

Effects of ionization and counterion binding on the surface areas of phosphatidic acids in monolayers

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Abstract At 24–26°C, force–area isotherms show that unionized dipalmitoyl phosphatidic acid forms a solid-condensed film while unionized egg and dioleoyl phosphatidic acids form liquid-expanded films. Surface area is a characteristic feature of a specific phosphatidic acid and the purity of a phosphatidic acid preparation can be established by the surface area of the unionized phosphatidic acid (acid subphase) at 17 dynes/cm (castor oil piston). Ionized dipalmitoyl phosphatidic acid desorbs from a monolayer at a measurable rate while ionized egg and dioleoyl phosphatidic acids desorb too slowly for rate studies. The apparent surface pK_2 for dipalmitoyl phosphatidic acid, calculated from desorption rates, is 9.4. Surface areas of the phosphatidic acids expand with ionization. Solid dipalmitoyl phosphatidic acid films expand only in the pK_2 region, showing one inflection point which indicates that the K_1/K_2 ratio is less than 100 and that, as a consequence of this ratio, the apparent surface pK_1 is greater than 7.4. Liquid egg and dioleoyl phosphatidic acid films have two inflection points, expanding in both the pK_1 and pK_2 regions. The apparent surface pK_1 and pK_2 values, calculated from inflection points in surface area data, are 3.5 and 8.0, respectively. Film expansion with phosphatidate anions is less than anticipated, showing the presence of weak transient hydrogen bonds. Expanded phosphatidate anion films are condensed by alkaline earth cations. The Ca^{2+} and Ba^{2+} salts of completely ionized phosphatidic acids collapse from monolayers, showing that the phosphatidate anion may function as an ionophore for the transport of alkaline earth ions.—**Patil, G. S., N. J. Dorman, and D. G. Cornwell.** Effects of ionization and counterion binding on the surface areas of phosphatidic acids in monolayers. *J. Lipid Res.* 1979. **20**: 663–668.

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A number of investigators have used phosphatidic acids to study the effects of ionization (1–6) and counterion binding (2–10) in model systems for membranes. Two general effects emerge from these studies. Ionization decreases the temperature (T_c) of the solid → liquid transition and alkaline earth coun-

terions increase the T_c of the ionized species. These effects on T_c are usually interpreted by the changes in surface area, A , induced by charge repulsion during ionization and charge neutralization with counterions (3–11). This interpretation has been expanded by Nagle (12) who suggests that weak transient hydrogen bonding is particularly important in phosphatidic acid systems. The surface areas of phosphatidic acids are reported in only a few studies which provide limited and sometimes widely varying data in the pH 5–8 range (13–15). In the present study, we use surface area in an examination of stability, ionization, and counterion binding in phosphatidic acid films.

MATERIALS AND METHODS

DL-Dipalmitoyl phosphatidic acid was purchased from Applied Science Laboratories (State College, PA). L- α -Dioleoyl phosphatidic acid and egg phosphatidic acid were purchased from Avanti Biochemicals (Birmingham, AL). Phosphatidic acids gave a single spot on thin-layer chromatographic plates (Quanta Q6, Quantum Industries, Fairfield, NJ) developed with chloroform–methanol–water 65:25:4 (v/v) and sprayed with molybdate (16). Surface areas for unionized phosphatidic acids were appropriate for the specified acyl chains. (Some commercial phosphatidic acid preparations that were not studied further gave unusually large surface areas, which indicated the presence of impurities.) Lipid phosphorus was measured by a standard procedure (17). Solvents were purified as previously described (18). Π - A isotherms were generated as previously described (18).

Constant pressure–variable area measurements were obtained with a floating barrier and piston oil as previously described (19). Phosphatidic acids

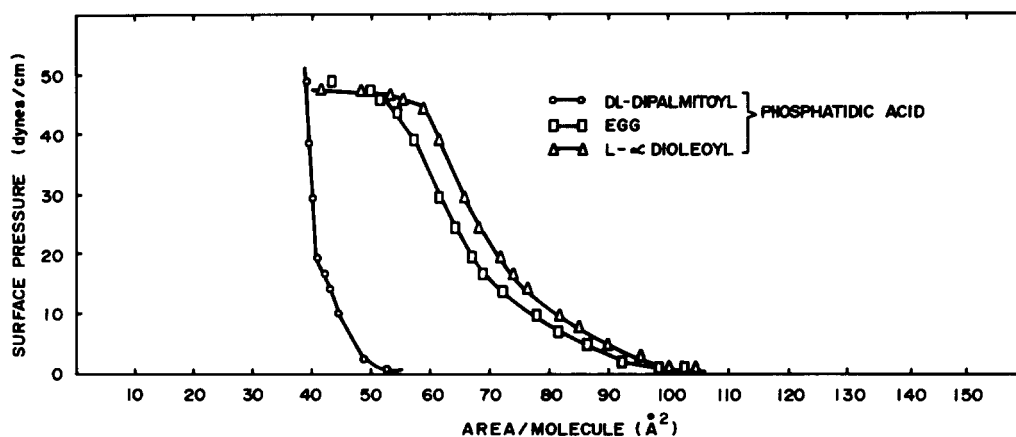


Fig. 1. Π - A isotherms for dipalmitoyl, egg, and dioleoyl phosphatidic acids on subphase containing 0.1 N HCL (pH 1.2). Films were compressed at 25 $\text{\AA}^2/\text{molecule}$ per min. Temperature was 24–26°C.

