Lateral Diffusion in Phospholipid Multibilayers Measured by Fluorescence Recovery after Photobleaching†

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ABSTRACT: The method of fluorescence recovery after photobleaching has been used to measure the temperature dependence of the lateral diffusion coefficients (D) of two fluorescent lipid analogues in phospholipid multibilayers of various compositions. The probes employed were 3,3′-dioctadecyloxocarbocyanine (diO-C_{18}(3)) and *N*-4-nitrobenz-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE). In fluid egg phosphatidylcholine multibilayers at 25 °C, D was about 4×10^{-8} cm^2/s for NBD-PE and 1.5×10^{-7} cm^2/s for diO-C_{18}(3) and was moderately temperature dependent (2-fold change over 10 °C). Equimolar cholesterol reduced D for NBD-PE in these multibilayers by a factor of 2. A greater than 100-fold decrease in D was detected in dimyristoylphosphatidylcholine multibilayers at approximately 23 °C, which coincides with the gel-to-liquid-crystalline transition temperature, T_m ($D \sim 5 \times 10^{-8}$ cm^2/s at $T > T_m$ to $D < 5 \times 10^{-10}$ cm^2/s at $T < T_m$). Equimolar cholesterol abolished this transition behavior, raising D below T_m and decreasing D above T_m. These results confirm and extend previous studies of lateral diffusion employing magnetic resonance and other optical techniques and give additional confidence in the fluorescence method.

Measurements of lateral diffusion of lipids in model and natural membranes have been made by magnetic resonance methods (for review, see Edidin, 1974). The purpose of this study was to measure the diffusion of fluorescent lipid analogues in multibilayers by fluorescence recovery after photobleaching[1] (FRAP). This method has been described in detail recently (Jacobson et al., 1976, 1977; Schlessinger et al., 1976; Koppel et al., 1976; Edidin et al., 1976) and is conceptually similar to earlier measurements of protein diffusion in red cell ghosts (Peters et al., 1974) and rhodopsin diffusion in rod outer segment membranes (Poo and Cone, 1974; Liebman and Entine, 1974). Successful measurement of lipid lateral diffusion in well-characterized lipid bilayer systems below and above their phase transition temperature (T_m) would support the usefulness of the FRAP methodology. In this report, we present evidence that the lateral diffusion of two different lipid analogues can be measured by the FRAP method in lipid multibilayers of various compositions. The measured diffusion coefficients are in accord with our qualitative expectations based on previous physical studies of bilayer membranes and in reasonable quantitative agreement with comparable previous measurements of lateral diffusion.

Materials and Methods

Preparation of Lipids. The phospholipids used in this study were isolated or synthesized in this laboratory and contained no detectable impurities as determined by thin-layer chromatography on silica gel H and a solvent of chloroform–methanol–7 M ammonia (230:90:15, v/v/v). Dipalmitoylphosphatidylcholine (1,2-dihexadecyl-*sn*-glycero-3-phosphorylcholine; DPPC) and dimyristoylphosphatidylcholine (1,2-ditetradecyl-*sn*-glycero-3-phosphorylcholine; DMPC) were synthesized according to Robles and Van Den Berg (1969) and purified on a silicic acid column. The analysis of fatty acid esters indicated more than 99% palmitic acid and myristic acid for DPPC and DMPC, respectively. Phosphatidylcholine (EPC) was isolated and was extracted from egg yolk as described (Papahadjopoulos and Miller, 1967). The phospholipids were stored at −50 °C as solutions in chloroform (10–20 μmol/mL) in ampoules sealed under nitrogen.

Other Chemicals and Fluorescent Probes. Palmitic acid and myristic acid (both puriss) used for the synthesis of the phospholipids were obtained from Fluka, Switzerland (puriss, >99%). Cholesterol was recrystallized twice from methanol before use. Silic acid was from Mallinckrodt (A-R, 100 mesh). All solvents and chemicals were reagent grade. Water was twice distilled, the second time in an all-glass apparatus. 3,3-Dioctadecyloxacarbocyanine (diO-C_{18}(3)) was a generous gift of Dr. A. S. Waggoner. *N*-4-Nitrobenz-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE) was purchased from Avanti Biochemicals (Birmingham, Ala.) and migrated as a single fluorescent spot using thin-layer chromatography on silica gel H with a solvent of $CHCl_3$–CH_3OH–NH_4OH (10:5:1, v/v/v). According to the supplier, NBD-PE was derived from EPC.

