# Ionic Properties of Acidic Lipids. Phosphatidylinositol*

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ABSTRACT: Clear dispersions of 8–10 mg of ammonium phosphatidylinositol or sodium phosphatidylinositol in 5 ml of water obtained by gentle sonic radiation were titrated in the pH range of 2.5–10.0. The initial pH was 6.1–6.5. The p$K'$ of phosphatidylinositol, which was 3.12 in the absence of electrolyte, was lowered to 2.50 in 0.08 M NaCl. The interaction of phosphatidylinositol with metallic cations was studied by three methods. (1) Hydrogen ion release: Dispersions of phosphatidylinositol were adjusted to pH 3.5 and increments of electrolyte were added. The quantity of $H^+$ released (measured by the quantity of tetramethylammonium hydroxide required to maintain pH 3.5) followed the order $Ca^{2+} > Mg^{2+} \gg K^+ > Na^+$. (2) Turbidity: The turbidity of dispersions was measured by the scattering ratio $I_{90°}/I_{0°}$. The effectiveness of the cations in increasing turbidity was $Ca^{2+} > Mg^{2+} \gg K^+ > Na^+ > Li^+ >$ choline chloride. (3) Coagulation: Dispersions of phosphatidylinositol were coagulated by the addition of salts. Analysis of cations in the coagula provided a measure of cation selectivity. The molar ratio of divalent cation to phosphatidylinositol of 0.5 indicated bridging of two phosphatidylinositol molecules by each $Ca^{2+}$ or $Mg^{2+}$. The selectivity ratios were $Ca^{2+}:Mg^{2+} \simeq 2.4$ and $K^+:Na^+ \simeq 1.2$. An apparent constant for the association of sodium phosphatidylinositol was obtained from data on cation–proton exchange at pH 3.5, $K_{Na}' = 6.9$. The viscosity of phosphatidylinositol dispersions in water was much greater than for other acidic lipids; however, a low concentration (2.5 mM) of NaCl produced a sharp decrease.

In our studies of the ionic properties of complex lipids, we have examined lipids with different acidic groups including phospholipids (Abramson *et al.*, 1964a,b) with two acid groups (phosphatidylserine and phosphatidic acid) and sulfatide (Abramson *et al.*, 1967) with a single acid group. In a continuation of these studies, we describe here the ionic properties of phosphatidylinositol, a phospholipid with one acid group.

Acidic lipids such as phosphatidylinositol may play a role in biologic membrane organization and function through the binding of proteins and cations. The participation of phosphatidylinositol in cation transport is indicated by studies of its turnover during glandular secretion (Hokin and Hokin, 1964) and during stimulation of nerve tissue (Hokin *et al.*, 1960; Larrabee *et al.*, 1963). Its involvement in mitochondrial contraction (Vignais *et al.*, 1964), in the biosynthesis of diphosphoinositide and triphosphoinositide (Saunders and Ballou, 1966), and as an auxiliary lipid in the hydrolysis of phospholipids by phospholipases (Weiss *et al.*, 1959; Dawson, 1958) also emphasize the importance of this compound.

## Methods

*Phosphatidylinositol.* This lipid was prepared either as the ammonium salt (ammonium phosphatidylinositol) or the sodium salt (sodium phosphatidylinositol) according to Colacicco and Rapport (1967). A typical preparation of ammonium phosphatidylinositol had the following analysis: P, 3.68%; molar ratios: ester:P 2.00, inositol:P 0.99, $NH_4^+$:P 0.86; fatty acid (mole %): $C_{16:0}$, 54.6; $C_{18:0}$, 3.8; $C_{18:1}$, 2.1; $C_{18:2}$, 36.4; others, 2.3. Analysis for metallic cations (Na, K, Ca, and Mg) with a Perkin-Elmer atomic absorption spectrometer showed that these were absent. The phosphatidylinositol was stored in chloroform–methanol (2:1, v/v) at −10°. A molecular weight of 850 for ammonium phosphatidylinositol (based on the fatty acid distribution) corresponded to a phosphorus content of 3.65%. The sodium salt was prepared from the ammonium phosphatidylinositol as previously described (Colacicco and Rapport, 1967).

*Preparation of Dispersions.* Solutions containing 8–10 mg of phosphatidylinositol in chloroform–methanol were evaporated to dryness under vacuum in a 20-ml glass tube, and 5 ml of distilled water was added. The suspension, cooled in ice, was exposed to gentle ultrasonic radiation for 1 min using an ultrasonic generator (Measurement and Scientific Equipment, Ltd., London). The clear dispersions which resulted had an initial pH of 6.1–6.5. Thin-layer chromatograms of portions extracted with chloroform–methanol showed very little degradation as a result of the ultrasonic radiation.

