Comment to the Editor

Lipid Diffusion, Free Area, and Molecular Dynamics Simulations

In a recent article in *Biophysical Journal*, Falck et al. (2004) present a molecular dynamics (MD) study of phosphatidylcholine (PC)/cholesterol (Chol) bilayers, focusing on the lipid packing and its relation to free area and lateral diffusion of lipids. A significant comparison is made between their MD results and our experimental diffusion measurements using fluorescence recovery after photobleaching (FRAP) and the analysis of those experiments using the free area model (Almeida et al., 1992). In contrast to our conclusion, Falck et al. state that the free area model does not quantitatively represent lipid diffusion. Furthermore, their simulations predict a much stronger effect of cholesterol on diffusion than found experimentally. In our opinion, as written, some of the statements of Falck et al. are prone to misinterpretation. The criticism of the free are model based on the MD simulations is flawed because comparison is made between different systems. If the proper systems are compared, the free area model actually predicts the correct result for lipid diffusion, whereas the MD simulations do not. In this Comment to the Editor, we present our views on the problem and clarify several of the issues. Finally, we attempt to resolve the apparent quantitative disagreement between the MD simulations and our experiments on lipid diffusion.

Lipid diffusion in phospholipid/cholesterol systems

A brief summary of experimental diffusion measurements and the phase diagram of phospholipid/cholesterol mixtures is useful to follow this discussion. Above the main phase transition temperature (T_m) of the phospholipid, its mixtures with cholesterol result in a phase diagram with three different regions: if the mol fraction of Chol (χ_{cho}) is $<\sim 0.1$ (depending on temperature), the system is in an liquid-disordered (ℓ_d) phase; above $\chi_{cho} \approx 0.30$, the system is in a liquid-ordered (ℓ_o) phase (which may also be called a phospholipid/cholesterol condensed complex region; McConnell and Radhakrishnan, 2003); and, in between those Chol concentrations, ℓ_d and ℓ_o phases coexist (Shimshick and McConnell, 1973; Ipsen et al., 1987; Sankaram and Thompson, 1990b; Vist and Davis, 1990; Almeida et al., 1992).

In our work (Almeida et al., 1992), the lateral diffusion coefficient of the phospholipid (D_L) in dimyristoylphosphatidylcholine (DMPC)/Chol mixtures drops by a factor of 2.2 from the ℓ_d phase DMPC, in the absence of cholesterol (Vaz et al., 1985), to the ℓ_o phase with at least $\chi_{cho} = 0.30$ (Almeida et al., 1992). This is in agreement with measurements by different investigators, over two and half decades, using different techniques, as shown in Table 1, which emphasizes DMPC/Chol because this is the system we examined. For the phospholipid/cholesterol systems listed here, the ratio of D_L in ℓ_d-phase phospholipid to D_L in ℓ_o-phase phospholipid/cholesterol is always between 2 and 4, with an average value of 2.7 ± 0.7. Korlach et al. (1999) also report a measurement at $\chi_{cho} = 0.60$ in dilauroylphosphatidylcholine (DLPC)/Chol. This measurement differs from the data reported by other investigators on other PC/Chol systems in that it is the only one that shows a significant decrease in D_L in the ℓ_o phase, upon increase in the cholesterol content beyond $\chi_{cho} = 0.30$.

In our measurements, D_L does not decrease with Chol content in the ℓ_d phase; a significant decrease is only observed when the χ_{cho} is high enough for the system to enter the ℓ_d–ℓ_o coexistence region (Almeida et al., 1992). That lack of dependence of D_L on χ_{cho} in the ℓ_d phase is also apparent in the data from McConnell's group (Rubenstein et al., 1979) for DMPC/Chol and egg PC/Chol, and in the data of Filippov et al. (2003) for chicken egg sphingomyelin (SM)/Chol. The onset of the decrease of D_L with χ_{cho} agrees well with the two-phase boundary of the phase diagram for DMPC/Chol (Almeida et al., 1992) and SM/Chol (Filippov et al., 2003). Data of Filippov et al. (2003) on DMPC/Chol and dioleoylphosphatidylcholine (DOPC)/Chol, on the other hand, show a monotonic decrease of D_L with Chol concentration even in the ℓ_d phase. These data of Filippov et al. (2003) appear to be also in conflict with earlier measurements by the same group in DOPC/Chol (Lindblom et al., 1981). Filippov et al. (2003) discuss these apparent discrepancies.

