounds.

The absorption band centered at 1030 to 1020 cm^{-1} and extending beyond 1000 cm^{-1} in the acid forms of octadecyl dihydrogen phosphate, dioctadecyl hydrogen phosphate, and phosphatidic acid may be attributed primarily to the unionized $\text{P}-\text{OH}$, since it is absent or much reduced in the mono-cation form of these compounds, and it continues to decrease in intensity on ionization of the second $\text{P}-\text{OH}$ in phosphatidic acid. This assignment agrees with the broader region of 1055 to 910 cm^{-1} suggested by Corbridge³⁰ and Thomas and Chittenden³² for $\text{P}-\text{OH}$.

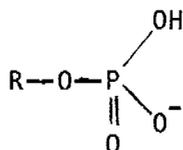
The salt forms of octadecyl dihydrogen phosphate and dioctadecyl hydrogen phosphate have a strong band in the 1110 to 1090 cm^{-1} region. There is no band in this region in the acid form, whereas the 1110 cm^{-1} band is the major phosphate absorption in sodium dioctadecyl phosphate. The series of spectra of phosphatidic acid show an increase in intensity in this region with increasing degree of ionization (Fig. 11). The increase in the 1100 cm^{-1} band parallels the decrease in the $\text{P}-\text{OH}$ band permitting the assignment of the absorption at 1100 cm^{-1} to the ionized $\text{P}-\text{O}^-$. There is some support for this in the studies of Corbridge and

Low³⁶ who show that acid salts of orthophosphates have their strongest band near 1100 cm^{-1} . However, there is no satisfactory agreement among the authors who have studied organic phosphates, and the regions assigned to $\text{P}-\text{O}^-$ are too broad to usefully identify the changes in ionization of phospholipids. Other bands that show striking differences between the acid and salt forms are at 3430 to 3330 cm^{-1} and at 3780 to 2630 cm^{-1} .

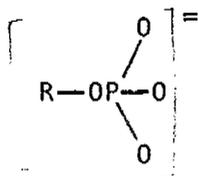
The band at 3430 to 3330 cm^{-1} is the OH absorption, and in the compounds discussed it is probably mainly due to bound water. This is strongest in the ionized forms of the compounds studied. The 2780 to 2630 cm^{-1} band has been well characterized as the $\text{P}=\text{OH}$ stretching frequency in highly associated molecules^{29,31,22}. In agreement with this, the acid forms of our compounds show a broad absorption band in this region with a maximum at about 2700 cm^{-1} , while the salt forms show decreased absorption, accompanied by an increased absorption at 3430 to 3330 cm^{-1} .

All phospholipids have a strong absorption in the 1070 to 1055 cm^{-1} region. This band is present at 1070 cm^{-1} in phosphatidyl-ethanolamine and at 1065 to 1055 cm^{-1} in lecithin and sphingomyelin. The absorption in this region appears to be relatively independent of ionic state, although the adjacent PO^- and $\text{P}=\text{OH}$ bands on either side undoubtedly overlap it and influence its intensity. There is complete agreement that this band is a $\text{P}-\text{O}-\text{C}$ stretch frequency²⁹⁻³².

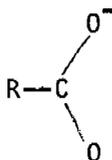
Concurring with other workers, we assign the 1235 cm^{-1} absorption to $\text{P}=\text{O}$ stretch^{29,30} primarily, with a possible contribution from $\text{P}=\text{OH}$ ^{22,30}. This band is present in all the compounds studied, although its intensity decreases in the salt forms, especially after the second OH group of phosphatidic acid has been neutralized. The decrease in the 1235 cm^{-1} band may be due not only to the loss of the $\text{P}=\text{OH}$ deformation absorption but also to the conversion of the structure



to a resonance structure



analogous to



Although Thomas³⁷ and Corbridge³⁰ have suggested that the absorption band at 1175 to 1160 cm^{-1} is due to $\text{C}=\text{O}-\text{P}$ we find it very weak in the spectra of octadecyl dihydrogen phosphate, dioctadecyl hydrogen phosphate, and the salts of these acids as well as in sphingomyelin, all of which are ester-free compounds. Therefore, we must conclude that the strong band at 1175 cm^{-1} in phosphatidic acid, phosphatidylserine, phosphatidylethanolamine, and lecithin is chiefly the well known ester $\text{C}=\text{O}-\text{C}$ absorption, and any small changes due to $\text{P}=\text{O}^-$ groups are masked by this much stronger band.

A summary of all of our assignments of absorption bands associated with the phosphate group is given in Table 8 for the acid and salt forms studied, and with this information we can now discuss the structures of the more complex phospholipids. It should be emphasized that the following discussion pertains only to solutions in CCl_4 and other nonpolar solvents. At this time, we cannot extrapolate these findings to ionic structures in aqueous media.

TABLE 8

Absorption		Acid forms	Salt forms
cm^{-1}	μ		
3430-3230	2.9-3.1		$\text{OH} (\text{H}_2\text{O})$
2780-2630	3.6-3.8	Bonded $\text{P}-\text{OH}$	
1250-1220	8.0-8.2	$\text{P}=\text{O} + \text{P}-\text{OH}$	$\text{P}=\text{O}$
1175-1160	8.5-8.6	$\text{C}=\text{O}-\text{C}$ ($\text{P}-\text{O}-\text{C}$)	$\text{C}=\text{O}-\text{C}$ ($\text{P}-\text{O}-\text{C}$)
1110-1090	9.0-9.2		$\text{P}=\text{O}^-$
1070-1055	9.3-9.5	$\text{P}-\text{O}-\text{C}$	$\text{P}-\text{O}-\text{C}$
1040-1000	9.6-10.0	$\text{P}-\text{OH}$	

The changes in the absorption bands of phosphatidic acid at 2630 cm^{-1} and 3430 cm^{-1} point to a strong association of the unionized acid form of this compound involving the $\text{P}-\text{OH}$ group, which is absent after conversion to the ionic form. Preliminary studies in our laboratory and the observations of others^{38,39} demonstrate that phospholipids exist as micelles in nonpolar solvents. Therefore, the polar groups of the lipid molecules must be directed away from the solvent and are closely associated by intermolecular bonding of these groups. Thomas, Chittenden, and Hartley²² found that the hydrogen bonding between phosphoric acid molecules was stronger than for carboxylic acids. On formation of the ionized phosphatidic acid, the polarity of the phosphate group increases further, and the bonding of water (possibly from contact with the aqueous

base solution) is then dominant. The reduction of the number of P--OH groups in the phosphatidic acid by neutralization of the second acid ionization produces the maximum increase in the 3430 cm^{-1} absorbance and maximum decrease at 2700 cm^{-1} .

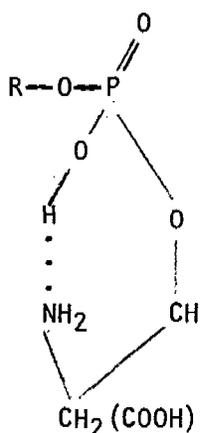
These lipids are recognized as existing as dipolar ions, and our data indicate that this structure persists in CCl_4 solution. The infrared spectra of these two lipids are essentially the same in all regions of phosphate absorption. The strong absorption at 1110 to 1090 cm^{-1} indicates an ionized phosphate in both compounds. There is no noticeable P--OH band at either 2700 cm^{-1} or 1040 to 1020 cm^{-1} . There is a strong bonded H_2O band at 3430 to 3230 cm^{-1} , characteristic of molecules containing the ionized phosphate group. Baer³⁴ has previously noted the molecule of strongly bound water in lecithin which is not present in phosphatidylethanolamine. The strong band at 1065 to 1055 cm^{-1} is ascribed to P--O--C groups.

The infrared spectrum of this molecule of phosphatidylethanolamine which contained 0.15 mole of cation per mole of phosphorus is more typical of those having unionized phosphate groups than of lecithin or sphingomyelin. There is a strong P--OH band in the 2700 cm^{-1} region, and no bound H_2O is present as shown by the lack of absorption at 3430 to 3330 cm^{-1} . The strongest phosphate band is at 1070 cm^{-1} with another strong band at 1040 cm^{-1} . The shift of the major band from 1110 to 1090 cm^{-1} as in lecithin or sphingomyelin to 1070 cm^{-1} and 1040 cm^{-1} supports our conclusion that the P--OH is essentially unionized. We assign the 1070 cm^{-1} band to P--O--C for this molecule and the 1040 cm^{-1} band to P--OH. Bellamy and Beecher²⁹ have also concluded that the presence of the P--OH band in the 2700 cm^{-1} region indicates that phosphatidylethanolamine does not exist as a dipolar ion.

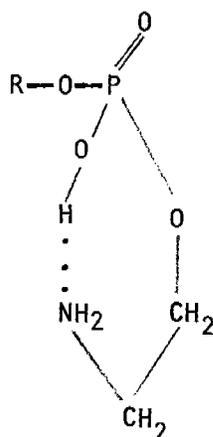
The phosphatidylserine compound can exist in a greater variety of ionic states than the other phospholipids, and the complexity of its series of spectra reflect this. There is no doubt that the cation-free form exists as the dipolar ion in aqueous micelles⁶. However, the ionic states could be different in CCl_4 solution. A study of a series of spectra of phosphatidylserine at increasing pH levels from 1.0 to approximately 6 show that with the addition of 1 eq of cation, ionization of the carboxyl group occurs; the resulting absorption band at 1640 cm^{-1} is due to the COO^- ion. The fact that the weaker carboxylic acid group is ionized rather than the P--OH indicates that the P--OH is bound in some way to the NH_2 , although without complete ionization taking place. Preparation phosphatidylserine-1 which had been brought to pH 1, which is just below the isoelectric point of pH 1.26, indicated an increase in P--OH groups which formed from the protonation of PO^- . This can be seen in the strong P--OH absorption at 2700 cm^{-1} and 1055 cm^{-1} (Fig. 12, Part 1 and Table 7). At the highest pH where the molecule contains 1.4 eq of cation per mole, the P--OH absorption is at a minimum at 2700 cm^{-1} while the band at 3430 cm^{-1} has increased. The PO^- band at 1100 cm^{-1} relative to the P--OH region at 1055 to 1000 cm^{-1} is much stronger than

at the lowest pH (Fig. 12, Part 3). These changes in the phosphate absorption with increasing pH appear to take place first on changing from the positive charged species to the isoelectric form, and secondly, at pH levels beyond the point where 1 eq of cation has been added. In the pH region between the isoelectric point and the first equivalence point, the ionization of the carboxyl group occurs without influencing the phosphate structures. Thus, we can say that at pH 1 the molecule contains unionized phosphate and is not predominantly in the dipolar ionic form in CCl_4 . As the pH is raised, the $\text{P}=\text{OH}$ bands decrease in intensity both in the 2700 cm^{-1} region and the 1055 to 1000 cm^{-1} region. Probably a family of ionic states exists at these intermediate pH values.

