LIMITED ROTATIONAL MOTION: RECOGNITION BY DIFFERENTIAL PHASE FLUOROMETRY*

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The rotational motions of fluorophores giving rise to a limited decay of the fluorescence polarization can be detected by measurements of diffrrential phase fluorometry. Like anisotropic rotations or, in general, rotations owing to more than one rotational rate, limited rotations give rise to a deficit in the maximum differential delay of the polarized components (tangent defect). It is shown that the amplitude allowed to the rotations, and the absolute rotational rate of a fluorophore of known lifetime and limiting polarization placed in an unknown medium can be extracted from the experimental data.

The rotational motion of fluorophores in homogeneous solvents is potentially unlimited, in that the angle determined by the directions of the transition moment in emission, at the times of excitation and emission has no upper limit. This free rotational motion is assumed in the original depolarization theory of Perrin [1] and in more recent extensions that describe the decay of the fluorescence polarization in real time, as studied by both the impulse response [2–4] and the harmonic analysis [8] methods.

In recent years the motions of molecules in heterogeneous, complex media have attracted much attention, particularly in connection with the phenomena of molecular diffusion in biological membranes [9]. These observations have not only practical value and important applications to biology and medicine, but afford an opportunity to test the more sophisticated extensions of the theory and to uncover aspects of molecular motion that would pass unnoticed in observations of homogeneous systems. A practical upper limit to the amplitude of molecular rotations arises as a result of high potential barriers. Phenomenologically a "square well" potential permitting isotropic rotations smaller than a stipulated maximum angle θ_{max}, would appear as the simplest case to treat, and one presumably sufficient to interpret the types of restricted rotations most likely to occur

* Dedicated to Professor Aleksander Jabłoński on the occasion of his 80th birthday.

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in practice. Observations of limited polarized decay that appear to fall in this category have been reported already [10–11]. We show below how differential polarized phase fluorometry may be used to study these cases.

1. Differential phase fluorometry of restricted rotations

Since the classical work of Perrin it has been known that the time dependent decay of the polarized components of the fluorescence $I_{\parallel}(t)$ and $I_{\perp}(t)$ is doubly exponential. Explicitly, [1]

$$I_{\parallel}(t) = \tfrac{1}{3}\exp-(\Gamma t)+\tfrac{2}{3}\, r_0 \exp-(\Gamma+6R)t,$$
$$I_{\perp}(t) = \tfrac{1}{3}\exp-(\Gamma t)-\tfrac{1}{3}\, r_0 \exp-(\Gamma+6R)t. \tag{1}$$

Γ is the rate of emission, R, the rate of rotation of the molecules, assuming spherical symmetry for the rotations, and $r_0 = 2P_0/(3-P_0)$, where P_0 is the polarization in the absence of rotations. If the motions are restricted so that the polarization cannot fall below a value P_∞ Eqs. (1) may be modified to give,

$$I_{\parallel}(t) = \tfrac{1}{3}(1+2r_\infty)\exp-(\Gamma t)+\tfrac{2}{3}(r_0-r_\infty)\exp-(\Gamma+6R)t,$$
$$I_{\perp}(t) = \tfrac{1}{3}(1-r_\infty)\exp-(\Gamma t)-\tfrac{1}{3}(r_0-r_\infty)\exp-(\Gamma+6R)t, \tag{2}$$

where $r_\infty = 2P_\infty/(3-P_\infty)$.

Equations (2) with $r_\infty = 0$ are commonly employed in the study of the free rotational motion of fluorophores. Recently Kinosita et al. [12] have used these equations with $r_\infty > 0$ to describe the apparantly limited rotations of fluorophores in bilayers.

We have introduced differential polarized phase fluorometry [8, 13], an extension of the phase methods first employed by Jabłoński [5] to study the free rotation of fluorophores. The differential phase delay Δ between the polarized components of the fluorescence excited by light of circular modulation frequency ω is given by [8]

$$\tan\Delta = \frac{\omega\tau r_0(2R\tau)}{\frac{1}{9}(1+2r_0)(1-r_0)(1+\omega^2\tau^2)+\frac{1}{3}(2+r_0)(2R\tau)+(2R\tau)^2} \tag{3}$$

