LATERAL DIFFUSION IN INHOMOGENEOUS MEMBRANES

MODEL MEMBRANES CONTAINING CHOLESTEROL

JOHN C. OWICKI, *Department of Biophysics and Medical Physics, University of California, Berkeley, California 94720*
HARDEN M. MCCONNELL, *John Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, California 94305 U.S.A.*

ABSTRACT The problem of lateral diffusion in inhomogeneous membranes is illustrated by a theoretical calculation of the lateral diffusion of a fluorescent lipid probe in binary mixtures of phosphatidylcholine and cholesterol under conditions of temperature and composition such that this lipid mixture consists of alternating parallel domains of fluid and solid lipid, having separations that are small compared with the distance scale employed in photobleaching experiments. The theoretical calculations clearly illustrate how inhomogeneities in membrane composition affecting the lateral motion of membrane components on a small (10–100 nm) distance scale can give complex diffusive responses in experiments such as fluorescence photobleaching that employ comparatively macroscopic distances (10–100 μm) for the measurement of diffusive recovery. The theoretical calculations exhibit the unusual dependence of the apparent lateral diffusion coefficient of a fluorescent lipid probe on lipid composition in binary mixtures of cholesterol and phosphatidylcholines as reported by Rubenstein et al. (1979, *Proc. Natl. Acad. Sci. U.S.A.*, **76**:15–18).

INTRODUCTION

There have been numerous studies of the lateral diffusion of membrane components in model membranes and in biological membranes. Such studies are relevant to problems of membrane structure and to biological functions such as hormone-receptor triggering and cell surface immunochemistry. For leading references, see Schlessinger et al. (1976*a*, *b*, 1978), and McConnell (1978). A problem which has not received adequate attention in earlier work is the role of membrane heterogeneity in the experimental and theoretical interpretation of diffusion data. The recent discovery of anisotropic lateral diffusion of fluorescent labels attached to the plasma membranes of mouse fibroblasts clearly points to an important role for membrane heterogeneity in measurements of molecular motion on cell surfaces, and also points to the existence of molecular interactions that have an anisotropic spatial distribution (B. Smith et al., 1979). Our paper gives a very brief general discussion of the problem of diffusion in inhomogeneous membranes from both an experimental and theoretical point of view. We then narrow the scope of the discussion to consider one specific model system, the lateral diffusion of a fluorescent lipid in binary mixtures of cholesterol and DMPC,[1] where there is evidence for lipid inhomogeneity on a microscopic scale (Copeland and McConnell, 1980).

[1]Abbreviations used in this paper: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; NBD-PE, N-4-nitrobenz-2-oxa-1,3-diazole phosphatidylethanolamine.

BIOPHYS. J. © Biophysical Society · 0006-3495/80/06/383/15 $1.00
Volume 30 June 1980 383–398

A major impetus for our work arose from a number of seemingly paradoxical and contradictory physical and immunochemical properties of lipid bilayer membranes composed predominantly of binary mixtures of cholesterol and phosphatidylcholines. Among the unusual physical properties of these binary mixtures there are, for example, the reported disappearance of the sharp endothermic heat absorption characteristic of DPPC at 42°C when cholesterol is included in the phospholipid bilayer at concentrations equal to and above 20 mol% (Mabrey et al., 1978; Estep et al., 1978), and the almost discontinuous increase in the lateral diffusion coefficient of a fluorescent lipid probe in DPPC bilayers when the cholesterol concentration is increased to 20% (Rubenstein et al., 1979). Although physical changes in phosphatidylcholine-cholesterol mixtures at 20 mol% were first detected from the resonance spectra of spin label probes included in these mixtures (Shimshick and McConnell, 1973), these changes are not large enough to indicate that 20 mol% represents a boundary between two distinct phases having markedly different structures (Rubenstein et al., 1980). Until recently it has not been possible for us to reconcile these and other physical properties of the binary mixtures.

To the above purely physical problems can be added a number of related immunochemical problems. Studies of certain specific antibody-dependent immunochemical responses to lipid-hapten containing phosphatidyl choline bilayer membranes have shown these responses to be larger for fluid membranes than for solid membranes. These responses include the activation of the first component of complement (Esser et al., 1979; Parce et al., 1980), the activation of neutrophils (Hafeman et al., 1979), and the binding and phagocytosis of membrane vesicles by RAW 264 macrophages (J. T. Lewis, D. Hafeman, and H. McConnell. Manuscript in preparation). The fluid and solid membranes employed for these immunochemical experiments were DMPC at 32–37°C, and DPPC at 32–37°C, respectively. There are immunological reasons to suspect that these differences are due to differences in the rates of lateral diffusion of antibody molecules bound to lipid haptens in these membranes. Paradoxically, however, the inclusion of much less than 20 mol% cholesterol in the otherwise solid DPPC membranes at 32–37°C results in a large enhancement of these immune responses (even though the reported lipid lateral diffusion coefficients are still very low when the cholesterol concentration is <20 mol%), and increasing cholesterol concentration from slightly below 20 mol% to slightly above 20 mol% (where the reported lipid lateral diffusion coefficients increase by an order of magnitude) brings about no large enhancement of immune response.

The pieces of these puzzles are now falling in place. Some time ago it was suggested (on the basis of freeze-fracture data) that binary mixtures of cholesterol and phosphatidylcholines might consist of alternating, parallel zones of fluid and solid lipid, when the temperature is less than the chain-melting transition temperature of the phosphatidylcholine, and the cholesterol concentration is <20 mol% (Kleemann and McConnell, 1976). More recently Copeland and McConnell (1980) have made a quantitative study of freeze-fracture electron micrographs of these binary mixtures, and have obtained evidence that such mixtures do consist of parallel, alternating zones: "ridges" consisting of pure phosphatidylcholine, and "plains" containing 20 mol% cholesterol (when the average cholesterol content of the sample is <20 mol%). From the data of Rubenstein et al. (1979) we draw the conclusion that the plains containing 20 mol% cholesterol must be fluid. The ridges of the pure phosphatidylcholine should then act as

barriers to lateral diffusion. Our paper shows that the lateral diffusion data of Rubenstein et al. (1979) are indeed compatible with this picture of the bilayer structure. The reader will appreciate how this unusual bilayer structure accounts at least qualitatively for the above-mentioned physical and immunochemical properties of these lipid mixtures. The sharp endothermic heat absorption corresponds to a melting of the ridges, the diffusion vs. composition behavior is explained in this paper, and there is no large change in spin label resonance spectra as cholesterol concentration is increased from just below to just above 20 mol%, since the ridges are only a minor component of the membrane in this concentration range. The absence of a sharp change in immunochemical response at 20 mol% is also clear since there is a negligible change in the fraction of fluid lipid at this concentration of cholesterol. On the other hand, when low concentrations of cholesterol are included in solid DPPC, the bilayer changes from all solid, to some fluid lipid, and there is a corresponding large effect on immunochemical response. The physical origin of this unusual bilayer structure remains to be discovered.

