# Lateral Diffusion in Phospholipid Bilayer Membranes and Multilamellar Liquid Crystals†

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ABSTRACT: The lateral-diffusion coefficients ($D$) of a fluorescent lipid analogue, 3,3′-dioctadecylindotricarbocyanine (diI), have been measured in black lipid membranes (BLMs), in large (20–50-$\mu$m diameter) solvent-free bilayer and multilayer membrane vesicles, and in multilamellar liquid crystals of dipalmitoylphosphatidylcholine, dimyristoylphosphatidylcholine, and egg lecithin. They show that $D$ changes by several orders of magnitude at the liquid-crystal transformations of the solvent-free bilayers and multilayers. In all BLMs, $D \approx 10^{-7}$ cm$^2$/s with only weak temperature dependence even near the putative phase-transformation temperatures, $T_t$. In the corresponding liquid crystals and large vesicles, $D \approx 10^{-8}$ cm$^2$/s above $T_t$, decreasing by about two orders of magnitude to $D \approx 10^{-10}$ cm$^2$/s below $T_t$. The changes of $D$ in bilayer vesicles suggest that the expected liquid-crystal phase transitions from smectic A to a higher ordered state do persist in isolated bilayers. Retained solvent in black lipid membranes formed by both Mueller–Rudin and Montal–Mueller methods appears to enhance lateral diffusion. A simple method of forming small numbers of large solvent-free vesicles is described.

The physical structure of lipid membranes affects the dynamics of various membrane processes in both cell membranes and model membrane systems. Thermotropic phase transformations of the phospholipid bimolecular layer have been implicated in profound changes of small-molecule permeability, binding, and solubility, the activity of membrane enzymes, membrane microviscosity, and rotational and translational diffusion (Chapman, 1975; Melchoir and Steim, 1976; Jacobson and Papahadjopoulos, 1976; Blok et al., 1976; Edidin, 1974). Close correspondence has been established between thermotropic transformations in cell membranes and in model systems consisting of liposomes, sonicated vesicles, and liquid crystals.

New capability for measurement of lateral diffusion of membrane components using fluorescence photobleaching recovery (FPR) techniques as described by Koppel et al. (1976) and Axelrod et al. (1976) is facilitating rapid progress in understanding the factors involved in the lateral mobility of cell-membrane components. Recent results include studies of the concanavalin A receptor on myoblasts by Schlessinger et al. (1976), the $F_c$ antibody receptor on mast cells by Schlessinger et al. (1977), the acetylcholine receptor on myotubes by Axelrod et al. (1977), and nonselectively labeled membrane proteins in myoblasts by Schlessinger et al. (1977), Jacobson et al. (1976), and Edidin et al. (1976). Earlier pioneering studies included an attempt to measure protein diffusion in erythrocyte ghosts by Peters et al. (1974) and measurements of rhodopsin diffusion in rod-outer-segment membranes by Poo and Cone (1974) and Liebman and Entine (1974). Measurements of the lateral diffusion of a fluorescent lipid analogue in a variety of mammalian cells showed little variation among cell types, history, or growth stage and suggested a common characteristic value of the self-diffusion coefficient of around $D \approx 10^{-8}$ cm$^2$/s with a modest (~3×) increase between 15 and 37 °C (see summary by Webb, 1977).

Recently, Fahey et al. (1977) have also measured the lateral-diffusion coefficients of fluorescent lipid analogues in planar bilayer lipid membranes (BLM)[1] using another new technique, fluorescence correlation spectroscopy (FCS), developed by Magde et al. (1972) (see also Elson and Magde, 1974; Magde et al., 1974; Koppel, 1974). These experiments showed that diffusion of certain lipid analogues in BLMs is about an order of magnitude faster than in the mammalian cell membranes. None of these measurements of lateral diffusion have, however, explored the consequences of phase transformations in membranes. Wu et al. (1977) had found, however, large changes of the lateral diffusion coefficient of lipid analogues associated with the phase transitions in multilamellar liquid crystals.

It was, therefore, the objective of this research to investigate the effects of phase transformation in phospholipid bilayers on lateral diffusion of lipid analogues in the model systems. The lateral-diffusion coefficients in planar membranes (BLMs) formed by thinning from lipid dispersions in hydrocarbon solvent (Mueller et al., 1972) and by pairing of monolayers spread on water surfaces (Montal and Mueller, 1972) were found to be remarkably insensitive to temperature in the vicinity of putative phase transitions, inferred from multilayer liquid-crystal behavior. Comparable diffusion measurements were, therefore, carried out on multilamellar, oriented, fully hydrated, phospholipid liquid crystals (MLLC) [formed by an adaptation of methods of Jost et al. (1971)] and large bi-

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[1] Abbreviations used are: BLM, black lipid membranes (i.e., planar lipid bilayer membranes); FCS, fluorescence correlation spectroscopy; FPR, fluorescence photobleaching recovery; MLLC, multilamellar liquid crystals; LBV, large bilayer vesicles; DPPC, dipalmitoylphosphatidylcholine; EPC, egg PC; DMPC, dimyristoylphosphatidylcholine; GMO, glycerol monooleate; C, cholesterol; diI, dioctadecylindotricarbocyanine; NBD-C12) *N*-4-nitrobenzo-2-oxa-1,3-diazolelaurate; M540, merocyanine-540.

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layer vesicles (LBV) [formed by a new method based on suggestions of Paul Mueller (private communication) and extensions of the methods of Reeves and Dowben (1969) and Melchior and Morowitz (1972)]. As will be seen, our data indicate substantial differences between BLMs and MLLC and large vesicle systems. Most of the discussion seeks to discern the origin of these differences.

## Materials and Methods

*Materials.* The lipids 1,2-dipalmitoylphosphatidylcholine (DPPC), 1,2-dimyristoylphosphatidylcholine (DMPC), egg phosphatidylcholine (EPC), glycerol monooleate (GMO), and 7-dehydrocholesterol (C) were purchased from selected commercial sources and were assayed for purity by thin-layer chromatography. 1,2-Dilaurylphosphatidylcholine (DLPC) was a gift of G. Feigenson. The fluorophores were 3,3′-dioctadecylindotricarbocyanine (diI), a gift of A. S. Waggoner; *N*-4-nitrobenzo-2-oxa-1,3-diazolelaurate (NBD-C12), a gift of P. Brynes; and merocyanine-540 (M540), purchased from Eastman Kodak.

