Lateral Mobility of Phospholipid and Cholesterol in the Human Erythrocyte Membrane: Effects of Protein–Lipid Interactions†

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ABSTRACT: The phospholipid and cholesterol derivatives *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and N^1-cholesterylcarbamoyl-N^8-(7-nitro-2,1,3-benzoxadiazol-4-yl)-3,6-dioxaoctane-1,8-diamine (NBD-Chol), respectively, were incorporated into egg phosphatidylcholine/cholesterol multilamellar liposomes, human erythrocyte ghost membranes, and multilamellar liposomes derived from extracted human erythrocyte membrane lipids. The lateral mobility of these probes in the plane of the various membranes was measured by using the fluorescence photobleaching recovery technique. NBD-PE and NBD-Chol manifested identical lateral mobilities in egg phosphatidylcholine/cholesterol multilamellar liposomes over the range of temperatures from 10 to 37 °C and the range of cholesterol mole fractions from 0.0 to 0.5, and in erythrocyte ghost membranes and erythrocyte membrane lipid-derived multilamellar liposomes over the range of temperatures from 15 to 37 °C. The weak temperature dependence of the lateral diffusion coefficients of the lipid probes in both artificial and erythrocyte ghost membranes is consistent with the lack of a phase transition in any of these systems over the temperature range studied. Both NBD-PE and NBD-Chol diffuse 4-fold faster in liposomes derived from extracted erythrocyte membrane lipids ($D = 8.0 \times 10^{-9}$ cm^2 s^{-1} at 37 °C) than in the ghost membranes themselves ($D = 2.1 \times 10^{-9}$ cm^2 s^{-1} at 37 °C), suggesting a significant restriction of lipid lateral mobility by membrane protein in the human erythrocyte membrane.

The fluidity of biological and artificial lipid bilayers is a fundamental tenet of modern membrane study (Singer & Nicolson, 1972). Lateral mobility of protein components in the plane of the membrane is critical for such membrane phenomena as patching and capping of surface immunoglobulin on lymphocyte cell surfaces (Schreiner & Unanue, 1976) and receptor-mediated endocytosis (Goldstein et al., 1979; King & Cuatrecasas, 1981); lateral diffusion of lipid in the membrane environment may allow proteins to undergo conformational changes necessary for receptor and transport functions. It has become increasingly evident that, in many biological membranes, transmembrane protein mobility is regulated by submembranous protein networks or cytoskeletons (Ash et al., 1977; Bourguignon & Singer, 1977; Flanagan & Koch, 1978; Fowler & Bennett, 1978; Golan & Veatch, 1980, 1982; Henis & Elson, 1981; Koppel et al., 1981; Nigg & Cherry, 1980; Schlessinger et al., 1977; Sheetz et al., 1980; Tank et al., 1982; Tokuyasu et al., 1979). Membrane-associated factors governing lipid mobility in biological membranes include cholesterol content (Searls & Edidin, 1981; Thompson & Axelrod, 1980), phospholipid composition (Axelrod et al., 1978), and protein content (Jacobson et al., 1981) of the membranes; these factors have been systematically studied in several reconstituted artificial bilayer systems (Alecio et al., 1982; Derzko & Jacobson, 1980; Rubenstein et al., 1979; Smith et al., 1979; Wu et al., 1977).

The role of membrane proteins in restricting lipid mobility in biological membranes is accessible experimentally by a comparison between the mobility of lipid in the native membrane and that in liposomes prepared from total lipid extracts of the native membrane. We have employed such a strategy to investigate the effect of membrane protein removal on the lateral mobility of lipids in the human erythrocyte membrane, using the technique of fluorescence photobleaching recovery (Axelrod et al., 1976; Koppel et al., 1976). Fluorescent lipid probes used in this study include *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE),[1] a phospholipid derivative, and NBD-cholesterol, a novel cholesterol derivative (Rando et al., 1982). We have previously demonstrated the ability of these derivatives to distinguish the lateral diffusion of phospholipid from that of cholesterol in the same model membrane system, that comprising dimyristoylphosphatidylcholine/cholesterol multilamellar liposomes (Alecio et al., 1982). In the present study, we extend these observations to a second model membrane system, composed of egg phosphatidylcholine/cholesterol multilamellar liposomes, and to the human erythrocyte membrane.

Materials and Methods

Egg yolk phosphatidylcholine (egg PC) and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) were purchased from Avanti Biochemicals. Cholesterol, pepstatin A, and sodium ethylenediaminetetraacetate (EDTA)

† From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, and the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520. *Received June 20, 1983.* This work was supported by National Science Foundation Grant PCM 74-27214 (to R.R.R.) and National Institutes of Health Grant AI 15311 (to W.R.V.). W.R.V. was supported by National Institutes of Health Research Career Development Award AI 00320, and D.E.G. was supported by National Institutes of Health Medical Scientist Training Program Grant 5-T32-GM-07205. This work represents part of a dissertation presented by D.E.G. to Yale University in partial fulfillment of the requirements for the Ph.D. degree. Portions of this work were presented at the International Workshop on the Biological Applications of Photobleaching Techniques, October 25–28, 1981, University of North Carolina, Chapel Hill, NC 27514.

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[1] Abbreviations: NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine; NBD-Chol, N^1-cholesterylcarbamoyl-N^8-(7-nitro-2,1,3-benzoxadiazol-4-yl)-3,6-dioxaoctane-1,8-diamine; egg PC, egg yolk phosphatidylcholine; EDTA, sodium ethylenediaminetetraacetate; PMSF, phenylmethanesulfonyl fluoride; FPR, fluorescence photobleaching recovery; DMPC, dimyristoylphosphatidylcholine.

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were from Sigma. Phenylmethanesulfonyl fluoride (PMSF) was purchased from Eastman. Sephadex G-25 was obtained from Pharmacia. NBD-Chol, N^1-cholesterylcarbamoyl-N^8-(7-nitro-2,1,3-benzoxadiazol-4-yl)-3,6-dioxaoctane-1,8-diamine, was prepared as described (Rando et al., 1982). Unless otherwise indicated, the buffer for hydration was composed of 10 mM sodium phosphate and 150 mM sodium chloride, adjusted to pH 7.2, which had been purged with nitrogen for at least 20 min.

