DETERMINATION OF LIPID ORDER PARAMETERS AND ROTATIONAL CORRELATION TIMES FROM FLUORESCENCE DEPOLARIZATION EXPERIMENTS

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1. Introduction

In discussing the properties of membrane lipids it is important to keep concepts related to the orientational order and to the dynamics of these molecules apart. The average conformation of the acyl chain or the order parameter of a segment are examples of static or time-averaged quantities. The rotational correlation time of a chain segment, its mobility or the viscosity it experiences during motion are notions which belong in the class of dynamic concepts. In many applications of spin label and fluorescence probes the terminology has become rather imprecise and concepts from these two areas have been used interchangeably. The word fluidity for example has been used with connotations of both order and motion. Until recently steady-state fluorescence depolarization measurements with fluorescent probes like 1,6-diphenyl-1,3,5-hexatriene(DPH) have been interpreted exclusively in terms of a microviscosity, i.e., given a dynamic interpretation. In a number of important contributions [1–4] it was shown that the rotational motion of DPH around axes perpendicular to its long axis is restricted and that the equilibrium orientational distribution of the probe is anisotropic. As shown below these results imply that the steady-state fluorescence anisotropy contains not only information on the dynamics (viscosity) but also on the statics (order) of the label and that the simple interpretation in terms of a microviscosity has to be revised.

It is the purpose of this paper to contribute to the interpretation of fluorescence depolarization experiments with fluorescent lipid probes and to clarify the concepts involved. A systematic approach is outlined which aims at the determination of the unknown orientational distribution function of the label and which enables one to construct this function from measurements of its moments without making any a priori assumptions. This represents an improvement over the currently popular 'cone model' in which the restricted motion is interpreted as wobbling within a cone and in which an unrealistic distribution function is assumed [5]. It is shown that the ratio r_∞/r_0 which has previously been called the 'degree of orientational constraint' [5] is in fact the square of the familiar order parameter S. The value of r_∞/r_0 is directly available from time-dependent fluorescence depolarization experiments. At the same time S is also the first and most important parameter in the characterization of the orientational distribution function. The dependence of the steady-state anisotropy on both the dynamic properties of the label and on its order is discussed and it is shown that r_∞ and thus also S can under certain conditions be determined from steady-state measurements alone. The previous expression for the calculation of the microviscosity can be corrected to take into account the contribution of the hindered rotation. This leads in general to a reduction in the viscosity value. The order parameters obtained from deuterium NMR and fluorescence depolarization show a surprisingly good correlation.

2. Theoretical background

2.1. *Orientational distribution function and order parameters*

We consider rod-like fluorescent probes like DPH or fluorescent fatty acids like *trans*-parinaric acid for

which the long axis of the fluorescing moiety makes a well-defined angle θ with the normal to the membrane. We are assuming that the label distribution is axially symmetric around the membrane normal. The only variable required to specify the orientational distribution of the label is thus θ. Let $f(\theta)\sin\theta\, d\theta$ be the probability that this angle has a value between θ and $\theta + d\theta$. We call $f(\theta)$ the orientational distribution function of the label. Since the average orientation of the lipid chains is normal to the membrane, it is to be expected that the preferential orientation of the rod-like probe molecules will be in the same direction. For DPH this is supported by experimental evidence [6]. $f(\theta)$ is therefore expected to be maximal around $\theta = 0$ and to decrease monotonically with increasing θ. Interest in $f(\theta)$ stems from the fact that it is generally believed that the orientational distribution of the label reflects the orientational distribution of the lipids in its neighborhood. Thus far the hindered rotation of DPH has been discussed in terms of a 'cone model', in which the long axis of the molecule can wobble freely within a cone of opening angle θ_c around the membrane normal, but cannot move outside this cone [5]. From an analysis of the data the cone angle θ_c and a diffusion constant for motion within the cone can be obtained [5]. These parameters however, are only meaningful within the context of this model, in which the a priori assumption is made that $f(\theta)$ is constant within the cone and zero outside. This is clearly an unrealistic and as we will see also an unnecessary assumption. We will take the opposite point of view. No assumptions on $f(\theta)$ are made. Instead the aim of our approach is to determine $f(\theta)$ as well as possible from experiments. Much of the formalism for such a model-free approach is available from the related field of liquid-crystal studies [7]. The function $f(\theta)$ can be expanded in the complete set of orthogonal Legendre polynomials $P_\ell(\cos\theta)$:

$$f(\theta) = \sum_{\ell=0}^{\infty} \frac{2\ell+1}{2} S_\ell P_\ell(\cos\theta) \qquad (1)$$

