Phosphatidic Acid Distribution on the External Surface of Mixed Vesicles†

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ABSTRACT: A new method has been developed to detect the distribution of phosphatidic acid on the external surface of mixed phospholipid vesicles. Some positive dyes undergo large absorbance changes when the spatial separation between two or more dye molecules is smaller than a critical distance. When these dyes interact with mixed phospholipid vesicles, the absorbance changes may be utilized to calculate the amount of phosphatidic acid molecules which, on the external surface, occupy nearby positions not exceeding the critical dye distance, i.e., the amount of paired phosphatidic acid molecules. This amount was found to be higher than that calculated by statistical methods, indicating that phosphatidic acid molecules tend to be associated, in spite of the electrostatic repulsion between negative groups. The dependence of the amount of paired phosphatidic acid molecules on the pH, phosphatidylcholine:phosphatidic acid ratio, and temperature has been also analyzed.

The problem of the lipid distribution in binary mixed membranes containing lipids of different nature, for charge or acyl chain length, has been extensively studied in the last few years. Transbilayer asymmetric distribution of phospholipids was observed in mixed vesicles and natural membranes (Michaelson et al., 1973; Litman, 1974; Berden et al., 1975; Gocdesky and Marinetti, 1973). Lateral phase separation and cluster formation were observed in phospholipid vesicles containing cholesterol (Darke et al., 1971; Shimshick and McConnell, 1973b), in phosphatidylcholine membranes containing phosphatidic acid or phosphatidylserine upon addition of divalent cations (Ohnishi and Ito, 1973, 1974; Ito and Ohnishi, 1974; Papahadjopoulos et al., 1974; Jacobson and Papahadjopoulos, 1975; Ito et al., 1975). However, even in the absence of divalent cations, phosphatidic acid seems to be at least partially segregated from phosphatidylcholine in mixed vesicles (Galla and Sackmann, 1975); addition of Ca^{2+} dramatically increases the extent of phase separation.

In the present work we have investigated the external surface distribution of phosphatidic acid in phosphatidylcholine–phosphatidic acid vesicles. The amount of paired phosphatidic acid molecules on the external surface was found to be higher than the amount predicted by statistical analysis. This amount was correlated with various parameters, such as phosphatidylcholine:phosphatidic acid ratio, pH, and temperature.

Materials and Methods

Formation of Phospholipid Vesicles. Phosphatidylcholine was isolated from chicken egg yolks according to Ansell and Hawthorne (1964). Phosphatidic acid was obtained from egg phosphatidylcholine by means of phospholipase D (Papahadjopoulos and Miller, 1967). The products obtained from these procedures showed a single component on thin-layer chromatography. Synthetic β,γ-dipalmitoyl-DL-α-glycerylphosphorylcholine was supplied by Sigma. To prepare the lipid dispersion, lyophilized phospholipids were suspended in a medium containing 0.05 M KCl, 0.2 mM EDTA–Tris,[1] and 10 mM Hepes at pH 7, at a concentration of approximately 3–5%. The suspension was then ultrasonically irradiated with a Branson Sonifier under a nitrogen atmosphere for 20 min in a jacketed vessel maintained at 2 °C by a constant-temperature circulating bath. Undispersed phospholipids and titanium particles were removed by centrifugation at 40 000*g* for 20 min at 4 °C. Phosphorus determination was made with the method of Ames and Dubin (1970).

Spectrophotometric Measurements. Absorbance spectra of the dyes were performed in a split beam Hitachi Perkin-Elmer spectrophotometer Model 124. Titration measurements were carried out by adding equal amounts of phospholipid vesicles to both reference and sample cells. Absorbance measurements of the dyes were carried out in a Coleman Perkin-Elmer spectrophotometer Model 55.

Dye absorption on the cuvette walls was minimized by equilibration of the system with a dye solution before each experiment.

Binding Experiments. Binding data were obtained by equilibrium dialysis and ultrafiltration techniques. The dialysis device consisted of two polycarbonate cells, embedded in a plexiglass support, separated by a cellular dialyzer membrane. The volume of the lower cell, provided with a magnetic stirrer, was 2 cm^3, that of the upper cell, 1 cm^3. Polycarbonate cells were used for the low binding of the dyes to this plastic material. The experiments were performed as follows: the cells were filled with a solution containing 5 mM buffer and variable amount of dye. Then a fixed amount of vesicles was added in the lower cell and the system was allowed to reach equilibrium. To increase the dialysis rate, the cellulose membrane was stretched by the application of a hydrostatic pressure and a longitudinal stretching in a controlled apparatus as described by Craig and Konigsberg (1961). The equilibration time at 20 °C was about 20 h. Once equilibrium was reached, aliquots of the solution of the upper and lower cells were analyzed in the

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[1] Abbreviations Used: EDTA, disodium ethylenediaminetetraacetate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; P_i, inorganic phosphate.