Labeling of Egg PC/Cholesterol Multilamellar Liposomes. The labeling procedure was modified from that of Smith et al. (1980) as follows. Solutions of egg PC and cholesterol (if required) in chloroform were mixed to give a total of 1.5 μmol of lipid. A solution of either NBD-PE or NBD-Chol (2.2 nmol, 0.15 mol %) in chloroform was added. After thorough mixing, the solvent was removed at 20 °C by using a rotary evaporator. The mixed lipids were then redissolved in fresh chloroform (200 μL) and again rotoevaporated to a thin film. Traces of solvent were removed by keeping the sample under a pressure of approximately 0.5 torr at 20 °C for at least 8 h. The thin lipid film was handled under nitrogen at all times to prevent oxidation of lipids. After thorough solvent removal, the lipid mixture was hydrated under nitrogen in 1 mL of buffer for 15 min at 40 °C and then vortexed vigorously for 15 s. This procedure produced a suspension of uniformly fluorescent multilamellar liposomes (diameter >30–40 μm) which was stored on ice and used in FPR experiments within several hours.

Preparation and Labeling of Multilamellar Liposomes from Extracted Erythrocyte Membrane Lipids. Erythrocyte membrane lipids were extracted and purified as described (Siakotos & Rouser, 1965; Turner & Rouser, 1974). Human erythrocytes were isolated from fresh human blood anticoagulated with acid citrate–dextrose by sedimentation at 1*g* in 0.9% NaCl and 0.75% (w/v) dextran 70 at room temperature for 60 min (Boyam, 1968). The resulting cells were washed 3 times in phosphate-buffered saline (10 mM sodium phosphate and 128 mM sodium chloride), pH 7.4, to which 1 mM EDTA had been added, at 4 °C. The erythrocytes were then lysed in 750 mL of 5 mM sodium phosphate, pH 7.4, to which 1 mM EDTA and 60 μM PMSF (dissolved at 100 mg/mL in methanol) had been added, for 30 min. Following lysis, the ghosts were pelleted by centrifugation at 21500*g* for 25 min and washed at least 4 times with 40 volumes of the lysis buffer. All operations were performed at 0–4 °C unless otherwise stated.

White erythrocyte ghosts in 5 mM sodium phosphate, 1 mM EDTA, 60 μM PMSF, and 1 μg/mL pepstatin A, pH 7.4, were then extracted twice with chloroform/methanol (2:1 v/v), once with chloroform/methanol (1:2 v/v), and finally with chloroform/methanol (7:1 v/v) saturated with aqueous ammonium hydroxide and all four extracts pooled. The product was chromatographed on a washed Sephadex G-25 column by using mixtures of chloroform/methanol/water to elute as described (Siakotos & Rouser, 1965). After chromatography, the solvent was removed by rotary evaporation and the residue stored in fresh chloroform under nitrogen at −20 °C. Total lipid phosphate (Ames, 1966) and total cholesterol (Courchaine et al., 1959; Zlatkis et al., 1963) were assayed as described.

Solutions of total extracted erythrocyte membrane lipids (3.4 μmol of total lipid) in chloroform and either NBD-PE or NBD-Chol (2.2 nmol, 0.06 mol %) in chloroform were mixed, and the solvent was removed at 20 °C by using a rotary evaporator. The labeled thin lipid film was then washed once in chloroform, dried, hydrated, and vortexed as described above, and the multilamellar liposomes were used in FPR experiments within several hours. Buffers for hydration included phosphate-buffered saline, pH 7.2 (see above), and 14 mM sodium phosphate, 1 mM EDTA, 60 μM PMSF, and 1 μg/mL pepstatin A, pH 7.4.

Labeling of Erythrocyte Ghosts. White human erythrocyte ghosts were prepared as described above with a final wash in 40 mM sodium phosphate, 1 mM EDTA, 60 μM PMSF, and 1 μg/mL pepstatin A, pH 7.4. Unless otherwise indicated, this buffer was used for all experiments with labeled ghosts.

NBD-PE Labeling. NBD-PE (110 nmol) was dried from a stock solution in chloroform, 200 μL of sodium phosphate buffer was added to the thin film, and the mixture was sonicated briefly at 10 °C until clarified. Packed ghosts (200 μL, 7.1 mg of protein/mL) were added, and the sample was stirred gently at 20 °C for 20 min. The labeled ghosts were washed twice in approximately 75 volumes of the phosphate buffer, resulting in uniformly fluorescent biconcave disks which were used immediately in FPR experiments or stored under nitrogen at 0 °C for up to 2 days before use. For analytical purposes, a small amount of labeled ghost sample was incubated at 4 °C in 50 volumes of 10 mM triethanolamine, pH 7.6, for 30 min and washed twice in the same buffer to remove all traces of inorganic phosphate. Total lipid phosphate (Ames, 1966) and total cholesterol (Courchaine et al., 1959; Zlatkis et al., 1963) were then assayed as described. Incorporated fluorophore was estimated from the absorbance at 462 nm of an ethanol extract of the labeled ghosts which had been dried for 5 h at 20 °C under vacuum, assuming an extinction coefficient of 19 500 cm^{-1} M^{-1} for the fluorophore in ethanol at 462 nm (R. Alecio, unpublished results).

NBD-Chol Labeling. NBD-Chol (410 nmol) was mixed with 80 μL of the total extracted erythrocyte membrane lipid solution (2.7 μmol of total lipid) in chloroform, and the solvent was removed at 20 °C by using a rotary evaporator. The labeled lipid film was washed once in chloroform and dried, and then hydrated and vortexed in 400 μL of phosphate buffer. The resulting labeled liposomes were stirred gently at 20 °C for 2 h with packed white erythrocyte ghosts in 40 mM sodium phosphate (400 μL, 7.1 mg of protein/mL), and the suspension was washed twice in approximately 75 volumes of phosphate buffer. This procedure yielded faintly fluorescent biconcave disks which were used immediately in FPR experiments.

