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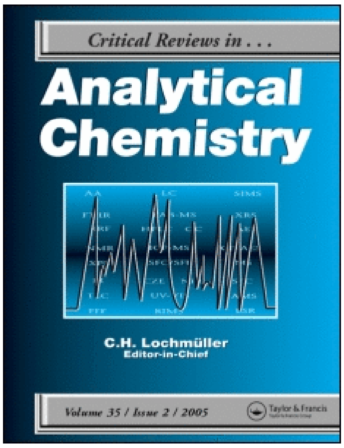

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Fluorescence Anisotropy: Theory and Applications of Rotational Depolarization*

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ABSTRACT: This review article presents the principal problems of fluorescence emission anisotropy in rigid and liquid isotropic solutions, as well as in partially ordered fluid systems (membranes), and indicates possible practical uses of these phenomena in physicochemical and biophysical investigations. It has been shown that fluorescence anisotropy provides a quantity of information on photophysical processes that occur in complex molecules. Thus, conclusions can be drawn with respect to molecular structure and properties, e.g., shapes, dimensions and conformational changes, electric dipole moments in excited states, relaxation of local temperature of excited molecules, as well as on the structure and internal dynamics of molecular systems.

KEY WORDS: Brownian dynamics, fluorescence anisotropy, fluorescence intensity decay, inertial effect, membranes, polarized fluorescence, rotational correlation time, rotational depolarization, rotational relaxation, two-photon excitation.

* In memory of Professor Aleksander Jabłoński.

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I. INTRODUCTION

It has generally been believed that the partial polarization of fluorescent light in rigid or highly viscous solutions was first observed by Weigert[1] in 1920, while in fact it was in 1909 that this phenomenon was mentioned: "Fluoreszenzlicht ist polarisiert, auch wenn das erregende Licht unpolarisiert war,"[2] in the Müller-Pouillets-Handbook.[2]

As a result of an anisotropic excitation of a rigid solution of organic luminescent molecules, the transition dipole moments (classic virtual oscillators), which are isotropically distributed, and the anisotropic fluorescence, i.e., the partial polarization of the fluorescence light, can be observed. This phenomenon has been termed "photoselection".[3] The fluorescence anisotropy is the source of much valuable information on photophysical processes occurring in complex molecules. Hence, conclusions can be drawn on the excitation energy migration between molecules in condensed systems,[4,5] the transition moment directions in absorption and emission,[6,7] the molecular structure and properties such as shapes, dimensions, and conformational changes,[8–13] electric dipole moments in excited states,[14–19] relaxation of local temperature of excited molecules,[20,21] as well as on the structure and internal dynamics of molecular systems.[22,23]

Polarized fluorescence spectroscopy has not yet become a routine method for the analyst, as already is in the field of the absorption and emission spectroscopy. In chemical, photophysical and biochemical investigations, the fluorescence methods are finding an increasing application in view of the fact that many of the natural physical processes are very fast, occurring in times of the order of nano- and picoseconds. These are primarily the relaxation phenomena such as the fluorescence decay of organic molecules in solutions, inter- and intramolecular excitation energy migration and emission anisotropy (light polarization) change due to molecular rotational motions during fluorescence decay.

The methods of fluorescence spectroscopy play a key role in structural investigations of dynamic properties of nucleic acids, proteins and biological membranes. From amongst numerous investigation methods, those deserving particular

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attention are based on investigating the fluorescence depolarization of artificially introduced or natural fluorescent probes. In recent years, this method has been refined, playing today an increasingly significant role. It permits the investigation of rapid rotational diffusion with a time constant of the order of nanoseconds, as well as in shorter and/or longer time intervals, depending on the probe and the experimental techniques employed.

Progress in polarization studies has first of all been made possible by the introduction of nano- and picosecond techniques[22,24–31] and improvement in the methods with continuous excitation owing to the employment of computer techniques.[32,33] This rapid development of experimental methods inspired, in turn, improvement in the theoretical description of the photoluminescence polarization in isotropic and anisotropic systems.

This paper focuses on problems underlying the theoretical bases of the depolarization phenomena due to free and restricted rotational motions (Brownian motions) of luminescent molecules in condensed media, as well as on the use of these phenomena for determining quantities characteristic of the molecular and macromolecular properties in media of different viscosities at different temperatures.

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II. PHOTOLUMINESCENCE ANISOTROPY

A. Anisotropy of Photoluminescence Radiation Field

Considerations regarding the photoluminescence anisotropy in solutions are based on the following assumptions:[34–36]

1. No phase relationships exist between the light exciting the photoluminescence in solution and that emitted by the luminescent molecules (LM), since the absorption and emission processes are time-separated (absorption proceeds in femtoseconds, whereas the mean lifetimes of LM are of the order of nanoseconds). Moreover, the molecules in the excited states are subject to various disturbances. This assumption is valid if the intensity of the excitation light is sufficiently low, i.e., the induced emission is negligible.
2. The emitting molecules are incoherent sources, so that no phase relationships exist between the light emitted by different molecules. In this case, the intensities of individual components of photoluminescence sum up.
3. The direction of the molecular transition moment responsible for the emission relative to the axis of the molecule does not depend on the method of excitation, but only on the molecular states between which the transition takes place.

The excitation by linearly polarized parallel light beam or by natural light is the most typical example of the anisotropic excitation of fluorescence in solutions. In such cases, the photoluminescence radiation field displays axial symmetry. Under excitation by linearly polarized light, the direction of the electric vector, **E**, of the excitation light constitutes the axis of symmetry which, upon excitation by natural light, is determined by the direction perpendicular to the plane of vibration of **E** in the excitation beam.

According to Jabłoński,[36] the anisotropy of the photoluminescence radiation field can be characterized by the emission anisotropy, r, defined as:

$$r^2 = \frac{\left(I_1 - I_2\right)^2 + \left(I_2 - I_3\right)^2 + \left(I_3 - I_1\right)^2}{2 \cdot I} \qquad (1)$$

where $I = I_1 + I_2 + I_3$ and I_1, I_2, and I_3 are relative intensities of the photoluminescence light components, with the vibrations of the electric vector being parallel to the three axes of the Cartesian system, the orientation of which is selected so that the greatest possible difference occurs between the strongest and the weakest component intensities. If I_1 denotes the strongest, and I_3 the weakest component, the orientation should be such that $I_1 - I_3$ attains its maximum value. Axial field symmetry requires equal intensities of components perpendicular to the symmetry axis.

For $I_2 = I_3$ from Equation 1 we obtain

$$r^2 = \left(\frac{I_1 - I_2}{I}\right)^2 \tag{2}$$

For example, in the case of excitation by linearly polarized light, I_1 is the component parallel to the electric vector **E** of the excitation light ($I_1 = I_{\parallel}$) and $I_2 = I_3 = I_{\perp}$ are components perpendicular to this direction (Figure 1). Hence, based on Equation 2:[37,38]

$$r = \frac{I_{\parallel} - I_{\perp}}{I} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \tag{3}$$

or with regard to $I_{\perp} = (I - I_{\parallel})/2$

$$r = \frac{3}{2} \cdot \frac{I_{\parallel}}{I} - \frac{1}{2} \tag{4}$$

The sign of r has been chosen so that $r > 0$ for $I_{\parallel} > I_{\perp}$, and $r < 0$ for $I_{\parallel} < I_{\perp}$.

