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CORRELATION BETWEEN THE ORDER PARAMETER AND THE STEADY-STATE FLUORESCENCE ANISOTROPY OF 1,6-DIPHENYL-1,3,5-HEXATRIENE AND AN EVALUATION OF MEMBRANE FLUIDITY

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In this paper it is shown that for 1,6-diphenyl-1,3,5-hexatriene there exists a simple analytical relation between the orientational order parameter and the steady-state fluorescence anisotropy. This relation is derived on semi-empirical grounds. The order parameter and the true microviscosity for membranes as calculated from steady-state measurements are evaluated. For biological membranes the estimation of the order parameter from steady-state experiments is feasible, but the evaluation of the true microviscosity is not reliable. Also, the physiological relevance of the order parameter is discussed.

Introduction

The most common fluorescence depolarization measurement is a steady-state experiment. Continuous illumination with monochromatic polarized light is used to excite fluorescent probes, such as 1,6-diphenyl-1,3,5-hexatriene, embedded in the lipid regions of the membrane sample. One measures the fluorescence intensities parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the polarization direction of the excitation light. The relevant parameter is the steady-state fluorescence anisotropy, defined as

$$r_s = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp}) \quad (1)$$

Recently, it has been shown [1,2] that r_s can be resolved into a static part, r_∞, and a dynamic part, r_f:

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

For a rodlike probe, such as diphenylhexatriene, which is supposed to be aligned with the acyl chains, the limiting fluorescence anisotropy, r_∞, is proportional to the square of the lipid order parameter, S [1,2]

$$r_\infty / r_0 = S^2 \quad (3)$$

where r_0 is the fluorescence anisotropy value in the absence of any rotational motion of the probe. For diphenylhexatriene, the theoretical value of $r_0 =$ 0.4. Experimental values lie between 0.362 and 0.395. Furthermore, it has been shown [1,2] that γ, defined as

$$\gamma = \phi/\tau \quad (4)$$

where ϕ is the rotational relaxation time and τ the fluorescence lifetime, satisfies the relation

$$\gamma = (r_s - r_\infty)/(r_0 - r_s) \quad (5)$$

The relaxation time, ϕ, refers to rotational diffusion of a probe molecule in an anisotropic environment, where the probe prefers to be oriented along the normal to the membrane plane. If the

membrane were similar to an isotropic liquid such as a mineral oil, and $r_\infty = 0$ were a good approximation, then ϕ could be translated into a 'microviscosity', η, [3,4] by applying classical hydrodynamic expressions of the Perrin type, that is:

Isotropic case

$$\eta = Vr_s/(r_0 - r_s) \tag{6}$$

where V is $C\tau T$; C is a geometrical factor and T is the absolute temperature. For diphenylhexatriene, $V = 2.4$ poise is a good approximation [3]. In the anisotropic case, ϕ could still be taken proportional to a viscosity parameter, but then the proportionality constant depends also on the order parameter [5–7]. This viscosity parameter is called the 'true microviscosity' or 'corrected viscosity', η_0. Recently an approximate expression has been proposed for the true microviscosity [7].

Anisotropic case

$$\eta_0 = V\frac{r_0(r_s - r_\infty)}{(r_0 - r_s)(r_0 - r_\infty)} = \eta - \frac{Vr_\infty}{r_0 - r_\infty} \tag{7}$$

which was also arrived at by Shinitzky and Yuli, based on different arguments [4]. It is the purpose of this paper to show that for diphenylhexatriene there exists a simple relation between the steady-state fluorescence anisotropy, r_s and the order parameter, S, allowing the calculation of S from steady-state measurements.

Furthermore, we wish to evaluate the order parameter, S, and the true microviscosity, η_0, for membranes as calculated from the simple steady-state measurements. We present a comprehensive experimental set of S and η_0 data of a large number of membranes for which r_s and r_∞ values are known from the literature. It is shown that for low r_s values, the estimation of S from r_s is inaccurate and the evaluation of S from r_s can be done with confidence for larger r_s values. This latter result is in agreement with the conclusion of Van Blitterswijk et al. [6]. The opposite is true for the true microviscosity; η_0 can be estimated accurately only from fluorescence anisotropy data for rather low r_s values. The inaccuracy in deriving η_0 from r_s increases strongly with increasing r_s.

