Mabrey, S., Mateo, P. L., & Sturtevant, J. M. (1978) *Biochemistry 17*, 2464–2468.
Marsh, D. (1974) *Biochim. Biophys. Acta 363*, 373–386.
McCammon, J. A., & Deutch, J. M. (1975) *J. Am. Chem. Soc. 97*, 6675–6681.
Oldfield, E., & Chapman, D. (1972) *FEBS Lett. 23*, 285–297.
Oldfield, E., Chapman, D., & Derbyshire, W. (1971) *FEBS Lett. 16*, 102–104.
Oldfield, E., Meadows, M., Rice, D., & Jacobs, R. (1978a) *Bicohemistry 17*, 2727–2740.
Oldfield, E., Gutowsky, H. S., Jacobs, R. E., Kang, S. Y., Meadows, M. D., Rice, D. M., & Skarjune, R. P. (1978b) *Am. Lab. 10*, 19.
Oldfield, E., Gutowsky, H. S., Hsung, J. C., Jacobs, R. E., Kang, S. Y., King, T. E., Meadows, M. D., & Rice, D. M. (1978c) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4657.
Petersen, N. O., & Chan, S. I. (1977) *Biochemistry 16*, 2657–2667.
Ranck, J. L., Mateu, L., Sadler, D. M., Tardieu, A., Gulik-Krzywicki, T., & Luzzati, V. (1974) *J. Mol. Biol. 85*, 249–277.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–418.
Seelig, A., & Seelig, J. (1975) *Biochim. Biophys. Acta 406*, 1–5.
Shimshick, E. J., & McConnell, H. M. (1973a) *Biochemistry 12*, 2351–2360.
Shimshick, E. J., & McConnell, H. M. (1973b) *Biochem. Biophys. Res. Commun. 53*, 446–451.
Sklar, L. A., Hudson, B. S., & Simoni, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1649–1653.
Slichter, C. P. (1978) *Principles of Magnetic Resonance* (2nd ed.) Springer-Verlag, West Berlin.
Solomon, I. (1958) *Phys. Rev. 110*, 61–65.
Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., & Smith, I. C. P. (1976) *Biochemistry 15*, 954–966.
Trauble, H., & Eibl, H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 214–219.
Tsong, T. Y. (1975) *Biochemistry 14*, 5415–5417.
Vega, S., & Pines, A. (1977) *J. Chem. Phys. 66*, 5624–5644.
Worcester, D. L., & Franks, N. P. (1976) *J. Mol. Biol. 100*, 359–378.
Wu, S. H., & McConnell, H. M. (1975) *Biochemistry 14*, 847–854.

Lateral Distribution of Negatively Charged Lipids in Lecithin Membranes. Clustering of Fatty Acids†

H. Hauser,* W. Guyer, and K. Howell

ABSTRACT: From ζ-potential measurements on multilamellar liposomes, it is concluded that negatively charged phospholipids incorporated in liquid-crystalline phosphatidylcholine bilayers are randomly distributed in the plane of the bilayer. Furthermore, the distribution of the negatively charged phospholipid between the two halves of the bilayer and over the concentrically arranged bilayers of the multilamellar structure is uniform. The same is true for spin-labeled fatty acids. In contrast, long-chain fatty acids appear to be clustered at neutral pH; i.e., they segregate into patches within the plane of the bilayer. Their carboxyl groups are fully ionized only at a pH $\geq$ 11, and charge repulsion leads then to a random distribution. At pH <7 the binding of $^{45}Ca^{2+}$ to arachidic acid monolayers is insignificant, and only at pH $\sim$10 does the amount of Ca^{2+} bound per fatty acid become comparable to that bound to phosphatidylserine monolayers at neutral pH. Ca^{2+} binding obviously parallels the deprotonation of the fatty acid carboxyl group in phosphatidylcholine bilayers. The pH dependence of the Ca^{2+} binding to fatty acid monolayers is similar to the pH–electrophoretic mobility relationship observed with fatty acid containing phosphatidylcholine liposomes. From the similarity in behavior of fatty acid monolayers and fatty acids present in bilayers, it is proposed that in the latter case fatty acids occur as patches of monolayers (clusters). Fatty acids and spin-labeled fatty acids also differ in their effect on the gel to liquid-crystalline transition of dipalmitoylphosphatidylcholine bilayers. The former are stabilizing as evident from an increase in the transition temperature while the latter have a destabilizing effect. Deuterated fatty acids behave like ordinary fatty acids in terms of clustering. The use of probing the structure and dynamics of phospholipid bilayers and membranes with deuterated fatty acids is therefore subject to criticism, particularly when large quantities of fatty acids have to be utilized as in deuteron magnetic resonance experiments. The information thus derived is likely to be affected by probe–probe interactions.

There is an increasing body of evidence indicating that in addition to the asymmetric distribution of lipids and proteins between the two halves of the membrane there is also a nonrandom (asymmetric) distribution of components within half (one monolayer) of the membrane. This segregation or clustering of membrane components into highly specialized regions is related to the fast lateral diffusion allowing the various components to come in contact and to interact with each other. The lateral distribution of fatty acids in bilayers and biological membranes is interesting from two points of view: (1) fatty acids occur, though to a small extent, in biological membranes as the result of phospholipid turnover and have been shown to affect the permeability and various transport functions of the mitochondrial membrane (Wojtczak, 1976) and (2) spin-labeled fatty acids have been used ex-

† From the Department of Biochemistry, Eidgenössische Technische Hochschule Zürich, ETH-Zentrum, CH-8092 Zürich, Switzerland. *Received October 12, 1978.* This work was supported by Swiss National Science Foundation Grant No. 3.116-0.77.