*Titrations.* Dispersions containing 8–10 mg of phosphatidylinositol in 5 ml were titrated with 0.050 or 0.100 N HCl or TMAOH[1] from pH 2.5 to 10.0 using a titri-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PI, phosphatidylinositol; TMAOH, tetramethylammonium hydroxide; TMACl, tetramethylammonium chloride; $TMA^+$, tetramethylammonium ion.

TABLE I: Binding of Cations by Phosphatidylinositol Studied by Coagulation.

| | | Cation Content in Coagulum ($\mu$equiv/$\mu$mole of lipid) | | | | | Cation Concn in Supernatant (mM) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | System | $Na^+$ | $K^+$ | $Ca^{2+}$ | $Mg^{2+}$ | Total | $Na^+$ | $K^+$ | $Ca^{2+}$ | $Mg^{2+}$ |
| IA | 0.18 M KCl<br>0.18 M NaCl | 0.41 | 0.52 | | | 0.93 | 210 | 190 | | |
| IB | 0.18 M KCl<br>0.18 M NaCl | 0.40 | 0.56 | | | 0.96 | 190 | 180 | | |
| IIA | 1.5 mM $CaCl_2$<br>1.5 mM $MgCl_2$ | 0.020 | | 0.64 | 0.28 | 0.94 | 1.1 | | 1.2 | 1.4 |
| IIB | 1.5 mM $CaCl_2$<br>1.5 mM $MgCl_2$ | 0.085 | | 0.65 | 0.30 | 1.03 | 1.0 | | 1.5 | 1.4 |
| III[a] | 3 mM $CaCl_2$ | | | 0.80 | | 0.80 | | | 2.2 | |
| IV[a] | 0.08 M NaCl<br>2.9 mM $CaCl_2$ | 0.21 | | 0.65 | | 0.86 | 65 | | 3.1 | |
| V | 0.15 M NaCl<br>5 mM $CaCl_2$ | 0.30 | | 0.68 | | 0.98 | 150 | | 4.7 | |
| VI | 5 mM $MgCl_2$ | 0.08 | | | 0.92 | 1.00 | 1.1 | | | 5.0 |
| VII | 0.046 M NaCl<br>0.123 M KCl<br>2.53 mM $CaCl_2$<br>7.10 mM $MgCl_2$ | 0.09 | 0.24 | 0.27 | 0.36 | 0.96 | 52 | 110 | 2.6 | 8.5 |
| VIII[a] | 0.08 M NaCl<br>0.08 M KCl<br>4 mM $CaCl_2$ | 0.21 | 0.27 | 0.36 | | 0.84 | 85 | 79 | 4.3 | |

[a] Ammonium phosphatidylinositol was used in these systems. All the other systems contained sodium phosphatidylinositol.

graph (Radiometer, Copenhagen). In all cases, a blank solution was titrated under identical conditions, and the values were used to obtain a corrected titration curve

*Cation-Proton Exchange.* Above pH 5.5 phosphatidylinositol contains no bound protons. As the pH is lowered and the concentration of the unionized acid is increased, added cations can exchange with $H^+$. Measurements were made at pH 3.5, where the value of $\alpha$ is 0.65–0.80 (depending on the ionic strength). Lower pH values were not studied in order to avoid degradation of the lipid. Also, small changes in $H^+$ concentration are more difficult to detect as the total $H^+$ concentration is increased. The ammonium salt of phosphatidylinositol in water was first brought to pH 3.5 by careful addition of 0.1 N HCl. Additions of 3 M KCl, NaCl, or TMACl were made by use of a microsyringe, which brought about an immediate release of $H^+$ ion. The microequivalent of $H^+$ released was determined from the microequivalent of TMAOH required to maintain constant pH (3.5). The TMAOH was added by means of a 0.1-ml microburet.

*Turbidimetric Measurements.* Aqueous dispersions of phosphatidylinositol were diluted with distilled water to a concentration of 0.2 mg/ml. These dispersions and all reagent solutions were suction filtered through 0.8-$\mu$ Millipore filters. Aliquots (9 ml) of these dispersions were mixed with 1 ml of 0.5 M Tris buffer and placed in semioctagonal cells in a Brice–Phoenix Universal light-scattering photometer. Serial additions of reagents were made with a microsyringe. Light intensities at 546 m$\mu$ were measured at 0, 90, and 45, 135° scattering angles.