Relation between the order parameter and the steady-state fluorescence anisotropy

An analytical relation between the order parameter and the steady-state fluorescence anisotropy, based on semi-empirical grounds, is derived using the following arguments:

(1) The theory of rotational diffusion of a rod-like object in an anisotropic potential [8–10] provides an expression for the product, $D_w\phi$, of the wobbling diffusion constant D_w multiplied by the effective rotational correlation time, ϕ. In the cone model [5,8], $D_w\phi$ is approximately proportional to $1-S^2$

$$D_w\phi \propto (1 - S^2) \tag{8}$$

(2) The wobbling diffusion constant will in general depend upon the order parameter, S [10,11]. A plot of the D_w data versus S^{-1} suggests a linear relationship

$$D_w^{-1} \propto S \tag{9}$$

(3) The fluorescence lifetime is also correlated with the order parameter [6,12]. Following Van Blitterswijk et al. [6] we assume the proportionality

$$\tau \propto (1 + S) \tag{10}$$

Eqns. 8 and 9 result in a relation between ϕ and S given by

$$\phi \propto S(1 - S^2) \tag{11}$$

By taking the derivative of $\phi(S)$ it is seen that ϕ has a maximum for $S = 0.58$. This is in excellent agreement with the experimental value found for different lipid systems [13].

Combining Eqns. 4, 10 and 11, we obtain

$$\gamma = S(1 - S) \tag{12}$$

where we have put the coefficient on the right-hand side equal to 1, which is close to the average of $\gamma/[S(1 - S)]$ for the 125 data points as mentioned below. This average is 1.09 ± 0.09. Substitution of Eqns. 3 and 12 in 5 gives our relation

$$r_s/r_0 = S/(1 + S - S^2) \tag{13}$$

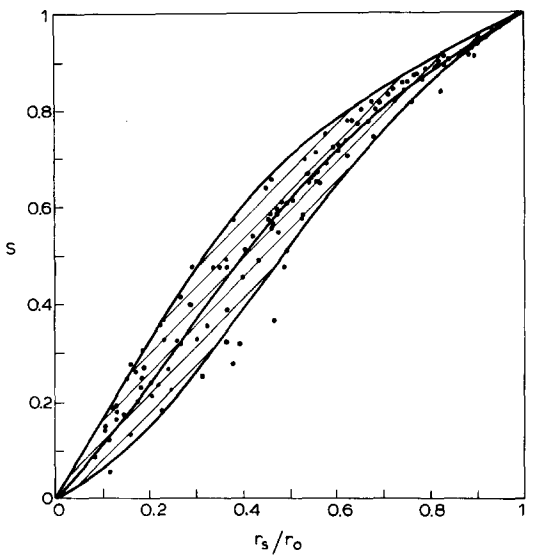

Fig. 1. The order parameter, S, as a function of r_s/r_0. The middle curve corresponds to Eqn. 14, the upper curve to $S + \Delta S$ and the lower one to $S - \Delta S$. The 125 data points (●) have been obtained from r_s, r_∞ and r_0 values in the literature for three different groups of membranes as mentioned in the text. The quoted r_0 values were taken, except for Ref. 21 (see Table I); r_s values of the membranes in Ref. 14 were calculated from ϕ, τ and r_∞ data using Eqns. 2 and 5; r_∞ values of the membranes in Ref. 16 were calculated from cone angle data. If the decay of the total fluorescence was double-exponential, we used $\tau = <\tau> = \alpha_1\tau_1 + \alpha_2\tau_2$.

In Fig. 1 we have plotted S as a function of r_s/r_0:

$$S = \frac{\left[1 - 2r_s/r_0 + 5(r_s/r_0)^2\right]^{1/2} - 1 + r_s/r_0}{2r_s/r_0} \qquad (14)$$

The middle curve in Fig. 1 corresponds with this equation, Combining Eqns. 3 and 14, we obtain an $r_\infty(r_s)$ relation, a nonlinear function in r_s. For $0.33 < r_s/r_o < 0.70$, r_∞/r_0 can be approximated by

$$r_\infty/r_0 = \tfrac{4}{3}r_s/r_0 - 0.28 \qquad (15)$$

in agreement with the result of Van Blitterswijk et al. [6]; for $r_0 = 0.4$, the difference between our r_∞ and theirs is 0.01.