were dissolved in *n*-hexane–chloroform 80:20 (v/v) and these solutions were applied in 100- μl aliquots to a Teflon Langmuir trough (50 \times 10 \times 1 cm). Castor oil, which generated 17 ± 0.7 dynes/cm, was used as the piston oil. The reference subphase for unionized phosphatidic acid was 0.1 N hydrochloric acid (pH 1.2). The reference subphase for completely ionized phosphatidic acid was 0.1 N sodium hydroxide (pH 12.8). Other subphases contained 0.1 M sodium chloride, 0.01 M Tris, and sufficient concentrated hydrochloric acid or sodium hydroxide to adjust the subphase to the specified pH. All subphases contained 0.01 mM ethylenediaminetetraacetic acid (EDTA) unless the subphase contained a specific alkaline earth salt. These subphases contained 0.1 N sodium hydroxide with either calcium chloride (Ca^{2+} salt) or barium chloride (Ba^{2+} salt) at a specified concentration. The temperature was maintained at 24–26°C.

RESULTS AND DISCUSSION

Π - A isotherms of solid and liquid condensed films

Coherent surface films exist in solid (gel) and liquid-condensed phases and each phase has characteristic surface properties (10, 18, 20, 21). At 24–26°C, Π - A isotherms (Fig. 1) show that unionized dipalmitoyl phosphatidic acid behaves as a solid phase while unionized egg and dioleoyl phosphatidic acids behave as liquid-condensed phases. Thus the Π - A isotherm for dipalmitoyl phosphatidic acid (Fig. 1) is very similar to the Π - A isotherm for unionized palmitic acid (18). Both isotherms have initial or limiting areas near 25 $\text{\AA}^2/\text{acyl}$ chain and both isotherms show transitions near 20 dynes/cm

and 20 $\text{\AA}^2/\text{acyl}$ chain. These features of a Π - A isotherm are characteristic of solid films that exist in several polymorphic crystalline forms (18). It is highly interesting that both palmitic acid and dipalmitoyl phosphatidic acid, compounds with hydrogen-bonding polar groups, form solid films at low Π whereas dipalmitoyl phosphatidyl choline forms a more expanded liquid-condensed film (10). Π - A isotherms for unionized egg and dioleoyl phosphatidic acid films (Fig. 1) are expanded, do not show a phase transition, and collapse to a stable plateau. These features of a Π - A isotherm are characteristic of liquid-condensed films (18, 20, 21).

Ionization and desorption of phosphatidic acid monolayers at constant Π

Monolayers that are stable in the unionized form are converted to unstable desorbing monolayers when the ionized form has appreciable solubility. The initial desorption process at constant pressure occurs within the first 5 min and this process is described by the equation (22–24):

$$K_i = - \frac{d \ln A}{d \sqrt{t}} \quad \text{Eq. 1}$$

where A is the film area, t is time, and K_i is the initial desorption coefficient. K_i varies directly with the surface concentration of the desorbing species (ionized form) when ionization and equilibration are fast quasi-equilibrium processes (22–25).

Liquid-condensed films of egg or dioleoyl phosphatidic acids form relatively stable monolayers. Surface area at a constant Π of 17 dynes/cm does not change over a 5–10 min interval when films are spread on acidic, neutral, and basic subphases (see Table 1). Dipalmitoyl phosphatidic acid also forms a relatively stable film on acidic and neutral subphases.

However, A of a dipalmitoyl phosphatidic acid film decreases when the film is spread on alkaline subphases. The change in A with time follows the rate equation (Eq. 1) developed for an ionization-desorption process. An initial desorption coefficient, K_i , may be calculated for each subphase pH in the alkaline pH range. The K_i data show that the dipalmitoyl phosphatidate film is less stable than the dioleoyl phosphatidate film, just as the palmitate film is less stable than the oleate film (23, 24).