THEORY

Lateral Diffusion in Inhomogeneous Membranes

In an inhomogeneous two-dimensional membrane, molecular lateral diffusion can be described by a diffusion tensor or dyadic $\boldsymbol{D}$,

$$\boldsymbol{D} = \mathbf{ii}D_{xx} + \mathbf{ij}D_{xy} + \mathbf{ji}D_{yx} + \mathbf{jj}D_{yy}. \tag{1}$$

Here $\mathbf{i}$ and $\mathbf{j}$ are unit vectors parallel to the laboratory fixed coordinate axes x,y. Membrane inhomogeneity can give rise to anisotropies in the elements of $\boldsymbol{D}$, i.e., $D_{xx} \neq D_{yy}$, as well as a dependence of the elements of $\boldsymbol{D}$ on the coordinates themselves, i.e., $D_{xx} = D_{xx}(x,y)$. The diffusion equation is

$$\frac{\partial c}{\partial t} = \nabla \cdot \boldsymbol{D} \cdot \nabla c, \tag{2}$$

where

$$\nabla = \mathbf{i}\frac{\partial}{\partial x} + j\frac{\partial}{\partial y}, \tag{3}$$

and $c(x,y)$ is the concentration of the diffusing substance. Some solutions for this diffusion equation for anisotropic and/or inhomogeneous diffusion dyadics $\boldsymbol{D}$ have been given by Carslaw and Jaeger (1959; see especially chapter 12), and by Crank (1975). Unfortunately, many of these solutions are not applicable to determinations of lateral diffusion in membranes because of the simplifying boundary conditions employed, and also the functional forms of $\boldsymbol{D}$ are usually inappropriate. In addition, analytical solutions are quite difficult for all but the simplest cases. The problem treated in this paper is related to earlier calculations bearing on diffusion in inhomogeneous systems and pulsed gradient nuclear magnetic resonance. See, for example, Tanner (1978) and references to earlier work contained therein. In this reference and others, complex diffusive behavior in inhomogeneous systems is sometimes represented by an effective time-dependent diffusion coefficient, $D(t)$. This is a formalism to be avoided for

biophysical problems where the physical and chemical properties of membranes may be authentic functions of the time.

Periodic Pattern Photobleaching

Collimated radiation that is passed through a periodic grid can be used to produce a corresponding periodic concentration gradient in a photosensitive target. This method has been used to produce periodic concentration gradients of spin labels (Sheats and McConnell, 1978) and fluorescent labels (Smith and McConnell, 1978) in membranes. For simplicity, our present discussion will be limited to periodic pattern fluorescence photobleaching and recovery. For a description of the earlier spot photobleaching and recovery method, see Axelrod et al. (1976), and Wu et al. (1977). The earliest photobleaching-recovery measurements of lateral diffusion on cell surface membranes were carried out by Poo and Cone (1974), and by Peters et al. (1974). Later we shall briefly indicate the relevance of our calculations for other techniques used in determining rates of lateral diffusion.

Most of our periodic pattern photobleaching experiments have employed a grid (opaque material on a transparent substrate) of parallel stripes. When the spatially periodic laser radiation is focused through a microscope objective, the photobleached membranes have a square wave concentration profile (except for usually minor optical diffraction effects). Typical experimentally employed repeat distances for these concentration stripes are P = 3–30 μm. As was pointed out previously, after "instantaneous" parallel stripe photobleaching, the concentration of fluorescent material varies according to the equation

$$c(x, t) = A_0 + \sum_n A_n \cos \Omega_n x [\exp(-\Omega_n^2 Dt)], \tag{4}$$

which is the solution to the one-dimensional diffusion equation for constant D,

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2}, \tag{5}$$

where $\Omega_n = 2\pi n/P$, and $n = 1,3,5, \ldots$. Note that because the concentration profile is assumed to have a square wave form, only odd values of n appear in the sum in Eq. 4. Thus, the second exponential term in Eq. 4 decays much more rapidly than the first and is usually not detected experimentally (Smith and McConnell, 1978). If one employed a rectangular rather than a square wave concentration profile, then both odd and even powers would appear in Eq. 4, an undesirable additional complication for the interpretation of experimental data. The ideal concentration profile is a simple cosine function where only a single exponential appears in Eq. 4. In some previous work, a two-dimensional grid of perpendicular stripes was employed to advantage to measure anisotropic diffusion on the surfaces of cell membranes (B. Smith et al., 1979). This is certainly a practical method for studying anisotropic diffusion if the diffusion dyadic is constant in space and time over the whole membrane surface, and the fluorescence intensity is low. We shall not consider this two-dimensional pattern photobleaching in the present paper because the basic ideas will be clear by discussing "one-dimensional" square wave concentration profiles.

In any technique for measuring lateral diffusion in membranes, such as pattern photobleaching, one must consider the distance and time scales over which molecular motion

takes place during the measurement, in comparison with the distance scale of inhomogeneities in D along with corresponding differences in time required for molecular motion over given distances.

Membrane Heterogeneity Due to Variations in Lipid Composition

We believe that hydrated binary mixtures of DMPC and cholesterol at temperatures below the chain melting transition temperature of DMPC (21°C) provide a concrete and informative example of the subtle interplay of inhomogeneous diffusion coefficients in membranes and their manifestation in experimental measurements of diffusion. This statement is thought to be true also for binary mixtures of DPPC and cholesterol. In a recent study of bilayers formed from binary mixtures of DMPC and cholesterol below the chain melting transition temperature of DMPC (21°C) it was found, using freeze-fracture electron microscopy, that the average distance between parallel ridges, characteristic of DMPC in the $P_{\beta'}$ phase, increases continuously with increasing cholesterol concentration, with no apparent change in ridge amplitude or shape (Copeland and McConnell, 1980). This effect is illustrated schematically in Fig. 1. The reciprocal of the distance between ridges has been found to approach zero linearly as the mole fraction of cholesterol in the sample approaches 20% (Copeland and McConnell. Manuscript submitted for publication). The freeze-fracture data are interpreted as indicating regions of "solid" DMPC (the ridges) and "fluid" regions containing 20 mol% cholesterol (the "plains" between the ridges). In other experiments using pattern photobleaching, it was found that the apparent diffusion coefficient of a fluorescent phospholipid NBD-PE increased precipitiously at 20 mol% cholesterol, at temperatures below the chain melting temperatures of DMPC (Rubenstein et al., 1979). These results are qualitatively consistent with the idea that the ridges are indeed nearly solid DMPC (where the diffusion of NBD-PE is low: $\sim 10^{-11}$–10^{-10} cm^2/s) and the plains are regions of "fluid" where the diffusion is at least an order of magnitude larger ($10^{-9} - 10^{-8}$ cm^2/s). In the freeze-fracture experiments, ridge separations in the range from 15 nm (0% cholesterol) to 100 nm (18% cholesterol) are observed (Copeland and McConnell, 1980). In the pattern photobleaching recovery experiments we are then confronted with the problem of diffusive recovery in a system in which the distance scale of inhomogeneity in the diffusion coefficient is much smaller than the spatial bleach period employed, say, $P = 3$–30 μm. We therefore consider the