*Analysis of Coagula.* A dispersion containing 3–5 mg of lipid (sodium phosphatidylinositol or ammonium phosphatidylinositol as indicated in Table I) in 4 ml was coagulated by the addition of a solution of the chloride of $K^+$, $Na^+$, $Mg^{2+}$, or $Ca^{2+}$ or mixtures of these at the appropriate concentration. The system was equilibrated by allowing it to stand for 12 hr at 2°, and then for 1 hr at 24°. The coagulum was separated by centrifugation. Details of the procedure for the treatment and analysis of the supernatant solution and the coagulum have been reported (Abramson *et al.*, 1967).

## Results

*Acid–Base Titrations.* The titration with HCl of 11.2 $\mu$moles of ammonium phosphatidylinositol in 5 ml of $H_2O$ is shown in Figure 1, curve I. In this system the concentration of $NH_4^+$ was approximately 2 mM. The point of half-neutralization calculated from the equivalence point indicates an apparent p*K* (p*K*′) of 3.12. The calculation assumes that phosphatidylinositol behaves as a polyelectrolyte dispersion with all ionizable groups exposed. The line relating log $\alpha/(1-\alpha)$ to pH, where $\alpha = (PI^-)/(PI_{total})$, has a slope of 1.56, typical of the charged surface of a polyelectrolyte. The

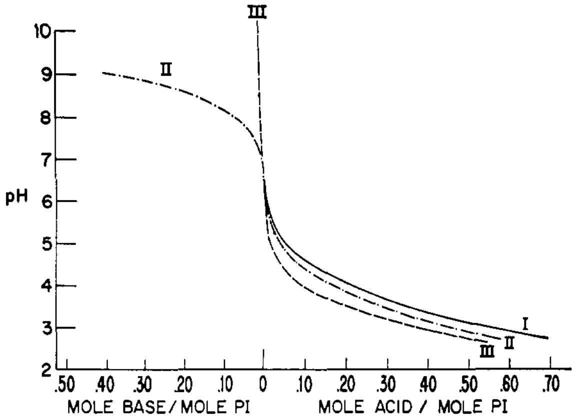

FIGURE 1: Titration of aqueous dispersions of phosphatidylinositol. (I) Ammonium phosphatidylinositol (11.2 $\mu$moles) in 5.0 ml of $H_2O$ titrated with 0.050 N HCl; (II) 11.2 $\mu$moles of ammonium phosphatidylinositol in 5.0 ml of $H_2O$ titrated with 0.050 N tetramethylammonium hydroxide; and (III) 11.8 $\mu$moles of sodium phosphatidylinositol in 5.0 ml of 3 mM NaCl titrated with 0.100 N HCl.

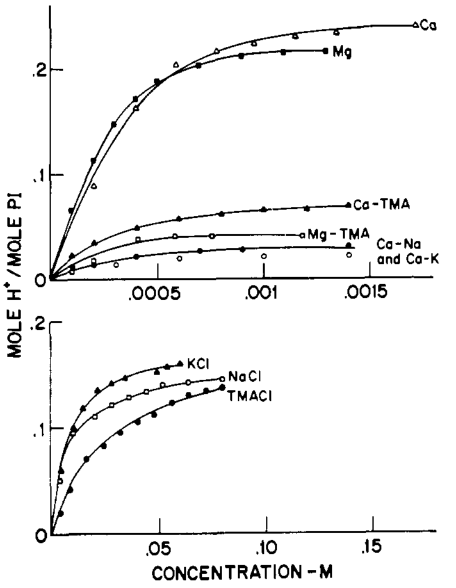

FIGURE 2: Hydrogen ion released from 10 to 11 $\mu$moles of ammonium phosphatidylinositol in 5 ml at pH 3.50. A constant pH was maintained by adding 0.050 N tetramethylammonium hydroxide after each salt increment.

titration of the ammonium phosphatidylinositol in water with tetramethylammonium hydroxide (Figure 1, curve II) gave a p$K'$ of 2.90. The effect of small concentrations of cations in lowering the p$K'$ is evident from the difference between the titrations in the presence and absence of added cation (compare curves I and II). At higher salt concentrations, this effect is not greatly increased, *e.g.*, in 0.1 M TMACl the p$K'$ for ammonium phosphatidylinositol is 2.83. In the titration of sodium phosphatidylinositol (Figure 1, curve III), the system was initially brought to pH 10.0 with NaOH to maintain constant metallic cation (3 mM $Na^+$). The p$K'$ of 2.76 calculated from this curve was slightly lower than that for ammonium phosphatidylinositol. In 0.08 M NaCl, the p$K'$ was 2.50, showing that $Na^+$ has a greater effect than $TMA^+$.