Fluorescence Photobleaching Recovery. The optics and electronics employed in fluorescence photobleaching recovery measurements have been described (Golan, 1982). Briefly, a 4-W argon ion laser (Spectra-Physics Model 164-08) tuned to 488 nm was used as the excitation source for a fluorescence microscope (Leitz Diavert) equipped for incident light (Ploem) illumination. The beam was focused to a waist at the secondary image plane of the microscope by a weak (f = 500 mm) planoconvex lens (Melles-Griot), and to another waist at the specimen plane by a 100× or 40× microscope objective. A pinhole (0.51-mm diameter) was placed in the light path at the secondary image plane of the microscope in order to improve the Gaussian beam profile.

Emitted light was collected by the objective and filtered by a dichroic mirror (Leitz TK510) and a suppression filter (Leitz K510) to eliminate most of the backscattered and reflected excitation light. The emission was then passed through a series of alignment lenses (Leitz MPV-1) and a monochromator (Schoeffel QPM 30) to an extended S-20 photomultiplier tube (EMI 9558) which was thermoelectrically cooled (Schoeffel DP-5001-2T) and driven by a stabilized high-voltage power

supply (Schoeffel M600). An adjustable field diaphragm placed in the image plane was used to discriminate against fluorescence from regions other than the sample plane of interest. The photocurrent was fed into an electrometer (Schoeffel M600) and the output read by an XY recorder operated in the time-base mode (Hewlett-Packard 7004B). A dark current of 1×10^{-9} A at a power of 1100 V was obtained with thermoelectric cooling.

A series of three microprocessor-controlled (MOS.KIM-1) electromechanical shutters (Ilex) were used in combination with an interferometer (Ealing) to control the measuring and bleaching pulses. The interferometer served to split the excitation laser beam into two parts. One part passed twice through a neutral-density filter (OD 2.0, Fish-Schurman), providing a measuring beam which had been attenuated by 10^4. The other part was normally stopped by a closed shutter, which could be opened on command to the microprocessor to deliver a brief (10–150 ms), unattenuated bleaching pulse. This arrangement allowed the production of bleaching pulses without movement of any optical elements, thus ensuring the coincidence of bleaching and measuring laser beams to a high degree of precision (Koppel, 1979; Barisas, 1980; Barisas & Leuther, 1979). Excitation Gaussian laser beam diameters at the sample plane were determined to be 1.8 and 4.4 μm for the 100× and 40× objectives, respectively (Axelrod et al., 1976). Photobleaching power at the sample was approximately 2 mW.

Samples containing multilamellar liposomes or erythrocyte ghosts in suspension were deoxygenated in a sealed, purged glovebag by a stream of nitrogen for 60 min at room temperature in the dark. One to two microliters of sample was sealed with epoxy on a microscope slide in the glovebag, as described (Golan et al., 1980). Deoxygenation served to eliminate adverse photochemical effects mediated by singlet oxygen (Nigg et al., 1979; Lepock et al., 1978; Sheetz & Koppel, 1979). Sample temperatures (T) during the FPR experiment were controlled to ±0.5 °C by using a water-jacketed (Lauda) aluminum microscope slide holder. Diffusion coefficients (D) and fractional recoveries of fluorescence [$f(\infty)$] were routinely calculated from FPR records by a simple three-point fitting procedure (Axelrod et al., 1976). In all cases, FPR data tracings for both fluorescent probes were well fit by theoretical curves describing lateral diffusion of a single diffusing species (Axelrod et al., 1976).

Results

Composition of Samples. It was surprisingly difficult to label the erythrocyte ghosts with a large enough concentration of NBD-Chol to perform the FPR experiment. Sonication of a suspension of the probe in phosphate buffer followed by incubation with ghosts did not result in detectable labeling (data not shown). Exchange of NBD-Chol from liposomes prepared from extracted erythrocyte lipids, a procedure employed with this probe in several other cell types with great success (R. R. Rando and F. W. Bangerter, unpublished experiments), proceeded only to a limited degree in the experiments reported herein involving erythrocyte ghosts. Such exchange most likely involves a water-soluble intermediate rather than a fusion event between liposome and ghost membrane (Backer & Dawidowicz, 1981; McLean & Phillips, 1981; Nichols & Pagano, 1981), implying that the problem with exchange in the present case occurs in either the release of NBD-Chol from the liposome or the insertion of the solubilized probe into the ghost membrane. The latter possibility is made less likely by the observation that the cholesterol content of erythrocyte ghost membranes can be varied markedly by equilibration with phospholipid dispersions containing the appropriate level of cholesterol (Lange et al., 1980). Since NBD-Chol does, in fact, readily exchange from simple phospholipid/cholesterol dispersions into cell membranes of several other types (R. R. Rando and F. W. Bangerter, unpublished experiments), there may well be a unique interaction between the probe and the particular mixture of lipids in the erythrocyte membrane which prevents efficient solubilization (release) of the cholesterol derivative from the erythrocyte lipid liposomes.

Egg PC/cholesterol multilamellar liposomes were prepared with fluorophore (NBD-PE or NBD-Chol) at a final concentration of 0.15 mol %. Multilamellar liposomes from extracted erythrocyte membrane lipids contained fluorophore at a final concentration of 0.06 mol %; these liposomes had a cholesterol/phospholipid (Chol/PL) molar ratio of 0.75. Erythrocyte ghosts labeled with NBD-PE were found to have a fluorophore concentration of 0.67 mol % and a Chol/PL molar ratio of 1.00. This ratio compares favorably with that found in unlabeled ghosts (1.11). It thus appears that labeling with fluorophore does not change the overall lipid composition of the erythrocyte membrane. The Chol/PL molar ratio and final concentration of fluorophore in NBD-Chol-labeled ghosts could not be determined quantitatively, given the necessity of adding the probe in the presence of exogenous lipid. It is expected, however, that exchange of the probe from erythrocyte lipids to ghosts would proceeed without significant change in the Chol/PL molar ratio of the resultant labeled ghosts. The final NBD-Chol concentration can be estimated to be 0.1–0.2 mol % in the labeled ghosts from a comparison of the fluorescence intensity of a typical NBD-Chol-labeled ghost with that of a typical NBD-PE-labeled ghost in the fluorescence microscope (data not shown).