Upon excitation by natural light, the distinguishable component is that perpendicular to the vibration plane of the electric vector from the excitation beam. If, in this case, I_2 is distinguishable, then $I_2 \neq I_1 = I_3$ and, based on Formula 2, one has

$$r_n = \frac{I_1 - I_2}{2I_1 + I_2} \tag{5}$$

The sign of r_n has been chosen so that $r_n > 0$ for $I_1 > I_2$ and $r_n < 0$ for $I_1 < I_2$. Definition 1 can also be used to characterize the anisotropy of the excitation radiation, which, for the cases considered above, amounts to $r = 1$ and $r = 1/2$, respectively.

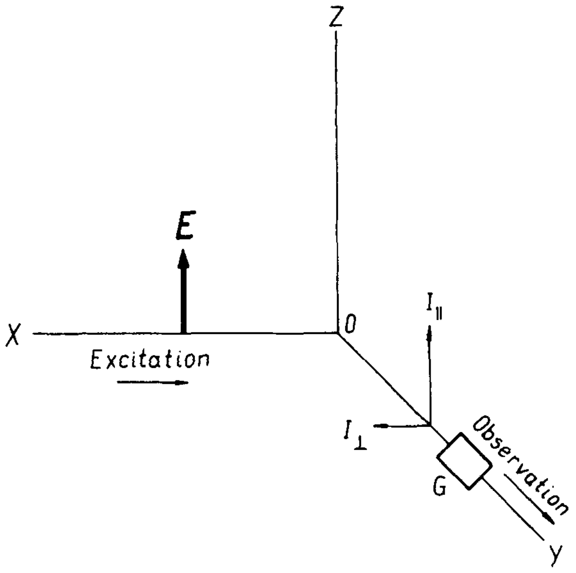

FIGURE 1. Schematic diagram for determining the emission anisotropy. The fluorescent solution is placed at 0. The exciting light reaches it from X and is polarized with electric vector **E** along OZ. The fluorescence emitted towards Y is observed and the emission intensities $I_{\parallel}$ and $I_{\perp}$ are measured by orienting the Glan prism G in two parallel and perpendicular positions relative to **E**, respectively.

It should be noted that the natural excitation light beam can be regarded as a superposition of two beams polarized in mutually perpendicular planes. In such a case, each beam excites the photoluminescence with anisotropy r given by Formula 3. As a result, two components $I_{\parallel}$, perpendicular to each other and to the direction of the excitation beam, and four components $I_{\perp}$ are created. The superposition of the two radiation fields yields $I_2 = 2I_{\perp}$ for the component parallel to the excitation beam, and $I_1 = I_3 = I_{\parallel} + I_{\perp}$ for two perpendicular components. The substitution of this result to Equation 5 gives the well-known Vavilov-Levshin Formula:[39]

$$r_n = \frac{I_{\parallel} + I_{\perp} - 2I_{\perp}}{2I_{\parallel} + 4I_{\perp}} = \frac{1}{2} \cdot \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} = \frac{r}{2} \tag{6}$$

Apart from the emission anisotropy, the concepts of the polarization degree

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{7}$$

or the depolarization degree

$$\rho = \frac{I_{\perp}}{I_{\parallel}} \tag{8}$$

are often used.

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The emission anisotropy is related to the polarization degree by:

$$r = \frac{2P}{3-P} = \frac{1-\rho}{1+2\rho} \tag{9}$$

From Equation 3 for components $I_{\parallel}$ and $I_{\perp}$ it follows that

$$I_{\parallel} = \frac{I}{3}(1+2r) \tag{10}$$

$$I_{\perp} = \frac{I}{3}(1-r) \tag{11}$$

For the emission anisotropy observed in a solution containing various groups of centers with different r_i and I_i (more than one fluorescent species) the following relation is obtained based on Equation 3:[38]

$$r = \frac{\sum_i r_i I_i}{\sum_i I_i} \tag{12}$$

Equation 12 is valid for the case of excitation by light polarized in plane, r, as well as for that by natural light, r_n.

B. Fundamental Emission Anisotropy

Let us assume that the photoluminescence of an isotropic rigid solution is excited by parallel linearly polarized light beam. Maximum fluorescence anisotropy, i.e., the so-called fundamental emission anisotropy, r_f, is observed if no depolarizing factors are present. According to the Vavilov-Levshin theory,[39] when the same transition dipole moment is responsible for both fluorescence light absorption and emission (corresponding to the so-called virtual electronic linear oscillator), which means that the excitation of the LM directly leads to the initial level for the fluorescence (i.e., for the excitation in long-wave absorption band), $r_f \equiv r_p = 0.4$ is obtained. In this case, r_f has been termed the principal emission anisotropy, denoted by r_p. The observation of negative emission anisotropy (negative polarization, $I_{\perp} > I_{\parallel}$), which corresponds to the excitation of a molecule to higher energy levels from which radiationless deactivation to the initial level of fluorescence band occurs,[40] inclined Perrin[41] to accept the assumption of different directions of the absorption and emission transition moments.

According to Expression 4, the ratio $I_{\parallel}/I$ should be calculated in order to obtain r_f. To this end, we shall employ a method simpler than the traditional one.

Let us assume that the emission transition dipole, **F**, makes an angle β with the absorption transition dipole, **A**, the angles between **A** and the Z-axis, and between **F** and the Z-axis being denoted by ω_1 and ω_2, respectively, for a given orientation of a LM in space. The direction of the electric vector, **E**, of the excitation light is consistent with the Z-axis (Figure 2). δ denotes the angle between planes incorporating OZ, **A**, and **A** and **F**. Angles ω_1, ω_2, β, and δ are related according to the following expression:[42]

$$\cos\omega_2 = \cos\omega_1 \cos\beta + \sin\omega_1 \sin\beta \cos\delta \tag{13}$$

In an isotropic solution, all orientations of vectors, **A** and **F**, determined by angles ω_1, ω_2, and δ, are equally probable. Since the total fluorescence intensity, $I = I_{\parallel} + 2I_{\perp}$, is proportional to E^2, the intensity component $I_{\parallel}$ is, according to Figure 2, given by

$$I_{\parallel} = I\langle\cos^2\omega_2\rangle \tag{14}$$

In rigid solutions with a low dye concentration, when no excitation energy migration takes place, $\langle\cos^2\omega_2\rangle = I_{\parallel}/I$ is time-independent. If, however, rotational molecular motions of the LM occur, as for example in liquid solutions, $\langle\cos^2\omega_2\rangle$ varies with time.

Upon squaring Equation 13 and averaging over the azimuthal angle δ ($\langle\cos\delta\rangle = 0$, $\langle\cos^2\delta\rangle = 1/2$), for a given absorption oscillator direction ω_1 we obtain the mean value of the emission oscillator:[11,43]

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$$\left\langle \cos^2 \omega_2 \right\rangle = \left(\frac{3}{2}\cos^2 \omega_1 - \frac{1}{2}\right)\cdot\left(\cos^2 \beta - \frac{1}{3}\right) + \frac{1}{3} \tag{15}$$

where β is constant for a given transition in the LM.

