# Lipid Lateral Diffusion in the Surface Membrane of Cells and in Multibilayers Formed from Plasma Membrane Lipids†

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ABSTRACT: In this project, the diffusion of a fluorescent lipid analogue, dihexadecylindocarbocyanine [diI-$C_{16}$(3)], was compared in several systems: the cell surface membrane of living human fibroblasts, the bottom surface membrane remaining after the top surface, nucleus, and cytoplasm were removed, multibilayers reconstituted from the whole cell lipids, and multibilayers reconstituted from plasma membrane (PM) lipids. One major purpose was to inquire about the effects of membrane proteins on the lateral mobility of lipids in the plasma membrane. The human fibroblasts selected for the study were highly spread and only slowly internalized the lipid analogue probe. Large numbers of these cells were fractionated on a sucrose step gradient to obtain plasma membranes. Lipids from this fraction and the entire cell were extracted and analyzed. Cell surface and multibilayer diffusion of diI-$C_{16}$(3) was measured by the fluorescence recovery after photobleaching technique. Diffusion coefficients ($D$) for diI-$C_{16}$(3) inserted into the living cell's surface membrane or bottom surface ghosts were similar, ranging from $\sim 3.5 \times 10^{-9}$ cm$^2$/s at 5 °C to $\sim 2 \times 10^{-8}$ cm$^2$/s at 37 °C with a change in slope at ~25 °C. For diI-$C_{16}$(3) incorporated into extracted PM lipid multibilayers, $D$ ranged from $\sim 5 \times 10^{-9}$ cm$^2$/s at 5 °C to over $6 \times 10^{-8}$ cm$^2$/s at 37 °C. When the probe was introduced into multibilayers formed from the total cell lipids, $D$ was about $9 \times 10^{-9}$ cm$^2$/s at 5 °C and $8.5 \times 10^{-8}$ cm$^2$/s at 37 °C. Both of the multibilayers showed evidence of undergoing a transition at ~10 °C, and a break in the diffusion curve occurred at 25 °C. Above 25 °C, the temperature dependence of $D$ was similar in all systems with activation energies ($E_a$) in the range of 5–7 kcal/mol. Below 25 °C, $E_a$ ranged from 9–11 kcal/mol for the intact cells and bottom surface ghosts to 17 kcal/mol for the PM lipid multibilayers. When the increased diffusion in whole cell lipid multibilayers relative to that in PM lipid multibilayers is considered, the decreased cholesterol to phospholipid ratio must play a major role, since other compositional differences appear minor. Cholesterol presumably alters not only the fluidity of the multibilayers (above 25 °C) but also the lateral lipid organization (below 25 °C), resulting in the steeper dependence of diffusion on temperature in this region. When the multibilayer results were compared to those from the actual PM, diffusion in the PM was nearly matched by that in the PM lipid multibilayer at 5 °C, but at 37 °C, diI-$C_{16}$(3) diffusion was 4 times faster in the multibilayer composed of PM lipids than in the PM itself. The membrane proteins in the PM could produce the observed reduction in diI-$C_{16}$(3) mobility by generally increasing membrane viscosity and/or binding the probe at protein–lipid interfaces.

Fluorescence recovery after photobleaching (FRAP),[1] has provided additional information about the mobility of membrane components in model membranes and in living cell membranes [for reviews, see Jacobson (1980) and Cherry (1979)]. Certainly, a large amount of evidence indicates that the lipids of biomembranes are arranged in a bilayer [see Singer & Nicolson (1972)], although specialized exceptions may exist (deKruijff et al., 1980). Photobleaching studies have indicated that lipid analogue probes can diffuse relatively rapidly in the plasma membranes (PM) of myoblasts (Schlessinger et al., 1976, 1977; Axelrod et al., 1978), lymphocytes (Dragsten et al., 1979), rodent fibroblasts (Struck & Pagano, 1980; Eldridge et al., 1980), neuroblastoma cells (deLatt et al., 1980), mouse eggs (Johnson & Edidin, 1978), and red blood cells (Thompson & Axelrod, 1980); these results are in consonance with both the fluid mosaic model (Singer & Nicolson, 1972) and earlier spin resonance measurements on sarcoplasmic reticulum (Scandella et al., 1972). One can next ask how different the lipid lateral mobility in a given plasma membrane is compared to a simple bilayer composed of its constituent lipids. This comparison would indicate the importance of complete membrane organization including the presence of proteins in determining the magnitude of the lipid lateral diffusion coefficient in the plasma membrane of eukaryotic cells. Thus, the design of this study was simply to perform FRAP experiments on living human fibroblast cells and then to perform similar experiments on multibilayers formed from lipids extracted from (1) a plasma membrane fraction and (2) the total cell. Analysis of the lipids from the two extracts was also performed, in order to extend this comparison.

## Materials and Methods

*Cell Culture.* For the FRAP experiments human neonatal foreskin diploid fibroblasts, strain BG-9, were cultured in 35-mm plastic Petri dishes (Falcon, Oxnard, CA) on glass cover slips as previously described (Jacobson et al., 1977). For the cell fractionation experiments, large numbers of cells were grown in monolayer culture in roller bottles with 850 cm$^2$ of growing area (Corning, Corning, NY) and in large plates (530

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[1] Abbreviations used: HF, human fibroblast cells; PM, plasma membrane; PML, plasma membrane lipid; WCL, whole cell lipid; diI-$C_{16}$(3), dihexadecylindocarbocyanine; DPH, diphenylhexatriene; FRAP, fluorescence recovery after photobleaching; $D$, diffusion coefficient; $w_s$, $e^{-2}$ radius of Gausian laser beam in specimen plane; $E_a$, activation energy; MEM, minimum essential medium; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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$cm^2$ of growing area; Nunc Products, Neptune, NJ). The medium used was Eagle's MEM with Earle's salts (GIBCO, Grand Island, NY) plus 10% heat-inactivated fetal calf serum (KC Biological, Kansas City, MO), penicillin (50 units/mL) and streptomycin (50 $\mu$g/mL).

