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Comparisons of steady-state anisotropy of the plasma membrane of living cells with different probes

James M. Collins and W. McLean Grogan

Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Campus of Virginia Commonwealth University, Richmond, VA (U.S.A.)

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We have used an extended Perrin equation which was in agreement with literature data for steady-state anisotropy (r_{SS}) for a wide variety of artificial and isolated biological membranes labeled with various probes (Van der Meer et al. (1986) Biochim. Biophys. Acta 854, 38–44 to obtain the static component (r_∞) for the intact plasma membranes of living cells. We show that lipid structural order parameters can be obtained for DPH and TMA-DPH in the plasma membranes of intact cells. We have examined the relationship between 'fractional limiting hindered anisotropy', r_∞/r_0, which is related to the lipid structural order parameter, of DPH, TMA-DPH, DPHpPC, and a series of depth-dependent probes (*n*-(9-anthroyloxy) fatty acids, with $n = 2$–16), using data from 19 cell types. There was a linear relationship between r_∞/r_0 values of DPH and TMA-DPH, but the relationship between either of these probes was non-linear with respect to DPHpPC or the series of fatty acid probes. The relationship between r_∞/r_0 values of DPHpPC and the series of fatty acid probes was linear, suggesting that they not only undergo similar motions in the membrane, but also experience similar types of restriction to motion, a type which is different from that experienced by DPH and TMA-DPH. We show that for the plasma membranes of living cells, 'second degree' order parameters can be estimated for DPH and TMA-DPH, and propose that the parameter r_∞/r_0, or the 'fractional limiting hindered anisotropy', analogous to a 'first degree' order parameter, can be estimated for DPHpPC and the depth-dependent fatty acid probes to evaluate the density of membrane packing.

Introduction

Since its introduction by Shinitzky et al. [1], the most widely used probe for the assessment of the physical state of the membrane has been 1,6-diphenyl-1,3,5-hexatriene (DPH). The apolar DPH is thought to insert into membranes with its long axis parallel to the acyl chains, near the bilayer center, although its precise location is uncertain [2]. The excitation and emission dipoles are parallel to the long axis of the molecule [3]. It wobbles as in a double-cone, about axes which lie in the plane of the membrane [4]. A more restricted wobble in cone type of motion would be expected with 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH), whose positively-charged quaternary amino group should anchor at the membrane surface in contact with water. However, the DPH moiety of the DPH-phosphatidyl choline derivative, 1-palmitoyl-2-([2-(4-[6-phenyl-*trans*-1,3,5-hexatriencnyl]phenyl)ethyl]carbonyl)-3-*sn*-phosphatidylcholine (DPHpPC) would be expected to rotate about the same axis as a fatty acyl hydrocarbon chain, i.e., about an axis perpendicular to the plane of the membrane [5].

Waggoner and Stryer [6] suggested that members of the (9-anthroyloxy) fatty acid (*n*-AF) series should tether the negatively-charged carboxyl group at the membrane surface in contact with water, and thus locate the anthracene ring at defined depths. The *n*-AF series insert into the membrane with the fatty acid chain parallel to the membrane phospholipid acyl chains, with the anthroyloxy group extended perpendicular to the long axis of the fatty acid chain; the excitation dipole is in the plane of the anthracene ring, perpendicular to its long axis, and the emission dipole is 30° to the excitation dipole [7]. There is little motion of the anthracene ring about the ester linkage [8]. The major type of motion is rotation about the long axis of the acyl chain. Thus, in membranes the *n*-AF

Correspondence: J.M. Collins, Box 614, MCV Station, Richmond, VA 23298-0614, U.S.A.

probes would obviously sense different kinds of restrictions to motion than would DPH or TMA-DPH, but might experience restrictions similar to those of DPH-pPC.