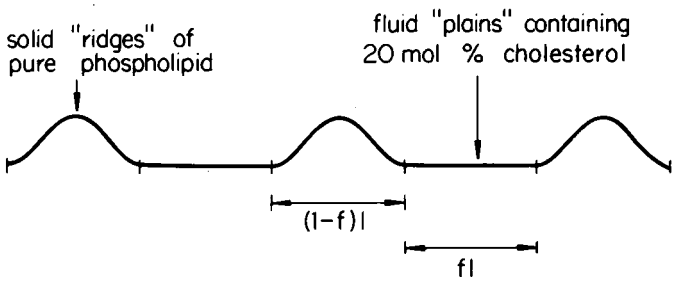

FIGURE 1 A schematic cross section of a phosphatidylcholine-cholesterol bilayer, showing "ridges" of pure phosphatidylcholine and "plains" of a mixture of phosphatidylcholine and cholesterol containing 20 mol% cholesterol. The plains are fluid (large diffusion coefficient, $D \sim 10^{-8}$ cm^2/s) and the ridges are solid (low diffusion coefficient, $D \sim 10^{-10}$ cm^2/s). A schematic view looking down onto the surface of a (photobleached) bilayer is shown in the following figure.

model for diffusive recovery sketched in Fig. 2. We assume two local isotropic diffusion coefficients, D_S and D_F. The diffusion coefficient D_S for NBD-PE in DMPC at 19°C is 10^{-10} cm^2/s; D_F is set equal to the diffusion coefficient of NBD-PE in DMPC-cholesterol mixtures where the mole fraction of cholesterol is $\gtrsim$ 20%; i.e., $D_F \sim 10^{-8}$ cm^2/s (Rubenstein et al., 1979). The fraction of the membrane surface area in the "fluid" regions is f, and the spatial period of the ridges is ℓ. The boundaries between the regions are assumed to be infinitely sharp, and it is assumed that there are no interfacial effects which act as barriers to diffusion.

The problem then is to relate D_F, D_S and the distances $f\ell$, $(1 - f)\ell$ to the results of a photobleach-recovery experiment where the bleach period is P, and the direction, x, of the bleach stripes makes an angle of θ with the x'-direction that is normal to the ridges.

The first question that must be answered is whether or not the results of this pattern photobleach recovery experiment can be accounted for in terms of one or more diffusion coefficients (i.e., an isotropic D, or a constant $D_\parallel$ and $D_\perp$.) Let us first consider the case of photobleach recovery where the bleach stripes are parallel to the ridges, i.e., net diffusion is perpendicular to the ridges.

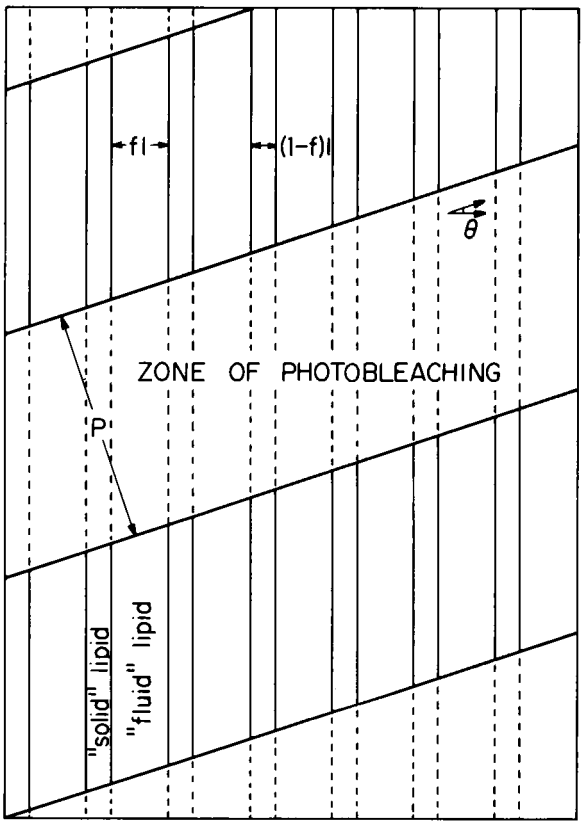

FIGURE 2 A schematic view of a phosphatidylcholine-cholesterol bilayer looking down in a direction normal to the bilayer surface. The "solid" phosphatidylcholine "ridges" have widths equal to $(1 - f)\ell$ and the fluid "plains" have widths $f\ell$. The zones of periodic photobleaching have widths of P. The stripe direction of bleaching makes an angle of θ with respect to the normal to the ridges. The drawing is not to scale in that the calculations in the text apply to the case when $P \gg \ell$.

In the Appendix it is shown that when $P \gg \ell$ the diffusion perpendicular to the ridges can be described by a diffusion coefficient $D_\perp$ given by the equation,

$$\frac{1}{D_\perp} = \frac{f}{D_F} + \frac{(1-f)}{D_S}. \tag{6}$$

For simplicity it is assumed that the number of molecules of NBD-PE in the two domains are proportional to f and $(1-f)$. That is, it is assumed that the concentration of NBD-PE is equal in the two phases at equilibrium. Explicit consideration of motion in the z-direction is omitted, but could be accounted for by an effective value of D_S.

A sufficient condition for the validity of Eq. 6 for a photobleach recovery experiment is that $P \gg \ell$. One can of course easily derive Eq. 6 for a constant flux of material normal to layered structures having different diffusion coefficients (Crank, 1975). However, it must be emphasized that this problem is not equivalent to the time-dependent photobleach recovery experiment; for example, no conditions analogous to $P \gg \ell$ is required for the validity of Eq. 6 for steady-state (constant flux) conditions (Crank, 1975).