*Cation–Proton Exchange.* The greatest effect resulted from the initial additions of salt, whereas smaller changes occurred on further increases in salt concentration (Figure 2, bottom). When the concentration of univalent cation reached 0.05 M, approximately 0.15 $\mu$equiv/$\mu$mole of lipid had been released, or about half the protons bound at pH 3.5 in the absence of salt. The $H^+$ ion released reached a maximum at 0.08 M univalent cation. KCl was slightly more effective than NaCl, whereas TMACl was less effective.

The effect of $MgCl_2$ and $CaCl_2$ was much more pronounced than that of the univalent salts. In 0.001 M divalent cation, 0.22 $\mu$equiv of $H^+$/$\mu$mole of phosphatidylinositol was released, with $CaCl_2$ producing a somewhat greater effect than $MgCl_2$ (Figure 2, top). In 0.1 M TMACl, $CaCl_2$ released up to 0.07 $\mu$equiv of $H^+$/$\mu$mole lipid; for $MgCl_2$, 0.05 $\mu$equiv of $H^+$ was released. Since the ionic strength was approximately constant, these values indicate relative binding affinities for $Ca^{2+}$ and $Mg^{2+}$. Further information on the relative effectiveness of different cations was obtained by the following experiments. Adding 0.1 M TMACl to ammonium phosphatidylinositol produced a release of 0.14 $\mu$equiv of $H^+$/$\mu$mole of lipid which appeared to be the maximum obtainable by this salt. However, the addition of alkali metal ions to these systems produced a further release of $H^+$ of approximately 0.04 $\mu$equiv/$\mu$mole of lipid, showing the greater effect of the alkali cation. The addition of $CaCl_2$ to systems that contained 0.08 M univalent cation produced an additional release of $H^+$. This was greater for the system containing 0.08 M TMACl than for the comparable concentration of alkali cation. The effect of TMACl may be due primarily to increased ionic strength and also to an exchange for $H^+$ in the electrical double layer. With the alkali ions there is an additional effect due to a weak binding to the lipid anion. The divalent cations exhibit a still greater effectiveness because of their charge and probably also because of their ability to bridge two phosphatidylinositol molecules.

*Turbidimetric Titrations.* Systems containing approximately 0.5 mM ammonium phosphatidylinositol had low turbidities, and on addition of salts, very little increase in turbidity was seen until the ionic concentrations were close to those that produced flocculation. The concentrations causing flocculation were characteristic of each cation (Figures 3 and 4). Potassium ion was more effective than other univalent cations and caused coagulation at 0.34 M, whereas sodium required 0.365 M. LiCl had little effect up to 0.5 M. No increase in turbidity was observed with tetramethylammonium or choline chloride up to 0.4 M. The divalent metallic cations produced sharp increases in turbidity at much lower concentrations, $CaCl_2$ at 1 mM and $MgCl_2$ at 3.6 mM (Figure 4). Univalent cations in the system reduced the effectiveness of the divalent cations, since higher concentrations of divalent cations were required to bring about an increase in turbidity in the presence of univalent cations (Figure 4). The more highly hydrated cations ($Na^+$ and $Li^+$) were more antagonistic toward $Ca^{2+}$ than the less hydrated $K^+$.

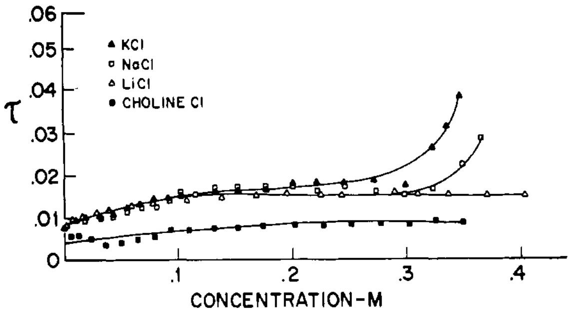

FIGURE 3. Turbidity of 0.5 mM ammonium phosphatidylinositol on addition of univalent cations; λ 546 mμ.

