line test assay method and is radiochemically pure as revealed by at least three chromatographic methods. Purity is also revealed by ultraviolet spectral analysis of the vitamin and provitamin. Its specific activity (26,000 dpm/IU) is sufficiently high to be exceedingly useful for experiments on the mechanism of vitamin D action and is specifically labeled (1,2 position) to be very useful in metabolic experiments. It is hoped that this preparation will provide an important tool to be used in our quest of the mechanism of action and metabolism of vitamin D.

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The Reactions of Cations with Aqueous Dispersions of Phosphatidic Acid. Determination of Stability Constants*

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ABSTRACT: The reactions of univalent and divalent cations with aqueous dispersions of phosphatidic acid (PA) produced by ultrasonic radiation were studied by measurements of the H^+ ion released. Apparent stability constants (K') were calculated from equivalents of tetramethylammonium hydroxide (TMAOH) needed to maintain constant pH on addition of alkaline earth metal chlorides ($MeCl_2$) or alkali metal chlorides (MeCl). Titration curves gave concentrations of ionic forms of PA available for reactions $Me^{2+} + PA^{2-} = MePA$; $Me^+ + PA^{2-} = MePA^-$, and $HPA^- = PA^{2-} + H^+$. Assuming absence of specific reaction with TMA^+, values of K' for formation of CaPA, MgPA, $LiPA^-$, $NaPA^-$, and KPA^- are estimated as 1.6×10^4, 0.97×10^4, 17, 16, and 9, respectively. These values agree with those calculated from drop in pH on adding salt and in the case of CaPA from turbidimetric measurement.

The importance of the reactions of univalent and divalent cations in aqueous media with acidic lipids in biological tissues is abundantly clear (Ansell and Hawthorne, 1964). It is believed that determining the constants for the association of cations with these compounds will aid in understanding their characteristics in a manner analogous to the success attained in the study of the solutions of metal chelates (Dwyer and Mellor, 1964). In addition, this provides an opportunity for elucidating the reactions of ions with colloidal aggregates.

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Phosphatidic acid was chosen for the study of its reactions with cations, because its structure is relatively simple compared to other phospholipids. As a dibasic acid, the functional characteristics of the two acid groups of phosphatidic acid are related; previously we measured their ionization constants and showed that in aqueous dispersions produced by ultrasonic radiation all the acid groups were exposed to the medium, and stoichiometric relations based upon the mass of the lipid could be applied (Abramson *et al.*, 1964b).

Several methods have been employed for the determination of the stability constants of phospholipids with dibasic cations. We have been able to estimate the stability constant of Ca^{2+} with phosphatidic acid using a turbidimetric technique (Abramson *et al.*, 1965). Hendrickson and Fullerton (1965) estimated the stability constants of complexes of phosphatidylserine and triphosphoinositides with Ca^{2+}, Mg^{2+}, and Ni^{2+} from the displacement of the titration curves.

A decrease in pH was observed when solutions of metallic salts were added to aqueous dispersions of acidic lipids by Christensen and Hastings (1940). They titrated cephalin emulsions in water and dilute NaCl and the displacement of the latter titration curve to lower pH levels was explained as the result of the formation of Na-cephalin. A similar effect was found in the titration of a synthetic α-palmitoyl-β-glycerophosphate. Dervichian (1955) in a study of lipid emulsions also reported a lowering in the pH of the system on adding solutions of salts. Studies in our laboratory have shown this effect upon the addition of various salts to dispersions of phosphatidic acid and phosphatidylserine (Abramson *et al.*, 1964a,b). We have used this hydrogen ion release to measure the stability constants of the products of the interaction of aqueous dispersions of phosphatidic acid with univalent as well as divalent cations. The binding is measured as a function of the amount of base required to maintain constant pH as salts are added. This study using micelles is similar to the measurements of stability constant of soluble complexes formed by metal ions and anions which have included metal chelates of biologic importance such as with adenosine phosphates (Smith and Alberty, 1956a,b).