The next problem to consider is the diffusion of molecules parallel to the ridges when the bleach stripes are perpendicular to the ridges. It is difficult to give a formal analytic solution to this problem similar to that given in the Appendix for $D_\perp$. However, a convincing order-or-magnitude argument can be given that the photobleach recovery behavior in this case is described by a diffusion constant $D_\parallel$,

$$D_\parallel = fD_F + (1-f)D_S. \tag{7}$$

This result follows from simple random-walk considerations (Davidson, 1962). If the molecules equilibrate rapidly between fluid and solid domains on the time scale of the diffusion measurement, they spend times in the two domains proportional to f and $(1-f)$. Then for motion parallel to (but not perpendicular to) the ridges, the mean-square displacements in time t in each type of domain are $<r^2>_F = 2D_F ft$, and $<r^2>_S = 2D_S(1-f)t$. The overall mean-square displacement is the sum of these, which gives Eq. 7.

We now establish conditions under which equilibration between fluid and solid domains is a good approximation. If we first neglect the exchange of molecules between solid and fluid domains, the fastest recovery is from molecules in the fluid domains, for which the time constant is $1/\Omega_1^2 D_F$, and the time constant for recovery of molecules in the solid domains is $1/\Omega_1^2 D_S$, for diffusion parallel to the ridges. If we now consider the exchange of molecules between the solid and fluid domains it is evident that corresponding transverse recovery-equilibration times will be no larger than of the order of the larger of $1/\omega_S^2 D_S$ or $1/\omega_F^2 D_F$, where $\omega_S = 2\pi/[(1-f)\ell]$ and $\omega_F = 2\pi/(f\ell)$. As long as the relaxation times $1/\Omega_1^2 D_F$ and $1/\Omega_1^2 D_S$ are long compared with these transverse relaxation times we can confidently assume concentration equilibrium between solid ridges and fluid plains, except possibly in small regions near the boundaries between the initially bleached and nonbleached regions. Illustrative calculations of the diffusive anisotropy $(D_\parallel/D_\perp)$ are given in Fig. 3 as a function of D_F/D_S and the fraction of fluid lipid f. Note that in the model of Copeland and McConnell (1980) the observed widths of the ridges are constant ($d_0 \simeq 15$ nm) to within the experimental error. Thus, $(1-f)\ell = d_0$ and $(1-f) = d_0/\ell$. Therefore, as $f \to 1$, $\ell \to \infty$, and our principal assumption $P \gg \ell$ cannot be valid. Our calculations thus cannot yield the correct asymptotic behavior as $f \to 1$; a theoretical calculation of this asymptotic behavior would probably be of

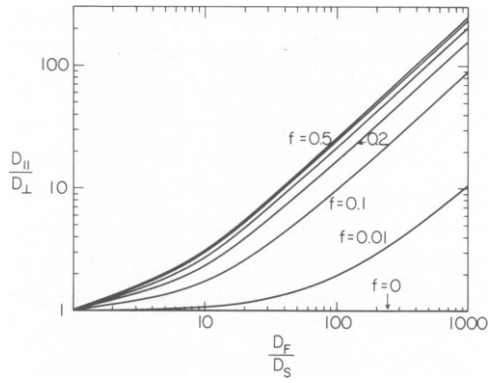

FIGURE 3 Anisotropy of macroscopic diffusion ($D_\parallel/D_\perp$) as a function of the ratio of diffusion coefficients in fluid and solid regions (D_F/D_S). Curves are presented for several values of f, the area fraction of fluid lipid. Curves for $f = 0.3$ and 0.4 are between the curves for $f = 0.2$ and 0.5. The curves are symmetric about $f = 0.5$; that is, curves for f_i and f_j are identical if $f_i = (1 - f_j)$. This follows from the equation $(D_\parallel/D_\perp) = 1 + (D_F/D_S + D_S/D_F - 2)(f)(1 - f)$ which can be derived from Eqs. 6 and 7 in the text.

little interest since the freeze-fracture electron micrographs indicate that the ridges begin to lose their regular spacing and linearity when this spacing is of the order of 0.1 μm. When $f = 1$ our calculation is correct. Note also that in the model of Copeland and McConnell (1980), f is proportional to the mole fraction X_c of cholesterol in the sample, $f = 5X_c$.

Because experiments are often carried out on multilamellar arrays of lipid bilayers containing fluorescent probes, it is important to consider the fluorescent recovery of an isotropic distribution of bilayer orientations (all values of θ between 0 and 180° in Fig. 2). It is also true that in some liposomes studied by freeze-fracture electron microscopy there are sometimes (but not always) multiple values of θ in a single liposome; pattern photobleach recovery experiments can be carried out on individual liposomes (L. Smith et al., 1979).

Let the unit vectors in the principal axis directions corresponding to $D_\perp$ and $D_\parallel$ be $\mathbf{i}'$ and $\mathbf{j}'$, respectively, so that the diffusion dyadic is

$$\boldsymbol{D} = \mathbf{i}'\mathbf{i}' D_\perp + \mathbf{j}'\mathbf{j}' D_\parallel. \tag{8}$$

Let $\mathbf{i}$ and $\mathbf{j}$ be unit vectors in the directions of the bleach stripes, and perpendicular to the bleach stripes, and use these vectors for the gradient term ∇ in Eqs. 2 and 3. The diffusion equation, Eq. 2, can then be written

$$\frac{\partial c}{\partial t} = D(\theta)\frac{\partial^2 c}{\partial x^2} + \cdots, \tag{9}$$

where

$$D(\theta) = D_\parallel \sin^2\theta + D_\perp \cos^2\theta, \tag{10}$$

and the $+\ldots$ denote terms involving $\partial^2 c/\partial y^2$ and $\partial^2 c/\partial x \partial y$. These terms are zero by the condition of rapid lipid equilibration between fluid plains and solid ridges and by the assumption of equal concentration of the fluorescent lipid in the two domains under equilibrium conditions. If the latter assumption were not correct, the concentration c in all the

equations would have to be multiplied by thermodynamic activity coefficients. The question of equal concentrations of probe in plains and ridges is discussed later.

