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Steady-state fluorescence polarization data in membranes. Resolution into physical parameters by an extended Perrin equation for restricted rotation of fluorophores

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An extended Perrin equation is derived applicable to the restricted rotation of fluorophores. The equation results in a relation between time-resolved (r_∞) and steady-state fluorescence anisotropy (r_s) data. This relation contains a parameter m, which expresses the difference between rotational diffusion in a lipid membrane and that in an isotropic reference oil having the same r_s value. The relation is in agreement with r_s, r_∞ literature data for a variety of artificial and biological membranes labeled with various probes. Cholesterol and fatty acyl unsaturation affect the value of m, but temperature does not. The results indicate that, as far as fluorescence depolarization is concerned, either liposomes of saturated phospholipids without cholesterol or liposomes of unsaturated phospholipids containing cholesterol are good model systems for biological membranes. The accuracy of estimating order parameters or rotational diffusion constants from r_s data is discussed. The formalism described here introduces a novel way of applying Arrhenius plots and allows for an unambiguous interpretation of r_s data.

Introduction

Since more than a decade the steady-state fluorescence polarization technique has been applied to biological and artificial membranes to estimate their lipid fluidity or to detect phase transitions as a function of temperature. In the early literature quantitative data were obtained by comparing fluorescence polarization values in membranes with those in an isotropic reference oil, using the Perrin equation [1]. In this manner the measured values in membranes were converted into 'anisotropy parameters', $((r_o/r_s)-1)^{-1}$ [2], or in 'microviscosity' units, $\bar{\eta}$ [1].

More recently, a more realistic interpretation of the steady-state fluorescence polarization data was given, in which both a dynamic and a static component of the measured anisotropy appear [3,4]. For the probe 1,6-diphenyl-1,3,5-hexatriene (DPH) the static component (r_∞) could be derived from the measured steady-state data (r_s) by an empirical relationship [5,6], thus allowing calculation of an order parameter in lipid membranes. Despite these new insights, there are nowadays still groups that erroneously keep expressing their measured fluorescence polarization data in the old units (perhaps by tradition or because order parameters, unlike the supposed microviscosities or anisotropy parameters, are as such not unambiguously related

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to phase transitions in membranes to be detected by Arrhenius plots [7]).

The present paper aims at a synthesis between the old and the new interpretation by describing an extended Perrin equation. The extension in this equation, expressed by the parameter m, represents the difference between the rotational diffusion of a fluorophore in a lipid membrane and that in an isotropic reference oil having the same steady-state fluorescence anisotropy. Empirical determination of m in various artificial and biological membranes leads to a relation between r_∞ and r_s which is in agreement with published data. The present derivation of the r_∞-r_s relation is an extension of the one introduced by Van Blitterswijk et al. [5] and Pottel et al. [6] and has several advantages, e.g.: (1) it is applicable not only to DPH, but to other fluorophores as well; (2) the variation from one type of membrane to another, which has been noted in some cases [8] can be taken into account; (3) the use of Arrhenius plots in terms of a modified anisotropy parameter can be justified for fluorescence depolarization data in membranes.

Theory

Time-resolved fluorescence anisotropy studies of rodlike probes such as 1,6-diphenyl-1,3,5-hexatriene (DPH) in membranes have demonstrated that the rotational motion of these probes is hindered and distinctly different from probe motions in isotropic oils [9,10]. The fluorescence anisotropy response of a membrane probe to an ideal flash illumination is shown in Fig. 1. Immediately after the flash the anisotropy equals r_o, the maximal fluorescence anisotropy, and decays towards a finite level, r_∞, the limiting fluorescence anisotropy. A useful approximation of this decay behaviour is [3,4]

$$r_t = r_\infty + (r_o - r_\infty)\exp(-t/\phi) \quad (1)$$

where r_t is the fluorescence anisotropy at time t after the flash and ϕ is the rotational correlation time.

