Lateral Diffusion in the Liquid Phases of Dimyristoylphosphatidylcholine/Cholesterol Lipid Bilayers: A Free Volume Analysis†

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ABSTRACT: The technique of fluorescence recovery after photobleaching is used to perform an extensive study of the lateral diffusion of a phospholipid probe in the binary mixture dimyristoylphosphatidylcholine/cholesterol, above the melting temperature of the phospholipid. In the regions of the phase diagram where a single liquid phase exists, diffusion can be quantitatively described by free volume theory, using a modified Macedo–Litovitz hybrid equation. In the liquid–liquid immiscibility region, the temperature dependence of the diffusion coefficient is in excellent agreement with current theories of generalized diffusivities in composite two-phase media. A consistent interpretation of the diffusion data can be provided based essentially on the idea that the primary effect of cholesterol addition to the bilayer is to occupy free volume. On this basis, a general interpretation of the phase behavior of this mixture is also proposed.

The properties of lipid bilayers composed of phospholipids and cholesterol have been studied over the past 2 decades. Two considerations have contributed to this continued interest. First, the lipid component of mammalian cell plasma membranes consists primarily of these two types of molecules (Rouser et al., 1968; Houslay & Stanley, 1982; Cullis & Hope, 1985). Second, these mixed bilayers are liquid crystals accessible to direct experimentation with techniques of physical chemistry. In order for quantitative studies to be possible, a knowledge of the phase diagram of these systems is essential. Binary mixtures of dimyristoylphosphatidylcholine (DMPC)[1] or dipalmitoylphosphatidylcholine (DPPC) and cholesterol have received much attention in this respect. Early studies using differential scanning calorimetry (DSC) (Mabrey et al., 1978; Estep et al., 1978) and electron spin resonance (ESR) (Shimshick & McConnell, 1973; Recktenwald & McConnell, 1981) indicated the existence of both solid–liquid and liquid–liquid immiscibility regions. Theoretical work (Ipsen et al., 1987, 1989) is in agreement with the general features of these experimental studies and suggests moreover the existence of a critical point in the liquid state of these mixtures. However, only recently have rather complete experimental phase diagrams become available for DMPC/cholesterol (Figure 1) and DPPC/cholesterol (Vist & Davis, 1990; Sankaram & Thompson, 1990a,b, 1991), obtained using nuclear magnetic resonance (NMR), DSC, and ESR techniques. Above the melting temperature (T_m) of DMPC (23.9 °C; Mabrey & Sturtevant, 1976), this system can exist in one of two possible liquid phases: a liquid-disordered (L_d) phase, at low cholesterol concentrations, and a liquid-ordered (L_o) phase, at high cholesterol concentrations, or these two phases can coexist at the same temperature and pressure (Figure 1). Recently, new models have been proposed for the molecular arrangements in each phase: in the L_d phase, the cholesterol molecules are believed to span the hydrocarbon core of both leaflets of the bilayer; in the L_o phase, cholesterol is thought to pack like the phospholipid molecules in each leaflet (Sankaram & Thompson 1990a, 1991).

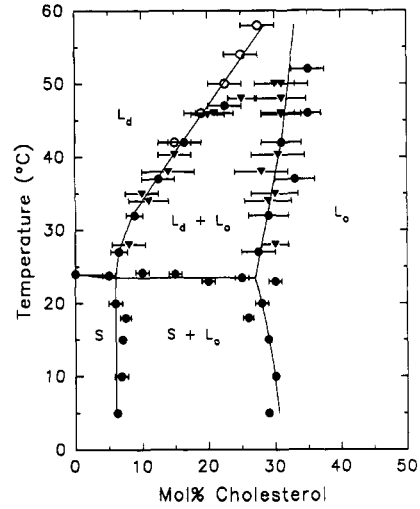

FIGURE 1: DMPC/cholesterol phase diagram. Filled points correspond to discontinuities in ESR experiments using a 5-PC spin-label (●) and a 16-PC biradical label (▼) (Sankaram & Thompson, 1991). (○) are breaks in the behavior of the diffusion coefficient (Figure 4). The lines drawn through the points represent our best judgement.

Prior to this knowledge of the phase diagram, there were several studies which examined lateral diffusion in phosphatidylcholine (PC)/cholesterol binary mixtures (Rubenstein et al., 1979; Alecio et al., 1982; Galla et al., 1979; Lindblom et al., 1981; Shin & Freed, 1989). Recently, we have used fluorescence recovery after photobleaching (FRAP) to study the connectivity of the liquid and solid phases in several lipid binary mixtures in the solid–liquid coexistence region (Vaz et al., 1989, 1990; Bultmann et al., 1991; Almeida et al., 1992). In the present study, we have used this technique to study

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; NBD-C(10)PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)didecanoylphosphatidylethanolamine; PC, phosphatidylcholine; FRAP, fluorescence recovery after photobleaching; ESR, electron spin resonance; NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; L_d, liquid-disordered (phase); L_o, liquid-ordered (phase); D, diffusion coefficient; E_a, activation energy; T_m, melting temperature.

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phospholipid lateral diffusion of *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)didecanoylphosphatidylethanolamine [NDB-C(10)-PE] in the liquid phases of DMPC/cholesterol in a systematic way. We are interested in understanding diffusion in the one-phase regions on the basis of free volume theory (Cohen & Turnbull, 1959; Macedo & Litovitz, 1964). It is generally believed that cholesterol causes an increase in the density of lipid bilayers or, in other words, a decrease in free volume. We can show that a modified Macedo–Litovitz diffusion equation provides a convenient means of understanding quantitatively the relation between the diffusion coefficient of a phospholipid and the concentration of cholesterol, through the molecular area occupied by this molecule in the plane of the bilayer. An interpretation is proposed of the DMPC/cholesterol phase diagram based on free volume. In the liquid–liquid immiscibility region, we attempt to determine the connectivity of the two phases with a simple analysis of the diffusion results based on the phase diagram. We find, however, that it is not possible to determine the line of connectivity in this two-phase region, due to the small difference between the diffusion coefficients in the two phases. On the other hand, the values of the diffusion coefficients in this region are explained exactly by combining their values at the boundaries of the phase diagram using the lever rule and current theories of transport properties of statistically isotropic, two-phase systems (Milton, 1981; Torquato, 1985; Kim & Torquato, 1990, 1991).

MATERIALS AND METHODS

DMPC was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was obtained from Serva Fine Biochemicals (Heidelberg, FRG). The purity of the lipids was confirmed by thin-layer chromatography. All lipids were used without further purification. Chloroform solutions of the lipids were prepared and stored at −20 °C. NBD-C(10)-PE was prepared as described before (Vaz & Hallmann, 1983).

