Asymmetry of Lipid Dynamics in Human Erythrocyte Membranes Studied with Impermeant Fluorophores†

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ABSTRACT: The synthesis, purification, and application of five membrane-impermeant derivatives of pyrene are described. Each probe consists of a membrane-impermeant moiety, either an oligosaccharide or glutathione, linked to pyrene via a connecting arm. Intact human erythrocytes and leaky ghost membranes prepared from them were treated with the probes to label, respectively, the outer membrane leaflet and both leaflets. Motional freedom of the pyrene fluorophores in the membrane was assessed by estimation of the steady-state polarization of fluorescence, the excited-state lifetime, and the excimer/monomer fluorescence intensity ratio. The fluorescence anisotropy of each impermeant derivative was lower in the outer as compared to the inner hemileaflet, whereas the corresponding excited-state lifetimes were similar. Excimer formation was consistently greater in the outer leaflet. The results demonstrate that the impermeant fluorophores experience greater motional freedom ("fluidity") in lipid domains of the outer as compared to the inner leaflet of the human erythrocyte membrane.

It is well established that the outer and inner leaflets of the erythrocyte membrane differ in lipid composition (Rothman & Lenard, 1977; Op den Kamp, 1979). In a number of animal species, the erythrocyte outer leaflet is relatively enriched in phosphatidylcholine and sphingomyelin, whereas the inner leaflet contains most of the phosphatidylethanolamine and essentially all of the phosphatidylserine. Lipid asymmetry has also been observed in other biological membranes, although the evidence is not as consistent or compelling as that reported for the erythrocyte (Op den Kamp, 1979). Given the widespread occurrence of lipid asymmetry, a number of investigators have sought to determine whether it implies a difference in physical state and relative motional freedom, i.e., "fluidity",[1] of the lipid components of the two leaflets. The issue is of considerable importance, since the physical state of the lipids is a significant determinant of the passive permeability of the membrane (de Gier et al., 1968; Papahadjopoulos et al., 1973) and is capable of modulating the functions of intrinsic membrane proteins responsible for many translocation and metabolic processes (Sandermann, 1978; Brasitus et al., 1979).

Rottem (1975) studied the electron spin resonance (ESR)[2] spectra of spin-labeled stearic acid probes in *Mycoplasma hominis* and *Acholeplasma laidlawii* and suggested that the outer leaflet is more fluid in each species. Wisnieski & Iwata (1977) compared a nitroxide derivative of decane, which is capable of "flip-flop" (Kornberg & McConnell, 1971) across the bilayer, with a glucosamine derivative of stearic acid 12-nitroxide, which is restricted in its ability to flip. Arrhenius studies of plasma membrane preparations derived from transformed mouse fibroblasts (LM cells) and embryonated chick cells (Newcastle disease virus envelopes) provided evidence for thermotropic transitions with different characteristic temperatures in the outer and inner leaflets. On this basis, the authors concluded that "at all temperatures, the outer membrane monolayer is probably less rigid than the inner monolayer." A similar conclusion for the LM cell plasma membrane was reached by Schroeder (1978), who studied the fluorescence polarization of β-parinaric acid in conjunction with selective quenching of the outer leaflet signal by reaction of exofacial amino groups with 2,4,6-trinitrobenzenesulfonic acid. ESR studies of human erythrocytes and ghost membranes treated with spin-labeled phospholipids, on the other hand, led Tanaka & Ohnishi (1976) to suggest "a more rigid phosphatidylcholine bilayer phase," presumably in the outer leaflet of this cell.

The present report describes the preparation of five membrane-impermeant, fluorescent derivatives of pyrene which were applied to assess lipid dynamics in the individual leaflets of the human erythrocyte membrane. The basic design of the probes, which has precedence in the membrane-impermeant maleimides (Abbott & Schachter, 1976) and spin-labels (Lepock et al., 1975; Mehlhorn & Packer, 1976; Wisnieski & Iwata, 1977), is that of a water-soluble, membrane-impermeant moiety, either glutathione or an oligosaccharide,

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[1] The term "lipid fluidity" as applied to anisotropic bilayer membranes has been used in different senses by various authors. Many investigators have applied it as a general term to express the relative motional freedom of the lipid molecules or substituents thereof, combining in the one term concepts of both rates of movement and extent of movement. As evaluated by steady-state fluorescence polarization of lipid fluorophores, this is equivalent to assessing "fluidity" via the fluorescence anisotropy, r, without further resolution of the components which determine r. Studies with 1,6-diphenyl-1,3,5-hexatriene have demonstrated that the rotations of this rodlike fluorophore are hindered in both artificial and natural membranes (Chen et al., 1977; Kawato et al., 1977; Veatch & Stryer, 1977; Glatz, 1978; Hildenbrand & Nicolau, 1979; Lakowicz et al., 1979a,b). Accordingly, the fluorescence anisotropy of such a probe is not described adequately by the Perrin equation (see text) but by a modified relationship (Jähnig, 1979; Heyn, 1979), $r = r_\infty + (r_0 - r_\infty)[\rho_c/(\rho_c + \tau)]$, where r_∞ is the limiting anisotropy observed after long time intervals in time-resolved anisotropy decay experiments, r_0 is the maximal limiting anisotropy, ρ_c is the correlation time, and τ is the excited-state lifetime. Jähnig (1979) and Heyn (1979) suggest that the term fluidity be applied only to changes in rates of rotation as assessed by ρ_c, whereas changes in r_∞ are related to an order parameter. For many current biological applications, changes in r, whether owing to ρ_c, r_∞, or both, are of significance, and a broad term is needed to designate them. We shall use the expression "motional freedom" in this general sense.

[2] Abbreviations used: ESR, electron spin resonance; PBH, 4-(1-pyrene)butyryl hydrazide; LPBH, pyrenebutyryl hydrazide derivative of lactose; RPBH, pyrenebutyryl hydrazide derivative of raffinose; SPBH, pyrenebutyryl hydrazide derivative of stachyose; GSH, glutathione; GS–PI, glutathione–pyrene I; GS–PII, glutathione–pyrene II; RBC, red blood cell; $NaDodSO_4$, sodium dodecyl sulfate; SH, sulfhydryl group.