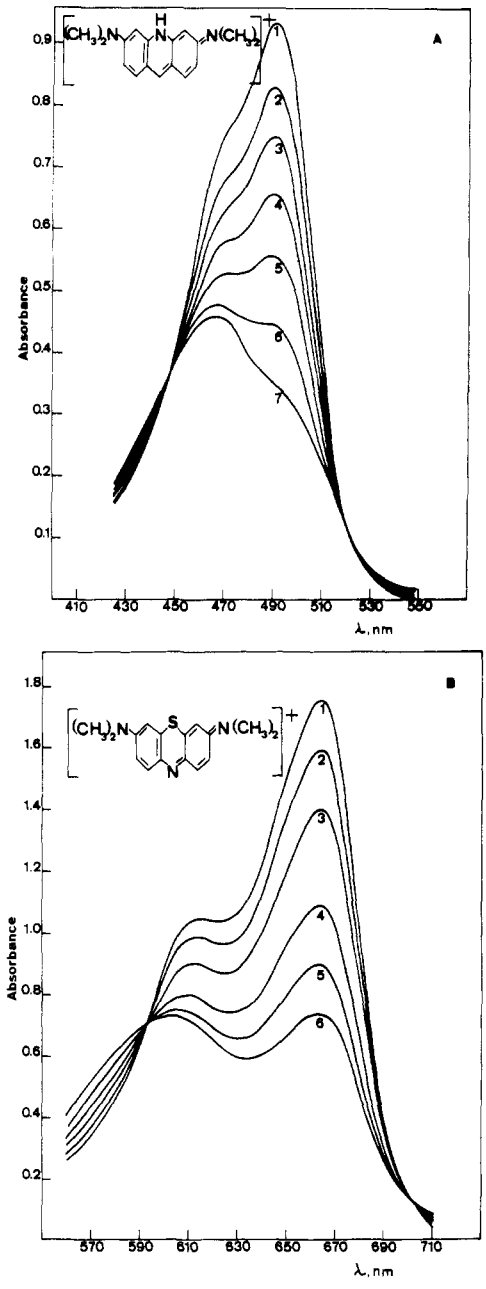

FIGURE 1: Dye spectral changes upon addition of phospholipid vesicles. The medium (2.5 mL) contained: 5 mM Hepes and 0.2 mM EDTA-Tris, pH 6. The vesicles were formed by dipalmitoylphosphatidylcholine–egg phosphatidic acid in the 3:1 ratio. (A) (1) Acridine orange, 18 μM; (2) 0.053 μmol of P_i; (3) 0.106 μmol of P_i; (4) 0.185 μmol of P_i; (5) 0.292 μmol of P_i; (6) 0.504 μmol of P_i; (7) 1.09 μmol of P_i. (B) (1) Methylene blue, 22 μM; (2) 0.106 μmol of P_i; (3) 0.292 μmol of P_i; (4) 0.530 μmol of P_i; (5) 0.740 μmol of P_i; (6) 1.27 μmol of P_i.

dye content by means of absorbance titration curves. To know the amount of dye in the lower cell, where phospholipids are present, the dye solution was added to methanol in a 1:3 ratio. The decrease in dielectric constant destroys the dye metachromatic behavior which depends on hydrophobic forces (Mukerjee and Gosh, 1970). The amount of dye bound to the cell walls and the dialysis membrane is the difference between the total amount of dye initially present and that present at equilibrium in the cells.

Ultrafiltration was performed with a syringe equipped with a membrane with 13 mm diameter and 7.5 nm pore size, provided by Gelman. The procedure for the analysis of the dye binding was the same used for the dialysis technique.

Resolution of the Scatchard plot into linear partial plots has been accomplished according to Ohnishi et al. (1972) using an Olivetti P602 computer.

Materials. Acridine orange was a product of Merck and purified according to Pal and Schubert (1962). Zn-free methylene blue was supplied by Baker and further recrystallized according to the same authors (Pal and Schubert, 1962). These two dyes were chosen for the following reasons: (i) their structure is very similar; (ii) the absorbance changes due to the dye–dye interactions are very large; (iii) the cationic form of the dye is not pH dependent (the pK_a of acridine orange is about 10.45 (Albert, 1966)); (iv) the maximum possible separation between nearest neighbor dyes where absorbance changes occur is less for methylene blue than for acridine orange (Stone and Bradley, 1967; Stone et al., 1963).

The molarity of the dyes was determined on the basis of titration curves, obtained by measuring the molar extinction coefficient of known amounts of dye. The molar extinction coefficient of a solution of 10 μM acridine orange at 492 nm was 54 200 ± 300, in agreement with the values reported (Zanker, 1952; Stone and Bradley, 1961, 1967; Furano et al., 1966; Myhr and Foss, 1971; Vitagliano et al., 1973). The molar extinction coefficient of a solution of 10 μM methylene blue at 664 nm was 83 000 ± 500, also in agreement with the values already reported (Bergmann and O'Konski, 1963).

Stock aqueous solutions of 1 mM acridine orange and methylene blue were maintained at 4 °C in the dark.

All experiments were performed at room temperature. Hepes was used in all experiments in view of the fact that this buffer, differently from others, does not interact with phosphatidic acid molecules (Schreier-Mucillo et al., 1973). The phospholipid vesicles were maintained at 4 °C and used no longer than a week after sonication.

Results

In Figure 1A and B are reported the spectral changes of the dyes acridine orange and methylene blue following the addition of increasing amounts of phosphatidylcholine–phosphatidic acid vesicles in the 3:1 ratio. The absorbance decrease, the relative increase of the maximum at the shorter wavelengths, and the presence of an isosbestic point, at 448 and 593 nm for acridine orange and methylene blue, respectively, are typical phenomena of the interaction of these dyes with polyanions (Stone et al., 1963; Stone and Bradley, 1967; Hammes and Hubbard, 1966; Blake and Peacocke, 1968; Saunders, 1968; Ikeda and Imae, 1971; Lagunoff, 1974). The metachromatic behavior of these and similar dyes was attributed to the stacking of two or more dye molecules bound to near sites (Michaelis, 1950). In the case of phospholipid vesicles, the spectral shifts may be attributed to the interaction of two or more dye molecules electrostatically bound to near phosphatidic acid molecules. With phosphatidylcholine vesicles, no spectral shifts were detected. The isosbestic point disappears when a relatively high amount of vesicles is added. This probably is due to an unbalanced light scattering signal in the sample and reference cells. However, at alkaline pH, where the second group of phosphatidic acid becomes dissociated, a smaller amount of vesicles is required to have a deviation from the isosbestic point. It seems, therefore, that not only the dimeric but also higher aggregate forms of the dye are present. When the amount of phosphatidic acid exceeds the amounts of dye present, the absorbance, after reaching a minimum, tends to return to the original level, especially in the case of acridine orange. This phenomenon, observed also with polyanions, was attributed to the redistribution of the dye molecules