A remark must be made concerning the value of r_0. Kinosita et al. [14–18] always use a fixed value of 0.395 for r_0. Other authors [19–21] let r_0 free in the curve fitting. This results in a value for r_0 which can be considerably low: even an experimental value for $r_0 = 0.1967$ is found [20]. The value of r_0 is very important as it has a drastic effect on r_∞, in contrast with r_s which is independent of r_0. As a consequence, no unique relationship exists between r_∞ and r_s. Therefore, to eliminate the influence of r_0, relative values are always considered.

Interpretation of data

In the last few years r_s/r_0 and r_∞/r_0 values for a large number of membranes, labelled with diphenylhexatriene, have been determined using time-resolved fluorescence anisotropy decay measurements [14–23] or differential polarized phase fluorimetry in combination with steady-state fluorescence anisotropy measurements [24,25]. For these membranes, the order parameter, S, and the true microviscosity, η_0, can be calculated according to Eqn. 3 and Eqn. 7, respectively, as a function of r_s/r_0.

The order parameter, S

The experimental values for 125 membranes are plotted as a function of r_s/r_0 in Fig. 1. These membranes belong to three different groups:
(1) model membranes (66 points) from one lipid component: dimyristoyl, dipalmitoyl-, dioleoyl-, palmitoyloleoyl-, palmitoyllinoleoyl- and palmitoylarachidonoylphosphatidylcholine at various temperatures from Refs. 14, 15, 24 and 25;
(2) model membranes (39 points) from dimyristoyl- and dipalmitoylphosphatidylcholine or egg yolk lecithin containing various amounts of cholesterol, from Refs. 16, 22 and 25;
(3) various biological membranes (20 points), listed in Table I, from Refs. 17 and 19–22.
Although these membranes differ considerably in temperature and composition, they all follow closely our relation $S(r_s/r_0)$, given by Eqn. 14, the middle curve in Fig. 1.

The microviscosity

Substitution of Eqns. 3 and 13 into 7, gives

$$\eta_0 = VS/(1+S) \qquad (15)$$

This equation, together with Eqn. 14, gives an $\eta_0(r_s/r_0)$ relation as plotted in Fig. 2. It is the

TABLE I

FLUIDITY PARAMETERS FOR VARIOUS BIOLOGICAL MEMBRANES

The types of membrane are listed with increasing r_s/r_0; S is calculated according to Eqn. 3, η_0 according to Eqn. 7 with $V = 2.4$ P. r_0 values for Ref. 21 are calculated from r_s, r_∞, ϕ and τ using Eqn. 5.

Type of membrane	Ref.	Temp. (°C)	r_0	r_s/r_0	r_∞/r_0	S	η_0 (P)
Sarcoplasmic reticulum (rabbit)	17	35	0.395	0.334	0.230	0.48	0.49
Mitochondria (rat liver)	17	35	0.395	0.349	0.230	0.48	0.57
Microsomal extracts (*Tetrahymena*)	20	39.5	0.2554	0.361	0.106	0.33	1.07
D17 (whole cells)	22	25	0.362	0.459	0.323	0.57	0.89
BHK21 (whole cells)	22	25	0.362	0.472	0.359	0.60	0.80
3T3-A31 (whole cells)	21	37	0.23	0.478	0.304	0.55	1.15
Microsomal extracts (*Tetrahymena*)	20	15	0.1967	0.486	0.230	0.48	1.55
L1210 (whole cells)	19	25	0.218	0.523	0.330	0.57	1.45
LM-fibroblasts (whole cells)	22	25	0.362	0.541	0.423	0.65	1.07
Erythrocytes (human) (plasmamembr.)	17	35	0.395	0.554	0.509	0.71	0.49
HeLa (whole cells)	22	25	0.362	0.555	0.445	0.67	1.07
Sarcoplasmic reticulum (rabbit)	17	10	0.395	0.557	0.428	0.65	1.22
Mitochondria (rat liver)	17	10	0.395	0.565	0.425	0.65	1.34
Py6-R1 (whole cells)	21	37	0.16	0.625	0.500	0.71	1.60
N-Egg (whole cells)	22	25	0.362	0.669	0.605	0.78	1.17
3T3 d (whole cells)	21	37	0.18	0.677	0.556	0.75	2.02
NDV-MDBK (viruses)	22	25	0.362	0.682	0.646	0.80	0.77
Erythrocytes (human) (plasmamembr.)	17	10	0.395	0.747	0.709	0.84	1.24
3T3-Py6 (whole cells)	21	37	0.18	0.762	0.667	0.82	2.88
SVT 2 (whole cells)	21	37	0.17	0.882	0.706	0.84	5.32