0006-2960/79/0418-3285$01.00/0

tensively as probes to investigate the molecular organization and motion of lipid bilayers and membranes. For the same purpose perdeuterated or selectively deuterated fatty acids have been recommended as nonperturbing molecular probes to explore the molecular architecture and dynamics of membranes. Knowledge of the lateral distribution of these probes within the plane of the bilayer is required for the successful interpretation of such measurements.

This paper deals with the lateral distribution (or mixing) of fatty acids in phosphatidylcholine bilayers. Evidence will be presented to show that under physiological conditions long-chain fatty acids are partially present as clusters (islands) within the liquid-crystalline domain of phosphatidylcholine bilayers whereas spin-labeled fatty acids are randomly distributed.

Experimental

Materials

Egg phosphatidylcholine (grade I), the monosodium salt of phosphatidylserine from bovine spinal cord, and cardiolipin from beef heart were purchased from Lipid Products (South Nutfield, U.K.). Monocetyl- and dicetylphosphoric acid were obtained from Albright and Wilson Ltd. (Birmingham, U.K.). Phosphatidylinositol was extracted from frozen peas and purified on DEAE-cellulose (Ansell & Hawthorne, 1964; Rouser et al., 1963). Dipalmitoylphosphatidylcholine was synthesized as described (Paltauf et al., 1971). The purity of the phospholipids was checked by thin-layer chromatography by applying 0.3–1 mg as a 1.5-cm band on silica gel H using chloroform–methanol–7 M ammonia (65:25:4, by vol) as the solvent. When necessary, the phospholipids were purified by preparative thin-layer chromatography. Triolein, stearic acid, oleic acid, arachidic acid, and dioleoylphosphatidylcholine were obtained from Applied Science Laboratories Inc. (State College, PA) and had a nominal purity of better than 99%.

Stearic acid spin-labels with the 4,4-dimethyl-3-oxazolidinyl group attached to carbon atom 5 (5-doxylstearic acid), carbon atom 12 (12-doxylstearic acid), and carbon atom 16 (16-doxylstearic acid) and the methyl ester of 16-doxylstearic acid were purchased from Syva (Palo Alto, CA). $^{45}CaCl_2$ (sp act. 1.0–1.6 kCi/mol) was obtained from the Radiochemical Centre (Amersham, U.K.).

Preparation of Phospholipid Dispersions. Unsonicated mixed dispersions of egg phosphatidylcholine containing acidic lipid were prepared at room temperature as follows. The desired quantities of phosphatidylcholine and the second lipid were dissolved in $CHCl_3$–CH_3OH (2:1, by vol), and the solution was dried down in a round-bottom flask by using a vacuum of $\sim 10^{-3}$ to remove the last traces of tenaciously retained organic solvent. The appropriate amount of aqueous solvent was added to the dried material, and the dispersion was produced by mechanical shaking for about 15 min. Unsonicated dispersions of L- or DL-dipalmitoylphosphatidylcholine were prepared as described above except that the dispersion was produced and stored before measurement at 45 °C. All procedures were carried out under N_2.

Electron micrographs of negatively stained and freeze-fractured samples of aqueous phosphatidylcholine dispersions containing up to ~10 mol % stearic acid revealed the presence of large, multilamellar structures similar to the ones observed with pure phosphatidylcholine. The presence of fatty acid in phosphatidylcholine bilayers (up to levels used in this study) did therefore not produce any disintegration of the multilamellar structures with concomitant formation of small micelles. This conclusion is consistent with the fact that >90% of the fatty acid could be spun down with the multilamellar liposomes at 1000–2000*g*. Furthermore, multilamellar liposomes of phosphatidylcholine and fatty acid were easily visible in the light microscope whereas small micelles would not have been detected.

Methods

Particle Microelectrophoresis. Electrophoretic mobilities were measured in a thermostated particle electrophoresis apparatus (Mk II, from Rank Brothers, Cambridge, U.K.). A thin-walled, horizontal, cylindrical cell of Pyrex glass (cross section 1.62 mm) was used together with four electrodes; two Pd electrodes fed from a constant-current supply served to generate the potential difference across the cell, and two Pt electrodes together with a high input resistance voltmeter were used to measure this potential difference. As the light source either a quartz–iodine lamp or a 3-mW He/Ne laser was used. The liposomes were viewed with a long working range objective at right angles to the direction of the incident light beam by using dark-field illumination. Unless otherwise noted, electrophoretic mobilities were measured at 25 ± 1 °C, and the values [in units of $cm^2/(V\ s)$] represent the average of about 40 measurements. In order to minimize electrode polarization, we made successive measurements in the opposite direction. Measurements carried out both in the upper and in the lower stationary layer agreed within the experimental error.

Other Techniques. The surface chemical techniques and the apparatus used to measure surface radioactivities of $^{45}Ca^{2+}$ have been described before (Hauser & Dawson, 1967; Hauser & Phillips, 1973). Electron spin resonance spectra were recorded on an X-band Varian E-104 A spectrometer equipped with a variable temperature accessory. Measurements were carried out at 25 ± 1 °C.

Results

Particle Electrophoresis of Phosphatidylcholine Liposomes. Egg phosphatidylcholine liposomes dispersed in 0.025 M NaCl had zero electrophoretic mobility over a wide pH range (Bangham & Dawson, 1959; Hauser & Dawson, 1967; Papahadjopoulos, 1968). Incorporation into phosphatidylcholine bilayers of acidic phospholipids such as phosphatidylserine and phosphatidylinositol produced a negative ζ potential as indicated by their electrophoretic mobility to the anode. Figure 1 shows the effect of acidic lipids upon the electrophoretic mobility of egg phosphatidylcholine liposomes. Up to a certain concentration of the acidic lipid, the electrophoretic mobility increased linearly. At concentrations > 5–10 mol %, deviations from linearity were observed except for stearic acid (Figure 1). The increment in electrophoretic mobility given by the slope of the linear relationship at zero concentration is summarized in Table I. There is a good correlation between the slope and the number of net negative charges of the lipid molecule. The slope for lipids with one net negative charge was about -0.22×10^{-4} $cm^2/(V\ s)$ per mol % lipid, and this value increased by an integral factor for each additional negative charge (Table I).