*BLM Formation.* To form BLMs by the Mueller–Rudin method (MR) (Mueller et al., 1962), a small droplet (0.2 $\mu$L) of suspension of lipid in *n*-alkane (hexane, hexadecane, or octane) was deposited on an electron-microscopy grid attached to a Teflon support in a glass chamber containing some pH 7 phosphate buffer (0.1 M $KH_2PO_4$ and 0.0058 M NaOH) or sometimes distilled water. The fluid level in the chamber was raised past the grid, leaving a population of BLMs attached to the grid by narrow Gibbs plateau borders of the lipid dispersed in solvent; the water was eventually allowed to overflow the cell, flushing away any free lipid droplets. Use of electron-microscopy grids of 100- or 200-mesh size as the bilayer support resulted in a large population of quite stable bilayers. To form BLMs by the technique of Montal and Mueller (1972) (MM), the lipid was first spread with hexane on a water surface in the same type of cell and allowed to stand for a few minutes for evaporation of most of the hexane; then the water–lipid surface was raised past the grid (S. H. White et al., 1976; Fahey et al., 1977). The MM technique has been shown by capacitance measurements to reduce solvent retention in the membrane (Berry et al., 1975; Benz and Janko, 1976).

We have tested our version of the MM techniques by capacitance measurements above the transition temperature and have found agreement with the aforesaid studies.

*Multilayer Formation.* Multiple bilayers, i.e., planar, multilamellar, fully hydrated smectic liquid crystals (MLLC), were formed by deposition on a flat glass substrate using variations of standard procedures (Jost et al., 1971; Badley et al., 1973; see also Badley, 1976). First, a thin layer of lipid dissolved in a mixture of chloroform–ethanol (2:1) at 10 mg/mL or in hexane at 1 mg/mL was spread on a glass slide, and the solvent was evaporated in a gentle flow of dry nitrogen for several hours. To facilitate uniform deposition, some of the slides were coated with an insoluble surfactant, *N*,*N*-dimethyl-*N*-octadecyl-3-aminopropyltrimethoxylilyl chloride usually called DMOAP and obtained originally from Dow Corning. The films were hydrated by either immersion in distilled water for at least 20 min with a minimum of mechanical movement or more slowly by exposure to saturated water vapor for several days at temperatures above the hydrocarbon chain-ordering temperature. During hydration in liquid water, some liposomes formed from the film but generally hydrated regions of smooth oriented multiple bilayers of adequate dimensions, say >0.2-mm square, remained. In the case of DLPC, excessive liposome formation was avoided by carrying out most of the hydration in saturated water vapor, following application of a little liquid water. Completion of hydration could be judged by observing that the diffusion coefficient of diI had reached its limiting value. Flat, uniform, oriented regions of the film were selected for diffusion measurements by observing the absence of birefringence and the uniformity of fluorescence over the visual field of the optical microscope. Since the fluorescent probe was uniformly distributed, the fluorescence intensity provided a measure of the film thickness that could be used to select regions of 100–1000 bilayers thickness. Control measurements of the diffusion coefficients of the lipid probe diI showed no effect of method of multilayer hydration, thickness, spreading solvent, or use of surfactant coating on the slide.

*Large Vesicle Formation.* Suitable large lipid bilayer vesicles (LBV) that are inherently solvent free were formed simply by taking advantage of the propensity of anhydrous multilayer liquid crystals to delaminate and re-form into vesicles on immersion in water, as mentioned above. To encourage formation of isolated single bilayer vesicles of rather large size, it was only necessary to limit carefully the ionic strength of the aqueous phase during hydration. To begin, a 50-$\mu$L drop of a solution consisting of 2 mg of lipid (doped with fluorophore at about $10^{-4}$ molecular fraction) per mL of chloroform–methanol solvent mixture (2:1) was spread on a clean glass slide, and the solvent was allowed to evaporate in a stream of dry nitrogen. (If necessary, the lipid solution was extracted with water or the powdered lipid was simply washed with deionized water to remove residual small ions.) After complete solvent evaporation, 0.1 mL of doubly deionized distilled water was flowed gently onto the lipid deposit at a temperature above the phase-transition temperature of the hydrated liquid crystal. Within a few hours some of the multilayer delaminated and re-formed into large lipid vesicles, some as large as 80-$\mu$m diameter, provided the ionic strength was low enough. Most of the vesicles were isolated bilayers, although sometimes smaller vesicles were enclosed in some of the larger; some multilayer vesicles were usually present. Frequently, nonspherical shapes appeared. To collect the vesicles, a 50-$\mu$m microslide consisting of a flattened capillary tube was inserted into the water droplet so that some of the vesicles and water were drawn into the microslide by capillary forces. Samples sealed into microslides with paraffin could be preserved for many months; sometimes substantial growth of the smaller vesicles seemed (conveniently) to occur. Vesicles were easily observed by fluorescence microscopy but could also be seen by transmission phase-contrast microscopy at 100 to 400 times magnification. The vesicle wall thickness, i.e., its number of bilayers, could be ascertained by measurements of the fluorescence. Since the concentration of fluorophore relative to lipid was known, the fluorescence intensity excited in a known illuminated area yielded the thickness via suitable calibration or comparison with the fluorescence of BLMs. More directly, the variance of FCS correlation spectra measurements was used to obtain directly the fluorophore concentration per unit membrane area and thus the membrane bilayer multiplicity independent of other calibrations. Many vesicles, particularly those that happened to be nonspherical, were seen to survive repeated passages through the phase-transformation temperature of the lipids.

*Fluorophore Incorporation.* The fluorophores diI and NBD-C12 were incorporated in the BLMs by dissolving appropriate amounts in suspensions or solutions with the lipid. The molar ratio of fluorophore to lipid in the suspensions and the ratio in the final membranes and films agreed within the experimental uncertainty of the fluorescence intensity assays.

The other fluorophore, M-540, was added to preformed membranes by dispersing it in the surrounding buffer at 0.1 $\mu$g/mL to introduce about 0.1 mol % M-540 in the membrane. Only dil was used in diffusion measurements. It was excited by the 520.8-nm line of a krypton ion laser. The fluorescence intensity of M-540 in the GMO BLMs and NBD-C12 in phospholipid BLMs was measured as a function of temperature in order to attempt a convenient indication of the occurrence of anticipated phase transitions. For this purpose, the fluorescence intensity was recorded as the temperature was varied using the photomultiplier, which was also used to record data for FCS and FPR measurements. The M-540 fluorescence was excited by the 568-nm line of the krypton laser and the NBD-C12 by the 476-nm line. The molar concentration ratio of dil and NBD-C12 to lipid was usually $\sim 5 \times 10^{-4}$, corresponding to $\sim 10^3$ dil molecules per $\mu m^2$ of bilayer. The volume distribution of all of the fluorophores within the membranes and films appeared uniform within the resolution of optical microscopy. The solubility of dil in water was too small for detectability by fluorescence techniques.