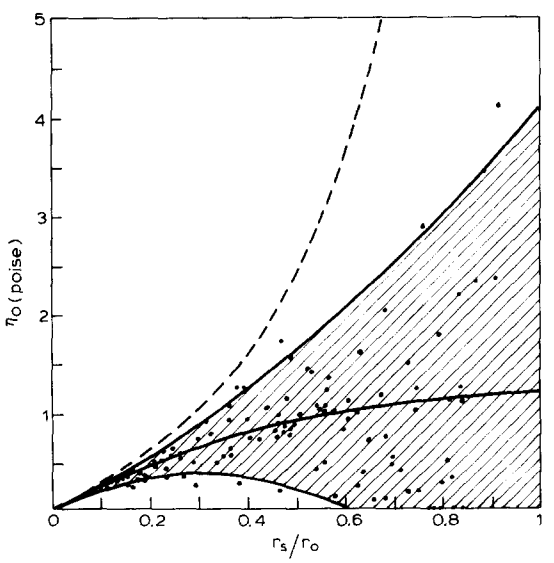

Fig. 2. The true microviscosity η_0 as a function of r_s/r_0. The middle solid curve corresponds to the $\eta_0(r_s/r_0)$ relation as described in the text, the upper solid curve to $\eta_0(r_s/r_0)+\Delta\eta_0$ and the lower one to $\eta_0(r_s/r_0)-\Delta\eta_0$. The data points (●) refer to the same membranes as used in Fig. 1, from the same r_s and r_∞ values. For comparison, the 'ordinary' viscosity, η (Eqn. 6), is also plotted (dashed line).

middle curve of the three solid lines in this figure, where we have also plotted the experimental η_0 values calculated from literature data for the same membranes as in Fig. 1. Three data points, however, from Refs. 14, 21 and 25 have a value for η_0 greater than 5 P and are not shown on the figure.

Estimation of errors

Comparing Figs. 1 and 2, we see that the deviations from the $S(r_s/r_0)$ relation are much smaller than the deviations from the $\eta_0(r_s/r_0)$ curve. Since the location of a data point in the $\eta_0 - r_s/r_0$ plane follows from its corresponding coordinates in the $S - r_s/r_0$ plane, it is clear that these scattering errors are interrelated. To quantify this connection, we have plotted the experimental deviation, ΔS, from the $S(r_s/r_0)$ curve as a function of r_s/r_0. The error, ΔS, is well described by

$$\Delta S/S = \tfrac{1}{2}\Delta r_\infty/r_\infty = A(1 - r_s/r_0)^2 \qquad (16)$$

We have chosen $A = 0.6$, so that 90% of the data

lie between the upper curve (corresponding to $S(r_s/r_0)+\Delta S$) and the lower one (corresponding to $S(r_s/r_0)-\Delta S$).

Using Eqns. 7 and 16 first and then Eqns. 3 and 13, we obtain $\Delta\eta_0$ at fixed r_s/r_0

$$\Delta\eta_0 = \left|\frac{\partial\eta_0}{\partial r_\infty}\right| \Delta r_\infty = V r_0 \Delta r_\infty/(r_0 - r_\infty)^2 = 2AV(r_s/r_0)^2 \quad (17)$$

$\eta_0(r_s/r_0)+2AV(r_s/r_0)^2$ is the upper solid curve in Fig. 2 and corresponds to the lower curve in Fig. 1, while $\eta_0(r_s/r_0)-2AV(r_s/r_0)^2$ describes the lower solid curve in Fig. 2 and corresponds to the upper curve in Fig. 1. In the region where r_s/r_0 is high, a small error in r_∞/r_0 has a negligible effect upon the order parameter, but results in an enormous error in the true microviscosity. Read-off errors for r_s and r_∞ from plots in the literature are responsible for an extra source of error, but they do not change the former conclusion. In Fig. 2 the 'ordinary' viscosity, η, according to Eqn. 6 is also plotted.

