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

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ABSTRACT: The fluorescence anisotropy and mean excited-state lifetime of 1,6-diphenyl-1,3,5-hexatriene, 12-(9-anthroyloxy)stearate, 2-(9-anthroyloxy)stearate, and pyrenedecanoic acid in the membranes of intact human erythrocytes, lysate suspensions, and ghost membranes were compared. The excited-state lifetime of each lipid fluorophore, estimated by single photon counting, is significantly shorter in the intact erythrocytes as compared to the lysates, owing to nonradiative energy transfer from the lipid fluorophore donors in the membrane to heme acceptors at the endofacial surface of the intact cell. The fluorescence observed in intact cell suspensions is thus weighted in favor of outer leaflet fluorophores, and estimates of the fluorescence anisotropy by steady-state fluorescence polarization indicate that all four fluorescent probes experience greater motional freedom in the outer as compared to the inner membrane leaflet. The results are in accord with prior studies of impermeant pyrene derivatives, which also indicate that the outer leaflet lipids have greater motional freedom.

The hemileaflets of the human erythrocyte membrane differ in their lipid composition (Rothman & Lenard, 1977; Op den Kamp, 1979), and we have recently reported a corresponding leaflet asymmetry in lipid motional freedom, or "fluidity"[1] (Cogan & Schachter, 1981). Experiments with a series of membrane-impermeant pyrene derivatives indicate that motional freedom of the fluorophore is consistently greater in the outer as compared to the inner leaflet. Inasmuch as evidence for a similar asymmetry in fluidity has been reported for *Myocoplasma* (Rottem, 1975), Newcastle disease viral envelopes from chick cells (Wisnieski & Iwata, 1977), and mouse LM cells (Wisnieski & Iwata, 1977; Schroeder, 1978), the fluidity difference may be a feature of many plasma membranes and point to a general functional requirement.

The impermeant pyrene fluorophores permit direct assessment and comparison of the hemileaflets of cellular plasma membranes (Cogan & Schachter, 1981). Since a single fluorophore such as pyrene, however, could partition preferentially into selected microdomains within each leaflet, we have examined further the asymmetry of leaflet fluidity in erythrocytes by using the permeant lipid fluorophores 1,6-diphenyl-1,3,5-hexatriene (DPH),[2] 2-(9-anthroyloxy)stearate (2AS), 12-(9-anthroyloxy)stearate (12AS), and pyrenedecanoic acid. Advantage was taken of nonradiative energy transfer (Förster, 1948, 1966; Wu & Stryer, 1972; Shaklai et al., 1977) from a lipid fluorophore donor in the membrane of an intact erythrocyte to heme acceptors at the endofacial (inner) surface. Since the efficiency of nonradiative energy transfer varies inversely with the sixth power of the distance between donor and acceptor (Förster, 1966), nonradiative energy transfer is expected to shorten the mean lifetime of the excited state ($\tau_F$) of donor fluorophores in membranes of intact erythrocytes as compared to lysates or washed ghosts. Further, in intact cells the excited-state lifetimes of inner leaflet fluorophores should be shortened disproportionately, and the fluorescence observed should represent disproportionately the outer leaflet fluorophores. The results described below confirm the expectation that the excited-state lifetime ($\tau_F$) of each lipid fluorophore listed above is shorter in the membranes of intact erythrocytes as compared to lysates or washed ghosts. Moreover, when account is taken of the corresponding fluorescence anisotropy values, as determined by steady-state fluorescence polarization, the results uniformly support the conclusion that lipid fluidity of the outer leaflet exceeds that of the inner leaflet.

## Experimental Procedures

*Erythrocytes and Membrane Suspensions.* Human erythrocytes were separated by centrifugation from the freshly drawn blood of normal donors or from recently outdated blood-bank blood. The buffy coat was discarded, and the cells were washed 3 times with an isotonic "wash buffer" composed of 5 mM sodium phosphate, pH 7.4, 145 mM NaCl, and 5 mM KCl. For the fluorescence studies, washed erythrocytes were suspended in the wash buffer to a hematocrit of 1.0%.