Lateral Mobility of NBD-PE and NBD-Chol in Egg PC Liposomes: Temperature Dependence. The two lipid probes manifested identical lateral mobility behavior in egg PC multilamellar liposomes (Figure 1). The lateral diffusion coefficients showed a very weak temperature dependence, as expected for diffusion in a heterogeneous mixture of various phosphatidylcholines. In particular, there was no recognizable phase transition over the temperature range 10–37 °C, and diffusion was rapid over the entire range of temperatures (Figure 1). NBD-PE and NBD-Chol diffusion coefficients in egg PC liposomes were identical with those in DMPC liposomes at temperatures greater than T_m, the main phase transition temperature for this homogeneous saturated phospholipid (Figure 1; Alecio et al., 1982).

Lateral Mobility of NBD-PE and NBD-Chol in Mixtures of Egg PC and Cholesterol: Composition Dependence. As in egg PC multilamellar liposomes, the phospholipid and cholesterol probes manifested identical lateral mobility behavior in multilamellar liposomes composed of egg PC and cholesterol over the entire composition range from $X_{Chol} = 0.0$ to $X_{Chol} = 0.5$ (Figure 2, Table I). Diffusion coefficients showed a gradual 2-fold decrease with increasing cholesterol mole fraction at both 15 and 37 °C; this may be compared with the 3-fold decrease exhibited by the two probes in binary mixtures of DMPC and cholesterol above the main phase transition temperature for the bulk phospholipid (Figure 2; Alecio et al., 1982).

Lateral Mobility of NBD-PE and NBD-Chol in Erythrocyte Ghost Membranes and Ghost Membrane Lipids. In multilamellar liposomes prepared from total lipid extracts of erythrocyte ghost membranes, NBD-PE and NBD-Chol manifested identical lateral mobilities within the temperature

Table I: Lateral Mobility of NBD-PE and NBD-Chol in Egg PC/Cholesterol Multilamellar Liposomes[a]

		NBD-PE			NBD-Chol		
temp (°C)	X_{Chol}[b]	$D \times 10^8$ (cm² s⁻¹)	$f(\infty)$	N	$D \times 10^8$ (cm² s⁻¹)	$f(\infty)$	N
10	0.0	1.8 ± 0.2	0.84 ± 0.09	4	1.7 ± 0.2	0.90 ± 0.02	4
	0.1	1.8 ± 0.2	0.88 ± 0.03	4	1.6 ± 0.2	0.84 ± 0.01	4
15	0.0	1.9 ± 0.3	0.88 ± 0.08	4	1.7 ± 0.3	0.92 ± 0.03	5
	0.1	1.8 ± 0.2	0.98 ± 0.01	4	1.8 ± 0.2	0.87 ± 0.02	4
	0.2	1.5 ± 0.2	0.90 ± 0.08	4	1.6 ± 0.2	0.89 ± 0.06	4
	0.3	1.1 ± 0.1	0.94 ± 0.02	4	1.1 ± 0.1	0.97 ± 0.02	3
	0.4	0.9 ± 0.1	0.94 ± 0.11	2	0.8 ± 0.0	0.91 ± 0.07	2
	0.5	1.0 ± 0.1	0.83 ± 0.14	3	1.0 ± 0.1	0.87 ± 0.04	4
21	0.0	2.1 ± 0.3	0.91 ± 0.04	4	2.3 ± 0.1	0.93 ± 0.08	4
	0.5	1.0 ± 0.1	0.95 ± 0.03	3	0.9 ± 0.1	0.97 ± 0.03	4
37	0.0	2.8 ± 0.3	0.87 ± 0.04	3	2.8 ± 0.2	0.94 ± 0.01	4
	0.1	2.2 ± 0.4	0.97 ± 0.01	4	2.6 ± 0.2	0.87 ± 0.01	3
	0.2	2.4 ± 0.3	0.94 ± 0.02	4	2.5 ± 0.6	0.87 ± 0.03	4
	0.3	1.8 ± 0.1	0.92 ± 0.01	4	2.0 ± 0.1	0.96 ± 0.04	3
	0.4	1.7 ± 0.1	0.90 ± 0.05	2	1.8 ± 0.5	0.96 ± 0.03	3
	0.5	1.6 ± 0.3	0.93 ± 0.04	3	1.4 ± 0.2	0.98 ± 0.02	4

[a] Measurements at a given cholesterol mole fraction were performed on identical liposome preparations, on the same day. Measurements are reported as mean ± SD. [b] Cholesterol mole fraction.

Table II: Lateral Mobility of NBD-PE and NBD-Chol in Extracted Erythrocyte Lipid Multilamellar Liposomes[a]

		NBD-PE			NBD-Chol		
buffer	temp (°C)	$D \times 10^9$ (cm² s⁻¹)	$f(\infty)$	N	$D \times 10^9$ (cm² s⁻¹)	$f(\infty)$	N
b	15	4.8 ± 0.2	0.91 ± 0.05	3	4.9 ± 0.4	0.98 ± 0.01	4
	21	5.6 ± 0.5	0.92 ± 0.04	2	5.2 ± 0.8	0.95 ± 0.02	4
	37	7.1 ± 1.4	0.88 ± 0.03	4	8.5 ± 0.7	0.88 ± 0.02	3
c	15	5.8 ± 0.4	0.90 ± 0.04	4	5.4 ± 0.1	0.93 ± 0.06	4
	21	5.7 ± 0.3	0.94 ± 0.02	4	5.4 ± 0.3	0.91 ± 0.04	4
	37	8.5 ± 0.7	0.95 ± 0.01	4	7.8 ± 0.6	0.94 ± 0.01	4

[a] Measurements are reported as mean ± SD. [b] PBS: 10 mM sodium phosphate and 150 mM NaCl, pH 7.2. [c] 14 mM sodium phosphate, 1 mM EDTA, 60 μM PMSF, and 1 μg/mL pepstatin A, pH 7.4.