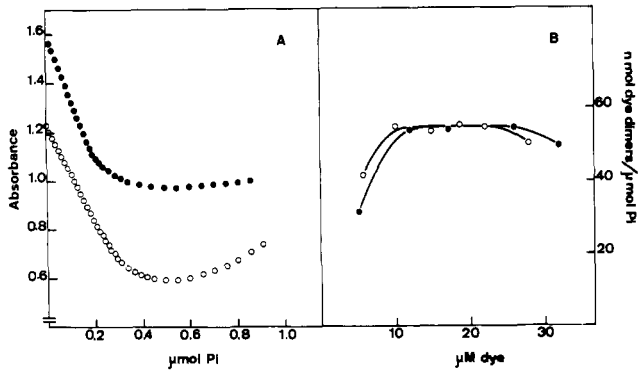

FIGURE 2: Dye spectrophotometric titrations with phospholipid vesicles. The medium and the vesicles were the same as in Figure 1. (A) Absorbance changes at 492 nm for 25 μM acridine orange (O—O) and at 664 nm for 20 μM methylene blue (●—●). (B) Amount of dye dimers at various acridine orange (O—O), or methylene blue (●—●) concentrations.

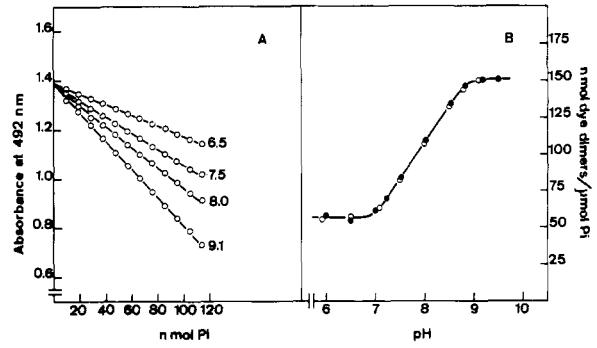

FIGURE 3: pH dependence of the amount of dimers of dye bound. The medium (2.5 ml) contained: 0.2 mM EDTA-Tris, 5 mM Tris (pH 9), and 28 μM acridine orange (O—O) or 22 μM methylene blue (●—●). The pH values were adjusted by adding increasing amount of HCl. The numbers in Figure 3A indicate the pH values. The phospholipid vesicles were the same as in Figure 1.

from an aggregate to a monomeric bound form over the negative groups of the polyanion. Let us assume that: (i) the dyes do not penetrate into the vesicles, and (ii) only one molecule of dye binds to one molecule of phosphatidic acid, at acidic pH (see Discussion). With high dye concentration, when all the negative groups of the external phosphatidic acid bind the dye, it is possible to calculate the amount of external paired phosphatidic acid molecules from the absorbance changes of the dye. If A is the absorbance of the dyes at their maximum, 492 and 664 nm for acridine orange and methylene blue, respectively, ϵ_f, ϵ_m, and ϵ_d the molar extinction coefficients for the free monomer dye f, the bound monomer dye m, and the bound dimer dye d, respectively, the absorbance of a 1-cm path length cuvette containing dye and phospholipid vesicles in a buffered solution is:

$$A = \epsilon_f[f] + \epsilon_m[m] + \epsilon_d[d] \quad (1)$$

The total dye concentration is:

$$D_t = [f] + [m] + 2[d] \quad (2)$$

If $\epsilon_f = \epsilon_m$, as already established (Stone and Bradley, 1961; Vitagliano et al., 1973), combining eq 1 with 2, we obtain the dimer concentration:

$$[d] = \Delta A/(2\epsilon_f - \epsilon_d) \quad (3)$$

where $\Delta A = \epsilon_m D_t - A$. For acridine orange, ϵ_f = 55 000 and ϵ_d = 14 000 at 492 nm; for methylene blue, ϵ_f = 83 000 and ϵ_d = 26 000 at 664 nm. It is possible that on the surface of the vesicles are present not only dye molecules in the dimer form, but also in n-meric form. However, the extinction coefficient of dimers at the maximum of the longer wavelength band differs only slightly from that of n-mers (Vitagliano et al., 1973). Therefore a n-mer is equivalent, from the absorbance point of view, to $n/2$ dimers.

Figure 2A shows the spectrophotometric titrations of the dyes with phosphatidylcholine–phosphatidic acid in the 3:1 ratio. The absorbance of the maximum at the longer wavelength decreases linearly with the amount of vesicles added, until an end point is reached, where the absorbance has a minimum. The slope of the straight line indicates that, for both dyes, the amount of paired phosphatidic acid molecules on the external surface of the vesicles is 110 ± 8 nmol/μmol P_i, i.e., about 70% of the total external phosphatidic acid. This value is higher than the value obtained by statistical analysis: 36 nmol/μmol P_i (see Appendix). The amount of paired phosphatidic acid molecules did not change if: (i) egg lecithin was used in place of dipalmitoyllecithin; and (ii) the vesicles were obtained by mixing phospholipids in lyophilized form instead of lyophilizing a mixture of phospholipids in organic solvent.

