Distribution of Negative Phospholipids in Mixed Vesicles†

Stefano Massari,* Donatella Pascolini, and Girolamo Gradenigo‡

ABSTRACT: Positive metachromatic dyes (methylene blue and acridine orange) interact with mixed vesicles containing phosphatidylcholine and negative phospholipids. The following results have been obtained: (1) the interaction occurs only with external negative phospholipids; (2) the dye to negative phospholipid stoichiometry is 1:1; (3) the presence of the dyes does not perturb the lipid distribution on the external surface. The dye absorbance changes can be utilized to calculate the amount of paired negative phospholipids on the external surface. This value, compared with that obtained from the statistical analysis, gives the degree of association of these lipids. As to the surface distribution of negative phospholipids, it has been found that: (1) in the case of phosphatidylserine and phosphatidylglycerol the amount of paired phospholipids is lower than that statistically calculated, in the case of phosphatidylinositol it is slightly higher, and in the case of phosphatidic acid it is the same. (2) The degree of association for the different phospholipids is: phosphatidylinositol > phosphatidic acid > phosphatidylserine > phosphatidylglycerol, at neutral pH and at low ionic strength. The sequence is not altered by changing the phosphatidylcholine/negative phospholipid ratio in the vesicles. (3) The association is higher when the vesicles are in the solid state and decreases at temperatures higher than the transition temperature of the vesicles. (4) The mixing properties seem to be related to the difference in polar head groups rather than in the hydrocarbon chain structure. Regarding the transbilayer distribution in mixed vesicles the charged component is preferentially distributed on the external layer at a high phosphatidylcholine/negative phospholipid ratio. At a low phosphatidylcholine/negative phospholipid ratio, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidic acid are preferentially distributed on the internal layer.

Much work has been recently done to elucidate the problem of lateral-phase separation of phospholipids in mixed vesicles. Using partition techniques of the spin-label 2,2,6,6-tetramethylpiperidinyl-1-oxy, a limited solid-phase miscibility was observed for many phospholipids (Shimshick and McConnell, 1973; Wu and McConnell, 1975) and a limited fluid-phase miscibility for only a few components (Lentz et al., 1976; Wu and McConnell, 1975). Similar results have been obtained by freeze-fracture electron microscopy (Vervegaert et al., 1973; Grant et al., 1974), fluorescence polarization (Bashford et al., 1976; Lentz et al., 1976), and differential scanning calorimetry (Mabrey and Sturtevant, 1976). Lateral-phase separation may be induced or influenced by external parameters, such as pH and divalent cation concentration (Jacobson and Papahadjopoulos, 1975; Ito et al., 1975; Galla and Sackmann, 1975). Lipid miscibility could play an important role in modulating enzyme activity, ion permeability, and membrane aggregation. Therefore, we undertook the study of several phospholipids in order to correlate molecular structural properties with the miscibility of these molecules on the surface of mixed vesicles.

The technique used in the present study is based on measuring the absorbance changes of positive metachromatic dyes, such as acridine orange and methylene blue, upon the addition of known amounts of vesicles (Massari and Pascolini, 1977). The amount of dye dimers formed is calculated from the absorbance changes and the molar extinction coefficients of the monomer and dimer dye. The dyes do not penetrate into the vesicles, and the dye-negative phospholipid stoichiometry is 1:1. The amount of dye dimers corresponds to the amount of external negative phospholipids occupying adjacent positions not exceeding the critical dye dimerization distance, about 9 Å (Massari and Pascolini, 1977).

Methods and Materials

Phospholipid vesicle spectrophotometric titrations and phosphorus determinations were made as previously described (Massari and Pascolini, 1977). The pH of the sonication medium was in the range of 6.5 to 7.5. Vesicle dispersions were maintained at 4 °C and used only within the first week after sonication to avoid collision-induced aggregation or coalescence of the vesicles and formation of multilamellar structures. Spectrophotometric titrations, carried out within the first week, gave a constant amount of dye dimers, indicating that the vesicle sizes did not change.

ESR spectra were obtained on a Varian E-12 spectrometer at X band equipped with a variable temperature accessory. In the range of 0–60 °C, the temperature was controlled with a variability of ±2 °C. Phosphatidylserine determination was obtained according to Litman (1973) by reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS[1]). In the latter case, sodium bicarbonate was used instead of Tris or Hepes buffers in the sonication medium.