In the present paper we are concerned with the steady-state fluorescence anisotropy, r_s, which is also indicated in Fig. 1. This value can be calculated from r_t by a time integration [3,4]. In the case of a single-exponential decay of the fluorescence, r_s can be expressed as a sum of two terms

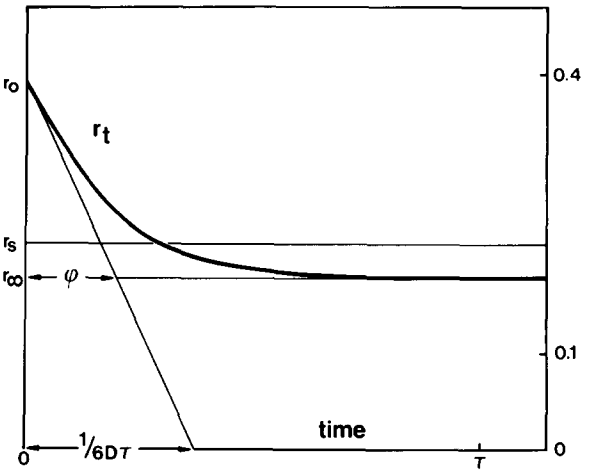

Fig. 1. The fluorescence anisotropy decay as a function of time (r_t) following a very short flash illumination at time zero. In the particular example shown, the maximal anisotropy (r_o) is 0.380, the steady-state fluorescence anisotropy (r_s) is 0.213, the limiting anisotropy (r_∞) equals 0.180; the fluorescence lifetime (τ) has been taken as the time unit, the rotational correlation time (ϕ) is 0.2 and the rotational diffusion constant (D) equals $0.44/\tau$.

$$r_s = r_\infty + r_f \quad (2)$$

The limiting fluorescence anisotropy, r_∞ reflects the range or amplitude of random librational motions, and r_f depends primarily on the rate of rotation and reflects essentially the frequency of the librational motions, although it depends on r_∞ as well

$$r_f = (r_o - r_\infty)/(1 + \tau/\phi) \quad (3)$$

$$r_\infty = r_1 S^2 \quad (4)$$

where τ is the fluorescence lifetime, S is the second rank orientational order parameter of the probe and r_1 is a factor depending on the geometry of the absorption and emission dipoles [11]. When at least one of these dipoles lies along the long axis of the probe, r_1 equals r_o, which is the case for rodlike probes, such as diphenylhexatriene. Although generally more than one lifetime and more than one correlation time are

needed to describe high quality time-resolved data [12], the equations used here present nevertheless a good approximation, if one of the lifetimes and one of the correlation times is dominant or if the times do not differ too much. For example, in the case of two lifetimes, $\tau_1 = 2$ ns and $\tau_2 = 10$ ns with preexponential factors 0.25 and 0.75, respectively, the difference in r_s is 6% compared to the case of one average lifetime of 8 ns, if one takes $r_o = 0.4$, $\phi = 2$ ns and $r_\infty = 0$. In the case of a larger r_∞, the difference becomes less and equals zero for $r_\infty = r_o$. As another example, consider the case of one lifetime, $\tau = 8$ ns, and two rotational correlation times, $\phi_1 = 1$ ns and $\phi_2 = 3$ ns with equal weights, for $r_o = 0.4$. This results in a r_s value differing only 4% from having one average $\phi = 2$ ns, if $r_\infty = 0$ and less if $r_\infty > 0$.

The time derivative of the fluorescence anisotropy immediately after excitation with a short flash is proportional to the wobbling diffusion constant D for rodlike probes [13]. In the approximation with a single correlation time, ϕ is related to D according to Ref. 14

$$\phi = (r_o - r_\infty)/(6Dr_o) \tag{5}$$

This relation, which is also indicated in Fig. 1, applies to rodlike probes with the absorption and/or emission dipole along the long axis. However, in the case that the deviation from cylindrical symmetry is small or the rotational diffusion constants differ not too much from the average value D, Eqn. 5 will be a good approximation as well.

It is of interest to compare two systems labeled with the same probe and having the same r_s value: one is a membrane suspension where the probe rotation is restricted, $r_s = r_f + r_\infty$, and the other is an isotropic reference oil with anisotropy r_s ($r_\infty = 0$). In the membrane r_f is inversily related to the (average) rotational diffusion constant D and, similarly, in the isotropic oil r_s to D_i, the rotational diffusion constant in this system. Because r_f is smaller than r_s, D must be larger than D_i whereas for D_i Perrin's equation holds [1]. Therefore, $6D\tau$ must be larger than $(r_o/r_s) - 1$, that is