Experimental Section

Phosphatidic acid was prepared by enzymatic degradation of egg lecithin (Sylvana) as previously described (Abramson *et al.*, 1964b). The product contains calcium bound to the lipid. It was found that acid dialysis and purification by use of a Unisil R column resulted in material that contained approximately 40% of the lyso form (contains one fatty acid chain). To avoid the degradation to the lyso form while removing Ca, the phosphatidic acid was passed through a cation-exchange column (Dowex 50-×8 using 10 g of resin/125 mg of lipid). The resin was first washed with 10 column volumes of 2 N NaOH and then with water until neutral. It was then flushed exhaustively with CH_3OH–$CHCl_3$ (1:1, v/v), then with CH_3OH, and this solvent displaced with CH_3OH–$CHCl_3$ (1:1, v/v). The lipid in a small volume of the latter solvent was placed in the column and eluted with 2 column volumes of the same solvent, then taken to dryness and dissolved in absolute ethanol.

The conversion of phosphatidic acid to the sodium form was then completed in order to reduce hydrolysis. The lipid dissolved in $CHCl_3$–CH_3OH (2:1, v/v) was partitioned with an aqueous solution of 0.1 N NaCl maintained at pH 8 with NaOH. The lower phase was separated, washed with a small volume of water maintained at pH 8, and stored at −70°. The analysis of the resulting material was: P, 1.3 μmoles/mg; ester–P ratio of 2.0 (showing no lyso form); N, 0.05%; Na, 1.3 μmoles/mg; K, 0.11 μmole/mg; and Ca, 0.049 μmole/mg.

All salts used were CP grade. Standard calcium chloride was prepared from weighed quantities of calcium carbonate and hydrochloric acid, then brought to pH 7 by minimal additions of tetramethylammonium hydroxide (TMAOH).[1] Tetramethylammonium chloride (TMACl) and TMAOH were from Eastman Kodak. TMACl was recrystallized from an ethanolic solution by the addition of diethyl ether. The water used in all experiments was redistilled from an all-Pyrex still and had a conductivity of 0.7 μmho/cm.

Dispersions of phosphatidic acid were prepared by ultrasonic treatment of a weighed amount of the lipid (7–10 mg) deposited on the bottom of a 20-ml tube and covered with 5 ml of water. These preparations were quite stable when brought to the neutral range. Titrations of representative dispersions were carried out with a Radiometer titragraph at 24 ± 1° in an enclosed tube with a rapid stream of CO_2-free nitrogen passed over the liquid. Earlier papers (Abramson *et al.*, 1964a,b) gave details of the preparation of the dispersions and of the titration procedure. Titrations were carried out in water and in 0.1 M TMACl using 0.0500 N TMAOH and 0.0500 N HCl for the titration cycle of each system.

Reactions with Cations. The lipid dispersion was brought to the desired initial ionic strength by the addition of a concentrated solution of TMACl and to the desired pH level by the addition of 0.0500 N TMAOH from a 0.1-ml Gilmont micropipet. Solutions of 0.05 M $CaCl_2$ or $MgCl_2$ or 4.0 N NaCl, LiCl, or KCl were added from a 0.1-ml micropipet starting with additions of 0.001 ml of the salt solution and increasing to 0.01-ml additions. Stirring and a CO_2-free atmosphere were provided as in the titrations. The resulting drop in pH took place rapidly. Base was added to reestablish the desired pH level. Although equilibrium was reached in most instances within 5 min, additional time (about 10 min) was given for

[1] Abbreviations used are HPA^-, and PA^{2-}, the anions formed by the release of successive protons from phosphatidic acid, PA. TMA^+ is the cation of tetramethylammonium chloride (TMACl), TMAOH, TMA hydroxide; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphates.

TABLE I: Apparent Stability Constants for Formation of MePA at pH 7.0 in 0.1 M TMACl at 24°.

Calcium						Magnesium		
Sample 1, $PA_T = 2.20 \times 10^{-3}$ M			Sample 2, $PA_T = 2.06 \times 10^{-3}$ M			$PA_T = 2.16 \times 10^{-3}$ M		
$Ca_T \times 10^{-4}$ M	$Ca^{2+} \times 10^{-4}$ M	$K' \times 10^4$	$Ca_T \times 10^{-4}$ M	$Ca^{2+} \times 10^{-4}$ M	$K' \times 10^4$	$Mg_T \times 10^{-4}$ M	$Mg^{2+} \times 10^{-4}$ M	$K' \times 10^4$
2.0	0.47	1.01	2.0	0.73	0.58	2.0	0.49	0.98
3.0	0.48	1.63	2.5	0.81	0.70	3.0	0.82	0.85
4.0	0.46	2.39	3.0	0.84	0.86	3.9	0.90	1.07
5.0	0.39	3.64	4.0	0.94	1.10	5.8	1.18	1.28
6.0	0.57	2.98	5.0	1.04	1.29	7.7	1.82	1.09
8.0	1.91	1.06	6.0	1.17	1.42	11.0	3.94	0.67
10.0	2.35	1.11	7.0	1.40	1.39	15.0	5.85	0.66
12.0	2.70	1.19	8.0	1.56	1.45	18.0	6.20	0.82
15.0	1.50	2.95	9.0	1.85	1.38	21.0	6.00	1.07
20.0	2.10	2.86	10.0	2.00	1.44	27.0	9.80	0.99
24.0	5.20	1.44	12.0	2.80	1.23	32.7	14.8	1.18
31.8	12.80	1.07	15.0	3.10	1.58			
38.6	19.50	2.60						
Av K'	1.99×10^4			1.20×10^4			0.97×10^4	

