Degree of Hydration and Lateral Diffusion in Phospholipid Multibilayers†

J. Thomas McCown, Evan Evans,* Sherra Diehl,‡ and H. Craig Wiles

ABSTRACT: The fluorescence recovery after photobleaching technique (FRAP) was used to measure the lateral diffusion coefficient of the fluorescent lipid analogue *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) in egg yolk phosphatidylcholine (EPC) multibilayers as a function of water content. Diffusion coefficients were determined relative to a reference state of constant degree of hydration. The use of relative measurements allows changes in diffusivity to be determined accurately without the need for precise measurement of laser beam intensity profiles. Over the range of hydrations investigated, an 8-fold decrease in the diffusion coefficient accompanies water removal from the multilamellar system. Because of the anisotropic structure of the multilamellar system, it is expected that the lateral diffusivity of the probe will depend on both the area per lipid molecule and the proximity of lipid head groups in opposite layers. Therefore, we have correlated the lateral diffusivity with both the area per molecule relative to the state of maximum surface condensation and the volume fraction of lipid head groups in the aqueous region. The area per molecule and volume fraction of head groups in the aqueous region were calculated from published X-ray diffraction plus gravimetric data. In the range of partial hydration, the area per molecule in the surface is a linear function of the volume fraction of lipid head groups in the aqueous region. Likewise, in this region, the lateral diffusivity exhibits linear correlations with both increase in area relative to the condensed state and decrease in volume fraction of head groups in the aqueous region. The diffusivity extrapolates to zero at an area per molecule ($\sim$54 Å^2) which corresponds to maximum surface condensation and at a volume fraction of head groups equal to 0.68 (where the volume of water is about 96 Å^3 per lipid molecule).

Numerous studies have appeared in which the fluorescence recovery after photobleaching (FRAP)[1] technique was employed to characterize the diffusivity of fluorescent lipid analogues in lecithin multibilayers (Wu et al., 1977, 1978; Fahey & Webb, 1978; Rubenstein et al., 1978; Smith & McConnell, 1979; Vaz et al., 1979; Owicki & McConnell, 1980). A frequent interest in many has been the determination of the effects of temperature on a variety of multibilayer systems, both binary (lecithin–water) phases and systems in which nonhomologous species (cholesterol, polypeptides) have been introduced. Apart from the effects of temperature, the relationship between diffusivity and other variables which characterize the thermodynamic state of lipid–water systems has received little attention. In the absence of external forces, the state of binary lipid systems is fixed by specification of temperature, pressure, and degree of hydration (composition). X-ray diffraction studies (Reiss-Husson, 1967; Small, 1967; LeNeveu et al., 1977) have shown that reduction of the degree of hydration leads both to a decrease in the bilayer separation distance and to a reduction in the surface area per lipid molecule. The surface condensation and decreased water gap produced by progressive water removal are expected to affect lipid diffusivity.

Here we report the dependence of the lateral diffusivity of the fluorescent lipid probe *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) upon the degree of hydration of egg yolk phosphatidylcholine (EPC) multibilayers at constant temperature. Diffusion coefficients are determined relative to their value at a fixed degree of hydration. The use of relative measurements circumvents the need for the precise determination of laser beam profiles.

Materials and Methods

Lipids. Both EPC and the probe NBD-PE were purchased from Avanti Biochemicals (Birmingham, AL). According to the manufacturer, both products migrate as a single spot on silica gel thin-layer chromatography plates. Lipids were stored as chloroform (EPC) or hexane (NBD-PE) solutions under argon at −20 °C.

Multibilayers were prepared by deposition of a chloroform–hexane solution (7:1 v/v) of EPC and NBD-PE on a coverslip with subsequent evaporation of the solvent at reduced pressure. The mole ratio of probe to lipid was maintained at 0.2%. Lipid films were allowed to hydrate at least 24 h in a water-saturated argon atmosphere. When viewed under crossed polarizers, dark homogeneous regions fringed by bright domain boundaries were observed. Such observations are characteristic of well-ordered, hydrated multibilayers (Powers & Pershan, 1977; Wu et al., 1977).