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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 used it as a general term to express the relative motional freedom of the bilayer lipid molecules or substituents thereof, combining in the one term concepts of both rate of movement and extent of movement. As evaluated by steady-state fluorescence polarization of lipid fluorophores, this amounts 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 rotations of this rodlike fluorophore are hindered in both artificial and natural membranes (Chen et al., 1977; Dale et al., 1977; Kawato et al., 1977; Kinosita et al., 1977; Veatch & Stryer, 1977; Glatz, 1978; Hildenbrand & Nicolau, 1979; Lakowicz et al., 1979a,b) and can be described by the relationship $r = r_\infty + (r_0 - r_\infty)[\tau_c/(\tau_c + \tau_F)]$, where $r_\infty$ is the limiting anisotropy observed after relatively long time intervals in time-resolved anisotropy decay experiments, $r_0$ is the maximal limiting anisotropy, $\tau_c$ is the correlation time, and $\tau_F$ is the excited-state lifetime (Heyn, 1979; Jähnig, 1979). Jähnig (1979) and Heyn (1979) suggest that the term fluidity be applied only in reference to $\tau_c$, *i.e.*, to rates of rotation, whereas $r_\infty$ is related to an order parameter. For many biological applications, changes in $r$ owing to changes in $\tau_c$, $r_\infty$, or both are of significance, and a general term is needed to designate such changes in $r$. We shall use the term "motional freedom" in this general sense.

[2] Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; 12AS, 12-(9-anthroyloxy)stearate; 2AS, 2-(9-anthroyloxy)stearate; $r$, fluorescence anisotropy; $r_\infty$, limiting fluorescence anisotropy after long time intervals in time-resolved anisotropy decay experiments; $\tau_c$, correlation time; $\tau_F$, excited-state lifetime; $R'_0$, apparent critical distance (Å) for 50% efficiency of nonradiative energy transfer from donor to acceptor.

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Fluorescent probe (see below) dissolved in absolute ethanol was added with rapid mixing to the suspension to give final concentrations of 10–15 μM probe and 1.0% ethanol. Control cell suspensions were treated with ethanol alone. After being shaken at 37 °C for 30–60 min, the cells were pelleted by centrifugation, washed 4 times with 1000 volumes of wash buffer, and suspended finally to hematocrit values of 0.02–0.05% for fluorescence estimations.

For comparison of the fluorescence of the intact cells with lysates or ghost membranes, 3.0-mL aliquots of the final cell suspensions (hematocrit 0.05%) loaded as described above were pelleted by centrifugation, and the cell pellets were lysed in 0.5 mL of 8 mM sodium phosphate, pH 7.4. After lysis the volume of each sample was adjusted to 3.0 mL by addition of wash buffer prior to estimation of the fluorescence. Thus the suspensions of intact cells and the lysates were comparable, except that the relatively high concentration of hemoglobin localized at the endofacial surfaces of the intact cells was greatly diluted in the ambient medium of the lysates. Following fluorescence estimations of the foregoing samples, the contribution of fluorescence in the ambient medium was checked routinely by centrifugation of the cells or of the lysed ghost membranes at 30000*g* for 20 min. The contribution of the ambient medium amounted to 0–5% of the total fluorescence of the cell or lysate suspension, and these small corrections were made in estimations of the fluorescence anisotropy described below. The results also indicate that in the whole cell suspensions fluorescence from the cytosol was negligible.

Hemoglobin-free ghost membranes were prepared from the lysates described above by washing the pelleted membranes twice in approximately 1000 volumes of 8 mM sodium phosphate, pH 7.4. When the membranes were resuspended in wash buffer, the fluorescence anisotropy of the washed membranes was identical within experimental error with that observed for the lysates.