Table III: Lateral Mobility of NBD-PE and NBD-Chol in Erythrocyte Ghost Membranes[a,b]

	NBD-PE			NBD-Chol		
temp (°C)	$D \times 10^9$ (cm² s⁻¹)	$f(\infty)$	N	$D \times 10^9$ (cm² s⁻¹)	$f(\infty)$	N
15	1.3 ± 0.2	0.79 ± 0.06	5	1.3 ± 0.1	0.81 ± 0.06	4
21	1.6 ± 0.4	0.77 ± 0.06	5	1.5 ± 0.1	0.75 ± 0.04	3
37	2.0 ± 0.3	0.80 ± 0.02	4	2.2 ± 0.7	0.68 ± 0.10	4

[a] Measurements are reported as mean ± SD. [b] Buffer comprised 40 mM sodium phosphate, 1 mM EDTA, 60 μM PMSF, and 1 μg/mL pepstatin A, pH 7.4.

range 15–37 °C. Diffusion coefficients and fractional recoveries of fluorescence had no dependence on ionic strength for either probe (Table II). As seen in the model systems egg PC and egg PC/cholesterol (1:1 mol/mol), the temperature dependence of the diffusion coefficient was small (less than 2-fold increase from 15 to 37 °C); fractional recoveries of the probes were greater than 0.90 in all cases (Figure 3, Table II).

Detailed comparisons between lateral mobility parameters for NBD-PE and NBD-Chol in erythrocyte ghost membranes were made in 40 mM sodium phosphate buffer. Under these conditions, the cholesterol and phospholipid probes exhibited identical lateral mobility between 15 and 37 °C (Figure 3, Table III). Again, the temperature dependence of the diffusion coefficient was small; fractional recoveries of fluorescence averaged 0.79 for NBD-PE and 0.75 for NBD-Chol in this buffer system.

There was no significant ionic strength dependence of the lateral mobility parameters of NBD-PE in erythrocyte ghost membranes. Buffers employed in this study included isotonic phosphate-buffered saline, pH 7.2, as well as 14 and 40 mM sodium phosphate to which 1 mM EDTA, 60 μM PMSF, and 1 μg/mL pepstatin A at pH 7.4 had been added (Table IV).

Table IV: Effect of Ionic Strength on the Lateral Mobility of NBD-PE in Erythrocyte Ghost Membranes[a]

buffer	temp (°C)	$D \times 10^9$ (cm² s⁻¹)	$f(\infty)$	N
b	15	1.0 ± 0.3	0.82 ± 0.03	4
	21	1.4 ± 0.2	0.82 ± 0.05	4
	30	2.1 ± 0.3	0.84 ± 0.00	3
	37	2.2 ± 0.4	0.84 ± 0.02	3
c	15	1.1 ± 0.2	0.88 ± 0.04	4
	21	1.4 ± 0.3	0.83 ± 0.02	5
	30	2.0 ± 0.5	0.86 ± 0.02	4
	37	2.2 ± 0.3	0.86 ± 0.04	5
d	15	1.3 ± 0.2	0.79 ± 0.06	5
	21	1.6 ± 0.4	0.77 ± 0.06	5
	37	2.0 ± 0.3	0.80 ± 0.02	4

[a] Measurements are reported as mean ± SD. [b] PBS: 10 mM sodium phosphate and 150 mM NaCl, pH 7.2. [c] 14 mM sodium phosphate, 1 mM EDTA, 60 μM PMSF, and 1 μg/mL pepstatin A, pH 7.4. [d] 40 mM sodium phosphate, 1 mM EDTA, 60 μM PMSF, and 1 μg/mL pepstatin A, pH 7.4.

Discussion

In this study, the lateral mobilities of the phospholipid derivative NBD-PE and the cholesterol derivative NBD-Chol in model membranes and human erythrocyte ghost membranes have been examined. Comparing NBD-PE and NBD-Chol mobility in egg phosphatidylcholine based multilamellar liposomes with that in dimyristoylphosphatidylcholine-based liposomes (Figures 1 and 2; Alecio et al., 1982), it can be seen that both probes exhibited relatively slow diffusion ($D < 10^{-9}$

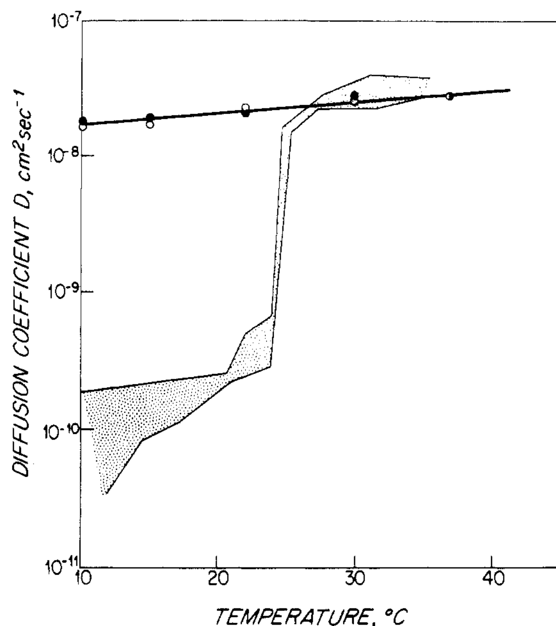

FIGURE 1: Lateral diffusion coefficient of NBD-phosphatidylethanolamine (closed circles) and NBD-cholesterol (open circles) in egg phosphatidylcholine multilamellar liposomes as a function of temperature. Lipid probes were added to a final mole fraction of 0.15%. Measurements at a given temperature were performed on identical liposome preparations on the same day. Each point represents the average of three to five independent measurements. Standard deviations for all points are within the width of the symbols. There is no significant difference between the diffusion coefficients of the two probes at any temperature examined. Fractional recoveries of fluorescence were 0.88 ± 0.06 (mean ± SD) for NBD-phosphatidylethanolamine and 0.83 ± 0.04 for NBD-cholesterol; these were not significantly different at any temperature. For comparison, the range of values of the lateral diffusion coefficient of the lipid probes in dimyristoylphosphatidylcholine multilamellar liposomes is shown [stippled area; see Figure 1 of Alecio et al. (1982)].