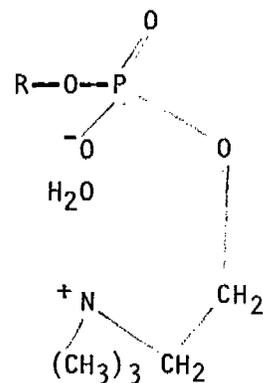
Based upon these changes in the $\text{P}=\text{OH}$ and $\text{P}=\text{O}^-$ groups as well as the changes in the bonded H_2O and intermolecular $\text{P}=\text{OH}$ bands, we suggest the following structures for the phospholipids in organic solvents. Although for convenience sake we have written them to indicate intramolecular bonding, it is probable that in the micelle these molecules would be involved in chains of intermolecular bonds utilizing the same groups as we have illustrated.



Phosphatidylserine
(at isoelectric point)



Phosphatidyleth-
anolamine



Lecithin or
sphingomyelin

These structures illustrate our interpretation that in lecithin and sphingomyelin there is complete transfer of the proton from $\text{P}=\text{OH}$ to the strongly basic quaternary ammonium group, releasing a molecule of water and resulting in an ionized phosphate group. In the cation-free forms of phosphatidylserine and phosphatidylethanolamine the $\text{P}=\text{OH}$ apparently does not completely transfer its proton to the weaker base in these molecules. Instead, a strong hydrogen bond (inter- or intramolecular) is formed. Ionic groups are not present, and $\text{P}=\text{OH}$ associations result rather than bonding to water. Only when phosphatidylserine contains more than 1 eq of cation do we find the characteristic absorption of ionized phosphate.

These structures also account for the persistence of the $\text{P}=\text{O}$ absorbance at 1235 cm^{-1} . Despite the partly ionized state of the phosphate group, the oxygen atoms are not equivalent, since they are bonded to a basic group, and a resonance structure does not result. Only in the series of phosphatidic acid (Fig. 11), do we see a decrease in intensity of this band with increased ionization.

The effects of the dielectric constant, the polarity, and the ability of the solvent to form hydrogen bonds undoubtedly exert major influences on the structures of these lipid molecules. It is, therefore, reasonable to anticipate that in an aqueous medium some differences could be detected in the nature of these polar groups.

Ion exchange; stability constants.

Perhaps the most significant portion of these studies is the measurement of the ion exchange properties of the acidic lipids by measurement of H^+ ion release. These studies were initially carried out in a qualitative manner; striking differences were found in the effect of different ions. The information obtained has made it possible to carry out the measurements in such a way that quantitative determinations of stability constants of cation-lipid complexes can be made.

Phosphatidylserine — The initial qualitative study was carried out on phosphatidylserine. 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 CaCl_2 produced a lowering 1.1 pH units in NaPS dispersions. 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. 13 and 14). The amount of NaCl and KCl needed to reduce the pH of the dispersion of NaPS in distilled H_2O by 1 pH unit was 100 -fold that of the amount of CaCl_2 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. 15). The curves for the addition of NaCl or KCl show a plateau at 0.5 M concentrations. With CaCl_2 at $3 \times 10^{-3}\text{ M}$, the release of H^+ is similar to that produced by 0.7 M NaCl .

With NaPS in water (Fig. 14), the addition of K , Na , and tetramethylammonium 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 quarternary ammonium cation is not bound to phosphate^{40,41}, it is reasonable to expect that Na^+ or K^+ are

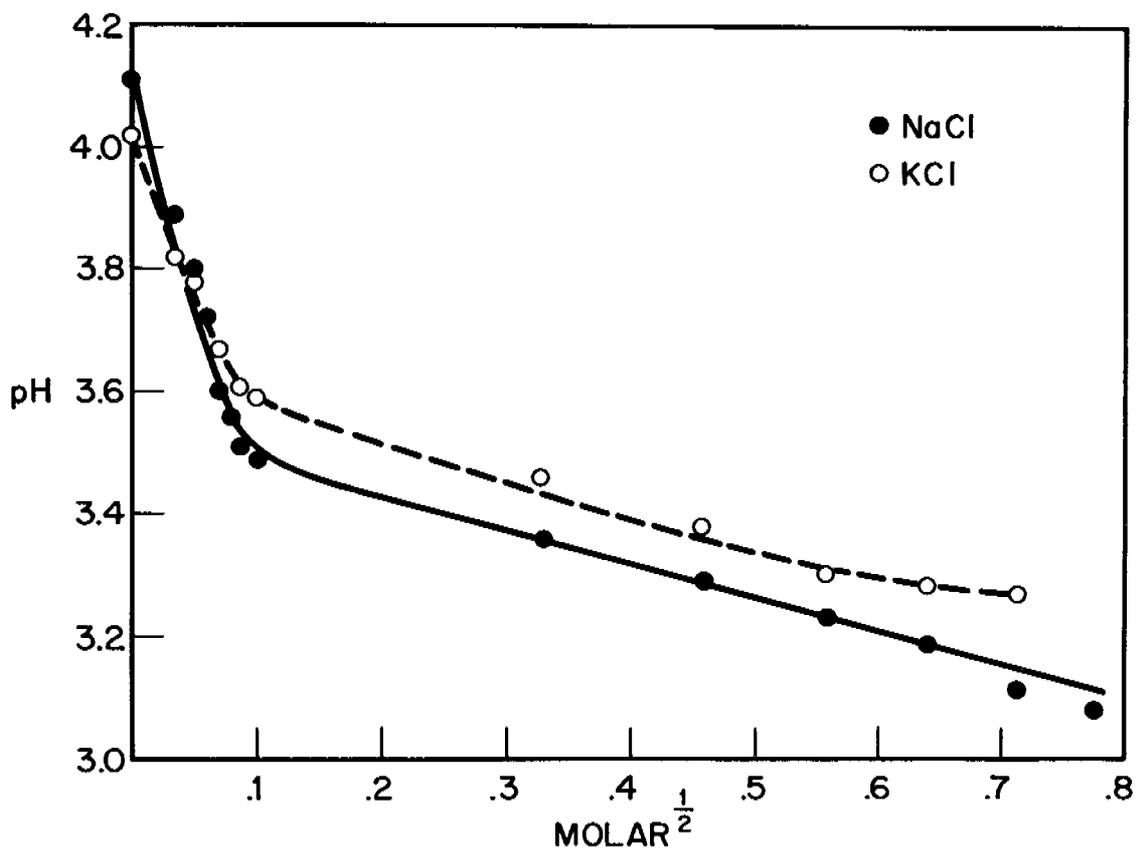


Fig. 13. The effect of added NaCl and KCl on pH of ultra-sonicated dispersions of HPS plotted as in Fig. 14. The dispersion contained 21 μ moles of HPS in 8 ml of distilled H₂O.

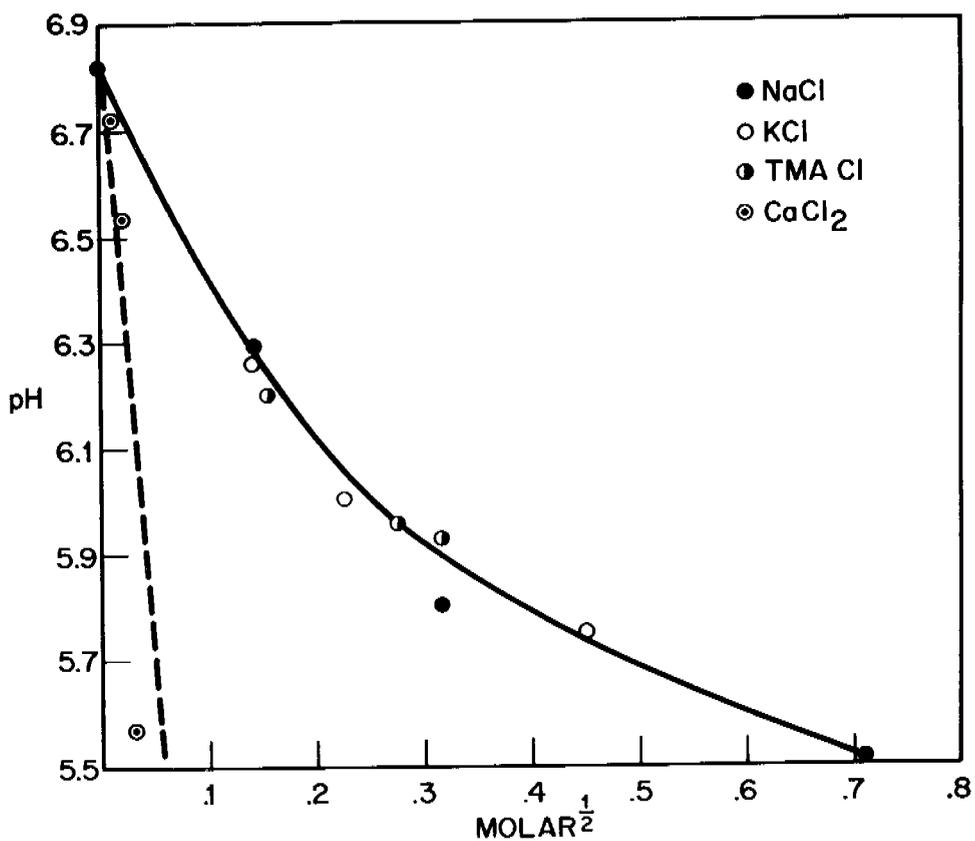


Fig. 14. Effect of added salts on pH of ultrasonicated dispersions of NaPS. The pH is plotted against the square root of the final molar salt concentration. The initial material contained 21 μ moles of NaPS in 8 ml of distilled H₂O. The difference in effect between additions of CaCl₂ and the univalent salts may be noted.