Preparation of Lipid Multibilayers and Liposomes. Hydrated phospholipids, when pressed between two flat surfaces, form highly ordered multibilayers with the bilayer planes parallel to the flat surfaces (Levine and Wilkins, 1971; Devaux and McConnell, 1972; Badley et al., 1973). In the text, we have referred to these structures as multibilayers, bilayers, or simply membranes; however, it must be borne in mind that the physical properties of multibilayers may not be identical with either the planar lipid bilayer or unilamellar lipid vesicles. About 100 μL of concentrated lipid and dye mixture (lipid: 30 μmol/mL; dye/lipid < 1/1000) was deposited on a cleaned glass slide and was then pumped under vacuum for 15 to 30 min to remove the

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[1] Abbreviations used are: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; EPC, egg phosphatidylcholine; diO-C_{18}(3), 3,3′-dioctadecyloxacarbocyanine; NBD-PE, *N*-4-nitrobenz-2-oxa-1,3-diazole phosphatidylethanolamine; diI-C_{18}(3), 3,3′-dioctadecylindocarbocyanine; T_m, gel to liquid crystalline phase transition temperature; FRAP, fluorescence recovery after photobleaching; %R, percent recovery; $\tau_{1/2}$, half-time for recovery; w_S, laser spot radius at specimen plane; D, diffusion coefficient.

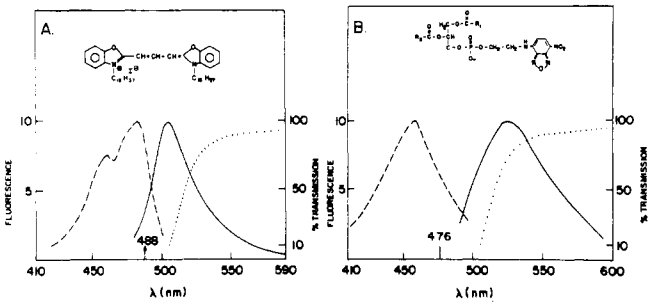

FIGURE 1: Chemical structures and excitation (- - -) and emission (—) spectra for diO-C_{18}(3) (panel A) and NBD-PE (panel B) when embedded in sonicated EPC vesicles. Transmission curve (dotted line; right ordinate) for cutoff filter K530 also is indicated along with laser lines used for excitation. Spectra were measured using an Aminco-Bowman spectrophotofluorimeter using a 12-nm excitation and emission bandpass (1-mm slits). For excitation spectra, emission monochromator was set at 550 and 525 nm for diO-C_{18}(3) and NBD-PE, respectively. For emission spectra, excitation monochromator was set at 450 and 430 nm for diO-C_{18}(3) and NBD-PE, respectively. Scattering contribution from unlabeled EPC suspensions was negligible.

residual solvents. The vacuum dried lipid film was hydrated by placing it in water saturated nitrogen gas for at least 48 h or by immersion (Badley et al., 1973) in phosphate buffered saline at pH 7.2 at 25 or 37 °C for 5 min with equivalent results for diO-C_{18}(3) labeled bilayers. All NBD-PE labeled bilayers and diO-C_{18}(3) DPPC bilayers were hydrated by immersion in buffer. In the case of buffer immersion, care was taken to remove the excess water on the slide to prevent formation of water bubbles and small lipid vesicles. A thin microscope coverglass was then placed on top of the lipid film and the slide-lipid-coverglass assembly pressed between two optical flats by a pressure of 75 psi for 15 to 30 s. The pressed sample was sealed with paraffin to prevent dehydration. After each measurement, the sealed sample was stored in a chamber containing water-saturated nitrogen. With this precaution, the sample was found to yield consistent and reproducible results for at least 1 week. EPC liposomes and sonicated vesicles were prepared as described before (Jacobson and Papahadjopoulos, 1975).

FRAP Measurements. Our instrument for measuring FRAP kinetics has been described in detail previously (Jacobson et al., 1977). The argon laser excited fluorescence microscope is similar to a filter fluorometer except that no excitation filter is required since the laser provides extremely narrow excitation lines. The uncorrected fluorescence excitation and emission spectra for both diO-C_{18}(3) and NBD-PE in sonicated EPC vesicles are shown in Figure 1A,B along with the structure of each probe. The laser line employed for excitation and the cutoff filter used to isolate the fluorescence are also shown. The TK510 dichroic beam splitting mirror was used in the Ploem illumination system (Lietz, Rockleigh, N.J.).