Discussion

We have confirmed the conclusion of Van Blitterswijk et al. [6] that there is a one-to-one correspondence between the order parameter, S, and the relative steady-state fluorescence anisotropy, r_s/r_0, allowing an evaluation of S from r_s/r_o data. This would suggest that the dynamic contribution to r_s/r_0 and therefore the true microviscosity, η_0, could also be estimated from fluorescence anisotropy measurements [4,26]. However, the latter conclusion is wrong, because of inaccuracy: while the spreading of the data around the $S(r_s/r_0)$ curve in Fig. 1 is quite small, the inaccuracy due to this scattering is strongly amplified in the $\eta_0-(r_s/r_0)$ plane, as is apparent from inspection of Fig. 2. Adopting the criterion that an error of 20% is the highest inaccuracy still acceptable, we find:

S from r_s/r_0 accurate for $r_s/r_0 > 0.4$;

not reliable for $r_s/r_0 < 0.4$;

η_0 from r_s/r_0 accurate for $r_s/r_0 < 0.2$;

not reliable for $r_s/r_0 > 0.2$.

Since the great majority of biological membranes have $r_s/r_0 > 0.4$, we conclude that S can be reliably evaluated from steady-state fluorescence anisotropy data for biomembranes, but η_0 can not. Most of the data have a r_0 value between 0.362 and 0.395. For biological membranes, however, there are eight points with a very low r_0 value, as seen in Table I. Three of these points do not fall between the upper and lower curve in Fig. 1. The other five are close to the lower curve in this figure. If we omit the eight points, then for biomembranes we can put $A = 0.4$ in Eqn. 16. Even this more optimistic choice for A does not alter the conclusion. In that case the evaluation of S is accurate for $r_s/r_0 > 0.3$ and the estimation of η_0 is reliable for $r_s/r_0 < 0.3$. The physical state of a biological membrane can be suitably described by the order parameter, S, but not by the true microviscosity, η_0.

The arguments leading to $\gamma = S(1-S)$ and from there to the $S(r_s/r_0)$ relation have some shortcomings [11,13]. This relation is rather unexpected and it must be emphasized that it could be obtained because of the apparent correlation of the lifetime of diphenylhexatriene with the order parameter, which is not easy to explain photophysically. However, the $S(r_s/r_0)$ relation works surprisingly well for all types of membrane, except for those where the fluorescence lifetime is considerably shortened due to Förster energy transfer, as is clearly the case for artificial membranes containing large amounts of cytochrome oxidase [18] and the purple membrane which is rich in bacteriorhodopsin [17]. For these membranes, the S values deviate from Eqn. 12. Eqns. 8–11 apply if no energy transfer occurs. It should be stressed that these equations are only rough approximations exhibiting certain trends that follow from theory and experiment. It is possible to arrive at improved $S(r_s/r_o)$ relations by taking into account not only the variation in γ due to the order parameter, but from other physical parameters as well, as proposed by Hare [13].

The present approach has the great advantage that it is simple and straight-forward and requires only a measurement of r_s. Moreover, it is empirically justified, as is shown by Van Blitterswijk et al. [6] and in the present paper. Our $S(r_s/r_0)$ relation could be very convenient in practice, because it allows for a direct evaluation of the order

parameter from steady-state fluorescence anisotropy measurements only. The accuracy of a particular S value can be estimated from Eqn. 16. We have taken a constant $V = 2.4$ P. However, if one calculates for each membrane its own V value, essentially the same pattern emerges as that shown in Fig. 2. Kinosita et al. [5,8,14–17] have calculated a 'viscosity in the cone', η_c. The spreading in the η_c data is of the same order as for the η_0 data.

Cholesterol is known to rigidify the membrane in the fluid phase [27]. The present discussion allows for a precision of this statement. Cholesterol does not make the membrane more viscous, but seems, on the contrary, to lower the true microviscosity, in agreement with the conclusions of Heyn et al. [1]. On the other hand, cholesterol increases the order parameter [1] and because S is believed to reflect the lipid packing in the membrane, cholesterol is in fact a 'condensor' of the membrane, in agreement with its effects on monolayers [28].

The physiological significance of the steady-state fluorescence anisotropy of diphenylhexatriene is not that it measures the membrane viscosity, but rather that it gives information on the packing [29,30]. Due to the simple geometry of diphenylhexatriene (absorption and emission dipoles aligned along the long axis) and to its location parallel to the fatty acyl chains (as has generally been assumed), only the packing-sensitive motional modes cause depolarization. The lipid composition and therefore the packing can change upon certain stimuli or as a result of artificial modifications and this has been shown to modulate cell function [26,27]. Such specific effects could be mediated through local lipid packing constraints which would affect membrane protein conformation and the exposition of membrane components [27,29].

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