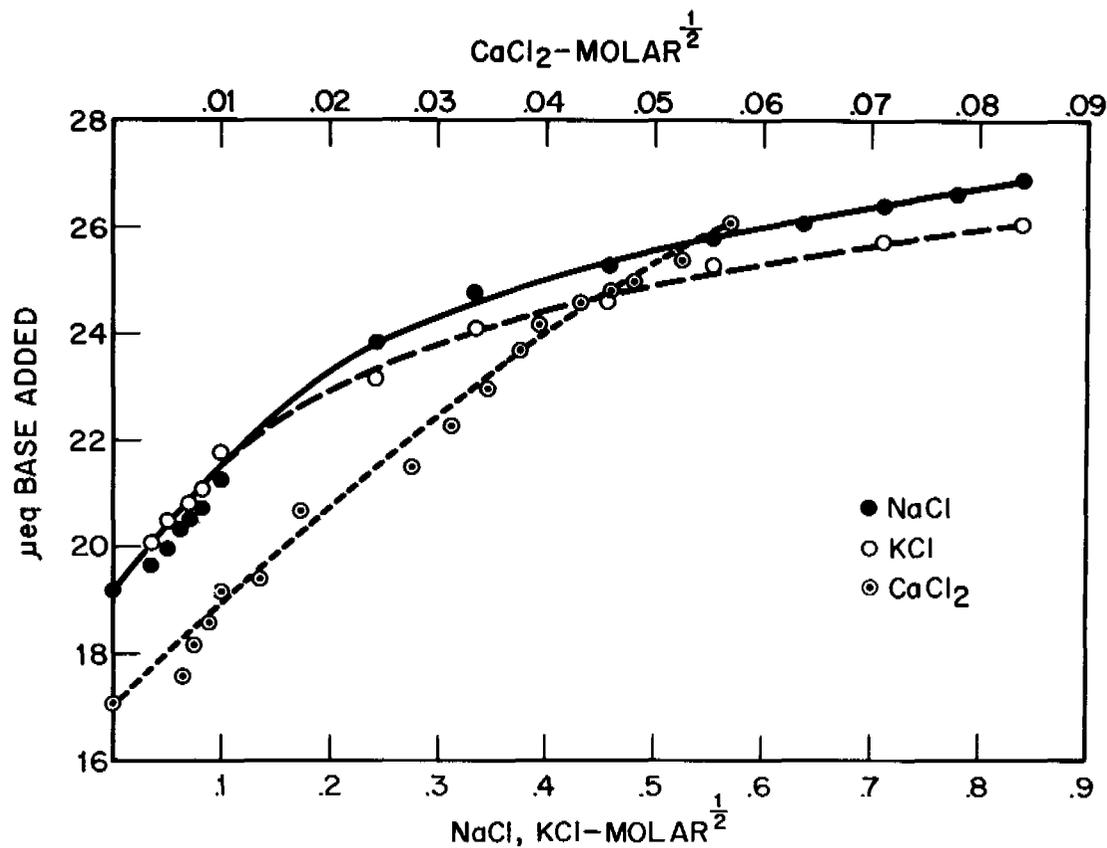


Fig. 15. Base required to maintain pH of dispersions constant at 7.0 during additions of neutral salt. The ultrasonicated dispersions of 21 μ moles of HPS were initially brought to pH 7 with NaOH or KOH. The microequivalents of base are plotted against the square root of the molar concentration. CaCl_2 shown on upper scale.

also not bound by phosphate. Addition of CaCl_2 to NaPS produced a marked release of acid (Figs. 14 and 15), with about 0.35 mole H^+ freed per mole of CaCl_2 , suggesting that Ca^{++} 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. 13). A reasonable assumption is that there is a preferential binding of Na over K; since in this case the source of H^+ is the carboxylic acid group, this is the binding site.

Phosphatidic acid -- Similar effects were noted with phosphatidic acid. The addition of small amounts (to 0.05 M) of NaCl or KCl to aqueous suspensions of phosphatidic acid at pH 3.9 lowered the pH by 0.1; an equal effect was produced by approximately 10^{-4} M CaCl_2 , showing a preferential binding of calcium by this lipid. In contrast, phosphatidylserine dispersions showed a considerable decrease in pH (from 4.2 to 3.5) on the addition of 0.05 M Na^+ or K^+ .

Figure 16 shows the release of H^+ produced by the addition of salts to dispersions of phosphatidic acid which had been brought to pH levels of 5 to 7 and maintained at that level by the addition of KOH or NaOH. The first addition of Na^+ , K^+ , or Ca^{++} released a considerable amount of H^+ ; further additions produced progressively smaller changes (Fig. 16). There was a considerably greater release of H^+ by Na^+ , K^+ , and Ca^{++} at pH 7 than at pH 5. It is known that monobasic and dibasic phosphate groups bind ions differently, and some of the effects observed here may be the result⁴². At pH 9, the effect was less than at pH 7, because most of the acid was already neutralized.

The striking effects of Ca^{++} on the release of H^+ can be seen in Fig. 16. The addition of 1 μmole of Ca^{++} to a 5-ml dispersion of approximately 13 μmoles of phosphatidic acid maintained at a constant pH 7 produced a release of slightly more than 1 μeq of H^+ . The addition of 2500 μmoles of either Na^+ or K^+ to a similar dispersion at pH 7 produced a release of 5 μeq of H^+ . A comparable effect was produced by the addition of 6.5 μmoles of Ca^{++} . A small part of this greater effect of Ca^{++} (200:1 in terms of equivalents) can be ascribed to the greater charge of the cation. However, the predominant effect here is undoubtedly due to the specific binding of Ca^{++} by phosphate.

In another series of experiments, identical dispersions were prepared containing 2×10^{-3} M phosphatidic acid in 0.1 N tetramethylammonium chloride. The systems were first brought to pH 7 with tetramethylammonium hydroxide. To each system small additions of metal chloride were made and the pH was noted after equilibrium was reached. With both univalent and divalent chlorides, the first addition produced the greatest lowering in pH with progressively smaller changes resulting on further equal additions. The order of effectiveness of the cations

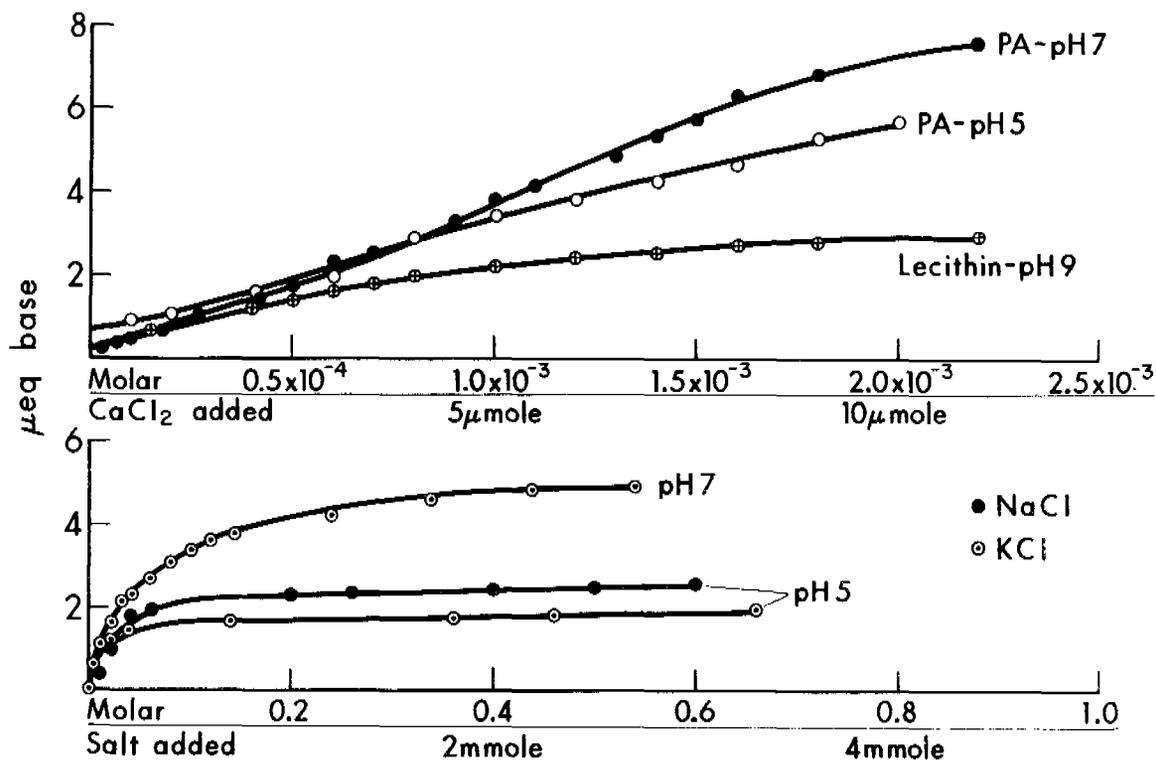


Fig. 16. Base required to maintain dispersions at constant pH during additions of neutral salts. The ultrasonically treated dispersions of acid-dialyzed phosphatidic acid were brought to the desired pH with NaOH or KOH. The upper panel shows the effect of CaCl_2 on phosphatidic acid at two pH levels. The lower panel shows the comparable effect of NaCl and KCl. Note that the scale of concentration of NaCl and KCl in the lower figure is 400 times that of CaCl_2 in the upper.

in releasing H^+ was $Ca^{+2} > Mg^{+2} > Li^+ > Na^+ > K^+ > TMA^+$. The different systems were lowered from pH 7 to pH 6 in 4×10^{-4} M $CaCl_2$, 5×10^{-4} M $MgCl_2$, 0.22 M $LiCl$, 0.35 M $NaCl$, 0.75 M KCl . $CaCl_2$ at 3.5×10^{-3} M brought the system to pH 4.25, while the lowest pH established on adding $MgCl_2$ was 5.25. The univalent chlorides did not depress the pH below 5.6. Of special interest in this connection is the fact that the addition of $CaCl_2$ brought the pH below the level of the equivalence point for the formation of the monobasic anion. Presumably H^+ ions can be released from the unionized acid by reaction with calcium but not with the other cations employed.

Stability constants.

The experimental procedure used for determining the stability constants for the cation-lipid complex was the following: A 5 ml dispersion of phosphatidic acid of known concentration was brought to 0.1 ionic strength by the addition of tetramethylammonium chloride and to the desired pH with the corresponding hydroxide. Small additions of a solution of the metal chloride were then made. After each addition, the amount of tetramethylammonium hydroxide needed to re-establish the initial pH was measured. In all cases, sufficient time was given for equilibrium to be attained. In this way, a series of values was obtained for the microequivalents of H^+ released at increasing cation concentrations. This procedure offers the following advantage in studies with polyelectrolytes: it permits obtaining a series of values for hydrogen ion-cation relationship at a pH level which may be selected for special interest. Where more than one acid group is present, the pH may be selected to study one group only. If the pH of the system is permitted to change the simple Henderson-Hasselbach relation cannot be employed, because the slope of $\log \frac{\alpha}{1-\alpha}$ vs. pH is greater than unity and may change with pH. As the concentration of cation increases, if the pH is permitted to drop, coagulation of the polyelectrolyte may follow with a change in surface and reaction characteristics.