with $\tau = 1/\Gamma$, the fluorescence lifetime. A more general equation for spherically symmetric rotations restricted to the lower limit of polarization P_∞ may be derived starting from Eq. (2). As discussed elsewhere [8], if P and Q are respectively the sine and cosine transforms of the impulse-response intensities, defined by

$$P_{\parallel} = \int_0^\infty I_{\parallel}(t)\cos\omega t dt; \quad P_{\perp} = \cdots$$
$$Q_{\parallel} = -\int_0^\infty I_{\perp}(t)\sin\omega t dt; \quad Q_{\perp} = \cdots \tag{4}$$

it follows that,

$$\tan\Delta = (P_{\parallel}Q_{\perp}-P_{\perp}Q_{\parallel})/(P_{\parallel}P_{\perp}+Q_{\parallel}Q_{\perp}) \tag{5}$$

performing the operations indicated by Eq. (4) with $I_{\parallel}(t)$ and $I_{\perp}(t)$ given by Eq. (2) and introducing the results in Eq. (5),

$$\tan \Delta = \frac{\omega\tau(r_0 - r_\infty)\,(2R\tau)}{\frac{1}{9}\,m_0(1+\omega^2\tau^2)+\frac{1}{3}\,S(2R\tau)+m_\infty(2R\tau)^2}, \tag{6}$$

where

$$m_0 = (1+2\,r_0)(1-r_0), \qquad m_\infty = (1+2\,r_\infty)\,(1-r_\infty), \qquad S = 2+r_0-r_\infty(4r_0-1). \tag{7}$$

It is easily derived from the last equation that the maximum value of the differential tangent is given by

$$\tan \Delta_{\max} = \frac{3\omega\tau(r_0 - r_\infty)}{S+2[m_0 m_\infty(1+\omega^2\tau^2)]^{1/2}} \tag{8}$$

and that it occurs at a value of $2\,R\tau$ given by,

$$x_0 \equiv 2R_0\tau = \tfrac{1}{3}\left(\frac{m_0}{m_\infty}\,(1+\omega^2\tau^2)\right)^{1/2}. \tag{9}$$

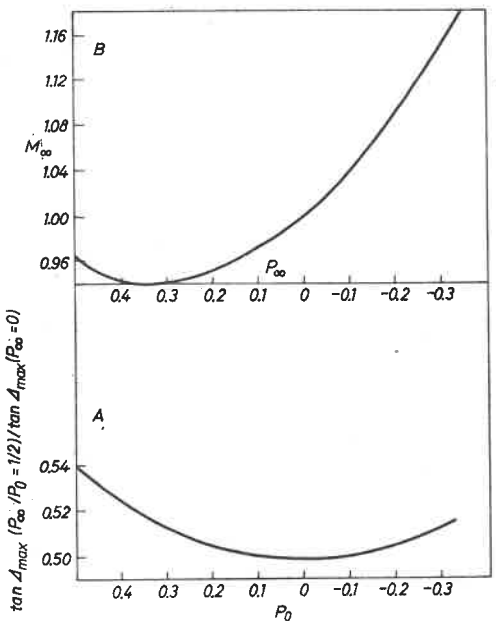

Fig. 1. (A) Dependence of M_∞ upon P_∞, (B) dependence of tangent defect upon P_0, when $P_\infty/P_0 = 1/2$

According to Eq. (9) the ratio of restricted an free rotational rates at $\tan \Delta_{\max}$ equals,

$$\frac{R_0(r_0, r_\infty)}{R_0(r_0, 0)} = m_\infty^{1/2}. \tag{10}$$

Figure 1 shows that this ratio differs from unity by less than app. $\pm 10\%$ for $0.5 > P_0 > -0.2$. Therefore the absolute rate of rotation can be determined within this accuracy without knowledge of r_∞, by simply setting $m_\infty = 1$ in Eq. (9). This small difference of the values of m from unity and the small contribution to the S term by the product $r_\infty(4r_0 - 1)$ would lead us to expect that

$$\frac{\tan \Delta_{\max}(r_0, r_\infty)}{\tan \Delta_{\max}(r_0, 0)} \simeq 1 - (r_\infty / r_0) \tag{11}$$

a simple proportionality between tangent defect and r_∞/r_0 (or P_∞/P_0). Actual detailed computation shows that this is indeed the case, independently of the values of $\omega\tau$. The greatest departure from the linearity indicated by the last equation occurs at $P_\infty/P_0 = 0.5$ and, as shown also in Figure 1, it amounts to less than 10% of the tangent defect. We conclude that even in systems of unknown viscosity one can obtain the absolute rotational rate of the included fluorophore, and the maximum amplitude of the allowed rotations within errors of less than 10% by the use of Eqs. (9) and (11) (with $m_\infty = 1$).