For these measurements, a low power objective was used (×10 or ×12) to provide a spot size of $2w_s$ of about 8 or 16 μm for NBD-PE and diO-C_{18}(3) measurements, respectively. Details of the spot radius (w_s) calculation have been described earlier (Jacobson et al., 1977). The calculation was checked by centering a solar cell at a known distance (z) below the laser waist formed at the specimen plane by the objective such that the expanding beam below the waist was completely intercepted by the solar cell. An iris diaphragm placed on top of the solar cell was closed to a radius (w_z) which reduced the detector signal to 0.865 of its full value and the iris diaphragm diameter measured. If the beam is assumed to have a Gaussian intensity profile, w_z is the $1/e^2$ radius of the beam at position z and is related to the radius (w_s) of the beam at its waist by

$$w_z^2 = w_s^2[1 + (\lambda z/\pi w_s^2)^2] \quad (1)$$

where λ is the laser wavelength. Agreement of the calculated radius and that arrived at by measurement of w_z and application of eq 1 was excellent. The laser was maintained at low excitation to ensure the output beam was in a single mode (TEM_{00}) with an approximately Gaussian intensity profile.[2] The short recovery times for lipid diffusion require faster amplifiers than are used for conventional steady-state photometry. For diO-C_{18}(3) FRAP measurements, we used a preamplifier (SLM Instruments, Champaign, Ill.) with a risetime (10 to 90%) of 200 ms; for NBD-PE diffusion an amplifier having a risetime (10 to 90%) of 10 ms was employed (ICElectronics, Amherst, N.Y.). When the recovery was distorted by the recorder response, a storage oscilloscope (Model 5115, Tektronix, Beaverton, Oreg.) was used. Bleaching times (T_B) and powers (I_B) are given in the captions to the figures. Bleaching power was measured by placing the thermal disc detector of a power meter (Model 210, Coherent Radiation, Palo Alto, Calif.) beneath the objective such that all the bleaching radiation was intercepted. It is desirable to keep $T_B < 0.10\tau_{1/2}$ in order to approximate the initial condition assumed in calculating D (Axelrod et al., 1976).

From a diffusion-limited fluorescence recovery curve, an estimation of the diffusion constant, D, can be obtained by the following relation (Axelrod et al., 1976):

$$D = (w_s^2/4\tau_{1/2})\gamma_D \quad (2)$$

where w_s is the laser beam radius at the $1/e^2$ intensity point, $\tau_{1/2}$ is the time required for the fluorescence to recover to half of its final intensity, and γ_D is a parameter determined by the initial extent of bleaching and had a value between 1.0 and 1.3 for our measurements. D was determined using eq 2 and subsequently used as the initial guess for a computer iteration program which provided a least-squares comparison of the measured curve with the theoretical curve computed from eq 12 of Axelrod et al. (1976). In most cases this roughly estimated D was well within 20% of the D value obtained by the elaborate least-squares data fitting process and also within the fluctuation range of D values measured among different regions of a sample and among samples prepared under different conditions. Therefore, D was routinely computed using eq 2.

We estimate uncertainties of ±15% in $\tau_{1/2}$ (for $\tau_{1/2} \gtrsim 500$ ms) and ±6% in w_S.[2] This leads to a propagated uncertainty in an individual determination of D from eq 2 of about ±20%; γ_D in eq 2 is not a sensitive function of K, the extent of photo-

[2] Deviations from an ideal Gaussian beam profile constitute a source of systematic error in FRAP measurements. For earlier studies (Jacobson et al., 1976), we measured the intensity of the laser beam as a function of r, the distance from the optical axis, in a plane normal to that axis at a known distance, z, below the laser waist formed at the specimen plane by the objective. If such data are plotted as log intensity vs. r^2, non-Gaussian behavior will be indicated by deviations from a straight line, with the slope of the straight line portion being $2/w_z^2$. Near the optical axis, the data could all be fitted by a straight line for all objectives. However, further away from the spot center, the plots exhibited upward curvature with these deviations being most pronounced for the higher power objectives (×100). Fortunately, for the low power objectives (×10, ×12), these non-Gaussian deviations did not appear significant. For an intermediate power objective (×32), we found that all of the log intensity vs. r^2 data, with the exception of very low intensities at large r, could be enveloped by straight lines whose differences in slope gave rise to an uncertainty in w_s of ±6%. We feel safe in quoting this uncertainty since we used even lower power objectives in this study.