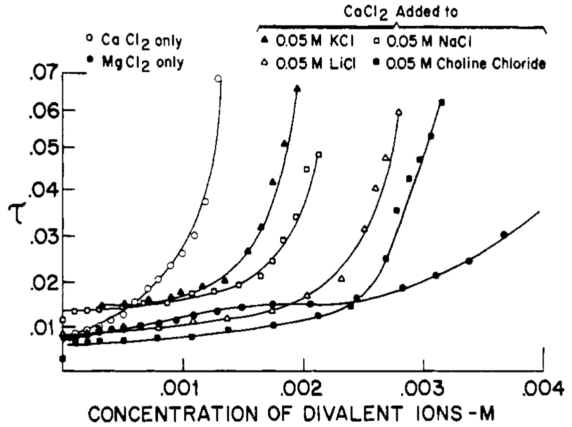

FIGURE 4: Turbidity of 0.5 mM ammonium phosphatidylinositol on addition of divalent cations; λ 546 mμ.

*Coagulation Studies.* For systems containing only alkali metal chlorides, relatively high concentrations of the salt were necessary for flocculation, whereas with alkaline earth metals, coagulation occurred in 3 mM $CaCl_2$ or 5 mM $MgCl_2$. Systems IA,B and IIA,B were designed to measure the relative binding of $Na^+$-$K^+$ and $Ca^{2+}$-$Mg^{2+}$ (Table I), respectively. For these systems, the coagulum was analyzed for metallic cation and phosphorus so that the cation content could be related more exactly to the micromoles of phosphatidylinositol. Values of the molar ratios (divalent metal: phosphatidylinositol = 0.5 and univalent metal: phosphatidylinositol = 1) of total cation content to lipid in the coagulum show the complete conversion of the lipid into the salt form. The values also indicate that the exchange of cations between lipid and solution is governed by the concentration of the ions and by a selectivity ratio.

In system IIA,B in which sodium phosphatidylinositol was used, the $Na^+$ found in the equilibrating solution results from its release by the more strongly bound alkaline earth cations. Systems IV and V show that in the presence of added concentrations of $Na^+$ in the solution, the binding for $Ca^{2+}$ is reduced compared with system III. In systems III and VI, the values of $Ca^{2+}$ and for $Mg^{2+}$ approaching 1 μequiv/μmole of lipid indicate that two phosphatidylinositol molecules are bound for each divalent cation. System VII was designed to determine the cations accepted by phosphatidylinositol in a solution whose ionic concentrations approximate those present within cells. At such relatively high concentrations of univalent metal ions, it was found necessary to increase the concentration of divalent cations in order to cause coagulation. The results show that the amount of $Na^+$ bound to the lipid under these conditions is small. System VIII indicates that the presence of a divalent cation does not alter the relative affinities of $K^+$ and $Na^+$.

Table II summarizes the results obtained with the three procedures used to measure the affinities of phosphatidylinositol for $K^+$ and $Na^+$ and for $Ca^{2+}$ and $Mg^{2+}$. Agreement among the three methods is evident. The effectiveness both in releasing protons and in causing increased turbidities show a ratio of $K^+$: $Na^+$ of 1.1. In evaluating the coagulation studies, a measure of the capacity for separation of the ions (separation factor) is given by $\alpha^{K^+}_{Na^+}$, where $\alpha^{K^+}_{Na^+} = (\bar{K})(Na^+)/(\overline{Na})(K^+)$ and $(\bar{K})$ and $(\overline{Na})$ are the microequivalents of the respective cation per milligram of coagulated phosphatidylinositol, and $(Na^+)$ and $(K^+)$ are the molar concentrations of these ions in the supernatant solution (Helfferich, 1962). The values for these separation factors range from 1.3 to 1.5. Similar comparisons for $Ca^{2+}$ and $Mg^{2+}$ show ratios of 1.4 for their effectiveness in proton exchange and 3.6 for increasing the turbidity. The separation factor, $\alpha^{Ca^{2+}}_{Mg^{2+}} = (\overline{Ca})(Mg^{2+})/(\overline{Mg})(Ca^{2+})$, for three systems ranged from 2.1 to 2.6.