The coefficients S_ℓ are integrals of the unknown function $f(\theta)$:

$$S_\ell = \int_{-1}^{+1} P_\ell(\cos\theta) f(\theta) d\cos\theta \qquad (2)$$

On symmetry grounds $S_\ell = 0$ for all odd ℓ. Thus only terms with even ℓ contribute to the sum in eq. (1). According to eq. (1) $f(\theta)$ is completely characterized by the set of coefficients S_ℓ. From eq. (2) it is clear that the S_ℓ are weighted averages of $f(\theta)$. Since the P_ℓ are polynomials of degree ℓ in $\cos\theta$, the S_ℓ can also be called the moments of the distribution. In the present context it is more appropriate however to call them order parameters. In the isotropic phase, i.e., for complete disorder, $f(\theta)$ is independent of θ and it follows from eq. (2) that $S_\ell = 0$ for all ℓ ($\ell \neq 0; S_0 = 1/2$). In a perfectly ordered phase with maximal anisotropy $f(\theta)$ is non-vanishing only at $\theta = 0$, and it can be easily shown from eq. (2) that all $S_\ell = 1$. The set of S_ℓ are thus indeed measures of the order or anisotropy and it is appropriate to call them order parameters. The traditional order parameter S equals S_2:

$$S = S_2 = \int_{-1}^{+1} \left(\frac{3\cos^2\theta - 1}{2}\right) f(\theta) d\cos\theta \qquad (3)$$

and appears as the coefficient of the first non-trivial term in the series. The higher order S_ℓ describe features of increasing subtlety in the orientational ordering. Provided a sufficient number of S_ℓ can be determined experimentally and provided the convergence is rapid enough, the truncated series consisting of the first few terms will represent an adequate approximation to $f(\theta)$. For nematic liquid crystals S_4 was shown to be considerably smaller than S_2 [8]. We expect the rate of convergence to be good at least for low order. In that case $f(\theta)$ is a slowly varying monotonically decreasing positive definite function. Since the number of zero's of the oscillating function P_ℓ increases with ℓ, the integrals S_ℓ are expected to decrease with ℓ.

On the basis of recent theoretical work (eq. (25) in [5]), it can be shown that for fluorescent probes with parallel absorption and emission transition dipole moments which coincide with the long axis of the molecule, the measured endvalue r_∞ and initial value r_0 in time-dependent fluorescence anisotropy experiments with membrane suspensions are related to the order parameter S in the following way:

$$\frac{r_\infty}{r_0} = S^2 \qquad (4)$$

This relationship between the experimental quantities r_0 and r_∞, and the model-free order parameter S has not been recognized before. Instead r_∞/r_0 was called the 'degree of orientational constraint' [5] and the cone model was introduced [5]. We propose to use measured r_∞ and r_0 for the calculation of the order parameter S according to eq. (4). Moreover, as has been shown [8,9], S_4 and S_2 can be determined from depolarization experiments on oriented bilayers. Taken together this means that a systematic approach of determining $f(\theta)$ by measurement of the S_ℓ is feasible.

2.2. *Time-dependent and steady-state fluorescence depolarization of rigid fluorophores embedded in lipid bilayers*

The fluorescence anisotropy $r(t)$ of the fluorescent probes DPH and parinaric acid in lipid bilayers does not decay to zero after flash excitation but reaches a constant non-zero value r_∞ [1–3]. This indicates that the rotational diffusion of the probe is hindered and that the equilibrium orientational distribution function is anisotropic. This is not surprising in view of the inherently anisotropic nature of the lipid bilayer in which these probes are embedded. The decay of $r(t)$ from its initial value r_0 to the final value r_∞ could be adequately described by a simple exponential decay characterized by a rotational correlation time τ_c [1–3]:

$$r(t) = r_\infty + (r_0 - r_\infty)\, e^{-t/\tau_c} \qquad (5)$$

Thus information on the dynamics (τ_c) and on the order ($r_\infty/r_0 = S^2$) can be obtained from time-dependent measurements in an easily separable way. More common and easier to perform are steady-state fluorescence depolarization experiments in which the sample is continuously illuminated. Assuming that the time dependence of r is correctly described by eq. (5), the steady-state anisotropy $\bar{r}$ can be easily obtained by integration with the fluorescence decay function (characterized by the fluorescence lifetime τ_F):

$$\bar{r} = r_\infty + (r_0 - r_\infty)\frac{\tau_c}{\tau_c + \tau_F} \qquad (6)$$