Figure 1 shows the time-dependent absorbance increase of TNBS in the presence of phosphatidylcholine–phosphatidylserine vesicles incubated at various pHs. The absorbance of TNBS reacting with the external phosphatidylserine increases more rapidly at a more alkaline pH (and at higher temperatures). At pH 9.5, a plateau is reached after 2 h. Measurements of external phosphatidylserine were performed at pH 9 at various time intervals in order to detect the absorbance value of the plateau. TNBS solutions were prepared immediately

† From the C.N.R.Unit for the Study of Physiology of Mitochondria and Institute of General Pathology, University of Padua, Padua, Italy. *Received November 21, 1977; revised manuscript received May 22, 1978.*

‡ Present address: Centro di Calcolo, Sezione Scientifica e Didattica, University of Padua, Padua, Italy.

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, disodium ethylenediaminetetraacetate; TNBS, 2,4,6-trinitrobenzenesulfonic acid; P_i, inorganic phosphate.

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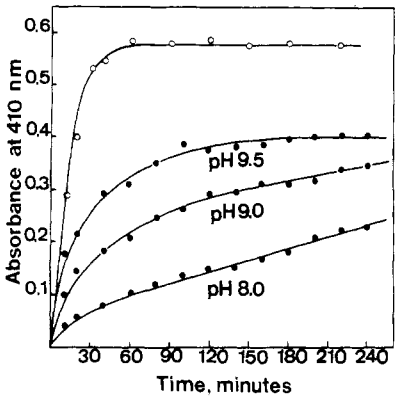

FIGURE 1: TNBS titration of external and total phosphatidylserine in mixed vesicles. The amount of external phosphatidylserine was measured by adding 1.2 μmol of P_i from phosphatidylcholine-phosphatidylserine vesicles in a 3:1 ratio to 2 mL of an aqueous solution containing 10 mM sodium bicarbonate buffer at various pHs and 0.05% TNBS. The reaction was terminated at various time intervals by adding 2 mL of 96% acid-propanol, and the absorbance was measured at 410 nm. Total phosphatidylserine content was measured by adding the same amount of vesicles to 2 mL of 1:1 propanol-aqueous solution containing buffer and TNBS. The reaction was terminated with 2 mL of 50% acid-propanol and the absorbance was measured. Corrections for the blank absorbance were made; temperature 20 °C: (○) total amount of phosphatidylserine; (●) external amount of phosphatidylserine.

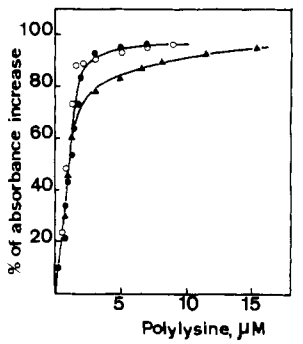

FIGURE 2: Inhibition of dye dimer formation by polylysine. In the ordinate is plotted the percentage of the absorbance increase due to the addition of polylysine (molecular weight 2000, degree of polymerization 9) with respect to the total absorbance change due to the addition of the vesicles to the dye-containing solution. The medium contained 0.2 mM EDTA-Tris, 5 mM Tris-Cl and 25 μM methylene blue: (●) 26 nmol of P_i from phosphatidylcholine-phosphatidylserine vesicles in a 2:1 ratio was added to the medium at pH 8; (▲) 135 nmol of P_i from phosphatidylcholine-phosphatidylglycerol vesicles in a 1.5:1 ratio was added to the medium at pH 7; (○) 70 nmol of P_i from phosphatidylcholine-phosphatidylinositol vesicles in a 1:1 ratio was added to the medium at pH 7.

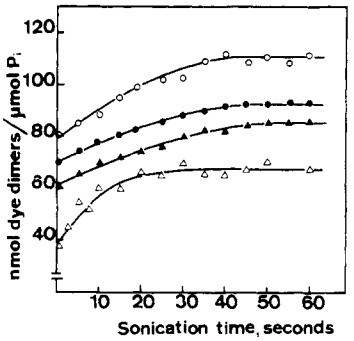

FIGURE 3: Amount of dye dimers at various time intervals of cosonication. The details of the method of cosonication are described elsewhere (Massari and Pascolini, 1977). The medium is the same as in Figure 2: (●) 27 nmol of P_i from phosphatidylcholine-phosphatidylserine vesicles in a 3:1 ratio (pH 9.5); (▲) 30 nmol of P_i from phosphatidylcholine-phosphatidylglycerol vesicles in a 3:1 ratio (pH 6.5); (○) 33 nmol of P_i from phosphatidylcholine-phosphatidylinositol vesicles in a 3:1 ratio (pH 7); (△) 66 nmol of P_i from phosphatidylcholine-phosphatidic acid vesicles in a 5.5:1 ratio (pH 6.3).

before each experiment.