*Bottom Surface and Plasma Membrane Fractionation.* Bottom surfaces of the BG-9 cells were prepared as follows for the FRAP measurements. Five-day-old confluent monolayer cultures grown on cover glasses were washed 3 times in PBS without $Ca^{2+}$ and $Mg^{2+}$. The cells were rinsed with 1 mM Tris–1 mM EDTA, pH 7.2, and incubated at room temperature in this hypotonic solution for 10 min. The cover glass was placed on a slide, and one piece of Whatman No. 2 filter paper was carefully placed over the culture. After 10 s was allowed for the paper to absorb all the fluid from the cover glass, the paper was removed leaving a substantial number of bottom surface fragments adhering to the cover glass. These fragments were washed 4 times with 1 mM Tris–1 mM EDTA and twice with PBS.

Cells were fractionated on the basis of the hypotonic treatment method of Kartner et al. (1977) with some modifications, to obtain the lipid samples. Eleven- to fourteen-day-old cultures were used; these cells had been confluent for 7–10 days and were used from the 29th to 35th passage (subcultivation ratio 1:2). Roller bottle cultures were washed 4 times with 0.9% NaCl containing 0.25 mM phenylmethanesulfonyl fluoride (PMSF) as a protease inhibitor in dimethyl sulfoxide ($Me_2SO$; 0.01% v/v final concentration) and then once with hypotonic solution (1.0 mM $NaHCO_3$ containing 0.25 mM PMSF). Following addition of 25 mL of fresh hypotonic solution, the bottles were kept rolling at room temperature for 10–30 min. The swollen cells were scraped off the growing surface with a large rubber policeman. Cell suspensions from the roller bottles were collected in a T-75 flask (Falcon), and homogenization was carried out at 4 °C by vigorous manual shaking of the flask. After roughly 20 shakes, ~2 $\mu$m diameter vesicles were observed in phase contrast with little clumping. This homogenate was used for the whole cell lipid extraction. For purification, the homogenate was centrifuged at 27000*g* for 20 min. The pellet was resuspended with 10% (w/w) sucrose containing 0.25 mM PMSF to a volume corresponding to 10 mL per roller bottle of cells ($1 \times 10^8$ cells). This suspension was layered on the top of a 10/30% (w/w) sucrose step gradient containing 0.25 mM PMSF–0.01% $Me_2SO$ in a 60-mL centrifuge tube and spun at 76000*g* for 2 h in an SW 25.2 rotor. The PM band at the 10/30% sucrose interface was harvested and washed twice with hypotonic solution. The final pellet was resuspended in the same solution and frozen. Several batches of plasma membrane preparations were pooled for the lipid extraction and assays to obtain enough material.

*Estimation of Plasma Membrane Purity.* As a membrane marker, the activity of 5′-nucleotidase was assayed according to Windell (1972). Inorganic phosphate release was measured by the method of Chen et al. (1956), and protein was determined by the Markwell et al. (1978) modified $NaDodSO_4$ Lowry procedure. 5′-Nucleotidase was shown to be a valid plasma membrane marker by comparing the activity of intact cells to that of lysed cells: 93% of the lysate activity was found in a comparable intact cell determination. Nuclear contamination was determined by the amount of deoxyribonucleic acid (DNA) present in the plasma membrane fraction, assayed according to Burton (1956). Ribonucleic acid (RNA) contamination was determined by Fleck & Munro (1962). The presence of mitochondrial contamination was assessed by measuring the succinate dehydrogenase activity per milligram of total protein according to King (1963).

*Lipid Extraction and Analysis.* The whole cell and plasma membrane lipids were extracted by the method of Bligh & Dyer (1959). All steps in the extraction procedure were performed under nitrogen to avoid any oxidation of lipids. After drying, the extracted lipids were dissolved in chloroform, ampuled under nitrogen, and stored at −20 °C for subsequent multibilayer preparation and lipid analysis.

Lipid separations were performed by thin-layer chromatography (TLC) on 10 × 10 cm silica gel plates with a combination of three organic solvent systems (Organisciak & Klingman, 1974). By this procedure neutral lipids, which migrate near the solvent front in the first solvent, can be subsequently separated and eluted from the same TLC plate as the phospholipids. After visualization and elution of lipids from the TLC plates, the plates were then resprayed with 50% sulfuric acid and charred in order to detect small amounts of triacylglycerols or free fatty acids, which might influence our results. None were found. Lipid phosphorus was quantitated with a modified nanomolar phosphorus assay based on the formation of a malachite green–phosphomolybdate complex (Organisciak & Noell, 1976). Cholesterol was determined in aliquots of the original lipid extract and on cholesterol and cholesterol ester eluates from TLC plates with a Liberman–Burchard assay or an enzymatic cholesterol oxidase technique (Sigma Chemical Co., St. Louis, MO).

Fatty acid methyl esters were prepared by transesterification with 14% methanolic boron trifluoride (Morrison & Smith, 1964) and separated by gas–liquid chromatography (GLC). Chromatography was performed isothermally at 185 °C on 6 ft by $^1/_4$ in. glass columns containing 10% diethylene glycol succinate on 80–100-mesh Supelcoport with helium as the carrier gas. Fatty acids were identified by comparison of retention times with standards and quantitated with the aid of an electronic integrator.

*Fluorescence Polarization.* The fluorescence polarization of diphenylhexatriene (DPH) embedded in multilamellar whole cell lipid vesicles was measured in an SLM 4800 fluorometer in the laboratory of Dr. B. Lentz by using conditions described in detail previously (Lentz et al., 1980).