The K_i -pH isotherm for dipalmitoyl phosphatidic acid at 24–26°C is presented in Fig. 2. The apparent pK_2 for dipalmitoyl phosphatidic acid was obtained from these data after they were recast in the linear form (24):

$$\frac{1}{K_i} = \frac{1}{K_{imax}} + \frac{[H^+]}{K_{imax}K_2} \quad \text{Eq. 2}$$

where $1/K_{imax}$ is the intercept and $1/K_{imax}K_2$ is the slope of the $1/K_i$ vs. $[H^+]$ plot. The apparent surface pK_2 of dipalmitoyl phosphatidic acid, calculated from Eq. 2, is 9.4 at 24–26°C.

In other studies, the pK_2 for saturated phosphatidic acids has generally been estimated from T_c measurements. These studies suggest that the pK_2 occurs between 7 and 9 (3–6). However, T_c data are complicated by the fact that temperature affects the packing mode of phosphatidic acid in the monolayer and any change in packing will change the apparent

TABLE 1. Effect of alkaline earth cations on the surface areas of phosphatidic acids at 17 dynes/cm and 24–26°C

Conditions ^a	Phosphatidic Acid	
	Dioleoyl	Egg
	surface area ($\text{\AA}^2/\text{molecule}$)	
0.1 N HCl		
0 min	74	69
5 min	74	68
0.1 N NaOH		
0 min	89	86
5 min	88	85
0.1 N NaOH		
+ 1 mM CaCl_2		
0 min	73	68
5 min	70	65
+ 3 mM CaCl_2		
0 min	71	67
5 min	60	55
0.1 N NaOH		
+ 1 mM BaCl_2		
0 min	78	73
5 min	74	68
+ 3 mM BaCl_2		
0 min	72	69
5 min	58	56

^a Surface area was recorded at 0 min and after compression for 5 min.

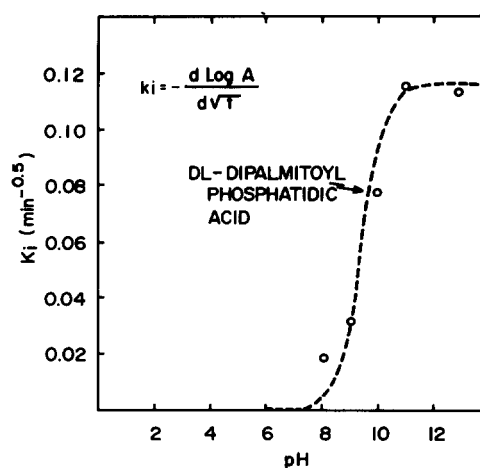


Fig. 2. K_i values for dipalmitoyl phosphatidic acid spread on the designated buffers (see text) and maintained at 17 dynes/cm with castor oil. Temperature was 24–26°C.

surface pK_a (24). For example, Jacobson and Papahadjopoulos (5) found that perylene polarization did not change in the pH 7–9 region when dipalmitoyl phosphatidic acid vesicles were maintained at 25°C. The polarization change indicated that the apparent pK_2 was 8.7 when the vesicles were maintained at 53.5°C. Our pK_2 and surface area data at 24–26°C are consistent with these observations.

Ionization and expansion of phosphatidic acid monolayers at constant Π

The apparent surface pK_1 of the carboxylic acid group in a solid film varies from 8.6 to 9.6 (24). Area-pH (A -pH) studies with saturated fatty acids at constant pressure (24, 26) show that ionization in a solid film has little effect on A until the pH approaches the apparent surface pK_1 . Surface area expands abruptly to a maximum in this region. These observations are explained by the formation of a condensed phase through short-range hydrogen bonds between the fatty acid anion and unionized fatty acid in the partially ionized acid-soap (24, 26–28). Surface area expands after the pK_1 through charge repulsion between anion molecules.

A -pH data for solid dipalmitoyl phosphatidic acid films (Fig. 3) are very similar to A -pH data for saturated long-chain fatty acid films (24, 26). The A -pH isotherm shows an abrupt increase in A near pH 8 and we may assume that the pK_1 for the saturated phosphatidic acid occurs in this region.

The A -pH isotherm for dipalmitoyl phosphatidic acid has another characteristic feature that strongly suggests that the pK_1 is near pH 8. Titration curves for dibasic acids will show two inflection points when the K_1/K_2 ratio approaches and exceeds 100 (29). The