*Diffusion Measurements.* To determine the lateral-diffusion coefficients of dil in the lipid films, we employed two techniques, fluorescence correlation spectroscopy (FCS) (Magde et al., 1972, 1974; Elson and Magde, 1974; Koppel et al., 1974) and fluorescence photobleaching recovery (FPR) (Axelrod et al., 1976). The apparatus described by Koppel et al. (1976) was used. The choice of method in the present research was dictated by the anticipated values of characteristic diffusion times. The FCS method was usually selected for fast diffusion) thus, for most of the BLM measurements and for large vesicles above the transition temperature. For all of the multilamellar films, and for large vesicles both above and always below $T_c$, FPR was used. Both methods are based on measurements of the dynamics of changes of fluorescence intensity due to changes in the number of fluorophores within a small illuminated area of the film. The fluorescence of dil was excited by an attenuated 520.8-nm krypton laser line ($\sim 10^{-5}$ W), focused on the film through the vertical illuminator of a fluorescence microscope to a spot of Gaussian intensity profile $I(r) = I_0 \exp(-2r^2/w^2)$, where usually $w = 1.1$ $\mu$m. The excited fluorescence was collected through the microscope optics and suitable filters and measured by photon counting with a sensitive photomultiplier, and the signal was processed as required for either the FCS or the FPR method described below.

*Fluorescence Correlation Spectroscopy.* In the FCS method the temporal correlations of the stochastic fluctuations of the fluorescence intensity due to random diffusion of the fluorophore in and out of the illuminated spot are analyzed to determine the diffusion coefficient of the fluorophore. The characteristic time scale $\tau_c$ of the fluctuations is just the characteristic time for diffusion through the beam. Assuming a constant diffusion coefficient $D$ for macroscopic transport of the fluorophore in two dimensions, fluctuations of the fluorophore concentration ($\delta c$) evolve according to the diffusion equation $\partial(\delta c)/\partial t = D\nabla^2\delta c$. In this case the normalized photocount autocorrelation function $g(\tau)$ is

$$g(\tau) = [\langle n(t)n(t+\tau)\rangle - \langle n(t)\rangle^2]/\langle n(\tau)\rangle^2 = [\langle N\rangle(1 + \tau/\tau_c)]^{-1}$$

where $n(t)$ is the number of photocounts in an interval at time $t$; the angular brackets signify long time average; $\langle N\rangle$ is the effective average number of independent fluorophore molecules within the beam. The characteristic correlation time for diffusion in two dimensions over a distance $w$ is $\tau_c = w^2/4D$. A linear fit of $[g(\tau)]^{-1}$ vs. $\tau$ yields $\langle N\rangle$, the intercept of the fit, and $\langle N\rangle/\tau_c$, the slope; thus, the correlation time $\tau_c$ and the diffusion coefficient $D$ are determined. In the experiments on BLMs and LBVs the ratio of dil to lipid in the forming solution was $5 \times 10^{-4}$, which corresponds to $\langle N\rangle \approx 2 \times 10^3$. The FCS experiments also yielded $\langle N\rangle \approx 2 \times 10^3$, indicating that dil diffused in the membrane as individual independent molecules.

*Fluorescence Photobleaching Recovery.* In the FPR method the fluorophore diffusion is determined by analysis of the transient recovery of the fluorescence by diffusion of fresh fluorophore from the surrounding film into the illuminated spot following irreversible photobleaching of some of the fluorophore in this spot with a brief intense pulse of the laser beam. The recovery transient is monitored with the laser beam attenuated by several orders of magnitude to prevent further bleaching. The time course of the fluorescence recovery is analyzed to determine a characteristic diffusive recovery time $\tau_{1/2} = \gamma_D w^2/4D$ and thus to determine the diffusion coefficient $D$ using $\gamma_D = 1.05$ and applying the methods of Axelrod et al. (1976). Values of $w$ for these experiments were either 1.2, 2.8, 3.4, 4.0, or 5.1 $\mu$m.

*Controls and Limitations.* Because the bleaching time can become a significant fraction of the recovery time for fast diffusion, $D \gtrsim 10^{-8}$ cm$^2$, noticeable recovery can occur during bleach and before the observations of the recovery process begin. Although comparisons of FCS and FPR experiments in this range have established their compatibility, the values measured by the FPR measurements for $D \gtrsim 10^{-8}$ may be underestimated by about 50%. Completeness of fluorescence recovery in the FPR experiments showed that all of the dil molecules were mobile.

To control the temperature rise due to absorption of the laser power in both FCS and FPR methods, the laser intensity and fluorophore concentration were limited. The power absorbed per bilayer was $<10^{-12}$ W during intensity measurements and $<10^{-8}$ W during the 0.5-s photobleach of FPR experiments. Axelrod (1977) has analyzed this problem theoretically. Although the heating rate during photobleaching is enormous, thermal conduction to the surroundings distributes the absorbed heat so rapidly that the films quickly ($\tau < 10^{-5}$ s) approach an asymptotically limited temperature rise of <0.1 °C, even in the least-favorable case occurring during the 0.5-s photobleach of some of the multilamellar liquid-crystal films. The temperature rise during the diffusion measurements themselves is $\lesssim 10^{-6}$ °C. Control measurements at higher fluorophore concentrations chosen to increase heat absorption about ten times yielded indistinguishable results.

Convective flow processes which might be anticipated in BLMs were excluded by several observations. First, the dependences of the characteristic diffusion times on laser beam size $w$ were measured and found to vary as $w^2$, as expected of diffusion and not as $w$ as expected of flow (Koppel et al., 1976; Fahey et al., 1977). Second, the shape of the FCS correlation functions was observed to be consistent with diffusion and not with flow. Convective flow at a velocity $v$ introduces a factor $\exp[-(v/w)^2\tau^2/(1 + \tau/\tau_c)]$ in the FCS correlation function. Thus, linearity of the on-line computer plot of the measured $[g(\tau)]^{-1}$ vs. $\tau$ through the range $0 > \tau \gtrsim 5\tau_c$ excludes flow effects on the determinations of $\tau_c$ to at least the stated experimental uncertainties. In one case only, DPPC at the highest temperatures, the experimental scatter due to loose liposomes in the aqueous phase was too large to allow this control procedure. Third, direct observation of particle motion on the membrane was made possible by the presence of occasional visible dust particles and of solvent microlenses during a brief period after membrane formation. Except during deliberate

perturbations of the membrane, the observed flow velocities did not exceed 0.02 $\mu$m/s, a rate that is too small to disturb the reported diffusion measurements.

The experiments also exclude contributions of solvent microlenses to the measured diffusion coefficients. Since the fluorophore partitions uniformly between the BLM and the lipid suspension, in solvent microlenses appear as bright fluorescent spots. If present, their Brownian motion could contribute significantly to the FCS correlation function. If so, however, their presence is signaled by excessively large values of variance of the correlation function due to the reduced number of independent mobile objects $\langle n \rangle$, for dil in BLMs indicated that it diffused as an ensemble of essentially independent molecules. This experiment does not exclude the presence of uniformly distributed solvent in BLMs; it excludes only structures in which the area density of dil molecules is locally enhanced.