Use of free area theory to analyze lipid diffusion

Falck et al. (2004) conclude their introduction by stating that free area theories correctly predict a reduction in diffusion caused by the addition of cholesterol to a PC membrane, ''but are not applicable to quantitatively describing lateral diffusion in lipid bilayers.'' Without qualification, this statement appears to apply both to MD simulations and experimental data. Further, in their discussion they add: ''In our opinion, one should at least not expect free area theory to yield quantitative results.'' These statements are contrary to our conclusions. First, Vaz et al. (1985) showed that the free area model can indeed fit the experimental diffusion data quantitatively, when a change in free volume is caused by

Submitted January 18, 2005, and accepted for publication March 25, 2005.

Address reprint requests to Paulo F. F. Almeida, Tel.: 910-962-7300; Fax: 910-962-3013; E-mail: almeidap@uncw.edu.

0006-3495/05/06/4434/05 $2.00

doi: 10.1529/biophysj.105.059766

TABLE 1 Comparison of diffusion coefficients in the ℓ_d and ℓ_o phases for a few phospholipid/cholesterol systems

System	X_{cho}	T (°C)	Phase	D (cm^2s^{-1})	Ratio	Method	Reference
DMPC	0	35	ℓ_d	7.5×10^{-8}	–	FRAP	Rubenstein et al. (1979)
DMPC/Chol	≥0.30	35	ℓ_o	3.0×10^{-8}	2.5	–	–
DMPC	0	26	ℓ_d	6.0×10^{-8}	–	FRAP	Alecio et al. (1982)
DMPC/Chol	≥0.30	26	ℓ_o	1.8×10^{-8}	3.3	–	–
DMPC	0	35	ℓ_d	7.6×10^{-8}	–	FRAP	Vaz et al. (1985)
DMPC/Chol	≥0.30	34	ℓ_o	3.5×10^{-8}	2.2	–	Almeida et al. (1992)
DLPC	0	25	ℓ_d	3×10^{-8}	–	FCS	Korlach et al. (1999)
DLPC/Chol	0.30	25	ℓ_o	1×10^{-8}	3	–	–
DMPC	0	35	ℓ_d	11×10^{-8}	–	pfg-NMR	Filippov et al. (2003)
DMPC/Chol	0.33	35	ℓ_o	3×10^{-8}	4	–	–
SM	0	55	ℓ_d	8×10^{-8}	–	pfg-NMR	Filippov et al. (2003)
SM/Chol	0.30–0.425	55	ℓ_o	3.5×10^{-8}	2.3	–	–
DOPC	0	30	ℓ_d	10×10^{-8}	–	pfg-NMR	Filippov et al. (2003)
DOPC/Chol	0.33	30	ℓ_o(?)	5×10^{-8}	2	–	–

a change in temperature. Second, Almeida et al. (1992), extended this study to the effect of cholesterol, as an obvious way of changing the free volume, using a version of the Macedo-Litovitz equation (Eq. 1) (Macedo and Litovitz, 1965) for the free volume theory (Cohen and Turnbull, 1959; Turnbull and Cohen, 1961, 1970). In fact, the agreement between free area theory and experiment is not only qualitative but also quantitative, as shown in Fig. 1, which presents data and theoretical curves for diffusion in pure DMPC and DMPC/Chol (χ_{cho} = 0.30, 0.40, and 0.50) as a function of temperature (Almeida et al., 1992). The theoretical curves were obtained by fitting the equation

$$D_L = A(a^{1/2})\, e^{-a_o/a_f}\, e^{-E_a/kT}, \quad (1)$$

to the experimental data using a simple least-squares analysis. The only free parameters in this procedure were the average cross-sectional, hard-core area (equivalent to the van der Waals volume) of cholesterol (a_o^{cho}), which was subtracted from the total area to obtain the free area (a_f), and the activation energy (E_a). The preexponential factor depends on the square root of the area over which diffusion occurs as described in detail by Almeida et al. (1992). We found that a value for a_o^{cho}=26.6 Å^2, for all DMPC/Chol compositions examined (χ_{cho} = 0.30, 0.40, and 0.50), yielded very good agreement with the experimental data; and that E_a = 2.7 kcal/mol for pure DMPC, and 1.9, 2.1, and 2.5 kcal/mol for DMPC/Chol 70:30, 60:40, and 50:50, respectively, gave the best fits. It is interesting that the value of 26.6 Å^2 is exactly the same as determined recently by MD simulations, which yielded 27 ± 1 Å^2 (Hofsaß et al., 2003; Khelashvili and Scott, 2004). Such nearly perfect agreement is certainly fortuitous, but it indicates that the value we arrived at is entirely reasonable.