Instrumentation. The basic methodology of the FRAP experiment has been given previously (Axelrod et al., 1976; Jacobson et al., 1976). In the experiments reported here, an argon ion laser (Coherent Radiation Model CR2) tuned to the 458-nm line was used for fluorescence stimulation. Typically the laser output level was 0.1 W. Bleach windows were opened by a solenoid-driven shutter with an attenuation of optical density 4. For a given experiment, the duration of bleach windows was less than 10% of the characteristic diffusion time, τ. Additional neutral density filters increased the total attentuation to an optical density of 5.5 throughout the observation phase of the experiment. After passing through a spatial filter, the laser beam was directed into the entrance aperture of the Ploem illuminator of a Leitz Ortholux II microscope. The microscope has been modified to accept a

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* Address correspondence to this author at the Department of Pathology, University of British Columbia, Vancouver, B.C., Canada V6T 1W5.

‡ Present address: Department of Electrical Engineering, Auburn University, Auburn, AL 36830.

[1] Abbreviations used: FRAP, fluorescence recovery after photobleaching; EPC, egg yolk phosphatidylcholine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine.

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negative lens used for fine positioning of the beam waist. A 10× objective was used to epiilluminate the sample.

Fluorescence from the illuminated sample region was imaged on the face of a photomultiplier tube (RCA C31034). The tube output was amplified and fed into a photon counter (Ortec Model 9315) and subsequently into the memory of a dedicated microprocessor. Count windows of 50-ms duration were used in all experiments.

Data Reduction. The theoretical treatment for recovery after photobleaching with a Gaussian beam profile has been given by Axelrod et al. (1976). For purely diffusive phenomena, the recovery half-time, τ_h, may be used to calculate the diffusion coefficient through the relationship

$$D = \gamma w^2/(4\tau_h) \quad (1)$$

where w is the beam waist radius (to the e^{-2} intensity point) and γ is a parameter relating the characteristic diffusion time to the half-time: $\tau = \tau_h/\gamma$. Axelrod's treatment allows the calculation of γ from the equilibrium fluorescence, F_0, and the fluorescence intensity immediately following the conclusion of specimen bleach, F_i. Typically, γ was 1.4 for the experiments reported.

The magnitude of a diffusion coefficient calculated with the above relationship is strongly dependent on the beam waist radius, a parameter difficult to measure directly. Provided w is constant, the ratio of the probe diffusion coefficient to that of a sample maintained at a specific reference state is independent of w, i.e.

$$\bar{D} = D/D_{ref} = \tau_{ref}/\tau \quad (2)$$

Thus relative diffusion coefficients may be calculated directly from the fluorescence recovery times. To facilitate this relative measurement, we constructed a twin sample chamber to be mounted on the microscope stage. One sample was maintained at a fixed state of hydration while the adjacent sample was equilibrated with water at different vapor pressures to achieve the desired degree of hydration. Bleach and recovery cycles were carried out on each sample to obtain the recovery time constants. This method yields two immediate advantages as compared to the conventional FRAP experiment: (1) uncertainties due to difficulties in the measurement of w are eliminated; (2) time-dependent changes in w due to long-term laser variations do not affect the result.

Though absolute magnitudes of diffusion coefficients are of intrinsic interest, more often the change in diffusivity in response to a specific variable is of greater interest in biological studies. This information is obtained directly through measurement of relative diffusion coefficients, and the difficulties previously mentioned may be avoided. The method can be adapted to a variety of experimental situations.