## Results

These experiments were designed to determine the effects of the thermotropic phase transitions on lateral diffusion in phospholipid membranes and liquid crystals of several types. Included are solvent-free bilayers and multilayers forming the walls of large vesicles (LBV), multilamellar liquid crystals (MLLC), and black lipid membranes (BLM). For convenience of comparison, results are presented for two groups: (A) BLMs and (B) all of the others.

*Lateral Diffusion with Weak Temperature Dependence in BLMs.* Temperature ranges selected for these measurements of lateral diffusion were intended to extend our previous measurements on BLMs (Fahey et al., 1977) to temperatures below the anticipated phase transitions insofar as possible to maintain stable membranes. The data encompass the temperatures of the known phase transitions in hydrated multilayer liquid crystals and in sonicated bilayer vesicles. Membranes were formed by the MM technique and by the MR technique using hexadecane as solvent in order to minimize retained solvent; membranes formed from hexane as solvent by the MR technique were also studied to appraise solvent effects.

The diffusion coefficients of dil in membranes containing primarily DPPC were measured from 28 to about 48 °C in three cases: (1) MM (low solvent) membranes of DPPC; (2) MM membranes of DPPC with 7-dehydrocholesterol at DPPC/C = 4:1; and (3) MR hexane membranes of DPPC. Typical data for three cases are shown in Figure 1. The essential result is that the large diffusion coefficients $D \approx 10^{-7}$ cm$^2$/s previously observed above the expected transition temperatures are retained throughout the temperature range. Addition of 20 mol % 7-dehydrocholesterol in DPPC in MM membranes seems to increase $D$ by a barely significant 25%. Diffusion in MR membranes formed from hexane appears to be about three times faster than in MM membranes.

These results motivated an attempt to determine whether remnants of a phase transition actually occurred at 41 °C, as expected in the MM and DPPC membranes, and as reported by Stark et al. (1972) in MR membranes of DPPC at about 30 °C. Faucon and Lussan (1973) had recently observed changes in fluorescence intensity on scanning through the phase-transition temperatures in liposomes with both single and multiple bilayer structures; therefore, we looked for changes in fluorescence output at the phase transition.

We found that the fluorescence intensity of dil invariably showed insignificant temperature dependence. On substituting NBD-C12, however, changes of fluorescence intensity with temperature were detectable. Small but ambiguous breaks in slope appeared in the vicinity of the anticipated phase-transition temperatures, at 41 °C in MM membranes and at 30 °C in the MR membranes formed from hexane as shown in the top of Figure 1.

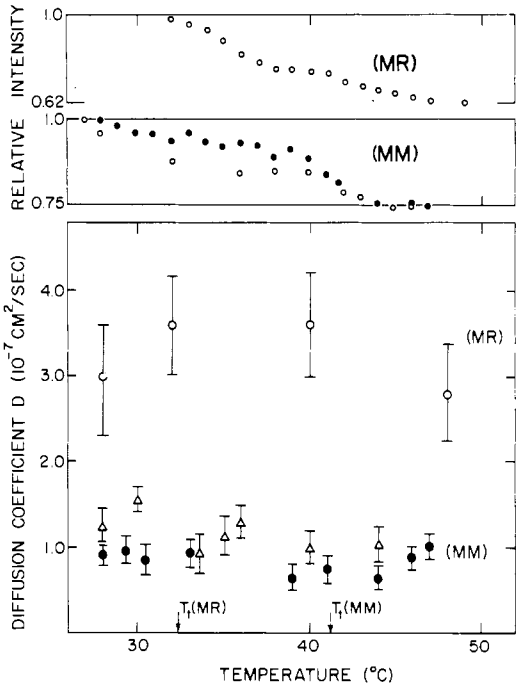


FIGURE 1: Temperature dependence of lateral diffusion of dil in BLMs of DPPC formed by the MM technique (●), the MR technique (○), and DPPC + 20 mol % cholesterol formed by the MM technique (△). Measurements by FCS. Also shown by small points are recordings of temperature dependence of the fluorescence intensity of NBD-C12 indicating changes in slope at the anticipated phase transition temperatures $T_t$ in both MM and MR membranes (see text).

The stability characteristics of the DPPC membranes also suggest that some sort of phase transition might actually occur in these BLMs. Bilayer membranes of DPPC could never be formed by the MM technique below 42 °C, although once formed they could be cooled. However, addition of 20 mol % 7-dehydrocholesterol, which is supposed to suppress the phase transition, enabled membrane formation at just above room temperature.

Using the MR technique, BLMs of DPPC could be formed from hexane above 30 °C and BLMs of DMPC were readily accomplished at room temperature. Unfortunately, DMPC membranes could not be formed at all by the MM technique or by the MR technique from hexadecane. The value of $D$(dil) in DMPC membranes formed by the MR technique remained in the range $1.4 \pm 0.3 \times 10^{-7}$ cm$^2$/s from 17 to 24 °C.

Diffusion of dil in GMO membranes was measured with relatively high precision over the relevant temperature range, as shown in Figure 2. GMO membranes could be formed readily by the MM and MR techniques from both hexane and hexadecane. Phase-transition effects at 16 °C had been well established in BLMs by measurements of specific capacitance (White, 1975) and reflectance (Pagano et al., 1973). To confirm the phase transition in our membranes, measurements of the intensity of a fluorescent probe were again used. Neither dil nor NBD:C12 show a significant temperature dependence of fluorescence intensity, but a merocyanine dye M540, that is better known as a fluorescent probe of membrane potential, did give an indication of the anticipated phase transitions. The relative fluorescence intensity of M540 in GMO is plotted at the top of Figure 2. Clear breaks in the slopes of the curves are evident at about 15 °C, close to the expected phase-transition temperatures.