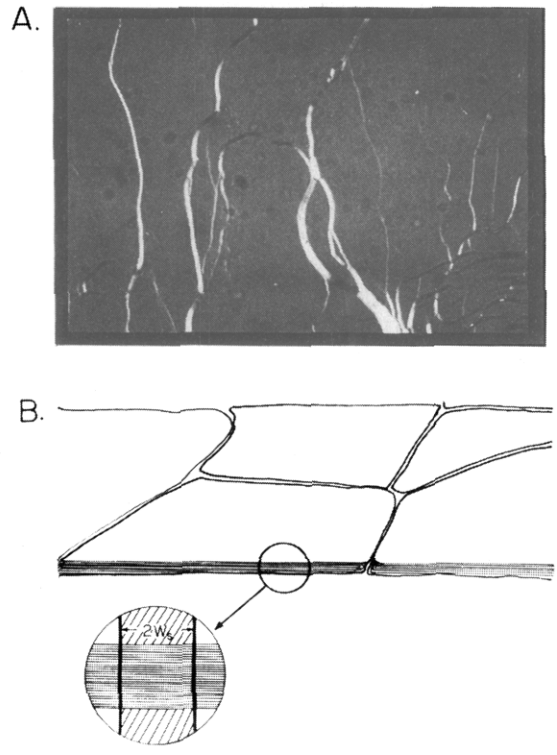

FIGURE 2: (A) Micrograph of DMPC multibilayer at 33 °C viewed under crossed polarizers and showing typical "block-like" domains. Scale: 1 cm = 50 μm. (B) Cross-sectional view of hypothesized structure for multibilayer specimen (see text). Circled detail depicts the larger number of parallel lamellae comprising the specimen and indicates the approximate relationship of the beam diameter ($2w_s$) to the total thickness of the multibilayer sample.

bleaching, for $K < 3$ (Axelrod et al., 1976) so its contribution to the error estimate is not important. A serious source of systematic error, particularly when smaller spot sizes are employed, is the failure to record the earliest portion of the recovery curve because of various instrumental delays. This effect results in K being underestimated (hence, a smaller γ_D) and $\tau_{1/2}$ being overestimated; both of these systematic errors will cause a smaller D to be calculated from eq 2. In the case of our NBD-PE data, an instrumental delay time of approximately 100 ms was corrected for by linearly extrapolating the earliest portions of the recovery curve back through zero time. This is justifiable provided the extrapolation is based on times not exceeding 0.25 to $0.5\tau_{1/2}$, depending on the K employed (Axelrod et al., 1976; Figure 2).

To obtain the temperature dependence of the lateral diffusion coefficient, a variable temperature stage (Leitz, Inc., Rockleigh, N.J.) was controlled by a circulating water temperature bath (Lauda K2/R) to ±0.1 °C. The stage was modified to provide better thermal geometry near its center. Sample temperature was monitored by a thermometer built into the stage or, in the case of NBD-PE measurements, by placing a surface thermocouple probe for a digital thermometer (Model BAT-8 with surface probe SST-1, Bailey Instruments, Saddle Brook, N.J.) directly on the coverglass. Before the FRAP measurement was performed, the sample was held at the desired temperature for at least 5 min to ensure thermal equilibrium.

Results and Discussion

Microscopic Examination of the Lipid Multibilayer Samples. Figure 2A shows a typical low power micrograph of a DMPC-multibilayer sample at 33 °C prepared as described above and obtained using a polarizing microscope. These images were also obtained with EPC, DMPC–cholesterol (1:1), and EPC–cholesterol (1:1) specimens. Typically, a granular or "block" structure was obtained with the "grains" ranging in size from 10 to 1000 μm. Larger "grains" were obtained when visible droplets of buffer were removed from the region surrounding the lipid deposit prior to the pressing process. Thus, it is likely that a large excess of hydrating buffer causes the formation of smaller vesicles in the specimen. Similar structures were observed on DPPC membranes except that the size of the flat blocks was substantially smaller than those of EPC and DMPC membranes. In the phase transition region, the boundaries on DMPC and DPPC membranes were very bright apparently due to greater birefringence of the boundary regions near T_m. Interference microscopy showed that the various multibilayer specimens were about 5 to 10 μm thick corresponding to approximately 1000 stacked bilayers.

In the flat, nonboundary regions uniform green fluorescence was observed in fluid membranes. With NBD-PE in particular, the boundary regions appeared brighter than the flat regions. Below that T_m of DMPC multibilayers labeled with diO-C_{18}(3), occasionally green clusters were observed in the fluorescent image of the specimen. Raising the temperature reduced both the size and the number of these patches and above the T_m they disappeared completely. The phenomenon was reversible; on cooling below T_m, the patches usually reappeared within a few minutes. These observations suggest that probe rich regions can occur on cooling below the T_m.

A model consistent with these data is given in Figure 2B. The multibilayer is viewed as a closely packed array of squashed, very large, multilamellar liposomes. The linear dimension of these liposomes parallel to the orienting surface is generally much greater than the thickness. The "grain boundaries" are regions where these liposomes meet. Particularly, with NBD-PE, the fluorophore is expected to have almost complete rotatory freedom so that the greater density of fluorophores in the boundary region leads to higher fluorescence here than in the flat areas. FRAP measurements made in "grainy" regions always give incomplete recovery, suggesting that these boundaries present an impediment to complete diffusional recovery. This result is consistent with the model depicting the boundaries as regions of contact between integral multilamellar liposomes. In such a situation, the infrequent jumping of probe from the outer bilayer of one liposome to the outer bilayer of an adjacent liposome which would be required for complete recovery would be expected to effectively prevent complete recovery on the time scale of these measurements. This model also indicates that we observe lateral diffusion averaged over the number of individual bilayers which define the thickness of the specimen.