*FRAP Experiments.* Photobleaching measurements were performed on multibilayers, prepared from aliquots of the extracted lipids, as previously described (Wu et al., 1977, 1978). For the cell surface experiments, human fibroblasts were grown on glass cover slips and labeled with 1.25–2.5 $\mu$g/mL diI-$C_{16}$(3) injected into PBS from a concentrated ethanolic dye solution. Final ethanol concentration was 0.5–1% (v/v). The cover slips were washed 3–4 times with PBS, inverted, placed on a microscope slide, and spaced with narrow strips of weighing paper to prevent the cells from being "squashed". The assembly was sealed with a paraffin–vasoline mixture to prevent dehydration. Labeling of the bottom surface fragments with diI-$C_{16}$(3) was accomplished by incubating the fragments (on cover slips) with 8 $\mu$g/mL probe for 10 min at 37 °C. The dye was diluted from a concentrated methanolic stock solution (to a final methanol concentration of 0.5% v/v). Following the incubation, the fragments were washed 5 times in PBS (without $Ca^{2+}$ or $Mg^{2+}$) and sealed as described above. For variation of the temperature of the specimen, a Leitz thermal stage (Wu et al., 1977) or a thermoelectric stage (Bailey Instruments, Saddle Brook, NJ) was employed.

In a comparative study, sources of systematic error must be considered. Beam radii, $w_s$, were calculated as previously

Table I: Lipid Composition of Whole Cell and Plasma Membrane Extracts from Human Fibroblast Cells

| | whole cell | plasma membrane |
|---|---|---|
| chol/TPL[a] (mole ratio) | 0.41 (0.36–0.44) | 0.57 (0.52–0.62) |
| PE[b] | 21.2 ± 1.6 | 21.7 |
| PC[b] | 53.5 ± 2.9 | 49.8 |
| PS[b] | 9.2 ± 0.5 | 6.6 |
| PI[b] | 5.6 ± 2.2 | 6.4 |
| SPH[b] | 10.6 ± 0.9 | 15.7 |

[a] Average cholesterol/total phospholipid molar ratio for whole cell $n = 3$ (range) and plasma membrane $n = 2$ lipid extracts, respectively. Cholesterol esters of whole cell lipid extracts represent no more than 13% of the total. [b] Average percent composition of whole cell ($n = 4$) ± SD and plasma membrane ($n = 2$) phospholipids. Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SPH, sphingomyelin.

described (Jacobson et al., 1977) to be ~4.1 μm for the 10× objective, used for the multibilayer results, and ~1.7 μm for the 25× objective, which was employed for the living cell studies. These beam diameters were confirmed in the specimen plane by a variation of the flow calibration measurement (Axelrod, et al., 1976) as previously described (Derzko & Jacobson, 1980). Such measurements also indicated that the specially selected glass neutral density filters employed (ND 4.0, ND 4.5, ND 5.0) did not shift the measuring beam by more than $0.3w_s$ from the photobleached "hole", thereby ensuring a maximmum of ~10% error in $D$ due to this effect [see Barisas (1980) and Derzko & Jacobson (1980)]. Several empirical tests also suggested that the differences to be presented are valid. First, the large cell size allowed the 10× objective to be employed, and results obtained by using this objective were consistent with those obtained by using the 25× objective. Second, over the 18-month duration of this study, the differences presented were reproducible. Finally, direct comparison of cellular FRAP data employing the three ND filters above was within experimental error (±15%), indicating that measuring beam shift did not affect the data presented here.

The question of photodamage is always a concern in FRAP experiments (Jacobson et al., 1978; Wolf et al., 1980). For lipid analogue diffusion, the strongest argument against appreciable artifact is the general agreement of the FRAP results with the magnetic resonance results in model membranes [see Wu et al. (1977) and Derzko & Jacobson (1980)] and with the fluorescence correlation spectroscopy results (a nonbleaching method) on myoblast plasma membranes (Schlessinger et al., 1977).

## Results and Discussion

*Cell Fractionation.* The mean plasma membrane fraction enrichment of 5′-nucleotidase activity following the fractionation protocol described above was 9.9-fold (range 9.3–11.9-fold; six trials). In addition, succinate dehydrogenase, a mitochondrial marker, was depleted 20-fold. Total succinate dehydrogenase activity was reduced to 0.5% of its value in the cell homogenate. Also RNA and DNA were depleted more than 10-fold. Electron micrographs showed typical membranous fragments similar to those reported by Kartner et al. (1977).

*Lipid Composition.* The lipid class compositions of whole cell (WC) and plasma membrane (PM) extracts are compared in Table I. As shown in row 1, the ratio of total cholesterol to total phospholipid in PM lipids was 39% higher than in WC lipids. In contrast, only minor differences in the glycerophospholipid composition of the two extracts were measured.

Table II: Total Fatty Acid Composition of Whole Cell and Plasma Membrane Lipid Extracts from Human Fibroblast Cells

| | mol % | |
|---|---|---|
| fatty acid[a] | whole cells | plasma membranes |
| 14:0 | 1.2 | 2.4 |
| 16:0 | 28.0 | 27.1 |
| 16:1 | 11.6 | 9.2 |
| 18:0 | 18.2 | 14.9 |
| 18:1 | 22.2 | 30.6 |
| 18:2 | 0.3 | 0.3 |
| 20:3 | 1.7 | 0.8 |
| 22:0 | 1.2 | 0.7 |
| 20:4 | 10.7 | 6.6 |
| 22:4 | 1.7 | 2.2 |
| 22:5[b] | 1.3 | 1.2 |
| 22:6 | 2.1 | 3.7 |
| | total % | |
| saturated | 48.5 | 45.1 |
| monounsaturated | 33.8 | 39.8 |
| polyunsaturated | 17.8 | 14.8 |
| unsaturates/saturates | 1.06 | 1.21 |

[a] Results represent the average of two separate determinations. Mol % composition calculated from area % composition by correction for fatty acid molecular weights ($c(18:0) = 1.0$). Less than 0.1% for 18.3, 20:0, and 20:5. [b] Total of $22:5^{\omega 6} + 22:5^{\omega 3}$.