From eq. (6) it is apparent that in steady-state experiments the information on the dynamics and on the order is no longer separable and that in general neither r_∞ nor τ_c can be determined from a measurement of $\bar{r}$. It is worthwhile to analyse eq. (6) a little closer. $\bar{r}$ is evidently a function of the ratio of τ_c and τ_F. In fig.1 $\bar{r}$ is plotted as a function of this ratio. When $\tau_c/\tau_F \ll 1$, i.e., when the rotational correlation time is short in comparison with the fluorescence lifetime, diffusion and depolarization can occur, r_∞ is reached and $\bar{r} = r_\infty$. However, when $\tau_c/\tau_F \gg 1$, the rotational correlation time is much longer than the fluorescence lifetime; the label rotation is effectively frozen on the scale of the fluorescence lifetime, no depolarization occurs and $\bar{r} = r_0$. In all other cases $\bar{r}$ lies between r_∞ and r_0. If from other measurements it is known that $\tau_c/\tau_F \ll 1$, then a measurement of $\bar{r}$ suffices to determine r_∞ and the order parameter S. Note from eq. (6) that the difference $(\bar{r} - r_\infty)$ is proportional to $(r_0 - r_\infty)$. Thus at constant τ_c/τ_F, the difference between $\bar{r}$ and r_∞ is largest for small r_∞ and smallest for large r_∞. This is also apparent from fig.1.

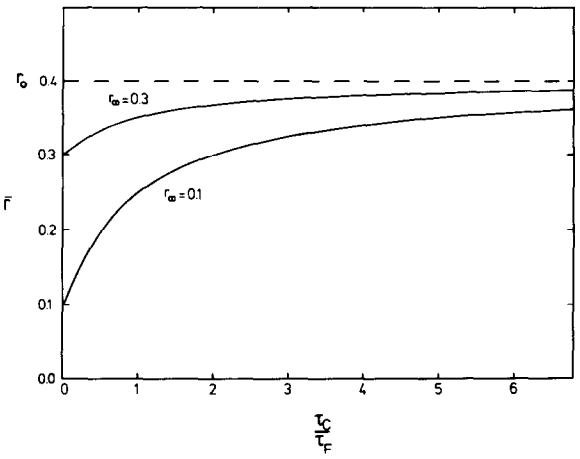

Fig.1. Dependence of the steady-state anisotropy $\bar{r}$ on the ratio of the rotational correlation time τ_c and the fluorescence lifetime τ_F. The curves were calculated according to eq. (6). For the lower curve $r_\infty = 0.1$, for the upper curve $r_\infty = 0.3$. r_0 was assumed to be 0.4 (for DPH values of 0.390 [4] and 0.395 [3] have been reported). $\bar{r}$ is approximately equal to r_∞ only when τ_c/τ_F is close to zero. The difference $(\bar{r} - r_\infty)$ is largest when r_∞ is small.

Equation (6) can be cast into a form which is more appropriate for a discussion of the dynamics of the probe motion (microviscosity):

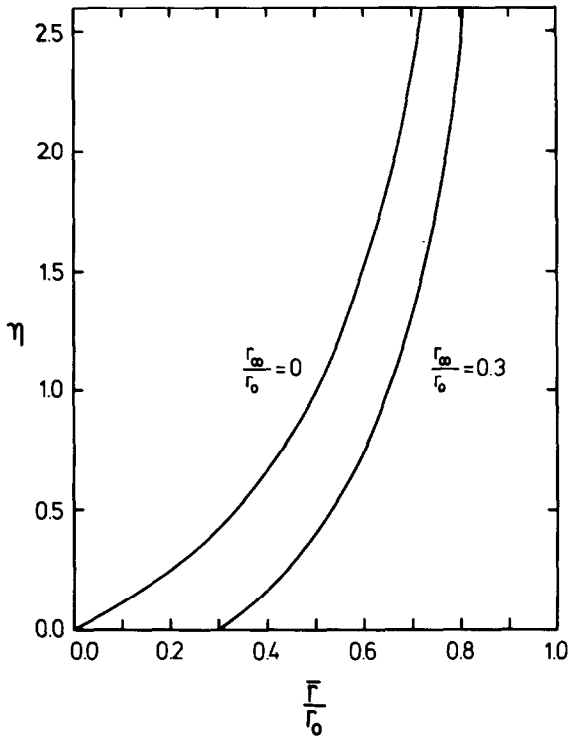

Fig.2. Dependence of the viscosity η (arbitrary units) on the steady-state anisotropy $\bar{r}$ (normalized by r_0) for $r_\infty/r_0 = 0$ and $r_\infty/r_0 = 0.3$. The curves were calculated on the basis of eq. (7). The effect of a non-vanishing r_∞ (order) is to reduce η.