Synthetic β,γ-dipalmitoyl-DL-α-glycerylphosphorylcholine, L-α-phosphatidyl-DL-glycerol from egg-yolk lecithin, and TNBS were supplied by Sigma. Phosphatidyl-L-serine from bovine brain and phosphatidylinositol from yeast were supplied by Koch-Light Laboratories. The latter lipid was purified by chromatography. Phosphatidylcholine from chicken egg yolks was obtained according to Ansell and Hawthorn (1964). 1-Palmitoyl-2-stearoylphosphatidylcholine carrying a spin-labeled oxazolidine ring at C-16 was a gift of Dr. G. A. Smith of the Department of Biochemistry in Cambridge. The phospholipid purity was checked by thin-layer chromatography: only a single spot was obtained for the phospholipids used. Unless specified, synthetic dipalmitoylphosphatidylcholine was used throughout the experiments.

The Monte Carlo simulation method was used to obtain the average number of phospholipid dimers. The lipid lattice positions occupied by a negative phospholipid were extracted casually from a rectangular distribution with a variability field from 1 to 10 000. The dimer number was an average of the values obtained by several simulations. The calculations were performed by a CDC Cyber 70/76 computer of the Centro di Calcolo Interuniversitario dell'Italia Nord-Orientale.

Results

As reported by Massari and Pascolini (1977), the absorbance changes of metachromatic dyes interacting with negatively charged lipid vesicles can be utilized to find the amount of dye dimers formed. At neutral pH, phosphatidylserine, phosphatidylglycerol, and phosphatidylinositol possess one net negative charge. The amount of dye dimers, in vesicles formed by phosphatidylcholine–negative phospholipid in a 3:1 ratio, is 47, 64, and 72 nmol/μmol of P_i, respectively. Mixed vesicles in which egg phosphatidylcholine was used in place of dipalmitoylphosphatidylcholine give the same amount of dye dimers, in the range of the experimental errors. The latter experiments were performed at 4 °C, where the vesicles approach the solid state. Figure 2 shows that the addition of increasing amounts of polylysine (molecular weight 2000, degree of polymerization 9) to mixed vesicles causes a progressive decrease of the amount of dye dimers. Total neutralization of the external negative charges, induced by a high amount of polylysine, completely inhibits the dye dimer formation. Since the large multicharged molecule of polylysine does not penetrate into the vesicles, it follows that the interaction between dyes and vesicles occurs only on the external surface. If this interpretation is correct, penetration of the dyes into the vesicles would cause an increase of the amount of dye dimers, consequent to the interaction between dyes and negative phospholipids of the internal layer. Figure 3 shows that cosonication of methylene blue with vesicles, at various time intervals, causes an increase of the amount of dye dimers, a plateau being reached after 1 min.

The presence of dyes may influence the surface distribution of the negative phospholipids. Ca^{2+}-induced lateral-phase separation in phospholipid bilayers formed by phosphatidylcholine and phosphatidylserine or phosphatidic acid was observed using spin-labeled phospholipids (Ohnishi and Ito, 1974; Galla and Sackmann, 1975). We used spin-labeled phosphatidylcholine to compare Ca^{2+} and dye effect on the lipid distribution. Figure 4A shows ESR spectra of variable molar