The linearity between absorbance and amount of vesicles added extends further with acridine orange than with methylene blue. This is probably due to the different sensitivity of the two dyes to the site distance (Stone et al., 1963; Stone, 1967). Since the distance between two paired phosphatidic acid molecules may be variable (see Appendix), methylene blue redistributes itself to those sites having an effective interdye distance which favors stronger binding and greater metachromasy, when the amount of paired phosphatidic acid molecules exceeds the amount of dye bound (Stone et al., 1963; Stone and Bradley, 1967). In Figure 2B is shown that the amount of paired phosphatidic acid molecules, calculated by the absorbance changes following the addition of 84 nmol of P_i of mixed vesicles in the 3:1 ratio according to eq 3, is independent of the dye concentration in the range 10–25 μM. At dye concentrations lower than 10 μM, the amount of paired phosphatidic acid molecules is lower because it exceeds the amount of dye bound. At dye concentrations higher than 25 μM, the amount of paired phosphatidic acid molecules decreases because, at these dye concentrations, free dye dimers are present in solution. Therefore, as soon as the dyes bind to the vesicles, free dimers dissociate into monomers giving an absorbance increase. The dimerization constants of acridine orange and methylene blue in aqueous solution are 2.4×10^4 and 2.2×10^3 M^{-1}, at room temperature, respectively (Zanker, 1952; Ghosh and Mukerjee, 1970). Using these values, it can be easily calculated that, at 30 μM, about 6.5 μM dimers for acridine orange and 1.5 μM for methylene blue are present.

At acidic pH, where all phosphatidic acid molecules are in monoprotonated form HPA^-, the binding of one dye molecule could modify the pK_a of the second group of phosphatidic acid, leading to the binding of two dye molecules:

$$2[dye^+] + [HPA^-] \rightleftharpoons [(dye)_2PA] + [H^+] \quad (4)$$

However, this mechanism should lead to a stoichiometric extrusion of hydrogen ions (Abramson et al., 1966). Adding 120 μM of acridine orange to 5 mL of solution containing 0.1 mM EDTA-Tris, 3.3 mM KCl, 0.66 mM Tris-Cl (pH 6.3) and 2 mM P_i of phosphatidylcholine–phosphatidic acid vesicles in the 3:1 ratio, the H^+ released was only 0.01 μequiv/μmol of external phosphatidic acid molecules, i.e., about 5% of the total amount of dye bound to the vesicles.

Figure 3B shows the pH dependence of the amount of dimers

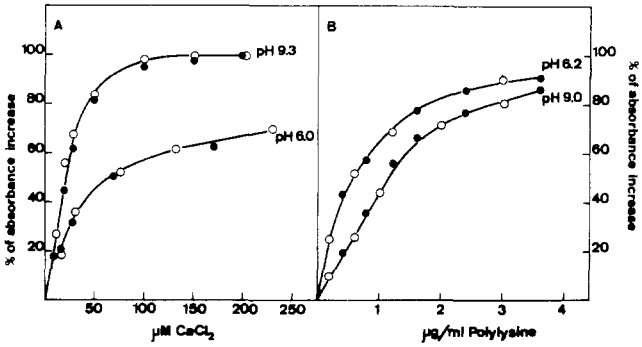

FIGURE 4: Effect of external cations on the absorbance changes of the dyes. The medium (2.5 mL) contained: 0.2 mM EDTA-Tris, 5 mM Tris, 0.38 μmol of P_i in A and 0.04 μmol of P_i in B of dipalmitoylphosphatidylcholine-egg phosphatidic acid vesicles in the 3:1 ratio and 25 μM acridine orange (O—O) or 25 μM methylene blue (●—●). The degree of polymerization of polylysine was 2000. The scale of the ordinate indicates the percent of absorbance increase of the dyes at their maxima after addition of external cations with respect to the initial absorbance change due to the mixing of dyes and vesicles.

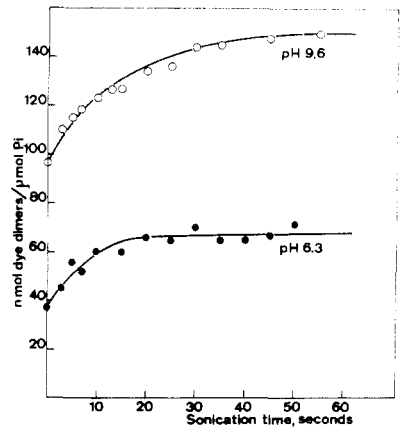

FIGURE 5: Dependence of the amount of dye dimers on the sonication time. (O—O) The medium (3 mL) contained: 5 mM Tris, 0.2 mM EDTA-Tris, 22 μM methylene blue, and 33 nmol of P_i of dipalmitoylphosphatidylcholine-egg phosphatidic acid vesicles in the 5.5:1 ratio. Final pH was 9.6. (●—●) The medium (3 mL) contained: 5 mM Hepes, 0.2 mM EDTA-Tris, 22 μM methylene blue, and 66 nmol of P_i of the same vesicles. Final pH was 6.3.

of bound dye, obtained by spectrophotometric titrations, as in Figure 3A for acridine orange. The molar extinction coefficient of the dye dimers does not depend on the pH of the medium. At alkaline pH, when phosphatidic acid is in double ionized form, each molecule can bind two dye molecules. Therefore the amount of dimers of bound dye should coincide with the amount of external phosphatidic acid molecules, which, in the case of 3:1 vesicles, is about 150 nmol/μmol P_i (see Appendix). This value and that obtained by spectrophotometric titrations are about the same. The spectral shifts of acridine orange may be used as a means of determining the pK_a value of the binding groups (Lagunoff, 1974). Fifty percent of dissociation of the second group of phosphatidic acid occurs at pH of about 8. pK_a values close to 8 were found by Abramson et al. (1964).

Figure 4 shows the effect of the addition of $CaCl_2$ and polylysine (degree of polymerization 2000) on the dye absorbance after mixing dye and vesicles. The cations cause an absorbance increase, probably due to competition with the dye for the external negative groups. At alkaline pH, 200 μM $CaCl_2$ induces a complete return of the absorbance to the initial level. At acidic pH higher Ca^{2+} concentration cannot be reached, because vesicle aggregation induced by high concentration of divalent cation has different light-scattering effects in the reference and sample cells. At alkaline pH the effect of polylysine is smaller than at acidic pH because part of the polylysine groups are uncharged.