FRAP Experiments. Multibilayers were prepared as described earlier (Vaz et al., 1989); 2–3 mg of lipid was mixed with the NBD probe at a probe to lipid molar ratio of 1:1000 in chloroform solution and allowed to stand for 30 min at room temperature. The solvent was then evaporated to a volume of about 0.1 mL and the solution deposited on a siliconized microscope slide at 80 °C, making a lipid film deposit of about 1 cm^2. The microscope slide was placed in a vacuum desiccator over anhydrous calcium chloride granules for 4 h and then warmed to 70 °C in an oven for 10 min. Hydration of the bilayers was carried out at this temperature by depositing a 75-μL drop of 10 mM phosphate buffer, pH 7.5, containing 50 mM KCl and 0.02% NaN_3 on the microscope slide and forcing it to spread over the lipid film with the aid of a cover slip. The samples, now in excess water, were incubated for 2 h at 70 °C, and finally cooled slowly to room temperature over about 4 h. The edge of the cover slip was sealed with a silicone paste (Bayer AG, Leverkusen, FRG) to prevent water evaporation. Samples were left in the dark for 2–15 days before measurements. The multibilayers used for FRAP experiments were selected by inspection with polarization microscopy. The FRAP measurements were made using a circular beam profile with a radius of 4 μm, with the 488-nm line of an argon ion laser. The laser output of 200 mW was split into a monitoring and a bleaching beam with an intensity ratio of 10^{-4}. A Zeiss Neofluar 6.3/0.2 objective was used in these experiments. The temperature of the sample was controlled by a Peltier temperature control unit (Cambridge Thermionic Corp., Cambridge, MA) built into the microscope stage; the temperature control is accurate to about ±0.15 °C. Further details of the apparatus can be found in a previous publication (Vaz et al., 1989). The measurements were begun at 58 °C. The samples were cooled by setting the control unit to a desired temperature and waiting for a period of at least 30 min before each measurement. When the temperature was changed by more than 2 °C, this was done in steps of 2 °C, waiting for about 10 min between each change. Only cooling scans were performed because in previous studies either there was no hysteresis present (Vaz et al., 1989) or the cooling scan represented the equilibrium curve (Almeida et al., 1992). The bleaching times were of the order of 30–60 ms. The fluorescence recovery was monitored for a period of 30 s to 2 min as appropriate, and the data were collected in a total of 1000 channels. To ascertain that no measurable bleaching was caused by the monitoring beam, the fluorescence intensity was measured for a period of time equivalent to that of an actual experiment, but without the bleach pulse. The recovery curves were fitted to the theoretical equations (Soumpasis, 1983) using the Simplex algorithm (Quantum Chemistry Exchange Program, J. P. Chandler, Oklahoma State University, Stillwater, OK). We allowed for the existence of more than one recovery component by using a linear ramp for the possible slow components, but this proved not to be necessary.

Theory. (a) Diffusion in the Liquid-Crystalline Phase of One-Component Lipid Bilayers. Vaz et al. (1985) showed that a free area model for diffusion in fluid systems, based on the free volume theory of Cohen and Turnbull (1959), can be used to describe quantitatively the diffusion process in lipid bilayers. The main weakness of this model is the use of several parameters to characterize the system, some of which are ultimately determined by the fit to the experimental diffusion data. We propose here a similar approach, reducing, however, the number of parameters to a minimum. The starting point is the Macedo–Litovitz hybrid equation (Macedo & Litovitz, 1964) which represents a refinement of the Cohen and Turnbull model. That equation was rederived on the basis of statistical mechanics (Chung, 1965) and provides a convenient way to describe the diffusion process. According to free volume theory (Cohen & Turnbull, 1959; Macedo & Litovitz, 1964), diffusion is limited by the occurrence of a free volume greater than a critical size next to a diffusing particle. Free volumes smaller than the critical size do not contribute to diffusion.

We consider a particle performing a random walk in two dimensions. At each diffusion step, the particle needs to have both a certain activation energy and a minimum free area to move into. In accordance with Macedo and Litovitz, the diffusion coefficient (D) in a two-dimensional surface is of the form

$$D = D'p(a)p(E) \tag{1}$$

where $p(a)$ is the probability that the diffusing particle will find a vacancy next to it of an area greater than a certain critical size and $p(E)$ is the probability that enough energy will be available at each diffusion step to overcome the interactions with neighboring molecules. These probabilities are given by

$$p(a) = \exp\left(-\frac{a_o}{a(T) - a_o}\right)$$

$$p(E) = \exp\left(-\frac{E_a}{kT}\right) \tag{2}$$

where $a(T)$ is the average area per molecule, which is a function of temperature, and a_o is the critical area which is essentially

the close-packed cross-sectional molecular area. The average free area per molecule in the plane of the bilayer is $a_f = a(T) - a_o$. E_a is the activation energy associated with diffusion, k is the Boltzmann constant, and T is the absolute temperature. A geometric factor (γ) is frequently used as a multiplier of the numerator in the power term in eq 2, i.e., $p(a) = \exp(-\gamma a_o/a_f)$. However, it was shown by McCarthy and Kozak (1982) that this is not necessary, at least if the interpretation of the critical area a_o is that given above. We shall adopt their point of view.

In order to obtain an expression for the preexponential factor D', we consider a particle performing a random walk in a two-dimensional lattice. This derivation follows that of McCarthy and Kozak (1982). The particle moves with an average velocity v, $v = \delta/\tau$, where δ is the distance between the lattice points and τ is the time per jump. In two dimensions, the average kinetic energy of a particle, E_k (thermal energy), is related to the average velocity of a particle of mass m by

$$E_k = kT = {}^1/_2 mv^2 \tag{3}$$

In two dimensions, the diffusion coefficient is given by

$$D' = \delta^2/4\tau \tag{4}$$

Substituting $v = \delta/\tau$ and eq 3 into eq 4 and rearranging terms, we obtain

$$D' = \frac{\delta}{2\sqrt{2}}\sqrt{\frac{kT}{m}} \tag{5}$$

Substituting eq 2 and 5 into eq 1, the diffusion coefficient is given by

$$D = \frac{\delta}{2\sqrt{2}}\sqrt{\frac{kT}{m}}\exp\left[\frac{-a_o}{a(T)-a_o} - \frac{E_a}{kT}\right] \tag{6}$$