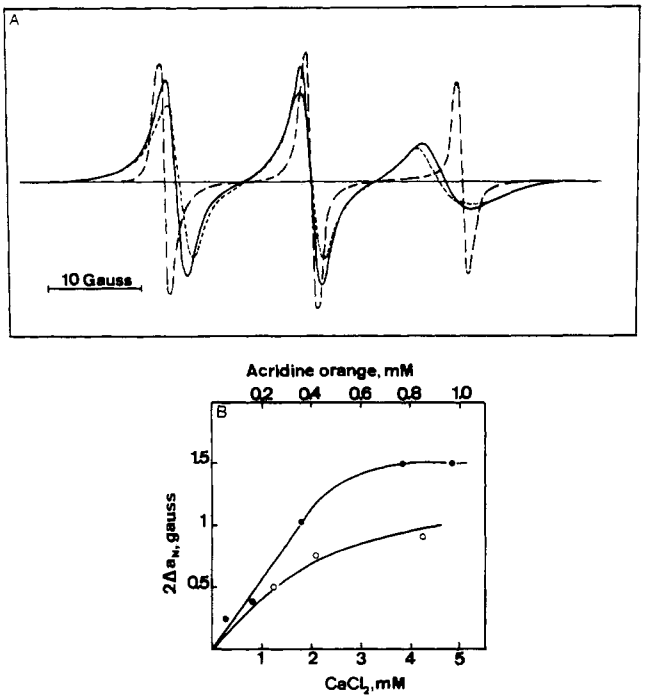

FIGURE 4: ESR spectral changes induced by $CaCl_2$ and acridine orange. The medium was 5 mM Tris-Cl (pH 8), 0.2 mM EDTA-Tris. About 2 mM phospholipid was present. Temperature was 20 °C. (A) ESR spectrum of phosphatidylserine vesicles with 1% of spin-labeled phosphatidylcholine (– –); ESR spectrum of phosphatidylserine vesicles with 10% of spin-labeled phosphatidylcholine (—); ESR spectrum of the latter vesicles in the presence of 5 mM $CaCl_2$ (- - -). (B) Hyperfine coupling constant change as a function of the concentration of $CaCl_2$ (●) and acridine orange. (○). Δa_N represents the change of the hyperfine coupling constant due to the addition of the cations.

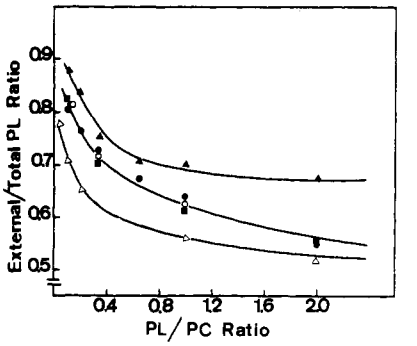

FIGURE 5: Dependence of the external/total ratio of the negative phospholipid on the vesicle composition. The medium was the same as in Figure 2. The experimental points were obtained by dividing the amount of dye dimers obtained at zero time and after 2 min of cosonication of vesicles and methylene blue (35 μM). Phosphatidylserine parallel measurements of the external/total ratio were carried out with TNBS as reagent (■): (●) phosphatidylserine, (▼) phosphatidylglycerol, (○) phosphatidylinositol, (▽) phosphatidylethanolamine (from Litman, 1973); PL = negative phospholipid, PC = phosphatidylcholine.

fractions of spin-labeled phosphatidylcholine in phosphatidylserine vesicles. The spectrum of the dilute sample exhibits a very sharp symmetric triplet, indicating a fast and almost isotropic tumbling of the label molecules. At higher spin content, the three lines are broadened, particularly the high-field line, and the hyperfine coupling constant decreases. Broadening of the triplet line and movement of the side bands toward the center of the spectrum were observed in the case of weak exchange and magnetic dipole–dipole interactions (Sackmann and Träuble, 1972). If $CaCl_2$ does induce lateral-phase separation, one would expect that the effect of the divalent cation is equivalent to that obtained by increasing the content of the spin-labeled molecules. In vesicles with a high content of spin-label, addition of $CaCl_2$ indeed induces a further broadening and a shift of a hyperfine coupling constant. Taking the double of the hyperfine coupling constant as an indicative parameter of the extent of spin–spin interaction, Figure 4B shows the change of this parameter as a function of the concentration of $CaCl_2$ and acridine orange. This dye also induces spectral changes similar to those obtained with $CaCl_2$, at lower concentrations. However, at 30 μM acridine orange, which is the dye concentration used in our experiments, the shift of the hyperfine coupling constant is practically negligible. We can therefore conclude that the dye, at low concentrations, does not perturb the lipid distribution on the external surface.