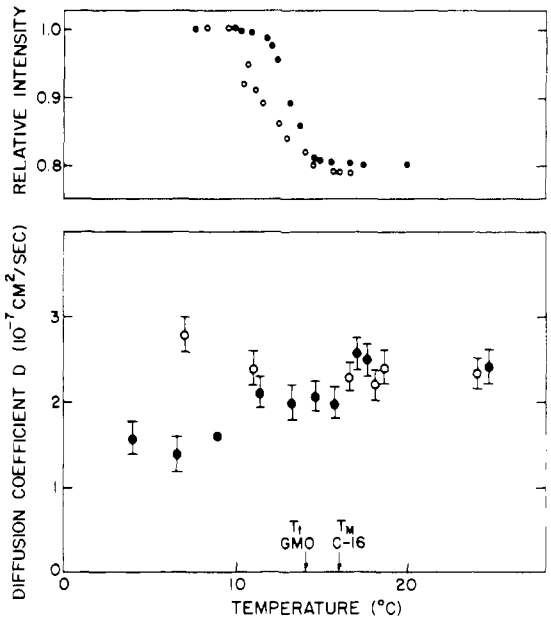


FIGURE 2: Temperature dependence of lateral diffusion of dil in BLMs of GMO formed by the MM technique (●) and the MR technique using hexadecane (○). The fluorescence intensity of M540 is also shown with a sharp change of slope observed near the anticipated phase-transition temperature $T_t$; $T_m$ is the melting point of hexadecane. Measured by FCS.

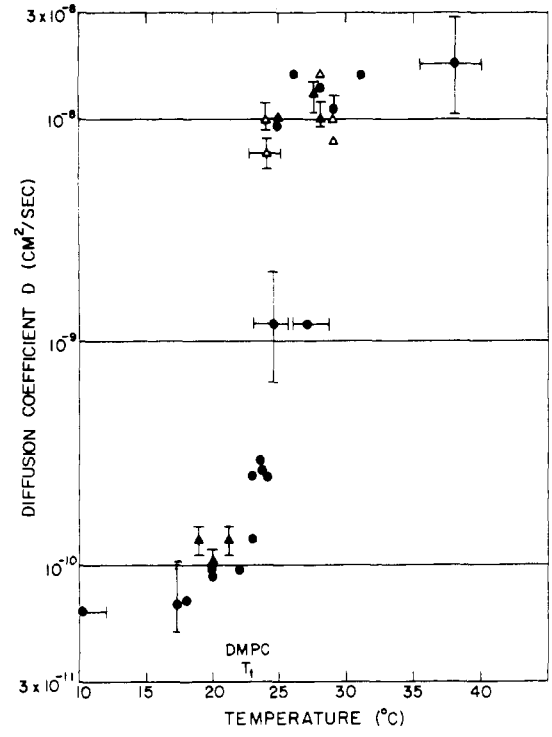


FIGURE 3: Temperature dependence of the lateral-diffusion coefficient of dil in DMPC: large bilayer vesicles, as measured by FCS (△), large bilayer vesicles, as measured by FPR (▲), MLLCs as measured by FPR (●). Typical uncertainties for various ranges of $D$ and $T$ and the two methods are indicated by error bars.

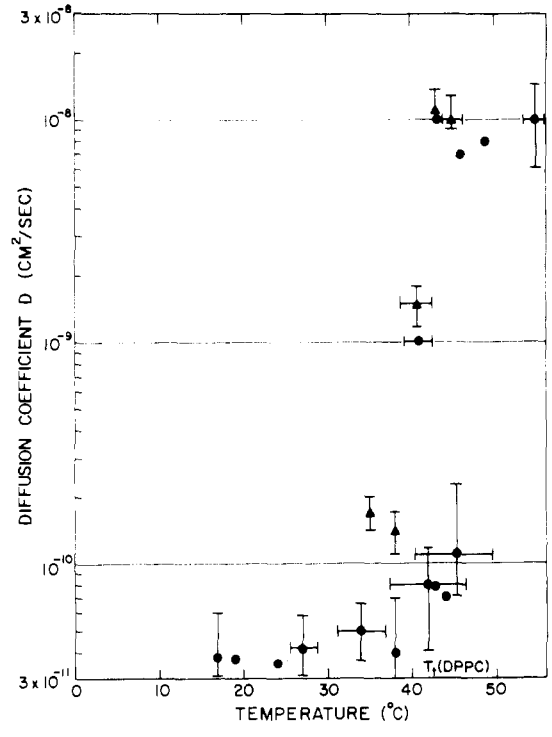


FIGURE 4: Temperature dependence of the lateral diffusion coefficient of dil in DPPC: large bilayer vesicles, as measured by FPR (▲), (b) MLLCs as measured by FPR (●). Typical uncertainties of $T$ and $D$ are indicated by error bars for the various ranges and methods of measurement.

The data on the diffusion coefficient of dil in MM membranes of GMO suggest a possible but barely perceptible drop at $T_t$ from about $2.4 \times 10^{-7}$ cm$^2$/s above to about $2.0 \times 10^{-7}$ cm$^2$/s just below. There appears to be no significant change of the diffusion coefficient in the sparse data on MR membranes of GMO prepared from hexadecane solvent. Nevertheless, the fluorescence intensity monitor suggests that something has changed around 14 °C; Pagano et al. (1972) suggest that a phase transition does occur in BLMs of GMO formed from hexadecane.

The central result of these observations on BLMs is the absence of significant changes of the diffusion coefficient on cooling through the anticipated phase-transition temperatures. Values of $D \simeq 2 \times 10^{-7}$ cm$^2$/s were again observed.

*Diffusion Changes at the Phase Transition in Solvent-Free Single-Bilayer Membranes and Multilayer Liquid Crystals.* Figure 3 displays on a logarithmic scale measurements of the diffusion coefficient $D$ of dil in hydrated MLLC and in large bilayer vesicles of DMPC as a function of temperature through the anticipated phase-transition temperature at $T_c \approx 23$ °C. A sharp change of more than 100-fold from $D \simeq 6 \times 10^{-11}$ cm$^2$/s well below $T_t$ to $D \simeq 1.5 \times 10^{-8}$ cm$^2$/s well above occurs over a range of less than 2 °C with the logarithmic center of the transition at about 24.5 °C. A phase transition at about 23.5 °C in DMPC and a precursor at about 17 °C have been well established by differential calorimetry and X-ray crystallography.

Similarly, in DPPC (Figure 4) diffusion was slow below 40 °C, where $D = 4 \times 10^{-11}$ cm$^2$/s, but it increased more than 100-fold to $D \approx 10^{-8}$ cm$^2$/s above about 43 °C. A major phase transition at about 42 °C and a precurser at about 38 °C are well established by scanning calorimetry and X-ray crystallography. The large vesicle data presented were measured on the single-bilayer vesicles described above. However, no differences in diffusion coefficients either above or below $T_t$ were detected between single bilayers and double- or triple-bilayer vesicles.

Three additional systems that were accessible only above the phase transitions showed values of $D$ comparable with those observed in DMPC and DPPC above $T_t$. In DLPC and EPC multilayers above their phase transitions, we found $D \simeq 2.0 \times 10^{-8}$ cm$^2$/s at room temperature; no decrease was noticed

on cooling to 10 °C. In large vesicles of EPC, $D \simeq 1.2 \times 10^{-8}$ cm$^2$/s at $T = 24$ °C.