small fluctuations to stabilize before again adding salt. However, at concentrations of $CaCl_2$ at which coagulation occurred, the pH dropped slowly, requiring several hours for each point.

At the completion of an experiment, the high concentration of cation caused sufficient coagulation of the lipid to permit separation by centrifugation. Thin layer chromatography using silica gel plates and as the developing solvent $CHCl_3$–CH_3OH–H_2O(70: 30:4, v/v/v) showed no degradation products and analysis revealed an ester–P ratio of 2:1 as in the original material.

Calculations. At pH levels above 5.40 (the first equivalence point in 0.1 M TMACl), the species of phosphatidic acid present are HPA^- and PA^{2-}. The addition of solutes with indifferent ions (quaternary ammonium) to the dispersions caused an exchange of cations with H^+ ions in the double layer surrounding the particle and an increase in self-ionization with a decrease in the pH of the system.

In experiments performed with this and other acidic lipids, the addition of TMACl produced smaller effects than equal concentrations of alkali metal and alkaline earth cations. This, together with the large diameter of the TMA^+, permits the assumption that the specific binding of the TMA^+ is nil and the greater effects shown by metallic cations can be attributed to either their smaller (hydrated) size and the resultant decrease in the potential of the double layer (Miller and Gregor, 1965) or to a specific binding of these cations by the anions of the lipid.

Cation binding can also be studied by a comparison of the titration curves of the acid in the presence and absence of a given concentration of the cation of interest. However, in the present investigations it was found that the displacements of the titration curves were not sufficiently large for precise calculations. Also, carrying the titrations to pH levels below neutral in the presence of cations in some instances caused coagulation of the dispersion. Another procedure we have used is the stepwise addition of salt with the determination of the resulting pH. This also causes coagulation as the salt concentration increases, accompanied by lower pH levels. However, stability constants calculated by this latter procedure provided a useful check for the values found.

The stability constants measured in this investigation were determined at a constant pH level, with increasing concentrations of the cation. Under the conditions of the experiments particle sizes in the dispersion do not change until the concentration of cations reaches relatively high values according to turbidity measurements (Abramson *et al.*, 1965). A series of values for the stability constant can be determined for a fixed pH, but with increasing cation concentrations.

The values listed in Tables I and II for pH 7 were computed by the method described below. The phosphatidic acid dispersion in TMACl was brought to pH 7 by the addition of TMAOH. Titration curves made under identical conditions showed that at this pH level the degree of neutralization was $\alpha = 0.15$ where

$$\alpha = \frac{(PA^{2-})}{(HPA^-) + (PA^{2-})} \quad (1)$$

Values for α were found by identifying the first equivalence point on the titration curve by its maximum

TABLE II: Apparent Stability Constants for Complexes (1:1) of Metal Cations with Phosphatidic Acid at $\mu = 0.1$; 24°.

	pH 7.0[a]	pH 6.5[c]	pH 6.0[c]
CaPA	1.60×10^{4}[b]	1.17×10^{4}	1.95×10^{4}
MgPA	0.97×10^{4}	1.49×10^{4}	1.10×10^{4}
$LiPA^-$	17.3	20.5	15.0
$NaPA^-$	15.8[b]	12.2	11.0
KPA^-	8.9[b]	9.9	6.42

[a] Determined at constant pH 7.0. [b] Average of sets of determinations on two samples. [c] Determined by drop in pH from 7.0.