*Fluorescent Probes.* The lipid-soluble fluorophores 1,6-diphenyl-1,3,5-hexatriene (Eastman Kodak Co.), DL-2-(9-anthroyloxy)stearic acid (Molecular Probes, Inc.), DL-12-(9-anthroyloxy)stearic acid (Molecular Probes, Inc.), and pyrenedecanoic acid (Molecular Probes, Inc.) were chosen for study after preliminary estimations of $R'_0$, the apparent critical distance for 50% efficiency of nonradiative energy transfer from each fluorescent donor to hemoglobin heme. The $R'_0$ values were calculated from the theory of Förster (1966) as described by Wu & Stryer (1972), by determination of the spectral overlap integrals and quantum yields and by assuming values of 1.4 for the refractive index of the medium and $^2/_3$ for $K^2$, the orientation factor for dipole–dipole transfer. This $K^2$ value assumes that the relative orientation of donor–acceptor pairs is completely randomized during the excited-state lifetime, an assumption which is questionable for a lipid fluorophore like DPH whose rotations are hindered in the erythrocyte ghost membrane (Glatz, 1978). Accordingly, the calculated $R'_0$ distances are only approximate apparent values subject to correction by more accurate estimates of $K^2$. The $R'_0$ distances (in angstroms) calculated for DPH, 2AS, 12AS, and pyrenedecanoic acid, respectively, were 38, 39, 35, and 36 Å. Shaklai et al. (1977) calculated a corresponding value for 12AS of 45 Å. Taking the width of the lipid bilayer as approximately 50 Å (Levine, 1976) and allowing for some distance between the acceptor heme residues at the endofacial surface and the inner leaflet lipids, it seemed reasonable to predict that nonradiative energy transfer would shorten the excited-state lifetime of each of the four lipid fluorophores in the membrane of the intact erythrocyte, with most of this effect on the fluorophores of the inner leaflet.

*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 a two-channel polarization spectrofluorometer (SLM Instruments, Champaign, IL) with a 1-cm light path, a 450-W xenon light source, and a monochromator to select peak excitation wavelengths of 360 nm (DPH), 385 nm (2AS, 12AS), or 345 nm (pyrenedecanoic acid). Emitted light was passed through Corning 3-75 glass filters (pyrenedecanoic acid) or through Corning 3-75 plus 3-74 glass filters (DPH, 2AS, 12AS). Fluorescence anisotropy, $r$, was calculated as $(I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$, where $I_\parallel$ and $I_\perp$ are the intensities of the emitted light oriented respectively parallel and perpendicular to the plane of polarization of the excitation beam. Scatter corrections were made routinely by testing control suspensions of intact erythrocytes, lysates, or washed ghost membranes which lacked fluorescent probe. For the lysates and washed ghosts the scatter corrections amounted to 0–3% of the fluorescent signals, and for the intact cell suspensions (hematocrit 0.02–0.05%) corrections ranged from 3% to 15%. Despite the higher scattering corrections required for the intact cell suspensions, the values of the fluorescence anisotropy were satisfactorily reproducible (see Results). Moreover, the fluorescence anisotropy values were independent of the cell suspension density from a hematocrit value of ~ 0.05% down to ~0.01% (see Figure 1).

The excited-state lifetime, $\tau_F$, was estimated as described previously (Cogan & Schachter, 1981) by time-resolved single photon counting (Photo-Chemical Research Associates, London, Ontario; Model 1000 single photon counter). Owing to light scattering by the suspension studied, we estimated only the major lifetime observed after suitable intervals (10–12 ns for DPH, 2AS, or 12AS; 30 ns for pyrenedecanoic acid) following the lamp pulse. Thus possible shorter-lived decay components were not identified or quantified.