Steady-state anisotropy measurements are easy to perform, and precise physical/molecular descriptions have become available. Initially steady-state anisotropy of membranes probes was interpreted as the effects of solvent drag on the motion of the probes and their behavior in membranes was modeled with reference to isotropic oils; i.e, r_{SS} measurements were converted to microviscosity units [1,9]. However, after the development of time-resolved anisotropy measurements, it became clear that whereas r_∞, the limiting anisotropy, indeed approaches zero in oils, it never reaches this value in membranes. Accordingly, a new form of the Perrin equation that adequately described behavior in membranes was formalized [10–13]:

$$r_{SS} = \frac{r_0 - r_\infty}{1 + \frac{\tau_F}{\tau_C}} + r_\infty \tag{1}$$

where r_{SS} = steady-state anisotropy, r_0 = maximal limiting anisotropy (i.e., where it cannot rotate, 0.362 for DPH, 0.285 for 12-AF), r_∞ = limiting hindered anisotropy (i.e., in a membrane the restriction of complete 90° rotation, τ_F = mean excited state lifetime (time of decay), and τ_C = correlation time (varies inversely with speed of rotation). r_{SS} can be separated into two components (by grouping the numerator and denominator terms in the above equation) as:

$$r_{SS} = r_F + r_\infty \tag{2}$$

where: r_F is the 'kinetic' component, mainly describing the *rate* of rotation, and r_∞ is the 'static' component, describing the *restriction* to rotation [10–13]. In isotropic oils and homogeneous model phospholipid bilayers, r_{SS} can reflect different motional parameters with different probes, i.e., with the rod-like DPH (and TMA-DPH, and DPHpPC), r_{SS} is mainly determined by r_∞, whereas r_{SS} of anthroyloxy probes mainly reflects τ_C, that is, speed of rotation (i.e., r_∞ values are low). However, over the range of lipid packing exhibited by biological membranes, r_{SS} of DPH [4,10–14] and the anthroyloxy fatty acid probes [15] is dominated by r_∞, i.e., r_{SS} mainly reflects restriction of rotation.

Kinoshita et al. [2] used a 'cone model' to describe the restriction to the wobbling motion of DPH. The opening angle θ of this cone around the membrane normal reflects the density of molecular packing (i.e., order) in the membrane and is related to r_∞ as:

$$r_\infty / r_0 = [1/2 \cos\theta(1 + \cos\theta)]^2 \tag{3}$$

This equation, called the 'degree of orientational constraint' by Kinoshita et al. [2], has recently been recognized as the square of the orientational order parameter [11,12,16] from DMR and ESR studies. Realizing this, the relation:

$$r_\infty / r_0 = S^2 \tag{4}$$

was derived for fluorescence depolarization of DPH in a liquid crystalline environment, independent of any model [11,12,16], and S (i.e., the square root of r_∞/r_0) is called the 'second degree lipid structural order parameter'. It is important to recognize that r_∞ is determined solely by the density of membrane packing, i.e., lipid structural order. Therefore, r_{SS} mainly reflects lipid structural order [12,13,15,16].

Van Blitterswijk et al. [13] demonstrated that the relationship between r_{SS} values for DPH (hence r_∞ values) and structural order parameters was linear over the range of literature values obtained with biological membranes. Recently Van der Meer et al. [15] have presented data showing that the following extended form of the new Perrin equation describes the static component of r_{SS}:

$$r_\infty = \frac{r_0 r_{SS}^2}{r_0 r_{SS} + \frac{(r_0 - r_{SS})^2}{m}} \tag{5}$$

where m = 1.71 for DPH, 1.19 for TMA-DPH, and 1.80 for the *n*-(9-anthroyloxy) probes, thus providing a means of calculation of r_∞ from r_{SS} measurements. A surprisingly good correlation was found between r_∞ values measured experimentally and calculated from this equation, for the *n*-(9-anthroyloxy) fatty acids, i.e., only about 4% deviation [15].

Methods

Literature values of r_{SS} and r_∞: Values for DPH in biological membranes were taken from Van Blitterswijk et al. [13]; for DPH and TMA-DPH in living macrophages from Petty et al. [17]; for TMA-DPH in biological membranes from Van der Meer et al. [15]; for a series of anthroyloxy fatty acid probes (n = 2, 3, 7, 9, 12, and 16) in model egg lipid membranes from Kutchai et al. [18].

Values of r_∞ were calculated using Eqn. 5 from r_{SS} values obtained for DPH, TMA-DPH, DPHpPC and a series of anthroyloxy fatty acid probes (n = 2, 3, 6, 7, 9, 12, and 16) with living cells: Portions of this data have been previously presented for HeLa [19] and young human leukocytes (neutrophils, lymphocytes, and monocytes [20]. Data not previously published was from sperm (three stages of maturity, caput, cauda, and

corpus), two types of human prostatic carcinoma cells, three types of HL-60 cells (undifferentiated, and differentiated to macrophage or granulocytes), rat hepatocytes (three different diets, control, high cholesterol, and low cholesterol), aged human leukocytes (neutrophils, lymphocytes and monocytes), and aged human fibroblasts. In using Eqn. 5 to calculate r_∞ values from r_{SS} values, we assumed that $m = 1.71$ for DPH, 1.19 for TMA-DPH, and 1.80 for the anthroyloxy probes [15]. We used the data of Parente and Lentz [5] to calculate a value for m of 1.45 for DPHpPC according to Van der Meer et al. [15].