slope. From this point the total number of equivalents of acid or base needed for reacting with the number of equivalents of PA present was indicated. The pH levels at fractional degrees of neutralization were interpolated between these points. With the addition of Ca^{2+} or other divalent cation Me^{2+}, the reaction is assumed to proceed in the following manner: $Me^{2+} + PA^{2-} \rightarrow MePA$. The resulting decreased concentration of PA^{2-} leads to the further ionization of HPA^-, releasing H^+. The TMAOH added to restore the system to the initial pH level then measures the H^+ released and also adds an equivalent concentration of TMA^+ to the solution; this concentration of added TMAOH is referred to as B. Converting all concentrations to a molar basis (eliminating brackets for convenience), the total phosphatidic acid PA_T is

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

Using Me_T for total metal-cation concentration in all forms

$$Me_T = Me^{2+} + MePA \quad (3)$$

For the electroneutrality after the addition of $MeCl_2$, ignoring the initial concentration of the supporting electrolyte, TMACl

$$H^+ + (1 + \alpha)PA_T + B + 2(Me_T - MePA) = OH^- + (1 - \alpha)(PA_T - MePA) + 2\alpha(PA_T - MePA) + Cl^- \quad (4)$$

It is assumed here that despite the formation of the metal–lipid complex, α remains unchanged at the given pH in a manner similar to that reported by Andelman *et al.* (1959) and Liu and Gregor (1965). The sum of the concentrations of the lipid in the ionic forms is then $PA_T - MePA$. At pH 7, $H^+ = OH^-$ and, since $Cl^- = 2Me_T$, eq 4 simplifies to

$$(1 - \alpha)MePA = B \quad (5)$$

In a similar manner, the release of H^+ by addition of an univalent cation Me^+ to the dispersion is assumed to be due first to formation of $MePA^-$ by the reaction

$$Me^+ + PA^{2-} = MePA^- \quad (6)$$

At higher concentrations of Me^+, Me_2PA may form.

Neglecting activity coefficients, the apparent stability constants for divalent cations are based upon

$$K' = \frac{(MePA)}{(Me^{2+})(PA^{2-})} = \frac{(MePA)}{(Me_T - MePA)\alpha(PA_T - MePA)} \quad (7)$$

MePA is found from eq 5. For univalent cations similar reasoning leads to

$$K' = \frac{(MePA^-)}{(Me^+)(PA^{2-})} = \frac{(MePA^-)}{(Me_T - MePA^-)\alpha(PA_T - MePA^-)} \simeq \frac{MePA^-}{(Me_T)\alpha(PA_T - MePA^-)} \quad (8)$$

since the concentration of $MePA^-$ formed is small relative to Me_T and $Me^+ \simeq Me_T$.

For experiments in which decreasing pH levels were measured with increasing salt concentration, the release of H^+ is equal to the equimolar decrease in HPA^- in the system. The initial concentration of HPA_1^- is $HPA_1 = (1 - \alpha_1)(PA_T)$. Here α_1 is read from the titration curve at pH 7 in the presence of TMA^+ only. The concentration of HPA^- after the addition of the metal chloride is $HPA_2^- = (1 - \alpha_1)PA_T - \Delta H^+$, where ΔH^+ is the hydrogen ion released in the pH change. On the basis of previous assumptions, the concentration of PA_2^{2-} at the lower pH level can now be found:

$$\frac{(PA_2^{2-})}{(HPA_2^-)} = \frac{(\alpha_2)}{(1 - \alpha_2)} \quad (9)$$

where α_2 is also read from the titration curve at the lower pH level. From the mass balance equations for phosphatidic acid and the metal, the apparent stability constants were computed from $K' = (MePA)/((Me^{2+})(PA^{2-}))$. Similar treatment was applied to the formation of $MePA^-$ by univalent cations Me^+.

Results and Discussion

The release of H ions by the addition of metallic chlorides to dispersions of phosphatidic acid is shown in Figures 1 and 2. In all cases, the systems contained 0.1 M TMACl and were maintained at a fixed pH level by the addition of TMAOH. To permit a proper com-

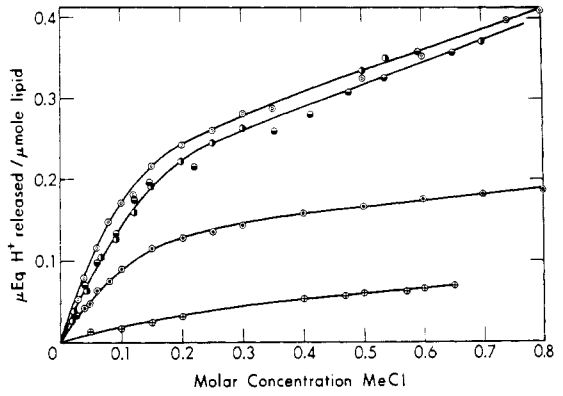

FIGURE 1: H^+ ion released on addition of univalent chlorides to 5-ml dispersions of 2×10^{-3} M phosphatidic acid maintained at constant pH 7.0 in 0.1 M tetramethylammonium chloride. Values for two determinations with NaCl are shown. ◎ = LiCl; ◒ = NaCl I, ◑ = NaCl II; ⊙ = KCl; ⊕ = TMACl.