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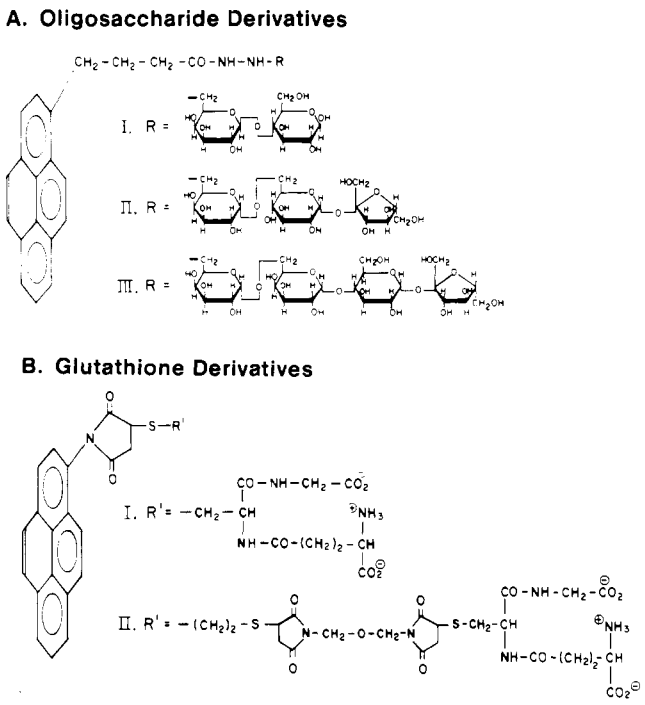

FIGURE 1: Structures of the impermeant pyrene-linked derivatives. The oligosaccharide derivatives (A) have membrane-impermeant substituents derived, respectively, from lactose (I), raffinose (II), and stachyose (III). The glutathione derivatives (B) differ from each other in the length and nature of the connecting arms.

linked to the pyrene fluorophore via an arm. The impermeant fluorophores were inserted into the membrane outer leaflet of intact human erythrocytes or into both leaflets of leaky ghost membranes. Estimations of the fluorescence polarization and extent of excimer formation (Vanderkooi & Callis, 1974; Galla & Sackmann, 1974; Dembo et al., 1979) indicate that the probes experience greater motional freedom in the outer as compared to the inner leaflet of the human erythrocyte membrane.

Experimental Procedures

Preparation of Oligosaccharide Derivatives. The structures of pyrene-linked derivatives of lactose, raffinose, and stachyose are illustrated in Figure 1A. For preparation of the compounds, the terminal galactosyl of each sugar was oxidized enzymatically at the C6 ($-CH_2OH$) position to an aldehyde and treated with pyrenebutyryl hydrazide (PBH) to form a Schiff's base, which was then reduced with borohydride to yield the oligosaccharide-substituted hydrazide. In a typical preparation, 49.5 μmol of the oligosaccharide was dissolved in 1.25 mL of 0.1 M potassium phosphate, pH 6.0. Approximately 10 units of *Dactylium dendroides* galactose oxidase (EC 1.1.3.9, Worthington Biochemical Corp.) dissolved in 1.25 mL of water were added, and the solution was stirred gently for 30 min at room temperature. The pH was then lowered to 5.6 with 0.1 N HCl and 16.5 μmol of 4-(1-pyrene)butyryl hydrazide (Molecular Probes, Inc., Plano, TX) dissolved in 2.5 mL of tetrahydrofuran added. The reaction mixture was stirred at 37 °C for 2 h and then overnight at room temperature. For reduction of the Schiff's base, the pH was raised to 8.3 with 1 N NaOH, 3.75 mg of $NaBH_4$ dissolved in 0.1 mL of ice-cold H_2O was added, and the mixt. was stirred for 30 min at room temperature and then 4 h at 4 °C. The pH was lowered to 5 with glacial acetic acid and the material concentrated on a rotary evaporator (40–45 °C) to 150–300 μL. The reaction yields were usually greater than 90%. The products were purified by thin-layer chromatography on 1-mm-thick silica gel plates (Brinkmann Instruments, Inc., SIL G-100 UV 254). Each plate was developed initially in $CHCl_3/CH_3OH$ solution (9/1 v/v) to remove unreacted PBH from the reaction product, which remained at the origin. After the plate dried, it was developed in the same direction in *n*-butyl alcohol/glacial acetic acid/water (4/1/2 v/v/v). In this solvent system, each of the pyrenebutyryl hydrazide derivatives of lactose (LPBH), raffinose (RPBH), and stachyose (SPBH) moved as a major fluorescent band with R_f values of 0.78, 0.35, and 0.20, respectively. Each fluorescent band was scraped and desorbed from the silica by three successive washes with 2 mL of ice-cold water. Nonfluorescent impurities extracted from the silica were removed by adsorbing the product on a small reverse-phase chromatography column (SEP-PAK C_{18}, Waters Associates, Milford, MA) followed by elution with CH_3OH/H_2O (3/1 v/v). Most of the methanol was then evaporated under a N_2 stream at room temperature, the final product was lyophilized and dissolved in an isotonic "wash" buffer (30 mM sodium phosphate, pH 7.4, 117 mM NaCl, and 2.8 mM KCl) to give a final concentration of 0.5–2.0 mM in terms of pyrene, and the stock solutions were stored frozen at −20 °C. Assay of the products yielded pyrene/oligosaccharide molar ratios of 0.85–1.1 for various preparations of LPBH, RPBH, and SPBH. Preparations of SPBH were also purified by high-pressure liquid chromatography using a reverse-phase C_{18} column (Waters Associates) and a linear gradient of CH_3OH in H_2O (50–100%, v/v). Experimental results were similar with SPBH purified by either thin-layer or high-pressure liquid chromatography.