Let $S(t)$ be the fluorescence intensity arising from fluorescent molecules in the initially photobleached regions depicted in Fig. 2, at time t after bleaching. It follows that the "recovery function" is given by an integral giving an angular average over θ,

$$\frac{S(\infty) - S(t)}{S(\infty) - S(0)} = \frac{2}{\pi} \exp(-\Omega_0^2 D_\perp t) \int_0^{\pi/2} d\theta \exp[-\Omega_1^2 (D_\parallel - D_\perp) t \sin^2\theta]$$

$$= \exp[-\Omega_1^2 (D_\parallel + D_\perp) t/2] I_0[\Omega_1^2 (D_\parallel - D_\perp) t/2], \tag{11}$$

where I_0 is the modified Bessel function of the first kind. The short-time behavior is given by the equation,

$$\frac{S(\infty) - S(t)}{S(\infty) - S(0)} = \exp[-\Omega_1^2 (D_\perp + D_\parallel) t/2] \tag{12}$$

The long-time behavior is given by the equation,

$$\frac{S(\infty) - S(t)}{S(\infty) - S(0)} = \exp(-\Omega_1^2 D_\perp t)/[\pi \Omega_1^2 (D_\parallel - D_\perp) t]^{1/2}. \tag{13}$$

These results are illustrated by the plots in Figs. 4–7. Note that these calculations do not include the rapidly decaying terms such as those discussed in connection with Eq. 4. Orientational averaging of diffusion in a different anisotropic system has been carried out independently by Callaghan et al. (1979). The calculated apparent diffusion coefficients D_{app}

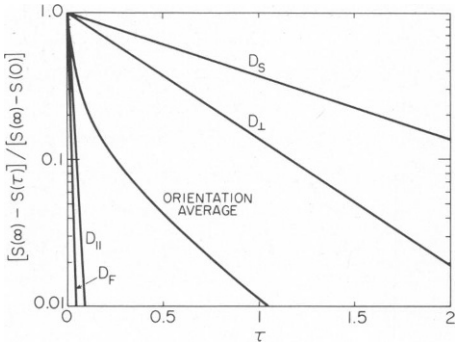

FIGURE 4

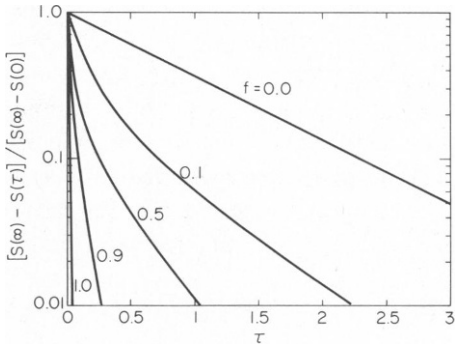

FIGURE 5

FIGURE 4 Fluorescence recovery after pattern photobleaching after normalized time, $\tau = \Omega_1^2 D_S t$, where D_S is the diffusion coefficient in the solid phase and $\Omega_1 = 2\pi/P$ where P is the bleach stripe period. The quantity $S(\tau)$ gives the fluorescence intensity arising from fluorescent molecules in the photobleached regions depicted in Fig. 2, at normalized time τ. Recovery curves are presented for lipid with diffusion coefficients D_S, $D_F = 100\ D_S$, and $D_\perp$ and $D_\parallel$ as given by Eqs. 6 and 7 in the text. Also given is the diffusive recovery calculated for a multilamellar system having an isotropic distribution (all values of θ in Fig. 2). See Eq. 9 in the text. Calculations are for $f = 0.5$.

FIGURE 5 Fluorescence recovery after pattern photobleaching for various area fractions f of fluid lipid. For definitions of τ and $S(\tau)$ see Fig. 4. In the present figure $D_F/D_S = 100$. Recoveries are orientation averages as obtained from Eq. 9.

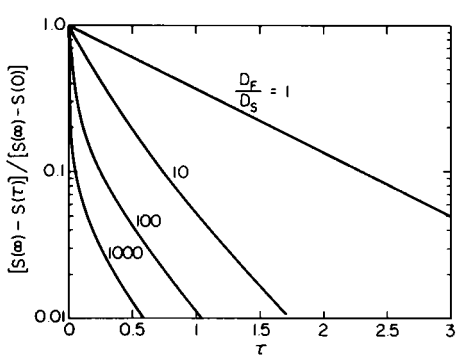

FIGURE 6

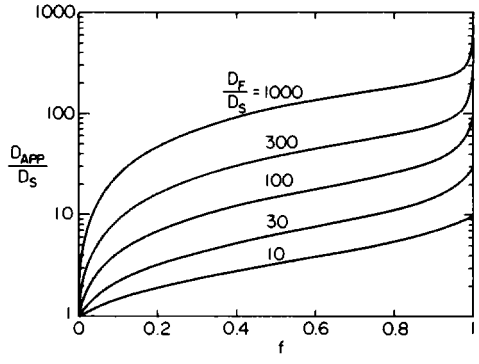

FIGURE 7

FIGURE 6 Fluorescence recovery after photobleaching for various ratios of D_F/D_S, with $f = 0.5$. See Fig. 4 for definitions of $S(\tau)$ and τ. Calculations refer to orientation averages obtained from Eq. 11.

FIGURE 7 Apparent diffusion coefficients D_{app} (given as D_{app}/D_S) as functions of f, the fraction of fluid lipid, for various values of D_F/D_S. The values of D_{app} were obtained from theoretical recovery curves for orientationally averaged multilamellar systems (Eq. 11) by fitting each curve to a single exponential decay using a least-square procedure. This is intended to mimic the experimental data analysis.

illustrated in Fig. 7 do mimic in a semiquantitative way the diffusion coefficients reported by Rubenstein et al. (1979) in binary mixtures of DMPC and cholesterol (and also in binary mixtures of DPPC and cholesterol) in the 0–20 mol% cholesterol concentration range, in that there is a substantial increase in the apparent diffusion coefficient as the concentration of cholesterol is increased in the range between 0 and 20 mol%, before the marked increase at 20 mol%, where $f = 1$. The calculation of these apparent diffusion coefficients is described in the legend to Fig. 7, and is intended to mimic an attempt to fit the experimental recovery rates to a single exponential. The calculated marked increase in D_{app} near $f = 0$ corresponding to low cholesterol concentrations (i.e., 1–2% cholesterol) was not detected by Rubenstein et al. (1979). The source of this discrepancy is not known, but could arise if the ridges were parallel but sometimes formed closed circles, ellipses, polygons, or other closed figures. The apparent diffusion coefficients in Fig. 7 were obtained by least-squares best fits of the recovery curves (Eq. 11) to single exponential decays. The method of data analysis used by Rubenstein et al. (1979) was to fit the logarithm of the recovery to a straight line. If the recovery is not truly exponential, the result obtained with the second procedure will depend significantly on how much of the "tail" of the recovery is analyzed, since this part of the recovery is weighted as heavily as is the initial (large amplitude) part. The net effect is that some of the discrepancies between the theoretical and experimental results may occur because the experimental data analysis technique was not very sensitive to a small component of fast recovery. Because of these complications we cannot exclude the possibility that there is a nonuniform distribution of the fluorescent lipid between fluid plains and solid domains in the system. In the case of spin-label lipids there is strong evidence against a highly nonuniform partitioning of the probes between the solid and fluid regions (Rubenstein et al., 1980).