We assumed that $r_0 = 0.362$ for DPH, TMA-DPH, and DPHpPC [9,5], and 0.285 for the *n*-(9-anthroyloxy) fatty acid probes with $n = 2, 3, 6, 7, 9, 12$, and 16 [21].

r_{SS} of living cells measured by flow cytometry: The method has been described in detail elsewhere [19–24]. Essentially, probe dispersals at concentrations of 0.5 μM are added 1:1 with equal volumes of living cells and analyzed starting after 1 min with a flow cytometer (EPICS V, Coulter Electronics, Hialeah, FL) equipped with a half-wave retarder optical element (used to depolarize the incident laser beam permitting balancing of photomultiplier tubes fitted with vertical and horizontal polarizers prior to measurements). Next, the intensities of vertically and horizontally polarized emissions are measured. The data are output as intensity of vertically (I_V) and horizontally (I_H) polarized emission, and as anisotropy (calculated by the flow cytometer's computer as: $r_{SS} = (I_V - I_H)/(I_V + 2I_H)$) over a time period from 1 to 30 min (using the flow cytometer's clock). Under the conditions used [19,20,22] (i.e., 0.5 μM probe dispersals added to equal volumes of cells), greater than 95% of cellular fluorescence was located in the cell periphery, presumably the plasma membrane, as judged by light microscopy, after 30 min for TMA-DPH, all members of the *n*-AF series, and DPHpPC. With DPH, the cell interior began to appear fluorescent after 8 min. For each of the cell types, kinetic analysis revealed that the fluorescence anisotropy, r_{SS}, reached constant values after about 5 min, with no significant decrease up to 30 min with TMA-DPH, all members of the *n*-AF series, and DPHpPC, indicating little probe redistribution during this period (i.e., the probes are located predominantly in the plasma membrane, not interior membranes). However, with DPH, r_{SS} values began to decrease after 8 min, and continued to decrease up to about 25 min, indicating probe redistribution to internal membranes. Accordingly, the anisotropy values at 5 min were used for the data reported herein for all of the probes. We have previously demonstrated that there was no significant difference between r_{SS} values obtained with the flow cytometer and those obtained with an SLM spectrofluorometer when living cells were labelled with DPH, TMA-DPH, and 12-AF [22].

Results

Fractional limiting hindered anisotropy of living cells

Representing limiting hindered anisotropy (r_∞) as r_∞/r_0 provides a means of assessing the fraction of the maximum resistance to motion, as when $r_\infty = r_0$ the rotational motion of the probe is absent. Representing steady-state anisotropy as r_{SS}/r_0 provides a means of comparing different probes with different r_0 values. Fig. 1A shows the relationship between our experimentally-measured r_{SS} (as r_{SS}/r_0) and our calculated r_∞ (as r_∞/r_0) values obtained with living cells with DPH and TMA-DPH, along with literature values (where r_{SS} and r_∞ were each experimentally-measured with isolated biological membranes [13]). The relationship is linear (linear correlation coefficient, $r = 0.997$) over the range of living cell plasma membranes. The two greatest DPH literature values (which are greater than those of any of the intact cells surveyed) are those of eye lens and retinal rod outer disk membranes, which have a remarkably high structural order [13], and the single literature TMA-DPH value is from Ref. 17. For these rod-like probes, where a model is known [2,4], we can calculate lipid structural order parameters, S(probe), by means of Eqn. 4; this is shown in Fig. 1B. Again, the relationship is linear ($r = 0.990$) over the range of living cell plasma membranes.

The relationship between our experimentally-measured r_{SS} (as r_{SS}/r_0) and our calculated r_∞ (as r_∞/r_0) values obtained with living cells using the *n*-(9-anthroyloxy) fatty acid probes, along with literature values (where r_{SS} and r_∞ were each experimentally-measured with model membranes [18] is shown in Fig. 1C. The relationship is linear ($r = 0.994$) over the range of living cell plasma membranes. The square root of the expression (r_∞/r_0) in Fig. 1C would not represent the second degree lipid structural order parameter as it is most likely that some relationship other than that of Eqn. 4 would be the appropriate one for the fatty acid probes.