$cm^2\ s^{-1}$) in membranes comprised mainly of "solid" lipid regions (i.e., DMPC/cholesterol liposomes at $X_{Chol} < 0.20$ and $T < T_m$) and relatively fast diffusion ($D > 10^{-9}$ $cm^2\ s^{-1}$) in membranes containing large amounts of "fluid"-phase lipid (i.e., DMPC/cholesterol liposomes at $X_{Chol} \geq 0.20$ and/or $T > T_m$, and all egg PC/cholesterol liposomes). Increasing temperature led to increases in the lateral diffusion coefficient of both probes in all liposome systems, with sharp 50-fold changes at the main phase transition temperature of the bulk phospholipid (T_m) in DMPC liposomes. The mole fraction of cholesterol (X_{Chol}) in binary mixtures of phospholipid (DMPC or egg PC) and cholesterol was directly related to the lateral diffusion coefficient of both probes at a temperature below T_m and inversely related to D at temperatures above T_m; sharp 10-fold increases in the diffusion rate at $X_{Chol} \sim 0.20$ and $T < T_m$ in DMPC/cholesterol liposomes were also observed. NBD-PE and NBD-Chol manifested identical lateral mobilities in all model systems except DMPC liposomes to which 5–20 mol % cholesterol had been added, at temperatures below T_m. Under these conditions, the cholesterol probe consistently diffused 60–80% faster than the phospholipid probe. Lateral mobility parameters for the phospholipid probe NBD-PE obtained in the present study agree, both qualitatively and quantitatively, with those presented in the literature for model membrane systems composed of egg PC and egg PC/cholesterol multibilayers (Derzko & Jacobson, 1980; Wu et al., 1977).

The concordance between the lateral mobilities of NBD-Chol and NBD-PE in the various model membranes under nearly all conditions implies that this novel fluorescent cho-

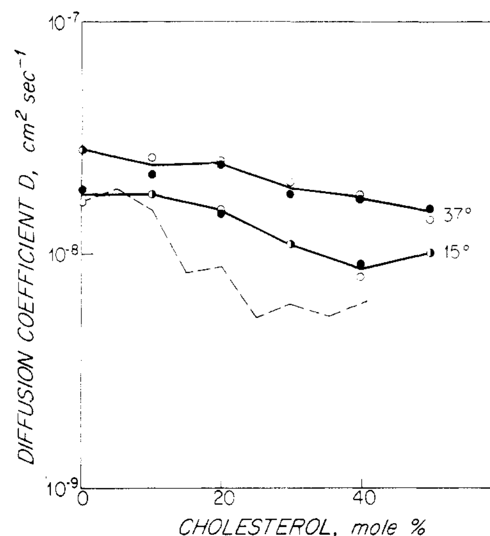

FIGURE 2: Lateral diffusion coefficient of NBD-phosphatidylethanolamine (closed circles) and NBD-cholesterol (open circles) in mixtures of egg phosphatidylcholine and cholesterol as a function of cholesterol mole fraction at two different temperatures. Measurements at any given lipid composition were performed on identical liposome preparations on the same day. Individual points represent the average of two to five independent measurements (see Table I). There is no significant difference between the diffusion coefficients of the two probes at any lipid composition at either temperature. For comparison, the mean value of the lateral diffusion coefficient of the lipid probes in binary mixtures of dimyristoylphosphatidylcholine and cholesterol above the main phase transition temperature for the bulk phospholipid is shown [dashed line; see Figure 2 of Alecio et al. (1982)].

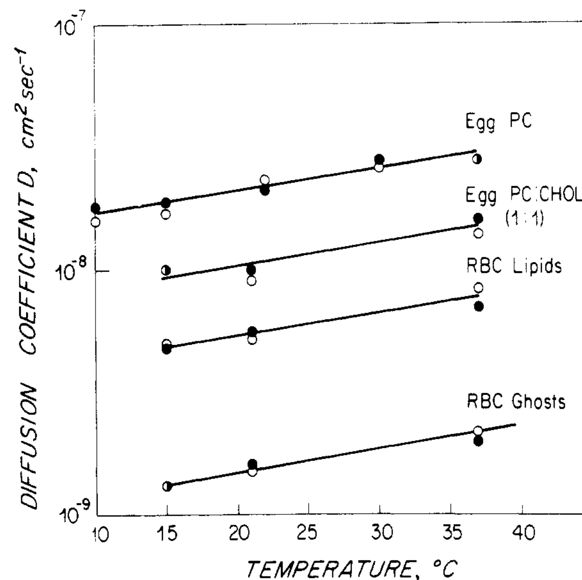

FIGURE 3: Lateral diffusion coefficient of NBD-phosphatidylethanolamine (closed circles) and NBD-cholesterol (open circles) in egg phosphatidylcholine multilamellar liposomes (egg PC), egg phosphatidylcholine/cholesterol (1:1 mol/mol) multilamellar liposomes [egg PC:CHOL (1:1)], multilamellar liposomes prepared from total lipid extracts of erythrocyte ghost membranes (RBC lipids), and erythrocyte ghost membranes (RBC ghosts) as a function of temperature. Lipid probes were added to final mole fractions of 0.15% in the egg PC and egg PC:CHOL (1:1) samples, 0.06% in the RBC lipids sample, and 0.67% (NBD-PE) or approximately 0.15% (NBD-Chol) in the RBC ghosts sample (see text). Measurements at a given temperature were performed on identical liposome or ghost preparations on the same day. Buffers comprised 10 mM sodium phosphate and 150 mM NaCl, pH 7.2, for all samples except RBC ghosts for which the buffer comprised 40 mM sodium phosphate, 60 μM PMSF, 1 mM EDTA, and 1 μg/mL pepstatin A, pH 7.4. Each point represents the average of two to five independent measurements (see Tables I–III). There is no significant difference between the diffusion coefficients of the two lipid probes under any conditions.