*Association Constants of Metal Ions with Phosphatidylinositol.* Association constants for the binding of the $PI^-$ anion with $Ca^{2+}$ and $Na^+$ were evaluated from the data on cation-proton exchange. Assuming the reactions to be $Na^+ + PI^- \rightarrow NaPI$ and $Ca^{2+} + PI^- \rightarrow CaPI^+$, the apparent constants were based on the relations $K_{Na}' = (NaPI)/(Na^+)(PI^-)$ and $K_{Ca}' = (CaPI^+)/(Ca^{2+})(PI^-)$. The systems were first brought to an ionic strength of 0.1 with TMACl and pH 3.5 with HCl. Although the addition of this salt causes a release of protons resulting from the change in ionic strength, and an exchange for protons in the electrical double layer, the possibility of the binding of the $TMA^+$ ion by the lipid cannot be ruled out. However, in the calculations, the concentration of unbound $PI^-$ was determined by using $1 - \alpha$ at pH 3.5 (where $\alpha = (PI^-)/(PI_{total})$) and which was obtained from the titration curve in 0.1 M TMACl. The calculation has been described (Abramson *et al.*, 1966). Reactions at higher concentrations of $Ca^{2+}$ (>1.5 mM) are influenced by the onset of flocculation. The constants were therefore calculated for concentrations below this (Table III). The trend in the calculated values of the constants for $CaPI^+$ may result from incipient aggregation and consequent unavailability of some phosphate groups for binding. The values obtained for the formation of NaPI show a greater constancy (Table IV). Flocculation with NaCl does not begin until much higher concentrations are reached.

For the reaction of phosphatidylinositol with $Ca^{2+}$ at neutral pH values it was possible to calculate an association constant by a different method, namely measurements of the turbidity of buffered systems following the addition of $Ca^{2+}$. This procedure utilized two equivalent dilute systems (approximately 1 mM) of phosphatidylinositol filtered through 0.8-μ Millipore filters buffered with 0.05 M Tris at pH 7.3. All reagents were also filtered.

TABLE II: Comparison of the Cation Affinity of Phosphatidylinositol Determined by Three Experimental Methods.

| | KCl | NaCl | $CaCl_2$ | $MgCl_2$ | Effectiveness<br>K:Na | <br>Ca:Mg |
|---|---|---|---|---|---|---|
| 1. Cation–proton exchange,[a] $\mu$equiv of $H^+$ released/ $\mu$mole of phosphatidylinositol | 0.16 | 0.14 | 0.07 | 0.05 | 1.1 | 1.4 |
| 2. Turbidity, mM concentration required to bring turbidity to $\tau$ 0.03[c] | 326 | 364 | 1.0 | 3.6 | 1.1 | 3.6 |
| | | | | | Separation Factor[b]<br>$\alpha^{K}_{Na}$ | <br>$\alpha^{Ca}_{Mg}$ |
| 3. Coagulation (see Table I) | | | | | | |
| System IA, IB | | | | | 1.4, 1.5 | |
| IIA, IIB | | | | | | 2.6, 2.1 |
| VII | | | | | 1.3 | 2.4 |
| VIII | | | | | 1.4 | |

[a] Constant pH (3.50). Addition of 0.08 M $K^+$ or $Na^+$ or 1.4 mM $Ca^{2+}$ or $Mg^{2+}$ at $\mu = 0.1$. [b] Separation factor is defined: $\alpha^{B}_{A} = (\bar{X}_B)(X_A{}^+)/(\bar{X}_A)(X_B{}^+)$, where $(\bar{X})$ is microequivalents of cation per milligram of coagulated phosphatidylinositol and $X^+$ is molar concentration of cation in supernatant. [c] On addition to 0.5 mM phosphatidylinositol.

One system was brought to 0.8 mM in sodium citrate while the other was brought to 2.4 mM in sodium chloride (equal $Na^+$ concentration). Additions of $CaCl_2$ were made to both systems and their turbidities were measured. To produce equal increases in turbidity, the system containing sodium citrate required higher concentrations of calcium than that containing sodium chloride. The difference in total calcium concentration between the two systems at the same turbidity determines the calcium bound to citrate (Table V). Earlier experiments have shown that the presence of the calcium citrate ion does not alter the turbidities. From the concentration of $CaCit^-$ and its association constant, $K = 1.62 \times 10^3$ (Hastings *et al.*, 1934), and the total citrate concentration, the $Ca^{2+}$ concentration can be calculated. The mass balance for total calcium gives the concentration of $CaPI^+$ and the mass balance for total phosphatidylinositol gives the concentration of $PI^-$. (No acid form of phosphatidylinositol remains at this pH.)

$$CaPI^+ = Ca_T - CaCit^- - Ca^{2+}$$

$$PI^- = PI_T - CaPI^+$$

Details of the procedure were given earlier (Abramson

TABLE III: Association Constant for $CaPI^+$. Cation–Proton Exchange at pH 3.5 in 0.1 M TMACl.