Preparation of Glutathione Derivatives. The structures of two pyrene-linked derivatives of glutathione are illustrated in Figure 1B. Compounds I and II differ in the length of the connecting arm and are termed, respectively, glutathione–pyrene I (GS–PI) and II (GS–PII). For preparation of GS–PI, 0.2 mmol of *N*-(1-pyrenyl)maleimide (Molecular Probes, Inc.) was dissolved in 25 mL of ethanol/acetone (1/1 v/v). To this solution was added 5 mL of 0.1 M $NaHCO_3$ followed by 0.23 mmol of glutathione (Sigma Chemical Co.) dissolved in 20 mL of water, the pH adjusted to 7.0 with 1 N NaOH, and the reaction mixture incubated under argon at 37 °C for 90 min. Alkylation of the glutathione (GSH) was complete as judged by the disappearance of the SH group (Ellman, 1959) and the appearance of fluorescence, since unreacted pyrenylmaleimide does not fluoresce (Wu et al., 1976). The mixture was evaporated to dryness in a rotary evaporator at 35–40 °C, the residue dissolved in glacial acetic acid, and the product precipitated by addition of acetone and chilling to −15 °C. The pellet obtained on centrifugation was reprecipitated once as just described and the resulting pellet dissolved in water and purified further by thin-layer chromatography, as detailed in the preceding section. After development in the *n*-butyl alcohol/glacial acetic acid/water system, the material gave a major fluorescent band corresponding to R_f = 0.24. Following elution (see above), the pyrene/glutathione molar ratio in the product varied from 0.94 to 1.14 in various batches.

For the preparation of glutathione–pyrene II (Figure 1B), glutathione–maleimide I, prepared as described previously (Abbott & Schachter, 1976), was treated with excess ethanedithiol and subsequently with pyrenylmaleimide as follows. A solution was prepared of 3.7 mmol of 1,2-ethanedithiol (Aldrich Chemical Co.) in 10 mL of ethanol/tetrahydrofuran/water (1.1/1.8/2.1 v/v/v). A second solution of 0.37 mmol of glutathione–maleimide I in 5 mL of 50% ethanol was prepared, adjusted to pH 7 with 1 N NaOH, and added to the ethanedithiol dropwise, with vigorous stirring, over a period

of 10 min at room temperature. After being stirred for an additional 40 min at room temperature, the mixture was extracted 5 times with 4 volumes of $CHCl_3$ to remove excess ethanedithiol, and the last $CHCl_3$ extract showed negligible SH. The residual aqueous phase, approximately 8 mL, was diluted to 24 mL with water and the pH adjusted to 6.8 with 1 M $NaHCO_3$. To it was added 24 mL of a solution of 0.26 mmol of *N*-(1-pyrenyl)maleimide in ethanol/acetone (1/1 v/v), and the reaction mixture was shaken overnight, under argon, at room temperature, in a vessel shielded from light. Subsequent purification followed the procedures described for GS–PI. Thin-layer chromatography in the *n*-butyl alcohol/glacial acetic acid/water system revealed three major fluorescent products corresponding to R_f values of 0.35, 0.37, and 0.40. After elution, the fraction of the total bound pyrene recovered in each band was, respectively, 0.32, 0.31, and 0.37, and the pyrene/glutathione molar ratio was 1.13, 0.98, and 1.13, respectively. The products appear to be closely related structures which may be stereoisomers, inasmuch as each succinimidyl ring in the GS–PII structure (Figure 1B) contains one asymmetric carbon linked to sulfur. The three chromatographically pure bands were used separately in the membrane experiments but yielded similar results.

Derivatives similar to GS–PI and GS–PII were also made by using *N*-(1-pyrenyl)iodoacetamide (Molecular Probes, Inc.) in place of the pyrenylmaleimide. Results obtained in erythrocyte experiments with the iodacetamide derivatives were comparable to those observed with the foregoing compounds.

Intact Erythrocyte Studies. Human erythrocytes separated by centrifugation from the freshly drawn blood of normal donors or from recently outdated blood-bank blood were washed by centrifugation 3 times with the isotonic "wash buffer" to remove plasma components and buffy coat. For assessment of the outer hemileaflet, intact erythrocytes were treated with GS–PI or GS–PII, and the fluorescence of the cell suspensions was determined directly, as follows. Washed cells suspended to a hematocrit of 1.0% in wash buffer were incubated with shaking in the presence of 200–400 μM fluorophore for 60 min at 37 °C. The cells were then pelleted by centrifugation at 2000*g* for 10 min and washed 4 times with 10 mL (2000 volumes) of wash buffer. Cells were finally diluted with wash buffer to a hematocrit of 0.05%, and the polarization of fluorescence was estimated as described below. Control cell suspensions prepared identically in the absence of fluorophore were used to correct for light scattering. Under the conditions of measurement, the scattering corrections amounted to 25–30% of the fluorescence signals. While these exceed the scattering corrections of 2–10% that we observe routinely with ghost membrane suspensions, the fluorescence anisotropy values of the intact cell suspensions were highly reproducible (see below) and did not vary significantly with dilution to hematocrit values of 0.01–0.02%.

The following procedure obviates the larger scattering corrections applicable to the cell suspensions, while it yields fluorescence anisotropy values for the outer hemileaflet which are quite similar to those observed directly with intact erythrocytes. Washed cells suspended to a hematocrit of 20% in wash buffer are incubated with shaking in the presence of 10–400 μM impermeant fluorophore. After 60 min at 37 °C, the suspensions are pelleted and washed 4 times as described above, and the final pellets are lysed by suspension in 200 volumes of cold (2–5 °C) 5 mM sodium phosphate, pH 8.0. The ghosts are washed 3 times by centrifugation (30000*g*, 15 min) in 200 volumes of the cold 5 mM sodium phosphate to yield membranes essentially free of hemoglobin and suitable for fluorescence measurements as described below. Preparations were maintained at 2–5 °C to minimize possible redistribution of the impermeant probes to the inner leaflet. The fluorescence anisotropy and excimer estimations to be described suggest that relatively little redistribution is evident at 2–5 °C for as long as 12 h. This may be a consequence of the relatively high partition coefficients, (membrane lipid)/(suspending medium water), observed for all five probes. Values for these coefficients, defined as (moles of probe per gram of membrane lipid)/(moles of probe per gram of water), approximated 10^5 (range of 0.4×10^5–12×10^5).