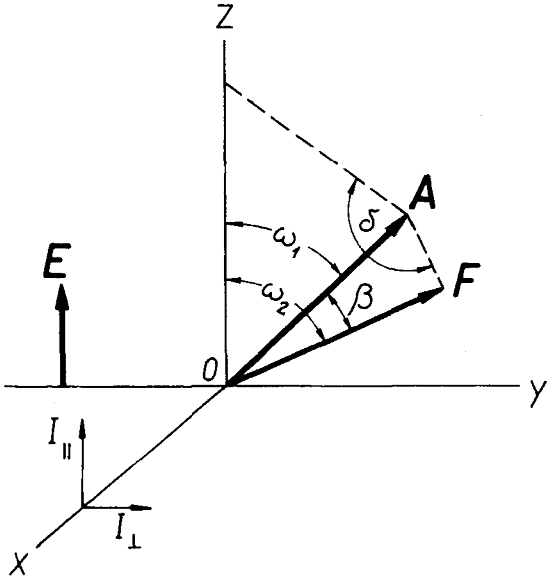

FIGURE 2. Geometry of the system. The exciting light **E** is polarized in the Z direction. The fluorescence is observed along X. Vectors **A** and **F** represent the directions of the absorption and fluorescence transition moments. δ is the angle between the planes formed by the OZ, **A** and **A,F** directions. The position of angles ω_1, ω_2, and β is evident from the figure.

The averaging over all directions ω_1 gives the mean value $\langle\langle \cos^2 \omega_2 \rangle\rangle$, which is related to the emission anisotropy

$$r(\omega_2) = \frac{3}{2}\frac{I_{\parallel}}{I} - \frac{1}{2} = \frac{3}{2}\left\langle\left\langle \cos^2 \omega_2 \right\rangle\right\rangle - \frac{1}{2} \tag{16}$$

The substitution of the averaged Equation 15 into Equation 16 yields

$$r(\beta, \omega_1) = \left(\frac{3}{2}\left\langle \cos^2 \omega_1 \right\rangle - \frac{1}{2}\right)\left(\frac{3}{2}\cos^2 \beta - \frac{1}{2}\right) \tag{17}$$

where

$$\left\langle \cos^2 \omega_1 \right\rangle = \frac{\int_0^{\pi/2} \cos^2 \omega_1 f(\omega_1)\, d\omega_1}{\int_0^{\pi/2} f(\omega_1)\, d\omega_1} \tag{18}$$

and $f(\omega_1)\, d\omega_1$ is the direction distribution in the excited state due to the polarized absorption. The probability distribution

$$f(\omega_1)\, d\omega_1 = \cos^2 \omega_1 \sin \omega_1\, d\omega_1 \tag{19}$$

determines the maximum selection that can be obtained using the optical excitation of an isotropic solution. This phenomenon is called photoselection. The substitution of Equation 19 into Equation 18 yields $\langle \cos^2 \omega_1 \rangle = 3/5$ and from Equation 17 the well-known Perrin Equation is obtained:[41]

$$r_f(\beta) = \frac{2}{5}\left(\frac{3}{2}\cos^2 \beta - \frac{1}{2}\right) \tag{20}$$

According to Equation 20, all possible theoretical values of r_f cover the range of

$$-0.2 \le r_f \le 0.4 \tag{21}$$

Principal emission anisotropy, $r_p = 0.4$, is obtained for $\beta = 0°$.

The experimental values of the emission anisotropy are always somewhat lower than r_p, owing to the depolarizing factors present in the luminescent solution. These are secondary fluorescence, excitation energy migration between LM, rotational motions of the LM, and their torsional vibrations. The value of the emission anisotropy measured experimentally (obtained by extrapolation to an infinitely high viscosity or in rigid solutions) has been termed the limiting emission anisotropy and denoted by r_0. Certain cases are known, such as the fluorescence of fluorocyclene ($r_0 = 0.109$),[44] benzene ($r_0 = 0.091$), and deuterobenzene ($r_0 = 0.081$),[45] when the value of r_0 markedly differs from that of r_p, which cannot be accounted for by the effect of the depolarizing factors mentioned above.

This fact inclined Jabłoński[34] to consider the possibility of further development of the theory of the fundamental emission anisotropy, by replacing linear oscillators with the spatial ones in which the dipoles of the same electronic transition are differently directed in a molecule. We shall discuss this theory on the grounds of the latest approach presented by Jabłoński.[36]

Let us consider the Cartesian coordinate system fixed rigidly on a LM and oriented so that the transition moment has the maximum value along one of the axes, and the minimum value—for another axis. Thus, the direction of the third axis is determined. These axes have been termed the principal axes of the spatial oscillator. The different spatial oscillators, which are differently oriented, are related to the absorption and emission processes. The power of absorption from the excitation light beam, with the electric vector parallel to one of the principal axes of the absorption spatial oscillator, is proportional to the square of the respective transition moment. The ratio of the powers (the principal "absorption intensities") is denoted by Γ_1: Γ_2: Γ_3, Γ_i being normalized so that $\sum_{i=1}^{3} \Gamma_i = 1$. Similarly, for the emission oscillator $\sum_{i=1}^{3} \Gamma'_i = 1$, the principal intensities of this oscillator are denoted by Γ'_i, Γ'_2, and Γ'_3. It is assumed that the solution is excited by linearly polarized light with electric vector **E** parallel to the Z-axis of a system fixed in space. For an isotropic solution, the total absorption intensity, I, is given by the following formula:

$$I = \left\langle \Gamma_1 \alpha_{1Z}^2 + \Gamma_2 \alpha_{2Z}^2 + \Gamma_3 \alpha_{3Z}^2 \right\rangle$$
$$= \frac{1}{3}(\Gamma_1 + \Gamma_2 + \Gamma_3) = \frac{1}{3} \qquad (22)$$

where α_{iz} are the cosines of angles between the principal axes of the absorption oscillator and the Z-axis. It can be assumed that the total emitted power is equal to the total absorbed power, since, in the case under consideration, the ratios of the principle emission intensities (which are independent of the photoluminescence yield of the solution) are of interest. According to the definition of the emission anisotropy (Equation 4) and to Equation 22, the following expression is obtained for the fundamental emission anisotropy:

$$r_f = \frac{1}{2}\left(9 \cdot I_{\parallel} - 1\right) \qquad (23)$$

where $I_{\parallel}$ is expressed by relative units the same as is I in Equation 22. It is therefore sufficient to calculate $I_{\parallel}$. The photoluminescence intensity component along the Z-axis parallel to **E** is given by

$$I_{\parallel} = \left\langle \sum_i \Gamma_i^2 \alpha_{iZ}^2 \sum_j \Gamma'_j \alpha'^2_{jZ} \right\rangle \qquad (24)$$

where the cosine of the angle between Γ'_j and Z amounts to

$$\alpha'_{jZ} = \sum_k \beta_{jk} \alpha_{kZ} \qquad (25)$$

β_{jk} is the cosine between Γ'_j and Γ_k.

Based on Equation 25 we obtain

$$\alpha'^2_{jZ} = \sum_k \beta_{jk} \alpha_{kZ} \sum_l \beta_{jl} \alpha_{lZ} \qquad (26)$$

The substitution of Equation 26 into Equation 24 yields:

$$I_{\parallel} = \sum_{i=1}^{3} \sum_{j=1}^{3} \sum_{k=1}^{3} \sum_{l=1}^{3} \Gamma_i \Gamma_j \beta_{jk} \beta_{jl} \left\langle \alpha_{iZ}^2 \alpha_{kZ} \alpha_{lZ} \right\rangle$$
$$= \sum_i \sum_k \Gamma_i G_k \left\langle \alpha_{iZ}^2 \alpha_{kZ}^2 \right\rangle$$
$$= \sum_i \Gamma_i G_i \alpha_{iZ}^4 + \frac{1}{15} \sum_{i \neq k} \Gamma_i G_k$$
$$= \frac{1}{15}\left(2 \cdot \sum_i \Gamma_i G_i + 1\right) \qquad (27)$$

since $\left\langle \alpha_{iZ}^2 \alpha_{kZ}^2 \right\rangle = \frac{1}{15}$ for $i \neq k$

and $\sum_{i,k} \Gamma_i G_i = \sum_i \Gamma_i \sum_k G_k = 1$

where $G_k = \sum_j \Gamma_j \beta_{jk}^2$

is the emission intensity component along the k-

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axis of the absorption oscillator (in Equation 27, all terms with $k \neq 1$ are eliminated).