Surface and Transbilayer Distribution. Figure 5 shows the external/total ratio of the negative phospholipid in vesicles of variable composition. The ratio was calculated by dividing the amount of dye dimers before and after cosonication of methylene blue with vesicles for 2 min. The different curvature of the internal with respect to the external layer may influence the pairing of the lipid molecules. The external/total dye dimer ratio therefore may differ from the external/total ratio of the negative phospholipid. However, the coincidence of the two ratios is supported by the following considerations: (1) At alkaline pH in mixed vesicles containing doubly charged phosphatidic acid, the amount of dye dimers after cosonication coincides exactly with the total amount of phosphatidic acid molecules present in the vesicles (Massari and Pascolini, 1977). The external/total ratio of dye dimers, calculated at alkaline pH, equals that of acidic pH. (2) The ratio measured by TNBS in phosphatidylcholine–phosphatidylserine vesicles is the same as the ratio obtained with the dye dimers. (3) In phosphatidylcholine–phosphatidylserine vesicles in the 1.5:1 ratio, the value of 0.67 calculated with both techniques coincides with the value of Berden et al. (1975) with the NMR technique. The ratio reported in Figure 5 for phosphatidylcholine–phosphatidylethanolamine vesicles is derived from Litman values (1973). For all negative phospholipids used, the external/total ratio increases by increasing the phosphatidylcholine content in the vesicles. This ratio depends on the vesicle size and on the asymmetric distribution of the negative phospholipid between the internal and external layer of the vesicles. The size of the vesicles may be dependent on the experimental conditions of preparation, such as sonication time, ionic strength, phospholipid source, and impurities (Andrews et al., 1975). Whatever the sizes of mixed vesicles are, they should approach that of phosphatidylcholine vesicles when the content of the negative phospholipid approaches zero. In other words, the external/total ratio of the negative phospholipid, in the absence of transbilayer asymmetry, should approach 0.60–0.65, the value of the external/total phosphatidylcholine in unfractionated vesicles (Berden et al., 1975). Since the values reported in Figure 5 are much higher, it seems that the negative phospholipids used prefer the external layer of the vesicles when their content in the vesicles is low. This result was unexpected.

Figure 6 shows the percentage of dye dimers at various mixing ratios with respect to the maximum obtainable in the case that all external negative phospholipids are paired. The dashed curve represents the percentage of paired negative phospholipids calculated statistically for an external/total ratio of 0.60 of the negative phospholipid.

Temperature Dependence. When egg phosphatidylcholine was substituted for dipalmitoylphosphatidylcholine in mixed vesicles, the amount of dye dimers, measured at 25 °C, was decreased (10–20%). At this temperature, the vesicles con-

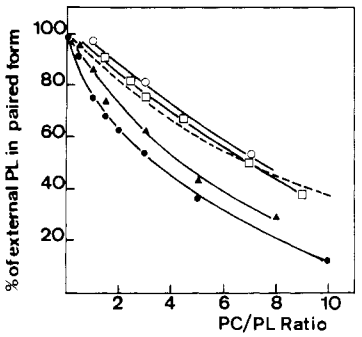

FIGURE 6: Degree of pairing of the negative phospholipid in vesicles of variable composition. The medium was the same as in Figure 2. The percentage of paired external phospholipid was calculated from the amount of dye dimers at neutral pH and the total amount of external negative phospholipid derived from Figure 5: (●) phosphatidylserine, (▲) phosphatidylglycerol, (○) phosphatidylinositol, (□) phosphatidic acid.

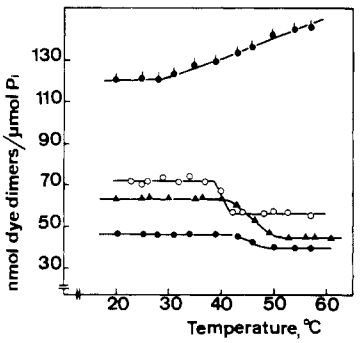

FIGURE 7: Dependence of the amount of dye dimers on the temperature. The medium used for spectrophotometric measurements consisted of 5 mM Tris-Cl, 0.2 mM EDTA-Tris, and 25 or 40 μM acridine orange at low and high temperatures, respectively. pH 7.3 was maintained constant at all temperatures. Aliquots of vesicles, maintained at 4 °C, were added to the medium at various temperatures: (●) phosphatidylserine, (▲) phosphatidylglycerol, (○) phosphatidylinositol. All vesicles were in a 1:3 ratio with phosphatidylcholine: (◐) phosphatidylserine (1:1 ratio).