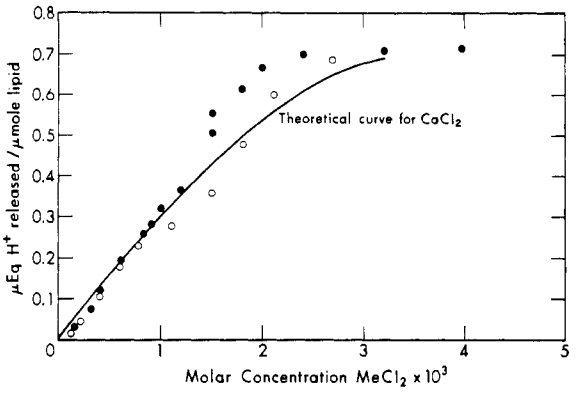

FIGURE 2: H^+ ion released on addition of $CaCl_2$ and $MgCl_2$ to 5-ml dispersions of 2×10^{-3} M phosphatidic acid maintained at constant pH 7.0 in 0.1 M tetramethylammonium chloride. The curve shows the anticipated release calculated by the use of a stability constant for CaPA of 1.6×10^4 (see text). Deviation from the curve occurs in region of coagulation. ● = $CaCl_2$; ○ = $MgCl_2$.

parison, the curve for TMACl was plotted to show the effect of TMACl added to a solution of 0.1 M TMACl. The effect of TMACl is markedly less than that of any of the other salts. The greater effects of the other salts is taken to represent specific effects of these cations in the relative order, Ca > Mg > Li > Na > K.

An interesting confirmation of the relative binding abilities was shown by experiments of the following nature. The addition of KCl was made to a dispersion of PA until the concentration reached 0.8 M and the release of H^+ became minimal. The addition of NaCl to this system now released additional H^+ in moderately large amounts. Similar effects were observed when $CaCl_2$ was added to a system brought to a high concentration in NaCl. Further indication of the strong binding for Ca^{2+} is shown in Figure 2 in which the release of H^+ approaches 0.725 μequiv of H^+/μmole of P. Since $1 - \alpha = 0.85$ at pH 7, this points to the great extent to which H^+ is exchanged for Ca^{2+} by the HPA^-; this occurs despite the heavy coagulation that takes place and the resulting difficulty of attaining equilibrium. Additional experiments show that for each of these cations, the release of H^+ is less at pH 6 than at 7. The addition of salts to dispersions of PA at pH 4.0 in 0.1 M TMACl led to relatively small releases of H^+. In 0.7 M NaCl the H^+ release was 0.093 μequiv/μmole of P. In 0.8 M KCl it was 0.076 μequiv/μmole of P. At this pH level $\alpha = HPA^-/H_2PA + HPA^- = 0.87$, and this H^+ could be accounted for by the increased ionization of H_2PA. However, on the addition of $CaCl_2$, 0.11 μequiv of H^+/μmole of P was released at 1.7×10^{-3} M $CaCl_2$. On further increasing the concentration of Ca^{2+}, the slope of the graph, plotting the H^+ released against $CaCl_2$ added, changed its form maximum of 0.17 μequiv of H^+ released/μmole of phosphorus.

The reactions we have postulated at pH levels above the first equivalence point are $Me^{2+} + PA^{2-} =$ MePA and $Me^+ + PA^{2-} = MePA^-$, with the release of H^+ coming from $HPA^- = PA^{2-} + H^+$, assuming the degree of disassociation is constant at a fixed pH. Tables I and II show that the stability constants calculated on the basis of these assumptions are reasonably constant for this type of colloidal system.