The common property of all of these observations on MLLCs and LBV is slow diffusion at $D \approx 10^{-10}$ cm$^2$/s below the main liquid-crystal phase transition and faster diffusion at $D \approx 10^{-8}$ cm$^2$/s above.

## Discussion

*Lateral Diffusion in MLLC and LBVs.* That there is a marked change in the diffusion coefficient of our fluorescent lipid analogue at the phase transition of the lipids in our MLLC is expected as a consequence of the structural changes at the phase transition that have been well characterized in MLLC (Janiak et al., 1976). Below the $T_t$ there is clear evidence of at least quasi-long-range order in the plane of the bilayer with a high order of symmetry. In the smectic A phase above $T_t$ only the short-range order of a two-dimensional fluid remains. Thus, the change of diffusion coefficient at $T_t$ should parallel the large changes associated with solid–liquid transitions.

A priori, lateral diffusion coefficients in the isolated bilayers of our large vesicles could not be predicted so easily. There is no published crystallographic data on the lateral order in single, isolated bilayers. The large heats of transition associated with the main crystallographic transition in MLLC do persist in the small (200-Å diameter) sonicated vesicles used for most calorimetry but are subject to uncertainties of interpretation (Suurkuusk et al., 1976). However, most of this effect is due to the change of intramolecular order in the hydrocarbon chains and not to the intermolecular order. Therefore, it does not necessarily imply a significant change of $D$. The calorimetric peaks in sonicated bilayer vesicles are relatively diffuse, suggesting a less cooperative phase transition, but it is not clear whether this effect should be attributed to isolation of the bilayers or to their curvature.

The behaviors of the lateral-diffusion coefficients in our bilayer vesicles were indistinguishable from those in MLLCs in the range of our experiments. Furthermore, no significant differences of $D$ between single bilayer and multilayer vesicles were detected. Therefore, we may infer that there is little essential structural difference between the isolated bilayers of large vesicles and multilayers in our experiments. However, this result does not exclude the possibility that the structures do differ in the details of their long-range order. Janiak et al. (1976) have shown that the bilayers in MLLC composed of DPPC and DMPC are puckered periodically just below $T_c$. It is not yet clear to us whether some analogue of this structure persists in isolated bilayers.

Above the phase transition, the dil diffusion coefficient in both large vesicles and MLLCs is in agreement with the values obtained by Devaux and McConnell (1972) on lateral diffusion of spin probe labeled lipids in MLLCs of EPC and lecithin. Their probe was a spin-labeled lipid. These results are also in agreement with values obtained by Cullis (1976) on sonicated vesicles in 85% glycerol solution. His value is derived from the NMR spectra of $^{31}$P in the phospholipid. Above the transition temperature, he finds $D \simeq 1.5 \times 10^{-8}$ cm$^2$/s and below the transition $D \simeq 1 \times 10^{-9}$ cm$^2$/s. The value below the transition is an order of magnitude greater than the dil diffusion coefficients that we measured. Other NMR studies (Brulet and McConnell, 1975; Kroon et al., 1976; Lee et al., 1973; Bloom et al., 1975; Roeder et al., 1976) yielded values of D above the transition temperature of less than $10^{-7}$ cm$^2$/s in vesicle systems. However, the interpretation of these NMR spectra in terms of a self-diffusion coefficient for a lipid molecule remains a controversial problem.

Fluorescence techniques have also been used to estimate lipid self-diffusion coefficients in various bilayer and multilayer systems. McGrath et al. (1976) using a fluorescence energy-transfer technique in MLLC systems have obtained values of $D \simeq 2.6 \times 10^{-8}$ cm$^2$/s for DLPC at room temperature and $D \simeq 5.3 \times 10^{-8}$ cm$^2$/s for dielaiadoyl phosphatidylcholine. Razi-Naqvi et al. (1974) and Behr et al. (1974) have obtained a measure of the lateral-diffusion coefficient of anthracene-labeled DPPC in sonicated DPPC vesicles by observing the bimolecular kinetics of energy transfer between triplet states of the fluorophore. They found $D \simeq 2 \times 10^{-7}$ above $T_t$ and $D \simeq 10^{-8}$ cm$^2$/s below $T_t$. The results on sonicated vesicles resemble our BLM measurements more closely than our large vesicle and MLLC measurements. They may reflect disorder due to the small radius of curvature of sonicated vesicles.

Concurrent with our studies, Wu et al. (1977) have measured the diffusion coefficient of a lipid analogue very similar to ours, 3,3′-dioctadecyloxytricarbocyanine, in MLLCs of DPPC, DMPC, and EPC and EPC–cholesterol mixtures. Their results on MLLCs generally agree with ours and show roughly a two-order of magnitude change in $D$ at the phase transition.

There are fundamental theoretical reasons to anticipate differences between bilayers and multilayers in the degree of intermolecular order. In strictly two-dimensional geometries, molecules with interaction potentials of the Lennard–Jones type cannot develop complete, conventional long-range crystalline order in equilibrium for fundamental physical reasons (Mermin, 1968). The structural consequences of this property of two-dimensional systems have been discussed by Kosterlitz and Thouless (1973) who show that substantial topological order and the possibility of phase transitions are retained. These results are suggestive of enhanced diffusion in two-dimensional systems. It is not clear just how germane or how large these effects might be. Many recent theories of the structural phase transition in individual bilayers suggest that substantial intramolecular disorder remains below the transition temperature. The fact remains that we observed little difference between isolated bilayers and MLLCs, suggesting that coupling between the layers does not strongly affect those aspects of order that control lateral diffusion.

*Black Lipid Membranes.* Our values of D in BLMs are quite different from those on large vesicles and MLLCs. In BLMs, $D \simeq 10^{-7}$ cm$^2$/s both above and below the published "gel to liquid-crystal" phase-transition temperature. This result has been observed even in BLMs that have been thought to contain negligible alkane solvent, i.e., in membranes formed by the MM method (Montal and Mueller, 1972; White et al., 1976) and by the MR method using hexadecane as the solvent (White, 1975; Benz and Janko, 1976). BLMs generated by these methods have greater specific capacitances than Mueller–Rudin BLMs generated from the smaller alkane solvents such as hexane or octane; therefore, it is thought that there is very little solvent between the monolayers that form the bilayer. However, the amount of solvent of any molecular weight that is interdigitated amongst the lipid molecules has not been determined. We have observed that dil diffusion coefficients in BLMs generated by the MR technique with the smaller alkane solvents are about two times greater than in the MM and MR–hexadecane generated BLMs (Fahey et al., 1977).