taining egg phosphatidylcholine are in the fluid state, whereas the vesicles containing a high content of dipalmitoylphosphatidylcholine are in the solid state. We would expect a phase-dependent change of the amount of dye dimers when mixed vesicles containing dipalmitoylphosphatidylcholine are incubated at various temperatures. Figure 7 shows the amount of acridine orange dimers as a function of temperature, using mixtures containing phosphatidylserine, phosphatidylglycerol, and phosphatidylinositol. The amount of dye dimers was obtained from the absorbance changes following the addition of aliquots of phospholipid vesicles maintained at 4 °C to the solution containing the dye at variable temperatures. With vesicles formed by phosphatidylcholine–negative phospholipid in a 3:1 ratio, the amount of dye dimers starts to decrease at a temperature of 39–42 °C, which is near the main transition temperature of dipalmitoylphosphatidylcholine (Ladbrook and Chapman, 1969). When the negative phospholipid content in the vesicles is increased (phosphatidylcholine–phosphatidylserine in 1:1 ratio, as shown in the figure, or phosphatidylcholine–phosphatidylinositol in 1:1 ratio, not shown), the amount of dye dimers starts to increase at a temperature of 25–30 °C. This is probably due to penetration of the dye molecules into the inner space of the vesicles. A marked increase in the permeability of liposomes to electrolytes or hydrophilic molecules was indeed observed over the solid–fluid phase transition (De Gier et al., 1968; Papahadjopoulos et al., 1973). The decrease of the amount of dye dimers above the transition temperature may be due (1) to a pK_a shift dependent on the solid–fluid phase transition (McDonald et al., 1976), (2) to a different spatial arrangement of the lipid polar head groups, or (3) to a decrease in the amount of paired negative lipids in the fluid phase. On the other hand: (1) The amount of dye dimers at 50 °C remains unchanged when the pH is varied between 6 and 7.5. (2) Acridine orange, in contrast to methylene blue, is only slightly sensitive to small distance changes between binding sites (Stone et al., 1963; Stone and Bradley, 1967). (3) The hyperfine coupling constant of the spin-labeled phosphatidylcholine in egg phosphatidylcholine vesicles shifts about 0.75 G, when the spectra are recorded at 20 and 2 °C. On the contrary, this shift is 1.75 G when the same amount of spin-labeled phosphatidylcholine is mixed with phosphatidylserine vesicles. Therefore, even in the absence of dyes, phsophatidylserine tends to aggregate when the vesicles approach the solid state.

Discussion

Binary mixtures containing phosphatidylcholine and phosphatidylserine or phosphatidic acid, phosphatidylinositol, and phosphatidylglycerol produce closed vesicles when exposed to ultrasonic radiation (Berden et al., 1975; Massari and Pascolini, 1977; Litman, 1973; Michaelson et al., 1973).

Our preparations also consist of closed vesicles, as deduced from Figures 2 and 3. The dyes interact with the external negative phospholipids and can reach the internal space only when the vesicles are disrupted by further sonication.

In the experiments of Figure 6, the amount of dye dimers approaches half of the amount of external negative phospholipids when the phosphatidylcholine content in mixed vesicles approaches zero. This indicates that the dye–negative lipid stoichiometry is 1:1. Lipid distribution on the external surface of mixed vesicles can be influenced by the addition of divalent cations (Ohnishi and Ito, 1974; Ito et al., 1975; Jacobson and Papahadjopoulos, 1975; Galla and Sackmann, 1975; Hartman et al., 1977). The dyes used might induce lipid clustering consequent either to the decrease of electrostatic repulsion between negative groups or to the hydrophobic attraction between two bound dye molecules. However, ESR experiments of spin-labeled lipid demonstrate that the dye molecules have a negligible effect on lipid aggregation at the concentration used. Since only one molecule of dye binds electrostatically to one molecule of negative phospholipid on the external surface without affecting the lipid distribution, the amount of dye dimers, obtained from the spectral changes, gives a quantitative value of the amount of paired negative phospholipids on the external surface.