This equation can be written in a more convenient form. First, we note that in a lattice $\delta = [a(T)]^{1/2}$. Second, we use the molecular weight M instead of the mass m with $m = M/N_A$, where N_A is Avogadro's number. Substituting the numerical constants (N_A, k) and using the appropriate factor to convert the result to the more familiar units of centimeters squared per second, we obtain

$$D = (3.224 \times 10^{-5})\sqrt{\frac{Ta(T)}{M}}\exp\left(\frac{-a_o}{a(T)-a_o} - \frac{E_a}{kT}\right) \tag{7}$$

Several concepts involved in this diffusion equation deserve comment. The way the effect of free area on diffusion was introduced, as determining the probability of finding a large enough vacancy next to the test molecule, is convenient in a formal manner for the purpose of derivation, but is probably too mechanistic. The free area is a property of the bilayer as a whole, and the important aspect for diffusion is the possibility of redistributing this free area. If we consider the entire membrane, this redistribution simply results from density fluctuations, without change in total energy. Locally, however, an energy change might be associated with the diffusion process. The merit of the Macedo–Litovitz over the Cohen–Turnbull equation is that it takes this possibility into account. The activation energy reflects the interactions of a lipid molecule with its environment, which include the lipid neighbors in the bilayer and the aqueous phase surrounding it. Vaz et al. (1987) showed that the surrounding phase has an important effect on the temperature dependence of the phospholipid diffusion coefficient.

(*b*) *Diffusion in the Cholesterol-Rich, Liquid-Ordered Phase.* In this phase, the cholesterol molecules are believed to be packed in each monolayer in the same way as the phospholipid molecules (Sankaram & Thompson, 1990b, 1991). The main effect of cholesterol is to decrease the available free area. The diffusion coefficient is still formally given by eq 7, but the free area and the activation energy may now be different. In particular, to obtain the area per phospholipid molecule, we have to subtract from the total area $a(T)$ the area na_o^{Chol} where n is the mole ratio of cholesterol to phospholipid and a_o^{Chol} is the molecular closed-packed area for cholesterol, or the area effectively occupied by the cholesterol molecules.

(*c*) *Diffusion in the Coexistence Region of L_d and L_o Phases.* In the liquid–liquid coexistence region, the diffusion coefficient is obtained by appropriately weighing the contributions from the two phases. Owicki and McConnell (1980) derived expressions for diffusion in inhomogeneous membranes which describe diffusion in the solid–liquid coexistence region. However, their model corresponds to a highly ordered system, which they believed was reflected in the experimentally observed rippled structures in bilayers in this region (Kleemann & McConnell, 1976; Copeland & McConnell, 1980). The liquid–liquid coexistence region probably corresponds better to a statistically isotropic mixture of phase domains. For such a system, in two dimensions, the diffusion coefficient in the mixture lies between the Hashin upper (D_{upper}) and lower (D_{lower}) bounds (Hashin, 1965; Milton, 1981):

$$D_{lower} = D_2\frac{1+f_1\beta_{12}}{1-f_1\beta_{12}}$$

$$D_{upper} = D_1\frac{1+f_2\beta_{21}}{1-f_2\beta_{21}} \tag{8}$$

where f_1, f_2, D_1, and D_2 are the fractions and the diffusion coefficients in phases 1 and 2. In these expressions, $D_1 > D_2$ and $\beta_{12} = (D_1 - D_2)/(D_1 + D_2)$. In principle, it is possible to assess the connectivity of the two phases from the dependence of these functions on the fractions of the two phases present, if the ratio D_1/D_2 is large enough. Experimentally, it would be necessary to observe that the diffusion coefficient in the mixture shows a crossover point from D_{upper} to D_{lower} at a particular fraction of one of the phases.

An improvement on these bounds have been proposed recently by Torquato (1985). The Torquato relation for the upper bound in two dimensions is given by

$$D_{upper} = D_1\frac{1+f_2\beta_{21}-f_1\xi_2\beta_{21}^2}{1-f_2\beta_{21}-f_1\xi_2\beta_{21}^2} \tag{9}$$

and was shown to be in excellent agreement with Brownian motion simulations (Kim & Torquato, 1990, 1991). Here $\xi_2 = f_2/3 - 0.05707f_2^2$ is a microstructure parameter (Kim & Torquato, 1990). The lower bound is simply obtained by interchanging the subscripts 1 and 2 everywhere.

Phospholipid Average Volumes and Cross-Sectional Areas. In order to use eq 7, it is necessary to know the average area per phospholipid as a function of temperature. The strategy used to obtain this information was the following: (1) determine the specific volume $v(T)$ as a function of temperature from dilatometric measurements; (2) obtain the bilayer thickness $d(T)$ as a function of temperature from the deuterium NMR order parameter and its temperature dependence. The area $a'(T)$ per phospholipid molecule as a function of temperature is then given by $a'(T) = fv(T)d^{-1}(T)$ Å^2 ($f = 2M$), where the factor 2 appears to account for having lipids in the two monolayers. In the case of the pure phospholipid, M is simply the molecular weight; in the phospholipid/cholesterol

mixtures, $M = M(\text{PC}) + nM(\text{Chol})$, where $M(\text{PC})$ is the molecular weight of DMPC, $M(\text{Chol})$ is the molecular weight of cholesterol, and n is the cholesterol to DMPC mole ratio. Notice that $a'(T)$ still includes, in the mixtures, the area occupied by cholesterol per phospholipid molecule.

The data of Nagle and Wilkinson (1978) for $v(T)$ were used for pure DMPC. For convenience in the calculations, data points obtained from their scan and from a short linear extrapolation of the final part of the scan were fitted to a second-order polynomial in T. For the DMPC/cholesterol mixtures, the corresponding data are not available in the literature. However, Melchior et al. (1980) have published scans of the specific volume as a function of temperature for DPPC/cholesterol mixtures. The phase diagrams of DMPC/cholesterol and DPPC/cholesterol are extremely similar, except for position along the temperature axis (Sankaram & Thompson, 1990a, 1991). Therefore, we calculated from the DPPC/cholesterol scans the corresponding curves for DMPC/cholesterol mixtures for the compositions 30:70, 40:60, and 50:50 by mapping the curves onto the DMPC transition region. This was performed by (1) a translation of the DPPC/cholesterol curves in the temperature axis by the difference in the transition temperatures of the two phosphoslipids (41.4–23.9 °C, Mabrey & Sturtevant, 1976), (2) rescaling the values of the specific volumes by the ratio of the changes in the specific volume of DMPC to DPPC, and (3) requiring that the curves of DMPC/cholesterol mixtures intercept the curve of pure DMPC at the phase transition at the same relative amplitudes of the change in specific volume observed in DPPC/cholesterol for the same cholesterol concentrations. The curves were approximated by a straight line which was extrapolated to higher temperatures. The approximation seems acceptable for cholesterol concentrations greater then 30 mol %, though it is not satisfactory below this value, due to the evident curvatures of the dilatometric scans. For pure DMPC, the function $v(T) = (-1.35 \times 10^{-5})T^2 + (9.30 \times 10^{-3})T - 0.595$ mL/g was found to give an accurate representation of the specific volume data above 25 °C (slightly above the melting temperature of DMPC, 23.9 °C). For the mixtures, the corresponding lines are represented by $v(T) = (1.12 \times 10^{-3})T + 0.624$ mL/g, $v(T) = (8.02 \times 10^{-4})T + 0.719$, and $v(T) = (5.58 \times 10^{-4})T + 0.793$, for DMPC/cholesterol 70:30, 40:60, and 50:50, respectively. These functions are plotted in Figure 2A.