FRAP Control Studies. Calculations of transport coefficients from FRAP kinetics are much simpler if spontaneous regeneration of fluorescence is negligible during the recovery time. The very slow recovery of diO-C_{18}(3) and NBD-PE fluorescence when these probes are embedded in membranes below their phase transition temperature suggests that this is the case. We have also shown that large, individual multilamellar EPC liposomes (~10 μm diameter) containing either probe can be bleached uniformly over their surface. Recovery can then be checked with a 2-μm diameter probe beam positioned at the center of the liposome; in no case was recovery observed for over 10 min. This experiment indicates that spontaneous regeneration of fluorescence from either probe is negligible during typical FRAP experiments.

It is possible that the brief, but intense, photobleaching pulse may produce some membrane damage. We have shown that

TABLE I: Lateral Diffusion Coefficients[a] of Two Fluorescent Lipid Analogues in Multibilayers of Different Composition.

	Lipid composition									
	EPC		EPC + cholesterol (1:1)		DMPC		DMPC + cholesterol (1:1)		DPPC	
	25 °C	37 °C	25 °C	37 °C	20 °C ($T < T_m$)	30 °C ($T > T_m$)	20 °C	30 °C	30 °C ($T < T_m$)	45 °C ($T > T_m$)
D (cm^2/s) for diO-C_{18}(3)	1.6×10^{-7}	3×10^{-7}			$\leq 1 \times 10^{-10}$	5.5×10^{-8}			$\leq 5 \times 10^{-10}$	7×10^{-8}
D (cm^2/s) for NBD-PE	4×10^{-8}	8×10^{-8}	1.8×10^{-8}	3.5×10^{-8}	$\leq 1.5 \times 10^{-10}$	8×10^{-8}	8×10^{-9}	2×10^{-8}		

[a] Data precision can be assessed from error bars in Figures 4 and 5; some of the values quoted are estimated by interpolation between points at proximate temperatures.

repeated photobleaching of the same spot yielded complete recovery and identical $\tau_{1/2}$ values within experimental uncertainty for each successive FRAP measurement indicating, at least, the absence of an accumulating photodamage artefact. Furthermore, the agreement is striking between D for diO-C_{18}(3) in EPC membranes and D for 3,3′-dioctadocylindocarbocyanine (diI-C_{18}(3)) in single EPC bilayers obtained by fluorescence correlation spectroscopy (Fahey et al., 1977) in which no photobleaching is required. Since these two probes are structurally very similar, this agreement suggests any photodamage produced in the FRAP method is not significantly affecting the results.

Local heating during the photobleaching pulse will be caused by nonradiative transfer of energy from the excited fluorophores to the environment. However, since the transition temperatures detected by the abrupt increase in the lateral diffusion coefficients are very close to the values measured by other physical techniques, one can presume that any generation of heat in the membrane is rapidly conducted away from the measurement area. If it were otherwise, one would expect the phase transition measured by this technique to occur at some temperature below the known T_m for these phospholipids.

The theory employed to calculate diffusion coefficients (Axelrod et al., 1976) from FRAP kinetics demands that K, a parameter characterizing the extent of photobleaching, be linearly proportional to T_B, the duration that the photobleaching beam irradiates the sample. We have measured K as function of T_B for diO-C_{18}(3) and NBD-PE embedded in multibilayers where diffusion was relatively slow. In such systems, where the inequality $T_B < \tau_{1/2}/10$ can be satisfied for a range of T_B's, the dependence of K on T_B was approximately linear for $T_B \leq 100$ ms.

Lateral Diffusion in Single Component Multibilayers. Diffusion coefficients for the two probes in multibilayers of varying composition which were calculated from the FRAP kinetics are summarized in Table I. The temperature dependence of D is presented in Figures 4 and 5 for the various bilayers studied.