Comparisons between DPH, TMA-DPH, DPHpPC, and the n-AF series

The relationship of individual r_∞/r_0 values of DPH and those of TMA-DPH, DPHpPC, 12-AF, and 16-AF obtained with living cells are shown in Fig. 2. These data are linked in the sense that each *Y*-axis data point corresponds to an *X*-axis DPH value for the same cell type. As can be seen there is a fairly linear relationship ($r = 0.988$) between values of TMA-DPH and DPH. The relationship between DPHpPC and DPH is nonlinear (Fig. 2, $r = 0.140$). This is expected as the DPH moiety of DPHpPC undergoes a completely different type of motion and should thus experience a different type of restriction to motion. It has been suggested that the average location of DPH in the membrane is

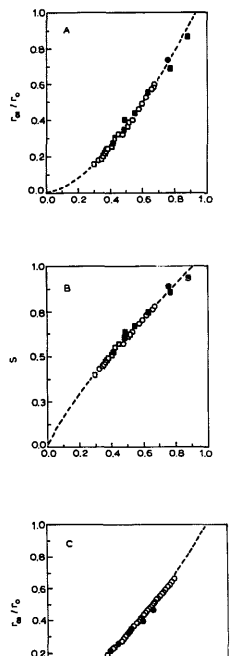

Fig. 1. The fractional limiting hindered anisotropy, r_∞/r_0 (Panels A and C) and the order parameter, S (panel B), as a function of r_{SS}/r_0.The r_{SS} values were obtained for the plasma membranes of living HeLa, young and aged human leukocytes, sperm, rat hepatocytes, HL 60, two human prostatic carcinoma cell lines, and aged human fibroblasts. the large symbols denote values of experimentally measured r_∞ and r_{SS} taken from the literature. The dashed lines show the best fit of the data when the curves are forced to intercept zero. Panels A and B: □, DPH intact cells; ■, DPH literature; ○, TMA-DPH intact cells; ●, TMA-DPH literature. Panel C: ○, n-AF intact cells; ●, n-AF literature.

similar to that of 12-AF [18]; however, no apparent relationship is noted with 12-AF ($r = 0.088$), nor 16-AF ($r = 0.096$) in Fig. 2, nor with any of the other n-AF probes as well as the averages of the n-AF probes (e.g., averaged values of those probes with $n = 2$–16, 2–9, 9–12, 9–16, etc; data not shown). As expected, when

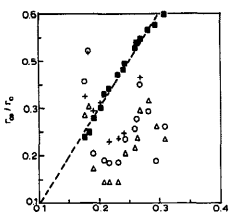

Fig. 2. The relationship between r_∞/r_0 values of DPH and TMA-DPH, DPHpPC, 12-AF and 16-AF of living cell plasma membranes. ■, TMA-DPH; +, DPHpPC; ○, 12-AF; △, 16-AF.

the data were plotted as r_{SS}/r_0, the same relationships as those seen in Fig. 2 were noted (data not shown).

The relationship between r_∞/r_0 values of DPHpPC and the n-AF probes obtained with living cells, are shown in Fig. 3. Again, the data are linked whereby each Y-axis data point corresponds to an X-axis DPH-pPC value for the same cell type. As can be seen, the relationship between DPHpPC and the average of the values obtained with the entire series of probes (i.e., $n = 2$–16) is linear ($r = 0.991$). Similar common relationships are obtained for other combin. ions of averaged values (i.e., for $n = 7$–12, $r = 0.999$; $n = 6$–12, $r = 0.998$; $n = 2$–9, $r = 0.993$; $n = 9$–16, $r = 0.989$, etc., data not shown). As expected, no similarities are noted (Fig. 3) with TMA-DPH ($r = 0.340$) and DPH ($r = 0.061$). Also as expected, when the data were plotted as r_{SS}/r_0,the same relationships as those seen in Fig. 3 were noted (data not shown).