The temperature dependencies of the bilayer thickness (d), calculated from the deuterium NMR order parameter (Sankaram & Thompson, 1990b), are 0.034 Å/°C for pure DMPC and 0.016 Å/°C for 2:1 and 1:1 DMPC/cholesterol (M. B. Sankaram, personal communication). This information, in combination with the value for the bilayer thickness at 37 °C (Sankaram & Thompson, 1990b), was used to calculate expressions for the reciprocal thickness as a function of temperature. These are $d^{-1} = (2.5 \times 10^{-5})T + (1.9 \times 10^{-2})$ Å^{-1} for pure DMPC and $d^{-1} = (9.6 \times 10^{-5})T + (2.1 \times 10^{-2})$ Å^{-1} for DMPC/cholesterol 2:1 and 1:1. The latter function was used for the DMPC/cholesterol mixtures 70:30, 60:40, and 50:50. The areas per phospholipid molecule are given by $a(T) = a'(T) - na_0^{\text{Chol}}$, where n is the cholesterol:phospholipid ratio, a_0^{Chol} is the effective cross-sectional area of cholesterol for which a value of 26.6 Å^2 was used (see below), and $a'(T)$ was calculated in the manner outlined above; for DMPC, $a'(T) = (7.58 \times 10^{-7})T^3 - (5.27 \times 10^{-5})T^2 + 0.36T - 25$ Å^2, and for DMPC/cholesterol mixtures, $a'(T) = (2.95 \times 10^{-5})T^2 + (8.22 \times 10^{-2})T + 36.7$ (70:30), $a'(T) = (2.36 \times 10^{-5})T^2 + (7.36 \times 10^{-2})T + 47$ (60:40), and $a'(T) = (1.87 \times 10^{-5})T^2 + (6.82 \times 10^{-2})T + 59.1$ (50:50). The areas $a(T)$ as a function of temperature are plotted in Figure 2B. Notice that, for the three mixtures, the areas are quite similar but considerably smaller than that of pure DMPC. However, among the mixtures, *the areas increase in the order 70:30 < 60:40 < 50:50*. The approximations involved in the calculation of the areas may affect the final values, but not the qualitative differences between them.

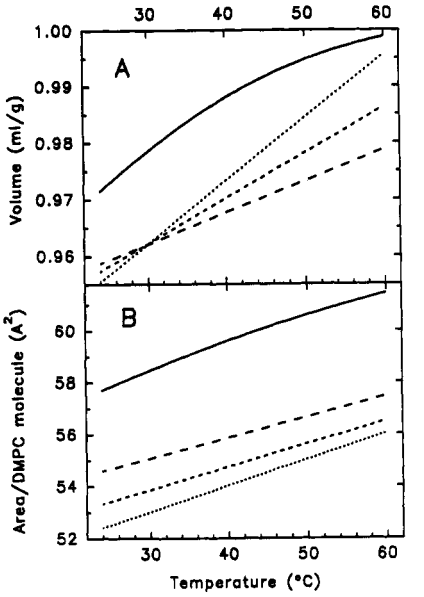

FIGURE 2: (A) Specific volume and (B) area per DMPC molecule as a function of temperature. Curves are for pure DMPC (solid line) and DMPC/cholesterol 70:30 (short dash), 60:40 (medium dash), and 50:50 (long dash). The area occupied by cholesterol (26.6 Å^2/molecule) has been subtracted in the three curves corresponding to the areas in the mixtures.

RESULTS AND DISCUSSION

FRAP Experiments. Fluorescence recovery curves were measured in DMPC/cholesterol mixtures with molar ratios of 85:15, 80:20, 75:25, 70:30, 40:60, and 50:50, for temperatures between 22 and 58 °C. The fluorescent phospholipid derivative NBD-C(10)PE was the probe used in these experiments because it partitions equally between the two liquid phases as estimated by fluorescence anisotropy partition measurements (data not shown). Figure 3 shows a typical FRAP curve for this binary system. In all cases, the recovery was found to be complete. The fit to the equations of Soumpasis (1983) was very good, except for some points at 24 °C and below where it was only slightly poorer. From the analysis of each fluorescence recovery curve, the value of the characteristic recovery time (τ) was obtained. The diffusion coefficient D was calculated from τ using the relation $D = \omega^2/4\tau$, where ω is the radius of the bleached spot (Soumpasis, 1983).

Diffusion Coefficient in DMPC/Cholesterol Mixtures as a Function of Temperature and Cholesterol Concentration above the T_m of DMPC. For some selected temperatures, from 26 to 58 °C, the diffusion coefficients are plotted as a function of cholesterol concentration in Figure 4. Our results are in good agreement, when comparison can be made, with those of Rubenstein et al. (1979). In general, the diffusion coefficient decreases with increasing cholesterol concentration. We notice, however, that between 50 and 58 °C the diffusion coefficient begins to decrease between 20 and 30 mol % cholesterol, indicating that a phase boundary is probably located in that region. This fact has lead us to modify the