lesterol derivative functions as a true probe of lipid (and more specifically, cholesterol) mobility [see Alecio et al. (1982) for further discussion of this point]. Further, this agreement suggests that any significant difference between the diffusion coefficients of NBD-Chol and NBD-PE in a particular membrane system reflects a real distinction between the environments of the bulk (unlabeled) cholesterol and phospholipid. Such a difference was found in a previous study for DMPC/cholesterol liposomes at cholesterol mole fractions between 5 and 20% and temperatures less than the main phase transition temperature for the phospholipid (Alecio et al., 1982). That this difference was due to the addition of bulk cholesterol to DMPC and not to temperature alone is evident from the observation that tracer quantities of NBD-PE and NBD-Chol had identical lateral mobilities in pure DMPC liposomes at temperatures both above and below T_m. Low concentrations of cholesterol (5–20 mol %), when added to gel-phase DMPC, thus appear to partition in the membrane such that the bulk of the cholesterol is in a different physical environment from the bulk of the phospholipid. Two models for the molecular packing in binary mixtures of DMPC and cholesterol have recently appeared in the literature; both suggest a lateral phase separation between solid regions containing pure phospholipid and fluid regions containing phospholipid + 20 mol % cholesterol, under the conditions $T < T_m$ and $X_{Chol} < 0.20$. The first model postulates a separation based on alternating parallel bands of solid and fluid lipid (Owicki & McConnell, 1980) and is supported by freeze–fracture electron microscopic studies (Copeland & McConnell, 1980); the second hypothesizes unoriented islands of solid and fluid lipid (Snyder & Freire, 1980). From the present data, it is impossible to choose between these competing models, since they both would predict only a small difference between the measured diffusion coefficients of cholesterol and phospholipid in DMPC/cholesterol multilamellar liposomes using the FPR technique. Thus, both the large discrepancy between the size of the putative lipid domains (0.01–0.1 μm width) and the diameter of the bleaching laser beam (2 μm) and the spatial averaging over all possible domain orientations in a multilamellar system tend to minimize the measured difference between the diffusion coefficients of the two probes.

In contrast to the results obtained in DMPC/cholesterol liposomes at $X_{Chol} < 0.20$ and $T < T_m$ (in which lipid lateral mobility is greatly slowed by significant amounts of solid-phase lipid), cholesterol and phospholipid have the same lateral mobility in all model membranes composed of fluid-phase lipid only. This result is of interest in light of the hypothesis that coexisting, immiscible fluid domains may be present in model membranes under certain conditions (Recktenwald & McConnell, 1981). The present observations would imply either that cholesterol is present to the same extent in all fluid domains, that lipids in all of the domains have the same lateral mobility, or that the FPR technique is not sensitive enough to detect small differences in microenvironment between adjacent fluid lipid domains.

On the basis of the studies of NBD-PE and NBD-Chol mobility in DMPC-based and egg PC based multilamellar liposomes, it might be predicted that cholesterol and phospholipid should have identical lateral mobilities in the erythrocyte ghost membrane, which contains a heterogeneous mixture of lipids that are not organized into laterally segregated domains (Golan & Veatch, 1980; Kinosita et al., 1981; Kleinfeld et al., 1981). Indeed, phospholipid and cholesterol probes are found to have identical lateral mobilities in the erythrocyte ghost membrane, under all conditions of ionic strength and temperature examined. There is a small dependence of the lipid lateral diffusion coefficient on temperature over the range 15–37 °C, with no suggestion of a "break point" that would signify a membrane lipid phase transition in this temperature range. At 37 °C, the diffusion coefficient of the lipid probes is 2.1×10^{-9} cm^2 s^{-1}, or approximately 4-fold greater than the fastest diffusion of the major erythrocyte transmembrane protein, band 3, in the same membrane (Golan & Veatch, 1982).

Diffusion data for lipids in the human erythrocyte ghost membrane obtained in the present study agree, both quantitatively and qualitatively, with those available from the literature. Thompson & Axelrod (1980) and Kapitza & Sackmann (1980) independently measured the lateral mobility of the lipid probe diI (3,3′-dioctadecylindocarbocyanine) in the human erythrocyte ghost membrane, the former over the temperature range −3 to 40 °C and the latter over the range 10–45 °C. Both groups reported a small temperature dependence of the diffusion coefficient (1.5–2-fold change between 15 and 37 °C measurements); the diI diffusion coefficient at 20 °C was approximately $(1.0–1.5) \times 10^{-9}$ cm^2 s^{-1} in both cases. More recently, Koppel et al. (1981) observed a diffusion coefficient for NBD-PE lateral mobility of 1.4×10^{-8} cm^2 s^{-1} in the mouse erythrocyte ghost membrane at 23 °C. The order of magnitude difference between this and the results described for the human erythrocyte ghost may reflect a real interspecies difference in membrane fluidity.