Ghost Membrane Studies. Both the inner and outer membrane leaflets were labeled by preparing ghost membranes from washed erythrocytes and suspending them in either 5 mM sodium phosphate, pH 8.0, or wash buffer to a membrane protein concentration of approximately 1 mg/mL. Impermeant fluorophore was added as described above, the suspensions were shaken for 60 min at 37 °C, and the loaded membranes were harvested and washed 3 times with 200 volumes of cold 5 mM sodium phosphate. These suspensions as well as the membranes obtained after loading the intact cells were adjusted to a protein concentration of 150–200 μg/mL for fluoresence readings.

Intact cells and ghost membranes were loaded with the permeant fluorophores pyrene or 10-(1-pyrene)decanoic acid (Molecular Probes, Inc.) as described above, except that the compounds were dissolved in absolute ethanol and added to the suspensions to yield a final ethanol concentration of 1% (v/v). Control suspensions were treated with ethanol alone.

Liposome Studies. Ghost membranes prepared as described above were extracted by the method of Folch et al. (1957), and the dried, extracted lipid was suspended in wash buffer to a final concentration of 3–4 mg/mL. By use of a Branson sonifier (Branson Sonic Power Co., Model 350), the suspensions were sonicated under N_2 for 5 min at 5 °C and for an additional 5 min while the temperature increased to 30 °C. After centrifugation at 20000*g* for 30 min, the supernatant dispersions (liposomes) were labeled by incubation with 25–100 μM fluorophore for 60 min at 37 °C. Labeled liposome membranes were separated from unbound probe in the aqueous medium by gel filtration at 25 °C through a column of Bio-Gel P-4 (Bio-Rad Laboratories). Both labeled and unlabeled dispersions were used for fluorescence studies.

Fluorescence Studies. Excitation and emission spectra (5-nm resolution) and estimations of total fluorescence intensity were obtained with a Perkin-Elmer MFP 2A spectrofluorometer. Steady-state fluorescence polarization measurements were made in an SLM polarization spectrofluorometer, as previously described (Schachter & Shinitzky, 1977) by using a 1-cm light path and the peak excitation wavelengths shown in Table I and 338 and 345 nm, respectively, for pyrene and pyrenedecanoic acid. Emitted light was passed through a Corning 3-75 filter. In all fluorescence measurements, the contribution of scattered light was subtracted. For polarization measurements, the suspension densities were standardized and minimized to avoid depolarization owing to the particulates.

The polarization of fluorescence was expressed as the fluorescence anisotropy, r, and the anisotropy parameter, $[(r_0/r) - 1]^{-1}$, was calculated by using values of the maximal limiting anisotropy, r_0, determined experimentally for each probe in propylene glycol at −60 °C (Table I). The anisotropy parameter varies inversely with the fluidity of the microenvironment and directly with ρ, the rotational relaxation time of the fluorophore, according to the Perrin equation, $[(r_0/r)$

Table I: Wavelength Maxima and Limiting Anisotropies of Pyrene Derivatives

derivative[a]	r_0[b]	medium	wavelength maximum (λ_{max}) (nm) excitation	emission monomer	emission excimer
pyrenebutyryl hydrazide	0.120	aqueous[c]	343	378	
		tetrahydrofuran	344	378	
		membranes	349	379	
lactose derivative (LPBH)	0.117	aqueous	345	379	
		tetrahydrofuran	346	378	468
		membranes	347	378	468
raffinose derivative (RPBH)	0.119	aqueous	345	379	
		tetrahydrofuran	346	378	468
		membranes	347	378	468
stachyose derivative (SPBH)	0.119	aqueous	344	377	
		tetrahydrofuran	346	378	468
		hexane	348	387	468
		membranes	347	378	468
glutathione-pyrene I	0.180	aqueous	348	385	
		1-butanol[d]	360	388	
		membranes	346	377	
glutathione-pyrene II	0.202	aqueous	346	377	
		tetrahydrofuran	352	378	464
		membranes	348	377	468

[a] Derivatives were tested at concentrations of 2–5 μM. [b] Maximal anisotropy was determined in propylene glycol at −60 °C. [c] Wash buffer of pH 7.4 (see Experimental Procedures). [d] 1-Butanol/H_2O (96/4 v/v).

$- 1]^{-1} = \rho/(3\tau)$, where τ is the mean lifetime of the excited state. The value of τ for each pyrene fluorophore in the erythrocyte membrane was determined experimentally by time-resolved single photon counting (Photo-Chemical Research Associates, London, Ontario; Model 1000 single photon counter), and an apparent rotational relaxation time, ρ', was calculated from the Perrin equation on the assumption that the depolarizing rotations were unhindered.[1]

The content of each fluorophore in the membrane was estimated fluorometrically. Sodium dodecyl sulfate ($NaDodSO_4$) to a final concentration of 1% (w/v) was added to each suspension and the sample clarified by heating at 100 °C for 3 min. The fluorescence intensity was estimated and compared to reference standards of each probe in 1% $NaDodSO_4$ or added incrementally to each sample. The lipids and proteins of the erythrocyte membrane were considered to be equal in mass (Pennell, 1974), and the partial specific volume of the lipids was taken as 1.

Other Methods and Materials. Absorption spectra were recorded in a Cary Model 15 spectrophotometer. The major extrinsic proteins of the endofacial surface of the erythrocyte membrane, i.e., erythrocyte spectrin and actin (Coomassie brilliant blue bands 1, 2, and 5), were extracted by treatment with 0.1 mM EDTA at pH 8.0, as previously described (Steck & Yu, 1973). At least 60–70% of these proteins were removed as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis (Abbott & Schachter, 1976). The sugar content of the oligosaccharide derivatives was estimated by the phenol–sulfuric acid method (Dubois et al., 1956), using each oligosaccharide as a standard. Glutathione was estimated by the ninhydrin reaction (Kabat & Mayer, 1961) and protein by the method of Lowry et al. (1951), using bovine serum albumin as the standard. The pyrene content of the fluorophores was quantified by optical absorbance in the range 340–342 nm with reference to appropriate standards of pyrenebutyryl hydrazide, pyrenylmaleimide, and pyrenyliodoacetamide.