Figure 5 shows the amount of dye dimers calculated from the absorbance change after sonication of vesicles and methylene blue. During the sonication, titanium is released into the solution, especially during extended sonication, and dye is partially adsorbed in the glass vessels and in the sonifier tip. Due to the titanium particles released, the absorbance increases. The absorbance was therefore corrected by substracting to the absorbance at 664 nm, the absorbance of the solution of 750 nm, where the molar extinction coefficient of the dye is negligibly small. The absorbance value of the dye in the absence of vesicles was obtained by diluting 0.5 mL of the solution with 2 mL of methanol. In apolar solvents both the vesicles and the dye dimers are destroyed; the initial absorbance value therefore may be calculated using a calibration curve. At alkaline pH (upper curve) the amount of dye dimers in 5.5:1 vesicles increases from 93 to 150 nmol/μmol P_i. The latter value is in good agreement with the total amount of phosphatidic acid molecules present. The percentage of external phosphatidic acid may be calculated by dividing the amount of dye dimers at zero time of sonication by the total amount of dye dimers at infinite time of sonication, both at alkaline pH. For mixed vesicles in the 5.5:1 ratio this percentage is about 62%; for vesicles in the 3:1 ratio, the value is 61%. For unfractionated phosphatidylcholine vesicles, the percentage of external phosphatidylcholine is about 65% (Berden et al., 1975). Our lower value for mixed vesicles may be explained either by the transbilayer asymmetrical distribution of phosphatidic acid, or by the greater size of mixed compared with phosphatidylcholine vesicles. If $CaCl_2$ is present during cosonication, absorbance change is not observed; if $CaCl_2$ is added after cosonication, the residual absorbance change corresponds only to the amount of internal phosphatidic acid molecules.

Figure 6 shows the Scatchard plot of the binding of acridine orange to the phospholipid vesicles, obtained at room temperature by dialysis and filtration experiments. The dashed lines are obtained by resolution of the Scatchard plot according to Ohnishi et al. (1972). The amount of low and high affinity sites is 108 and 50 nmol/μmol P_i, respectively; the instability constants are 35 and 3.5 μM, respectively. The total amount of sites agreed with the total amount of external phosphatidic acid molecules (150 nmol/μmol P_i), and the amount of high affinity sites agreed with the amount of phosphatidic acid molecules which occupy nearby positions, as derived from the absorbance measurements (110 nmol/μmol P_i). The difference in stability constants between dye bound to one molecule and paired molecules of phosphatidic acid is due to the well-known tendency of the metachromatic dyes to occupy preferentially adjoining rather than monomeric sites (Pal and Schubert, 1962; Young et al., 1967).

Figure 7 shows the dependence of the external paired phosphatidic acid molecules on the phosphatidylcholine–phosphatidic acid ratio, as inferred from the spectrophotometric titrations. In Figure 7A, the points represent the experimental values obtained by titrations at alkaline pH with both dyes, whereas the solid line is the theoretical value of the external amount of phosphatidic acid molecules. The two values are about the same. In Figure 7B the points represent the experimental values obtained by titrations at acidic pH with both dyes, whereas the solid line is the statistical calculation of the amount of paired phosphatidic acid molecules. The

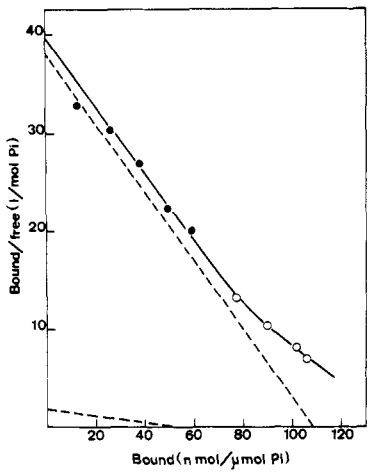

FIGURE 6: Scatchard plot of the binding of acridine orange to the vesicles. The medium (2.5 mL) contained 5 mM Hepes and 0.2 mM EDTA-Tris, pH 6. (●—●) Obtained by dialysis experiments; the dye concentration varied between 2 and 10 μM; 0.4 μmol of P_i. (○—○) Obtained by filtration experiments; the amount of P_i varied between 0.1 and 0.4 μmol; 18 μM dye. The vesicles were formed by dipalmitoylphosphatidylcholine-egg phosphatidic acid in the 3:1 ratio.

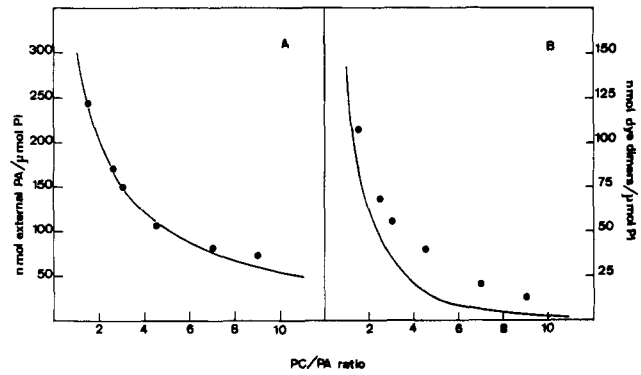

FIGURE 7: Dependence of the amount of dimers of dye bound on the phosphatidylcholine-phosphatidic acid ratio. The medium was the same as in Figure 1, except for the pH values. (A) Nanomoles of external phosphatidic acid/μmol of P_i as deduced by spectrophotometric titrations at pH 9.3 with both acridine orange and methylene blue. (B) Nanomoles of dye dimers/μmol of P_i as deduced by spectrophotometric titrations at pH 6 with both dyes. For the solid lines, see explanation in the text. PA, phosphatidic acid; PC, phosphatidylcholine.

deviation from the values expected by the statistical predictions is present in all the tested ratios, but is higher at high phosphatidylcholine-phosphatidic acid ratios (Galla and Sackmann, 1975).