Multibilayer Hydration. The degree of multibilayer hydration was controlled by exposure of the lipid systems to a closed, circulating argon atmosphere of known relative humidity (defined as the ratio of the partial pressure of water to its saturation pressure, P/P_0). Humidity was maintained at the desired level by bubbling argon through aqueous sulfuric acid solutions. Tables which relate acid concentration to the vapor pressure of water may be found in the International Critical Tables. The relationship between the degree of hydration of lipid–water systems and the relative humidity of the equilibrating atmosphere has been explored previously (Elworthy, 1961; Lundberg et al., 1978). Each well of the sample chamber was connected to an independent environmental control system. Fully hydrated specimens were placed in the chamber and allowed to equilibrate an additional 24 h before experiments were commenced. Samples were changed after each experiment at a given humidity. A reference state of 94.5% relative humidity was chosen to avoid the possibility of free water accumulation in the system. All experiments were carried out at a temperature of 23 ± 2 °C.

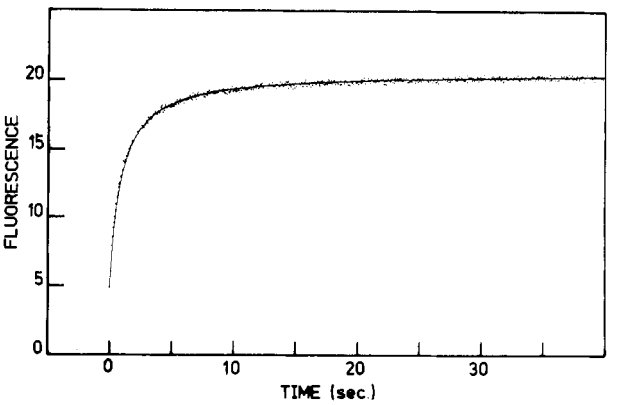

FIGURE 1: Fluorescence recovery kinetics for NBD-PE in fully hydrated EPC multibilayers. Solid line is the theoretical fit obtained by use of experimental fluorescence intensities prior to bleaching and immediately after the conclusion of bleach. Ordinate units are arbitrary.

Typically the fluorescence recovery kinetics of both specimens could be recorded within about 7 min. As the microscope stage was repositioned between recordings, it was important that both specimens be placed in the same position relative to the laser beam waist. For minimization of variations in w due to the repositioning, each sample was placed in a position yielding maximum fluorescence intensity (as determined by the average of at least four trial positions). A sample of 32 experiments conducted in this fashion led to a standard deviation of 8% in the characteristic diffusion time. Thus, relative diffusion coefficients are estimated to be accurate to within approximately 15%.

Results and Discussion

A typical fluorescence recovery is shown in Figure 1. Superimposed is the recovery calculated with a single-parameter (τ) least-squares fit. The calculated curve was generated with the recovery equation given by Axelrod et al. (1976) and the experimental values of F_i and F_0. The characteristic time obtained from the fit was 0.65 s while that calculated from the recovery half-time was 0.60 s. Generally the two methods of calculation were in agreement to within 10%. To within experimental error, fluorescence intensities which followed bleaching returned to the level observed prior to bleach. On the basis of a diffraction-limited spot radius (Barrett & Adams, 1968; Dickson, 1970), a lower limit of approximately 10^{-8} cm^2/s was calculated for the diffusion coefficient of NBD-PE in fully hydrated EPC multibilayers, a result comparable to that observed by other authors (Wu et al., 1978; Fahey & Webb, 1978). In a sample of 32 experiments the average relative diffusion coefficient obtained with both specimens exposed to the reference environment differed by only 2% from the expected ratio of 1. In addition, no hysteresis was observed for samples cycled through the lowest humidity investigated.

In Figure 2, the relative diffusion coefficient is plotted as a function of the logarithm of relative humidity. Over the range investigated, a smooth decrease of approximately a factor of 8 is observed. The relationship between relative humidity and degree of hydration may be used to correlate diffusivity with lipid water content. Figure 3 displays the dependence of degree of hydration on humidity as determined by gravimetric methods. Also shown is the composition at full hydration deduced from X-ray diffraction investigations.