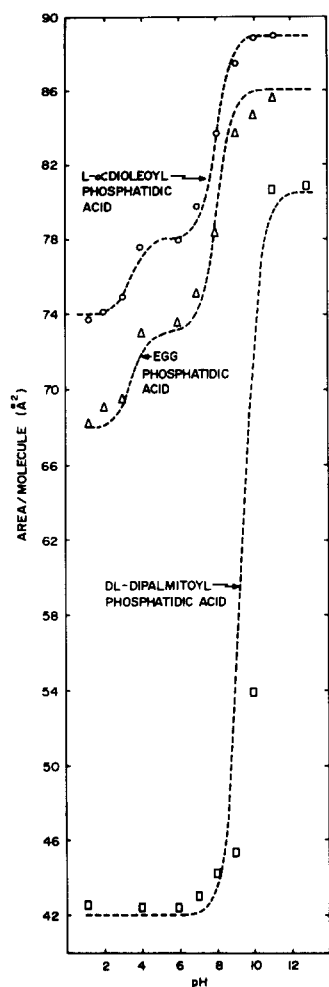


Fig. 3. Surface areas for dipalmitoyl, egg, and dioleoyl phosphatidic acids on the designated buffers (see text) maintained at 17 dynes/cm with castor oil. Temperature was 24–26°C. Dashed lines are calculated from the surface area at the pK. Midpoint surface areas are used for egg and dioleoyl phosphatidic acids. The surface area at pK₂, obtained from K_i-pH data, is used for dipalmitoyl phosphatidic acid.

A-pH isotherm for dipalmitoyl phosphatidic acid has only one inflection point (Fig. 3). Since the pK₂ for this acid is 9.4, only one inflection point will be found if the pK₁ approaches 8, a value consistent with film expansion.

A-pH data (Fig. 3) for unsaturated phosphatidic acids show two inflection points that yield apparent surface pK₁ and pK₂ values of 3.5 and 8.0, respectively. These pK values are in close agreement with titration (1) and surface potential (2) measurements for unsaturated phosphatidic acids. The pK₁ and pK₂ values for unsaturated phosphatidic acids are somewhat higher than the pK₁ and pK₂ values for phosphoric acid, 2 and 7, respectively, showing that surface charge density has a small but significant effect in these liquid-expanded films. However, changes in apparent sur-

face pK values in liquid-expanded phosphatidic acid films are much smaller than the change in the apparent surface pK value in the solid phosphatidic acid film.

Nagle (12) has suggested that weak-transient hydrogen bonds may exist in completely ionized phosphatidic acids. These bonds should decrease *A* in the completely ionized film and indeed experimental *A*-pH data (Fig. 3) show that *A* is less than expected with completely ionized phosphatidic acids. The *A* for unionized dipalmitoyl phosphatidic acid, 21 Å²/acyl chain (Fig. 3) is very similar to the *A*, 20 Å²/molecule, for unionized palmitic acid measured under identical conditions (30). The palmitate anion expands to 44 Å²/molecule with complete ionization (30) while the dipalmitoyl phosphatidate anion expands to only 40 Å²/acyl chain with complete ionization (Fig. 3). The *A* for unionized dioleoyl phosphatidic acid, 37 Å²/acyl chain (Fig. 3), is very similar to the *A*, 36.5 Å²/molecule, for unionized oleic acid measured under identical conditions (30). The oleate anion expands to 53 Å²/molecule with complete ionization (30) while the dioleoyl phosphatidate anion expands to only 44.5 Å²/acyl chain with complete ionization (Fig. 3). Thus *A*-pH data with saturated and unsaturated phosphatidic acids show that weak-transient hydrogen bonding forces may have significant effects on the properties of phosphatidate anions in surface films.

Physical parameters such as melting or boiling points are not available as criteria of purity for complex lipids. Surface areas in these lipids are established by the cross-sectional areas of the acyl chains. For example, the surface areas/acyl chain for some commercial dipalmitoyl and dioleoyl phosphatidic acids (Fig. 3) are the same as the surface areas/molecule of pure palmitic and oleic acids measured under identical conditions (30). Surface areas/acyl chain were increased as much as 33% in other commercial phosphatidic acid preparations, probably through contamination with diglycerides. These data suggest that the surface areas of an unionized lipid, measured under standard conditions, is a simple physical criterion of purity.

Counterion binding and stability of phosphatidic acid monolayers at constant Π

Dioleoyl and egg phosphatidate anions form stable and expanded films when they are spread on an alkaline subphase (Table 1). These expanded films are condensed through charge neutralization and the initial *A* (0 min) approaches the value for the unionized films when alkaline earth cations are added to the alkaline subphase (Table 1). The Ba²⁺ cation, as predicted (24), is less firmly bound (higher *A*) than the Ca²⁺ cation (lower *A*) to the strong anionic field. This

difference is most apparent at a lower cation concentration (Table 1) where competition with 100 mM Na⁺ is probably significant. Phosphatidate anion-alkaline earth complexes are unstable and a significant decrease in surface area occurs over a 5-min interval. The decrease in surface area resembles the collapse of the stearate anion-alkaline earth cation complex that we have reported previously (31). Film collapse occurs more rapidly on 3 mM alkaline earth subphases where *A* data show that the alkaline earth cations are more closely associated with the phosphatidate anion films. Several investigators (32, 33) have suggested that the phosphatidate anion may act as an ionophore for alkaline earth cations. Film collapse with phosphatidate anion-alkaline earth complexes shows that the complexes are removed from the interface, a process that supports the phosphatidic acid-ionophore hypothesis.



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