At room temperature egg phosphatidylcholine-egg phosphatidic acid vesicles are in the fluid state. Egg phosphatidic acid will decrease the transition temperature of dipalmitoylphosphatidylcholine (about 41 °C, Ladbrook and Chapman, 1969; Jacobson and Papahadjopoulos, 1975) and will broaden it. It is possible therefore that dipalmitoylphosphatidylcholine-egg phosphatidic acid vesicles are in the fluid state also at room temperature. In order to check if the amount of dye dimers increases below the phase transition, we have measured this amount at various temperatures from 7 to 55 °C. Dipalmitoylphosphatidylcholine-egg phosphatidic acid vesicles in the 3:1 ratio were incubated for 2 h at the initial temperature and, for each 2 °C temperature interval, the sample was heated for 10 min. In the experiments at higher temperature, the dye concentration was increased, to counteract the decrease of the dimerization constant (Zanker, 1952). The amount of dye dimers remains constant at all temperature interval. This is unexpected in view of the fact that lateral phase separation will occur for some phospholipid mixtures below the phase transition (Shimshick and McConnell, 1973a). However, no evidences have been reported that phase separation occurs in solid state between phosphatidylcholine and phosphatidic acid.

Discussion

The spectral shifts of positive metachromatic dyes, acridine orange and methylene blue, in the presence of phospholipid vesicles containing net negative charged groups, are probably due to the interaction of the dye molecules electrostatically bound to nearby negative groups (Massari, 1975). In absence of negative charged groups, i.e., with phosphatidylcholine vesicles, no spectral changes were observed. Moreover, by increasing the net negative charge, either by increasing the pH or decreasing the phosphatidylcholine-phosphatidic acid ratio, the spectral shifts were parallelly enhanced. Part of the spectral shifts may be due to the internal negative charged groups. However, at room temperature, a movement of dyes across the membrane is unlikely for the following reasons: (i) external nonpenetrating cations, such as Ca^{2+} and polylysine, compete with the dyes to the external sites and tend to abolish the dye spectral shifts; (ii) cosonication of dyes and vesicles results in an increase in the amount of dye dimers. At alkaline pH the total amount of phosphatidic acid molecules is the same as the amount of dye dimers; (iii) the mixing time between dyes and solution containing vesicles is of the order of 1 s, followed by an absorbance decrease which is maintained for a long time; it is unlikely that dye penetration (and perhaps collision-induced rupture of the vesicles) takes place in this very short time, at room temperature. It seems therefore that the spectral shifts reflect the interaction between dye molecules and external negatively charged groups.

Acridine orange, methylene blue, and other metachromatic dyes were shown to form a 1:1 stoichiometric complex with binding sites (Stone and Bradley, 1961, 1967; Overath and Träuble, 1973). These dyes indeed were used to calculate the amount of binding sites for a large variety of polyanions (Stone and Bradley, 1967; Stone et al., 1963). In the case of the vesicles, however, the stoichiometry may be different from 1:1. A surface potential may increase the free concentration of dye molecules at the interface with respect to the bulk concentration. Due to the high concentration at the interphase, the dye can aggregate giving a high amount of dimers. According to the Graham equation (McLaughlin et al., 1971), phosphatidylcholine-phosphatidic acid vesicles in the 3:1 ratio, incubated in 5.2 mM Tris, give a surface potential of 136 mV. In a charge screening mechanism, the dye concentration is too low to affect this potential. Therefore, by increasing the dye concentration, more and more dye molecules should concentrate at the interface. However, as shown in Figure 2B, the amount of dye dimers reaches a plateau at very low dye concentration. It seems therefore that an absorption or binding mechanism is involved in the dye dimers formation. Hydrophobic molecules, such as 8-anilino-1-napthalenesulfonate and atebrine, absorb or bind to the vesicles (McLaughlin et al., 1971; Massari, 1975). For methylene blue and acridine orange a direct evidence for a dye-site stoichiometry of 1:1 is: (i) the sharp coincidence between the total amount of binding sites derived from the Scatchard plot and the amount of external phosphatidic acid molecules; (ii) the sharp coincidence between the amount of binding sites calculated from the absorbance changes at alkaline pH and the amount of external phosphatidic acid molecules.

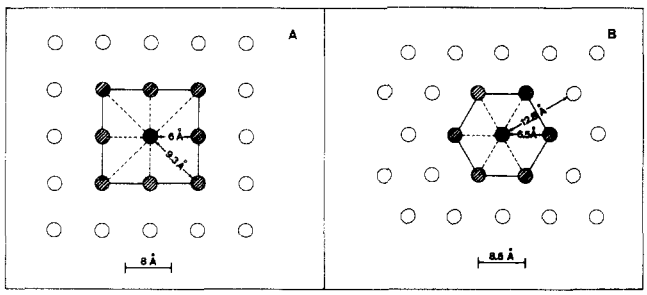

FIGURE 8.

If only one molecule of dye binds electrostatically to one molecule of phosphatidic acid on the external surface of the vesicles, then the spectral shifts are a consequence of a dye-dye interaction of two or more dye molecule bound to near negative charged groups. It is possible therefore to obtain, from the spectral shifts, the amount of paired phosphatidic acid molecules on the external surface of the vesicles.