If more accurate values of P_∞ and R_0 are required these may be computed by iteration, employing r_∞, given by Eq. (11) to obtain better estimates of m_∞ and S entering in Eqs. (9) and (10).

2. *Distribution of* $\tan \Delta$ *over* $2R\tau$

It will be noticed that the integral of $\tan \Delta$ over $2R\tau$ does not converge, but the integral

$$\Sigma \equiv \int_{-\infty}^{+\infty} \tan \Delta \, d \log (2R\tau) \tag{12}$$

converges to the values

$$\Sigma = (6\omega\tau(r_0 - r_\infty)/\sqrt{T}) \left(\frac{\pi}{2} - \tan^{-1} \frac{S}{\sqrt{T}} \right), \quad (\text{if } T > 0)$$

$$\Sigma = (3\omega\tau(r_0 - r_\infty)/\sqrt{-T}) \ln \left(\frac{Q + \sqrt{-T}}{Q - \sqrt{-T}} \right), \quad (\text{if } T < 0) \tag{13}$$

with

$$T = 4m_0 m_\infty (1 + \omega^2\tau^2) - S^2, \quad Q = 4m_0(1 + \omega^2\tau^2) + S. \tag{14}$$

If Eqs. (8) and (13) are employed to compute $\tan \Delta_{\max}$ and Σ, for fixed r_0 and $\omega\tau$, it is seen that both decrease monotonically as r_∞ increases from 0 to r_0, but that the ratio $\Sigma/\tan \Delta_{\max}$ remains virtually constant, so that we expect the hald-width of the distribution of $\tan \Delta$ over $R\tau$ to be independent of R_0 and r_∞.

The actual half-width of the distribution is obtained by combining Eqs. (8) and (6). If $\tan \Delta$ in the latter equation is replaced by $\frac{1}{2} \tan \Delta_{\max}$ obtained from (8), we get after simplification,

$$m_\infty x_{1/2}^2 - \tfrac{1}{3}\{S + 4[m_0 m_\infty (1+\omega^2\tau^2)]^{1/2}\} + \frac{m_0}{9}(1+\omega^2\tau^2) = 0. \tag{15}$$

The last equation permits computation of $x_{-1/2}$ and $x_{1/2}$ the two values of $2R\tau$ at which $\tan \Delta = \frac{1}{2} \tan \Delta_{\max}$. At any fixed $\omega\tau$ the ratio $x_{1/2}/x_{-1/2}$ for any value of P_∞, differs from the same ratio for $P_\infty = 0$ by less than 10% on account of the small variation of S and m_∞ with P_∞. The half-width of the plot of $\tan \Delta$ against $2R\tau$ decreases as $\omega\tau$ increases

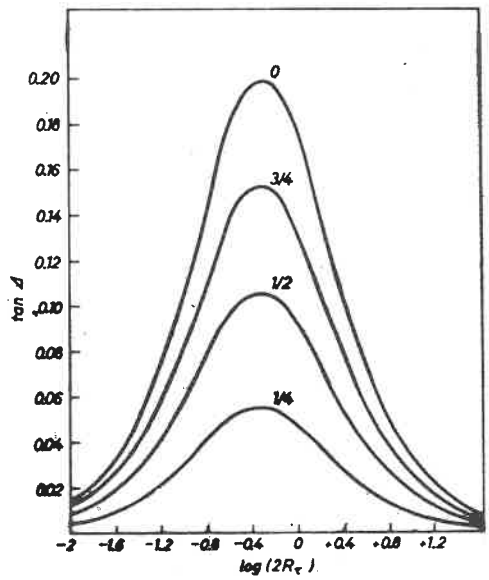

Fig. 2. Plots of $\tan \Delta$ against $\log (2R\tau)$ for $\omega\tau = 1, P_0 = 0.45$ and values of P_∞/P_0 indicated on the curves. The figure shows clearly the relative invariance of $2R\tau$ at $\tan \Delta_{\max}$, and the constancy of the half-widths of the $\tan \Delta$ distribution

and between $\omega\tau = 0.5$ and $\omega\tau = 2$ the ratio $x_{1/2}/x_{-1/2}$ drops from approximately 35 to 25. The relative invariance of the half-width with P_∞ may be used to determine the thermal coefficient of the viscosity of an unknown medium by the use of a fluorophore with known τ and P_0 (Fig. 2).