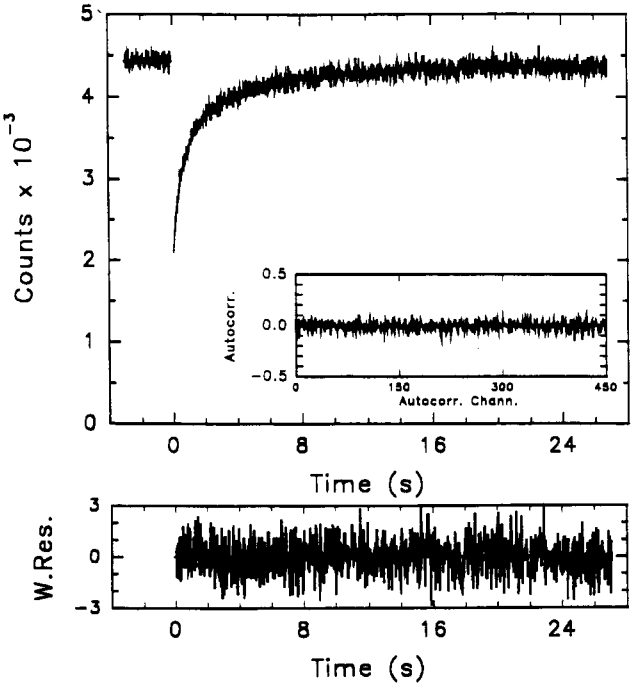

FIGURE 3: Typical experimental fluorescence recovery curve. This curve corresponds to a DMPC/cholesterol 80:20 mixture at 34 °C, in the middle of the L_d–L_o coexistence region as given by the phase diagram. The parameters given by the fit are the following: τ, 0.68 s; recovery, 99.6%; slope, 0.0 cps; χ^2, 1.0.

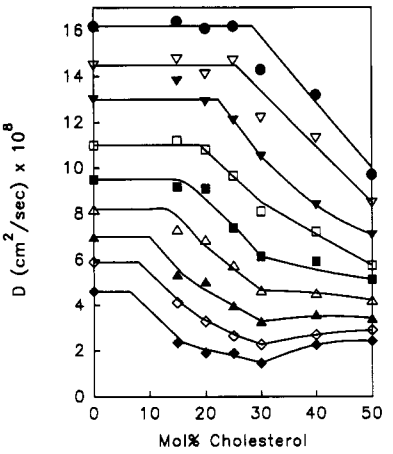

FIGURE 4: Diffusion coefficient as a function of cholesterol mole percent for several different temperatures (in degrees centrigrade): 58 (●), 54 (▽), 50 (▼), 46 (□), 42 (■), 38 (△), 34 (▲), 30 (◇), and 26 (◆). The points for pure DMPC are not experimental but are taken from the curve in Figure 5A, because the experimental points were obtained at other temperatures. The lines are drawn through the points to guide the eye. They are horizontal up to the liquid immiscibility region in accordance with the experimental evidence available (see text).

phase diagram (open circles in Figure 1), extending the two-phase region to higher temperatures than suggested by the ESR data alone. Moreover, for cholesterol concentrations greater than 30 mol % and up to 34 °C, the diffusion coefficient actually *increases with increasing cholesterol content.*

The values of D as a function of temperature (T) are plotted in Figures 5 and 6. Figure 5 shows the data corresponding to the one-phase regions of the phase diagram: L_d phase, pure DMPC [data from Vaz et al. (1985)], and L_o phase, DMPC/cholesterol 70:30, 60:40, and 50:50. Figure 6 shows the data for the two-phase region (L_d + L_o), DMPC/cholesterol 85:15, 80:20, and 75:25. Diffusion depends strongly on temperature, showing a nonlinear increase with T. The average ratio of D in pure DMPC to D in 70:30 DMPC/cholesterol

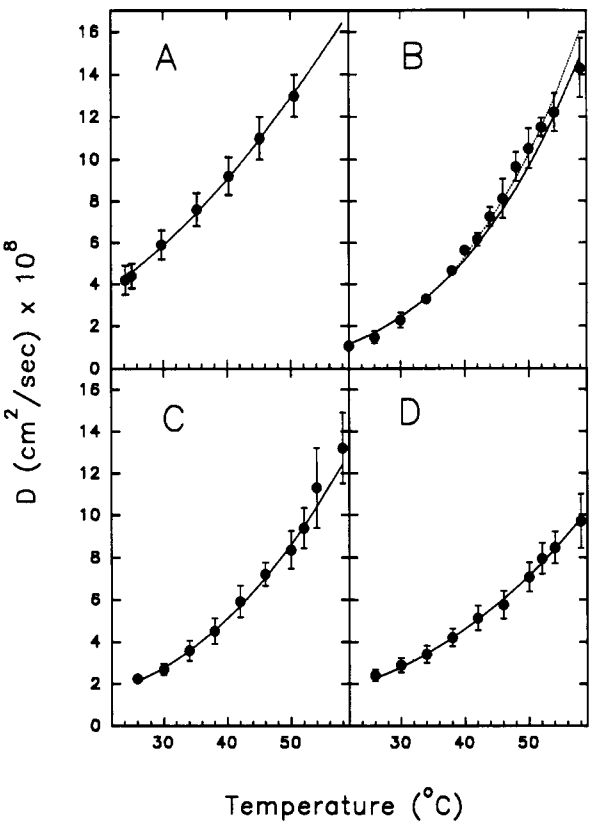

FIGURE 5: Diffusion coefficients as a function of temperature in the one-phase regions of the phase diagram (Figure 1). L_d phase, pure DMPC (A); L_o phase, DMPC/cholesterol 70:30 (B), 60:40 (C), and 50:50 (D). Data for DMPC are taken from Vaz et al. (1985). For the mixtures, the points are averages of eight measurements in two different samples, and the error bars are the corresponding standard deviations. The lines are nonlinear least-squares fits to eq 7.

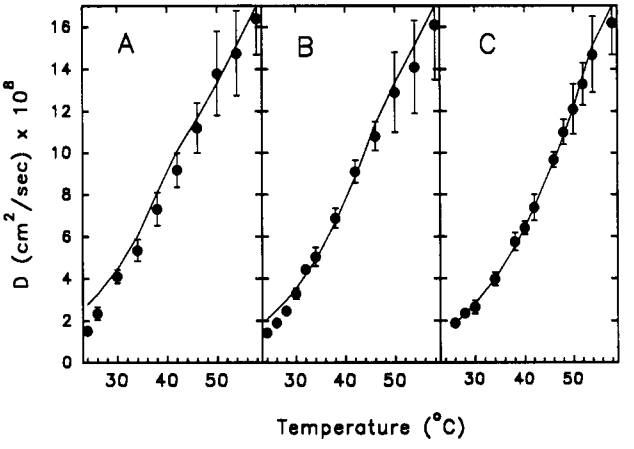

FIGURE 6: Diffusion coefficient D as a function of temperature in the L_d + L_o coexistence region for DMPC/cholesterol 85:15 (A), 80:20 (B), and 75:25 (C). The points are averages of eight measurements in two different samples, and the error bars are the corresponding standard deviations. The error bars on the low-temperature points are buried within the symbols. The lines are calculated according to eq 9 and the fractions of each phase given by the phase diagram at each temperature point.

is approximately 1.5. It varies between 3 at low temperatures and 1 at high temperatures.