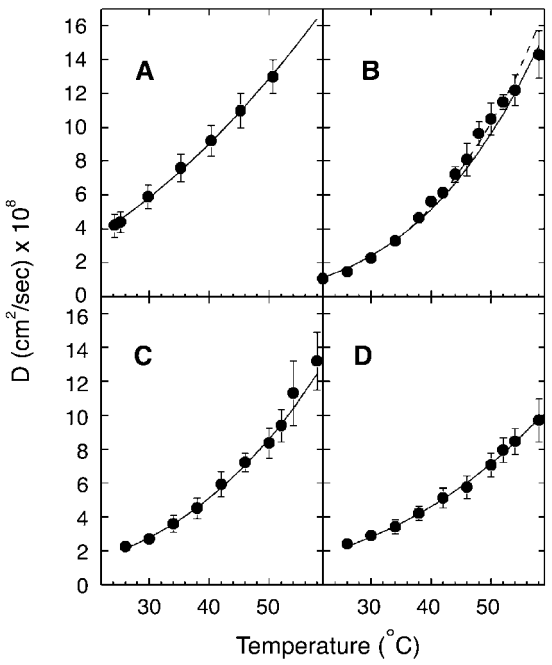

FIGURE 1 Diffusion coefficients (D_L) in DMPC (*A*), and DMPC/Chol 70:30 (*B*), 60:40 (*C*), and 50:50 (*D*) measured by FRAP. The lines are fits of the equations derived from free area theory (Eq. 1) to the data. Reprinted with permission from Almeida et al. (1992). Copyright 1992 American Chemical Society.

However, Falck et al. (2004) write, ''it seems reasonable to expect that E_a should increase with cholesterol content. Experimental results (Almeida et al., 1992) do support this idea but are partly contradictory. This is, however, probably due to the fitting procedure used.'' First, we cannot discern any contradiction in our experimental results: there are no two measurements of the same observable that yield different values. If we understand Falck et al. correctly, they are not questioning the fitting method itself (least squares) but the possible effects of the preexponential factor and the use of constant (temperature-independent) values for the hard-core, cross-sectional areas of DMPC (a_o^{pc}) and cholesterol (a_o^{cho}). In our work, a_o^{pc} was taken as the close-packed area per lipid in gel state DMPC (45 Å^2) and a_o^{cho} was an adjustable parameter in the fits. Slightly different choices for the

preexponential factor have a very minor effect because of its weak (square root) dependence on the area. Reasonable variations in this term lead to no more than ~0.1 kcal/mol changes in the values obtained for E_a for the different systems that we examined and are insufficient to alter the ranking of E_a values in these four mixtures. Indeed, we noted that E_a for DMPC and DMPC/Chol 50:50 are essentially the same (Almeida et al., 1992). With the same E_a, it was then suggested that the effect of cholesterol content in the ℓ_o phase (50:50 mixture) should be equivalent to a shift in temperature, which was shown to be the case (Fig. 2). The meaning of a minimum in E_a for a mixture with $\chi_{cho} = 0.3$ deserves some discussion, which is postponed until the next section. Before concluding this summary we should note that the data of Filippov et al. (2003) yield a different temperature dependence as a function of cholesterol, with a monotonically increasing E_a with cholesterol content in DMPC/Chol. However, their E_a values were obtained with simple Arrhenius plots, not with Eq. 1; therefore, in this form, they cannot be directly compared with ours.

MD simulations, free area, and the timescale of diffusion

The MD simulations of Falck et al. (2004) and their analysis by ''slicing'' the bilayer at various levels along the normal have made clear an important difficulty of free area theory when applied to diffusion in lipid bilayers: the fact that the free volume is not constant across the bilayer height. Therefore, the assignment of a value to a_o^{pc} and a_o^{cho} is conceptually complicated. Free area theory defines an average cross-sectional area for a lipid and treats diffusion in the bilayer as two-dimensional, which is not strictly correct. Movement of the lipids into free volumes at different levels along the bilayer normal would reasonably be expected to contribute to diffusion, probably softening the barriers to displacements along the bilayer plane. Essentially, the free area model ignores all these complications and a_o^{pc} and a_o^{cho} are then, to some extent, operational parameters. Apart from that conceptual difficulty with free area theory (which we share), Falck et al. appear to have two major problems when comparing their MD simulations with our experiments and their interpretation using free area theory: 1), the magnitude of the decrease in the diffusion coefficient when Chol content is increased from $\chi_{cho} \approx 0$ to $\chi_{cho} \approx 0.30$; and 2), our observation of a minimum in E_a for DMPC/Chol 70:30, compared to pure DMPC and DMPC/Chol 60:40 and 50:50.