Results

Spectral Characteristics. Values for the excitation and emission wavelength maxima of the pyrene derivatives in various solvents and for the maximal limiting anisotropy, r_0, in propylene glycol at −60 °C are listed in Table I. Figure 2 illustrates excitation and emission spectra of RPBH, which are essentially identical with those of LPBH and SPBH. Although the excitation and monomer emission spectra of all five impermeant derivatives were relatively insensitive to the solvent polarity, a small red shift in the peak excitation wavelength accompanied a decrease in solvent polarity (Table I). As expected, the excitation maxima of the oligosaccharide derivatives and of GS-PII in situ in the membranes corresponded to the less polar environment. The membrane values

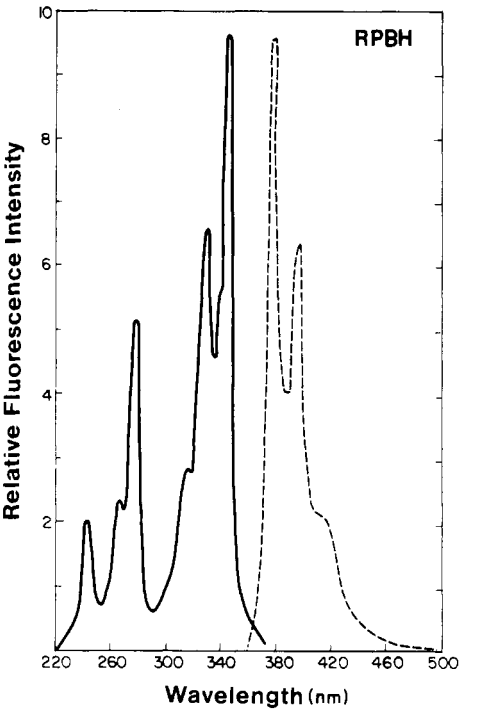

FIGURE 2: Uncorrected excitation (—) and emission (– – –) spectra of RPBH. The sample consisted of 1.5 μM RPBH in wash buffer (see Experimental Procedures). For the excitation spectrum, the fluorescence was monitored at 378 nm, and for the emission spectrum, the solution was excited at 344 nm.

Table II: Fluorescence Anisotropy of Impermeant Pyrene Derivatives in Intact Erythrocytes and Leaky Ghost Membranes

derivative	fluorescence anisotropy,[a] r erythrocytes	ghosts	P
glutathione-pyrene I	0.091 ± 0.001	0.147 ± 0.003	<0.001
glutathione-pyrene II	0.044 ± 0.002	0.089 ± 0.005	<0.02

[a] Values are means ± the standard error (SE) for three different samples of human erythrocytes and ghosts prepared from them. P values for the differences are based on the paired t test. Anisotropy values were determined at 25 °C.

for GS–PI, however, did not show a red shift, suggesting that the probe localizes closer to the aqueous interface of the membrane. This conclusion is further supported by the relatively high anisotropy and lack of excimer formation noted below and by the relatively short distance between the glutathione moiety and pyrene in GS–PI (Figure 1B).

The monomer emission spectra of the oligosaccharide derivatives in aqueous buffers were quite similar to that of PBH, but in less polar media such as tetrahydrofuran, tetrahydrofuran–mineral oil mixtures, or hexane, the emission spectra of the impermeant probes showed strong excimer fluorescence, whereas that of PBH did not. The excimer fluorescence observed for the amphipathic probes is probably due to the formation of inverted micelles, with the polar moieties shielded from the solvent.

Fluorescence Anisotropy. The fluorescence anisotropy values observed for the impermeant glutathione derivatives in intact erythrocytes (outer leaflet) as compared to those for leaky ghost membranes (both leaflets) are listed in Table II. Values for the outer leaflet alone are considerably less than those for the combined leaflets, both for GS–PI ($P < 0.001$) and for GS–PII ($P < 0.02$). Moreover, the anisotropy differences are not ascribable to differences in probe concentration. It is well-known that overlap of the excitation and emission spectra of the pyrene fluorophore can lead to concentration-dependent quenching of fluorescence polarization; (Weber, 1954; Knox, 1968). The fluorophore concentration in the outer leaflet of the intact cells (Table I), expressed as the probe/lipid molar ratio, ranged from 0.003 to 0.007, whereas the corresponding range for the combined leaflets of the leaky ghosts was 0.007–0.011.

The preceding observation that the fluorescence anisotropy estimated in the outer leaflet is less than that in the inner leaflet is supported further by comparisons of membranes freed of hemoglobin and derived from either intact cell loaded (outer leaflet) or leaky ghost loaded (both leaflets) preparations. In each experiment, intact erythrocytes and leaky ghosts prepared from them were treated initially with various concentrations of impermeant fluorophore, as described under Experimental Procedures. Thereafter, washed membranes were prepared from the intact cells and from the leaky ghost loaded preparations, and the results of fluorescence anisotropy estimations are shown in Table III. The mean anisotropy values for

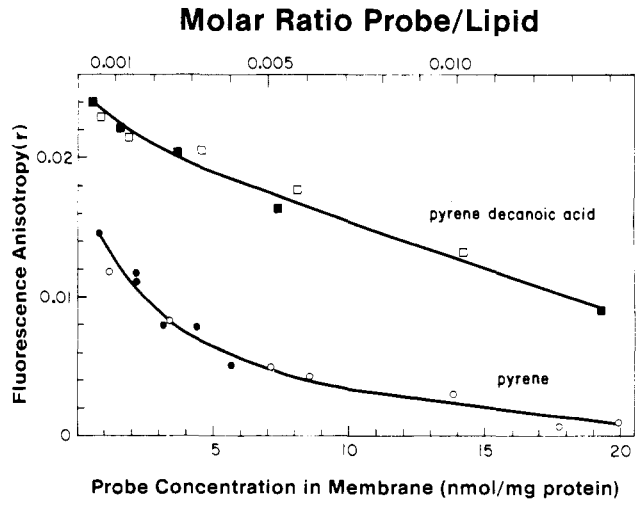

FIGURE 3: Fluorescence anisotropy of pyrene (lower curve) and pyrenedecanoic acid (upper curve) in erythrocyte-loaded (■, ●) and ghost-loaded (□, ○) membranes. Two of three experiments with pyrene and one of two experiments with pyrenedecanoic acid are shown. Probe concentrations in the membrane were estimated as described under Experimental Procedures, and fluorescence anisotropy was determined at 25 °C.