The effect of stearic acid on the electrophoretic mobility of phosphatidylcholine liposomes depended on the pH (Figure 2). No electrophoretic mobility was observed up to pH ~6. At pH ~8 stearic acid generated a negative ζ potential, but the increment in electrophoretic mobility was only about half of that measured for phospholipids with one net negative charge (Figure 1 and Table I). At pH 11.6 the electrophoretic mobility approached the value measured for phosphatidyl-

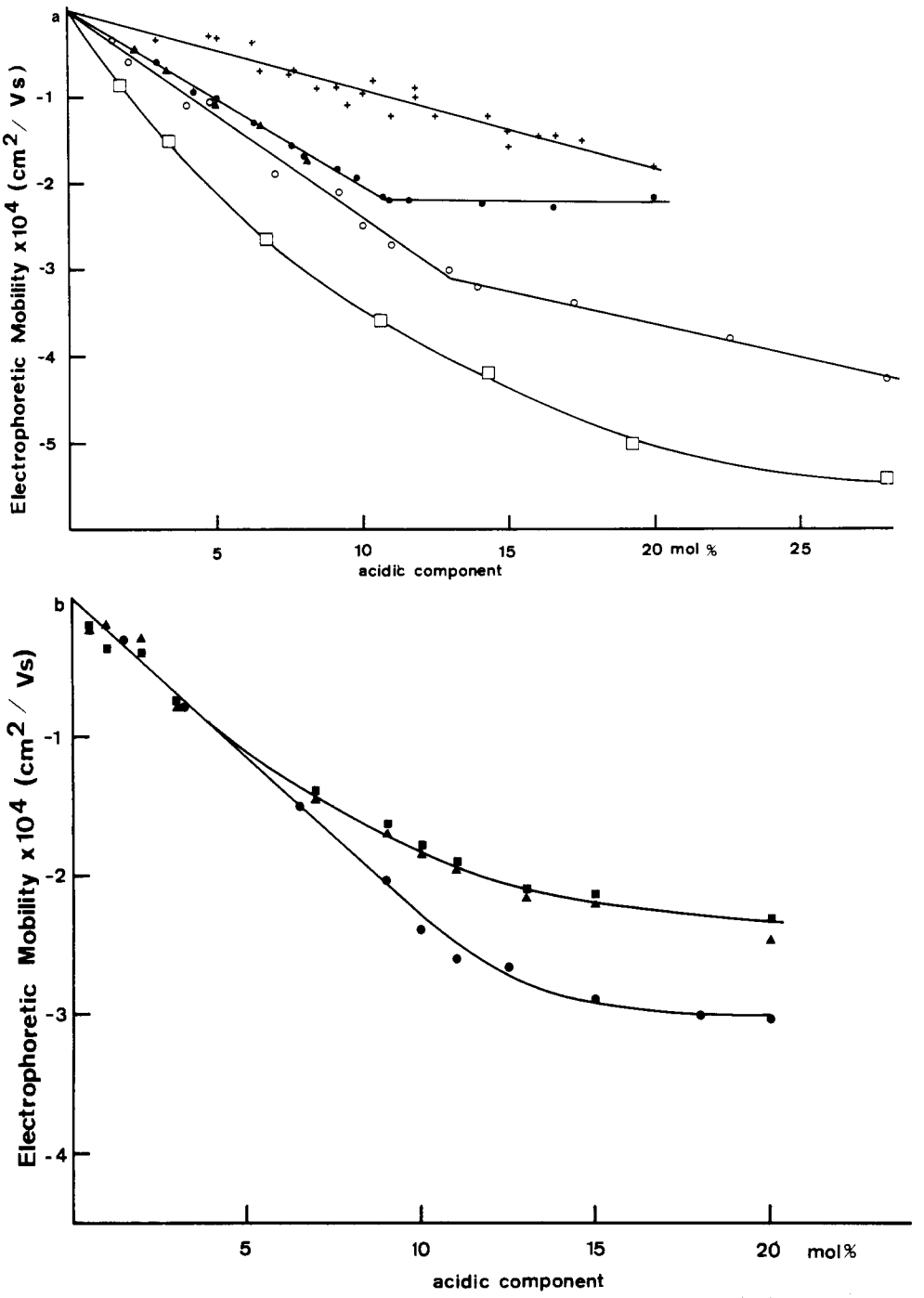

FIGURE 1: Electrophoretic mobility [$cm^2/(V\ s)$] of egg phosphatidylcholine liposomes containing acidic lipids. The lipids (0.13–0.67 mM) were dispersed either in 0.025 M NaCl, pH 5–6, in 0.025 M NaCl to which NaOH was added to adjust the pH or in 0.025 M NaCl and 5 mM sodium phosphate, pH 8. Within the experimental error of the measurement, there was no difference in the mobility of phosphatidylserine containing liposomes when dispersed in 0.025 M NaCl and 5 mM sodium phosphate, pH 8, or when dispersed in 0.025 NaCl to which NaOH was added to bring the pH to 8. (a) (+) Stearic acid, pH 8.1; (○) phosphatidylserine, pH 5.5 and pH 8.0; (●) dicetylphosphoric acid, pH 5.5; (▲) stearic acid, pH 11.6; (□) cardiolipin, pH 5.5. (b) (●) 5-Doxylstearic acid; (▲) 12-doxylstearic acid; (■) 16-doxylstearic acid; all at pH 8.0. The solid lines are least-squares fits to the experimental points. All measurements were carried out at 25 ± 1 °C.

choline liposomes containing acidic phospholipids with one net negative charge (cf. Figures 1 and 2 and Table I). At this pH there was still no contribution to the electrophoretic mobility arising from phosphatidylcholine itself [cf. Bangham & Dawson (1959)]. Palmitic acid, oleic acid, and various deuterated fatty acids incorporated in phosphatidylcholine liposomes behaved similarly to stearic acid.