In the following analysis, we first consider the behavior of the phospholipid diffusion coefficient in the one-phase regions of the phase diagram (L_d and L_o in Figure 1) and interpret the results using the modified Macedo–Litovitz equation. Next, we utilize the information thus obtained to try to understand diffusion in the two-phase region (L_d + L_o in Figure 1) in

conjunction with the Torquato relation (eq 9). Finally, we present a general interpretation of the phase behavior of the DMPC/cholesterol system in terms of free volume.

Analysis of the Temperature Dependence of the Diffusion Coefficient with the Macedo–Litovitz Equation in the One-Phase Regions. In the regions of the phase diagram where only one phase exists, we have used the modified form of the modified Macedo–Litovitz equation, to analyze the diffusion results. The experimental values of D in pure DMPC and in DMPC/cholesterol 70:30, 60:40, and 50:50 were fitted to eq 7 with a nonlinear least-squares analysis. The close-packed cross-sectional area of the DMPC molecule was taken to be the value observed in the gel phase, $a_o = 45$ Å^2 (Wiener et al., 1989). In the case of pure DMPC, the energy of activation (E_a) was the only parameter that was allowed to vary during the fitting procedure. A value of $E_a = 2.7$ kcal/mol was obtained. In the case of the cholesterol-containing mixtures in the L_o phase (30, 40, and 50 mol % cholesterol), the additional parameter a_o^{Chol} was chosen so as to simultaneously minimize the residuals in the three compositions. A value of $a_o^{Chol} = 26.6$ Å^2 was found to give the best overall fits. The corresponding values of E_a are 1.9, 2.1, and 2.5 kcal/mol for DMPC/cholesterol 70:30, 60:40, and 50:50, respectively. In the case of the 70:30 composition, the fit was done using only the values of D from 22 to 38 °C. After this temperature, an appreciable fraction of L_d phase is present, according to the phase diagram (Figure 1): at 40 °C, the L_d phase fraction is already 5%. The fits obtained are represented by the solid lines in Figure 5. Notice that, for the 70:30 mixture, the beginning of the deviation from the fit coincides very well with the low-temperature boundary of the two-phase region. This deviation will be discussed in more detail when we analyze the results in the two-phase region. In DMPC/cholesterol 60:40, the two highest temperature points have an error substantially greater than the others and were not included in the fit shown, though their inclusion would result only in a very minor change in the parameters.

It is clear that the modified Macedo–Litovitz eq 7 can be used to describe quantitatively and conveniently lateral diffusion in the DMPC/cholesterol system, in the one-phase regions of the phase diagram (Figure 5). The two parameters that enter this equation, the free area and the energy of activation, are sufficient for a description of the diffusion process and its dependence on temperature and cholesterol concentration. This observation forms the basis of our subsequent interpretation of the results presented.

It is our contention that the most important effect of cholesterol is to occupy free volume in the membrane. There are two components to this process. First, the free area in the plane of the bilayer is reduced; second, there are changes in the molecular packing in the membrane. The effect on free area is taken into account explicitly in eq 7; the effect on molecular packing is reflected in the activation energy. The idea of the importance of the free volume in phosphatidylcholine/cholesterol interactions is not new (Galla et al., 1979; Straume & Litman, 1988). In fact, the so-called "condensing effect" of cholesterol has been known for many years (Leathes, 1925). It has not, however, been shown to explain the observations related to the dynamics of phosphatidylcholine/cholesterol mixtures in a quantitative manner. In this paper, we demonstrate that this is possible.

It is interesting that, while the system is completely in the liquid-disordered (L_d) cholesterol-poor phase, addition of cholesterol does not lead to a change in the phospholipid diffusion coefficient (Figure 4), in apparent disagreement with what we have just proposed. Our measurements cover only cholesterol concentrations from 15 to 50 mol %, but the data available for lower cholesterol contents support this assertion [Rubenstein et al., 1979; Alecio et al., 1982; see also Lindblom et al. (1981)]. Thus, if the Macedo–Litovitz eq 7 is valid in this phase, addition of cholesterol to a DMPC bilayer cannot result in a decrease in the free area *useful* for phosphatidylcholine diffusion while the system is completely in the liquid-disordered phase. One way of understanding this observation is with reference to the packing of cholesterol in the L_d phase proposed by Sankaram and Thompson (1990b, 1991), where the cholesterol molecule *spans the nonpolar core of the two monolayers* simultaneously. There is almost no net change in free volume upon cholesterol addition because the volume occupied in one monolayer is essentially generated in the apposing monolayer. According to this explanation, cholesterol has essentially no effect on the distribution of free volume in the membrane. An alternative possibility is that, up to a certain cholesterol content (depending on temperature), the cholesterol molecules occupy preferentially small free volumes, which are below the critical volume for phospholipid diffusion; their removal would not affect the diffusion coefficient of a phospholipid molecule. This second hypothesis predicts that cholesterol alters the distribution of free volumes. The distribution becomes skewed toward larger free volumes, the smaller free volumes of the original one having been eliminated or reduced. According to this view, although the diffusion of phospholipid molecules does not change, the diffusion coefficient of cholesterol should decrease, because the critical volume (or cross-sectional area) for its diffusion is lower and occupancy of small volumes thus results in removal of *useful* volume for cholesterol diffusion. The observation (Shin & Freed, 1989) that in a phosphatidylcholine/cholesterol system the diffusion coefficient of a cholestane spin-label is significantly reduced by addition of cholesterol while that of a phospholipid spin-label remains unchanged indicates that at least the process corresponding to the second interpretation occurs.