The higher amount of paired phosphatidic acid molecules in respect to the statistical predictions may be a consequence of a phospholipid redistribution following the binding of the dyes to the vesicles. With the phospholipid vesicles in a high dynamic state, two phosphatidic acid-dye complexes, initially far from each other, can occupy near positions in view of the fact that the bound dye decreases the electrostatic repulsion between two negative groups and increases the attraction between two complexes in consequence of the dye-dye aggregation. There are three lines of evidence against this interpretation: (i) if the dye induces a reorganization of the lipid distribution, the amount of paired phosphatidic acid molecules should depend on the amount of dye bound to isolated phosphatidic acid molecules, i.e., on the total amount of dye. However, as shown in Figure 2B, this amount does not vary in the range of dye concentrations between 10 and 30 μM. (ii) The amount of isolated phosphatidic acid molecules increases, increasing the phosphatidic acid-phosphatidylcholine ratio; one would expect, in the case of a reorganization of the lipid distribution, a higher amount of paired phosphatidic acid molecules at higher phosphatidic acid-phosphatidylcholine ratios with respect to the statistical predictions. However, the discrepancy between the experimental and the statistical data is higher at low phosphatidic acid-phosphatidylcholine ratios; (iii) a reorganization of the lipid distribution induced by the dye should lead to a cooperativity in the binding curve. However, the Scatchard plot is linear.

Galla and Sackmann (1975), using spin-labeled lecithin, showed that, in mixed vesicles in the fluid state, lateral phase separation occurs between phosphatidylcholine and phosphatidic acid. However, the presence of a nitroxide group in the acyl chain may influence the degree of randomization of the mixture; moreover the changes in the external frequency do not give a real indication of phase separation if no correlation is made between amount of paired phosphatidic acid calculated statistically and that derived by the experimental data. In the present work we have confirmed that the two phospholipids do not mix statistically, and that phosphatidic acid molecules tend to be paired on the external surface of the vesicles. Clearly nothing can be said about the cluster formation of one component with respect to the other, because the dye spectral change method detects only the presence of two neighbor phosphatidic acid molecules.

Partial immiscibility between two components in binary mixtures may depend on the chain length of the phospholipids. Galla and Sackmann (1975), however, found lateral phase separation in phosphatidylcholine-phosphatidic acid vesicles even when the two phospholipid components have the same chain length (dipalmitoyl phospholipids). The deviation from statistical distribution seems to depend therefore on the different structure of the phosphatidylcholine and phosphatidic acid polar head groups.

From the present work we cannot rigorously exclude the possibility that the phosphatidylcholine-phosphatidic acid ratio varies from vesicles to vesicles, preferring the phosphatidic acid molecules to be situated in vesicles rich of phosphatidic acid rather than of phosphatidylcholine. If this were the case, the amount of paired external phosphatidic acid molecules could coincide with the amount calculated statistically. However, the conclusion that the electrostatic repulsion between negative groups is not a prevailing force to account for the interaction between two phosphatidic acid molecules would still remain valid.

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Appendix

In order to arrive at the dye spectral changes, the interplanar distance between adjacent dye molecules must be sufficiently close so that electronic interaction (π electrons) between neighbors is possible. It is generally agreed that the maximal site distance may not exceed 9-10 Å (Stone et al., 1963; Shoenber and Moore, 1964; Stone, 1967). The area of a phospholipid head group in phosphatidylcholine or phosphatidylcholine-phosphatidic acid vesicles is about 64 Å^2 (Gulik-Krzywicki et al., 1967; Johnson et al., 1971; Träuble and Haynes, 1971; Johnson, 1973; Papahadjopoulos, 1973). The two-dimensional lattice representing the external surface of the vesicles may be described as in Figure 8A and B. In both cases the surface area per phospholipid molecule is 64 Å^2, but, if the "unit cell" is square, the distance between two neighbor phospholipid molecules is 8 Å, whereas, if the unit cell is hexagonal, the distance is 8.5 Å. Taking into account the capacity of rotation of the phosphoric group around the P-O bond, the minimal distance between two negatively charged oxygens is about 6 and 6.5 Å, respectively. In Figure 8A and B, the black circles represent a phosphatidic acid molecule; the dashed circles represent the possible positions of another phosphatidic acid molecule which can be detected by the dye-dye interaction.

On the hypothesis that there is no interaction between the phospholipid molecules, it is possible to calculate statistically the amount of phosphatidic acid molecules which occupy two positions in the lattice where dye-dye interaction can be detected. In mixed vesicles the phosphatidic acid-phosphatidylcholine ratio of the unit cell may assume the values i/j, where $2 \leqslant i \leqslant 9$, $0 \leqslant j \leqslant 7$, and $i + j = 9$ in the case of a square unit cell, and $2 \leqslant i \leqslant 7$, $0 \leqslant j \leqslant 5$, and $i + j = 7$ in the case of a hexagonal unit cell. The probability Pij of a configuration i/j, taking into account that the phosphatidylcholine-phosphatidic acid ratio of the whole system is a/b, is:

$$Pij = \binom{a}{i}\binom{b}{j} \Big/ \binom{a+b}{i+j} \qquad (5)$$

For each configuration i/j, the probability $P'ij$ to have at least one phosphatidic acid molecule in one of the dashed positions of the unit cell is:

$$P'ij = [i!j!(i - 1 + j)!]/[(i + j)!j!(i - 1)!] \quad (6)$$

Then the total probability P to have paired phosphatidic acid molecules on the external surface is:

$$P = \sum_{2}^{i+j}{}_{i} (PijP'ij) \quad (7)$$

For instance, if $a/b = 3/1$, $P \cong 0.23$ in the case of the square unit cell, and $P \cong 0.20$ in the case of the hexagonal unit cell. The probability to have three phosphatidic acid molecules which occupy dye detectable positions may be calculated taking as unit cell a cell which includes all the possible positions. In the case of 3/1 vesicles, this probability is very small, contributing only 6 and 9%, respectively, to the total probability. If about 60% of the phospholipid molecules are external, the amount of paired phosphatidic acid molecules of 1 μmol of P_i of phosphatidylcholine–phosphatidic acid in the ratio a/b is:

$$\frac{6 \times 10^{-6}}{(a/b + 1)} P \quad (8)$$

in mol/μmol of P_i. If $a/b = 3$, the amount of paired phosphatidic acid molecules is about 36 nmol/μmol of P_i.

References

Abramson, M. B., Katzman, R., Gregor, H., and Curci, R. (1966), *Biochemistry 5,* 2207.