DISCUSSION

In this paper we have demonstrated how local variations in diffusion coefficients with spatial frequencies of order ω can affect diffusion coefficients based on experiments utilizing spatial

frequencies $\Omega \ll \omega$. Before we further discuss these complexities, it is important to emphasize that many diffusion measurements in model membranes do not exhibit these complexities. For example, consider various measurements of the lateral diffusion of spin-labeled phospholipids in lipid multibilayers. Some experiments have been carried out under steady-state conditions where spin exchange line broadening is used to measure the collision rate of spin-labeled lipids, and from this collision rate the diffusion coefficient is inferred (Devaux et al., 1973; Träuble and Sackmann, 1972). In these experiments the effective time and distance scales are of the order of 10^{-7} s and 10 nm. In other experiments employing nonuniform concentration distributions of spin labels (Devaux and McConnell, 1972; Sheats and McConnell, 1978), times of the order of many hours (e.g., 10 h) and distances of the order of millimeters have been employed. Excellent agreement has been obtained among such diverse measurements that cover time ratios of the order of 10^{10} and distance ratios of the order of 10^5. Also, using pattern photobleaching methods, it has been established that for fluid as well as solid lipid bilayer membranes, very similar results are obtained irrespective of whether one employs coplanar lipid multilayers, or predominantly single-shelled lipid vesicles (L. Smith et al., 1979). Having noted that homogeneous lipid bilayer systems do yield closely similar diffusion coefficients for a number of experimental techniques employing a wide range of spatial frequencies and times (as well as a variety of probe molecules), we now return to the more complex model considered in the present work.

In the model studied here it is assumed that except at solid-fluid boundaries the local diffusion coefficient is constant and isotropic, small in the solid phases and large in the fluid phases. In the photobleaching diffusion recovery experiment this model yields an anisotropic diffusion dyadic, with constant $D_\parallel$ and $D_\perp$, parallel and perpendicular to the "solid" lipid ridges. The model of course assumes that the solid ridges have a coherence length that is large compared with the photobleach period. Freeze-fracture electron microscopic studies indicate that this is often so, but not always (Copeland and McConnell, 1980; Kleemann and McConnell, 1976; Blok et al., 1977). Sometimes disclination defects cause surface patterns with characteristic dimensions comparable to the bleach period. The effects of these on diffusion may depend in a complicated way on the details of the spatial distribution of the defects. In those instances in which the ridges form closed (e.g., hexagonal) figures, the observed diffusion coefficient vs. cholesterol concentration is most likely to follow the sharp $D_\perp$ vs. composition behavior expected from Eq. 6. The diffusive behavior discussed in the present work should certainly also be considered in the analysis of the deuterium nuclear resonance spectra of deuterated lipids in binary mixtures of phosphatidylcholine and cholesterol (Jacobs and Oldfield, 1979; Kuo and Wade, 1979). It has already been shown that exchange of lipids between "fluid" and "solid" lipid domains can have large effects on nuclear resonance spectra (Brûlet and McConnell, 1976).

Inhomogeneities in lipid composition and "fluidity" in biological membranes are known (I. Smith et al., 1979) but are of course only one potential source of anisotropic motion and complex diffusive behavior. Lateral molecular motion modified by so-called anchorage modulation effects (Edelman, 1976; Nicholson, 1976) could also give rise, for example, to anisotropic, inhomogeneous diffusion. To give a specific example, suppose that a transmembrane protein diffuses through a lipid bilayer, binds to, and dissociates from, submembraneous binding sites. If the binding sites are isotropically distributed, have site-site distances that are small compared with the photobleach period P, and have binding and dissociation kinetics

that are fast compared with the time required to diffuse the distance P, then the photobleach recovery in this system should be accounted for by an apparent isotropic diffusion coefficient $D = XD_0$ where X is the fraction of the time the diffusing molecule is free (not bound to a binding site) and D_0 is the diffusion constant in the absence of binding sites. Obviously, if the binding sites are anisotropically distributed, but the other conditions mentioned above apply, the protein diffusion dyadic $\boldsymbol{D}$ will be anisotropic, and possibly also inhomogeneous, i.e., $D_{xx}(x, y)$. Other sources of complex diffusion on cell membranes are also present; for example, the surfaces of some cells are far from planar. In spite of these possible complexities in the analysis of diffusion data in cell membranes, there is every reason to hope that much valuable biophysical information can be obtained from such studies.

The authors are most indebted to Dr. Dean Hafeman, Professor John Ross, Dr. Barton Smith, and Mr. Robert Weis for helpful discussions.

This work was supported by National Science Foundation grant PCM 77-23586. Dr. Owicki was supported by a National Institutes of Health Postdoctoral Fellowship in the laboratory of Professor H. M. McConnell during some of the period of this research.

Received for publication 23 October 1979 and in revised form 7 February 1980.

APPENDIX

Analysis of Diffusion Perpendicular to Ridges

Consider first a two-dimensional homogeneous material, with diffusion coefficient D, which is bounded at $x = 0$ and L but is unbounded in y. We only treat the one-dimensional problem of diffusion along the x-direction.

Initially ($t = 0$) the concentration of diffusant is constant in the material. As a mathematical convenience we will take $c(x, t)$ to be relative to this initial concentration, so $c(x, 0) \equiv 0$. Because only relative concentrations are important for diffusion, this does not restrict the problem. Boundary conditions are applied to the concentration and/or the flux $J(x, t) = -D\partial c/\partial x$ at $x = 0$ and $x = L$.

After a Laplace transform with respect to time, the diffusion equation for this system is, in matrix notation (Carslaw and Jaeger, 1959):

$$\begin{pmatrix} c(x, p) \\ J(x, p) \end{pmatrix} = \boldsymbol{M}[x, p; D] \begin{pmatrix} c(0, p) \\ J(0, p) \end{pmatrix} \tag{A1}$$

where p is the transform variable conjugate to t,

$$\boldsymbol{M}[x, p; D] \equiv \begin{pmatrix} \cosh(xq) & -\sinh(xq)/Dq \\ -Dq \sinh(xq) & \cosh(xq) \end{pmatrix} \tag{A2}$$

and $q \equiv (p/D)^{1/2}$. The boundary conditions at $x = L$ enter by requiring Eq. A1 to hold at $x = L$.