By substituting Equation 27 into Equation 23 we obtain the required expression for the value of the fundamental emission anisotropy

$$r_f = \frac{3}{5}\sum_{i=1}^{3}\Gamma_i G_i - \frac{1}{5} \tag{28}$$

As seen from Equation 28, since $o \leq \Sigma_i \Gamma_i\, G_i \leq 1$, the values of the fundamental emission anisotropy, r_f, cover the interval of

$$-\frac{1}{5} \leq r_f \leq \frac{2}{5}$$

If the same transition moment is responsible for the absorption and emission ($\Gamma_i = G_i$), the principle emission anisotropy is given by

$$r_p = \frac{3}{5}\sum_{i=1}^{3}\Gamma_i^2 - \frac{1}{5} \tag{29}$$

Expression 28 yields the values of the fundamental emission anisotropy, r_f, predicted in earlier theories.[39,41] For $\Gamma_1 = 1$ and $\Gamma_2 = \Gamma_3 = 0$, for totally anisotropic oscillator, $r_p = 0.4$ is obtained according to Equation 29, i.e., the same value as that obtained by Vavilov and Levshin[39] based on their theory. Let us consider some particular cases:

1. If $\Gamma_1 = \Gamma_2 = \Gamma_3 = 1/3$ and $G_1 = 1$, $G_2 = G_3 = 0$, or if the absorption and emission oscillator are fully isotropic ($\Gamma_1 = \Gamma_2 = \Gamma_3 = 1/3$, $G_1 = G_2 = G_3 = 1/3$), $r_f = 0$ is obtained from Equation 28. This result seems evident, since all molecules, irrespective of the orientation, are excited in the same way and emit isotropic photoluminescence independent of the degree of excitation.
2. For a symmetric planar oscillator, when $\Gamma_1 = \Gamma_2 = G_1 = G_2 = 1/2$ and $\Gamma_3 = G_3 = 0$, $r_p = 0.1$ is obtained from Equation 29. This case is relevant to the fluorescence of benzene and its derivatives.[46]
3. If completely anisotropic absorption and emission oscillators form an angle β, then $\Gamma_1 = 1$, $\Gamma_2 = \Gamma_3 = 0$, $G_1 = \cos^2\beta$, which, after substitution to Equation 28, leads to the Perrin Equation 20. It should be noted that Equation 20 has been generalized by Perrin[42] to include also the case of spatial oscillators. The expression obtained differs from Equation 20 only in the presence of the mean value of $\cos^2\beta$.

Based on the investigations of the emission anisotropy and lifetimes of different components of photoluminescence of solutions, a number of interesting conclusions can be drawn if one assumes that for a certain kind of LM the value of the fundamental emission anisotropy is known. This value can be determined using Equation 28. The information that can be obtained from such investigations concern torsional vibrations and the Brownian rotations of LM, their solvation shells, and the relaxation times of local temperatures of the luminescent centers.

C. Influence of Torsional Vibrations of Luminescent Molecules on the Fundamental Emission Anisotropy

From among depolarization factors in a rigid isotropic fluorescent solution (e.g., the rotational diffusion of LM and the radiationless excitation energy migration between LMs) only torsional vibrations of LM are difficult to eliminate by suitable arrangement of experimental conditions. As already assumed by Perrin,[41] the vibrations performed by a molecule are one of the factors affecting the magnitude of the observed limiting emission anisotropy, r_0, of polyatomic complex molecules. In the case of simple biatomic molecules, the molecular symmetry is retained upon the change of the vibrational state,[46] whereas for complex molecules, deformation vibrations spoil the molecular configuration, and the relation between the oscillator and the molecule loses its rigidity. Thus, the degree of the oscillator anisotropy decreases, resulting in decrease in the fluorescence anisotropy. The following expression was obtained by Perrin[34,41] for the limiting emis-

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sion anisotropy

$$r_0 = \frac{2}{5}\left(1 - \frac{3}{2}u\right)^2 \tag{30}$$

where $u = \langle \sin^2 \gamma \rangle$ is the mean square of the sine of the angle γ between the mean direction of the equilibrium position and an instantaneous direction.

Torsional vibrations occur both in rigid and liquid media.[47–50] The vibration period, which in liquids is most certainly irregular, is very short as compared to the mean fluorescence lifetime, τ. Therefore, the effect of torsional vibrations upon the emission anisotropy is manifested only at an instant of excitation ($t \approx 0$), resulting in the reduction in the anisotropy of the absorption and emission spatial oscillators.

Therefore, Γ_i and G_i in Equation 28 should be replaced with[51]

$$\Gamma_i' = \sum_{j=1}^{3} \Gamma_j \left\langle \alpha_{ij}^2 \right\rangle$$

$$G_i' = \sum_{j=1}^{3} G_j \left\langle \alpha_{ij}^2 \right\rangle \quad (i = 1,2,3) \tag{31}$$

where $\langle \alpha^2_{ij} \rangle$ is the mean square of the cosine of the angle between the mean direction of the *i*th axis (equilibrium position) and the instantaneous direction of the *j*th axis (j = 1,2,3). In the case under consideration, the value of the fundamental emission anisotropy changed by the torsional vibrations will be termed the limiting emission anisotropy. Hence, we obtain

$$r_0 = \frac{3}{5} \sum_{i=1}^{3} \Gamma_i' G_i' - \frac{2}{5} \tag{32}$$

We shall further limit our considerations to the case:

$$1 - \left\langle \alpha_{ii}^2 \right\rangle << 1 \text{ and } \left\langle \alpha_{ij}^2 \right\rangle << 1 \quad (i \neq j)$$

assuming for simplification:

$$\left\langle \alpha_{ij}^2 \right\rangle = \left\langle \alpha_{ik}^2 \right\rangle \text{ for } k \neq j$$

Assume that $\langle \alpha^2_{ii} \rangle = 1 - u$, where $u = \langle \sin^2 \gamma \rangle$ is the dispersion of the sine of the angle between the direction of the *i*th axis and its equilibrium position, and $\langle \alpha^2_{ij} \rangle = u/2$ ($i \neq j$). Under these assumptions, and based on Equation 31, we obtain:[52]

$$\Gamma_i' = \Gamma_i \left(1 - \frac{3}{2}u\right) + \frac{u}{2}$$

$$G_i' = G_i \left(1 - \frac{3}{2}u\right) + \frac{u}{2}$$

and, upon substitution to Equation 32, the limiting emission, r_0, is expressed by the Equation

$$r_0 = \frac{3}{5}\left[\sum_i \Gamma_i \cdot G_i \left(1 - \frac{3}{2}u\right)^2 - \frac{1}{3}\left(1 - \frac{3}{2}u\right)^2\right]$$

$$= \frac{3}{5}\left(\sum_i \Gamma_i \cdot G_i - \frac{1}{3}\right) \cdot \left(1 - \frac{3}{2}u\right)^2 = r_p \cdot \left(1 - \frac{3}{2}u\right)^2 \tag{30'}$$

which is equivalent to Equation 30, when the same linear oscillator is responsible for the light absorption and emission ($\Gamma_i = G_i$) and $\Gamma_1 = 1$, $\Gamma_2 = \Gamma_3 = 0$ (in this case from Equation 29 it follows that $r_p = 2/5$).