In contrast to ordinary fatty acid, the three spin-labeled fatty acids (see Materials) produced the same increment in electrophoretic mobility as was observed with negatively charged phospholipids at least at concentrations < 5 mol % (Figure 1b and Table I). At higher spin-label concentrations, deviations from linearity occurred (for 12- and 16-doxylstearic acid at concentrations > 5 mol % and for 5-doxylstearic acid at concentrations > 10 mol %). As expected, the methyl ester of 16-doxylstearic acid did not produce a negative ζ potential when incorporated in phosphatidylcholine liposomes.

A difference in the behavior of ordinary and spin-labeled fatty acids was also observed when these compounds were incorporated in bilayers of L- or DL-dipalmitoylphosphatidylcholine. The increment in electrophoretic mobility measured with stearic acid containing bilayers dispersed in

Table I: Particle Microelectrophoresis of Egg Phosphatidylcholine Liposomes

acidic phospholipid added	pH[a]	increment in electrophoretic mobility[b] × 10^4	maximum no. of net negative charges per molecule
phosphatidylserine	5.5	−0.24	1
phosphatidylserine	8.1	−0.22	1
dicetylphosphoric acid	5.5	−0.21	1
phosphatidylinositol	5.5	−0.21	1
phosphatidylinositol	8.0	−0.20	1
cardiolipin	5.5	−0.42	2
phosphatidic acid	8.0	−0.42	2
monocetylphosphoric acid	5.5	−0.42	2
triphosphoinositide[c]	5.5	−1.08	5
palmitic acid	8.0	−0.11	1
palmitic-2,2-d_2 acid	8.0	−0.125	1
oleic acid	8.0	−0.12	1
stearic acid	5.5	0.00	1
stearic acid	8.0	−0.90	1
stearic acid	11.6	−0.22	1
stearic-d_{35} acid (perdeuterated)	8.0	−0.12	1
5-doxylstearic acid	5.5	−0.23	1
5-doxylstearic acid	8.0	−0.23	1
12-doxylstearic acid	8.0	−0.23	1
16-doxylstearic acid	8.0	−0.23	1
methyl ester of 16-doxylstearic acid	8.0	0	0

[a] Unsonicated egg phosphatidylcholine liposomes were dispersed in 0.025 M NaCl, pH 5.5, or in 0.025 M NaCl, 5 mM sodium phosphate, pH 8.0; the pH of 11.6 was obtained by adding NaOH to 0.025 M NaCl. [b] The increment in electrophoretic mobility (in $cm^2/(V\ s)$ per mol % of acidic lipid) was derived from the initial slope at zero concentration of the solid lines relating the electrophoretic mobility to the mole fraction of acidic lipid (Figure 1). The solid lines of Figure 1 are least-squares fits to the experimental data which gave a standard deviation of 0.01–0.02 $cm^2/(V\ s)$ per mol %. [c] Taken from Hauser & Dawson (1967).

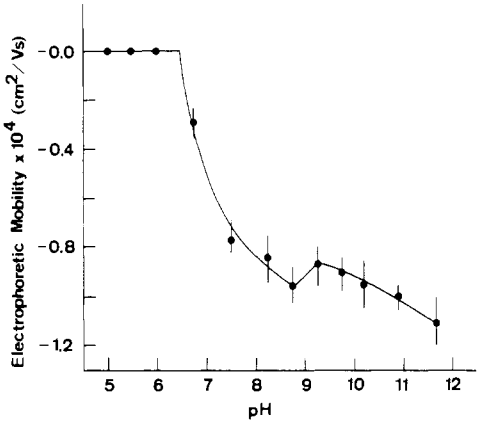

FIGURE 2: Electrophoretic mobility [$cm^2/(V\ s)$] of egg phosphatidylcholine liposomes (1.33 mM) with 5 mol % stearic acid incorporated in the bilayer as a function of pH. The lipid was dispersed as described under Methods in 0.025 M NaCl to which NaOH was added to adjust the pH. The hump in the curve at pH ~9 was reproducible; however, the mechanism underlying this phenomenon is unclear at present.