A careful titration of the phosphatidic acid dispersion was made in 0.1 M tetramethylammonium chloride using tetramethylammonium hydroxide and HCl for the titration cycle. The corrected titration curve gave values for α for different pH levels.

In the calculation of the stability constants several assumptions were necessary. The addition of tetramethylammonium chloride produced smaller changes in pH and turbidity than did equal concentrations of metallic cations. The large diameter of the tetramethylammonium ion minimizes its tendency to bind to charged surfaces. It was, therefore, assumed that the tetramethylammonium ion did not show any specific binding for the lipid. Its effect was due to changing ionic strength. Hence, the greater effects of other cations could be ascribed to their specific interactions with the phosphatidic acid. It was further assumed that the formation of the metal-lipid complex did not alter the titration characteristics of the uncombined lipid.

From the titration curve of phosphatidic acid in 0.1 M tetramethylammonium chloride it is shown that at pH levels above 5.5, the lipid exists in the form of the monobasic acid anion*, HPA⁻ and its conjugate base, PA⁻². At pH 7, α in this medium is 0.15 where $\alpha = \frac{PA^{-2}}{HPA^{-} + PA^{-2}}$. The total cation associated with the phosphatidic

acid at this pH level is $(1 + \alpha) PA_T$. The addition of Ca⁺² or other divalent ion is assumed to react with the anion PA⁻². $Me^{+2} + PA^{-2} \rightarrow MePA$. The resulting decreased concentration of PA⁻² leads to the release of H⁺ by the reaction $HPA^{-} \rightarrow PA^{-2} + H^{+}$. The addition of TMAOH to return to the initial pH gives a measure of the H⁺ ion released and also adds an equivalent concentration of tetramethylammonium ion TMA₁⁺ to the system. After the addition of Me⁺², the equations for mass balance are

$$PA_T = HPA^{-} + PA^{-2} + MePA \quad 1$$

$$Me_T = Me^{+2} + MePA \quad 2$$

PA_T-MePA gives the lipid in ionized form. The equation for electron neutrality is (the 0.1 M TMACl is neglected since it is assumed it does not bind):

$$H^{+} + (1+\alpha)PA_T + TMA_1 + 2 (Me_T - MePA) = OH^{-} + (1+\alpha) (PA_T - MePA) + 2\alpha (PA_T - MePA) + Cl^{-} \quad 3$$

All values are in molar concentration.

At pH 7, $H^{+} = OH^{-}$ and $Cl^{-} = 2 Me_T$. Equation (3) may be simplified to

$$(1 - \alpha) MePA = TMA_1 \quad 4$$

In a similar manner, the reaction of univalent cations Me⁺ is assumed to form initially MePA⁻ with the possible formation of Me₂PA at higher concentrations of Me⁺.

The apparent stability constants K' (because of the neglect of activity coefficients) are based upon the equations

$$K' = \frac{(MePA)}{(Me^{+2})(PA^{-2})} \quad 5$$

since from

$$(2) \quad Me^{+2} = Me_T - MePA$$

 *Abbreviations used are: PA, phosphatidic acid; HPA⁻ and PA⁻², the anions formed; MePA, the metal complex; PA_T, total lipid in all forms; Me_T, total cation in all forms; TMAOH, tetramethylammonium hydroxide, and TMACl, tetramethylammonium chloride.

and

$$\alpha = \frac{PA^{-2}}{HPA^{-} + PA^{-2}}$$

from

$$(1) \quad \alpha = \frac{PA^{-2}}{PA_T - MePA}$$

Since

$$K' = \frac{(MePA)}{(Me_T - MePA) \alpha (PA_T - MePA)}$$

Using

$$(4) \quad MePA = \frac{TMA_1^+}{1 - \alpha}$$

For univalent cations, similar reasoning leads to

$$K' = \frac{(MePA^{-})}{(Me_T - MePA^{-}) \alpha (PA_T - MePA^{-})}$$

Here, however, the concentration of $MePA^{-}$ formed is small relative to Me_T and in most instances Me_T may be used as the concentration of the ion.

The reactions with $CaCl_2$ and $MgCl_2$ in 0.1 M TMACl were at approximately constant ionic strengths, since the concentrations of these cations did not exceed 4×10^{-3} M. Table 9 shows the values calculated for duplicate determinations of K' the apparent stability constant for CaPA and for MgPA with increasing cation concentrations. The agreement between the value obtained for CaPA by this procedure and the values obtained by means of turbidimetric measurements at pH levels ranging from 7.2 to 9.6 is extremely good.

Table 10 shows the values for the apparent stability constants for the univalent cations forming the complex $MePA^{-}$ in 0.1 M TMACl. These values are for systems at increasing ionic strength and show a definite decrease with increased salt concentration. When plotted against the square root of the concentration of the added salt the values remain reasonably constant until roughly .08 M MeCl then they decrease reaching a limiting value at high salt concentrations. This decrease in value of the constant results in part from the shift of the titration curve to lower pH levels with increasing ionic strength reaching a limiting value at high ionic strengths.

TABLE 9

Ca sample 1, $PA_T = 2.20 \times 10^{-3} M$			Ca sample 2, $PA_T = 2.06 \times 10^{-3} M$			Mg, $PA_T = 2.16 \times 10^{-3} M$		
Ca_T (molar)	Ca^{+2} (molar)	K'	Ca_T (molar)	Ca^{+2} (molar)	K'	Mg_T (molar)	Mg^{+2} (molar)	K'
2.0×10^{-4}	4.7×10^{-5}	1.01×10^4	2.0×10^{-4}	7.3×10^{-5}	$.58 \times 10^4$	2.0×10^{-4}	4.9×10^{-5}	$.98 \times 10^4$
3.0×10^{-4}	4.8	1.63	2.5×10^{-4}	8.1×10^{-5}	.70	3.0	8.2	.85
4.0	4.6	2.39	3.0×10^{-4}	8.4×10^{-5}	.86	3.9	9.0	1.07
5.0	3.9	3.64	4.0×10^{-4}	9.4	1.10	5.8	1.18×10^{-4}	1.28
6.0	5.7	2.98	5.0	1.04×10^{-4}	1.29	7.7	1.82	1.09
8.0	1.91×10^{-4}	1.06	6.0	1.17	1.42	1.1×10^{-3}	3.94	.67
1.0×10^{-3}	2.35	1.11	7.0	1.40	1.39	1.5	5.85	.66
1.2×10^{-3}	2.70	1.19	8.0	1.56	1.45	1.8	6.20	.82
1.5×10^{-3}	1.50	2.95	9.0	1.85	1.38	2.1	6.00	1.07
2.0	2.10	2.86	1.0×10^{-3}	2.00	1.44	2.7	9.8	.99
2.4	5.20	1.44	1.2	2.80	1.23	3.27	1.48×10^{-3}	1.18
3.18	1.28×10^{-3}	1.07	1.5	3.10	1.58			
3.86	1.95	2.60						

Av. $K' = 1.99 \times 10^4$ Av. $K' = 1.20 \times 10^4$ Av. $K' = .97 \times 10^4$

PA_T = 2.16 X 10⁻³ M

PA_T = 2.10 X 10⁻³ M

PA_T = 2.10 X 10⁻³ M

PA_T = 2.16 X 10⁻³ M

PA_T = 2.46 X 10⁻³ M

Li_T (molar) K'

Na sample I
Na_T (m) K'

Na sample II
Na_T (m) K'

K sample I
K_T (m) K'

K sample II
K_T (m) K'

.008	15.3	.019	16.0	.019	16.2	.02	10.0	.02	8.00
.010	14.2	.039	15.4	.039	16.4	.04	9.40	.04	8.34
.020	13.1	.068	14.0	.058	16.3	.06	9.46	.06	8.07
.030	15.4	.089	13.2	.089	14.3	.08	8.63	.08	7.60
.040	17.3	.119	12.8	.119	15.2	.10	8.20	.101	7.20
.050	18.5	.148	13.2	.148	13.7	.15	7.31	.15	6.50
.060	18.0	.198	12.2	.218	10.9	.20	6.28	.20	5.94
.080	17.8	.25	10.8	.287	10.4	.25	5.30	.25	5.38
.10	17.5	.30	10.1	.415	8.19	.30	4.70	.30	5.00
.12	15.6	.50	8.70	.478	8.05	.40	3.94	.40	4.37
.15	12.7	.70	7.44	.537	7.78	.50	3.42	.50	3.86
.20	14.0			.594	8.40	.60	3.03	.60	3.44
.25	12.6			.65	7.65	.70	2.71	.70	3.10
.30	11.8					.80	2.47	.80	2.90
.35	10.6								
.40	9.40								
.50	8.86								
.60	8.55								
.75	10.2								
.79	10.2								

K' at = .1
17.3

k' at = .1
16.2

K' at = .1
8.8

The values for the apparent stability constants for CaPA and MgPA show a reasonable degree of constancy over an extended range of concentrations of the cation, for duplicate experiments and for experiments performed at other pH levels. These results support the view that these dispersions of acidic lipids have all the ionic groups exposed to the aqueous medium, and stoichiometric relations can be formulated for the reaction of the lipid with dissolved ions.

The addition of NaCl to dispersions of phosphatidic acid of concentrations in the order of 2×10^{-3} M at pH 7 caused coagulation when the salt concentration reached 0.25 M. It is shown in this investigation that in this medium, 0.22 to 0.25 eq H^+ /mole lipid has been released. Contrary to this, the addition of KCl to a concentration of 0.8 M does not produce coagulation, except on standing. At this concentration, only 0.19 eq H^+ /mole lipid is released. It may be reasoned that the coagulation takes place when the fraction of lipid bound to cation reaches a definite value, when the negative charges of the polyanion are reduced to the point where repulsive forces do not maintain the stability of the dispersion. Furthermore, the cation could coordinate with more than one lipid without further release of H^+ , but in a manner that would bind surfaces together.

The apparent stability constants for the binding of cations by phosphatidic acid shows interesting similarities to other phosphate anions of biologic and non-biologic origin. Smith and Alberty found values for ATP binding univalent cations in 0.2 M ionic strength that are close to the values found for phosphatidic acid. Our constants for Mg^{+2} and Ca^{+2} binding with phosphatidic acid are in agreement with the values reported by DiStefano and Neuman¹⁹ and others for ATP.