Torsional vibrations are only possible if the LM behave as rigid bodies placed in the field of external forces, i.e., when the intramolecular forces (binding the atoms within molecules) considerably dominate the intermolecular forces. Those latter are sufficient, however, to keep the molecules near the equilibrium orientation. Such conditions exist in liquids with high viscosities or in glazes. With increasing temperature, the torsional vibrations are transformed into rotational motion.

The quantum-mechanical theory on torsional vibrations of molecules was developed by Hanus.[53] As shown for small amplitudes,[53] the torsional vibrations about the three axes of inertia can be treated, to a certain approximation, as mutually independent. The torsional vibrations of a molecule can, in this case, be resolved into three components, and the vibrations along the axis parallel

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to the direction of the linear oscillator (the same oscillator being responsible for the absorption and emission) do not depolarize the fluorescence light.

Jabłońsk[48,49] has formulated the following expression for the dispersion of the sine of the angle between the instantaneous direction of the transition dipole and its mean direction ($kT >> \hbar\omega$)

$$u \equiv \langle \gamma^2 \rangle \approx \frac{\hbar}{I \cdot \omega} + \frac{2kT}{I \cdot \omega^2} \tag{33}$$

$\langle \sin^2 \gamma \rangle \approx \langle \gamma^2 \rangle$ since $u = \langle \sin^2 \gamma \rangle$ and $\gamma << 1$. Taking into account Expression 33, Equation 30′ assumes the following form:[54]

$$r_0 = r_p (A - BT)^2 \tag{34}$$

where

$$A = 1 - \frac{3\hbar}{2I \cdot \omega}, \quad B = \frac{3k}{I \cdot \omega^2}$$

By plotting $(r_0/r_p)^{1/2}$ as a function of temperature T, the effective molecular moment of inertia, I, and the mean frequency, ω, of torsional vibrations can be determined according to Equation 34.

Jabłoński's theory (Equation 34) was verified experimentally for anthracene by Grzywacz[55] and Bauer.[56] The determined mean frequencies of torsional vibrations for anthracene[55] and for 9,10-diphenylanthracene[57] are $\omega = 4.1 \times 10^{12}\ s^{-1}$ and $\omega = 1.5 \times 10^{12}\ s^{-1}$, respectively. Torsional vibrations and the Brownian rotations of LM, leading to partial depolarization of fluorescence of acriflavine and acridine yellow in cyclohexanol and glycerol, were investigated by Heldt.[58]

The effect of temperature (103 K < T < 303 K) upon the limiting fluorescence anisotropy, r_0, of 2,2′-*p*-phenylenebis(5-phenyloxazole) (POPOP) was investigated in a cellulose acetate film.[59] A slow increase in r_0 was observed when reducing the temperature (Figure 3). Since $u << 1$, Equation 34 may be replaced by the following approximate formula

$$\frac{r_p - r_0}{r_p} = A' + B'T \tag{35}$$

where

$$A' = \frac{3\hbar}{I \cdot \omega} \quad \text{and} \quad B' = \frac{6k}{I \cdot \omega^2}$$

The angle γ between the mean direction of the equilibrium position and the instantaneous direction amounts to 8.9° and 5.35° for T = 293 K and T = 103 K, respectively, for ω determined below.

As can be readily seen, u (Equation 34) does not vanish when temperature approaches 0 K. However, Formulas 34 and 35 are true only for $kT \rangle\rangle \hbar\omega$, and therefore the value A′ cannot be evaluated by the extrapolation of $r_0(T)$ for $T \rightarrow 0$ K. Plotting the left-hand side of Equation 35 as a function of T for $r_p = 0.4$, one obtains a straight line with a slope B′ (Figure 3). The moment of inertia I can be calculated from the known structure of the molecule. The resulting mean moment of inertia with respect to the axis perpendicular to the long axis of the POPOP molecule is $I = 19.7 \times 10^{-44}$ kg m^2, being considerably smaller than that determined from the rotational depolarization in a liquid solvent.[60,61] The value of B′ (Figure 3) obtained is $2.4 \times 10^{-4}\ K^{-1}$ and, hence, $\omega = 1.3 \times 10^{12}\ s^{-1}$ and the moment of torsion acting on the molecule is $M = I\omega^2 = 3.3 \times 10^{-19}\ m^2\ kg\ s^{-2}$. The value of ω seems quite reasonable. In rigid solutions, where the LM does not rotate, the torsional vibrations are the only depolarizing factor in the solution, immediately following excitation during thermal relaxation ($T' \approx 4.7 \times 10^{-12}$ s).

The Jabłoński theory of the effect of torsional vibrations on the emission anisotropy, r_p,[47,48] was also discussed by Johansson[62] for perylene and the perylenyl derivatives. The following factors should always be taken into account when measuring the effect of temperature on the limiting fluorescence anisotropy, in frozen or rigid solutions: (1) short- and long-wave absorption bands should be well separated in order to avoid simultaneous excitation in two bands;[63–65] (2) the change of the solution temperature is accompanied by that in the dielectric constant and the refractive

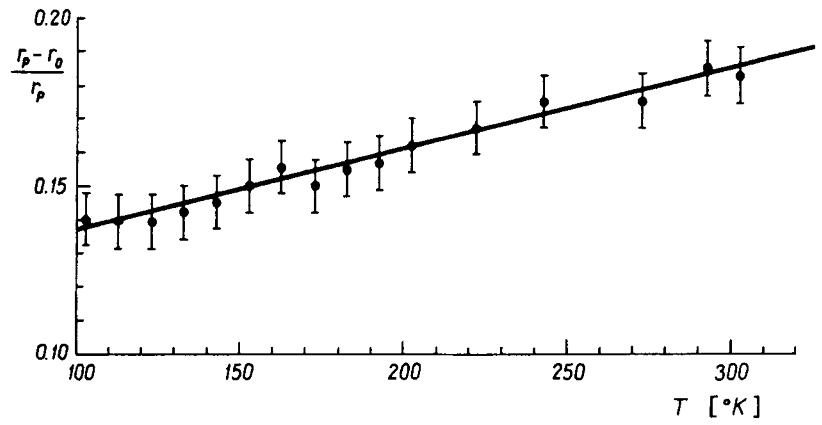

FIGURE 3. Dependence of $(r_p - r_0)/r_p$ on temperature for POPOP in cellulose acetate film, according to Equation 35. (From Kawski, A. et al. *Z. Naturforsch. Teil A* **1985,** *40,* 559. With permission.)

index, which results in a shift of the absorption and fluorescence bands; (3) phosphorescence or delayed fluorescence are undesired; (4) the mean lifetime, τ, of the LM should not vary in the relevant temperature range.

III. EFFECT OF DEPOLARIZATION FACTORS UPON THE DECAY OF FLUORESCENCE COMPONENTS

As has been known from previous considerations, the maximum fluorescence anisotropy is observed in the absence of depolarization factors. Under excitation with linearly polarized light in the long-wave absorption region of LM distributed isotropically in rigid transparent medium, a limiting emission anisotropy, r_0, close to the theoretical value of $r_p = 0.4$, is observed. Whether or not the discriminated direction of the transition moments of LM, resulting from photoselection during the fluorescence decay, is maintained, depends on the properties of LM themselves (first of all upon mean lifetime, τ), as well as on sufficiently high viscosity of the solvent chosen for the LM.