The major glycerophospholipids of the PM and WC extracts were phosphatidylcholine, which represented 54 and 50%, and phosphatidylethanolamine, which accounted for 21 and 22% of the lipid phosphorus, respectively. Phosphatidylserine, phosphatidylinositol, and sphingomyelin were present in both of the extracts in smaller amounts. In these experiments lysoglycerophospholipids represented no more than 1.0% of the total lipid phosphorus recovered from each of the thin-layer plates.

As shown in Table II, the fatty acid compositions of WC and PM lipids were similar. The major saturated fatty acids of both the WC and PM extracts were palmitic (16:0) and stearic (18:0) acids. Total saturated fatty acids of WC and PM extracts were 48 and 45 mol %, respectively. The major monounsaturated fatty acids in both extracts were oleic (18:1) and palmiteolic (16:1) acids. When the WC lipids were compared to the PM lipids, palmiteolic acid was slightly lower in the PM lipids, while oleic acid was over 8% higher in the PM lipids. A slight decrease in the concentration of total polyunsaturates was measured in the PM lipids which was attributable to a 4% decrease in arachidonic acid (20:4) going from the WC to the PM lipids. Overall, the ratio of total unsaturates to saturates was 1.21 and 1.06 for the PM and WC extracts, respectively.

*Cell Staining with diI-$C_{16}$(3).* Figure 1A shows the staining pattern when the BG-9 human fibroblast cells are labeled in the manner described above. The stain appears uniform with little evidence of internalization and localization within intracellular organelles. The brighter region reflects the partial overlapping of adjacent cells. It should be noted that when red cells are stained in a similar manner with diI-$C_{18}$(3), fluorescence polarization measurements on the labeled cells are consistent with the probe's chromophoric head group oriented parallel to the membrane surface and its acyl chains oriented normal to the membrane surface (Axelrod, 1979).

The small circle in Figure 1A shows the size of a measurement area, i.e., the $1/e^2$ diameter of the Gaussian profile laser beam ($2w_s \simeq 3.4$ μm), in relation to the typical size of the cell. The BG-9 cells are highly spread, and we typically

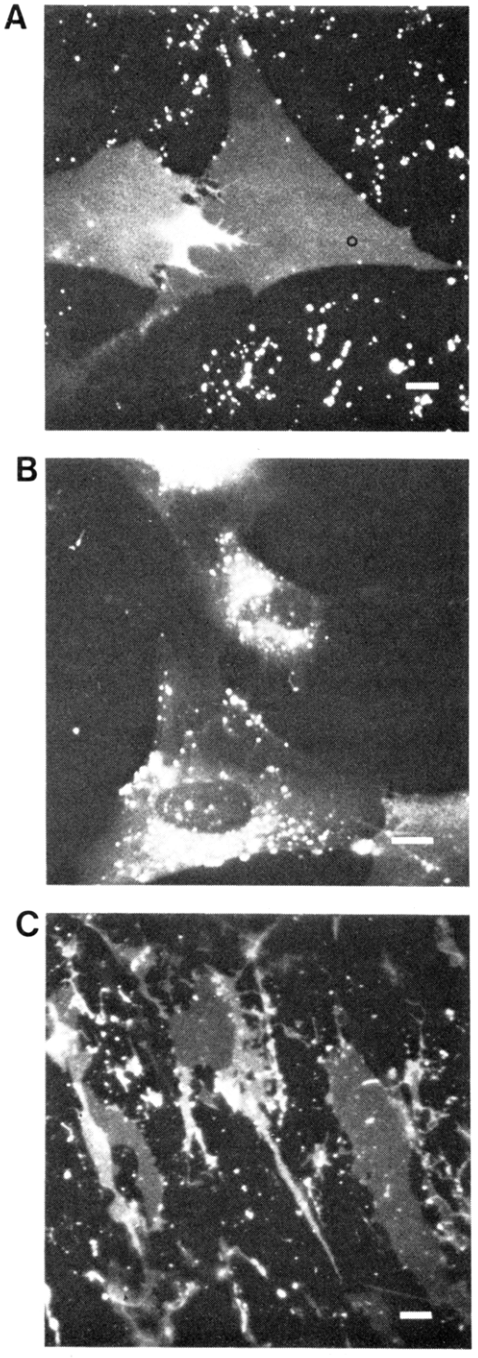


FIGURE 1: Fluorescence photomicrographs of cells and bottom surfaces labeled with diI-$C_{16}$(3) as described in the text. Bars indicate 10 $\mu$m. (A) HF cells 30 min after dye injection with cells maintained at 25 °C. (B) HF cells 2 h after dye injection with the cells maintained at 37 °C. (C) HF bottom surfaces.

measured diI-$C_{16}$(3) diffusion over flat, thin regions of the cell, far from the nucleus. Since we performed the FRAP experiment with a 25× objective on two cell surfaces separated by a distance on the order of 1 $\mu$m or less, the beam diameters were nearly identical on top and bottom cell surfaces.

Figure 1B shows that eventually diI-$C_{16}$(3) fluorescence appears in a perinuclear localization presumably reflecting accumulation within intracellular organelles. However, this image appears after 2 h at 37 °C, and images such as seen in Figure 1A persist for 30–40 min after the initial introduction of the probe. Even after 2 h, a veil of fluorescence at the cell periphery suggests that some of the probe is still in the PM. Figure 1C shows the staining pattern of the bottom surface fragments at slightly lower magnification. Note the brighter

Table III: Lipid Analogue Lateral Diffusion in Plasma Membranes and Multibilayers Made from Extracted Cell Lipids

| sample | $D^a$ (×$10^{-8}$ cm$^2$/s) for diI-$C_{16}$(3) 5 °C | 20 °C | 37 °C |
|---|---|---|---|
| PM of living cell | 0.35 | 0.8 | 1.6 |
| PM of cell bottom surfaces | 0.39 | 1.05 | 2.1 |
| PM fraction lipid multibilayers | 0.51 | 2.5 | 6.3 |
| whole cell lipid multibilayers | 0.91 | 3.8 | 8.5 |

[a] Taken from the least-squares fit of data presented in Figure 2.