It would seem important to test for relationships similar to those of Figs. 2 and 3 in model membrane

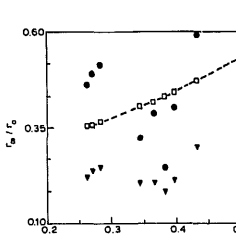

Fig. 3. The relationship between r_∞/r_0 values of DPHpPC and the average of those n-AF probes from 2–16, TMA-DPH and DPH of living cell plasma membranes. □, average of $n = 2$–16 AF probes; ●, TMA-DPH; ▼, DPH.

systems. However, the only linked data of which we are aware is that of Kutchai et al., (1983) where DPH and the *n*-AF series were used with DPPC, DOPC, POPC, and egg PC membranes. As expected, the relationship between r_∞/r_0 of DPH vs. r_∞/r_0 of the *n*-AF series was weak, i.e., $r = 0.725$ (data not shown).

Discussion

There is little doubt that when used in non-perturbing amounts (i.e., $< 1.0\ \mu M$), the anthroyloxy moieties of the series of *n*-AF probes locate at a graded series of depths in the outer hemi-leaflet of the membrane of model lipid bilayers (e.g., 25, 26, 28), of isolated beef mitochondria [28], and of living cells [29]. Furthermore, the recent adaptation of commercial flow cytometers for polarization studies, allows the use of living cells by circumventing many of the problems with static fluorometry, such as depolarization by scatterers, photobleaching, and use of probes with low quantum yields [19,20,22–24]. Thus, this technique permits anisotropy measurements of intact membranes, i.e., attached to living cells, rather than of isolated membranes, i.e., that have possibly been perturbed by removal from cells. We have observed with cuvette spectrofluorometry that r_{SS} values are about 40% higher with living cells than those values obtained with isolated membranes (plasma mixed with internal) (Collins and Grogan, data not shown).

The physiological significance of r_{SS} measurements is not that they reflect viscosity, but that they give information on lipid structural order parameters, i.e., on the density of packing. As r_∞ never reaches zero in a (anisotropic) membrane (e.g., r_∞/r_0 in Fig. 1, A and C), as it does in (isotropic) oils, the outmoded practice of converting anisotropy values to microviscosity units should be abandoned [10–13,15]. Data obtained with living cells (Fig. 1) are linear over a wide range of membrane rigidities and are in general agreement with data presented by Van Blitterswijk et al. [13] with model and isolated biological membranes, suggesting that the same principles regarding r_{SS} measurements apply, i.e., r_{SS} reflects lipid structural order.

r_∞/r_0 values of DPH and TMA-DPH over a wide range of different plasma membranes should have a common relationship (as seen in Fig. 2), as they have similar excitation and emission dipoles [3] undergo similar kinds of motion, i.e., wobble in a cone, about axes which lie in the plane of the membrane [4], and thus should experience similar types of restriction to motion [10–13]. Conversely, r_∞/r_0 values of DPHpPC and the *n*-AF members should not be closely related to those of DPH (as was seen in Fig. 2), as these probes experience a type of motion different from that of DPH. Like the phospholipid acyl hydrocarbon chains [30,31], the DPH moiety of DPHpPC [5] and the anthroyloxy moiety of the *n*-AF members [8] rotate about long axes perpendicular to the plane of the membrane.

As positioned in the membrane, individual members of the *n*-AF series would sense different portions of the larger region sensed by DPHpPC, i.e., the centers of the anthroyloxy rings of 7-AF and 16-AF align almost precisely with the centers of the first and second phenyl rings of the DPH moiety of DPHpPC, respectively; even with 2-AF, the member of the series with the anthroyloxy moiety at the surface, partly in contact with water and partly in the lipid phase [25,26], the lipid phase portion of the anthroyloxy ring aligns close to the first phenyl ring (from consideration of CPK molecular models – data not shown). Therefore, if DPHpPC and the *n*-AF probes experience similar types of restriction to motion, then their r_∞/r_0 values should be closely related over the wide range of values obtained with different membranes (as was seen in Fig. 3), irregardless of the differences [3,7] in emission and excitation dipoles. The closeness of the relationship is not due to the wrong value of m for DPHpPC in Eqn. 5, as use of *m*-values ranging from 1.19 to 1.80 only moderately affected the linearity seen in Fig. 3 (i.e., *r* ranged from 0.986 to 0.999, data not shown).