Strong emission anisotropy of weakly fluorescing erythrosine in alcohols and water was observed by Levshin[66] and Vavilov.[67] Also for this dye, Perrin[41] found a very short fluorescence lifetime, $\tau = 84$ psec, by employing an indirect method of rotational depolarization. Similar behavior was also observed later for Michler's ketone in ethanol at 295 K.[68] In recent years, very strong anisotropic fluorescence was observed for substituted *trans*-stilbenes in nonpolar and polar low-viscosity solvents (*n*-heptane, toluene, benzene, acetonitrile, dimethylformamide, *n*-propanol) at 293 K[69,70] and for ω-substituted 4-dimethylamino-*trans*-styrenes in alkane solutions at 293 K.[71] In that latter case,[69–71] the fluorescence lifetime, τ, was measured by direct methods. Table 1 summarizes the values of r and τ for several selected substances.

If the fluorescence intensity, I, decays according to the exponential law*

$$I(t) = I_0 e^{-t/\tau} \qquad (36)$$

and the emission anisotropy is a function of time, r(t), then, according to Equations 10 and 11, Jabłoński obtained:[38,72–75]

$$I_{\parallel}(t) = \frac{I_0}{3}[1 + 2r(t)]e^{-t/\tau} \qquad (37)$$

$$I_{\perp}(t) = \frac{I_0}{3}[1 - r(t)]e^{-t/\tau} \qquad (38)$$

---

* Equation 36 is only correct when the fluorescence level is directly excited by light absorption, i.e., with no other intermediary levels.

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TABLE 1

The Fluorescence Anisotropies, r, and Mean Lifetimes, τ (in 10^{-12} s) for Several Derivatives of *trans*-Stilbene and *trans*-Styrene in Solvents at 293 K

Compound	Pentane $\eta = 0.247 \times 10^{-3}$ Pa·s		Hexane $\eta = 0.326 \times 10^{-3}$ Pa·s		Benzene $\eta = 0.63 \times 10^{-3}$ Pa·s		Chlorobenzene $\eta = 0.90 \times 10^{-3}$ Pa·s		Ref.
	r	τ	r	τ	r	τ	r	τ	
4-Isocyanate-4′-dimethylamino-*trans*-stilbene	—	—	—	—	0.2923	16	0.3356	<10	69
4-Isocyanate-4′-methoxy-*trans*-stilbene	—	—	—	—	0.2594	87	0.3535	<10	69
4-Dimethylamino-ω-diphenylthiophosphinyl-*trans*-styrene	0.2950	3	—	—	—	—	—	—	71
4-Dimethylamino-ω-diphenylphosphinyl-*trans*-styrene	0.2850	4	—	—	—	—	—	—	71
4-Dimethylamino-ω-methylsulfonyl-*trans*-styrene	0.1644	18	0.1725	19	—	—	—	—	71

and the following relation for the mean duration of I(t):

$$\tau = \frac{1}{3}\left[\tau_{\parallel}\left(1+2\langle r\rangle\right)+2\tau_{\perp}\left(1-\langle r\rangle\right)\right] \qquad (39)$$

The decay of the individual components does not follow the exponential law, depending on the course of function r(t) and, in general, does not follow the simple exponential law. Equation 39 is independent of the explicit form of r(t) and allows us to evaluate τ, if the values $\tau_{\parallel}$, $\tau_{\perp}$, and ⟨r⟩ are known.

The fluorescence emission anisotropy can be lowered as a result of thermal relaxation of the luminescent center in its environment (according to Jabło´nski,[47–49] by torsional vibrations occurring in a very short time after excitation), and then by rotational motions of LM. At higher concentrations of LM, the emission anisotropy further decreases due to intermolecular excitation energy migration. The depolarization factors named, excluding the torsional vibrations, can be eliminated by the arrangement of suitable experimental conditions.

Thus, the emission anisotropy, r(t,n,T), determined experimentally is affected by numerous statistically independent factors, and can be represented by a product of different time-dependent functions:[76]

$$r(t,n,T) = r_0 W_r(t,T) W_m(t,n) \qquad (40)$$

In this expression, r_0 is the observed limiting emission anisotropy, embracing the contribution from initially rapidly depolarizing relaxation processes induced by intramolecular vibrations (torsional vibration, according to Jabłoński). $W_r(t,T)$ represents the reduction in r_0 resulting from thermal rotational motions of LM ($W_r(t,0) = 1$). $W_m(t,n)$ refers to the reduction in r_0 due to the resonance energy migration (n is the concentration of LM, i.e., the number of molecules per 1 cm^3; for $n \to 0$, $W_m(t,0) = 1$).

Thermal rotational motions of LM, as well as the excitation energy migration, distinctly affect the intensity components $I_{\parallel}(t)$ and $I_{\perp}(t)$ by altering the emission anisotropy, r(t), during the decay of fluorescence (Equations 37 and 38).

IV. ROTATIONAL FLUORESCENCE DEPOLARIZATION OF SPHERICAL MOLECULES

A. Time Decay of Emission Anisotropy

It has been assumed so far that rotational motions of LM do not occur in solutions, which is valid in highly viscous or rigid media. In liquid

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solvents, however, the LM perform Brownian rotations due to thermal motion. Let us first consider, according to Perrin's theory, the Brownian rotational motions of a spherical molecule associated with a transition dipole moment, μ.[41,77,78] The orientation of a LM in space is defined by spherical angles ϑ_0 and φ, determining the position of a selected direction which is the direction of the dipole transition moment μ. If, at the initial instant, the direction of the transition moment μ (consistent with the direction of axis 3 in Figure 4) is given by $\vartheta_0 = \varphi_0 = 0$, then after time t the anisotropy of the excited LM is described by the probability distribution $P_t(\vartheta,\varphi)$. As a result of isotropic Brownian motion, the probability $P_t(\vartheta,\varphi)$ will be symmetrical with respect to the initial position of vector μ, thus being independent of angle φ. The distribution function of vector μ over the angles should satisfy the following normalization condition:

$$\int_0^{2\pi} d\varphi \int_0^{\pi} P_t(\vartheta,\varphi)\sin\vartheta\, d\vartheta = 2\pi\int_0^{\pi} P_t(\vartheta,\varphi)\sin\vartheta\, d\vartheta' = 1 \qquad (41)$$

The change of $P_t(\vartheta,\varphi)$ in time is described by the following diffusion equation:

$$\frac{\partial P_t(\vartheta,t)}{\partial t} = D\left\{\frac{1}{\sin\vartheta}\frac{\partial}{\partial\vartheta}\left(\sin\vartheta\frac{\partial P_t(\vartheta,t)}{\partial\vartheta}\right)\right\} \qquad (42)$$

where D is the time-dependent diffusion coefficient of rotational motion. For spherical molecules, rotational diffusion coefficient, D, is related to the solvent macroscopic viscosity, η, and the volume of the LM, V, by the Stokes-Einstein formula:

$$6D = \frac{kT}{V\eta} \qquad (43)$$

where k is the Boltzmann constant and T is the absolute temperature.