$$6D\tau = (r_o/r_s) - 1 + m \tag{6}$$

where m has a positive value. The parameter m expresses the difference between the rotational diffusion of the probe in the membrane and that in the isotropic reference oil. Eqn. 6 is our extended perrin equation, briefly introducted before [15]. It means that a restricted fluorophore must rotate faster than an unrestricted one in order to have the same r_s value. Substituting Eqn. 3 into Eqn. 2, eliminating ϕ using Eqn. 5 and next D using Eqn. 6 and solving for r_∞ yields the following r_∞-r_s relation

$$r_\infty = r_o r_s^2 / \left(r_o r_s + (r_o - r_s)^2/m\right) \tag{7}$$

This equation is an extension of the $r_\infty - r_s$ relations introduced by Van Blitterswijk et al. [5] and Pottel et al. [6]; for $m = 1.5$ Eqn. 7 is essentially identical to the $r_\infty(r_s)$ function of Pottel et al. [6], and for $m = 1.5$ and $r_o = 0.4$ Eqn. 7 is very close to the relation of Van Blitterswijk et al. [5]. The present relation applies not only to diphenylhexatriene, but to other fluorophores as well and it is not one unique relation, but rather represents a set of $r_\infty(r_s)$ functions, so that there is a different relation for each particular m value, as is shown in Fig. 2. All the curves corresponding to Eqn. 7 lie between the line for $m = 0$ ($r_\infty = 0$), which value refers to the Perrin equation, and the line for $m = \infty$ ($r_\infty = r_s$), in which case the rotational diffusion does not contribute at all to the fluorescence anisotropy. A plot of r_∞ versus r_s is curvilinear in all other cases (see Fig. 2). A small

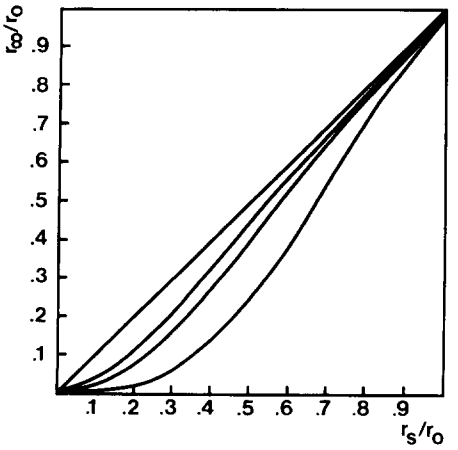

Fig. 2. Four examples of plots of r_∞/r_o versus r_s/r_o, derived from Eqn. 7, for $m = \infty$, $m = 4$, $m = 2$ and $m = 0.5$, from the upper to the lowest curve, respectively.

m means that the curvature of the r_∞-r_s relation is strong, and the r_f or the rotational correlation time ϕ has a large contribution to r_s (Eqns. 2, 3). If m incrases the r_∞-r_s curvature becomes less, approaches the straight line $r_\infty = r_s$ (the upper line in Fig. 2) and the contribution of r_f to r_s becomes relatively small.

Methods

Literature r_s, r_∞ data have been fitted to Eqn. 7 using a non-linear least-squares analysis. The best fitting value for m has been calculated by minimalizing [16]

$$\sigma^2 = \Sigma\left((r_\infty/r_o)_o - (r_\infty/r_o)_c\right)^2/(N-1) \tag{8}$$

where the summation runs over all data and the subscripts o and c refer to observed and calculated values, respectively. N is the number of data points. The standard deviation for m is obtained from Ref. 16

$$\sigma_m = \sigma/\sqrt{\Sigma\left(\partial(r_\infty/r_o)/\partial m\right)^2} \tag{9}$$

If m is known, the (average) rotational diffusion constant D can be estimated from r_s using Eqn. 6. The error in m results in an error in D, which can be calculated according to

$$\Delta D/D = |\partial D/\partial m|\sigma_m = \sigma_m/\left((r_o/r_s)-1+m\right) \tag{10}$$

The error in D increases with increasing r_s. The error in S which can be estimated from r_s using Eqns. 7 and 4, decreases with increasing r_s

$$\Delta S/S = \tfrac{1}{2}|\partial r_\infty/\partial m|\sigma_m = \sigma_m(r_o - r_s)^2/\left(m^2 r_o r_s + m(r_o - r_s)^2\right) \tag{11}$$