$$\tau_c = \tau_F \left(\frac{\bar{r} - r_\infty}{r_0 - \bar{r}} \right) \qquad (7)$$

τ_c is proportional to the viscosity η. In the standard recipe for the calculation of the microviscosity from $\bar{r}$, the fact that the rotational motion of the label is restricted was ignored [10]. This corresponds to setting $r_\infty = 0$ in eq. (7). From eq. (7) it follows that when the hindered rotation is taken into account, the resulting viscosity values are smaller than those obtained under the assumption $r_\infty = 0$ for all values of $\bar{r}$. This is illustrated in fig.2, where the viscosity is plotted as a function of $\bar{r}/r_0$. It follows from eq. (7) that the error made by the neglect of r_∞ is proportional to r_∞. It can thus be concluded that in particular in those cases where r_∞ is significantly different from zero (below the phase transition of the lipids, or above the transition but in the presence of cholesterol or protein) very substantial errors are made in η when $r_\infty = 0$ is assumed (see fig.2). In principle these errors can be corrected on the basis of eq. (7) when r_∞ is known.

3. Applications and outlook

3.1. *Lipid bilayers*

A large number of time-dependent and steady-state fluorescent depolarization experiments with DPH in pure lipid systems have been performed [1–4,10,11]. The fluorescence lifetime in these systems is 7–9 ns, the rotational correlation time is of the order of 1 ns. Whereas both τ_F and τ_c decrease with increasing temperature, the ratio is approximately temperature-independent. The condition $\tau_c/\tau_F \ll 1$ is satisfied and r_∞ can be set equal to $\bar{r}$ except perhaps above T_c where $(r_0 - r_\infty)$ is large (T_c is the temperature of the lipid gel to liquid-crystalline phase transition). The order parameter S can thus be determined to a good approximation from steady-state measurements alone. For a number of lipids data on r_∞ are available as well [1,3,4]. It is therefore of great interest to compare the DPH order parameter with the lipid order parameter as determined by deuterium NMR (DMR). Figure 3 shows the results of such a comparison for DPPC. The local order parameter of a particular chain segment as determined by DMR decreases towards the end of the chain [12]. DPH which is ~13 Å long in its extended all-*trans* form will at best sense some average order parameter of the hydrocarbon segments in its environment. Nevertheless the correlation which is best for carbon-10 and carbon-12 is striking. With parinaric acid, a naturally occuring fatty acid which can be incorporated in phosphatidylcholine, a similar hindered rotational diffusion as described by eq. (5) has been observed [13]. Not only is this probe more lipid-like, its fluorescent moiety is at the end of the hydrocarbon chain and its depth in the bilayer is well-defined. It is clearly the label of choice for measurements of this type.

3.2. *Lipid systems containing cholesterol*

A consistent set of time-dependent fluorescent depolarization data is available [4,14,15]. Above T_c cholesterol increases r_∞ (and thus the order) and

decreases τ_c. Below T_c, both r_∞ and τ_c are reduced. The effect of cholesterol on the lipid order parameter is known from numerous DMR studies [16–18], which show that above T_c the order is increased. Figure 3 shows that the correlation between the cholesterol-induced changes in order parameter determined by these two methods is quite good.