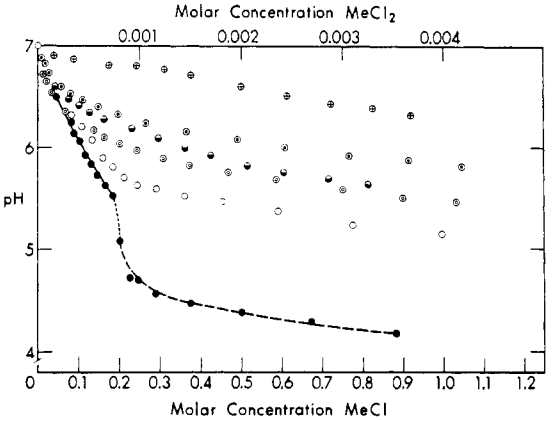

FIGURE 3: Effect of added salt on the pH of 5-ml dispersions of phosphatidic acid (2×10^{-3} M) in 0.1 M tetramethylammonium chloride. The dispersions, adjusted to pH 7.0, show progressive decreases in pH on increased salt concentration. The greater effect of alkaline earth cations (upper scale) is evident. Discontinuity in curve for $CaCl_2$ occurs with coagulation of the dispersion. ⊕ = TMACl; ◎ = KCl; ◒ = NaCl; ⊙ = LiCl; ○ = $MgCl_2$; ● = $CaCl_2$.

Similar displacement experiments were performed with a preparation that contained the lyso form of phosphatidic acid. Although the titration curve for this

material gave values of α at pH 7 and 6 that were smaller than for the preparation used for determining the stability constants reported here, constants for the metal binding showed reasonable agreement for the two preparations.

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. The systems containing LiCl, NaCl, or KCl were not at constant ionic strength. The constants for the univalent cations forming $MePA^-$ show a definite trend to lower values at higher salt concentrations. Since all systems contained initially 0.1 M TMACl, there is little change in ionic strength at the lowest added salt concentrations and the progressive decrease in K' at salt concentrations greater than approximately 0.08 M may be explained as the result of the increasing ionic strength of the systems.

It had been observed earlier (Abramson *et al.*, 1964b) that 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. The present investigation indicates that in this medium 0.22–0.25 H^+/mole of 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 equiv of H^+/mole of lipid is released. It may be reasoned that the coagulation takes place when the fraction of lipid bound to cation reaches a definite value, or when the negative charge of the polyanion is reduced to the point where repulsive forces do not maintain the stability of the dispersion. These are classical colloid phenomena.

Figure 3 shows the results of experiments in which 5-ml dispersions of phosphatidic acid, approximately 2.0×10^{-3} M and containing 0.1 M TMACl, were first brought to pH 7.00 with TMAOH. Small additions of salt solutions were made, and the resulting pH noted after coming to apparent equilibrium. The relative effects are Ca > Mg > Li > Na > K. The enhanced effect of LiCl appears greatest at low concentrations. For binding to take place between the hydrated cation and the negatively charged surface group, it is necessary that the hydration shell of the cation be penetrated on approaching the phosphate ion. Since the observed order of effectiveness of the alkali metal ions parallels the decreasing size of their crystallographic radii and increased hydration, this penetration presumably occurs. A similar order of binding of these cations by other phosphate systems (Bregman and Murata, 1952) may be interpreted in a like manner. The extreme effect of $CaCl_2$ is shown strikingly by its graph. A noteworthy observation is that the addition of $CaCl_2$ continues to release H^+ at pH levels below that for the conversion of PA^{2-} to HPA^-. At these levels, the H^+ could come from the reaction $Ca^{2+} + HPA^- = CaPA + H^+$, with a direct exchange of Ca^{2+} for H^+ or by the reaction $Ca^{2+} + HPA^- = CaHPA^+$. The decrease in concentration of HPA^- may then lead to the further ionization of H_2PA with release of H^+ ion. In Figure 2, we show the calculated μequiv of H^+ that should be released at pH 7 on addition of $CaCl_2$. This is obtained by the use of a stability constant of 1.6×10^4. Excellent agreement between the observed and calculated H^+ ion release is seen in the region before coagulation. Greater than anticipated release of H^+ apparently occurs with coagulation at a concentration of $CaCl_2$ of about 1.0×10^{-3} M. When the dispersions are coagulated, surface charges are shielded or neutralized and HPA^- groups on the surface increase in self-ionization.

Values for the stability constants for the complexes formed by univalent and divalent cation were also calculated from the pH change. Table II gives these constants for the final pH 6.5 and 6.0. Uncertainties in estimating the value for α near the equivalence point makes this procedure less satisfactory than the method at constant pH.