For rodlike fluorophores like DPH the relationship between the fluorescence anisotropy, $r$, and the excited-state lifetime, $\tau_F$, is given by $r = r_\infty + (r_0 - r_\infty)[\tau_c/(\tau_c + \tau_F)]$, where $r_\infty$ is the limiting anisotropy observed after relatively long time intervals in time-resolved anisotropy decay experiments, $r_0$ is the maximal limiting anisotropy, and $\tau_c$ is the correlation time (Heyn, 1979; Jähnig, 1979). In the calculations below (Results), we used $r_0$ values of 0.390 for DPH (Lakowicz et al., 1979a), 0.290 for 2AS and 12AS (Tilley et al., 1979), and 0.143 for pyrenedecanoic acid, estimated in propylene glycol at −60 °C as previously described (Cogan & Schachter, 1981). Values of $r_\infty$ for DPH, 2AS, and 12AS in human erythrocyte membranes were estimated by time-resolved fluorescence anisotropy decay using a photon counting apparatus in the laboratory of Dr. Jane Vanderkooi (Vanderkooi et al., 1974). Suspensions of washed ghost membranes loaded with each fluorophore as described above were examined at 23 °C and $r_\infty$ values of 0.144 (DPH), 0.028 (2AS), and 0.022 (12AS) obtained. In agreement with prior results (Glatz, 1978) DPH rotations are considerably hindered in the erythrocyte ghost membrane. Rotations of 2AS and 12AS, by comparison, are relatively unhindered.

## Results

*Comparison of Intact Erythrocytes and Lysates.* Initial experiments were designed to test the feasibility of characterizing the fluorescence of suspensions of intact erythrocytes at various hematocrit values. The cells were treated with DPH,

Table I: Excited-State Lifetime and Fluorescence Anisotropy Studies

| probe[a] | excited-state lifetime,[c] $\tau_F$ (ns) | | | fluorescence anisotropy, $r$ | | |
|---|---|---|---|---|---|---|
| | intact erythrocytes[b] | lysates | $P$[d] | intact erythrocytes | lysates | $P$[d] |
| diphenylhexatriene | 6.0 ± 0.6 (4) | 9.8 ± 0.1 (4) | <0.005 | 0.257 ± 0.006 (10) | 0.251 ± 0.005 (10) | ns |
| 12-anthroylstearate | 7.6 ± 0.1 (4) | 11.0 ± 0.1 (4) | <0.001 | 0.133 ± 0.001 (10) | 0.115 ± 0.001 (10) | <0.001 |
| 12-anthroylstearate[e] | 7.3 ± 0.0 (4) | 9.9 ± 0.3 (4) | <0.003 | 0.086 ± 0.003 | 0.085 ± 0.002 (6) | ns |
| 2-anthroylstearate | 7.0 ± 0.1 (4) | 8.2 ± 0.1 (4) | <0.001 | 0.154 ± 0.003 (9) | 0.153 ± 0.002 (9) | ns |
| 2-anthroylstearate[e] | 6.1 ± 0.2 (4) | 8.0 ± 1.1 (4) | <0.025 | 0.095 ± 0.003 (7) | 0.103 ± 0.002 (7) | <0.013 |
| pyrenedecanoate[f] | | | | | | |
| monomer | 73 ± 2 (4) | 86 ± 3 (4) | <0.05 | 0.004 ± 0.001 (4) | 0.009 ± 0.001 (4) | <0.01 |
| excimer | 69 ± 1 (4) | 87 ± 4 (4) | <0.005 | | | |

[a] Cells treated with 10–15 μM probe as described under Experimental Procedures, except as indicated below. [b] Cell suspensions correspond to hematocrit of 0.05%. [c] Values are means ± SE; values in parentheses are the number of preparations tested. [d] $P$ values for differences between cells and lysates as determined by the paired $t$ test; ns, nonsignificant. [e] Cells treated with 50 μM probe. [f] Monomer fluorescence monitored at 397 nm and excimer fluorescence at 465 nm in the lifetime studies; fluorescence anisotropy values are for combined monomer and excimer fluorescence. All estimations were at 23 °C.