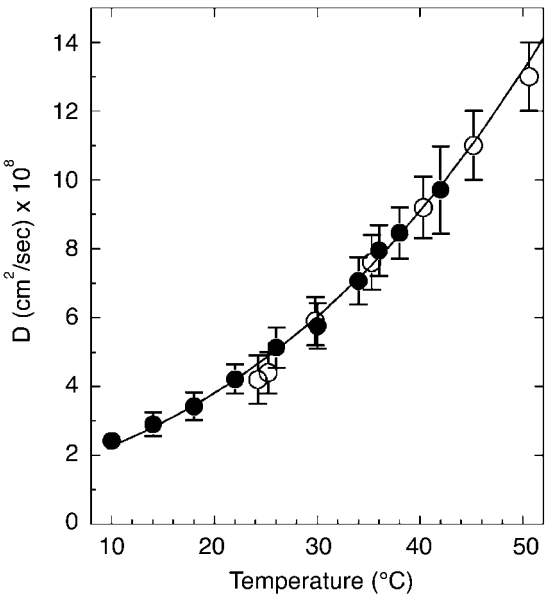

FIGURE 2 Diffusion coefficients (D_L) in DMPC (○) and DMPC/Chol 50:50 (●). The values of D_L for the equimolar mixture were shifted by −16°C. The curve is the same as in Fig. 1 *D*, but also shifted by −16°C and extrapolated to higher temperatures. Reprinted with permission from Almeida et al. (1992). Copyright 1992 American Chemical Society.

With regard to the first problem, Falck et al. base their conclusions on the fact that their MD simulations show a reduction of a factor of 10 in the lipid diffusion coefficient (D_L) when χ_{cho} is increased from 0.047 to 0.297 (Falck et al., 2004). However, if free area theory were correct, they calculate that D_L should be reduced by a factor of 3 at most. As shown above, in experimental measurements, the effect of cholesterol content on D_L is consistently a reduction by a factor of 2–3, up to $\chi_{cho} \approx 0.50$, for all measurements including those of Korlach et al. (1999) for $\chi_{cho} = 0.30$. Therefore, experimentally, the ratio of D_L in the ℓ_d to the ℓ_o phase agrees very well with the prediction of free area theory, as estimated by Falck et al. (2004). In support of their MD results, Falck et al. cite a measurement by Korlach et al. (1999) in DLPC/Chol using fluorescence correlation spectroscopy (FCS), which gives a reduction of 10 upon addition cholesterol to a final $\chi_{cho} = 0.60$. This decrease is a feature of the data of Korlach et al. (1999) for $\chi_{cho} = 0.60$, but has not been observed by other investigators. To the best of our knowledge, all other studies have shown that above $\chi_{cho} = 0.30$ the lipid diffusion coefficient does not vary much. In any case, the comparison made by Falck et al. (2004) was with MD simulations of dipalmitoylphosphatidylcholine (DPPC)/Chol containing $\chi_{cho} = 0.297$, so the relevant experimental data are those for $\chi_{cho} = 0.30$, not 0.60; and at $\chi_{cho} = 0.30$ D_L is reduced by a factor of 3 (as predicted by the free area model), not 10 (as predicted by the MD simulations).

Why then do the MD simulations show a stronger effect of Chol on D_L? One possibility is that this is due to the timescale of the simulations, which was 100 ns (Falck et al., 2004). This is a long time for an MD simulation, but is still short for diffusion. With a typical $D_L = 5 \times 10^{-8}$ cm^{2}s^{-1}, the area explored by a lipid molecule in 100 ns is 200 Å^2, which is only three times the average cross-sectional area per phospholipid in a fluid bilayer, ~65 Å^2. Another way of looking at the problem is that this timescale allows only for

three ''jumps'' if lipid diffusion is viewed as a random walk on a lattice. This is certainly not long-range diffusion. In our opinion, this is probably the main the reason for the apparent discrepancy between the MD simulations and the experimental data obtained with techniques that measure long-range diffusion, such as FRAP, FCS, and pulsed field gradient (pfg)-NMR. The differences in measurements of long- and short-range diffusion have been addressed previously (Vaz and Almeida, 1991). In addition, could it be that the force fields currently available for MD simulations do not correctly model the water-membrane interfacial region? Falck et al. (2004) point out that their simulations, as well as any other united-atom MD simulations, cannot reproduce the behavior of the experimental ^{2}H-NMR order parameter for the deuterons on the second carbon of the *sn*-2 chain (Sankaram and Thompson, 1990a; Seelig and Seelig, 1975), which are near the interface. A third possibility is that the experimental, long-range D_L in PC/Chol systems is affected by phospholipid–Chol complex formation, which could occur on timescales beyond the reach of the current MD simulations.