We are searching for the following form of the mean value

$$u(t) = \langle\cos^2\vartheta\rangle = 2\pi\int_0^{\pi} \cos^2\vartheta P(\vartheta,t)\sin\vartheta\, d\vartheta \qquad (44)$$

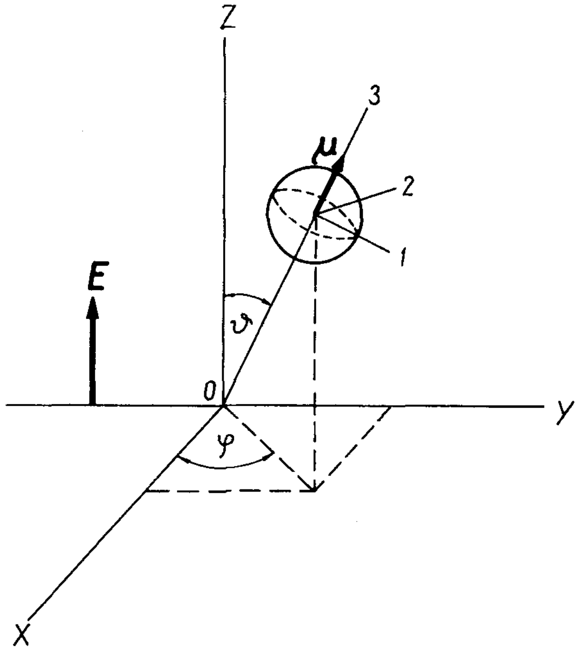

FIGURE 4. Geometry of the system.

The knowledge of the explicit form of function $P(\vartheta,t)$ is not indispensable to this end. It is sufficient to differentiate Equation 44 over time:

$$\frac{d}{dt}u(t) = 2\pi\int_0^{\pi} \cos^2\vartheta\frac{\partial P(\vartheta,t)}{\partial t}\sin\vartheta\, d\vartheta \qquad (45)$$

and to substitute Expression 42 and integrate twice over time. As a result, the following equation is obtained

$$\frac{d}{dt}u(t) = -6D\left[u(t) - \frac{1}{3}\right] \qquad (46)$$

the solution to which, taking into account initial condition u(0) = 1, is

$$u(t) = \langle\cos^2\vartheta\rangle = \frac{1}{3}\left(1 + 2e^{-6Dt}\right) \qquad (47)$$

The orientation of Brownian molecules can be found with the help of nine direction cosines, $C_{ij} = \cos(i,j)$, which determine the position of three rectangular coordinates of a sphere at any instant t, relative to the initial position of these coordinates (at t = 0). Evidently, the following

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relationships exist between the direction cosines $\langle C^2_{ii}\rangle = \langle C^2_{jj}\rangle = \langle C^2_{kk}\rangle = \langle \cos^2 \vartheta\rangle$ and $\langle C^2_{ij}\rangle = \langle C^2_{ik}\rangle$ in view of the equivalent significance of two axes relative to the third one during isotropic rotational Brownian motion. Taking into account the normalization condition for direction cosines

$$\left\langle C^2_{ii}\right\rangle + \left\langle C^2_{ij}\right\rangle + \left\langle C^2_{ik}\right\rangle = 1$$

and Equation 47, we obtain

$$\begin{aligned} \left\langle C^2_{ii}\right\rangle &= \frac{1}{3}\left(1 + 2e^{-6Dt}\right) \\ \left\langle C^2_{ik}\right\rangle &= \frac{1}{3}\left(1 - e^{-6Dt}\right) \end{aligned} \tag{48}$$

Expressions 48 describe the basic properties of the rotational Brownian motion of a spherical LM, and can be employed to describe the fluorescence anisotropy in solution.

Let us first consider the pulse excitation (δ-function excitation). Fluorescence intensity components, $I_z(\Omega,t)$ and $I_y(\Omega,t)$, emitted by molecules after time t, which at the initial instant (after the cessation of excitation) had defined orientations, are related with the initial fluorescence intensity components I_{x0}, I_{y0}, and I_{z0} for t = 0 by

$$\begin{aligned} I_z(\Omega,t) = \alpha(t)\Big\{&I_{x0}(\Omega)\left\langle C^2_{xz}\right\rangle + I_{y0}(\Omega)\left\langle C^2_{yz}\right\rangle \\ &+ I_{z0}(\Omega)\left\langle C^2_{zz}\right\rangle\Big\} \end{aligned} \tag{49}$$

$$\begin{aligned} I_y(\Omega,t) = \alpha(t)\Big\{&I_{x0}(\Omega)\left\langle C^2_{xy}\right\rangle + I_{y0}(\Omega)\left\langle C^2_{yy}\right\rangle \\ &+ I_{z0}(\Omega)\left\langle C^2_{zy}\right\rangle\Big\} \end{aligned} \tag{50}$$

where $\alpha(t) = C \cdot 1/\tau \cdot e^{-t/\tau}$ is the factor of proportionality expressing the fluorescence decay law. The z- and y-components of fluorescence intensities emitted by an ensemble of LM can be obtained by integrating Equations 49 and 50 over all initial orientations of the excited molecules. Hence, we obtain

$$I_z(t) = \alpha(t)\left\{I_{x0}\left\langle C^2_{xz}\right\rangle + I_{y0}\left\langle C^2_{yz}\right\rangle + I_{z0}\left\langle C^2_{zz}\right\rangle\right\} \tag{51}$$

$$I_y(t) = \alpha(t)\left\{I_{x0}\left\langle C^2_{xy}\right\rangle + I_{y0}\left\langle C^2_{yy}\right\rangle + I_{z0}\left\langle C^2_{zy}\right\rangle\right\} \tag{52}$$

where

$$I_{j0} = \int_\Omega I_{j0}(\Omega)\, d\Omega$$

Since the excitation light is linearly polarized, $I_{x0} = I_{y0}$ and Equations 51 and 52, with Expressions 48 being taken into account, will assume the following form:

$$I_z(t) = \alpha(t)\frac{1}{3}\left\{2\left(1 - e^{-6Dt}\right)I_{y0} + \left(1 + 2e^{-6Dt}\right)I_{z0}\right\} \tag{53}$$

$$I_y(t) = \alpha(t)\frac{1}{3}\left\{\left(2 + e^{-6Dt}\right)I_{y0} + \left(1 - e^{-6Dt}\right)I_{z0}\right\} \tag{54}$$

If, as a result of excitation, the fluorescence anisotropy at t = 0 amounts to

$$r_0 = \frac{I_{z0} - I_{y0}}{I_{z0} + 2I_{y0}}$$

then, after t, based on Equations 3, 53, and 54 we obtain the time decay emission anisotropy

$$r(t) = r_0 e^{-6Dt} \tag{55}$$

Formula 55 implies that the fluorescence anisotropy decays according to a simple exponential law under the assumption that thermal motions of LM are governed by the rules of rotational Brownian motion of spherical molecules (isotropic rotators). This is the Perrin Equation for the time-dependent fluorescence measurements.

B. The Steady-State Anisotropy of Fluorescence

The measurements of the fluorescence anisotropy are frequently carried out at continuous excitation. Therefore, the time-averaged value of function r(t) (Equation 55) should be calculated. Assuming that the total fluorescence light intensity I(t) decays according to the exponential law, given by Equation 36, and r(t) is expressed by Equation 55, the well-known Perrin formula is obtained:[41,77,78]

$$\langle r\rangle = \frac{\int_0^\infty r(t)\cdot I(t)\,dt}{\int_0^\infty I(t)\,dt} = \frac{r_0}{1+6D\cdot\tau} \qquad (56)$$

where the rotational diffusion coefficient 6D is expressed by Equation 43.