In a study of the turbidities of dispersions of acidic lipids it was shown by Abramson, et al.⁴³ that in the presence of a soluble complexing anion, calcium is in equilibrium with both the polyanion and the dissolved anion. It is interesting to consider the system of acidic lipid in an aqueous medium containing ATP and Ca^{+2} , in which calcium is bound to both anions in ratios determined by their stability constants and concentration. A change in concentration of ATP such as a conversion to ADP, which has a stability constant significantly lower than that of ATP, will release Ca^{+2} with a consequent increased binding by the lipid and with the attending changes in charge and structure.

The effect of $CaCl_2$ on the dispersion of phosphatidic acid may be explained in terms of a strong ionic attraction between the divalent cation and negatively charged phosphate sites. The rapid decrease in charge that occurs as Ca^{+2} is bound permits particles to approach each other. Now coordinate bonds can be formed between the Ca bound to one surface and donor oxygen atoms of an adjoining surface. This bond is established without release of H^+ or change in charge. The coagulum is

now held together, Ca bridging adjacent surfaces, by stronger bonds than in the case of univalent cations and cannot be reversed except by more forceful methods, such as by using ion exchange resins or by bringing to low pH levels, or high concentration of univalent cations.

It is interesting to compare the stability constant obtained here for calcium binding to phosphatidic acid with that already reported based on measurements of turbidity. Although the two procedures involve measurements that are completely different, good agreement is obtained in the values for the constant.

Monolayers.

The similarities between the properties and reactions of lipids in a membrane structure and in a monomolecular film is readily perceived. A series of investigations of the force-area characteristics of monolayers of purified lipids at the water-air interface has undertaken to study the changes in structure that take place with changing pH and cation concentrations of the aqueous phase.

For those experiments, a modified Langmuir hydrophil balance was used.

Monolayer films of phosphatidylserine were obtained by depositing a hexane solution of the lipid on the substrate surface and allowing the volatile solvent to evaporate. Minimal volume ($\sim .1$ ml/400 sq. cm) of lipid solution was used to obtain uniform spreading. The lipid solution was delivered from a 0.1 milliliter micrometer feed micropipette. The measurements of one dimensional surface pressure were made to $\pm .036$ dynes/cm. Area measurements were made to $\pm .15$ sq. cm.

Sufficient time at each interval of compression was allowed so that the molecules could attain their equilibrium arrangement and a stable surface pressure.

The pH of the substrate was measured by means of a Beckman pH meter whose electrodes were immersed in the trough solution. The substrate was prepared from reagent grade chemicals and double distilled water. The final distillation of the water was done in an all glass still to prevent metal contamination. The trough was enclosed in a plastic box with facilities for external operation to prevent atmospheric carbon dioxide from reacting with the substrate solution at the higher pH values.

Extensive studies were made of monomolecular films of phosphatidylserine. The typical force-area isotherm exhibited the characteristics of an expanded film with the increase of pressure beginning at a film area corresponding to a molecular area of approximately 100 \AA^2 . The minimum area for the condensed film was from 53 \AA^2 to 44 \AA^2 and the collapse pressure was from 36 dynes/cm to 46 dynes/cm.

Changing the pH of the aqueous medium produced pronounced effects on the molecular areas of the film. A comparison of the areas under different conditions was made possible by determining the molecular area of the lipid in the condensed film at zero pressure. This was obtained by drawing the tangent to the curve in the condensed region and extrapolating to zero pressure.

Films deposited on doubly distilled water showed a minimum in area at pH 5. The molecular area then being 60 \AA^2 . This area increases to 74 \AA^2 at pH 10.2 and to 72 \AA^2 at pH 1.75. In 0.001 M CaCl_2 the minimum area was at pH 5, but was somewhat greater (67 \AA^2) than in water. In CaCl_2 , the area also increased with increasing and decreasing pH.

Striking differences were detected in aqueous systems containing 0.1 M molar NaCl, KCl, or tetramethylammonium chloride. In these salt solutions, minimum area appeared at pH 6. The area, however, was greater than in the absence of salt. In all systems containing the univalent metal chloride, the molecular area rose steeply from pH 6 to pH 9.5, reaching values of 94 \AA^2 per molecule. The increase in area with decreased pH was less, reaching 78 \AA^2 at pH 1.2.

An increase in area at pH levels above 5 or 6 can be attributed to the expansion of the phosphatidylserine molecule as the linkage between the phosphate and amine groups is disrupted by the removal of a proton. In the presence of univalent cations, the exchange of a cation for the proton facilitates this release with the resulting increase in the negative charge of the molecule and its expansion. In a contrary fashion, the action of calcium reduces this effect presumably by binding together phosphate groups to bring molecules closer together.

Study of the properties of a model lipid membrane.

We are now studying a model system developed by Mueller, et al.¹ By using the technique described by these investigators, we are able to make in vitro from brain phospholipids a membrane of approximately 60 \AA in thickness, which in all probability is a bimolecular phospholipid structure of similar form to that generally attributed to the plasma membrane of cells. We are principally concerned, at present, with the electrical properties of these phospholipid membranes which are as follows: the membranes have a resistance of approximately $10^7 \Omega/\text{cm}^2$ and a capacitance of $0.5 \mu\text{F}/\text{cm}^2$. The system behaves fairly linearly up to potentials of about 150 mv. , at which point the membrane usually breaks. The capacitance of this membrane thus resembles that of the plasma membrane, but the resistance is much higher. This rupture may be interpreted as dielectric breakdown.

The addition of various substances such as proteins, detergents, etc. to the solutions on either side of the membrane produces modifications

of this basic electrical response. Of particular interest is the effect produced by an as yet unidentified material named by Mueller et al. EIM (excitatory inducing material). When this substance is added to one side of the membrane, the resistance decreases from $10^7 \Omega/\text{cm}^2$ to $10^4 \Omega/\text{cm}^2$ or less, depending on the concentration used. Thus, the resistance resembles that of the plasma membrane. Of more importance, however, is the fact that in this state the system shows electrical excitability. In the face of a constant current which brings the potential across the membrane initially to about 15 mv., the resistance regeneratively rises by five-fold or more, so that the potential goes from 15 mv. to 75 mv. or greater. The system remains in this state until the current is removed, at which time the potential returns to 0. This response is indefinitely repeatable on a given membrane. If now, in addition, protamine is also added to the solution, the membrane potential will rise and fall during the passage of an appropriate current to give what phenomenologically appears to be an "action potential".

We are attempting to purify and characterize (as described below) this EIM, in the hope that once learning the nature of the molecule that produces these profound changes in membrane response, we shall be able to discuss intelligently the physical mechanisms responsible for these events.

In each step of purification of excitatory inducing material (EIM), the activity of the material being studied is assayed by its effect on the resistance of the model lipid membrane, that is, a combination of lowered membrane resistance and the regenerative resistance change in response to an imposed current. A unit of activity is taken as the minimum weight of material added to our standard 4 ml membrane chamber that produces a fall of resistance to $10^4 \Omega/\text{cm}^2$.

We have been able to produce crude EIM according to the method of Mueller¹. This involves the incubation of egg white with a culture of aerobacter cloacae ATTC 961, grown in thioglycolate medium. After incubation at 37° overnight, the culture is adjusted to pH 5.5 and centrifuged, the supernatant readjusted to pH 7 and lyophilized. Using this method, we have obtained consistently material whose activity is one unit per 22 to 33 μ grams dry weight.

Experiments were performed in order to characterize EIM. The following data were obtained:

Exhaustive dialysis of crude EIM against distilled H₂O, in 4° C. cold room did not result in any decrease in activity of the retentate and the activity was not removed by filtering the retentate. Heating a solution of crude EIM to 74° C. for 10 minutes inactivated it. A solution of crude EIM is stable in pH range 3.5 to 11, at least for 1 hour; pH's below 3.5 cause rapid permanent loss of activity. Vigorous agitation of a crude EIM solution for 2 hours at 40 C. causes inactivation. EIM is destroyed by proteolytic enzymes.

For the above reasons, (non-dialyzability, solubility in aqueous solvents, heat lability, pH lability, susceptibility to surface in-activation), we are reasonably confident that EIM activity resides in a protein.

Attempts were then made to isolate the active material. Ammonium sulfate fractionation yielded in the 30 to 60 per cent saturation fraction, material with a specific activity 4 times that of the crude material. The recovery of total activity was about 90 per cent.

Fractionation of crude EIM on DEAE cellulose was carried out, the complete separation of egg white on this exchanger having been described by Mandeles⁴⁴. The protein concentration of each fraction was measured by use of the ultraviolet spectrophotometer at a wave length of 280. The first major protein peak is lysozyme, the second conalbumin, and the third ovalbumin as determined by their visible and ultraviolet absorption spectra (using a Perkin-Elmer recording spectrophotometer). This follows closely the chromatogram of egg white as reported by Mandeles. EIM activity was not detected in these fractions, however, but was found in subsequent fractions eluted after the pH of the developing glycine-phosphate-salt buffer was decreased below 6.0. The protein concentration of the eluate was low. In one tube with only 15 μ grams/ml of protein, the activity was equal to that of a 1500 μ grams/ml crude solution. Thus, a hundred-fold purification was achieved.

Recovery of activity was about 15 per cent. This relatively low recovery may be due to its undetected presence in dilute concentration in other tubes, its incomplete elution from column or its inactivation (denaturation) during chromatography. Since the elution of activity was between pH 5.7 and 5.2 and ionic strength 0.16 and 0.20, it is probable that its isoelectric point at this ionic strength is in this range. A more direct determination of iso-ionic point will be carried out using electrophoresis on acrylamide gel.

The separation of DEAE was repeated with a much larger column and protein load using a stepwise elution technique confirming the previous results. The elution of activity was again between pH 5.8 and 5.1 and ionic strength 0.17 and 0.21. The over-all purification was somewhat less (as expected with stepwise elution), and the percentage recovery about the same.

Experiments were performed to determine the factors necessary for production of EIM, both for understanding the mechanism and as an aid in the separation, for if a simpler system could be used as substrate, the separation should be easier. We have carried one strain of aerobacter over ten bimonthly passages on protein free media without any decrease in its ability to produce EIM when inoculated into the proper media. It

was found that EIM is present in small amounts in the filtrate of a 24 hour growth of aerobacter cloacea 961 in thioglycolate medium. This would indicate that it is a bacterial product rather than an altered egg protein. Since there was no measurable nucleic acids in the filtrate and the concentration did not increase on prolonged incubation, EIM is probably an extracellular protein.