Results and Discussion

A great number of r_s, r_∞ data from the literature for several membranes doped with various probes have been analyzed and fitted to Eqn. 7. Table I compiles the results. The majority of the data refer to the probe diphenylhexatriene, used in several types of artificial and biological membranes. It is noteworthy that essentially the same m values (about 1.8) were found for diphenylhexatriene in saturated phospholipids without cholesterol (Table I, 2nd line), in unsaturated phospholipids mixed with cholesterol (3rd and 5th line) and in biological membranes (4th line). This similarity may indicate that these particular artifical membranes (liposomes) are good model systems for biological membranes with respect to the diphenylhexatriene motions. On the other hand, the single-component unsaturated phospholipids (Table I, first line) show a much lower value ($m = 0.58$), whereas saturated phospholipids mixed with cholesterol (6th and 7th line) show m values as high as 7 for diphenylhexatriene.

The meaning of distinct magnitudes of m for the various types of membranes has been explained at the end of section Theory. Thus, if one compares unsaturated and saturated phosphatidylcholines at a given (low) degree of structural order (at the same r_∞), the former phospholipids show the larger r_s and r_f (larger ϕ, lower rotational rate of the diphenylhexatriene probe) because of their lower m value (Table I, lines 1 and 2, respectively). This seems in contradiction with the conception that acyl chain unsaturation has a fluidizing effect and decreases r_s. However, this can be explained by the different temperatures to which the two types of liposomes presumably have been exposed to in order to reach the same r_∞, i.e. the saturated phosphatidylcholine to the higher temperature. The high m value for liposomes consisting of saturated phosphatidylcholines and cholesterol (Table I, lines 6 and 7) in comparison to the former compounds only (line 2), means that cholesterol at a given r_∞ decreases r_s and r_f (smaller ϕ, higher rotational rate of diphenylhexatriene molecules). Again, this seems in contradiction with the conception that cholesterol decreases lipid fluidity and increases r_s in the liquid-crystalline state, but a similar reasoning as above can be given in this case.

An alternative interpretation of the distinct magnitudes of m can be given in terms of the type of orientational distribution of the fluorophore, i.e. cone-like [13] or non-cone-like. It has been shown that the steady-state fluorescence anisotropy (r_s) is very sensitive to this orientational distribution and that a small proportion of diphenylhexatriene molecules may be oriented parallel to the plane of the membrane (in between the bilayer leaflets),

TABLE I

FITTING PARAMETERS FOR MEMBRANES AND PROBES

Values are listed for a variety of artificial and biological membranes labeled with various probes; m has been obtained for every type of membrane by fitting r_s, r_∞ data to Eqn. 7, minimalizing σ^2 (Eqn. 8); the values for m, σ and σ_m (Eqn. 9) refer to this minimum; N is the number of data points. The data were obtained from the references indicated.

Probe [a]	Membranes [b]	Ref.	N	$1000\sigma^2$	$m \pm \sigma_m$
DPH	DOPC, POPC, PLPC, PAPC (10–60°C)	17, 18	25	1.32	0.58±0.05
DPH	DMPC, DPPC (5–60°C)	10, 17–19	41	0.92	1.7 ±0.1
DPH	Egg PC:Chol, Egg PC: Egg PE(1:1):Chol ($0.2 \leqslant C/PL \leqslant 1$) (25°C)	20	17	0.11	1.76±0.06
DPH	biological membranes [c]	20, 21	12	0.87	1.7 ±0.2
DPH	DOPC: Chol ($0 < C/PL < 1$) (25°C)	8	13	1.72	1.9 ±0.3
DPH	DPPC: Chol ($0 < C/PL < 1$) (47°C)	8	8	0.36	7 ±1
DPH	DMPC: Chol ($C/PL = 0.33$; 0–60°C), DPPC: Chol ($C/Pl = 0.5$; 5–60°C)	18, 22	22	0.05	7.7 ±0.6
TMA-DPH	DPPC (28–53°C), DMPC (10–38°C)	23	15	0.11	0.63±0.01
TMA-DPH	POPC, SOPC (5–35°C)	23	22	0.75	1.19±0.09
tPnA	DPMC (39°C), DPPC (47.5 and 59°C),	25, 26	8 [d]	2.52	1.6 ±0.3
MtPnPC	DPPC (21, 38, 47.5 and 59°C)	25,26	8 [d]	1.93	1.0 ±0.2
n-AS, 16-AP	DOPC: Chol, POPC: Chol, Egg PC: Chol ($0 \leqslant C/PL \leqslant 1$; 25°C), DPPC: Chol ($0 \leqslant C/PL \leqslant 1$; 47°C)	8	183	0.42	1.80±0.04