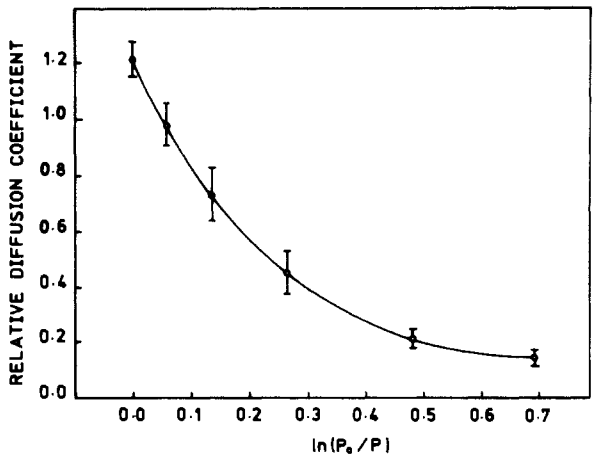

FIGURE 2: Relative diffusion coefficient plotted as a function of the natural logarithm of the inverse relative humidity of the equilibrating atmosphere. The magnitude with both specimens exposed to the reference humidity (unity expected) was found to be 0.98 experimentally. Data points are the average of from 5 to 32 separate FRAP experiments.

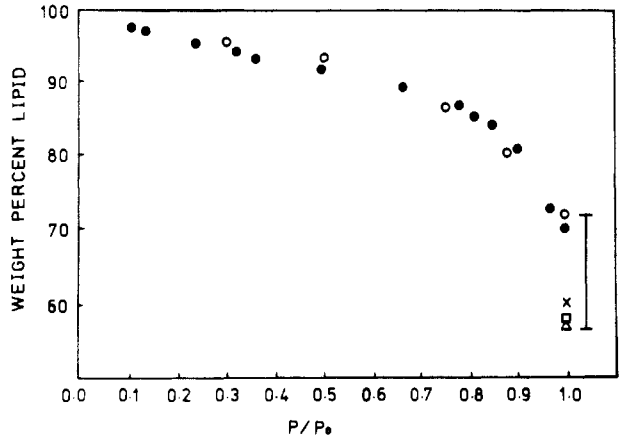

FIGURE 3: Weight percent lipid vs. relative humidity of the equilibrating atmosphere for EPC multibilayers [● (Elworthy, 1961), ○ (Lundberg et al., 1978)]. The three remaining points [× (Reiss-Husson, 1967), □ (LeNeveu et al., 1977), △ (Small, 1967)] represent the weight fractions at which no further change in the bilayer repeat distance is observed by X-ray diffraction. The bar delimits the range of uncertainty associated with the composition of fully hydrated systems.

Particularly noteworthy is the discrepancy observed in the composition of fully hydrated systems. The use of X-ray diffraction criteria (full hydration being defined as the point at which the bilayer repeat distance becomes independent of water content) led to a significantly higher estimation of the water content than that obtained in the vapor hydration investigations (full hydration assumed for systems in equilibrium with saturated water vapor). There are additional discrepancies in the reported X-ray data at the fully hydrated state.

Because the structure of the multilamellar system is anisotropic, we except the lateral diffusivity to depend on both the area per lipid molecule in the surface and the water gap between bilayers. In the range of partial hydration, the aqueous region is a solution of lipid head groups and water. Figure 4 is a schematic illustration adapted from Gary-Bobo & Rigaud (1975) which illustrates the head-group conformation and the approximate dimensions of the aqueous region in comparison to the mass-average water gap thickness deduced from X-ray diffraction plus gravimetric data. Clearly, the phosphorylcholine groups form a concentrated solution in the aqueous region. Indeed, it is the colligative property of these groups which opposes the removal of water from the multilamellar phase. The effect of the water removal is to produce a reduced pressure in the water phase that is offset by a repulsive stress normal to the bilayer and a lateral compressive stress in the bilayer surface (Parsegian et al., 1979; Evans & Skalak, 1979). The induced state of stress in the aqueous region is isotropic and depends on the concentration of head groups in the aqueous region. Therefore, the effect of proximity of the bilayers on the lateral diffusivity may be represented by the dependence on volume fraction of phosphorylcholine groups in the aqueous region.