0.025 M NaCl and 5 mM sodium phosphate, pH 8, at 45 °C was $(-0.16 \pm 0.02) \times 10^{-4}$ $cm^2/(V\ s)$ per mol % fatty acid while the increment observed with 5-doxylstearic acid under the same conditions was $(-0.27 \pm 0.02) \times 10^{-4}$ $cm^2/(V\ s)$ per mol %. Compared to the values measured at 25 °C (cf. Table I), the increase in electrophoretic mobility at 45 °C is partly accounted for by changes in viscosity and the dielectric constant with increasing temperature. Figure 3 gives the temperature dependence of the electrophoretic mobility of DL-dipalmitoylphosphatidylcholine liposomes containing either ~10 mol % stearic acid (top) or ~10 mol % 5-doxylstearic acid (bottom). Both systems give sigmoidal curves similar to the plot of excess enthalpy–temperature obtained in differential scanning calorimetry experiments. The inflection points of the heating curves (full symbols) were approximately 45 and 39 °C, respectively; those of the cooling curve were about 1–2 °C lower. The presence of stearic acid caused an increase in the phase transition temperature while the incorporation of spin-label produced a decrease. Below 40 °C the value of the electrophoretic mobility of stearic acid containing liposomes decreased to zero while the mobility of liposomes containing spin-label was still about -1×10^{-4} $cm^2/(V\ s)$. The first cycle gave different heating and cooling curves (shown in Figure 3); in subsequent cycles the cooling curve almost coincided with the heating curve. The irreversible change during the first cycle is probably due to some fatty acid (spin-label) being squeezed out at the phase transition. That this amount is less than 10% can be shown by ESR and by incorporating stearic acid ^{14}C-labeled in the CO position. After cooling the lipid dispersion below the phase transition temperature, we spun the lipid down at 2000*g*; counting the pellet and the supernatant showed that more than 90% of the counts remained associated with the liposomes.

Monolayer Studies. No $^{45}Ca^{2+}$ was bound to arachidic acid monolayers below pH 6 as determined from surface radioactivity measurements. $^{45}Ca^{2+}$ binding became only significant at pH >9 (cf. Figure 4 which also gives the details of the experimental conditions). Using the calibration curve (inset, Fig. 4), we converted surface radioactivities to Ca^{2+} surface concentrations which, knowing the area per fatty acid molecule, can be expressed as the mole ratio of Ca^{2+}/fatty acid. The area per molecule was taken as 0.2 nm^2 as determined from the force–area curve (not shown). At pH 9.5 one Ca^{2+} was bound on the average to 90 arachidic acid molecules while for phosphatidylserine monolayers the same Ca/lipid mole ratio was observed at pH ~6.

The affinity for $^{45}Ca^{2+}$ to mixed monolayers consisting of either synthetic phosphatidylcholines or triolein and fatty acids was determined by using the same surface radioactivity techniques. Under the experimental conditions described in the footnotes of Table II, pure monolayers of the synthetic phosphatidylcholines and triglyceride had no affinity for $^{45}Ca^{2+}$. Similarly, mixed monolayers consisting of dipalmitoylphosphatidylcholine and various fatty acids (palmitic, stearic, or oleic acid), of dioleoylphosphatidylcholine and oleic acid, or of triolein and oleic acid were found to bind no $^{45}Ca^{2+}$

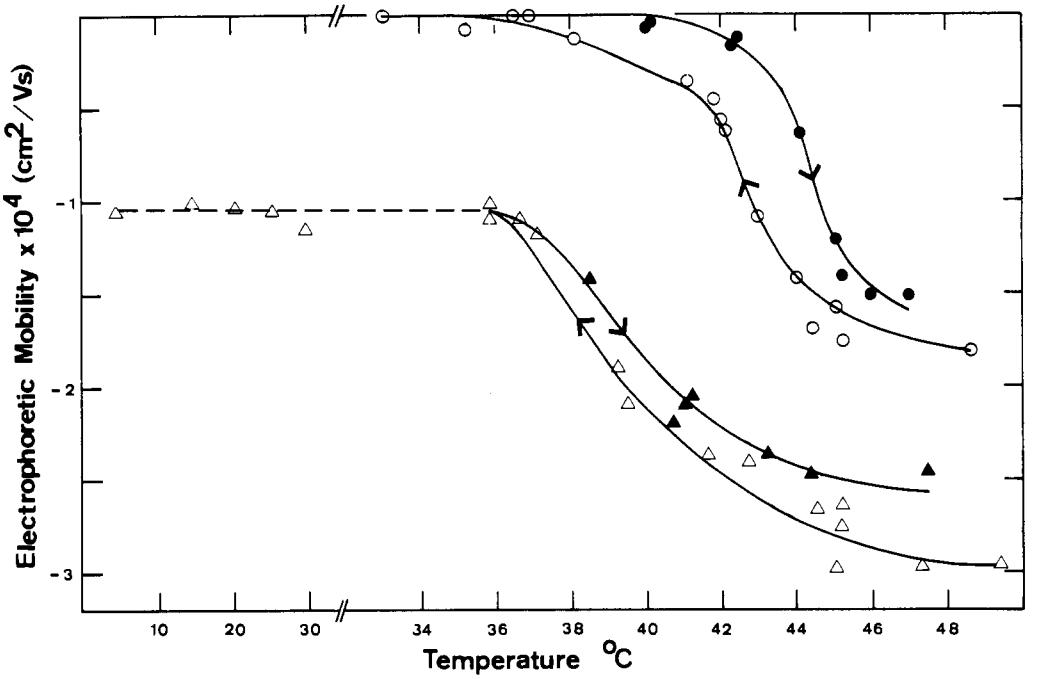

FIGURE 3: Electrophoretic mobility in [cm^2/(V s)] of L- or DL-dipalmitoylphosphatidylcholine liposomes (0.17 mM) containing 10 mol % stearic acid (top curve) and 10 mol % 5-doxylstearic acid (bottom curve) as a function of temperature. The lipid dispersions were prepared as described under Methods by using 0.025 M NaCl and 5 mM sodium phosphate, pH 8, as the solvent.