Bacteria were grown in individual semi-pure commercial fractions of egg white dissolved in egg white dialysate in the same concentration as found in whole egg white. Preliminary data reveal that the culture filtrates from heat denatured egg white proteins are inactive, and that the production of EIM is dependent on the concentration of protein in the medium. The filtrate of conalbumin (ovotransferrin) had the highest activity (about one-half that of whole egg white on an absolute basis, but five times the specific activity). Globulin, ovalbumin, lysozyme followed in decreasing order of activity, and ovomucoid (ovotrypsin inhibitor) was totally inactive. The bacteria were also grown on other proteins dissolved in egg white dialysate. The filtrate from bovine serum albumin media was much less active than whole egg white or the egg white proteins. Bovine gamma globulin and rabbit transferrin were totally inactive.

One byproduct of our experiments on the mechanism of production of EIM has been the high yield on conalbumin, whose iso-ionic point is relatively distant from the apparent isoelectric point of EIM (as determined by DEAE chromatography). This should allow a simple one step separation of the greatest bulk of contaminating protein from the active material, either on DEAE or carboxymethyl (CM) cellulose. However, it has been found that the EIM loses stability and is more quickly inactivated as it increases. This has led to difficulty in achieving our objective of isolation of EIM. One property of EIM that may materially help in establishing conditions of increased stability is the strong tendency of EIM to absorb to lipids, not only the lipid of the membrane but also to lipid micelles. The protective effect of such micelles upon EIM is now being investigated.

EXPERIMENTAL METHODOLOGY

Preparation of lipids.

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 Maltaner⁴⁵, followed by silicic acid column fractionation, and explained in detail elsewhere⁴⁶. From 60 to 80 μ g 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 Na₂CO₃ and developed with a solution of CHCl₃-CH₃OH-H₂O, 70:30:4 by volume, and both preparations ran as single spots. The lipid spots were identified by staining with 7,2-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 described⁴⁷ 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. In Preparation II, there was less than 1% plasmalogen and lipid galactose.

Preparation of HPS, NaPS, and Na₂PS — HPS was prepared by washing the extracted lipid with 0.1 N HCl and dialyzing it for 24 hours against distilled H₂O. NaPS was prepared according to the method of Folch, Lees, and Sloane-Stanley⁴⁸ by dissolving the extracted lipid in CH₂Cl-CH₃OH-H₂O, 86:14:1 by volume ("lower phase"), and washing with CHCl₃-CH₃OH-H₂O, 3:48:47 by volume, containing 0.14% NaCl. Na₂PS was prepared by washing the lipid in the "lower phase" with the NaCl of the "upper phase"⁴⁸ 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 divalent cations, and Cl by the method of Lowry et al.⁴⁹. The content of Ca plus Mg and Cl was negligible. The monovalent cations present are shown in Table II.

TABLE II

	Na	K
	meq/mole P	
HPS	0.10	0.02
NaPS	0.95	0.15
Na ₂ PS	1.97	0.01

Sonication of PS — An aliquot of the solution containing 15 to 60 mg of PS in chloroform was transferred to a 20-ml tube used for sonication. The chloroform was removed by evaporation for 24 hours in vacuum at room temperature, 5 ml of freshly boiled, double distilled, or deionized water were added, and a dispersion was formed by sonication in an ice bath for 5 minutes at 10 kc with a ultrasonic generator (Measurement and Scientific Equipment, Ltd., London, England).

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 μ . The suspensions remained stable for several weeks when stored at 4 $^{\circ}$ C. When sonicated in dilute acid or in salt solutions, the suspensions were turbid and in some instances coagulated.

PA preparation — Phosphatidic acid was prepared by enzymatic degradation of egg lecithin (Sylvania)⁵⁰. The enzyme used was obtained by homogenization of 100 g of Savoy cabbage in 150 ml of water⁵¹. Coarse solids were removed by pressing through silk and then centrifuging at 13,000 X g for 3 minutes under refrigeration. The supernatant liquid (30 ml) was combined with lecithin (400 mg), sodium acetate buffer (25 ml), pH 5.6, 1 M CaCl₂ (5 ml), ether (30 ml), and chloroform (1 ml) and shaken for 2 hours at room temperature. Phosphatidic acid was removed by extracting this mixture with 100 ml of ether. The extraction was repeated. The ether was evaporated with gentle warming in a nitrogen stream, and the material was redissolved in 5 ml of chloroform-methanol, 2:1, and partitioned with 1 ml of 0.04% CaCl₂ to remove water-soluble substances introduced by the crude enzyme. The residual lecithin and a chromogen still present were separated from the phosphatidic acid on a silicic acid (Unisil) column, 200 to 325 mesh, the phosphatidic acid being eluted by chloroform-methanol, 65:35 (v/v).

Dialysis of PA — The enzymatic preparation of phosphatidic acid with phospholipase D requires the presence of excess Ca⁺⁺, which remains as calcium phosphatide in the final product despite the numerous washings. To remove residual calcium, phosphatidic acid was dried, suspended in 0.1 N HCl (200 mg of lipid in 5 ml), transferred to a cellophane bag, and dialyzed against 0.005 M HCl for 24 hours. The contents were centrifuged at 13,000 X g, and the precipitate was redissolved in chloroform-methanol, 2:1, and partitioned with 0.2 volume of water. The lower phase lipid was then in the acid form suitable for dispersion.

Chemical analysis — The methods of chemical analysis were similar to those above. Measurements were carried out on three preparations of phosphatidic acid. They all ran as single spots both on ascending silicic acid paper chromatograms developed with isobutyl ketone-glacial acetic acid-water, 40:25:5 (v/v/v)⁵², stained with rhodamine G, and, on thin layer chromatograms with a basic plate prepared from a silica gel G in a slurry within 0.01 M Na₂CO₃, developed with a solution of chloroform-methanol-water, 70:30:4. The spots were identified by charring with sulfuric acid. The preparations contained 3.9% phosphorus and less than 0.04% nitrogen as determined by Nessler's reaction and had a carboxylic ester to phosphorus⁵³ molar ratio of 1.95. The sodium and potassium content, determined by internal standard flame photometry on nitric acid digests and calcium by EDTA titration at pH 13 with calcein as an indicator⁵⁴ are reported in Table I.

Lipid chromatography was done on thin layers of silica gel G with chloroform-methanol-water, 70:30:4 by volume and chloroform-methanol-concentrated ammonium hydroxide, 14:6:1 by volume⁵⁵ as developing solvents. Spots were detected by iodine vapor or by spraying with 50% H₂SO₄ followed by heating.

Infrared spectra were recorded with a Perkin-Elmer model 237 grating spectrophotometer. Samples were examined in both CCl₄ and C₂Cl₄ solutions in 1-mm cells at solute concentrations of 5 to 8 mg per ml, and as Nujol mulls. Infrared analyses gave the same spectrum for all three preparations in agreement with published spectra³⁵.

Sonic treatment.— Dispersions of phosphatidic acid were prepared by vacuum evaporation of chloroform solutions of the lipid in a 20-ml glass tube. This left a layer of solid deposited on the base of the tube. Freshly boiled deionized water (5 ml; conductivity, 1 μmho per cm) was added, and sonic treatment was carried out at 10 kc for 5- to 7-minute periods with an ultrasonic generator (Measurement and Scientific Equipment, Ltd., London). The system was cooled by a bath of crushed ice. To obtain complete dispersion of all solid from the deposit, it was necessary to use a rod, the diameter of which was close to that of the tube.

Several preparations of phosphatidic acid showed atypical characteristics. These required longer periods of treatment to produce reasonably clear dispersions, ones which also were at higher pH levels (approximately 4.9) than the other dispersions. They showed a reduced buffering capacity, approximately 0.3 of that shown by the "normal" preparations. When some of these preparations were redialyzed against dilute acid, they produced stable dispersions at a pH of 3.10 and titrated in a normal manner. These preparations were now free of calcium.

Mono-octadecyl dihydrogen phosphate and dioctadecyl hydrogen phosphate -- These compounds and their monosodium salts were prepared and purified by the procedures of Brown, Malkin, and Maliphant⁵⁶. Their properties were as shown in Table 12.

Phosphatidylethanolamine -- Synthetic L-α- dipalmitoylphosphatidylethanolamine was obtained from Calbiochem. The purest of several preparations was selected. It migrated as a single spot in the thin layer systems. Chemical analysis was 4.39% phosphorus; mole ratio of nitrogen to phosphorus, 0.98; mole ratio of ester to phosphorus, 2.05. The cation content was 0.145 eq of sodium per mole of phosphorus, 0.003 mole of calcium per mole of phosphorus, and no potassium.

Lecithin -- Synthetic L-α- dipalmitoylphosphatidylcholine was also selected from several lot numbers obtained from Calbiochem. It migrated as a single spot in the thin layer systems. Chemical analysis showed 3.55% phosphorus; mole ratio of phosphorus to nitrogen, 1.07; mole ratio of ester to nitrogen, 2.00.

Sphingomyelin — The starting material for the preparation of this lipid was a lipid fraction from beef brain gray matter. The cholesterol and cerebrosides had been removed by fractionation on a silicic acid column. A solution of 2.5 g of this fraction, containing phospholipids and sulfatides, was prepared in 190 ml of chloroform-methanol (2:1), 8 ml of 1% HgCl_2 , and 2 ml of glacial acetic acid. After heating for two hours at 37° to hydrolyze enol ethers, 40 ml of 1% disodium ethylenediaminetetra acetic acid was added, and the solutions mixed. The upper phase was discarded, and the lower phase washed once with "upper phase"⁵⁷, equal in volume to that removed. The lower phase was evaporated to dryness under vacuum, dissolved in 180 ml of methanol and 20 ml of 1 N NaOH. This solution was heated for 6 hours at 37° to hydrolyze esters, then diluted with 360 ml of chloroform, and 88 ml of H_2O . The upper phase, containing fatty acid salts and glyceryl phosphoryl bases, was discarded. The lower phase was washed once with "upper phase", and evaporated to dryness to give 503 mg of a mixture of fatty aldehydes, fatty acids, sulfatides, and sphingomyelin. This total mixture was fractionated on a column of 50 g of Unisil silicic acid (Clarkson Chemical Company). Eight fractions of 200 ml each were collected. The fatty aldehydes and fatty acids were eluted with chloroform and chloroform-methanol (8:2). Sulfatides were eluted with chloroform-methanol (7:3). After removing some minor unidentified components with chloroform-methanol (6:4, 5:5, and 4:6), a very pure sphingomyelin fraction (163 mg) was eluted with chloroform-methanol (3:7 and 2:8). This material was completely homogeneous by thin layer chromatography without further purification. Chemical analysis yielded 4.03% phosphorus; 3.76% nitrogen; mole ratio of nitrogen to phosphorus, 1.95; ester and galactose were not detectable. This method is given in some detail, because it is a rapid and simple way of preparing all of the sphingolipids (if total lipids are used at the start) in one procedure with high purity. Gray matter lipids were used in this particular case, but, of course, white matter lipids are a richer source of sphingolipids.