From the bilayer repeat distances determined by X-ray diffraction, the area per lipid molecule and bilayer separation distance may be calculated as a function of degree of hydration and, hence, as a function of the humidity of the environment. With the assumption that the lipid and water densities are 1 g/cm^3 and both components are volumetrically incompressible (Liu & Kay, 1977), the area per lipid molecule is given by

$$A = 2V/\phi d \tag{3}$$

and the water gap between bilayers is

$$d_w = (1 - \phi)d \tag{4}$$

where V is the volume per lipid molecule (1267 Å^3; Small, 1967), ϕ is the weight fraction lipid, and d is the bilayer repeat distance. Obviously, these are mass-average dimensions. As is shown in Figure 4, the water gap d_w is not the same as the thickness of the aqueous region. (It is assumed that the acyl chains are a dense liquid with negligible water content, which is reasonable because of their hydrophobic character.) Similarly, the volume fraction, ν, of polar head groups in the aqueous region is calculated with the relation

$$\nu = V_{HG}/(d_w A + V_{HG}) \tag{5}$$

where V_{HG} is twice the volume of a phosphorylcholine group (one group for each surface). The difficulty is to evaluate the appropriate head group volume; here, we will use twice the value, 204 Å^3, given by Small (1967).

Since we anticipate the diffusivity to approach zero at the state of maximum surface condensation (e.g., near the L_α–L_β liquid crystalline–crystalline phase transition), we choose the area dilation fraction, α, relative to the area per molecule at maximum condensation to represent the intensive dependence on area per lipid molecule in the surface

$$\alpha \equiv \frac{A - A_c}{A_c} \tag{6}$$

where A_c is the area per molecule for maximum surface condensation. From X-ray data extrapolated to zero water content and the minimum area per molecule for surface pressure–area curves of synthetic lecithin monolayers (with similar fatty acid chain composition: Van Deenan et al., 1962), the value for A_c is about 54 Å^2. Using the values for A_c, V_{HG}, and the X-ray diffraction data (Reiss-Husson, 1967; Small, 1967; LeNeveu et al., 1977), we have calculated the area dilation fraction, α, and volume fraction of head groups, ν, as a function of hydration. Figure 5 presents the dependence of α and ν on the logarithm of the vapor pressure ratio (inverse of the relative humidity). The fractional area dilation is found to be a linear function of the head-group concentration in the aqueous region, as is shown in Figure 6. Figure 6 demonstrates the bilayer compression which accompanies dehydration (analogous to elastic compression of a sponge with water removal).

Correlating the results for lateral diffusivity (Figure 2) as a function of hydration with the calculated area dilation and volume fractions (Figure 5), we see that the lateral diffusivity