FRAP kinetics for DMPC membranes labeled with diO-C_{18}(3) were measured for temperatures ranging from 9 up to 48 °C. These kinetics are shown in Figure 3 for 22.7 °C ($T < T_m$) and 28 °C ($T > T_m$). Figure 4 (panel A) shows the temperature dependence of the measured diffusion constant for diO-C_{18}(3) in DMPC membranes. The data were the superposition of the measurements performed on two different samples and the agreement is excellent. The weak temperature dependence of the diffusion constants above 24 °C is characterized by $\Delta D/\Delta T \sim 1.7 \times 10^{-9}$ cm^2 per s per °C. Below 22 °C, no substantial fluorescence recovery was observed and a comparison of the measured curve with the theoretical prediction indicated a diffusion constant lower than 5×10^{-10} cm^2/s. As the temperature approached 22 °C, a slow but steady increase in recovery rate was observed. From 22 to 24 °C the recovery rate increased sharply corresponding to an increase in diffusion constant from below 5×10^{-10} cm^2/s at 22 °C to 4×10^{-8} cm^2/s at 24 °C. In the transition region the fluorescence recovery behaved erratically; usually the recovery

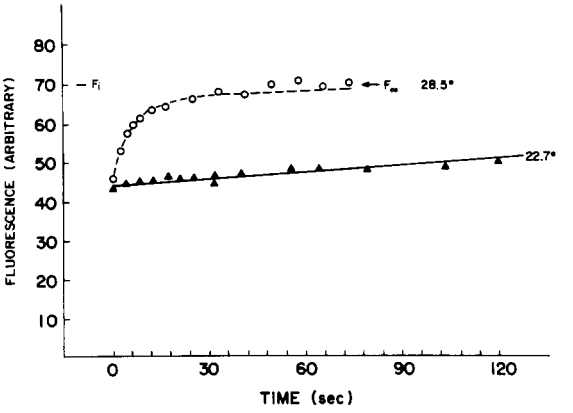

FIGURE 3: FRAP kinetics for diO-C_{18}(3) in DMPC multibilayers for T just below T_m (22.7 °C; ▲) and T above T_m (28.5 °C; ○). T_B = 100 ms. Broken line is the theoretical fit to 28.5 °C data using $D = 5 \times 10^{-8}$ cm^2/s; solid line is the theoretical fit to 22.7 °C using $D = 5 \times 10^{-10}$ cm^2/s.

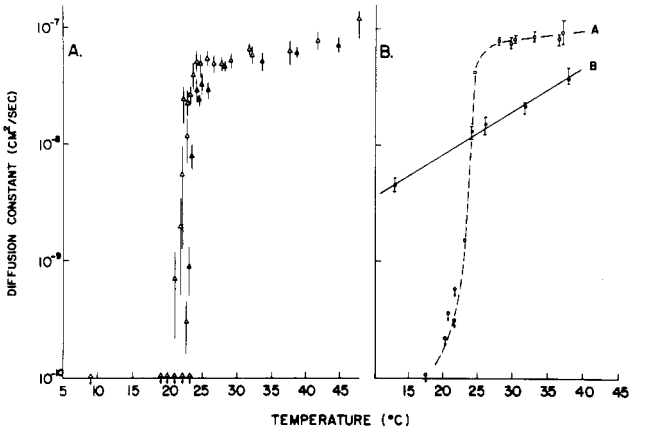

FIGURE 4: Lateral diffusion coefficients vs. temperature for two probes in DMPC and DMPC-cholesterol (1:1) multibilayers. Points are mean diffusion coefficients calculated from several FRAP curves and error bars represent maximum spread of these values. Panel A: diO-C_{18}(3) in DMPC multibilayer; open and filled triangles indicate points from independent experiments. T_B = 100 ms; bleach power was approximately 15 mW; bleaching beam was attenuated by 10^5 for measuring. Panel B: (Curve A) NBD-PE in DMPC multibilayer; (curve B) NBD-PE in DMPC-cholesterol (1:1) multibilayer. T_B = 40 ms (curve A) or 80 ms (curve B); bleach power was 2.5 mW; bleaching beam was attenuated by 10^5 for measuring.

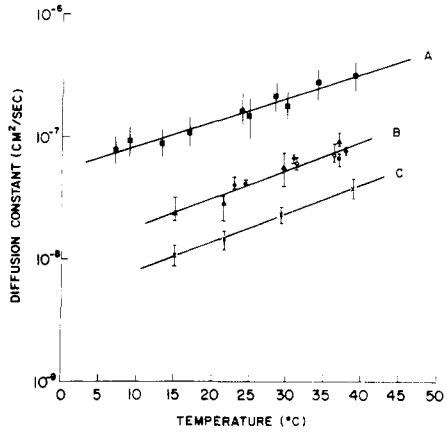

FIGURE 5: Lateral diffusion coefficients vs. temperature for two probes in EPC and EPC-cholesterol (1:1) multibilayers. (A) (■—■) diO-C_{18}(3) in EPC multibilayer. (B) (○, ◐, ●, ▲), NBD-PE in EPC multibilayer: the different circled symbols represent results from independent experiments. (C) (X—X) NBD-PE in EPC-cholesterol (1:1) multibilayer. Points are mean diffusion coefficients calculated from three to seven FRAP curves; error bar represents maximum spread in these diffusion coefficients. FRAP measurement parameters were as described in Figure 4.

was less than 100% complete, but occasionally more than 100% recovery was observed meaning that the final intensity (F_∞) was higher than the initial intensity (F_i). Samples prepared under different conditions showed slightly different transition ranges; however, they always fell within a narrow range between 22 and 24 °C. Above 24 °C the fluorescence recovery was always 100% complete and the recovery rate increased slowly with the temperature.