Abramson, M. B., Katzman, R., Wilson, C. E., and Gregor, H. P. (1964), *J. Biol. Chem. 239,* 4066.

Albert, A. (1966), in The Acridines, Arnold, E., Ed., London, LTD London, p 170.

Ames, B. N., and Dubin, D. T. (1970), *J. Biol. Chem. 235,* 769.

Ansell, G. B., and Hawthorne, J. N. (1964), in Phospholipids, Chemistry, Metabolism and Function, Vol. 3, BBA Library, Amsterdam, Elsevier, p 88.

Berden, J. A., Barker, R. W., and Radda, G. K. (1975), *Biochim. Biophys. Acta 375,* 186.

Bergmann, K., and O'Konski, C. T. (1963), *J. Phys. Chem. 67,* 2169.

Blake, A., and Peacocke, A. R. (1968), *Biopolymers 6,* 1225.

Craig, L. C., and Konigsberg, W. (1961), *J. Phys. Chem. 65,* 166.

Darke, A., Finer, E. G., Flook, A. K., and Phillips, M. C. (1971), *FEBS Lett. 18,* 326.

Furano, A. V., Bradley, D. F., and Childers, L. G. (1966), *Biochemistry 5,* 3044.

Galla, H. J., and Sackmann, E. (1975), *Biochim. Biophys. Acta 401,* 509.

Ghosh, A. K., and Mukerjee, P. (1970), *J. Am. Chem. Soc. 92,* 6408.

Gocdesky, S. E., and Marinetti, G. V. (1973), *Biochem. Biophys. Res. Commun. 50,* 1027.

Gulik-Krzywicki, T., Rivas, E., and Luzzati, V. (1967), *J. Mol. Biol. 27,* 383.

Hammes, G. G., and Hubbard, C. D. (1966), *J. Phys. Chem. 70,* 1615.

Ikeda, S., and Imae, T. (1971), *Biopolymers 10,* 1743.

Ito, T., and Ohnishi, S. (1974), *Biochim. Biophys. Acta 352,* 29.

Ito, T., Ohnishi, S., Ishinaga, M., and Kito, M. (1975), *Biochemistry 14,* 3064.

Jacobson, K., and Papahadjopoulos, D. (1975), *Biochemistry 14,* 152.

Johnson, S. M. (1973), *Biochim. Biophys. Acta 307,* 27.

Johnson, S. M., Bangham, A. D., Hill, M. W., and Korn, E. D. (1971), *Biochim. Biophys. Acta 233,* 820.

Ladbrooke, B. D., and Chapman, D. (1969), *Chem. Phys. Lipids 3,* 304.

Lagunoff, D. (1974), *Biochemistry 13,* 3982.

Litman, B. J. (1974), *Biochemistry 13,* 2844.

Massari, S. (1975), *Biochim. Biophys. Acta 375,* 22.

McLaughlin, S. G. A., Szabo, G., and Eisenman, G. (1971), *J. Gen. Physiol. 58,* 667.

Michaelis, L. (1950), *J. Phys. Chem. 54,* 1.

Michaelson, D. M., Horwitz, A. F., and Klein, M. P. (1973), *Biochemistry 12,* 2637.

Mukerjee, P., and Gosh, A. K. (1970), *J. Am. Chem. Soc. 92,* 6419.

Myhr, B. C., and Foss, J. G. (1971), *Biopolymers 10,* 425.

Ohnishi, S., and Ito, T. (1973), *Biochem. Biophys. Res. Commun. 51,* 132.

Ohnishi, S., and Ito, T. (1974), *Biochemistry 13,* 881.

Ohnishi, T., Masoro, E. J., Bertrand, H. A., and Yu, B. P. (1972), *Biophys. J. 12,* 1251.

Overath, P., and Träuble, H. (1973), *Biochemistry 12,* 2625.

Pal, M. K., and Schubert, M. (1962), *J. Am. Chem. Soc. 84,* 4384.

Papahadjopoulos, D. (1973), in Form and Function of Phospholipids, Ansell, G. B., Dawson, R. M. C., and Hawthorne, J. N., Ed., Amsterdam, Elsevier, p 143.

Papahadjopoulos, D., and Miller, N. (1967), *Biochim. Biophys. Acta 135,* 624.

Papahadjopoulos, D., Poste, G., Schaeffer, B. E., and Vail, W. J. (1974), *Biochim. Biophys. Acta 352,* 10.

Saunders, A. M. (1968), *Stain Technol. 43,* 207.

Schreier-Mucillo, S., Butler, K. W., and Smith, I. C. P. (1973), *Arch. Biochem. Biophys. 159,* 297.

Shimshick, E. J., and McConnell, H. M. (1973a), *Biochemistry 12,* 2351.

Shimshick, E. J., and McConnell, H. M. (1973b), *Biochem. Biophys. Res. Commun. 53,* 446.

Shoenber, M. D., and Moore, R. D. (1964), *Biochim. Biophys. Acta 83,* 42.

Stone, A. L. (1967), *Biochim. Biophys. Acta 148,* 193.

Stone, A. L., and Bradley, D. F. (1961), *J. Am. Chem. Soc. 83,* 3627.

Stone, A. L., and Bradley, D. F. (1967), *Biochim. Biophys. Acta 148,* 172.

Stone, A. L., Childers, L. G., and Bradley, D. F. (1963), *Biopolymers 1,* 111.

Träuble, H., and Haynes, D. H. (1971), *Chem. Phys. Lipids 7,* 324.

Vitagliano, V., Costantino, L., and Zagari, A. (1973), *J. Phys. Chem. 77,* 204.

Young, M. D., Phillips, G. O., and Balazs, E. A. (1967), *Biochim. Biophys. Acta 141,* 374.

Zanker, V. (1952), *Z. Phys. Chem. 199,* 225.