[a] Abbreviations of probes: DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-(trimethylamino)phenyl)-6-phenyl-1,3,5-hexatriene; tPnA, *trans*-parinaric acid; MtPnPC, 1-myristoyl-2-*trans*-parinaroyl-*sn*-glycero-3-phosphocholine; *n*-AS, *n*-anthroyloxystearate ($n = 2$, 7, 9 or 12); 16-AP, 16-(9-anthroyloxy)palmitate.

[b] Abbreviations: DMPC, DPPC, DOPC, DEPC, POPC, SOPC, PLPC, PAPC, eggPE, liposomes of dimyristoyl-, dipalmitoyl-, dioleoyl-, dielaidoyl-, palmitoyloleoyl-, stearoyloleoyl-, palmitoyllinoleoyl-, palmitoylarachidonyl-, egg yolk phosphatidylcholine, egg yolk phosphatidylethanolamine; Chol, cholesterol; C/PL, cholesterol/phospholipid molar ratio. Temperature (range) indicated.

[c] Biological membranes: rabbit sarcoplasmic reticulum, rat liver mitochondria, human erythrocyte plasma membranes at 10 and 35°C, from Ref. 21; whole cells of D_{17}, BHK21, LM-fibroblasts, HeLa, N-egg and NDV-MDBK (viruses) at 25°C from Ref. 20.

[d] Two r_∞ values from Ref. 24 have been used, the so-called $r_\infty^{(fit)}$ and $r_\infty^{(tail)}$.

rather than in a cone perpendicular to it, especially so in a highly fluid state (e.g. in liposomes of pure unsaturated phosphatidylcholines) [12,14]. This could result in the smaller m value for the latter type of phospholipids, corresponding to the downward shift of curvature in Fig. 2.

We found two exceptional m values of about 3.5 (not included in Table I), calculated from data of Kutchai et al. [8], for mixtures of (unsaturated) POPC or egg PC with cholesterol. It is not clear why these values are different from the many others for similar systems (Table I, lines 3 to 5, from Refs. 8, 20, 21). It could possibly be related to a difference in methodology.

One example of the least-squares fits to Eqn. 7 listed in Table I is shown in Fig. 3. This particular case refers to DMPC and DPPC at various temperatures (Table I, 2nd line), yielding $m = 1.7$. It is clear that the value for m is insensitive to temperature changes, because the low-temperature (high r_s/r_o) data fit the same line as the high-temperature (low r_s/r_o) data.

For the probe trimethylamino-diphenylhexatriene (TMA-DPH) the fitted data for several single-component phospholipids [23] yield m values which are different from those for diphenylhexatriene: The unsaturated phosphatidylcholines show higher values, while the saturated phosphatidylcholines show lower values (Table I, lines 8 and 9). These contrasting results for these two probes may be partly related to different fluorescence lifetimes, that of TMA-DPH being smaller than that of diphenylhexatriene, especially so in the liquid-crystalline state (the phospholipids with unsaturated acyl chains) [23,24]. Further, TMA-DPH is most probably anchored at the lipid-water

interface by its polar head, and is therefore more periferally located in the lipid bilayer than diphenylhexatriene. The data are consistent with the interpretation that acyl chain unsaturation increases the m value (increases the rotational rate) at the membrane surface, whereas it decreases m in the interior of the bilayer.

Only a few data are available for parinaric acid and its phosphatidylcholine derivative [25,26], yielding m values of 1.6 and 1.0, respectively, with a relatively poor fit to Eqn. 7. In contrast, a surprisingly good correlation between r_∞ and r_s is found for the anthroyloxy fatty acids (Table I, last line). Kutchai et al. [8] have published the relation

$$r_\infty = 0.0036 + 0.298\, r_s + 2.86\, r_s^2 \quad (r_o = 0.311) \tag{12}$$

for these probes, having a 'goodness of fit' of 1000 $\sigma^2 = 0.31$, which is slightly better than the corresponding fit in Table I. However, the one of Kutchai et al. [8] contains three adjustable parameters, while ours has only one, that is m.