Direct evidence about the structural changes associated with phase transitions in model membranes is available from electron-diffraction experiments using a moist stage by Hui and co-workers (Hui et al., 1974, 1975; Hui, 1976). They observed some in-plane order below the anticipated phase-transition temperatures of phospholipid BLMs formed by the MM technique using chloroform as the spreading solvent. If the

TABLE I: diI Diffusion Coefficients.

| lipid | system | $D$ (cm$^2$/s) $T > T_t$ | $D$ (cm$^2$/s) $T < T_t$ | phase transition temp ($T_t$), °C |
|---|---|---|---|---|
| DPPC | MM BLM | $0.9 \times 10^{-7}$ | $0.9 \times 10^{-7}$ | 42[a] |
| DPPC | MR (hexane) BLM | $3.2 \times 10^{-7}$ | $3.2 \times 10^{-7}$ | 32[a] |
| DPPC | MLLC | $1.0 \times 10^{-8}$ | $0.4 \times 10^{-10}$ | 42 |
| DPPC | large vesicles | $1.0 \times 10^{-8}$ | $1.4 \times 10^{-10}$ | 42 |
| DMPC | MR (hexane) BLM | $1.3 \times 10^{-7}$ | | <18 |
| DMPC | MLLC | $1.6 \times 10^{-8}$ | $1.0 \times 10^{-10}$ | 23.5 |
| DMPC | large vesicles | $1.1 \times 10^{-8}$ | $1.0 \times 10^{-10}$ | 23.5 |
| GMO | MM BLM | $2.5 \times 10^{-7}$ | $2.0 \times 10^{-7}$ | 16 |
| GMO | MR (hexadecane) BLM | $2.2 \times 10^{-7}$ | $2.2 \times 10^{-7}$ | 14[b] |
| EPC | MM BLM | $1.7 \times 10^{-7}$ | | |
| EPC | MLLC | $2.2 \times 10^{-8}$ | | −7 to −15[c] |
| EPC | large vesicles | $1.2 \times 10^{-8}$ | | |
| DLPC | MLLC | $2.6 \times 10^{-8}$ | | 0[c] |

[a] Suggested by fluorescence intensity of NBD-C12 as reported in the text and as reported by Stark et al. (1972). [b] Suggested by fluorescence intensity of M540 as reported in text and as reported by White (1975). [c] See Chapman (1975).

membranes generated by our adaptation of the standard techniques are comparable, then we are led to the conclusion that vestiges of the phase transition persist as suggested weakly by our fluorescence intensity observations. Since we have also clearly seen huge effects of the phase transition in the solvent-free large bilayer vesicles, we must conclude that the orders of magnitude enhancement of lateral diffusion in BLMs must be attributed directly to retained solvent.

Solvent lenses, i.e., local "precipitates" of solvent within the membrane, could accelerate lateral transport of diI. However, they were rarely seen even in our MR membranes because the small membrane size allowed the lenses to move rapidly to the plateau border and vanish there. The amplitudes of the correlation functions in our FCS experiments routinely assured the exclusion of moving lenses, since they showed that the number of independent diffusing objects was always equal to the number of fluorophore molecules. In the presence of lenses or clusters, the amplitude of the correlation function is enormously increased. Therefore, we discount this model of the effect and tend to prefer to suppose that interdigitated solvent molecules may be present in substantial concentrations. We think that this material would not have been detected in the experiments of Benz and Janko (1976).

*diI as a Lipid Analogue in Lateral Diffusion.* It remains to assess the validity of the lipid analogue diI in providing a measure of intrinsic features of lateral diffusion in phospholipids and in particular its suitability for approximation of the self-diffusion of the phospholipid.

How well the lateral diffusion of the fluorescent lipid analogue diI approximates the self-diffusion of the lipid depends on the accuracy with which diI mimics the lipid. The polarizability, amphipatic chemistry, shape, and size and the van der Waals figure of diI do mimic the phospholipids rather well. diI has two adjoining 18-carbon hydrocarbon chains joined by the fluorescent, polar head group. That the fluorescence dipole lies parallel with the plane of the bilayers has been shown by measurements of fluorescence polarization of the closely analogous oxycarbocyanine in phospholipid multilayers by Badley et al. (1973; see also Badley, 1976) and in BLMs by P. R. Dragsten (private communication). Thus, the only likely position of the hydrocarbon tails of diI is parallel with the hydrocarbon tails of the phospholipids within the membrane. We visualize diI molecules as substituting for phospholipids in the two-dimensional arrays forming the bilayers and, like the phospholipid, diffusing as a roughly cylindrical rod that remains immersed in, and essentially perpendicular to, the bilayer.

Various experimental observations indicate the similarity of diI diffusion to lipid self-diffusion. The time constant for transfer from one side of a membrane to the other (flip-flop) is slow and comparable with the phospholipids themselves (P. F. Dragsten, private communication). diI appears to partition into lipid membranes from solvent suspensions approximately in proportion to its concentration relative to the lipid, and it dissolves in membranes as a uniformly distributed solute. The FCS experiments showed that each diI molecule diffuses as an independent object. However, clustering of diI molecules has been noted in oxidized cholesterol membranes by Wolf et al. (1977). The possibility that diI diffuses along the water-lipid interface by hopping into the water phase and back is excluded by its extremely small solubility in water—too small for detectable fluorescence—compared with its ready solubility in lipid. Simple theoretical estimates indicate that the diffusion coefficients associated with this rate would be negligible in this case. Fahey et al. (1977) found that dipalmitoylphosphatidylethanolamine labeled at the polar head group with rhodamine diffuses in BLMs with a diffusion coefficient near to that of diI, actually about 50% faster. Wu et al. (1977) have obtained comparable diffusion results in multilayer liquid crystals of DMPC with NBD-PE and an oxycarbocyanine dye that is quite similar to diI.

It may be germane to mention the reasons we have not attempted to deduce a membrane viscosity from our diffusion measurements. The usual Stokes-Einstein relations for three-dimensional diffusion are not applicable. An approximate hydrodynamic theory for two-dimensional diffusion of rigid rods that extend through the membrane has been worked out by Saffman and Delbruch (1975) and Saffman (1976) but is also not precisely applicable to our experiments because our lipid probe molecule only penetrates halfway through the membrane.

## Conclusion

Our data are summarized in Table I. The self-diffusion coefficient of the fluorescent lipid analogue, diI, is about $10^{-10}$ cm$^2$/s below the gel to liquid-crystal phase-transition temperature in both single bilayers and in multilamellar liquid crystals of DPPC and DMPC. At the phase transition temperature, the diffusion coefficient increases abruptly (within two degrees) to $D \simeq 10^{-8}$ cm$^2$/s. In BLMs formed from al-

kane solvents using the standard Mueller-Rudin and Montal Mueller techniques, the diffusion coefficient is about $10^{-7}$ $cm^2/s$ whether above or below the published phase-transition temperature. For comparison, the diffusion coefficient of dil in the plasma membrane of a variety of mammalian cells in culture at room temperature is about $10^{-8}$ $cm^2/s$ (Schlessinger et al., 1976; 1977; Axelrod et al., 1977; see summary by Webb, 1977).