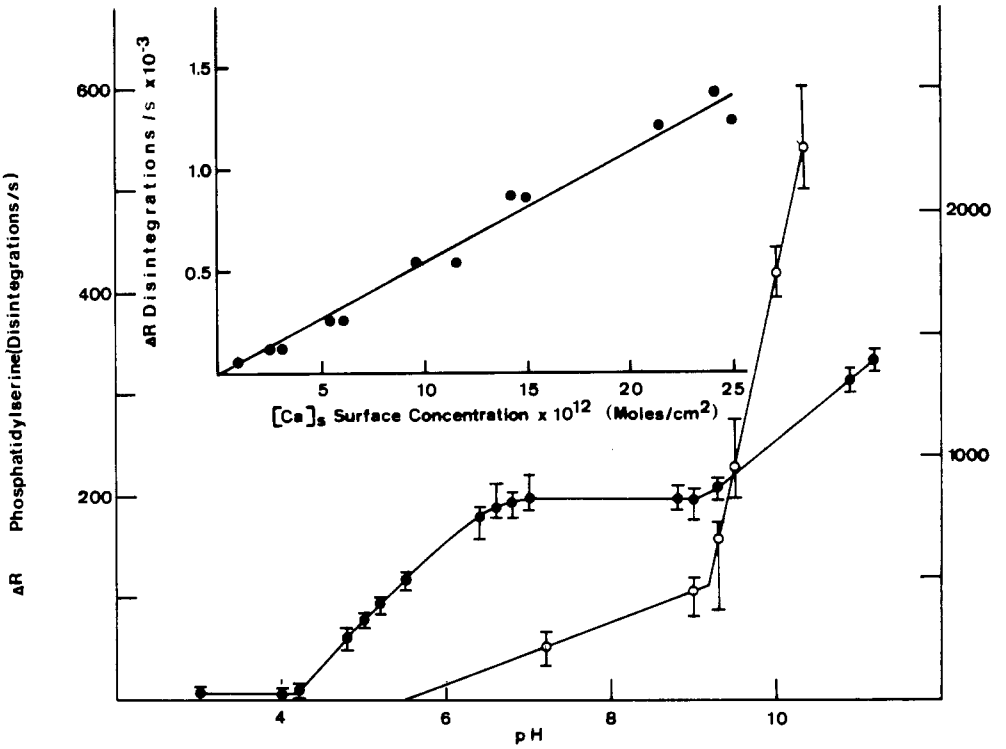

FIGURE 4: $^{45}Ca^{2+}$ binding to monolayers of arachidic acid (○) and of ox brain phosphatidylserine monosodium salt (●) as a function of pH. Lipids were spread from hexane–ethanol solutions on 0.01 M NaCl containing 0.13 μM $^{45}CaCl_2$ (sp act. 1.56 kCi/mol); the pH was adjusted by adding small amounts of HCl or NaOH. The lipid film was originally spread to a surface pressure below the liftoff point of the isotherm and was then compressed to a final pressure of π = 12 mN m^{-1}. The surface radioactivity was measured as described before (Hauser & Dawson, 1967; Hauser & Phillips, 1973). The bars represent the spread of three to six experiments. Inset: calibration curve relating the surface radioactivity ΔR (disintegrations/s) to the actual surface concentration of $^{45}Ca^{2+}$ (mol/cm^2). The plot is valid for a specific radioactivity of 10^3 Ci/mol. A value $\Delta R = 10^3$ corresponds to a surface concentration of $^{45}Ca^{2+}$ of 19.5 pmol/cm^2.

at pH 7.0 and molar ratios of lipid/fatty acid up to 1. In contrast to these results, mixed monolayers of phosphatidylserine and triolein behaved as one would expect from a random distribution of the acidic phospholipid in the inert triglyceride (Table II). As more triolein was added, spacing out the negative charges, the amount of $^{45}Ca^{2+}$ bound to one phosphatidylserine molecule (i.e., the mole ratio of $^{45}Ca^{2+}$/lipid) decreased (Table II).

Discussion

Lateral Distribution of Negatively Charged Phospholipids in Liquid-Crystalline Egg Phosphatidylcholine Bilayers. It

Table II: $^{45}Ca^{2+}$ Binding to Mixed Monolayers of Triolein and Phosphatidylserine

mole ratio of triolein/-phosphatidylserine[a]	no. of phosphatidylserine molecules per cm^2	mole ratio of Ca^{2+}/lipid	K_A[b] (M)
1:1	5.9×10^{13}	3.5×10^{-3}	2.7×10^4
2:1	3.6×10^{13}	2.8×10^{-3}	2.1×10^4
3:1	2.5×10^{13}	2.6×10^{-3}	1.0×10^4

[a] Experimental conditions: mixed monolayers were spread on 0.01 M NaCl solutions, pH 7.0, containing 0.13 μM $^{45}Ca^{2+}$ and compressed to a surface pressure $\pi = 12$ mN m^{-1}. [b] The apparent binding constant of the interaction between phosphatidylserine and $^{45}Ca^{2+}$ was calculated as described by Hauser et al. (1976).