It should be noted that the present analysis is based upon the assumption that the simple model of Eqn. 1 is correct. This approximation may not be valid for every probe. Particularly in the case of the anthroyloxy fatty acids, the in-plane and out-of-plane rotations (i.e., the plane of the fluorophore) may differ appreciably, so that the model of Eqn. 1 is invalid. The seeming relation between r_∞ and r_s for these probes may alternatively reflect a correlation between the in-plane and out-of-plane rotations..

The error in the rotational diffusion constant D due to an incertainty in the m value increases if r_s becomes larger (Eqn. 10). Taking, for example, $m = 2$ and $\sigma_m = 0.6$, we have $\Delta D/D = 5\%$ for $r_s = 0.1\, r_o$ and $\Delta D/D = 26\%$ for $r_s = 0.8\, r_o$. The error in the order parameter S, on the other hand, decreases with increasing r_s (Eqn. 11). For example, with $m = 2$ and $\sigma_m = 0.6$, one finds $\Delta S/S = 12\%$ at $r_s = 0.1\, r_o$ and only $\Delta S/S = 0.5\%$ at $r_s = 0.8\, r_o$. From these trends one can conclude that the estimation of S for diphenylhexatriene is accurate for high r_s and the estimation of the rate of rotation is reliable only for low r_s values, in agreement with previous findings of Pottel et al. [6].

As discussed above and illustrated in Fig. 3, temperature changes do not affect the value of the

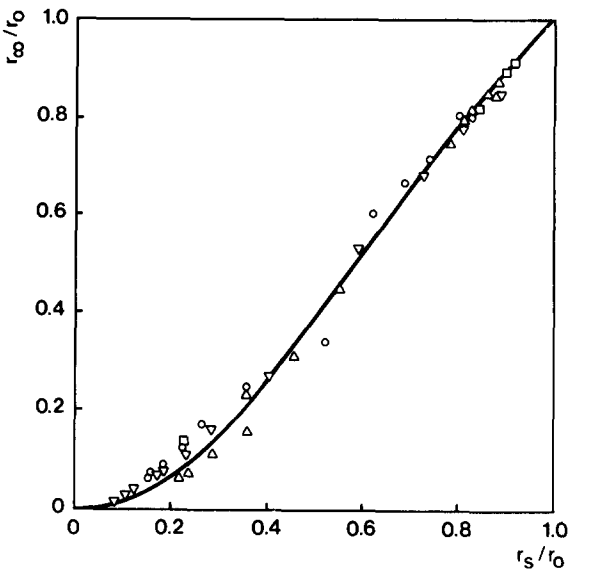

Fig. 3. The best fit to Eqn. 7 for the data in the 2nd line of Table I, yielding $m = 1.7$ and referring to the probe diphenylhexatriene in liposomes of DPPC (□), DPPC (○), DMPC (▽) and DPPC (△) at various temperatures between 5 and 60°C from Refs. 17, 18, 19 and 10, respectively.

m parameter. Accordingly, the extended Perrin equation (Eqn. 6) suggests a novel way to construct Arrhenius plots: by plotting $\ln((r_o/r_s) - 1 + m)^{-1}$ versus $1/T$ for a few estimates of m, so that the slopes are proportional to activation energies of diffusion. This presentation may be helpful in detecting phase transitions in membranes as a function of temperature and is an improvement over the Arrhenius plot with the old units [1,7], which does not yield a sound activation energy, because it is not corrected for the static component of the fluorescence anisotropy.

The r_∞-r_s relation discussed in this paper differs from the one discussed by Hare [27] in that the fluorescence lifetime does not appear in the formula and no assumptions on the lifetime have been made. We do not agree with his statement that a lifetime measurement is always necessary for estimating an order parameter [27]. However, changes in the lifetime may introduce an error in m. Applying the present method to the data for the colored membranes of Ref. 28, where the fluorescence lifetime is shortened due to energy transfer [6], we find that the r_∞-r_s relation for

these membranes can be properly described by $m \pm \sigma_m = 6 \pm 2$.

In conclusion, it can be stated that the present method to describe correlations between time-resolved and steady-state fluorescence anisotropy data has the advantage that it is applicable not only to diphenylhexatriene, but to other fluorophores as well, that the use of Arrhenius plots can be justified and that it is based upon an extended Perrin equation, which may be helpful in discussing the dynamics of fluorophores undergoing restricted rotations.

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