Table IV: Activation Energies for Lipid Analogue Diffusion in Plasma Membranes and Multibilayers Made from Extracted Cell Lipids

| sample | $E_a$ (kcal/mol)[a] $0 < T <$ 25 °C | 25 °C $< T <$ 40 °C |
|---|---|---|
| PM of living cell | 9.0 | 6.0 |
| PM of cell bottom surfaces | 10.7 | 5.2 |
| PM fraction lipid multibilayers | 17.0 | 6.2 |
| whole cell lipid multibilayers | 11.7[b] | 7.2 |

[a] Calculated from the least-squares fits of data in Figure 2. [b] 10 °C $< T <$ 25 °C.

regions of some of the fragments, probably indicating incomplete removal of the top surfaces in certain cases. As would be expected, this image did not change with time. Furthermore, diI-$C_{16}$(3) lateral diffusion was similar in these fragments and the living cell (see below). On the basis of the fluorescence images and the agreement of the lateral diffusion results between the intact cell and bottom cell surface, we conclude that diI-$C_{16}$(3) is located predominantly at the cell surface for times on the order of 30 min after injection of the dye.

*Diffusion of diI-$C_{16}$(3).* The lateral diffusion data obtained are given in Figure 2 and summarized in Tables III and IV. The lower curve in Figure 2A is that for diI-$C_{16}$(3) diffusion in the HF plasma membrane, ranging from $\sim 3.5 \times 10^{-9}$ cm$^2$/s at 5 °C to $\sim 1.6 \times 10^{-8}$ cm$^2$/s at 37 °C; these results were obtained over a period of 15 months at varying cell densities in asynchronous cultures. The upper curve (solid) gives the temperature scan for diI-$C_{16}$(3) diffusion in the bottom surfaces of HF cells. The overlap in the two sets of data suggests that internalization of diI-$C_{16}$(3) was not appreciable. The diffusion coefficients of lipid analogues in various cell surface membranes at room temperature range from about $1.5 \times 10^{-9}$ (red blood cell ghost; Thompson & Axelrod, 1980) to $1.5 \times 10^{-8}$ cm$^2$/s (lymphocytes; Dragsten et al., 1979). Our values ($\sim 1 \times 10^{-8}$ cm$^2$/s) fall in the upper part of this range.

In Figure 2B, the upper curve gives the diffusion coefficient for diI-$C_{16}$(3) in whole cell lipid (WCL) multibilayers: the diffusion coefficient ranges from $\sim 9.7 \times 10^{-9}$ cm$^2$/s at 5 °C to $\sim 8.5 \times 10^{-8}$ cm$^2$/s at 37 °C with a discontinuity at $\sim$10 °C (dotted portion) and a "kink" at $\sim$25 °C. The data were remarkably reproducible over a period of almost 1 year (see the legend of Figure 2), indicating that the culture system remained constant with regard to lipid diffusion. The middle curve was obtained for diI-$C_{16}$(3) diffusion in multibilayers prepared from plasma membrane lipids. It shows intermediate behavior with $D$ at 5 °C $\sim 5 \times 10^{-9}$ cm$^2$/s, close to that of