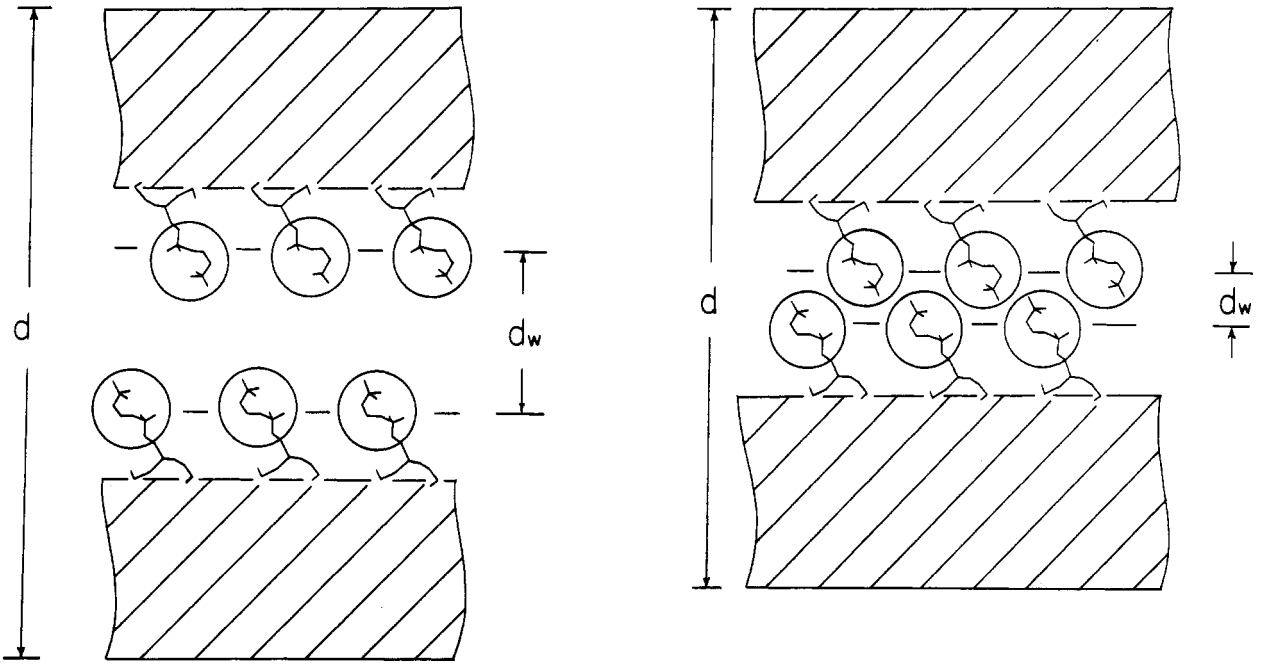

FIGURE 4: Schematic illustration adapted from Gary-Bobo & Rigaud (1975) of bilayer geometry at two states of hydration: (a, left) 26 wt % water (d = 54 Å, d_w = 14 Å) and (b, right) 9% water (d = 50 Å, d_w = 4.6 Å). The bilayer repeat distance d is taken from the midpoint of an acyl chain region (hatched area) to the midpoint of the next. Circles approximate the spherical volume excluded by phosphorylcholine. The water gap dimension, d_w, is the mass-average thickness determined from the weight fraction of water times d.

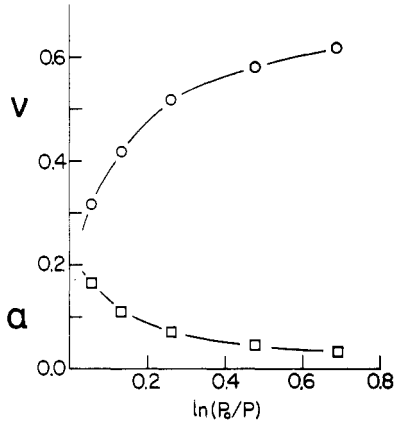

FIGURE 5: Volume fraction, ν, of polar head groups in the aqueous region and area dilation, α, relative to maximum condensation plotted vs. the logarithm of the vapor pressure ratio. Points are the average values of the three X-ray diffraction studies referenced in the text.