has been shown that phospholipids such as phosphatidylserine, dicetyl phosphate, and phosphatidylinositol bear one net negative charge in the pH range 5–8 (Hauser & Dawson, 1967; Papahadjopoulos, 1968; Hauser et al., 1976). Incorporation of such phospholipids in isoelectric phosphatidylcholine bilayers produced a uniform increment in the electrophoretic mobility of $(-0.22 \pm 0.02) \times 10^{-4}$ cm^2/(V s) per mol % (average of Table I ± standard deviation). There is good evidence that under the conditions of our experiments phosphatidylserine is randomly distributed in liquid-crystalline phosphatidylcholine bilayers (Ohnishi & Ito, 1974; Ito et al., 1975). Hence, this value of −0.22 is characteristic for a negatively charged lipid which is randomly distributed in the plane of the bilayer. Furthermore, the uniform increment measured for different, negatively charged lipids can only be reconciled with an even distribution of the lipid between the two halves of the bilayer as well as over the various, concentric layers of the multilamellar liposome. Such an even distribution seems reasonable considering the large size of multilamellar liposomes. Increment measurements as described above can thus be used as a quick test to decide whether or not a negatively charged lipid is randomly distributed in the plane of the bilayer. The departure from linearity (Figure 1) at higher concentration of acidic lipid could be due to saturation of the phosphatidylcholine bilayer with the excess acidic lipid forming a separate phase. That this is the correct explanation in the case of spin-labeled fatty acids (Figure 1b) can be shown by ESR. For all three curves shown in Figure 1b, the point of deflection coincides with the appearance in the ESR spectrum of a signal arising from free spin-label. It is seen from Figure 1b that at room temperature egg phosphatidylcholine bilayers can accommodate more of 5-doxylstearic acid than of the other two spin-labels. The deviation from linearity of the cardiolipin curve is difficult to explain because it has been shown (Rand & Sengupta, 1972) that mixed dispersions of cardiolipin and phosphatidylcholine in H_2O form a single lamellar phase. A possible explanation could be that cardiolipin produces a high surface potential leading to a decrease in surface pH and consequently to a reduction in the degree of ionization of charged groups at the surface.

Lateral Distribution of Fatty Acid in Phosphatidylcholine Bilayers. In contrast to negatively charged phospholipids, stearic acid did not produce a negative ζ potential when incorporated in phosphatidylcholine bilayers at pH <6 (Table I). Between pH 6 and 11 the electrophoretic mobility of stearic acid containing liposomes was always smaller than that of liposomes containing an equivalent amount of phosphatidylserine. Only at a pH > 11 did the increment in electrophoretic mobility approach that measured for negatively charged phospholipids (cf. Figure 1 and Figure 2 and Table I). The fact that at pH <11 stearic acid did not contribute its full charge to the interfacial region of the bilayer could be explained as follows: (1) the fatty acid molecules are fully ionized and randomly distributed, but they are inserted in the bilayer such that the carboxyl group does not contribute its full charge to the ζ potential in the plane of shear, (2) the fatty acid molecules are randomly distributed but the carboxyl groups are only partially ionized, and (3) the fatty acid molecules are clustered, forming separate domains within the liquid-crystalline phosphatidylcholine bilayer, and as a result the ionization of the fatty acid –COOH group is partially suppressed. The first possibility is unlikely considering that spin-labeled fatty acids are inserted such that they contribute one charge per molecule to the surface charge density (ζ potential) of the bilayer (Table I). The second one can be ruled out on the basis that the pK of carboxyl groups both in the bulk phase as well as in the interface at surface concentrations ≥ 70 Å^2/–COOH group is between 4 and 5 (Hauser et al., 1976). The most likely explanation is therefore the third one, that fatty acids are not randomly distributed in the plane of the bilayer. The monolayer data are in support of this explanation. Comparing the binding of $^{45}Ca^{2+}$ to phosphatidylserine and arachidic acid monolayers, it is clear that the –COOH group of phosphatidylserine is fully ionized at pH ~6 while that of the fatty acid is fully ionized only at pH >10. This finding is consistent with surface potential measurements on fatty acid monolayers showing that ionization of the –COOH groups is complete at pH >10 (Goddard, 1974; Rosano et al., 1969; Patil et al., 1972; Patil & Cornwell, 1977). The pH dependence of the $^{45}Ca^{2+}$ binding to fatty acid monolayers (Figure 4) is clearly paralleled by the pH dependence of the electrophoretic mobility of fatty acid containing liposomes (Figure 2); in both types of structures (monolayers and bilayers) fatty acids appear to be fully ionized only at pH >10. From the striking similarity in behavior of fatty acids in monolayers and bilayers, it is concluded that at neutral pH fatty acids incorporated in phosphatidylcholine bilayers behave as if they were present as patches of monolayers, i.e., that they are segregated into clusters. The drastic shift in pK of fatty acid –COOH groups when present in phosphatidylcholine bilayers can now be rationalized. In close-packed monolayers or clusters the surface charge density is as high as one charge per ~0.20 nm^2, and this tends to accumulate counterions, particularly H^+, in the interfacial region, thus shifting the apparent pK to higher values.

That oleic acid, which is liquid at room temperature, behaved similarly to stearic acid emphasizes the role of the –COOH group in clustering. Intermolecular hydrogen bonding might be important in stabilizing the fatty acid aggregates. As seen in Figure 3 fatty acid clustering occurs both above and below the phase transition temperature T_c of dipalmitoylphosphatidylcholine, suggesting that the physical state of the bilayer is not the determining factor. However, the decrease to zero of the mobility below the T_c point (Figure 3, top curve) indicates that parameters such as cluster size and the equilibrium between monomers and clusters are affected by the physical state of the bilayer. From our experiments no conclusion regarding the size of the cluster or the equilibrium between monomers and clusters can be drawn. Work on this problem is currently in progress.

The observation that the transition temperature T_c is raised when fatty acids are incorporated in dipalmitoylphosphatidylcholine bilayers (cf. Figure 3) is in agreement with differential scanning calorimetry data on mixtures of palmitic acid and dipalmitoylphosphatidylcholine (Jain & Wu, 1977;

Mabrey & Sturtevant, 1977) and myristic acid and dimyristoylphosphatidylcholine (Kantor & Prestegard, 1978; Usher et al., 1978). From the phase diagrams which Mabrey & Sturtevant (1977) and Kantor & Prestegard (1978) derived from their calorimetric data, the lateral separation of a pure fatty acid phase can be ruled out. This, however, is not at variance with the clustering of fatty acids proposed in this paper provided the average cluster size is sufficiently small (10–20 molecules) so that phosphatidylcholine bilayers containing randomly distributed clusters of fatty acids can be treated as a single phase (J. Sturtevant, personal communication).