2AS, or 12AS as described above, portions were used to prepare lysates, and dilutions of the cell suspensions and lysates were examined to estimate the total fluorescence intensity and fluorescence anisotropy. Figure 1 illustrates the results of a typical experiment with DPH. The total fluorescence intensity increased linearly with the packed cell volume from 0.009% to 0.05%. Throughout this range the fluorescence intensity ratio of lysate/cell suspension was 1.95 ± 0.07 (mean ± SE), and the fluorescence anisotropy of the cell suspensions and lysates remained constant within experimental error; mean ± SE values of $r$ for the cells and lysates, respectively, were 0.245 ± 0.0006 and 0.241 ± 0.005. Similar experiments with 2AS and 12AS gave comparable results. The lysate/cell suspension ratio of total fluorescence intensity was 1.61 ± 0.10 and 1.90 ± 0.06, respectively, for 2AS and 12AS, and the fluorescence anisotropy values (see below) were independent of the cell suspension density up to ~0.05%. The differences above in fluorescence intensity between cell suspensions and lysates could result in part from nonradiative energy transfer to heme in the intact erythrocyte suspensions. Since such a mechanism would shorten the excited-state lifetime (Wu & Stryer, 1972), systematic studies of $\tau_F$ and of the fluorescence anisotropy, $r$, were undertaken to compare cell suspensions and lysates, and the results are summarized in Table I. In accord with energy transfer to heme in the intact erythrocyte, the $\tau_F$ values for DPH, 2AS, 12AS, and pyrenedecanoic acid (monomer and excimer fluorescence) are significantly shorter in the cell suspensions as compared to the corresponding lysates. Mean ratios of $\tau_F$ in lysate/cell suspension for DPH, 2AS, 12AS, and pyrenedecanoic acid monomer and excimer fluorescence, respectively, were 1.63, 1.17, 1.45, 1.18, and 1.26.

The foregoing results indicate that energy transfer to heme shortens the excited-state lifetime of lipid fluorophores in the erythrocyte membrane. Since the energy-transfer process affects disproportionately those fluorophores in the inner leaflet, it is instructive to compare further the fluorescence anisotropy observed in a whole cell suspension, weighted in favor of the outer leaflet, with that of the lysate, which is representative of both leaflets. In the case of DPH and 2AS, for example, $\tau_F$ is significantly shorter in the intact cells as compared to the lysate membranes, whereas the corresponding $r$ values are not significantly different (Table I). From the relationship of $r$ to $\tau_F$ indicated by the equation under Experimental Procedures, it follows that for these lipid fluorophores either $\tau_c$, $r_\infty$, or both must be less in the outer as compared to the inner leaflet. A similar conclusion is justified for all four lipid fluorophores studied, as indicated by the values shown in Table II. Here we have calculated $\tau_c$ for the lysate

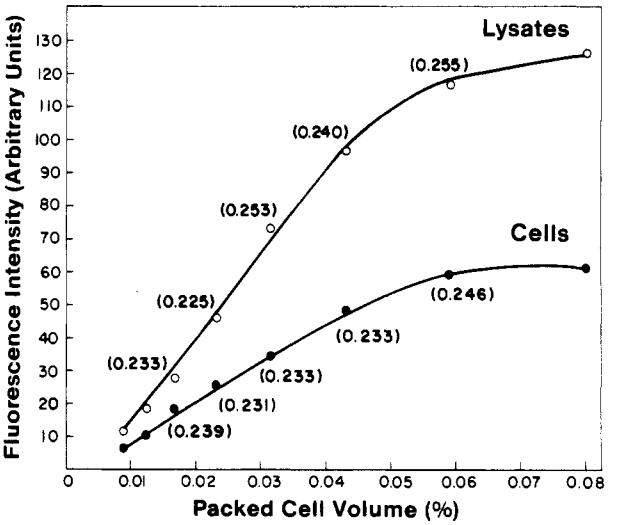


FIGURE 1: Total fluorescence intensity (arbitrary units) and fluorescence anisotropy (values in parentheses) of 1,6-diphenyl-1,3,5-hexatriene in membranes of intact erythrocyte suspensions (●) and lysates prepared from them (○). Human erythrocytes were treated with 15 μM DPH in ethanol (Experimental Procedures), washed, and suspended to various packed cell volumes, as shown. Lysates corresponding to each packed cell volume were prepared for comparison. Scatter corrections for the fluorescence anisotropy estimations (Experimental Procedures) were less than 15% for the intact cell suspensions and less than 3% for the lysates. After the cells or the lysate membranes were pelleted by centrifugation, negligible fluorescence (<2%) was recovered in the supernatant solutions.