Both NBD-PE and NBD-Chol diffuse 4-fold faster in liposomes prepared from chloroform/methanol extracts of erythrocyte ghost membranes ($D = 8.0 \times 10^{-9}$ cm^2 s^{-1}) than in the ghost membranes themselves. The mole fraction of cholesterol in the extracted lipid liposomes (0.43) differs only slightly from that in the ghost membranes (0.53), and the composition of extracted erythrocyte phospholipids obtained by the present methods (Turner & Rouser, 1974) is virtually identical with that reported by others in the literature (Rand & Luzzati, 1968). On the basis of the model system studies, such a small difference in membrane composition would not be sufficient to account for a 4-fold change in the lipid diffusion coefficient. It appears, rather, that the presence of membrane proteins restricts the lateral mobility of both phospholipid and cholesterol in the erythrocyte ghost membrane. This restriction could be mediated through a nonspecific reduction in membrane fluidity by the addition of membrane proteins to a pure lipid bilayer (Fraley et al., 1979; Hong & Hubbell, 1972; Rottem et al., 1970; Sefton & Gaffney, 1974), analogous to the "soldifying" effect of cholesterol on pure phospholipid bilayers above the main phase transition temperature. Alternatively, a specific interaction between both phospholipid and cholesterol and some slowly diffusing or immobile integral membrane protein might exist, leading to a slowing of the lipid diffusion rate. It is unlikely, however, that molecules as different as phospholipid and cholesterol should interact in exactly the same manner with membrane protein to produce the same degree of slowing of both lipid components.

A theoretical treatment of lipid lateral diffusion in membranes containing impermeable species such as gel-phase lipid or protein components has recently appeared (Saxton, 1982). Assumptions used in this theory include a random distribution of solid lipid or protein domains in a two-dimensional fluid membrane under steady-state conditions, and the absence of specific interactions between the diffusing lipid and the impermeable domains. This treatment may be applied to the human erythrocyte ghost membrane, in which transmembrane protein occupies an area fraction of approximately 0.17.[2] The

theoretically derived ratio between lipid diffusion coefficients in a protein-containing membrane whose fluid phase occupies an area fraction of 0.83 and the equivalent fluid-phase membrane without protein is 0.29 [see Figure 2 of Saxton (1982)]. The results of the present study ($2.1 \times 10^{-9}/8.0 \times 10^{-9} = 0.26$) are in excellent agreement with the theoretical calculation, suggesting that erythrocyte membrane proteins are, indeed, impermeable to and lacking in specific interactions with bulk-phase membrane phospholipid and cholesterol.

Restriction of lipid lateral mobility by membrane protein has also been shown by Jacobson et al. (1981), who compared the diffusion of the lipid probe diI in plasma membranes of human fibroblasts with that in multibilayers reconstituted from fibroblast plasma membrane lipids. Between 25 and 40 °C, diI diffusion was 4 times faster in the extracted lipid multibilayers than in the plasma membranes themselves, a difference identical with that observed in the present study. Qualitatively similar results were also obtained in studies of fluorescence polarization of the membrane probe diphenylhexatriene, in which greater anisotropy changes (interpreted as faster rotation) were observed in extracted membrane lipid bilayers from mouse LM cells (Gilmore et al., 1979) and human lymphocytes (Johnson & Kramers, 1978) than in the plasma membranes themselves.

While the total lipid composition of the extracted erythrocyte lipid multibilayers appears not to differ significantly from that of erythrocyte ghosts, the same cannot be claimed for the compositional asymmetries in lateral and transverse lipid organization of the two bilayer systems. It is well established that the various phospholipid classes are asymmetrically distributed in specific halves of the native erythrocyte membrane bilayer: choline-containing phospholipids (phosphatidylcholine, sphingomyelin) predominate in the outer monolayer while amino-containing phospholipids (phosphatidylethanolamine, phosphatidylserine) dominate the inner leaflet [reviewed in Op den Kamp (1979)]. Further, it is unlikely that such an asymmetry would spontaneously develop in multibilayers formed from thoroughly mixed total lipid extracts. It is therefore probable that, aside from their obvious differences in protein content, the extracted erythrocyte lipid multibilayers differ from the ghost membranes in at least their transverse organization of lipids. The importance of this difference in controlling lipid diffusion rates is unknown. Henis et al. (1982) labeled turkey erythrocyte membranes either externally and internally or externally alone with NBD-PE; they found differences in the lateral mobility of the probe in the two samples at various temperatures and interpreted these data as reflecting differences between the physical states of outer vs. inner leaflet lipids. Another report investigating the role of lipid asymmetry in regulating the physical properties of a natural biomembrane concerned the temperature dependence of lateral-phase separation in the rod outer segment membrane (Sklar & Dratz, 1980), a phenomenon which appears not to be present in the cholesterol-rich erythrocyte membrane (Golan & Veatch, 1980; Kinosita et al., 1981; Keinfeld et al., 1981).

Phospholipid and cholesterol diffusion in liposomes prepared from extracted erythrocyte lipids ($X_{\text{Chol}} = 0.43$) is very similar to that in the model liposome system egg PC + 50 mol % cholesterol. Phospholipid and cholesterol mobilities are identical within each membrane system, and the lateral diffusion coefficients exhibit the same small temperature dependence in the two membranes. The absolute diffusion coefficient of the lipid probes is approximately 2-fold greater in the egg PC/cholesterol membrane than in the extracted erythrocyte lipid membrane; this difference can probably be ascribed to the unusual lipid composition of the erythrocyte membrane.

In the present study, we have investigated the restricting effect of membrane protein on the lateral mobility of both phospholipid and cholesterol in the human erythrocyte membrane. The agreement between lateral mobility measurements on phospholipid and cholesterol derivatives in this membrane supports the notion that lipid domains and phase transitions in the membrane are absent over a wide range of temperatures. Future studies will explore in detail the relationships between transmembrane protein and lipid mobility in the native erythrocyte membrane and in membrane states in which the erythrocyte cytoskeleton has been specifically perturbed.

Acknowledgments

We thank Dr. Jordan Pober for critical review of the manuscript.

Registry No. NBD-Chol, 80685-24-1; cholesterol, 57-88-5.

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[2] This calculation assumes the erythrocyte membrane surface area to be 150 μm^2, the number of band 3 dimers per erythrocyte to be 5×10^5, and the radius of a single band 3 dimer to be 4 nm in its intramembranous portion. The equivalent radius of glycophorin, the other major erythrocyte transmembrane protein, is approximately 0.25 nm, and this molecule may therefore be ignored for the purposes of the calculation.

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