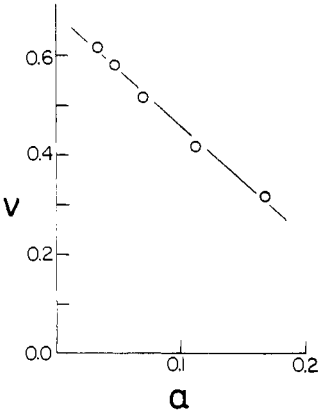

FIGURE 6: Polar head-group volume fraction plotted as a function of surface area dilation.

exhibits essentially linear relations with each variable, as shown in Figure 7 for the range of partial hydration. Because both area per molecule and bilayer spacing are reduced simultaneously by dehydration, it is not possible to use this experiment alone to separate the dependence of lipid diffusivity on the surface condensation from the head-group concentration. However, some interesting features are observed. As already noted, the relations are nearly linear. These extrapolate to zero (diffusivity) at the condensed area per molecule (~54 Å^2) as anticipated and a volume fraction of head groups equal to 0.68. At the state of maximum condensation, the chains are almost fully extended with little rotometric disordering and the lipids are tightly packed. Thus, the lipid translational mobility is severely limited. On the other hand, the volume fraction intercept corresponds to a water volume of 96 Å^3 per lipid molecule. This is equivalent to about three water molecules per lipid. Hence, the head groups are tightly crowded (perhaps interdigitated), with the layers coalesced as shown in Figure 4.

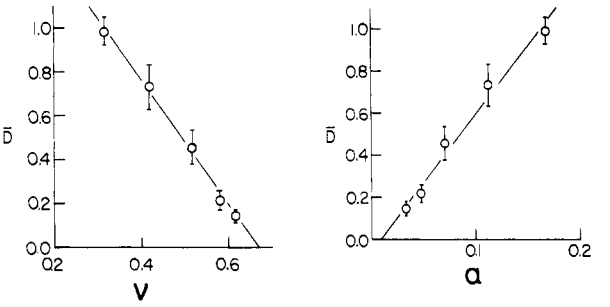

FIGURE 7: (a, left) Correlation of the relative diffusion coefficient $\bar{D}$ with polar head-group volume fraction and (b, right) correlation with surface area dilation. Note both relationships are essentially linear for the range of partial hydration.

Although the effects of proximity of the bilayers cannot be directly separated from the effects of surface condensation in this study, there is a method to isolate the dependence of lateral diffusivity on surface dilation at constant temperature. The approach is to measure the lipid diffusivity in a large single-walled EPC vesicle. Since the vesicle bilayer has fixed mass, the area per molecule can be forced to increase by aspiration of the vesicle with a suction micropipet. The experimental

method has been used by Kwok & Evans (1981) to measure the elastic area compressibility of a single EPC bilayer. An interesting result was that the elastic area compressibility of the single bilayer was nearly the same as the value deduced from analysis of equilibrium dehydration of multilayers in the range of partial hydration (Evans & Skalak, 1979). This indicates that the surface compressibility was essentially unaffected by proximity of the bilayers. If the lipid diffusivity depends only on area per molecule to the extent shown in Figure 7, we would anticipate about a 20–30% increase in diffusivity for a 3–4% area dilation (which is the limit where the vesicles are typically lysed). We are presently pursuing this study.

The important conclusion is that the lateral diffusivity in multibilayer systems is a strong function of the activity of the water phase, or water content, even though the acyl chains remain in the fluid state. It is expected that this property will significantly affect the kinetics of processes that involve membrane–membrane contact, e.g., fusion and vesiculation events.