membranes containing each of the probes. (The $r_\infty$ values were estimated experimentally for DPH, 2AS, and 12AS, as indicated under Experimental Procedures. The $r_\infty$ value for pyrenedecanoic acid was assumed to be zero.) On the premise that motional freedom, as assessed by $\tau_c$ and $r_\infty$, is similar in both leaflets, predicted $r$ values were calculated for the cell suspensions from the calculated $\tau_c$ and estimated $r_\infty$ values of the lysate membranes and from the $\tau_F$ values observed for the cells. As indicated in Table II, the $r$ value so predicted for each probe is significantly greater than the value observed experimentally. The results, therefore, contradict the premise of identical motional freedom in the two leaflets and support the hypothesis that lipid probes in the outer leaflet have relatively lower $\tau_c$ or $r_\infty$ values, i.e., greater motional freedom.

The four lipid fluorophores examined yield a concordant general result as stated above. Some differences in the fluorescence parameters, however, are illustrated in Tables I and II and merit comment. The correlation time of 12AS in lysate membranes is less than that of 2AS (Table II), in

Table II: Correlation Times in Lysate Membranes and Fluorescence Anisotropy of Intact Erythrocyte Suspensions

| probe[a] | preparation | mean correlation time,[b] $\tau_c$ (ns) | fluorescence anisotropy, $r$, of cell suspensions | | |
|---|---|---|---|---|---|
| | | | predicted[c] | obsd[d] | $p$[e] |
| diphenylhexatriene | lysate | 7.5 | | | |
| | cells | | 0.281 | 0.257 ± 0.006 (10) | <0.005 |
| 12-anthroylstearate | lysate | 5.9 | | | |
| | cells | | 0.139 | 0.133 ± 0.001 (10) | <0.005 |
| 2-anthroylstearate | lysate | 7.5 | | | |
| | cells | | 0.163 | 0.154 ± 0.003 (9) | <0.005 |
| pyrenedecanoate | lysate | 5.8 | | | |
| | cells | | 0.010 | 0.004 ± 0.001 (4) | <0.01 |

[a] Cells treated with 10–15 μM probe as described under Experimental Procedures. [b] Values calculated from the equation under Experimental Procedures, using the appropriate values of $r_0$, $r_\infty$, $\tau_F$, and $r$ for lysate preparations. The $r_\infty$ for pyrenedecanoate was not estimated and assumed to be zero. [c] Anisotropy values calculated from the equation under Experimental Procedures, using the $r_\infty$ and $\tau_c$ values of the lysates and the $\tau_F$ values of the cell suspensions (Table I). [d] Values are means ± SE; the values in parentheses are the number of preparations tested. [e] $P$ for the difference between the observed and predicted as determined by the paired $t$ test. All estimations were at 23 °C.

confirmation of prior studies of artificial (Bashford et al., 1976; Cadenhead et al., 1977; Tilley et al., 1979) and natural membranes (Brasitus & Schachter, 1980; Livingstone & Schachter, 1980), which indicate that the anthroyl fluorophore of 2AS localizes closer to the aqueous surfaces of the bilayer in a lipid environment of lower fluidity. The fluorescence anisotropy of 2AS and 12AS in cell and lysate membranes was relatively independent of the loading concentration up to 15–20 μM (Experimental Procedures). At higher loading concentrations, however, the values of $r$ decreased (Table I), perhaps owing to perturbation of the bilayer (Cadenhead et al., 1977).