| $Ca_{total}$ (mM) | $CaPI^+$ (mM) | $Ca^{2+}$ (mM) | $K'$ |
|---|---|---|---|
| | $PI_{total}$ = 2.16 mM | | |
| 0.58 | 0.44 | 0.14 | $2.2 \times 10^3$ |
| 0.77 | 0.46 | 0.31 | $1.1 \times 10^3$ |
| 0.96 | 0.48 | 0.48 | $0.85 \times 10^3$ |
| 1.16 | 0.51 | 0.65 | $0.74 \times 10^3$ |
| 1.35 | 0.54 | 0.81 | $0.69 \times 10^3$ |
| | $PI_{total}$ = 2.18 mM | | |
| 0.60 | 0.42 | 0.18 | $1.8 \times 10^3$ |
| 0.77 | 0.44 | 0.33 | $1.1 \times 10^3$ |
| 0.96 | 0.46 | 0.50 | $0.78 \times 10^3$ |
| 1.15 | 0.48 | 0.67 | $0.59 \times 10^3$ |
| 1.34 | 0.50 | 0.84 | $0.50 \times 10^3$ |

TABLE IV: Association Constant for NaPI. Cation–Proton Exchange at pH 3.5 in 0.1 M TMACl.

| $Na^+_{total}$ (mM) | NaPI (mM) | $K'$ |
|---|---|---|
| | $PI_{total}$ = 2.06 mM | |
| 7.6 | 0.06 | 5.7 |
| 15.2 | 0.08 | 8.9 |
| 30.3 | 0.13 | 7.4 |
| 45.2 | 0.19 | 7.5 |
| 60.1 | 0.23 | 7.0 |
| 82.0 | 0.27 | 6.1 |
| 104 | 0.32 | 5.9 |
| 133 | 0.34 | 6.9 |
| 168 | 0.48 | 6.1 |
| 189 | 0.59 | 7.1 |
| | | Av $K'$ = 6.9 |

TABLE V: Association Constant for $CaPI^+$. Turbidimetric Measurements.[a]

| $Ca_{total}$ (mM) | $CaCit^-$ (mM) | $Ca^{2+}$ (mM) | $CaPI^+$ (mM) | $PI^-$ (mM) | $K'$ |
|---|---|---|---|---|---|
| 0.62 | 0.30 | 0.37 | 0.25 | 0.68 | $1.0 \times 10^3$ |
| 0.87 | 0.36 | 0.51 | 0.36 | 0.57 | 1.2 |
| 1.14 | 0.41 | 0.65 | 0.49 | 0.44 | 1.7 |
| 1.60 | 0.50 | 1.04 | 0.60 | 0.33 | 1.7 |
| 1.78 | 0.52 | 1.15 | 0.63 | 0.30 | 1.8 |

[a] $PI_{total} = 0.93 \times 10^{-3}$ M in 0.05 M Tris (pH 7.3) and 2.4 mM NaCl.

*et al.*, 1965). The difference between the values obtained by the two methods may reflect the effect of pH.

*Viscosity.* Relevant to the effect of ions upon the properties of phosphatidylinositol in aqueous media were some observations on its viscosity. This was measured on filtered dispersions of ammonium phosphatidylinositol at 25.0° using an Ostwald viscometer. Serial dilutions were made with distilled water to give concentrations of 1.5–0.5 mM, and the viscosity at each concentration was measured. A marked increase in the reduced viscosity ($\eta_{sp}/c$) was seen upon dilution. However, bringing the solution to 2.5 mM in NaCl produced a sharp decrease in viscosity. A similar effect was also obtained with $CaCl_2$ but not with choline chloride. The increase in $\eta_{sp}/c$ on dilution may be explained by the decrease in concentration of gegenions with the resultant expansion of the electrical double layer and swelling of the lipid particles. However, the addition of metallic cations lowers the effective charge of the surface and thus causes a decrease in volume and in hydration of the particles with a consequent decrease in viscosity.

## Discussion

Phosphatidylinositol forms very clear, stable dispersions in water when subjected to mild ultrasonic radiation. The exceptionally low turbidities of these dispersions may be attributed to the small size and high degree of hydration of the particles. Near the neutral pH range, in the absence of added cations, the particles are highly charged, and thus highly dispersed. Phosphatidic acid, which is also highly charged under similar conditions, forms dispersions with turbidities appreciably higher than those of phosphatidylinositol. This indicates the effectiveness of the polar groups of the inositol moiety in phosphatidylinositol in increasing the hydration and dispersion in aqueous media, although some differences in length and unsaturation of the fatty acid chains may play a small part.