GS–PI and GS–PII observed in the membranes derived from the intact cell loaded preparations (Table III) agree closely with the values obtained by direct observation of the intact cells (Table II); the leaky ghost loaded preparations in Tables II and III are also essentially identical. Further, the data in Table III demonstrate that the pattern of lower fluorescence anisotropy in intact cell loaded as compared to leaky ghost loaded preparations also obtains for the three oligosaccharide derivatives. The probe/lipid molar ratios in these experiments were varied from 0.0005 to 0.003 with little effect on the fluorescence anisotropy values and no effect on the consistent differences between the intact cell loaded and leaky ghost loaded preparations. Hence, the characteristic difference in fluorescence anisotropy between the two hemileaflets is not ascribable to concentration-dependent quenching of the fluorescence polarization of any of the impermeant probes.

The foregoing experiments indicate that impermeant pyrene derivatives inserted into the outer leaflet of the intact erythrocyte retain this selective distribution through subsequent steps of osmotic lysis and the preparation of washed membranes under the conditions described under Experimental Procedures. Membrane-permeant pyrene fluorophores, on the other hand, would be expected to distribute readily between the two hemileaflets, and the fluorescence anisotropy values should be independent of the route of loading. As a test of this prediction, erythrocytes and leaky ghosts were treated with pyrene or pyrenedecanoic acid, and washed membranes were prepared and tested as described in the preceding paragraph. The results shown in Figure 3 demonstrate that at a given probe/lipid molar ratio the fluorescence anisotropy value of each probe is independent of the route of loading. The results

Table III: Fluorescence Polarization Studies of Erythrocyte-Loaded and Ghost-Loaded Membranes

probe	no.[b]	probe/lipid molar ratio range	fluorescence anisotropy,[a] r RBC loaded	ghost loaded	P	anisotropy parameter, $[(r_0/r)-1]^{-1}$ RBC loaded	ghost loaded	P
GS-PI	10	2×10^{-4}–3×10^{-3}	0.107 ± 0.005	0.137 ± 0.004	<0.001	1.68 ± 0.19	3.21 ± 0.35	<0.01
GS-PII	24	2×10^{-4}–5×10^{-3}	0.048 ± 0.001	0.074 ± 0.002	<0.001	0.31 ± 0.01	0.59 ± 0.03	<0.001
LPBH	4	3×10^{-4}–2×10^{-3}	0.018 ± 0.001	0.029 ± 0.001	<0.01	0.18 ± 0.01	0.33 ± 0.01	<0.01
RPBH	10	3×10^{-4}–2×10^{-3}	0.014 ± 0.001	0.023 ± 0.001	<0.001	0.13 ± 0.01	0.24 ± 0.01	<0.001
SPBH	12	2×10^{-4}–3×10^{-3}	0.012 ± 0.001	0.022 ± 0.002	<0.01	0.11 ± 0.01	0.23 ± 0.02	<0.01

[a] Values are means ± SE. P values are based on the paired t test. [b] Number of erythrocyte-loaded and ghost-loaded preparations examined. Anisotropy estimations were at 25 °C.

Table IV: Excited-State Lifetimes in Erythrocyte-Loaded and Ghost-Loaded Membranes

probe	no. of preparations	probe/lipid molar ratio range	mean lifetime,[a] τ (ns)		app rotational relaxation time, ρ' (ns)			
			RBC loaded	ghost loaded	RBC loaded (a)	ghost loaded (b)	P	(b)/(a) ratio
GS-PI	6	2×10^{-4}–6×10^{-3}	103 ± 5	97 ± 3	519 ± 59	933 ± 101	<0.01	1.8
GS-PII	10	8×10^{-4}–6×10^{-3}	101 ± 2	105 ± 1	95 ± 3	186 ± 8	<0.001	2.0
LPBH	4	3×10^{-4}–2×10^{-3}	124 ± 2	122 ± 2	69 ± 4	120 ± 2	<0.001	1.7
RPBH	6	4×10^{-4}–2×10^{-3}	130 ± 4	134 ± 1	51 ± 6	96 ± 5	<0.001	1.9
SPBH	4	2×10^{-4}–3×10^{-3}	121 ± 4	118 ± 3	40 ± 3	80 ± 6	<0.001	2.0

[a] Values are means ± SE for the indicated number of preparations; estimates were at 25 °C. The ρ' values are calculated from the Perrin equation on the assumption that the rotations are not hindered (see text footnote 1).

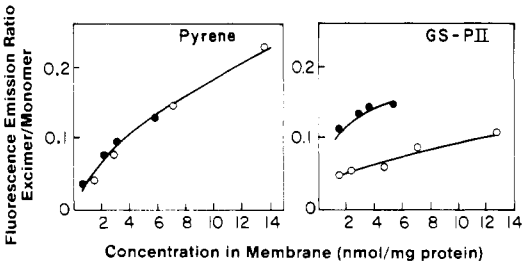

FIGURE 4: Excimer/monomer fluorescence ratio as a function of pyrene or glutathione–pyrene II concentration in erythrocyte-loaded (●) and ghost-loaded (○) membranes. Flushing with argon had no significant effect on the excimer fluorescence in the loaded membranes. Spectra were obtained at 25 °C. The values shown are for single experiments with each probe.