In phosphatidylcholine/negative phospholipid vesicles in a 3:1 ratio, the amount of "phospholipid dimers" calculated statistically is about 55 nmol/μmol of P_i for an external/total ratio of 0.6 of negative phospholipids. This value is higher than that calculated in the previous paper (Massari and Pascolini, 1977). The statistical value previously found was uncorrect. The asymmetrical distribution of the negative phospholipids between the external and internal surfaces of the bilayer leads to a different phosphatidylcholine/negative phospholipid ratio on the external surface. In vesicles where the phosphatidylcholine–negative phospholipid ratio is 3:1 on the external layer, the sequence of increasing association of the negative phospholipid with respect to the statistical predictions is: phosphatidylinositol (+10%) > phosphatidic acid (0%) > phosphatidylserine (−25%) > phosphatidylglycerol (−30%). The numbers in parentheses represent the percentage of deviation of the experimental values from the statistical values.

The different values of paired negative phospholipids point out the great heterogeneity of forces which may play a role on

lipid–lipid interaction, such as entropic forces (Galla and Sackmann, 1975), intermolecular or water-mediated hydrogen bonds, and electrostatic repulsion or attraction between charged groups.

Phosphatidic acid, phosphatidylserine, and phosphatidylinositol may be preferentially distributed on the internal layer of mixed vesicles (Berden et al., 1975; Michaelson et al., 1974; Litman, 1974), whereas phosphatidylglycerol prefers the external layer (Michaelson et al., 1973). The polar head groups of these lipids, which are smaller than the choline head group, seem to play the most important role in determining the observed asymmetry. The difference in curvature between the outside layer of the vesicle would represent the driving force for the asymmetric behavior (Berden et al., 1975). The distribution of phosphatidylglycerol has been interpreted as being due to the charge repulsion between adjacent molecules (Michaelson et al., 1973). We have found, however, that all negative phospholipids used prefer the external layer when their content in the vesicles is low. Theoretical considerations based on electrostatic energy minimization in mixed charged vesicles led Israelachvili (1973) to the conclusion that the external layer should have a higher density of charged lipids than the internal layer. The problem, therefore, could be approached from a different point of view. As a general rule, charged lipids are preferentially distributed on the external layer, but some other forces compel the charged lipid toward the internal layer when they are present in high content in the vesicles.

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References

Andrews, S. B., Hoffman, R. M., and Borison, A. (1975), *Biochem. Biophys. Res. Commun. 65,* 913.

Ansell, G. B., and Hawthorne, J. N. (1964), in Phospholipids, Chemistry, Metabolism and Function, Vol. 3, BBA Library, Elsevier, Amsterdam, p 68.

Bashford, C. L., Morgan, C. G., and Radda, G. K. (1976), *Biochim. Biophys. Acta 426,* 157.

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

De Gier, J., Mandersloot, J. G., and Van Deenen, L. M. M. (1968), *Biochim. Biophys. Acta 150,* 666.

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

Grant, C. W., Hong-Wei Wu, and McConnell, H. (1974), *Biochim. Biophys. Acta 363,* 151.

Hartmann, W., Galla, H. J., and Sackmann, E. (1977), *FEBS Lett. 78,* 169.

Israelachvili, J. N. (1973), *Biochim. Biophys. Acta 323,* 659.

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

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

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

Lentz, B. R., Barenholtz, Y., and Thompson, T. E. (1976), *Biochemistry 15,* 4529.

Litman, B. J. (1973), *Biochemistry 12,* 2545.

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

Mabrey, S., and Sturtevant, J. M. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3862.

MacDonald, R. C., Simon, S. A., and Baer, E. (1976), *Biochemistry 15,* 885.

Massari, S., and Pascolini, D. (1977), *Biochemistry 16,* 1189.

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

Michaelson, D. M., Horwitz, A. F., and Klein, M. P. (1974), *Biochemistry 13,* 2605.

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

Papahadjopoulos, D., Jacobson, K., Nir, S., and Isac, T. (1973), *Biochim. Biophys. Acta 311,* 330.

Sackmann, E., and Traüble, H. (1972), *J. Am. Chem. Soc. 94,* 4492.

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

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.

Vervegaert, P. H. J., Verkleij, A. J., Elbers, P. F., and Van Deenen, L. L. M. (1973), *Biochim. Biophys. Acta 311,* 320.

Wu, H., and McConnell, H. M. (1975), *Biochemistry 14,* 847.