The aggregated state of lipids in an aqueous medium does not obscure discernment of the first and second dissociations of the diprotic phosphate group, as can be seen by comparing the titration curves of phosphatidylinositol (monoprotic) and phosphatidic acid (diprotic). The situation is similar to that existing with anionic resins, for which Bregman and Murata (1952) have shown that titration of the phosphonous acid resins parallels the titration of the first ionization group of phosphonic acid resins.

The dispersion containing 2 mM sodium phosphatidylinositol had a pH of 6.1–6.5 in the absence of salts and the titration curves show essentially complete neutralization of the acid groups at this pH. Therefore, the surface of the particle is highly charged, and at low total concentration, $Na^+$ ions are localized to a great extent in the region surrounding the negatively charged surface. On adding salts to these systems, cations enter the electric double layer and bring about a decrease both in the repulsions between the negatively charged groups and in the hydrated volume of the particles. This is also seen in the sharp decrease in viscosity caused by metallic cations. Organic cations do not produce this effect.

When dispersions of ammonium phosphatidylinositol were brought to pH 3.5 some of the molecules were in the unionized form while the ionized form was associated with either $NH_4^+$ or $H^+$ ions in the double layer. The addition of salts led to a decrease in the pH of the aqueous phase. This may result from several processes: increased ionization of the acid caused by the increase in ionic strength, exchange of metallic cation and $H^+$ in the double layer, and specific reaction of the cation with phosphatidylinositol which releases $H^+$. For all salts used, the first small additions (10 mM univalent ion or 0.2 mM divalent ion) produced the largest release of $H^+$ ions. This did not vary greatly for different ions of the same charge, and thus did not suggest a significant measure of specific interaction. In contrast, the difference between ions that are evident at higher concentrations do indicate specific interactions with the lipid. This is confirmed by the results of the cation analysis in the coagulation experiments (Table I).

The relative affinities of phosphatidylinositol for $K^+$ and $Na^+$ as well as for $Ca^{2+}$ and $Mg^{2+}$ were established by three independent methods, which gave results in good agreement (Table II). The release of $H^+$ ion and the increase in turbidity can be viewed as phenomena related primarily to the binding or close association of a cation with the polyanion.

The binding of alkali cations with phosphatidylinositol followed the same order as with sulfatide, namely, $K > Na > Li$, indicating a basic similarity in the ionic properties of these monoprotic acids. The selectivity of sulfatide has been attributed to the lower polarizability of the sulfate group compared with water (Bregman, 1953). In this respect, monoprotic phosphate shows a greater similarity to sulfate than to diprotic phosphate (in phosphatidic acid) which had the selectivity Li > Na > K.

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# The Sterols of *Ochromonas danica* and *Ochromonas malhamensis*[*]

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ABSTRACT: The sterols of *Ochromonas danica* have been identified as ergosterol, brassicasterol, 22-dihydrobrassicasterol, clionasterol, poriferasterol, and probably 7-dehydroporiferasterol. By contrast *Ochromonas malhamensis* contains only poriferasterol as the major sterol component. In addition, evidence has been obtained for the occurrence of cycloartenol and 24-methylenecycloartanol in *O. danica* and *O. Malhamensis.*

The chemical composition of the phytoflagellates is of importance phylogenetically as these organisms are algal animals and the lipids of *Ochromonas* species have proved particularly interesting in this regard. Thus only chlorophyll a was found in *Ochromonas danica* (Allen *et al.*, 1960a) and the only carotene identified was β-carotene (Allen *et al.*, 1960b). The fatty acid composition yielded large amounts of α-linolenic as well as γ-linolenic and arachidonic acids (Haines *et al.*, 1962). A unique sulfatide has been isolated from its lipids (Mayers and Haines, 1967), while the chloroplast sulfonolipid has also been identified (Miyachi *et al.*, 1966).

Although the sterols of *O. danica* and *Ochromonas malhamensis* have been investigated several times, a positive identification of all the sterols has not been achieved to date. Stern *et al.* (1960) reported ergosterol (III) in *O. danica* on the basis of its ultraviolet spectrum, and this was later verified (Aaronson and Baker, 1961). On the basis of gas–liquid partition chromatography and silver nitrate thin-layer chromatography, Halevy *et al.* (1966) confirmed the presence of ergosterol (III) and reported that the major sterol of *O. danica* was stigmasterol (VII). A number of additional unidentified sterols were also observed by gas–liquid partition chromatography.

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