In the liquid-ordered (L_o), cholesterol-rich phase, above the T_m of DMPC, the calculated molecular areas available per phospholipid molecule *increase* with increasing cholesterol concentration (see Figure 2B; DMPC/cholesterol mixtures 70:30, 60:40, and 50:50). This is in agreement with the *decrease* in the deuterium NMR order parameter observed in going from a 2:1 to a 1:1 DMPC/cholesterol mixture (Sankaram & Thompson, 1990b). The saturable free area having been occupied, accommodation of more cholesterol is only possible by a lateral expansion of the lipid bilayer. In this region of the phase diagram, the diffusion activation energies for the three DMPC/cholesterol mixtures vary in the order 70:30 < 60:40 < 50:50. The activation energy reflects the strength of the intermolecular interactions. Therefore, the packing in the bilayer improves upon cholesterol addition in this composition range. It is instructive to compare Figures 2A and 7. The specific volumes of the DMPC/cholesterol mixtures (Figure 2A) as a function of temperature show a crossover region around 30 °C, and the same is observed with the diffusion coefficients (Figure 7) around 34 °C. The interplay of the free area and the activation energy explains this behavior. At low temperatures (<30 °C), the specific volumes (reciprocal densities) vary in the same order as the molecular areas: 50:50 > 60:40 > 70:30 (Figure 2). An increase in temperature has a larger effect on the densities of the mixtures where the intermolecular cohesion forces (activation energy) are smaller; therefore, the 70:30 mixture shows

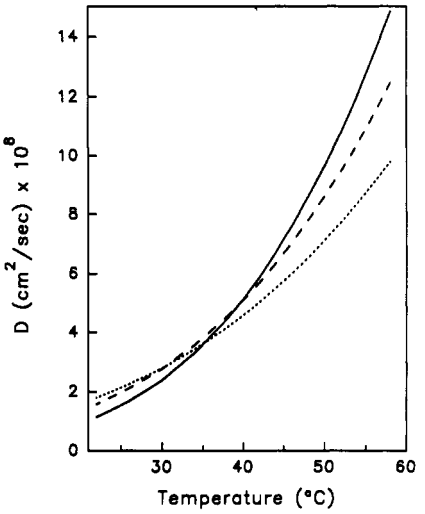

FIGURE 7: Diffusion coefficient as a function of temperature for DMPC/cholesterol 70:30 (solid line), 60:40 (dashed line), and 50:50 (dotted line). The curves are the fits from Figure 5. It is evident that there is a strong parallelism with the behavior of the specific volume shown in Figure 2A.

the largest change. At high enough temperature, addition of cholesterol results in an increase in density (Figure 2A) and a decrease in the diffusion coefficient.

Analysis of the Behavior of the Diffusion Coefficient in the Two-Phase Region of the Phase Diagram. Phospholipid diffusion in the liquid immiscibility region (L_d + L_o, Figure 1) of the DMPC/cholesterol system can be understood on the basis of the phase diagram and on the previous analysis in each of the liquid phases. We assume that the diffusion coefficient in each of the coexisting liquids is given by the diffusion coefficient in the phases with the compositions specified by the two boundaries of the immiscibility gap. For the L_d phase, we used the values of the diffusion coefficient of pure DMPC given by the curve in Figure 5. All the experimental evidence available indicates that diffusion in the L_d phase is the same as in pure DMPC (Rubenstein et al., 1979; Alecio et al., 1982; see Figure 4). The phase boundary at the L_o side runs to a very good approximation along the 30 mol % cholesterol line up to approximately 40 °C. Therefore, up to this temperature, we used the curve describing diffusion at this composition to represent diffusion in the L_o phase at the boundary of the immiscibility gap. Above 40 °C, this representation is replaced by a linear combination of the diffusion coefficients at 30 and 40 mol % cholesterol, weighted by taking into account the position of this phase boundary in the cholesterol mole percent axis. The fractions of the two coexisting phases were calculated from the phase diagram according to the lever rule (Moore, 1986). If this simple analysis is correct, diffusion in the L_d + L_o region should be described by the Torquato relation for a statistically isotropic distribution of domains in a two-phase system in two dimensions (see *Theory*, eq 9), taking the L_d phase as phase 1 and the L_o phase as phase 2. D_{upper} given by this equation is plotted in Figure 6 together with the experimental points of D for the DMPC/cholesterol mixtures 85:15, 80:20, and 75:25. It is apparent that the agreement is excellent, except for a deviation at low temperatures in the 85:15 and 80:20 compositions. This deviation is beyond the experimental uncertainty. It is also not accounted for by the Torquato lower bound (plot not shown) which coincides with the upper bound for most of the temperature range. The difference between the Torquato bounds is maximum at the low-temperature end but amounts to only 5%. The fact that the fit is best for the 75:25 composition and worst for the 85:15 composition suggests that this deviation is due to the finite width of the solid–liquid-phase transition of DMPC which extends to temperatures above the horizontal line in the phase diagram. The phospholipid fluorescent probe used in this study is insoluble in the solid phase. Solid-phase clusters act as barriers to diffusion (Saxton, 1987; Almeida et al., 1992) and thus lead to values of D smaller than predicted by eq 9.

The reason why the Torquato upper and lower bounds are essentially coincident (in fact, the same is true for the Hashin bounds, eq 8) is that the ratio of the diffusion coefficients in the two liquid phases is ≤ 3 at any temperature. Consequently, it is not possible to assess the connectivity of the L_d and L_o phases, unless a probe is used which partitions exclusively into one of the liquid phases [NBD-C(10)PE shows no phase preference between the two].

It was noticed that in DMPC/cholesterol 70:30 (Figure 5B) there is a systematic deviation of the experimental diffusion values from the fit at temperatures above 40 °C. The reason is that a mixture with this composition, which at lower temperatures is completely in the L_o phase, enters the two-phase region at 40 °C. The deviation is very well accounted for by the Torquato relation for D_{upper} (eq 9) taking into consideration the fractions of the phases given by the phase diagram as drawn in Figure 1 (dotted line in Figure 5B).

General Interpretation of the Phase Behavior of the DMPC/Cholesterol Mixture. A general interpretation of the DMPC/cholesterol phase diagram based on free volume can now be proposed. This free volume is reflected on the free area in the plane of the bilayer, so that the discussion which follows could also be given in terms of area rather than volume. The fundamental idea is that, above the T_m of the phospholipid, the bilayer can exist in two general states which differ in their allowed free volume ranges. Just slightly above T_m, the L_d phase is the high free volume state and the L_o phase is the low free volume state. The variance of a statistical distribution of free volumes increases with the total free volume. Thus, the L_d phase has a broad distribution of free volumes whereas the L_o phase has a relatively narrow one. Therefore, addition of cholesterol, leading to the $L_d \rightarrow L_o$ transition, decreases volume fluctuations in the bilayer.