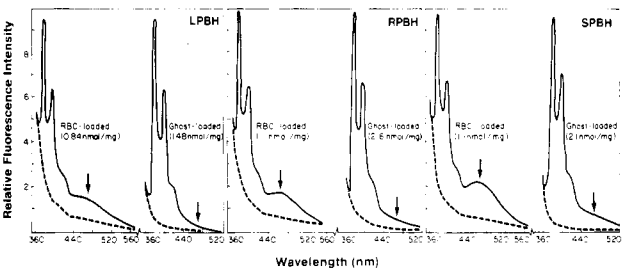

FIGURE 5: Emission spectra of LPBH, RPBH, and SPBH in erythrocyte-loaded and ghost-loaded membranes. The broken line in each instance represents the scattering pattern of a probe-free, control membrane suspension. The arrows designate the peak wavelength of the excimer fluorescence, 468 nm, and the membrane probe concentrations are shown. The excimer/monomer fluorescence ratio was calculated as the ratio of the emission intensity at 468/378 nm. Flushing with argon had no significant effect on the excimer fluorescence in the loaded membranes. Spectra were taken at 25 °C.

also illustrate concentration-dependent quenching of the fluorescence polarization of both fluorophores.

The data of Tables II and III also indicate that values of the fluorescence anisotropy and the anisotropy parameter, $[(r_0/r) - 1]^{-1}$, of GS–PI consistently exceed those of GS–PII in comparable preparations. One possible explanation is that the shorter connecting arm in GS–PI constrains the pyrene moiety to localize in lipid regions which are closer to the aqueous interfaces of the membrane and of lower fluidity (Bashford et al., 1976; Cadenhead et al., 1977).

Excited-State Lifetime Studies. The mean excited-state lifetime, τ, of each impermeant fluorophore in membranes derived from erythrocyte-loaded and ghost-loaded preparations was estimated, inasmuch as lifetime differences could underly the differences in fluorescence anisotropy. In contrast to the consistent differences in fluorescence anisotropy (Table III), the τ values for each probe listed in Table IV do not differ significantly with the route of loading. Accordingly, it is reasonable to suggest that the observed anisotropy differences signify greater motional freedom[1] of the probe molecules in the outer as compared to the inner membrane leaflet. Inasmuch as our results do not further resolve "motional freedom" into the components of rate of rotation vs. extent of rotation (Chen et al., 1977; Kawato et al., 1977; Veatch & Stryer, 1977; Lakowicz et al., 1979a,b; Heyn, 1979; Jähnig, 1979), a true rotational relaxation time cannot be calculated from the Perrin relationship. Table IV does include, however, values of an apparent rotational relaxation time, ρ', calculated on the assumption of unhindered rotations. The ratios of ρ' in ghost-loaded as compared to RBC-loaded membranes fall in a relatively narrow range, 1.7–2.0, for all five probes, whereas the absolute values of ρ' vary over 10-fold for the different compounds.

Excimer Fluorescence. Formation of the pyrene excimer is diffusion controlled and dependent on the fluidity of the microenvironment, and several investigators have utilized the excimer fluorescence to assess the fluidity of bilayer membranes (Galla & Sackmann, 1974; Vanderkooi & Callis, 1974; Dembo et al., 1979; Galla & Hartmann, 1980). Accordingly, it was of interest to compare the excimer fluorescence of erythrocyte-loaded and ghost-loaded membrane preparations, and Figure 4 shows the results of studies with GS–PII and pyrene in which the excimer/monomer fluorescence intensity ratio (Galla & Sackmann, 1974; Vanderkooi & Callis, 1974) is plotted against probe concentration in the membrane. As expected, excimer fluorescence increases with the concentration of each compound. Pyrene, however, a permeant compound, yields excimer/monomer ratios which are independent of the route of loading, whereas the ratios for the impermeant GS–PII are considerably greater in the erythrocyte-loaded (outer leaflet) as compared to the ghost-loaded preparations. The oligosaccharide derivatives, LPBH, RPBH, and SPBH, also show greater excimer fluorescence in erythrocyte-loaded membranes, as illustrated in Figure 5. In contrast to the other impermeant fluorophores, GS–PI yielded little or no excimer fluorescence under the conditions of our experiments.

Table V: Fluorescence Anisotropy of Impermeant Pyrene Fluorophores in Sonicated Dispersions of Erythrocyte Membrane Lipid

probe	no. of preparations	fluorescence anisotropy,[a] r	$[(r_0/r) - 1]^{-1}$
RPBH	2	0.018	0.18
SPBH	2	0.017	0.17
GS-PI	1	0.079	0.78
GS-PII	2	0.053	0.36

[a] Mean values are shown for the indicated number of preparations. Anisotropy estimations were at 25 °C.

Liposome Studies. Sonicated dispersions of erythrocyte membrane lipid were loaded with impermeant fluorophores as described under Experimental Procedures, and the resulting fluorescence studies are summarized in Table V. Values of the fluorescence anisotropy and $[(r_0/r) - 1]^{-1}$ for RPBH, SPBH, and GS–PII fall between the corresponding values for

the erythrocyte-loaded and ghost-loaded membrane preparations (Tables II and III), whereas the GS–PI values are somewhat less those observed in the membranes. Again, the values for GS–PI exceed those of GS–PII.

Extraction of Extrinsic Proteins. For determination of whether the asymmetry of fluorescence anisotropy in the outer and inner leaflets is due to the influence of spectrin and erythrocyte actin, major extrinsic proteins of the endofacial surface (Steck, 1974), ghost membranes were loaded with GS–PII, RPBH, or SPBH as previously described, and the membranes were extracted with 0.1 mM Na_2EDTA, pH 8, to remove most of the Coomassie brilliant blue stained bands 1, 2, and 5 (Experimental Procedures). Removal of the proteins had no significant effect on the fluorescence anisotropy of any of these probes.