Lateral Distribution of Spin-Labels in Phosphatidylcholine Bilayers. From a comparison of Figure 1a,b and the values of the increment in electrophoretic mobility (Table I), it is clear that fatty acid spin-labels behave differently from ordinary fatty acids: they are randomly distributed in the plane of liquid-crystalline phosphatidylcholine bilayers. This has also been established unequivocally from ESR spin-label work. Clustering of spin-label would lead to spin exchange and line broadening which is clearly not observed at the molar ratios of phospholipid/label > 100 commonly used in spin-label work. The difference in behavior of fatty acids and spin-labels is also evident from their effects on the transition temperature of dipalmitoylphosphatidylcholine bilayers. While the presence of ~10 mol % stearic acid produced a significant increase in T_c, the same amount of spin-label caused a decrease in T_c by a few degrees (Figure 3). Below the transition temperature the extent of stearic acid clustering seems to increase as evident from the decrease to zero in electrophoretic mobility (Figure 3, top curve). The transition to the gel state seems to induce some clustering of the spin-label as suggested by the decrease in electrophoretic mobility between 46 and 36 °C (Figure 3, bottom curve). The reproducibility of the curves in Figure 3 indicates that the rearrangement of the spin-label in that temperature range is reversible. The exact details of the rearrangement of the spin-label are unknown and are presently being studied by ESR techniques.

The difference in behavior of spin-labeled and ordinary fatty acids is probably due to the bulky oxazolidine ring not allowing for close packing of the label molecules. This interpretation is consistent with monolayer studies of Cadenhead et al. (1975), who reported significant differences in the molecular packing of monolayers of 12-doxylstearic acid and the parent molecule stearic acid. While long-chain fatty acids formed condensed monolayers at all pressures, monolayers of spin-labels were highly expanded (gaseous).

Fatty Acids as Molecular Probes. Since deuterated fatty acids behave like ordinary fatty acids in terms of clustering, their use as probes is subject to criticism. In such a situation, information derived from the probe molecule would be determined or at least modified by probe–probe interactions. The latter effect would critically depend on the probe/lipid mole ratio. The problem is clearly demonstrated by the chain ordering effect of stearic acid as evident from the increase in the phase transition temperature of dipalmitoylphosphatidylcholine (Figure 3, top curve). Seelig & Seelig (1977) reported a similar effect of palmitic acid on bilayers of 1-palmitoyl-2-oleoylphosphatidylcholine deuterated in the C-10 position. Using deuterium NMR measurements, these authors showed that at 27 °C the order parameter S_{mol} increased from 0.32 in the pure bilayer to 0.44 in the bilayer containing 15 wt % palmitic acid. In this concentration range the probe molecule itself may modify the packing and dynamics of the bilayer. Deuterium magnetic resonance measurements using deuterated fatty acid as probes are usually carried out at high label concentrations (5–10 mol %) to improve signal to noise ratios, and in this case the measurement can be expected to be troubled by probe–probe interactions. Therefore, the usefulness of deuterated fatty acids as molecular probes of the bilayer structure in models or biological membranes has to be carefully tested in each case.

Acknowledgments

The deuterated compound palmitic-2,2-d_2 acid was a gift of Dr. J. Seelig.

References

Ansell, G. B., & Hawthorne, J. N. (1964) in *Phospholipids*, p 96, Elsevier, Amsterdam.

Bangham, A. D., & Dawson, R. M. C. (1959) *Biochem. J. 72*, 486.

Cadenhead, D. A., Kellner, B. M. J., & Müller-Landau, F. (1975) *Biochim. Biophys. Acta 382*, 253.

Goddard, E. D. (1974) *Adv. Colloid Interface Sci. 4*, 45.

Hauser, H., & Dawson, R. M. C. (1967) *Eur. J. Biochem. 1*, 61.

Hauser, H., & Phillipis, M. C. (1973) *Chem., Phys. Appl. Surf. Act. Subst., Proc. Int. Congr., 6th, 1972, 2*, 371.

Hauser, H., Darke, A., & Phillips, M. C. (1976) *Eur. J. Biochem. 62*, 335.

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

Jain, M. K., & Wu, N. M. (1977) *J. Membr. Biol. 34*, 157.

Kantor, H. L., & Prestegard, J. H. (1978) *Biochemistry 17*, 3592.

Mabrey, S., & Sturtevant, J. M. (1977) *Biochim. Biophys. Acta 486*, 444.

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

Paltauf, F., Hauser, H., & Phillips, M. C. (1971) *Biochim. Biophys. Acta 249*, 539.

Papahadjopoulos, D. (1968) *Biochim. Biophys. Acta 211*, 467.

Patil, G. S., & Cornwell, D. G. (1977) *J. Lipid Res. 18*, 1.

Patil, G. S., Matthews, R. H., & Cornwell, D. G. (1972) *J. Lipid Res. 13*, 574.

Rand, R. P., & Sengupta, S. (1972) *Biochim. Biophys. Acta 255*, 484.

Rosano, H. L., Christodoulou, A. P., & Feinstein, M. E. (1969) *J. Colloid Interface Sci. 29*, 335.

Rouser, G., Kritchevsky, G., Heller, D., & Lieber, E. (1963) *J. Am. Oil Chem. Soc. 40*, 425.

Seelig, A., & Seelig, J. (1977) *Biochemistry 16*, 45.

Usher, J. R., Epand, R. M., & Papahadjopoulos, D. (1978) *Chem. Phys. Lipids 22*, 245.

Wojtczak, L. (1976) *J. Bioenerg. Biomembr. 8*, 293.