In summary, we have confirmed the observations of Van Blitterswijk et al. [13] and Van der Meer et al. [15] with artificial and isolated biological membranes and extended them to living cell plasma membranes (Fig. 1A). We have shown that 'second degree' order parameters for the plasma membranes of living cells can be estimated for DPH and TMA-DPH, for which an adequate model is known (Fig. 1B). Although we are presently uncertain as to the proper model for the *n*-AF probes, the potential wealth of information that can be obtained with these probes (i.e., membrane structural dynamics at different depths) far outweighs any current disadvantages in ability to estimate second degree order parameters. The parameter r_∞/r_0, or the 'fractional limiting hindered anisotropy', is analogous to a 'first degree' order parameter, is obviously related to lipid structural order, and is linearly related to r_{SS} over a wide range of living cell membranes (Fig. 1C). Thus, for the *n*-AF series, this parameter can be used to evaluate the density of membrane packing at different depths into the plasma membrane.

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References

1 Shinitzky, M., Dianoux, A.C., Gitler, C. and Weber, G. (1971) Biochemistry 10, 2106–2113.

2 Kinoshita, K., Kawato, S. and Ikegami, A. (1977) Biophys. J. 20, 289–305.
3 Andrich, M.P. and Vanderkooi, J.M. (1976) Biochemistry 15, 1257–1261.
4 Kawato, S., Kinoshita, K. and Ikegami, A. (1977) Biochemistry 16, 2319–2324.
5 Parente, R.A. and Lentz, B.A. (1985) Biochemistry 24, 6178–6185.
6 Waggoner, A.S. and Stryer, L. (1970) Proc. Natl. Acad. Sci. USA 67, 579–589.
7 Badley, R.A., Martin, W.G. and Schneider, H. (1973) Biochemistry 12, 268–275.
8 Johns, S.R., Willing, R.I., Thulborn, K.R. and Sawyer, W.H. (1979) Chem. Phys. Lipids 24, 11–16.
9 Shinitzky, M. and Barenholz, Y. (1978) Biochem. Biophys. Acta 515, 367–394.
10 Hildenbrand, K. and Nicolau, C. (1977) Biochim. Biophys. Acta 553, 365–377.
11 Heyn, M.P. (1979) FEBS Lett. 108, 359–364.
12 Jähnig, F. (1979) Proc. Natl. Acad. Sci. USA 76, 6361–6365.
13 Van Blitterswijk, W.J., Van Hoeven, R.P. and Van der Meer, B.W. (1981) Biochim. Biophys. Acta 644, 323–332.
14 Dale, R.E., Chen, L.A. and Brand, L. (1977) J. Biol. Chem. 252, 7500–7510.
15 Van der Meer, W.B., Van Hoeven, R.P. and Van Blitterswijk, W.J. (1986) Biochim. Biophys. Acta 854, 38–44.
16 Lipari, G. and Szabo, A. (1980) Biophys J. 30, 489–506.
17 Petty, H.R., Niebylski, C.D. and Francis, J.W. (1987) Biochemistry 26, 6314–6318.
18 Kutchai, H., Chandler, L.H. and Zavoico, G.B. (1983) Biochim. Biophys. Acta 5736, 137–149.
19 Collins, J.M., Dominey, R. and Grogan, W.M. (1990) J. Lipid Res. 31, 261–270.
20 Collins, J.M., Scott, R.B. and Grogan, W.M. (1990) J. Cell. Physiol. 144, 42–51.
21 Schachter, D. and Shinitzky, M. (1977) J. Clin. Invest. 59, 536–547.
22 Collins, J.M. and Grogan, W.M. (1989) Cytometry 10, 44–49.
23 Grogan, W.M. and Collins, J.M. (1990) Guide to Flow Cytometry Methods: A Laboratory Manual, pp. 1–228, Marcel Dekker, New York.
24 Fox, M.H. and Delohery, T.M. (1987) Cytometry 8, 20–25.
25 Podo, F. and Blasie, J.K. (1977) Proc. Natl. Acad. Sci. USA 74, 1032–1036.
26 Thulborn, K.R. and Sawyer, W.H. (1978) Biochim. Biophys. Acta 511, 125–140.
27 Blatt, E., Chatelier, R.C. and Sawyer, W.H. (1984) Photochem. Photobiol. 39, 477–483.
28 Chatelier, R.C. and Sawyer, W.H. (1985) Eur. Biophys. J. 11, 179–185.
29 Collins, J.M. and Grogan, W.M. (1991) Cytometry, in press.
30 Hubbell, W.L. and McConnell, H.M. (1971) J. Am. Chem. Soc. 27, 314–326.
31 Seelig, J. (1977) Q. Rev. Biophys. 10, 355–418.