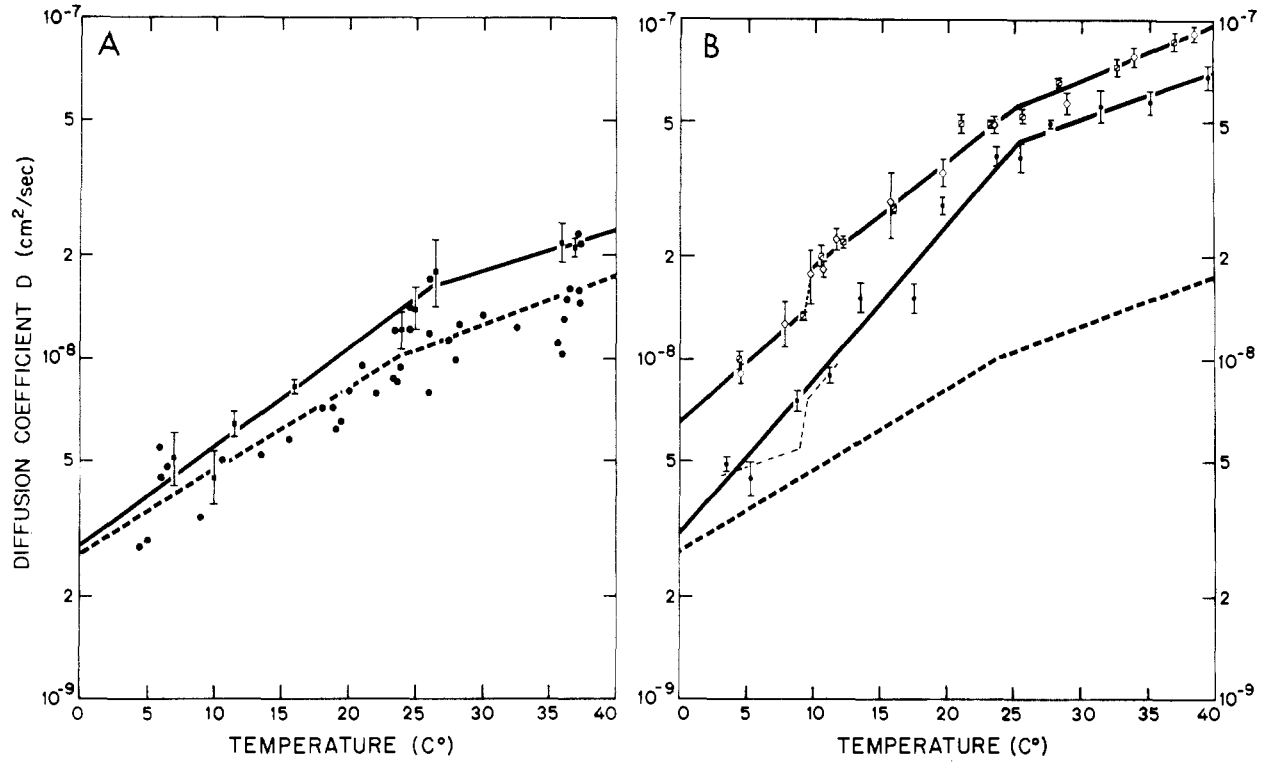


FIGURE 2: (A) (Top curve) (■) diI-$C_{16}$(3) diffusion in bottom surface ghosts formed from HF cells as described in the text. Bar represents ±SEM. (Bottom curve) (●) diI-$C_{16}$(3) diffusion in intact HF cells. Data were collected over 15 months on asynchronous cultures at varying densities with each point representing three to seven FRAP experiments. For clarity, error bars were removed; typical SD's were ±15% of the mean value. (B) (Top) (◇—□) diI-$C_{16}$(3) diffusion in multibilayers formed from the total cellular lipid extract. The two symbols (◇, □) give the results of data from two independent experiments 12 months apart. In this case, the error bars represent the maximum spread in the $D$ values calculated from the set of FRAP measurements. (Middle) (●) diI-$C_{16}$(3) diffusion data in multibilayers formed from the lipids extracted from the PM fraction. In this case, data from four independent experiments were collected from temperature intervals ≤ 2.5 °C and averaged; the error bars represent ±SEM. The dotted segment near 10 °C represents a feature seen in several individual experiments but which disappears in the averaging process . (Bottom curve) (---) Cellular data transferred from (A) for ease in comparison.

the actual plasma membrane value, and $D$ at 37 °C ~6.3 × $10^{-8}$ $cm^2/s$, close to the value of the whole cell lipid multibilayer. These data also showed a discontinuity at ~10 °C and a change in slope at 25 °C but were somewhat more variable than WCL data. For reference, the cell PM data from Figure 2A are given by the lower dotted line.

Regarding temperature dependence of diI-$C_{16}$(3) diffusion, Table IV gives activation energy values ($E_a$) for the systems examined. For the sake of analysis, the data from all of the systems were assumed to undergo a change in slope at ~25 °C. In fact, this change may be spread over a larger temperature range. Above 25 °C, all the $E_a$ values fall in the range of 5–7 kcal/mol. Below 25 °C, $E_a$ values increase to between 9 and 12 kcal/mol in all systems except the PML multibilayers which has an $E_a$ of 17 kcal/mol, reflecting the increase in diffusion rates in this system from values near the intact plasma membrane rates at 5 °C to near the rates of the WCL multibilayer at 25 °C.

The temperature dependence of DPH emission anisotropy was measured in multilamellar vesicles composed of the whole cell lipids, to assist in the interpretation of the temperature dependence for lateral diffusion in the multibilayer specimens. The anisotropy vs. temperature curve (not presented) showed a slight sigmoidicity with the region of steepest slope occurring between 11 and 28 °C. These temperatures approximately correspond to the discontinuity and kink, respectively, in the WCL multibilayer lateral diffusion data.

*Comparative Diffusion in the Systems Examined.* A discussion of the results in Figure 2 requires an explanation of the relative magnitudes of the $D$'s as well as the differences in their dependence on temperature. Consider first the difference between diI-$C_{16}$(3) lateral diffusion in multibilayers made from the whole cell lipids and the PM fraction lipids in terms of the lipid analysis given in Tables I and II. Head group and acyl chain compositional differences seem, in themselves, to be minor unless lateral distribution differences exist between the two multibilayers. If one considers fatty acid composition alone, PML multibilayers would be considered more fluid than WCL multibilayers on the basis of the ratio of total unsaturated to saturated fatty acids (Table II); this is in contradiction to the results in Figure 2B.

The major compositional difference is the higher cholesterol to phospholipid ratio in the PM lipids. Above 25 °C, diffusion is reduced by ~33% in the PM multibilayer relative to the WCL multibilayer—a reduction roughly consistent with the graded effect of added cholesterol on the diffusion of lipid analogues (Rubenstein et al., 1979) and peptides (Wu et al., 1978) in egg phosphatidylcholine membranes. Diffusion activation energies in this temperature range (6–7 kcal/mol) are similar to those for lipid lateral diffusion in fluid model membranes (Wu et al., 1977; Derzko & Jacobson, 1980). Below 25 °C, the slope for both $D_{PML}$ ($E_a$ = 17 kcal/mol) and $D_{WCL}$ ($E_a$ = 11.7 kcal/mol) is steeper; in fact, at 5 °C, $D_{PML}$ is reduced enough that diffusion in PML multibilayer approximates that in the actual PM.

It is curious that an apparent discontinuity occurs in both multibilayer samples at ~10 °C. The PML multibilayers occasionally displayed morphological changes similar to those observed at the phase transition of synthetic lipid multibilayers (Derzko & Jacobson, 1980), whereas the WCL multibilayer specimens never displayed such morphological changes. Computer curve fitting (Derzko & Jacobson, 1980) of the WCL multibilayer data gave one-component diffusion throughout the temperature range examined. In the PML

multibilayer, the situation was more complex. Above the discontinuity, one-component diffusion was observed. Below the discontinuity, two-component diffusion was observed, but only below 7 °C and only in ~50% of the cases analyzed. In such circumstances, ~70% of the analogue diffused at a rate of $\sim 3 \times 10^{-9}$ cm$^2$/s while the remainder displayed a diffusion coefficient of $9 \times 10^{-9}$ cm$^2$/s. (Note this occasional two componentness is not indicated in Figure 2B.) Recovery was complete in all cases.