When cholesterol is added to the L_d phase beyond the concentration which saturates the small free volumes, a transition occurs, from the disordered to the ordered phase, when the free volume falls below a certain critical value. The same effect is obtained if the system is originally far above the T_m and at cholesterol concentrations between 10 and 25 mol % and is cooled down into the L_d + L_o phase region. The decrease in free volume leads to the appearance of the L_o phase. The process would be equivalent to a glass transition [see, for example, Grest and Cohen (1981)] if the molecular changes induced by this volume decrease would not lead to phase separation. We now examine this aspect. Consider again the bilayer above T_m in the L_d phase. Addition of cholesterol eventually reduces the free volume in the bilayer, and the fraction of *trans* conformers in the acyl chains of the phospholipid molecules consequently increases. In small regions of higher local cholesterol concentration, a larger fraction of phospholipid molecules will have their acyl chains in a more rigid conformation. As proposed by Sankaram and Thompson (1990b, 1991), *the thickness mismatch following this process leads to the phase separation* of an L_o phase with about 30 mol % cholesterol. In this phase, each cholesterol molecule spans only one of the two monolayers as do the phospholipid molecules. More cholesterol can be added to it,

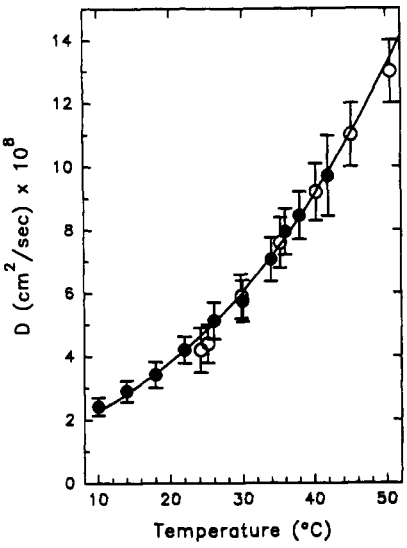

FIGURE 8: Diffusion coefficients for pure DMPC (○) and DMPC/cholesterol 50:50 (●). The values of D for the equimolar mixture were shifted by −16 °C. The curve is the same as in Figure 5D, but also translated −16 °C along the temperature axis and extended to higher temperatures.

resulting now in an area expansion in the plane of the bilayer (Figure 2B) but also in better packing approaching that existing in the L_d phase, as indicated by the increase in the diffusion activation energy upon cholesterol addition beyond 30 mol %.

It has been proposed (Shin & Freed, 1989) that, in phosphatidylcholine bilayers, addition of cholesterol and a decrease in temperature are ultimately equivalent. Moreover, the change in the bilayer free volume has been suggested to be the factor common to these two processes (Straume & Litman, 1987; Mitchell et al., 1990). We have seen that, within each phase (L_d or L_o), this is not necessarily the case. However, if we consider the transition from the L_d to the L_o phase, the results and analysis presented so far agree with this idea. Notice that the activation energy for diffusion in DMPC/cholesterol 50:50 (2.5 kcal/mol) is very similar to that observed in pure DMPC (2.7 kcal/mol). This suggests that the molecular packing is similar in the two situations. Thus, the difference in the diffusion coefficient upon addition of cholesterol is exclusively due to a change in free area. This change can also be attained by a simple shift in temperature. Figure 8 illustrates this point: we have shifted the data points corresponding to DMPC/cholesterol 50:50 by −16 °C, without any shift in the D axis. It seems evident that the curve describing the behavior of the diffusion coefficient in the mixture also describes diffusion in the pure phospholipid.

In conclusion, taken globally, the diffusion measurements reported herein strongly support the DMPC/cholesterol phase diagram determined by ESR with the small modification introduced (Figure 1). As depicted, the phase diagram is consistent with the occurrence of a critical point in this system as proposed in theoretical work (Ipsen et al., 1987, 1989). Its location at a temperature of 65–70 °C and a cholesterol concentration in the neighborhood of 30–35 mol % is in agreement with the diffusion data and the extrapolated specific volume curves (Figure 2A). Recall that a necessary condition for the critical point is that the densities of the two phases be equal. The interpretation of the thermodynamic and diffusional behavior in terms of free volume theory is self-consistent.

Although the present study does not allow for any conclusions relating to the connectivity properties in the two-liquid region of the phase diagram ($L_d + L_o$), this topic remains of utmost importance. We have established that a bilayer-spanning lipid fluorescent probe has a partition coefficient $K_p = 4$ in favor of the L_d phase (P. F. F. Almeida, unpublished observations) which compares to $K_p = 1$ for the probe used in this study. The reason for this differential behavior is probably that this bilayer-spanning probe has several ring systems in the two hydrocarbon chains which are difficult to accommodate by an ordered phospholipid phase (solid or L_o). Integral membrane proteins will very likely present an even rougher surface to the lipid hydrocarbon region. We expect, therefore, that they will prefer the L_d phase as well, and in addition will have partition coefficients even higher than the bilayer-spanning lipid. In fact, Smith et al. (1980) have studied the lateral diffusion of a fluorescein-labeled integral membrane protein (M-13 coat protein) in DMPC/cholesterol multibilayers, at a few temperatures, using periodic pattern photobleaching. Above the T_m of DMPC, they found that the apparent diffusion coefficient has a minimum at ~20 mol % cholesterol at 26 °C and at ~25 mol % cholesterol at 32 °C. No straightforward explanation of this observation was presented by them. We would like to propose that this minimum corresponds to the point of percolation for the L_d phase in the $L_d + L_o$ coexistence region. This suggestion is based on the observation that the percolation point is often associated with a maximum in the FRAP recovery time, or a minimum in the apparent diffusion coefficient (Almeida et al., 1992). If our suggestion is correct, the two first points of the line of connectivity in the $L_d + L_o$ region have already been determined. A complete study of this problem would be extremely valuable. From a biological point of view, it is essential to determine the partitioning of membrane proteins between these two liquid phases. The phase diagrams of sphingomyelin/cholesterol and phosphatidylcholine/cholesterol binary mixtures are very similar (Sankaram & Thompson, 1990a). In many mammalian cell plasma membranes, the outer leaflet of the bilayer is composed for the most part of sphingomyelins, phosphatidylcholines, and cholesterol (Rouser et al., 1968; Cullis & Hope, 1985; Houslay & Stanley, 1982). The sphingomyelin species occurring in these membranes have melting temperatures around 40–50 °C and the phosphatidylcholines around −10–0 °C. The cholesterol concentration varies between 25 and 35 mol %. It seems therefore very probable that at 37 °C many biological membranes may exist in a liquid-ordered–liquid-disordered coexistence region. If this is so, and if integral membrane proteins are not soluble in the L_o phase, in the region of the phase diagram where the L_d phase is disconnected, reactions involving integral proteins cannot occur over the entire membrane area. The effect of compartmentalization on chemical reactions in the plane of the bilayer has been recently discussed (Thompson et al., 1992; Melo et al., 1992). If the cell membrane, by means of small metabolically induced changes in its composition, could cross the line of connectivity in a controlled manner, this mechanism would be a powerful and energetically inexpensive way of regulating protein interactions and reactions in the plane of the lipid bilayer.

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