For the NBD-PE labeled DMPC bilayers, similar lateral diffusion behavior was recorded (Figure 4B, curve A). Very slow diffusion occurred below T_m ($D < 5 \times 10^{-10}$ cm^2/s), while rapid diffusion ($5 \times 10^{-8} < D < 1 \times 10^{-7}$ cm^2/s) with a weak temperature dependence was observed above T_m. Above T_m, the equivalence of NBD-PE and DMPC diffusion will probably be affected by the difference in acyl chain length between probe and host lipid.

For diO-C_{18}(3) labeled DPPC membranes, the measurements were performed between 24 and 53 °C. Similar fluorescence recovery behavior as in DMPC membranes was observed except the transition regions among different samples were scattered over a somewhat wider temperature range between 36 and 41 °C possibly due to preparation difficulties; however, the transition region of most samples occurred in a narrow range of 3 °C. Above the T_m, diffusion was again rapid. FRAP kinetics at 44 °C could be fitted very well with a $D = 5.2 \times 10^{-8}$ cm^2/s (data not shown). Between 41 and 53 °C, D increased slowly with temperature (data not shown) with a coefficient $\Delta D/\Delta T \sim 2.2 \times 10^{-9}$ cm^2 per s per °C, a value slightly higher than that in DMPC membranes.

Since many other physical measurements have indicated that the gel to liquid crystalline transition for hydrated DMPC and DPPC occurs at about 24 and 41 °C, respectively (Chapman et al., 1967; Hinz and Sturtevant, 1972; Jacobson and Papahadjopoulos, 1975), this work indicated that the rapid lateral diffusion of lipids above T_m of DMPC and DPPC is markedly inhibited when these membranes are cooled below their T_m. Similar results have been reported in a preliminary account of a FRAP study using the probe diI-C_{18}(3) in DMPC and DPPC multibilayers (Fahey and Webb, 1977).

As shown in Figure 5, lateral diffusion in EPC bilayers labeled with either lipid analogue in the range 10 to 40 °C is rapid ($\sim 2 \times 10^{-8}$ to 3×10^{-7} cm^2/s), moderately temperature dependent (8 kcal/mol $< E_a <$ 10 kcal/mol) and shows no phase transition behavior. The latter characteristic is expected as T_m for EPC is about −15 °C (Ladbrooke and Chapman, 1969). The lateral diffusion coefficient for diO-C_{18}(3) (Figure 5, curve A) is surprisingly fast ($\sim 1.6 \times 10^{-7}$ cm^2/s at 25 °C). As pointed out above, this large value agrees with an independent measurement of diI-C_{18}(3) diffusion in planar EPC bilayers by fluorescence correlation spectroscopy (Fahey et al., 1977). However, the lateral diffusion coefficient for NBD-PE (Figure 5, curve B) in the same membranes was about fourfold lower than that for diO-C_{18}(3). Since the structure of diO-C_{18}(3) is less like a phospholipid than NBD-PE which, in fact, has identical acyl chain composition as the host EPC (according to the Avanti Biochemicals Catalog), we presume that its more rapid diffusion is due to the perturbation it causes in the normal phospholipid packing in the bilayer or because some of the probe is located in a membrane position, perhaps the midplane, where faster lateral diffusion is possible.

Effect of Cholesterol. The effect of cholesterol on NBD-PE diffusion in DMPC-cholesterol bilayers (1:1 molar ratio) is shown in Figure 4B (curve B). Cholesterol is seen to abolish transition behavior, raising D at 20 °C from $< 2 \times 10^{-10}$ cm^2/s to about 8×10^{-9} cm^2/s (interpolated value) while reducing D above T_m from 8×10^{-8} cm^2/s to about 2×10^{-8} cm^2/s. NBD-PE diffusion in EPC-cholesterol bilayers (1:1 molar ratio) is reduced by about a factor of 2 at all temperatures tested (Figure 5; curve C).