The sudden decrease in diffusion rate at ~10 °C could be explained by a generalized transition decreasing $D$ by a factor or two. However, since the DPH anisotropy data show only a slight change in slope in this region, a bulk transition seems extremely unlikely.

As an alternative hypothesis, as the sample is cooled below 25 °C, a lateral phase separation may ensue with the probe distributing in a characteristic fashion among the various phases present. In this view, the segregation of the probe reaches an abrupt completion point at 10 °C. These separating phases may consist of small "islands" and/or contiguous domains; in no case, however, would the islands be immobile since recovery is always complete. In addition, our visual observations indicate these phases would be beneath optical resolution. A multiple phase equilibrium is strongly suggested by the detection of two diffusing components such as observed in the PML multibilayer below 10 °C. In cases where only one diffusing component is observed, either the diffusion coefficients in the several postulated phases are not separated enough to detect individual components in the FRAP curve or rapid probe exchange between the phases results in an average $D$ weighted by the amount of probe in each phase (see eq 1 below). Finally, it should be noted that on the basis of pronounced sensitivity of lateral diffusion to structural transformations in model systems (Wu et al., 1977; Rubenstein et al., 1979), the photobleaching techniques might be expected to reveal features in mixed lipid bilayers which are barely noticed by other spectroscopic measurements, such as the DPH depolarization method.

For our purposes the key comparison is between probe diffusion in the actual PM and in the PML multibilayers. While at 5 °C $D_{PM}$ and $D_{PML}$ are within ~30% of each other, between 25 and 37 °C $D_{PML}$ is almost a factor of 4 greater than $D_{PM}$. In comparable studies in which the rotational diffusion of diphenylhexatriene was monitored by measuring both steady-state polarization and lifetime, qualitatively similar results were obtained. The fluidity of bilayers formed from PM lipids was greater than that in PM vesicles for both mouse LM cells (Gilmore et al., 1979) and human lymphocytes (Johnson & Kramers, 1978). A similar fluidity comparison between the rod outer segment membranes and its lipids yielded the same conclusion (Sklar et al., 1979).

Several factors must be considered in interpreting the difference between diffusion in the PM and the PML multibilayer. These include the influence of cell surface morphology on FRAP kinetics, the presence of membrane (glyco-) proteins in the PM, and the potentially scrambled lateral and transverse lipid organization in the PML multibilayer relative to the PM.

Computer simulations (Aizenbud & Gershon, 1980) have indicated a villous cell surface morphology could account for an ~50% decrease in $D$. We have confirmed by scanning electron microscopy that nonmitotic HF cells have relatively few microvilli (evidence not shown). Furthermore, experimental studies on lymphocytes indicate a negligible effect of microvilli on lipid probe diffusion (Dragsten et al., 1979). On these bases, surface morphology should not be an important factor in explaining the observed differences between the PM and the PML multibilayer.

Membrane proteins could play a role in the reduction of diffusion in the PM. First, there is ample evidence that membrane proteins can generally reduce bilayer fluidity both in natural membranes [see, for example, Rottem et al. (1970), Sefton & Gafney (1974), Moore et al. (1978), and Fraley et al. (1979)] and in reconstituted systems [see, for example, Hong & Hubbell (1972) and Gomez-Fernandez (1979)]. Second, while the concept of a boundary layer of lipid surrounding membrane proteins may no longer be a tenable interpretation [see Chapman et al. (1979) and references cited therein], if diI-$C_{16}$(3) specifically binds to certain membrane (glyco-) proteins, its diffusion will be retarded. If bound probe is in fast exchange with the bulk lipid bilayer, the diffusion coefficient will be a weighted average of protein and lipid diffusion rates (E. Elson, personal communication) according to

$$D = f_L D_L + f_P D_P \quad (1)$$

where the subscripts L and P refer to bilayer lipid and protein, respectively, and the $f$'s represent the fractions of the analogue associated with the bulk bilayer and protein. For example, if 50% of the probe is associated with slowly diffusing or immobile protein, $D$ will be reduced by ~50%.

Regarding the temperature dependences of lateral diffusion above 25 °C, the $E_a$ value for $D_{PM}$ is similar to that for the PML and WCL multibilayers. This similarity supports the contention that diffusion in the PM takes place in a relatively homogeneous fluid lipid phase although it is possible that diI-$C_{16}$(3) does not have long enough acyl chains to partition into gel phase domains coexisting with fluid lipid bilayers (Klausner & Wolf, 1980).

Below 25 °C, the temperature dependence of $D$ is much greater in the PML multibilayer ($E_a$ = 17 kcal/mol) than in the PM itself ($E_a$ = 9 kcal/mol), and extrapolations of the two curves will actually cross each other slightly below 0 °C. One possibility is that the steep temperature dependence of $D$ in the PML multibilayer, presumably reflecting a thermal lateral phase separation, is attenuated in the PM by the presence of proteins which could disrupt longer range lipid ordering required for the full phase separation. The convergence of the PML multibilayer diffusion results with the plasma membrane mobility results at low temperatures may be a consequence of membrane protein segregation leading to lipid bilayer regions, largely free of proteins, which are approximately modeled by the PML multibilayer.

Another difference between the two systems is the potentially scrambled lateral and transverse organization of the lipids in the PML multibilayer. In this regard, only when Sklar & Dratz (1980) simulated, by using model membranes, the rod outer segment bilayer with respect to both lipid composition and transbilayer asymmetry, did the temperature dependence of lateral phase separation behavior in the model system approximate that observed in the natural membrane.

## Summary and Conclusions

(1) At 5 °C, the "fluidity" of PM lipids, arranged in a bilayer, is nearly low enough to account for the probe lateral diffusion coefficient in the PM itself. At growth temperatures, diffusion in the PML multibilayer is nearly 4 times faster than in the PM. Diffusion in multibilayers formed from whole cell lipids is more rapid at all temperatures studied than that in the PML multibilayers.