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References

Axelrod, D. (1977), *Biophys. J. 18,* 129.

Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E. L., and Webb, W. W. (1976), *Biophys. J. 16,* 1055.

Axelrod, D., Ravdin, P., Koppel, D. E., Schlessinger, J., Webb, W. W., Elson, E. L., and Podleski, T. R. (1977), *Proc. Natl. Acad. Sci. U.S.A. 73,* 4594.

Badley, R. A. (1976), in Modern Fluorescence Technology, Wehry, E. C., Ed., New York, N.Y., Plenum Press.

Badley, R. A., Martin, W. G., and Schneider, H. (1973), *Biochemistry 12,* 268.

Behr, J. P., Chapman, D., and Razi-Naqvi (1974), *Biochem. Soc. Trans. 2,* 960.

Benz, R., and Janko, K. (1976), *Biochim. Biophys. Acta 455,* 721.

Berry, R., Frolich, O., Lauger, P., and Montal, M. (1975), *Biochim. Biophys. Acta 394,* 323.

Blok, M. C., van Deenen, L. L. M., and DeGier, J. (1976), *Biochim. Biophys. Acta 433,* 1.

Bloom, M., Burnell, E. E., Volic, M. I., and Weeks, G. (1975), *Chem. Phys. Lipids 14,* 107.

Brulet, P., and McConnell, H. M. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1451.

Chapman, D. (1975), *Q. Rev. Biophys. 8,* 185.

Cullis, P. R. (1976), *FEBS Lett. 70,* 223.

Devaux, P., and McConnell, H. M. (1972) *J. Am. Chem. Soc. 94,* 4475.

Edidin, M. (1974), *Annu. Rev. Biophys. Bioeng. 3,* 179.

Elson, E. L., and Magde, D. (1974), *Biopolymers 13,* 1.

Fahey, P. F., Koppel, D. E., Barak, L. S., Wolf, D. E., Elson, E. L., and Webb, W. W. (1977) *Science 195,* 305.

Faucon, J. F., and Lussan, C. (1973), *Biochim. Biophys. Acta 307,* 459.

Hui, S. W. (1976), *Chem. Phys. Lipids 16,* 9.

Hui, S. W., Cowden, M., Papahadjopoulos, D., and Parsons, D. F. (1975), *Biochim. Biophys. Acta 382,* 265.

Hui, S. W., Parsons, D. F., and Cowden M., (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 5068.

Jacobson, K., and Papahadjopoulos, D. (1976), *Biophys. J. 16,* 549.

Jacobson, K., Wu, E. S., and Poste, G. (1976), *Biochim. Biophys. Acta 433,* 315.

Janiak, M. J., Small, D. M., and Shipley, G. G. (1976), *Biochemistry 15,* 4575.

Jost, P., Libertini, L. H., Hebert, V. C., and Griffith, O. H. (1971), *J. Mol. Biol. 59,* 77.

Koppel, D. E. (1974) *Phys. Rev. A 10,* 1938.

Koppel, D. E., Axelrod, D., Schlessinger, J., Elson, E. L., and Webb, W. W. (1976), *Biophys. J. 16,* 1315.

Kosterlitz, J. M., and Thouless, D. J. (1973), *J. Phys. 6,* 1181.

Kroon, P. A., Kainosho, M., and Chan, S. T. (1976), *Biochim. Biophys. Acta 433,* 282.

Lee, A. G., Birdsall, N. J. M., and Metcalf, J. C. (1973), *Biochemistry 12,* 1650.

Liebman, P., and Entine, G. (1974), *Science 185,* 457.

Magde, D., Elson, E. L., and Webb, W. W. (1972), *Phys. Rev. Lett. 29,* 705.

Magde, D., Elson, E. L., and Webb, W. W. (1974), *Biopolymers 13,* 29.

McGrath, A. E., Morgan, C. G., and Radda, G. K. (1976), *Biochim. Biophys. Acta 426,* 173.

Melchior, D. L., and Steim, J. M. (1976), *Annu. Rev. Biophys. Bioeng. 5,* 205.

Mermin, N. D. (1968), *Phys. Rev. 176,* 250.

Montal, M., and Mueller, P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3561.

Mueller, P., Rudin, D. O., Tien, H. T., and Westcott, W. C. (1962), *Nature (London) 194,* 979,

Pagano, R. E., Cherry, R. J., and Chapman, D. (1972), *Science 181,* 557.

Peters, R., Peters, J., Tews, L., and Bahr, W. (1974), *Biochim. Biophys. Acta 367,* 282.

Poo, M. M., and Cone, R. (1974), *Nature (London) 247,* 438.

Razi-Naqvi, K., Behr, J. P., and Chapman, D. (1974), *Chem. Phys. Lett. 26,* 440.

Reeves, J. P., and Dowben, R. M. (1969), *J. Cell Physiol. 73,* 49.

Roeder, S. B. W., Burnell, E. E., Kuo, A. L., and Wade, C. G. (1976), *J. Chem. Phys. 64,* 1848.

Saffman, P. G. (1976), *J. Fluid Mech. 73,* 593.

Saffman, P. G., and Delbruck, M. (1976), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3111.

Schlessinger, J., Axelrod, D., Koppel, D. E., Webb, W. W., and Elson, E. L. (1977), *Science 195,* 307.

Schlessinger, J., Koppel, D. E., Axelrod, D., Jacobson, K., Webb, W. W., and Elson, E. L. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 2409.

Schlessinger, J., Metzger, H., Webb, W. W., and Elson, E. L. (1976), *Nature (London) 264,* 550.

Stark, G., Benz, R., Pohl, G. W., and Janko, K. (1972), *Biochim. Biophys. Acta 266,* 603.

Suurkuusk, J., Lentz, B. R., Barenholtz, Y., Biltanen, R. L., and Thompson, T. E. (1976), *Biochemistry 15,* 1393.

Webb, W. W. (1977), in Electrical Phenomena at the Biological Membrane Level, Amsterdam, Elsevier Scientific.

White, S. H. (1975), *Biophys. J. 15,* 95.

White, S. H., Peterson, D. C., Simon, S., and Yafuso, M. (1976), *Biophys. J. 16,* 481.

Wolf, D. E., Schlessinger, J., Elson, E. L., Webb, W. W. Blumenthal, R., and Henkart, P. (1977), *Biochemistry 16,* 3476-3483.

Wu, E. S., Jacobson, K., and Papahadjopoulos, D. (1977), *Biochemistry 16,* 3936.