The values for the apparent stability constants in Tables I and II show a reasonable degree of constancy: over an extended range of concentrations of the cation, for duplicate experiments, for preparations containing the lyso form and for similar ones that did not, for experiments based upon the decrease in pH levels, and with the values for CaPA calculated from turbidimetric measurements (Abramson *et al.*, 1965). These results support the view that these dispersions of acidic lipids have all their ionic groups exposed to the aqueous medium, and stoichiometric relations can be formulated for the reaction of the lipid with dissolved ions.

The presence of a relatively large percentage of the lyso form does not appear to alter to any great extent the reactivity of the ionogenic group. The major difference in the properties of these preparations appears to be that the lyso form has a somewhat higher pK_a, resulting in smaller values for α at the pH levels studied. This is understandable, since if the structures of the dispersed particles in the two systems are different, the surface charge densities will differ.

The apparent stability constants for the binding of cations by phosphatidic acid show interesting similarities to other phosphate anions of biologic and nonbiologic origin. Smith and Alberty (1956a) measured stability constants for the adenosine 5-phosphates, finding that in 0.2 ionic strength solutions for AMP, ADP, and ATP, in tetramethylammonium bromide, the apparent stability constants for complexing with potassium were 1.8, 4.5, and 8.5, respectively. Our value of 8.9 places PA close to ATP, and suggests that the surface charge of PA has the same effect as the chain potential of the di- and triphosphates. There are, however, significant differences between the relative binding constants for the alkali metal cations with the soluble and insoluble phosphates. With ADP and ATP, binding constants for Na are about 25% greater than for K, but PA shows an increase of about 80% at pH 7. The two groups of compounds differ in that ADP and ATP have one or two monobasic phosphate groups with a terminal dibasic acid while PA has only the dibasic configuration. These structural differences may account for the differences in binding we observe.

Our values for the binding of Ca^{2+} with PA agree

well with those reported by DiStefano and Neuman (1953) for binding with ATP. Smith and Alberty (1956b) reported substantially lower values for ATP binding.

The values given here for the stability constants and the method of computation must be viewed in light of some of the assumptions and simplifications made. Although it has been shown that the H^+ activity at a negatively charged surface is greater than in the bulk aqueous phase, the work here is based upon measurements of H^+ and cation concentrations in the bulk phase. We calculate apparent constants because of our inability to determine the surface concentrations and also the activity coefficients for the ionic species. Although a theoretical flaw exists in this approach, it nevertheless has justification by providing a means for evaluating and comparing different systems in terms of measurable quantities. In a like manner, it is assumed that tetramethylammonium chloride is an indifferent salt with no specific binding of the cation to the lipid surface. Although our experiments indicate that the tetramethylammonium ion produces much smaller effects (release of H^+ ion, change in turbidity) than metallic cations, this does not *a priori* indicate an absence of some less pronounced specific binding. In their studies of the complex formation of adenosine phosphates with cations, Smith and Alberty (1956a,b) showed that for quaternary ammonium salts, as the length of the hydrocarbon chains increased, the specific binding with the complexing ion decreased. The results of Smith and Alberty are quite consistent with the theoretical and experimental results of Miller and Gregor on the role of counterion size in electrostatic interactions and do not require the assumption of any specific binding on the part of tetralkylammonium ions. In our studies, tetraethyl- or tetrapropylammonium chloride was not used because of the danger of the penetration of the longer hydrocarbon chains into the surface structure of the dispersed lipid.

Our method of calculation assumed that the binding of cation to phosphate did not alter the ionization characteristics of the remaining lipid molecules. There is some basis for this in our titration studies of phosphatidic acid containing calcium. These curves are similar to those free of calcium except that the base capacity is reduced by the strong binding of the calcium. Nevertheless, it is to be anticipated that some alteration of the ionization characteristics of the dispersed acid should result as the binding of metal cations to the surface proceeds with a consequent decrease in surface charge. Our assumption of a constant value for α at constant pH ignores these changes which cannot yet be evaluated properly.

It is interesting to compare the stability constant obtained here ($K' = 1.6 \times 10^4$) for calcium binding to phosphatidic acid with that already reported based on measurements of turbidity ($K' = 1.39 \times 10^4$) (Abramson *et al.*, 1965). Although the two procedures involve measurements that are completely different, good agreement is obtained in the values for the constant.

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