3. Average amplitude of the depolarizing rotations.

The average amplitude $\langle\theta\rangle$ of the rotations responsible for the depolarization of the fluorescence at any given value of R can be obtained from the simple equations

$$\langle\cos^2\theta\rangle = \cos^2\theta_{\max} + \sin^2\theta_{\max}/(1+6R\tau)$$

$$\cos^2\theta_{\max} = [1+2(r_\infty/r_0)]/3, \quad \langle\theta\rangle = \cos^{-1}\langle\cos^2\theta\rangle^{1/2}. \tag{16}$$

If $P_\infty = 0$, $\cos^2 \theta_{max} = 1/3$ and the amplitude of the average rotations that achieve complete depolarization is close to 55°. Setting $m_\infty = 1$ in Eq. (15), and $\omega\tau = 0.5$ or alternatively $\omega\tau = 2$, we obtain mean rotations $\langle\theta\rangle$ of 20° and 28° respectively at $x_{-1/2}$, 36° and 43° at x_0, and 49° and 51° at $x_{1/2}$. Thus, when rotations are free, differential phase fluorometry reflects primarily the molecular rotations of medium and large amplitude. The rotations of small amplitude contribute predominantly only in the region of $2R\tau < x_{-1/2}$ where the precision of the measurements is much less satisfactory on account of the small tangents. These facts need to be kept in mind in comparing the rotational properties of molecules revealed by differential phase fluorometry with those deduced by the use of other methods which may weigh differently the small and large rotations.

In cases of restricted rotations the mean amplitude of the rotations computed from differential phase data are proportionally scaled down. For $\omega\tau = 1$ and $P_\infty/P_0 = 1/2$ the values of $\langle\theta\rangle$ at $x_{-1/2}$, x_0 and $x_{1/2}$, are respectively 15°, 27°, and 34°. For $P_\infty/P_0 = 0.9$ they are 7°, 12°, and 15°. These values are to be compared with 22°, 39°, and 50° for $P_\infty = 0$. It will be noticed that the amplitude of the rotations increases, between $x_{-1/2}$ and $x_{1/2}$ by a factor of 2.1–2.3 in all three cases.

4. Characterization of restricted rotations

The variation of the ratio T/η (absolute temperature/viscosity) of pure liquids, over a limited temperature range, can be represented with an accuracy sufficient for our purpose by an equation of the form $T/\eta = (T_0/\eta_0) \exp \alpha(T - T_0)$. Here T_0 is an arbitrary temperature, η_0 the corresponding viscosity, and α is evidently the temperature coefficient of the rotational rate of a sphere suspended in the liquid. A similar relation may be expected to apply to heterogeneous media like lipid bilayers or even biological membranes, and as a result a simple plot of $\tan \Delta$ against temperature will have similar shape as one of $\tan \Delta$ against $\log 2R\tau$. From such plots P_∞, R_0, and α may be obtained as explained, on the assumption that the rotations are isotropic, whether free or restricted, and that the fluorophores constitute a homogeneous population as regards spectroscopic and rotational properties. Anisotropic rotations can give rise to tangent defects, but even extreme anisotropy produces tangent defects smaller than 35% [8]. Therefore, larger tangent defects can be attributed unequivocally to restricted rotations. A tangent defect can result also from supperposition of effects owing to several molecular species with appreciably different rotational rates, but unlike restricted rotations these result in a much broadened distribution of $\tan \Delta$ over $\log 2R\tau$. A distinction between these cases is possible when the thermal coefficient of the viscosity of the medium is known independently, an unlikely case with lipid bilayers or membranes.

In conclusion, certain cases of restricted rotations may be characterized unequivocally from observations of differential phase fluorometry alone, but those involving only a modest tangent defect require further spectroscopic or chemical information to be distinguished from cases of anisotropic motion or from motions due to a heterogeneous fluorophore population.

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