Next consider a modification of this system in which there are alternate domains ("plains") of fast ($D = D_F$) and domains ("ridges") of slow ($D = D_S$) diffusion, of widths $f\ell$ and $(1 - f)\ell$, respectively, parallel to the y-axis, as in Fig. 2. At any boundary $x = n\ell$ ($n = 1, 2, 3, \ldots$) between pairs of bands, the analog of Eq. A1 is (Carslaw and Jaeger, 1959):

$$\begin{pmatrix} c(x, P) \\ J(x, p) \end{pmatrix} = \{\boldsymbol{M}[(1 - f)x/n, p; D_S]\boldsymbol{M}(fx/n, p; D_F)\}^n \begin{pmatrix} c(0, p) \\ J(0, p) \end{pmatrix}. \tag{A3}$$

We will show that

$$\lim_{n\to\infty} \{M[(1-f)x/n, p; D_S]M(fx/n, p; D_F)\}^n = M(x, p; D_\perp), \tag{A4}$$

where

$$\frac{1}{D_\perp} = (f/D_F) + [(1-f)/D_S]. \tag{A5}$$

In other words, in the limit of infinitely narrow bands, the composite material behaves as a homogeneous material with diffusion coefficient $D_\perp$ for diffusion perpendicular to the bands.

The proof is straightforward but algebraically tedious. First we find the eigenvalues λ_+, λ_- and corresponding eigenvectors (α_+, β_+), (α_-, β_-) of the product $M\,(1-f)x/n,p;D_S]\,M\,[fx/n,p;D_F]$:

$$\begin{aligned}
\lambda_\pm &= \{\mathscr{E}_1 - \mathscr{E}_2 \pm [(\mathscr{E}_1 - \mathscr{E}_2)^2 - 16d^2]^{1/2}\}/4d \\
\alpha_\pm &= [\mathscr{E}_1/(1+d) - \mathscr{E}_2/(1-d)]/d - 2\lambda_\pm \\
\beta_+ &= \beta_- = (D_F q_F)\cdot(\mathscr{S}_1 - \mathscr{S}_2),
\end{aligned} \tag{A6}$$

where

$$\begin{aligned}
\mathscr{E}_1 &\equiv (1+d)^2 \cosh\{[fq_F + (1-f)q_S]x/n\} \\
\mathscr{E}_2 &\equiv (1-d)^2 \cosh\{[fq_F - (1-f)q_S]x/n\} \\
\mathscr{S}_1 &\equiv (1+d) \sinh\{[fq_F + (1-f)q_S]x/n\} \\
\mathscr{S}_2 &\equiv (1-d) \sinh\{[fq_F - (1-f)q_S]x/n\} \\
d &\equiv \sqrt{D_S/D_F} \\
q_F &\equiv (p/D_F)^{1/2} \\
q_S &\equiv (p/D_S)^{1/2}
\end{aligned}$$

The eigenvalues and eigenvectors can be used to express the matrix product as a similarity transformation of a diagonal matrix:

$$M[(1-f)x/n, p; D_S]M(fx/n, p; D_F) = \frac{1}{(\alpha_+\beta_- - \alpha_-\beta_+)} \begin{pmatrix} \beta_- & -\alpha_- \\ -\beta_+ & \alpha_+ \end{pmatrix} \begin{pmatrix} \lambda_+ & 0 \\ 0 & \lambda_- \end{pmatrix} \begin{pmatrix} \alpha_+ & \alpha_- \\ \beta_+ & \beta_- \end{pmatrix}. \tag{A7}$$

It follows from elementary considerations of linear algebra that

$$\{M[(1-f)x/n, p; D_S]M(fx/n, p; D_F)\}^n \equiv 1/(\alpha_+\beta_- - \alpha_-\beta_+) \begin{pmatrix} \beta_- & -\alpha_- \\ -\beta_+ & \alpha_+ \end{pmatrix} \begin{pmatrix} \lambda_+^n & 0 \\ 0 & \lambda_-^n \end{pmatrix} \begin{pmatrix} \alpha_+ & \alpha_- \\ \beta_+ & \beta_- \end{pmatrix}. \tag{A8}$$

Carrying out the matrix multiplication on the right-hand side of Eq. A8 expanding in powers of $(1/n)$, and taking the limit $n \to \infty$, one recovers Eq. A4.

As $n \to \infty$, any point in the material falls arbitrarily close to a boundary between bands, so the proof holds throughout the material. Although it was given for initial concentrations independent of x, the

proof is true more generally. It holds for piecewise constant initial conditions (one treats each constant section separately, then joins the solutions at the boundaries). Since any integrable function can be approximated to arbitrarily good accuracy by a piecewise constant function of fine enough mesh, the proof also can be shown to hold for very general initial conditions. If the concentration is a function of y, of course, the problem is two-dimensional and cannot be treated by the methods given here.

Finally, we note that deviations from homogeneous behavior for large but finite n could be obtained from Eqs. A3 and A8 using perturbation techniques.

REFERENCES

AXELROD, D., D. E. KOPPEL, J. SCHLESSINGER, E. ELSON, and W. W. WEBB. 1976. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* **16**:1055–1069.

BLOK, M. C., L. L. M. VAN DEENEN, and J. DE GIER. 1977. The effect of cholesterol incorporation on the temperature dependence of water permeation through liposomal membranes prepared from phosphatidylcholines. *Biochim. Biophys. Acta.* **464**:509–518.

BRÜLET, P., and H. M. MCCONNELL. 1976. Kinetics of phase equilibrium in a binary mixture of phospholipids. *J. Am. Chem. Soc.* **98**:1314–1318.

CALLAGHAN, P. T., K. W. JOLLEY, and J. LELIEVRE. 1979. Diffusion of water in the endosperm tissue of wheat grains as studied by pulsed field gradient nuclear magnetic resonance. *Biophys, J.* **28**:133–142.

CARSLAW, H. S., and J. C. JAEGER. 1959. Condution of Heat in Solids. 2nd ed. Oxford University Press, Oxford. 319–326.

COPELAND, B. R., and H. M. MCCONNELL. 1980. The rippled structure in bilayer membranes of phosphatidylcholine and binary mixtures of phosphatidylcholine and cholesterol. *Biochim. Biophys. Acta.* In press.