With regard to the second problem, the minimum in E_a for DMPC/Chol 70:30, which results from the analysis of our diffusion data using free area theory (Eq. 1), Falck et al. (2004) find this result unexpected and attribute it to an incompleteness of free area theory. This is possible. Perhaps what appears as an activation energy in that analysis has contributions that the theory does not treat or does not treat adequately. As a type of mean-field theory, its treatment of fluctuations, which are critical for diffusion, is certainly not complete. Nevertheless, with all its imperfections, free area theory has successfully described lipid diffusion in a quantitative way in the experimental systems that we have examined (Vaz et al., 1985; Almeida et al., 1992). The theory is certainly simple; however, simplicity in a theory is not necessarily a weakness. The real test of a theory is its ability to describe experimental data. As stated by Feynman (1963), ''The principle of science, the definition, almost, is the following: the test of all knowledge is experiment. Experiment is the sole judge of scientific 'truth'.''

Yet, another possibility is that the minimum in E_a for DMPC/Chol 70:30 is real and reflects some important property of the system. If phospholipid/cholesterol systems are understood on the basis of a phase diagram, $\chi_{cho} = 0.30$ essentially corresponds to the composition of the ℓ_o phase in equilibrium with the ℓ_d phase in the two-phase region. This may not be a coincidence and may reflect some special property of 2:1 PC/Chol mixtures. If an interpretation of the behavior of phospholipid/cholesterol systems in terms of complex formation is preferred, as proposed by McConnell and collaborators in recent work (see, for a review, McConnell and Radhakrishnan, 2003) this composition corresponds to a pressure cusp (minimum) in the phase diagrams of phospholipid/Chol systems, which has been interpreted by them as indicative of the formation of a phospholipid/cholesterol 2:1 condensed complex. An interesting observation by Chong (1994), on the basis on our calculated mean areas per DMPC as a function of cholesterol content (Almeida et al., 1992), is that the average value of a DMPC cross-sectional area per chain at 35°C is reduced from 29.5 Å^2 in pure DMPC to 26.7 Å^2 in 70:30 DMPC/Chol. This is exactly the same as the value found for a_o^{cho}, 26.6 Å^2, which, because it corresponds to a rigid molecule (cholesterol) is not expected to change with temperature (Chong, 1994; McConnell and Radhakrishnan, 2003). Therefore, packing may be especially good and the exchange between PC chains and cholesterol may be especially easy at $\chi_{cho} = 0.30$. This could be reflected in an smaller apparent E_a. Finally, as we have suggested (Almeida et al., 1992), these variations in E_a may reflect changes in hydration of the bilayer, which may not be monotonic with χ_{cho} when comparing one phase with the other (ℓ_d and ℓ_o), although they would be expected to be monotonic within each phase when the cholesterol content is changed, as is observed.

This work was supported in part by grant GM59205 from the National Institutes of Health to the University of Virginia/PFFA.

REFERENCES

Alecio, M. R., D. E. Golan, W. R. Veatch, and R. R. Rando. 1982. Use of a fluorescent cholesterol derivative to measure the lateral mobility of cholesterol in membranes. *Proc. Natl. Acad. Sci. USA*. 79:5171–5174.

Almeida, P. F. F., W. L. C. Vaz, and T. E. Thompson. 1992. Lateral diffusion in the liquid phases of dimyristoylphosphatidylcholine/cholesterol lipid bilayers: a free volume analysis. *Biochemistry*. 31:6739–6747.

Chong, P. L.-G. 1994. Evidence for regular distribution of sterols in liquid crystalline phosphatidylcholine bilayers. *Proc. Natl. Acad. Sci. USA*. 91: 10069–10073.

Cohen, M. H., and D. Turnbull. 1959. Molecular transport in liquids and glasses. *J. Chem. Phys*. 31:1164–1169.