Physico-chemical measurements.

To study the reaction of phosphatidylserine 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 $24 - 25^\circ$.

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 solid 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. It was immediately titrated down-scale with 0.0500 M or 0.100 M HCl to pH 2.75 to 3.25, the metallic cation concentration being unchanged during this titration. 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.

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 μ 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.

Absorption spectra of HPS, NaPS, and Na₂PS were obtained by means of a Perkin-Elmer model 237 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 tetrachlorethylene were obtained. The range from 4000 cm⁻¹ (2.5 μ) to 625 cm⁻¹ (16.0 μ) was studied in all cases.

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.

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 N 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 8.6, 0.1 N 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

$$\mu = dKA/l Rmt$$

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°.

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.

Dispersions of 9 to 14 mg of phosphatidic acid in 5 ml of deionized water were titrated at 24-25° in a 20-ml tube with a Radiometer apparatus; automatic and also manual readings of the pH were taken by periodically interrupting the titration to verify the pH readings. Glass and calomel electrodes, as well as tubes for delivering reagent and CO₂-free nitrogen, passed through a plastic cap which fitted into the glass titration tube. A rapid stream of N₂ through the system provided stirring and a CO₂-free atmosphere. For many of the experiments, the system was first titrated with either 0.0500 N KOH or NaOH to pH 10.2 to 10.5 and then titrated to pH 3.2 to 2.9 with 0.0500 N HCl. When the effects of added salt were to be studied, the desired salt concentration was established by adding the requisite volume of a concentrated solution or a weighed amount of solid so as not to alter the phosphatidic acid concentration. The titration cycle was then repeated.

Titration of blanks in water or salt solutions were performed under identical conditions, and the volumes of reagent used for the blank at a given pH level was subtracted from the volumes for the lipid titrations to give corrected titration curves. The magnitude of these corrections were from 0 to 10% in the pH range 5 to 9, and 20% at the extremes of the titrations.

Small additions of NaCl, KCl, or CaCl₂ were made to the suspensions of phosphatidic acid under nitrogen, and the resulting pH was measured. For concentrations of salt greater than 0.1 M, weighed amounts of the solid were added. In another series of experiments, the suspension was brought to a desired pH by the addition of base; the addition of salt then produced

a drop in pH. The pH level was re-established by the addition of base. In all cases, the initial drop in pH occurred rapidly, and a stable value was reached in 5 to 10 minutes.

The stability of dispersions of phosphatidic acid in media of different pH and salt concentrations was measured by first establishing the desired pH and then adding measured amounts of appropriate solutions of the salt. For higher concentrations of NaCl or KCl, weighed amounts of the solid were added. After each addition, the mixture was stirred by a stream of nitrogen, and the pH was adjusted to the original level. Increase in particle size was followed by measuring the optical density at 546 m μ with a Beckman B spectrophotometer.

Measurements of viscosity were made of dispersions at varying pH, salt concentration, lipid concentration, and temperature. A 2-ml Ostwald viscosimeter was used. The viscosity of the medium was used as a standard. Measurements were made at 7.8 $^{\circ}$ and at 25.0 $^{\circ} \pm 0.2^{\circ}$.

Conductance measurements were made of the dispersions of phosphatidic acid over the concentration range from 7.8×10^{-6} mg per ml to 2 mg per ml. The conductance was measured at 25.0 $^{\circ}$ by use of a dipping electrode and a 1000-cycle signal balanced by an impedance bridge (Electro-Measurements, Inc.). After each change of concentration, the system was stirred with nitrogen until stable values were obtained.

Structure of micelles and comparison of lipids.

The sonication of phosphatidylserine 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.⁵⁸

The average molecular weight was calculated from light scattering. For five dilutions of the same NaPS dispersion, light intensities at 0 $^{\circ}$ and 90 $^{\circ}$ gave the turbidity, τ , which increased linearly with concentration (Fig. 17). The change in refractive index with concentration (dn/dc) measurements permitted the calculation of the Hc/τ ratio; this parameter, plotted against c and extrapolated to 0 concentration, gives $1/M$ as an intercept.

The average molecular weight, 4×10^6 , is in the same range as that reported by Saunders, et al.³ 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 \AA^2 ⁵⁹, and postulating a micellar structure consisting of 2 disk-shaped lamellae

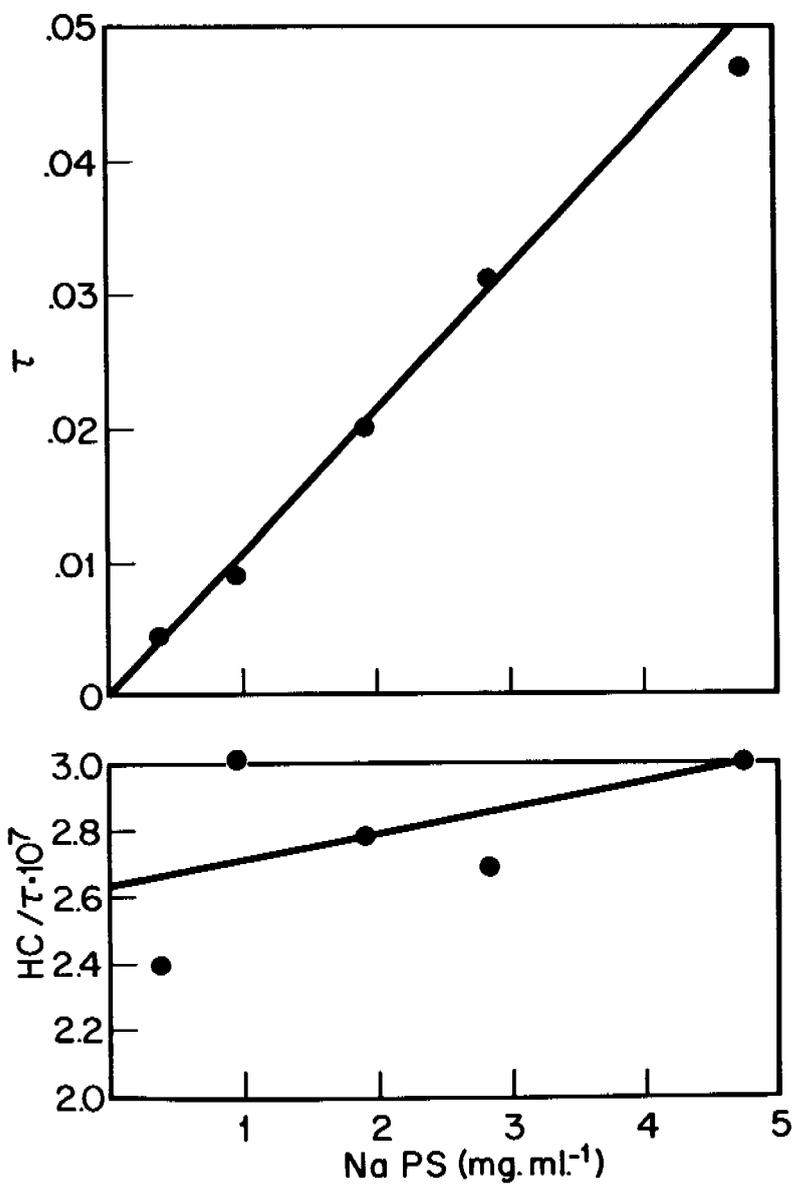


Fig. 17. Light scattering of ultrasonicated dispersions at different concentrations. Upper graph shows (turbidity) as a function of milligrams per ml of NaPS. In lower graphs, the extrapolation of $HC/\tau \cdot 10^7$ to 0 concentration gives the reciprocal of the molecular weight.

(each containing on average 2400 molecules), it is possible to calculate an area of $12 \times 10^4 \text{ \AA}^2$ and a diameter of 400 \AA 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^{60, 12}.

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.

Studies of the properties of phosphatidic acid micelles form an interesting comparison with previous studies of micelles of the acidic lipid phosphatidylserine⁶. A striking similarity is the orientation of the molecules in the micelles. In the case of phosphatidylserine, the molecular weight of the micelles was found to be of the order of 4×10^6 . Hence, they contain between 5000 and 6000 molecules, and the degree of orientation required thereby is indeed striking.

The effects of Ca^{++} are quite different; with phosphatidylserine micelles, titration curves over the entire range of α values can be obtained in the presence of CaCl_2 , whereas this is not possible with phosphatidic acid. The inability to titrate phosphatidic acid in the presence of Ca^{++} reflects an internal arrangement of the micelles which makes calcium hydrogen phosphatide molecules unavailable, as well as a strong binding of the Ca^{++} to the phosphate groups.

Preliminary electron micrographs have been obtained for the calcium precipitates of phosphatidic acid. These show stacks of bimolecular disks of about the size predicted. It is assumed that in the absence of calcium, the individual micelle consists of a single disk, but we have been as yet unable to obtain electron micrographs of the sonicated micelles.

A plot of the molar conductance (expressing concentrations in moles of phosphatidic acid per liter) versus the square root of the concentration showed a sharp drop in the molar conductance, similar to that found for weak or polymeric electrolytes. As expected, no sharp break in the curve indicative of a critical micelle concentration was found. The observed change in conductance may be ascribed to changes in the dissociation of the bound sodium ions.

Titration curves of a comparable molecule such as glycerophosphoric acid show no colloid or polyelectrolyte effect, the inflections

of the titration curve being well defined with slopes of unity. Phosphatidylserine shows a very marked polyelectrolyte effect with only minimal inflections revealed at the equivalence points. Phosphatidic acid is intermediate in this regard; hence, the electrostatic interaction of the charged groups in phosphatidic acid must be less than those of phosphatidylserine.