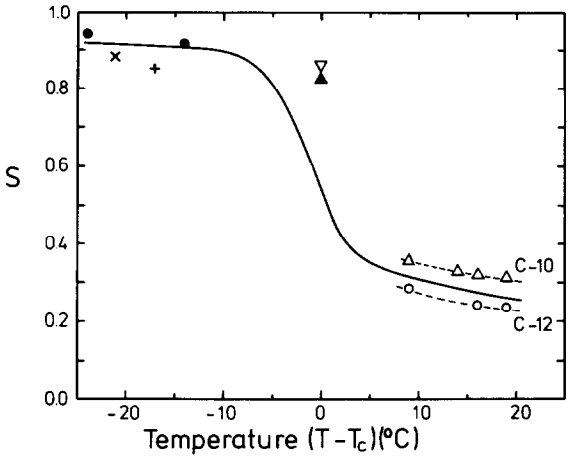

Fig.3. Comparison of the order parameter S obtained from fluorescence depolarization experiments and from DMR. In order to allow comparison of lipids having different T_c, the order parameter S is plotted as a function of the reduced temperature ($T - T_c$). The continuous curve (——) represents the order parameter for sonicated DPPC vesicles, calculated according to eq. (4) from the r_∞ values for DPH [4] (using $r_0 = 0.390$). The DMR order parameters for DPPC selectively deuterated at carbon-10 (△) and carbon-12 (○) as determined in [12] are shown for comparison. Since the latter data refer to unsonicated multilamellar dispersions which have a steeper transition than the sonicated vesicles, only DMR data well above T_c are shown. Below T_c no DMR data on selectively deuterated DPPC are available. Instead two theoretical values for carbon-12 of DPPC are shown (•), calculated according to a mean field theory which gives an excellent fit to the data above T_c (J.-P. Meraldi, J. Schlitter and J. Seelig, personal communication). (x) refers to the DMR order parameter of perdeuterated potassium palmitate determined in [26]. This value represents an average order parameter along the chain. The same holds for (+) for perdeuterated DPPC [27]. The value of (+) was calculated from the first moment of the spectrum. The addition of 30 mol% cholesterol leads to a large increase in the order parameter of carbon-10 of dimyristoylphosphatidylcholine (▲, [17]). The corresponding order parameter calculated from the r_∞ measurement of DPH [4] is represented by (▽) and is in very good agreement with the NMR result.

3.3. *Biological membranes*

The emerging picture is that the anisotropy for membranes is always higher than that of the extracted lipids [19,20]. This has almost invariably been interpreted as a protein-induced increase in microviscosity. According to eq. (6) however both dynamic and order effects have to be considered. Figure 4 shows an example of the effect of proteins: with decreasing lipid:protein ratio $\bar{r}$ increases above T_c (see also [21,22]). This protein-induced effect can be interpreted either as an increase in τ_c (slowdown of the motion between absorption and emission) or as an increase in r_∞ (increase in order). Whereas τ_c is ~1 ns for pure lipids, 2–4 ns have been reported for protein-containing membranes [23–25]. It seems therefore that the presence of proteins leads to an increase in τ_c. The condition $\tau_c << \tau_F$ is no longer satisfied and r_∞ will be less than $\bar{r}$ (see fig.1). For a calculation of S, r_∞ has to be measured directly. Nevertheless the changes in $\bar{r}$ of fig.4, are too large to be explained by an increase in τ_c alone. It is likely that this protein also leads to an increase in lipid order.

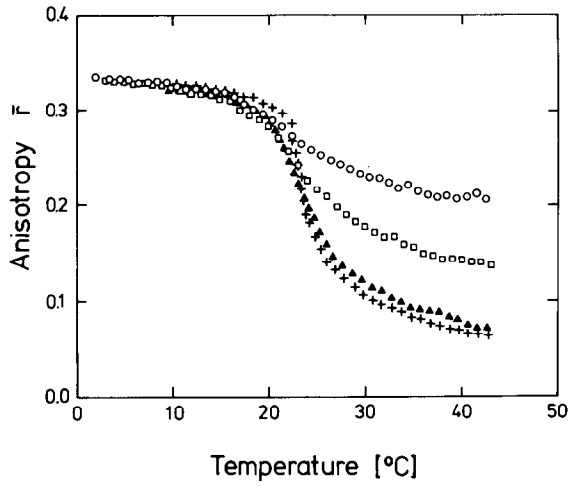

Fig.4. Temperature dependence of the fluorescence anisotropy $\bar{r}$ of DPH incorporated in unilamellar bacteriorhodopsin–dimyristoylphosphatidylcholine (DMPC) vesicles of various phospholipid:protein ratios. The vesicles were prepared as in [28]. Molar phospholipid:bacteriorhodopsin ratios, 49 (○); 87 (□); 231 (▲). The steepest transition is observed with protein-free vesicles (+). The DMPC T_c is 23°C. The molar DMPC:DPH ratio was ~1000.

3.4. *Outlook*

The preliminary comparison between the order parameter of DPH and that of the lipids shows an encouraging correlation. Further work should be done in particular with probes whose location in the bilayer is known. Deuterium NMR gives invaluable information on local order parameters that can never be matched by fluorescence depolarization measurements. The extended chromophores used in such experiments sense an average order parameter of the adjacent lipid chains. Although the information content of the latter order parameters is less than that of DMR, it may nevertheless be quite valuable. No deuterated lipids are required and the data can be readily obtained using standard instrumentation. An additional advantage is that information on S_4, which is not available from DMR measurements, is in principle available from fluorescence depolarization measurements on oriented bilayers.

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