Falck, E., M. Patra, M. Karttunen, M. T. Hyvönen, and I. Vattulainen. 2004. Lessons of slicing membranes: interplay of packing, free area, and lateral diffusion in phospholipid/cholesterol bilayers. *Biophys. J*. 87: 1076–1091.

Feynman, R. P., R. B. Leighton, and M. Sands. 1963. The Feynman Lectures on Physics, Vol. 1. Addison-Wesley, Reading, MA.

Filippov, A., G. Orädd, and G. Lindblom. 2003. The effect of cholesterol on the lateral diffusion of phospholipids in oriented bilayers. *Biophys. J*. 84:3079–3086.

Hofsaß, C., E. Lindahl, and O. Edholm. 2003. Molecular dynamics simulations of phospholipid bilayers with cholesterol. *Biophys. J*. 84: 2192–2206.

Ipsen, J. H., G. Karlström, O. G. Mouritsen, H. Wennerstrom, and M. J. Zuckermann. 1987. Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim. Biophys. Acta*. 905:162–172.

Khelashvili, G. A., and H. L. Scott. 2004. Combined Monte Carlo and molecular dynamics simulation of hydrated 18:0 sphingomyelin-cholesterol lipid bilayers. *J. Chem. Phys*. 120:9841–9847.

Korlach, J., P. Schwille, W. W. Webb, and G. W. Feigenson. 1999. Characterization of lipid bilayer phases by confocal microscopy and fluorescence correlation spectroscopy. *Proc. Natl. Acad. Sci. USA*. 96: 8461–8466.

Lindblom, G., L. B. A. Johansson, and G. Arvidson. 1981. Effect of cholesterol in membranes. Pulsed nuclear magnetic resonance measurements of lipid lateral diffusion. *Biochemistry*. 20:2204–2207.

Macedo, P. B., and T. A. Litovitz. 1965. On the relative roles of free volume and activation energy in the viscosity of liquids. *J. Chem. Phys.* 42:245–256.

McConnell, H. M., and A. Radhakrishnan. 2003. Condensed complexes of cholesterol and phospholipids. *Biochim. Biophys. Acta.* 1610:159–173.

Rubenstein, J. L. R., B. A. Smith, and H. M. McConnell. 1979. Lateral diffusion in binary mixtures of cholesterol and phosphatidylcholines. *Proc. Natl. Acad. Sci. USA.* 76:15–18.

Sankaram, M. B., and T. E. Thompson. 1990a. Modulation of phospholipid acyl chain order by cholesterol: a solid-state ^{2}H-nuclear magnetic resonance study. *Biochemistry.* 29:10676–10684.

Sankaram, M. B., and T. E. Thompson. 1990b. Interaction of cholesterol with various glycerophospholipids and sphingomyelin. *Biochemistry.* 29:10670–10675.

Seelig, A., and J. Seelig. 1975. Bilayers of dipalmitoyl-3-sn-phosphatidylcholine: conformational differences between the fatty acid chains. *Biochim. Biophys. Acta.* 406:1–5.

Shimshick, E. J., and H. M. McConnell. 1973. Lateral phase separations in binary mixtures of cholesterol and phospholipids. *Biochem. Biophys. Res. Commun.* 53:446–451.

Turnbull, D., and M. H. Cohen. 1961. Free-volume model of the amorphous phase: glass transition. *J. Chem. Phys.* 34:120–125.

Turnbull, D., and M. H. Cohen. 1970. On the free-volume model of the liquid-glass transition. *J. Chem. Phys.* 52:3038–3041.

Vaz, W. L. C., and P. F. F. Almeida. 1991. Microscopic versus macroscopic diffusion in one-component fluid phase lipid bilayer membranes. *Biophys. J.* 60:1553–1554.

Vaz, W. L. C., R. M. Clegg, and D. Hallmann. 1985. Translational diffusion of lipids in liquid-crystalline phase phosphatidylcholine multibilayers: a comparison of experiment with theory. *Biochemistry.* 24:781–786.

Vist, M. R., and J. H. Davis. 1990. Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: ^{2}H-nuclear magnetic resonance and differential scanning calorimetry. *Biochemistry.* 29:451–464.

Paulo F. F. Almeida,* Winchil L. C. Vaz,† and T. E. Thompson‡

**Department of Chemistry and Biochemistry*
University of North Carolina at Wilmington
Wilmington, North Carolina 28403 USA

†*Departamento de Química*
Universidade de Coimbra
3004-535 Coimbra, Portugal

‡*Department of Biochemistry and Molecular Genetics*
University of Virginia
Charlottesville, Virginia 22908 USA