Comparison with Previous Lateral Diffusion Results in Bilayers. In this section, we restrict ourselves to discussing comparable data on lipid analogue diffusion in bilayer membranes. Our FRAP results with NBD-PE in EPC bilayers yield a diffusion coefficient at 25 °C about twofold higher than obtained with a spin-labeled PC in a comparable system (Devaux and McConnell, 1972). Similarly, a lower limit for D in EPC bilayers is given as about 10^{-8} cm^2/s by analysis of proton magnetic resonance (Lee et al., 1973). This agreement is reasonable considering the variety of experimental systems. On the other hand, diO-C_{18}(3) diffusion is clearly faster at 25 °C (1.6×10^{-7} cm^2/s) in EPC bilayers than the diffusion of other analogues whose structure more nearly approximates a phospholipid. In DPPC vesicles, between 45 and 50 °C ($T > T_m$) lateral diffusion coefficients of $\sim 4 \times 10^{-8}$ cm^2/s have been measured by spin resonance (Brulet and McConnell, 1975) and ^{31}P NMR (Cullis, 1976), again within a factor of about two of our result using diO-C_{18}(3) (Table I). For DPPC below T_m, Cullis (1976) reports an upper limit for D of about 10^{-9} cm^2/s which is somewhat higher than our upper limit of 5×10^{-10} cm^2/s for either probe in saturated lecithins below T_m. Results for other extrinsic lipid-like probes in fluid membranes range from 3×10^{-8} cm^2/s for a spin-labeled androstane in EPC bilayers at 50 °C (Trauble and Sackmann, 1972) to 1.4×10^{-7} cm^2/s for pyrene in DPPC bilayers (Galla and Sackmann, 1974) also at 50 °C. Thus, it seems that diffusion coefficients in the range of 10^{-8} to a few times 10^{-7} cm^2/s can be expected for apolar or amphipathic substances in the intermediate (say, 300 to 3000) molecular weight range which are embedded in fluid bilayer membranes.

The general effect of cholesterol inclusion into bilayer membranes in 1:1 or 1:2 molar ratios is to impede rate processes above the T_m of the native membrane while preserving the fluid state below the T_m (for review, see Oldfield and Chapman, 1972). Our results with NBD-PE in DMPC bilayers are in agreement with this generalization. Quantitatively, cholesterol reduces the D for NBD-PE by a factor of two to four for membranes above their T_m (Table I). Similar reductions in D have been reported for spin-labeled lecithin in

EPC–cholesterol bilayers (Devaux and McConnell, 1972), for EPC diffusion in EPC–cholesterol vesicles (Cullis, 1976), and for pyrene in DPPC–cholesterol (2:1) bilayers (Galla and Sackmann, 1974). Rotational diffusion of perylene in equimolar EPC–cholesterol bilayers is similarly impeded (Jacobson and Wobschall, 1974).

The moderate temperature dependence of lateral diffusion in fluid EPC bilayers (8 kcal/mol $< E_a <$ 10 kcal/mol) and the insignificant effect of cholesterol on this temperature dependence is in accord with a recent study of lateral diffusion in sonicated EPC vesicles by ^{31}P NMR (Cullis, 1976). In general, the agreement of bilayer lateral diffusion coefficients determined from independent measurements on similar model systems increases our confidence in the FRAP technique; furthermore, all of these results provide essential background for the interpretation of lipid diffusion results in cellular systems.

Lipid Diffusion in Biomembranes. Measurement of lipid probe diffusion in biomembranes by spin resonance and optical methods has also yielded D's in the range of 10^{-8} to 10^{-7} cm^2/s. For example, FRAP measurements for diI-C_{18}(3) in myoblast membranes give a value of $D = 8 \times 10^{-9}$ cm^2/s at 25 °C (Schlessinger et al., 1977). Electron spin resonance measurements yield diffusion coefficients for spin-labeled fatty acids and lecithins ranging from 3×10^{-8} cm^2/s for *E. coli* membranes at 40 °C (Sackmann et al., 1973) to 6×10^{-7} cm^2/s for sarcoplasmic reticulum at 37 °C (Scandella et al., 1972) to 1×10^{-7} cm^2/s for microsomal membranes at 30 °C (Stier and Sackmann, 1973). The rapidity of diffusion at physiological temperatures is interesting and lends further support to earlier speculations (Adam and Delbruck, 1968; Devaux and McConnell, 1972; Keith and Snipes, 1974) that lateral membrane transport of amphipatic and apolar molecules may be an important intracellular transport mechanism for signaling, control, and biosynthetic purposes. FRAP measurements should provide useful data on lipid lateral diffusion in biomembranes on the single cell level. However, slow unidirectional lipid flows superimposed on this rapid diffusion, such as postulated by Bretcher (1976), would probably go undetected, since the recovery will be dominated by the faster diffusional process.

Acknowledgments

We thank Dr. Eric Mayhew for help with the interference microscopy and Ms. Joy Jacobson for help with an illustration.

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