CRANK, J. 1975. The Mathematics of Diffusion. 2nd ed. Oxford University Press, London. 414.

DAVIDSON, N. 1962. Statistical Mechanics. McGraw-Hill Book Company, New York. 284.

DEVAUX, P., and H. M. MCCONNELL. 1972. Lateral diffusion in spin-labeled phosphatidylcholine multilayers. *J. Am. Chem. Soc.* **94**:4475–4481.

DEVAUX, P., C. J. SCANDELLA, and H. M. MCCONNELL. 1973. Spin-spin interactions between spin-labeled phospholipids incorporated into membranes. *J. Magn. Resonance.* **9**:474–485.

EDELMAN, G. M. 1976. Surface modulation in cell recognition and growth. *Science (Wash. D.C.).* **192**:218–226.

ESSER, A. F., R. M. BARTHOLOMEW, J. W. PARCE, and H. M. MCCONNELL. 1979. The physical state of membrane lipids modulates the activation of the first component of complement. *J. Biol. Chem.* **254**:1768–1770.

ESTEP, T. N., D. B. MOUNTCASTLE, R. L. BILTONEN, and T. E. THOMPSON. 1978. Studies on the anomalous thermotropic behavior of aqueous dispersions of dipalmitoylphosphatidylcholine-cholesterol mixtures. *Biochemistry.* **17**:1984–1989.

HAFEMAN, D. G., J. W. PARCE, and H. M. MCCONNELL. 1979. Specific antibody-dependent activation of neutrophils by liposomes containing spin-label lipid haptens. *Biochem. Biophys. Res. Commun.* **86**:522–528.

JACOBS, R., and E. OLDFIELD. 1979. Deuterium nuclear magnetic resonance investigation of dimyristoyllecithin-dipalmitoyllecithin and dimyristoyllecithin cholesterol mixtures. *Biochemistry.* **18**:3280–3285.

KLEEMANN, W., and H. M. MCCONNELL. 1976. Interactions of proteins and cholesterol with lipids in bilayer membranes. *Biochim. Biophys. Acta.* **419**:206–222.

KUO, A.-L., and C. G. WADE. 1979. Lipid lateral diffusion by pulsed nuclear magnetic resonance. *Biochemistry.* **18**:2300–2308.

MABREY, S., P. L. MATEO, and J. M. STURTEVANT. 1978. High-sensitivity scanning calorimetric study of mixtures of cholesterol with dimrysitoyl- and dipalmitoylphosphatidylcholine. *Biochemistry.* **17**:2464–2468.

MCCONNELL, H. M. 1978. Relation of lateral molecular motion in membranes and immune response. *Harvey Lect.* **72**:231–251.

NICOLSON, G. 1976. Transmembrane control of the receptors on normal and tumor cells. I. Cytoplasmic influence over cell surface components. *Biochim. Biophys. Acta.* **457**:57–108.

PARCE, J. W., H. M. MCCONNELL, R. M. BARTHOLOMEW, and A. F. ESSER. 1980. Kinetics of antibody-dependent activation of the first component of complement on lipid bilayer membranes. *Biochem. Biophys. Res. Commun.* **93**:235–242.

PETERS, R., J. PETERS, K. H. TEWS, and W. BÄHR. 1974. A microfluorimetric study of translational diffusion in erythrocyte membranes. *Biochim. Biophys. Acta.* **367**:282–294.

POO, M.-M., and R. A. CONE. 1974. Lateral diffusion of rhodopsin in the photoreceptor membrane. *Nature (Lond.)* **247**:438–441.

RUBENSTEIN, J. L. R., J. C. OWICKI, and H. M. MCCONNELL. 1980. Dynamic properties of binary mixtures of phosphatidylcholines and cholesterol. *Biochemistry.* **19**:569–573.

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. U.S.A.* **76**:15–18.

SCHLESSINGER, J., W. W. WEBB, E. L. ELSON, and H. METZGER. 1976*a*. Lateral motion and valence of Fc receptors on rat peritoneal mast cells. *Nature (Lond.)* **264**:550–552.

SCHLESSINGER, J., D. E. KOPPEL, D. AXELROD, K. JACOBSON, W. W. WEBB, and E. L. ELSON. 1976*b*. Lateral transport on cell membranes: mobility of concanavalin A receptors on myoblasts. *Proc. Natl. Acad. Sci. U.S.A.* **73**:2409–2418.

SCHLESSINGER, J., Y. SCHECHTER, P. CUATRECASAS, M. C. WILLINGHAM, and I. PASTAN. 1978. Quantitative determination of lateral diffusion coefficients of the hormone- receptor complexes of insulin and epidermal growth factor on the plasma membrane of cultured fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **75**:5353–5357.

SHEATS, J., and H. M. MCCONNELL. 1978. A photochemical technique for measuring the lateral diffusion of spin-labeled phospholipids in membranes. *Proc. Natl. Acad. Sci. U.S.A.* **75**:4661–4663.

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.

SMITH, B. A., and H. M. MCCONNELL. 1978. Determination of molecular motion in membranes using periodic pattern photobleaching. *Proc. Natl. Acad. Sci. U.S.A.* **75**:2759–2763.

SMITH, B. A., W. R. CLARK, and H. M. MCCONNELL. 1979. Anisotropic molecular motion on cell surfaces. *Proc. Natl. Acad. Sci. U.S.A.* **76**:5641–5644.

SMITH, IAN C. P., K. W. BUTLER, A. P. TULLOCH, H. H. DAVIS, and M. BOOM. 1979. The properties of gel state lipids in membranes of *Acholeplasma laidlawiii* as observed by ^{2}H NMR. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **100**:57–61.

SMITH, L. M., J. W. PARCE, B. A. SMITH, and H. M. MCCONNELL. 1979. Antibodies bound to lipid haptens in model membranes diffuse as rapidly as the lipids themselves. *Proc. Natl. Acad. Sci. U.S.A.* **76**:4177–4179.

TANNER, J. E. 1978. Transient diffusion in a system partitioned by permeable barriers. Application to NMR measurements with a pulsed field gradient. *J. Chem. Phys.* **69**:1748–1753.

TRÄUBLE, H., and E. SACKMANN. 1972. Structure of a steroid-lecithin system below and above the lipid-phase transition. *J. Am. Chem. Soc.* **94**:4499–4510.

WU, E.-S., K. JACOBSON, and D. PAPAHADJOPOULOS. 1977. Lateral diffusion in phospholipid multibilayers measured by fluorescence recovery after photobleaching. *Biochemistry.* **16**:3936–3941.