The pK's of the ionized phosphate group in phosphatidic acid are considerably different from that of phosphatidylserine. With phosphatidylserine, the additional esterification of the phosphate and the presence of basic amino group gives a pK₁ for the phosphate group below an isoelectric point of pH 1.2. In contrast, the pK₁ of phosphatidic acid is 3.4. This effect of esterification of phosphate group is well known⁶¹. Another effect of the amino acid esterified to the phosphate group in phosphatidylserine is to provide a dipole structure which causes the micelle to unravel and change its shape at high pH levels as manifested in viscosity studies⁶. This change in viscosity at high pH is not observed with phosphatidic acid.

The effects of specific ions on these two molecules are similar for Na⁺ and K⁺, in that some difference in the ability of these two ions to coagulate the micelles is observed; there is but a slight difference in their effects in ion exchange and titration curves. With both molecules, Ca⁺⁺ has a very much more marked effect than Na⁺ or K⁺. In the case of phosphatidylserine this was of the order of 200:1 on a molar basis. However, with phosphatidic acid, the additional effect of the firmly bound calcium was to make that part of the acid nontitrable at available pH levels. Related to these effects are the observations that phosphatidic acid is often obtained as a calcium salt from tissue pulp, whereas phosphatidylserine is usually obtained as a sodium potassium salt⁴⁸. Furthermore, it was found necessary to use extensive acid dialysis to remove the calcium completely from the phosphatidic acid.

The titration data do give quantitative information concerning the amount of sodium and potassium associated with the molecule at different pH values. It can be seen from both the data of Table 1 and Fig. 4 that the association of Na⁺ and K⁺ is about 1.2 equivalents of cation per phosphate group at pH 7.4. This, plus the additional factor of very strong preference of phosphatidic acid for Ca⁺⁺ suggests that, indeed, phosphatidic acid would be a poor molecule to act as a carrier in the earlier scheme of Hokin and Hokin⁶². Their scheme required at least 2, preferably 3, sodium atoms attached to the phosphatidic acid molecule in order to account for the known efficiency of the system in terms of sodium atoms carried per phosphate group involved⁶³. The enzymatic data presented by these two workers indicating the markedly increased turnover of phosphatidic acid in situations in which sodium transport is

stimulated may, perhaps, be interpreted as an indication that this molecule is an intermediate either in the formation of another, more efficient but more labile component of the transport system or as a transducer producing a conformational change in a carrier protein as these authors now postulate.⁶⁴

Micelles were produced by adding distilled water to the solid lipid which was deposited on the lower surface of a 20 ml tube by vacuum evaporation of the CCl_4 solvent and then exposed to 10 kc. ultrasonic radiation for 5 minutes. The system was kept cold by a crushed ice bath. The dispersions, which had a pH of 3.2 to 3.5, were diluted with doubly distilled water (conductivity 1 micro mho cm^{-1}) to a lipid concentration from 2.0 to 2.75×10^{-4} M, about 0.02%. All suspensions and solutions were first clarified by ultrafiltration through a 0.80 μ millipore filter.

On standing the dispersions of phosphatidic acid showed a progressive increase in turbidity and were not used after 48 hours. For a series of experiments, four or five 9 ml aliquots of each diluted dispersion were employed.

Each system was buffered with tris at pH 7.2 or 8.8 by adding 1 ml of buffer, bringing the total volume to 10 ml with a buffer concentration of 0.05 M. One series of experiments at pH 9.6 was made using ammonium chloride buffer. To obtain the desired salt concentration, either the requisite volumes of 1 M or 3 M solutions of chlorides of univalent cations or 0.01 M CaCl_2 or MgCl_2 were added. Small additions of these salt solutions were made with a 0.1 ml Hamilton Syringe. Temperatures were at $24^\circ \pm 1^\circ \text{C}$.

Light scattering was measured by a Brice Phoenix photometer using both 438 $\text{m}\mu$ and 546 $\text{m}\mu$ light. A 30 ml semioctagonal cell was used to measure light intensities at 0° , 90° , 45° , and 135° . Readings were taken with a Keithley electrometer, which was found to have numerous advantages over a galvanometer. Although changes in turbidity took place rapidly after the addition of the salt solution, readings were taken after a 10-minute interval. The turbidity increased on standing only when it was high and heavy coagulation had begun.

The readings obtained with the suspension did not need to be corrected, since the scattering by the solvent and the solutions used was extremely small.

CONCLUSION

Studies of the ionic properties of ultrasonicated micelles of phosphatidic acid and phosphatidylserine have been carried out. These studies include titration curves, ion exchange properties, electrophoresis, viscosity, light scattering, coagulation by salts and infrared spectra. Publications resulting from these studies are the following:

Abramson, M.B., Katzman, R. and Gregor, H.P.: Aqueous Dispersions of Phosphatidylserine. Ionic Properties. *J. Biol. Chem.*, 239:70, 1964.

Abramson, M.B., Norton, W.T. and Katzman, R.: Study of Ionic Structures in Phospholipids by Infrared Spectra. *J. Biol. Chem.*, 240:2389, 1965.

Abramson, M.B., Katzman, R., Wilson, C.E. and Gregor, H.P.: Ionic Properties of Aqueous Dispersions of Phosphatidic Acid. *J. Biol. Chem.*, 239:4066, 1964.

Abramson, M.B., Katzman, R. and Curci, R.: Turbidimetric Studies of the Interaction of Aqueous Micelles of Phosphatidic Acid with Cations. *J. Coll. Sci.*, 20:777, 1965.

It has been found possible to study the physical chemistry of complex acidic lipids by use of ultrasonicated dispersions. These complex acidic lipids are components of cell membranes, and have important functions in regard to the movement of ions across these membranes. Consequently, it has been felt useful to systematically study the ionic properties of these lipid dispersions.

Phosphatidylserine - Dispersions of the monosodium form in distilled water consisted of micelles with an average molecular weight of 4×10^6 . 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_2O 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.

Phosphatidic acid -- Stable aqueous dispersions of phosphatidic acid were prepared by ultrasonic treatment. When freed from Ca^{++} these dispersions were at pH 3.05 to 3.29. The titration curves showed well defined inflection points with a pK_1 of 3.8 and a pK_2 of 8.6 in water and a pK_1 of 3.0 and a pK_2 of 8.0 in 0.1 N NaCl or KCl .

The stoichiometry of the titrations indicate that all of the acid groups in these micelles are oriented so as to be available to the aqueous medium. Sodium and potassium salts of phosphatidic acid were prepared, and dispersions of these gave pH values consistent with their cation content and titration curves. At pH 7.4, the micelles contain 1.2 μeq of sodium or potassium per μmole of phosphorus.

The presence of Ca^{++} either in the phosphatidic acid preparation or in the medium reduced the number of groups that could be titrated, indicating both stronger binding of this cation than of Na^+ or K^+ and probable rearrangement of the micelle.

Additions of neutral salt solutions to dispersions of this lipid lowered the pH of the system. The effect of CaCl_2 , which was approximately 500 times that of KCl or NaCl on a molar basis, was such that the micro-equivalents of H^+ released approached the micromoles of Ca^{++} added. Small additions of CaCl_2 at pH 5 produced an increase in optical density of the dispersions. Similar effects required much larger concentrations of NaCl , while the action of KCl was minimal at this pH and was extremely slow at pH 7.

Phosphatidic acid groups present in micellar dispersions of lecithin and phosphatidic acid were titrable, indicating that all of the phosphatidic acid groups were on the surface of the micelles.

Infrared studies -- The infrared spectra of octadecyl dihydrogen phosphate, dioctadecyl hydrogen phosphate, and phosphatidic acid are compared with those of their sodium salts. The regions showing significant changes in absorption are at 3430 to 3330 cm^{-1} , 2780 to 2630 cm^{-1} , 1230 cm^{-1} , 1100 cm^{-1} , and 1070 to 1030 cm^{-1} . The increased absorption at 3430 to 3330 cm^{-1} upon salt formation is attributed to the OH of water bound to the ionized species and accompanied by a corresponding decrease in absorption at 2780 to 2630 cm^{-1} ; this resulting from the decrease in intermolecular associations of the P--OH groups. The major region of absorption of the phosphate occurs at 1100 to 950 cm^{-1} . Here, the maximum absorption of the P--OH in the unionized form is at 1030 cm^{-1} , while the PO^- group in the salt forms give a strong absorption at 1100 cm^{-1} . The P=O group is seen to absorb at 1250 to 1220 cm^{-1} .

The spectra of lecithin and sphingomyelin are interpreted as showing a fully ionized phosphate group bonded to water with no association of P—OH groups. The major absorption band at 1100 cm^{-1} is indicative of the PO^- group.

Phosphatidylethanolamine and phosphatidylserine in CCl_4 show bonded P—OH groups and a stronger absorption in the region of P—OH (1075 to 1050 cm^{-1}) than of the PO^- (1100 cm^{-1}). Structures for phosphatidylserine, phosphatidylethanolamine, and lecithin are proposed on the basis of the spectral interpretations. A method for the preparation of sphingolipids is described.

The interactions of buffered systems of phosphatidic acid with cations were studied by turbidimetric titrations. At pH 7.2, increased turbidity occurs in 0.2 N LiCl or 0.4 N NaCl , however, KCl and tetramethylammonium chloride produce no change up to concentrations of 0.75 N . In 10^{-5} M CaCl_2 the turbidity increases and rises steeply to $2 \times 10^{-4}\text{ M}$. Somewhat higher concentrations are necessary with MgCl_2 . Additions of univalent cations to phosphatidic acid containing Ca reduces the turbidity by exchange with Ca.

In systems containing sodium citrate, the concentrations of CaCl_2 required to produce given turbidity changes are greater than in comparable systems containing NaCl . This gives a measure of the concentration of CaCit^- formed. From the known formation constant of CaCit^- , the Ca^{+2} concentration in equilibrium with the anions of citric and phosphatidic acids is calculated. This permits the evaluation of the formation constant of CaPA . Values obtained at pH 7.2, 8.8, and 9.6 are 1.2×10^4 .

The fate of these lipids as membrane constituents is being studied in the model membrane of the Mueller-Rudin type consisting of a bimolecular leaflet of lipids between two aqueous ion solutions. The electrical properties of this membrane have been confirmed as well as the existence of a material — excitatory inducing material — capable of converting these electrical properties to those similar to that of the plasma membrane. We have demonstrated this excitatory inducing material to be a protein and are now producing it by simplified methods and have purified it 100-fold. This work which is in an early stage of development may eventually provide the basis for use of chemicals resembling these natural lipids in artificial membranes.

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