Discussion

The foregoing studies describe the synthesis, purification, and application of glutathione- and oligosaccharide-linked derivatives of pyrene, impermeant fluorophores designed to probe selectively the outer hemileaflets of intact cell membranes. There is considerable evidence that the bulky, hydrophilic glutathione and oligosaccharide substituents are unable to permeate the membranes of human erythrocytes and certain other cell types (Wendel & Flohé, 1970; Lepock et al., 1975; Mehlhorn & Packer, 1976; Abbott & Schachter, 1976; Wisnieski & Iwata, 1977). Although the presence of hemoglobin can pose difficulties in fluorescence studies, we were nonetheless able to estimate with good reproducibility the fluorescence anisotropy of the glutathione-linked derivatives in intact human erythrocytes. Comparisons with leaky ghost membranes indicate clearly that the fluorescence anisotropy of the probes is significantly less in the outer as compared to the inner leaflet of the membrane. This major conclusion is supported further by additional experimental observations. A procedure was developed to load the outer leaflet of the intact cells and thereafter to prepare hemoglobin-free membranes in order to minimize scatter corrections and to control for the possible effects of osmotic lysis on erythrocyte membrane components. The close correspondence of the fluorescence anisotropy values observed directly in intact cells (Table II) to the values in membranes derived from loaded cells (Table III) indicates that the probe molecules retained to a significant degree their localization in the outer leaflet. Comparisons of membranes derived from loaded, intact erythrocytes with those loaded as leaky ghosts confirmed the asymmetry in fluorescence anisotropy for all five impermeant fluorophores (Table III). Moreover, pyrene and pyrenedecanoic acid, permeant compounds, yielded no asymmetry of fluorescence anisotropy in comparable experiments (Figure 3).

Inasmuch as the observed leaflet asymmetry in fluorescence anisotropy is not the result of systematic differences in excited-state lifetimes (Table IV), it is reasonable to conclude that each impermeant pyrene fluorophore experiences greater motional freedom in the outer as compared to the inner leaflet. To what extent this difference in motional freedom represents a difference in the rate of rotation of the probe, as assessed by the rotational relaxation time, or a difference in the degree of hindrance of the rotation, as assessed by the r_∞ value[1] (Heyn, 1979; Jähnig, 1979; Lakowicz et al., 1979a,b), has not yet been determined. The finding that the excimer fluorescence of the impermeant derivatives is also consistently greater in the outer leaflet (Figures 4 and 5), on the other hand, is consistent with the suggestion that the outer leaflet lipids are more fluid. Pyrene excimer formation is dependent on the fluidity of the environment and has been used to assess the microviscosity of erythrocyte membrane lipids (Vanderkooi & Callis, 1974; Dembo et al., 1979) and the jump frequency (related to the lateral diffusion coefficient) of pyrene fluorophores in these membranes (Galla & Hartmann, 1980). It is further significant that the asymmetry of fluorescence anisotropy of the hemileaflets was observed over a range of low concentrations of the impermeant probes, where concentration-dependent quenching of the polarization did not occur. Thus, the lower anisotropy values of the outer leaflet do not result from concentration of the probe molecules in restricted lipid domains.

The suggestion that membrane lipids exhibit lateral heterogeneity within each leaflet (Jain & White, 1977; Klausner et al., 1980) makes it pertinent to ask if the impermeant pyrene derivatives monitor the bulk or only a small fraction of the membrane lipid domains. Several observations suggest that the fluorescence signals are representative of a significant fraction of the membrane lipids. Liposome studies (Table V) yield anisotropy values for RPBH and SPBH which correspond very closely to those of the intact membranes (Table III), and the corresponding values for GS–PII are also in fair agreement. In Figure 4, the excimer/monomer ratios of GS–PII in the outer leaflet correspond closely to those of pyrene when allowance is made for a difference in distribution, i.e., GS–PII is distributed in the outer leaflet alone and pyrene in both leaflets. For example, the excimer/monomer ratios of GS–PII at a membrane concentration of 3 nmol/mg of protein are identical with that of pyrene at a membrane concentration of 6 nmol/mg of protein. Finally, recent studies in our laboratory indicate that enrichment or depletion of erythrocyte membrane cholesterol markedly affects the fluorescence anisotropy of the impermeant pyrene derivatives (M. Flamm and D. Schachter, unpublished experiments) and that the fluorescence of these probes in the outer leaflet responds to a thermotropic lipid transition at 15–20 °C, which others have observed with different techniques (Zimmer & Schirmer, 1974; Cullis & Grathwohl, 1977; Kapitza & Sackmann, 1980). The conclusion that the outer leaflet of the human erythrocyte membrane provides lipid domains of greater motional freedom than the inner leaflet is similar to that reached in prior studies of the plasma membranes of *Mycoplasma* (Rottem, 1975), mouse LM cells (Wisnieski & Iwata, 1977; Schroeder, 1978), and Newcastle disease viral envelopes derived from embryonated chick cells (Wisnieski & Iwata, 1977). This asymmetry in the leaflets, therefore, may be a general feature of the plasma membranes of many prokaryotic and eukaryotic cells. It is noteworthy, on the other hand, that Tanaka & Ohnishi (1976) interpreted spin-label studies of human erythrocytes to indicate that the outer leaflet contains a less fluid phosphatidylcholine phase which randomizes on osmotic lysis in the absence of added magnesium. However, the principal probe employed, phosphatidylcholine containing a stearic acid 12-nitroxide substituent, was not shown to localize preferentially in the outer leaflet, and it is noteworthy that ~24% of the membrane phosphatidylcholine is in the inner leaflet (Rothman & Lenard, 1977; Op den Kamp, 1979).

Despite many studies of the lipid composition of erythrocyte membranes (van Deenen & de Gier, 1974; Rothman & Lenard, 1977; Op den Kamp, 1979), prediction of the relative fluidity of the individual leaflets is difficult (van Dijck et al., 1976) because the hemileaflet distribution and the influence of a number of factors, including cholesterol, the glycolipids, the degree of saturation and chain length of fatty acid residues, the membrane proteins, and the ionic environment, have not been defined completely. An initial report that cholesterol content is greater in the outer leaflet (Fisher, 1976), for ex-

ample, was subsequently disputed (Blau & Bittman, 1978). The application of impermeant fluorophores such as those described in this report can provide insights into the lipid dynamics of the individual hemleaflets and their significance in membrane function.

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