(2) Activation energies ($E_a$) for diI-$C_{16}$(3) lateral diffusion in all the systems examined are 5–7 kcal/mol above 25 °C.

Below 25 °C, $E_a$'s are in the range of 9–12 kcal/mol for all systems except the PML multibilayer for which $E_a$ is 17 kcal/mol.

(3) When diffusion in the PML and WCL multibilayers is compared, the outstanding compositional difference is the increased cholesterol/phospholipid ratio in the PM lipids with the other differences being minor. Above 25 °C, the $D_{PML}/D_{WCL}$ can be accounted for by the known effects of cholesterol on diffusion and fluidity in simple model systems. Below 25 °C, the steeper temperature dependences of $D$ in both multibilayers suggest that lateral phase separation behavior is occurring.

(4) The difference in diffusion rates for diI-$C_{16}$(3) between the PM and PML bilayer at the higher temperatures presumably is accounted for by the presence of (glyco-) proteins in the PM. Membrane proteins could generally decrease bilayer fluidity or specifically bind the lipid analogue. These effects, singly or in concert, could reduce the lateral diffusion in the PM relative to a bilayer composed of its lipids. In addition, the inability to reproduce the plasma membrane lipid's lateral and transverse organization in the PML multibilayer may be important in accounting for the observed differences.

Viewed from a broader perspective, the agreement between diI-$C_{16}$(3) lateral diffusion in the plasma membrane and in simpler bilayers constructed from the PM lipids suggests that, with regard to lateral lipid lateral mobility, the fluid mosaic model (Singer & Nicolson, 1972) provides an adequate qualitative description. Relatively minor modification based on current thinking about protein–lipid interactions should account for the differences observed. These results are to be contrasted to those emerging from a comparison of (glyco-) proteins in cell membranes and in reconstituted bilayers. Such results indicate that protein diffusion in bilayers is at least 1 order of magnitude faster than in cell surface membranes with a substantial fraction of the labeled material being immobile [see Jacobson (1980) and references cited therein]. It is then at the level of protein mobility that more extensive modification of the original fluid mosaic model must be developed, presumably through structures peripheral to the membrane (Singer, 1974; Nicolson et al., 1977; Jacobson & Wojcieszyn, 1981).

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# Lateral Diffusion of Photopigments in Photoreceptor Disk Membrane Vesicles by the Dynamic Kerr Effect†

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ABSTRACT: The lateral diffusion of photopigment molecules in the photoreceptor disk membranes, osmotically swollen into spherical vesicles, has been investigated by dynamic Kerr effect measurements. Upon application of a rapidly reversing bipolar electric field to dilute aqueous suspensions of bovine disk membrane vesicles, the birefringence transient shows a characteristic rise and a deep dip corresponding to the first and second pulses, respectively. The birefringence transient is ascribed to the slowly induced dipole moment caused by electric field induced displacement of the photopigment distribution on the vesicular surface. The lateral translational diffusion coefficient is estimated from the time constant of the slowly induced dipole as $D = (3.3 \pm 1.2) \times 10^{-9}$ cm$^2$ s$^{-1}$. When spermine, a cationic tetraamine, is bound to the disk membrane vesicles, the relaxation time of the slowly induced dipole is shown to become longer, indicating that the birefringence mechanism is indeed due to the field-induced photopigment displacement.

The fluid mosaic model of a biomembrane accentuates the dynamic processes of the two principal components, integral proteins and phospholipids (Singer & Nicolson, 1972), on the bilayer lamellae. The processes involving integral proteins are the translational (lateral) and rotational diffusions, and these are shown to be closely related to physiological functions of a biomembrane [see, for example, a review by Edidin (1974)]. Hence, the lateral diffusion process of photopigment molecules in the photoreceptor disk membrane of the rod outer segment of vertebrate retina which is one of the first ever studied by microspectrophotometry (Poo & Cone, 1974; Liebman & Entine, 1974), has attracted significant attention in the literature. Beside this technique, there are numerous other techniques employed to study the intramembrane protein dynamics for a variety of membrane systems such as cell fusion (Frye & Edidin, 1970; Fowler & Branton, 1977; Schindler et al., 1980), spread of the fluorescent spot (Edidin & Fambrough, 1973), electrophoresis (Poo & Robinson, 1977; Poo et al., 1979), and fluorescence photobleaching–recovery (Peters et al., 1974; Jacobson et al., 1976; Schlessinger et al., 1976; Edidin et al., 1976; Smith & McConnell, 1978; Koppel, 1979; Koppel et al., 1980). Common to all of the above is the scheme of first perturbing the membrane system with optical probes and subsequently following the relaxation toward equilibrium by the dynamic process under study.

We report here a novel technique without chemical perturbation to study the lateral diffusion of photopigment molecules on the disk membrane vesicles (DMV).[1] The technique is an old one, hitherto used mostly for the rotatory diffusion of macromolecules in dilute, called the field-induced birefringence transient method or often shortened as the dynamic Kerr effect method [for a recent review, see O'Konski (1976)]. To the best of our knowledge, this is the first instance of its application to intramembrane dynamic processes. We do not imply, however, that we are the first ones to conceive of its application to the problem on hand. In fact, some 20 years ago, Tinoco & Yamaoka (1959) have suggested a possibility of studying the lateral diffusion of a mobile ion over the surface of axially symmetric macromolecules dissolved in aqueous solution. They have formulated the theory of electric field induced birefringence of macromolecules which exhibit a slowly induced dipole (SID) moment ascribed to the field-driven displacement of ions in addition to the permanent and usual induced dipole moments. By "slowly" and "usual", we mean that the time rate of inducing a dipole is comparable to and much faster than that of the global rotation of the body in question. Their theory points out that the time constant of the SID can be estimated if it is comparable to that of the

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[1] Abbreviations used: ROS, rod outer segment; DMV, disk membrane vesicles; SID, slowly induced dipole; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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