References

Axelrod, D., Koppel, D. E., Schlessinger, J., & Elson, E. (1976) *Biophys. J. 16*, 1055.
Barrett, J. J., & Adams, N. I. (1968) *J. Opt. Soc. Am. 58*, 311.
Dickson, L. D. (1970) *Appl. Opt. 9*, 1854.
Elworthy, P. H. (1961) *J. Chem. Soc.*, 5385.
Evans, E. A., & Skalak, R. (1979) *CRC Crit. Rev. Bioeng. 4*, 380.
Fahey, P. F., & Webb, W. W. (1978) *Biochemistry 17*, 3046.
Gary-Bobo, C. M., & Rigand, J. L. (1975) *Colloq. Int. C.N.R.S. 246*, 121.
Jacobson, K., Derzko, Z., Wu, E.-S., Hou, Y., & Poste, G. (1976) *J. Supramol. Struct. 5*, 565 (417).
Kwok, R., & Evans, E. (1981) *Biophys. J.* (in press).
LeNeveu, D. M., Rand, R. P., Parsegian, V. A., & Ginell, D. (1977) *Biophys J. 18*, 209.
Liu, N.-I., & Kay, R. L. (1977) *Biochemistry 16*, 3484.
Lundberg, B., Svens, E., & Ekman, S. (1978) *Chem. Phys. Lipids 22*, 285.
Owicki, J. C., & McConnell, H. M. (1980) *Biophys. J. 30*, 383.
Parsegian, V. A., Fuller, N., & Rand, R. P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2750.
Powers, L., & Pershan, P. S. (1977) *Biophys. J. 20*, 137.
Reiss-Husson, F. (1967) *J. Mol. Biol. 25*, 363.
Rubenstein, J. L. R., Smith, B. A., & McConnell, H. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 76*, 15.
Small, D. (1967) *J. Lipid Res. 8*, 551.
Smith, B. A., & McConnell, H. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2759.
Wu, E.-S., Jacobson, K., & Papahadjopoulos, D. (1977) *Biochemistry 16*, 3936.
Wu, E.-S., Jacobson, K., Szoka, F., & Portis, A. (1978) *Biochemistry 17*, 5543.
Van Deenen, L. L. M., Houtsmuller, U. M. T., DeHaas, G. H., & Mulder, E. (1962) *J. Pharm. Pharmacol. 14*, 429.
Vaz, W. L. C., Jacobson, K., Wu, E.-S., & Derzko, Z. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5645.

Characterization by Infrared Spectroscopy of the Bilayer to Nonbilayer Phase Transition of Phosphatidylethanolamines†

Henry H. Mantsch,* Adèle Martin, and David G. Cameron

ABSTRACT: A Fourier-transform infrared spectroscopic study of the thermotropic behavior of egg yolk phosphatidylethanolamines is reported. Two phase changes were monitored, the gel to liquid-crystalline acyl chain melting transition, centered at 12 °C, and a transition from the liquid-crystalline to the inverted hexagonal phase, centered at 28 °C. It is demonstrated that the gel to liquid-crystalline phase transition results in a large increase in the conformational disorder of the acyl chains in the bilayer and that the nonbilayer phase contains a still higher degree of conformational disorder. It is shown that the transition to the inverted hexagonal phase is promoted by highly unsaturated acyl chains. A model is developed for the bilayer to nonbilayer phase transition in which it is proposed that the driving force which triggers this phase transition is the introduction of a degree of conformational disorder so high that the integrity of the bilayer surface can no longer be maintained, due to the volume requirements of the acyl chains. A number of previously reported data are rationalized in terms of this hypothesis.

The physical properties of lipids provide information of fundamental significance to the determination of the structural and functional properties of biomembranes. With this aspect in mind, there have been numerous physical studies of lipid bilayer structures in the gel phase (Figure 1A) and in the liquid-crystalline phase (Figure 1B). However, there are certain naturally occurring lipids, particularly the phosphatidylethanolamines, which also form nonbilayer structures, such as the inverted hexagonal (H_{II}) phase (Figure 1C). The macromolecular properties of this H_{II} phase have been well characterized by differential scanning calorimetry (Cullis & de Kruiff, 1978) and by X-ray (Luzatti et al., 1968; Rand et al., 1971) and freeze–fracture techniques (Cullis et al., 1978a). However, studies employing spectroscopic methods capable of monitoring the molecular properties have been so far limited to ^{31}P NMR (Cullis & de Kruiff, 1976, 1978) and ^{2}H NMR (Gally et al., 1980; Taylor & Smith, 1981). The nature and particularly the driving force of the transition from the lamellar

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