## Discussion

The foregoing experiments employ four lipid fluorophores which differ in their chemical structure, fluorescence properties, and localization in the membrane lipid bilayer. Despite these differences, however, all four probes yield results which support the hypothesis that lipid motional freedom, or "fluidity",[1] is greater in the outer as compared to the inner leaflet of the human erythrocyte membrane. The experimental evidence rests on the occurrence of nonradiative energy transfer to heme on the endofacial surface of the intact erythrocyte and on the relationship between $r$ and $\tau_F$ expressed by the equation under Experimental Procedures (Heyn, 1979; Jähnig, 1979). That nonradiative energy transfer to heme occurs from each of the probes studied seems clear from the consistent shortening of the excited-state lifetime shown in Table I on comparison of intact erythrocytes with the corresponding lysates. Moreover, the dependence of the efficiency of energy transfer on the inverse sixth power of the distance between donor and acceptor (Förster, 1966; Stryer & Haugland, 1967) implies that the fluorescence observed in suspensions of intact cells is weighted in favor of outer leaflet fluorophores. The modified Perrin relationship between $r$ and $\tau_F$ (Experimental Procedures) is applicable to DPH and similar rodlike fluorophores (Heyn, 1979; Jähnig, 1979), and our results with this probe indicate clearly that the intact cell fluorescence (disproportionately outer leaflet) derives from molecules which experience greater motional freedom (decreased $\tau_c$ or $r_\infty$) than that of the membrane pool as a whole.

The anthroyloxy and pyrene fluorophores studied are disk shaped, and the applicability of the modified Perrin relationship (Experimental Procedures) to such structures has not been established rigorously. Moreover, anisotropic rotations of these probes may well occur in the membrane bilayer (Tilley et al., 1979). The comparisons with these probes of intact cell and lysate fluorescence, however, fully support the findings with DPH. In addition to the quantitative considerations summarized in Table II, it is indicative that the shortened $\tau_F$ values observed consistently in intact erythrocytes as compared to those of lysates are associated with no significant change (2AS) or a decrease (pyrenedecanoic acid) in fluorescence anisotropy (Table I).

In interpreting the results of our studies, which compare intact erythrocytes with lysates or ghost membranes, it is important to consider whether the observed differences in fluorescence could result from changes in lipid composition and fluidity owing to osmotic lysis or other steps of the membrane isolation procedure. This possibility is examined experimentally in the preceding study of impermeant pyrene fluorophores (Cogan & Schachter, 1981), which demonstrates directly that the outer leaflet fluidity of intact erythrocytes does not differ significantly from that of ghost membranes prepared from them. (These results also indicate that membrane protein degradation in the course of osmotic lysis and membrane isolation does not account for the observed differences in fluorescence. Moreover, examination of the membrane proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed no evident degradation, and treatment of membranes with exogenous trypsin or Pronase yielded no consistent change in fluidity as assessed with DPH.)

In concert with prior observations (Cogan & Schachter, 1981) the present results lead to the conclusion that lipids of the outer leaflet of the human erythrocyte membrane exhibit greater motional freedom than those of the inner leaflet. Evidence of a similar asymmetry in *Mycoplasma* membranes (Rottem, 1975), Newcastle disease viral envelopes (Wisnieski & Iwata, 1977), and mouse LM cells (Wisnieski & Iwata, 1977; Schroeder, 1978) raises the possibility that the asymmetry is a general feature of the plasma membranes of many prokaryotic and eukaryotic cells. 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, 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 erythrocyte membrane lipids (van Deenen & de Gier, 1974; Rothman & Lenard, 1977; Op den Kamp, 1979), prediction of the relative fluidity of the individual leaflets from the known composition is difficult (van Dijck et al., 1976) because the hemileaflet distribution and the influence of a number of factors, including cholesterol, the glycolipids, the membrane proteins and the ionic environment,

have not been defined completely. Further studies should help clarify the particular differences in lipid composition or lipid–protein interactions which underly the asymmetry of leaflet fluidity, as well as its significance for membrane function and cell structure.

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