Equations 37, 38, and 55 imply that:[74,75]

$$I_\parallel(t) = \frac{I_0}{3}\left[1+2r_0e^{-6Dt}\right]e^{-t/\tau} \qquad (57)$$

$$I_\perp(t) = \frac{I_0}{3}\left[1-r_0e^{-6Dt}\right]e^{-t/\tau} \qquad (58)$$

or, with 6D calculated from Equation 56, is

$$I_\parallel(t) = \frac{I_0}{3}\left[e^{-t/\tau}+2r_0e^{-(r_0/\langle r\rangle)(t/\tau)}\right] \qquad (59)$$

$$I_\perp(t) = \frac{I_0}{3}\left[e^{-t/\tau}-r_0e^{-(r_0/\langle r\rangle)(t/\tau)}\right] \qquad (60)$$

The mean durations of these components are[73,74]

$$\tau_\parallel = \tau\frac{r_0+2\langle r\rangle^2}{r_0+2r_0\langle r\rangle} \qquad (61)$$

$$\tau_\perp = \tau\frac{r_0-\langle r\rangle^2}{r_0-r_0\langle r\rangle} \qquad (62)$$

The Jabłoński Equations 59 to 62 were checked experimentally by Szymanowski[79] and Kessel[80] but the agreement appeared to be rather qualitative. The disagreement between Jabłoński's theory and the previous experimental results resulted from the insufficient accuracy of methods for measuring polarization and mean decay times. As shown by Bauer[81] and Bauer and co-workers,[82] there exists a very good agreement between the observations made for uranine solutions in glycerol diluted with methyl alcohol (Figure 5) and sodium-bis-ortho-stilbene-sulfonate (stilbene 3) in mixed glycerol and methanol solvents, and the Jabłoński Equations 61 and 62.

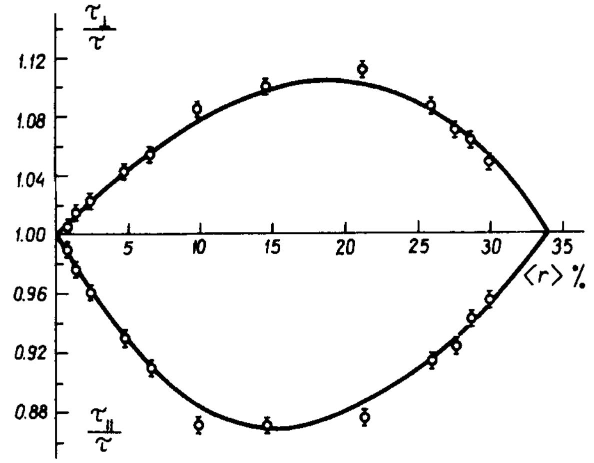

FIGURE 5. Experimental and theoretical (solid line) values of $\tau_\parallel/\tau$ and $\tau_\perp/\tau$ for fluorescence of uranine solutions in glycerol diluted with methyl alcohol. (From Bauer, R. K. *Z. Naturforsch. Teil A* **1963,** *18,* 718. With permission.)

The results of the measurements for stilbene 3 are presented in Figure 6. The quite good fit between the calculated (Equations 61 and 62) and measured values of $\tau_\parallel/\tau$ and $\tau_\perp/\tau$ means that the assumption of an exponential decay of the fluorescence anisotropy (Equation 55) is justified. It was therefore concluded that stilbene 3 molecules, which are rather elongated in shape, behave in solution like quasi-spherical particles. This allows calculation of the effective volume of the LM with its solvation shell. Similar measurements for uranine solutions in glycerol diluted with water have shown that the difference between the theoretical and experimental values of $\tau_\parallel/\tau$ and $\tau_\perp/\tau$ is greater than that observed for fluorescence of uranine in glycerol with methyl alcohol.

Perrin Equation 56 is frequently given as

$$\frac{r_0}{\langle r\rangle} = 1+\frac{3\tau}{\tau_R} \qquad (63)$$

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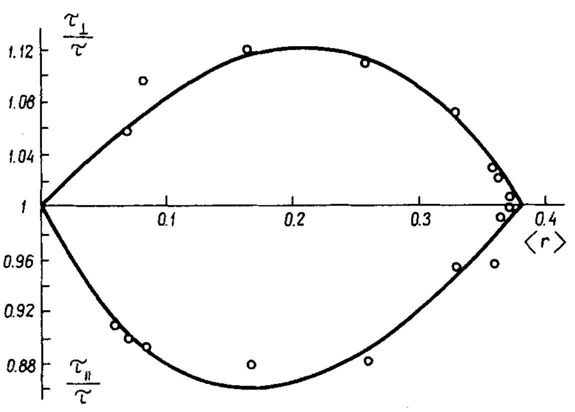

FIGURE 6. Mean decay time ratios of polarized fluorescence components for stilbene 3 in mixed glycerol and methanol solvents vs. ⟨r⟩. Solid line was calculated for $r_0 = 0.375$. (From Bauer, R. K. *Z. Naturforsch. Teil A* **1980,** *35,* 1319. With permission.)

where $\tau_R = 1/2D$ is the rotational relaxation time and

$$\tau_c = \frac{\tau_R}{3} = \frac{V\eta}{kT} = \frac{1}{\varphi} \qquad (64)$$

the rotational correlation time of the LM (φ is the probability of depolarization by Brownian motion). Equations 56 and 63, having been verified many times,[41,78,83–95] are employed for determining the value of r_0, as well as the effective volume V of the LM with its solvation shell. The parameter V is related to the molecular weight, M, and the hydration, h, of the LM by

$$V = \frac{M(v+h)}{N} \qquad (65)$$

where v represents the partial specific volume of the LM and N is Avogadro's number.[24]

In the investigations carried out by Bauer and Szczurek,[96] a linear relation between $1/\langle r\rangle$ and $1/\eta$ was indeed found for uranine in glycerol diluted with methyl alcohol, while a serious deviation from linearity was observed with water as a diluent. Jabłoński[97] suggested that, apart from Brownian rotations of LM, the depolarization is also caused by their torsional vibrations. The rotations of the dye molecules influence the course of r(t) and the torsional vibrations only reduce the initial value of r(t).

By taking the additional depolarization due to torsional vibrations of LM into account, Jabłoński[97] obtained the following expression:

$$\frac{1}{\langle r\rangle} = \frac{1+\frac{kT}{V\eta}\tau}{r_0 - \frac{\alpha}{\eta}} \qquad (66)$$

where α is a constant to be determined from the experimental curves. For $\alpha = 0$, Equation 66 does not differ from Equation 63.

Figures 7 and 8 show the results as plots of $1/\langle r\rangle$ vs. $1/\eta$, obtained by Bauer and Szczurek[96] for uranine in solutions of glycerol diluted with anhydrous methanol and water. In the first case (Figure 7), the experimental points lie on the Perrin straight line (Equation 63). Those for the uranine solutions in glycerol diluted with water exhibit deviations from linearity which become more prominent at low values ⟨r⟩ (Figure 8). The extrapolation of the experimental curve yields the limiting value of the emission anisotropy for solution in glycerol + water: $r_0 = 0.298$. The constants r_0 and α may be chosen so as to obtain the best fit to the experimental curve. For glycerol + water, $\alpha = 0.078$ has been obtained. The values of r_0 and α were substituted into Equation 66 and the calculated curve was traced, showing an excellent agreement with the experimental results. The direction of the deviation (see Figure 8) from linearity is in accordance with the experiments of Pringsheim and Vogels.[98] The torsional vibrations depend on the nature of the solvation shell of the LM, and therefore r_0 is not a molecular constant, but depends on the nature of the solvent.

C. Decay and Rotation under Cosinusoidal Excitation

1. General Considerations

A general expression for curves describing the time dependence of the intensity of fluores-

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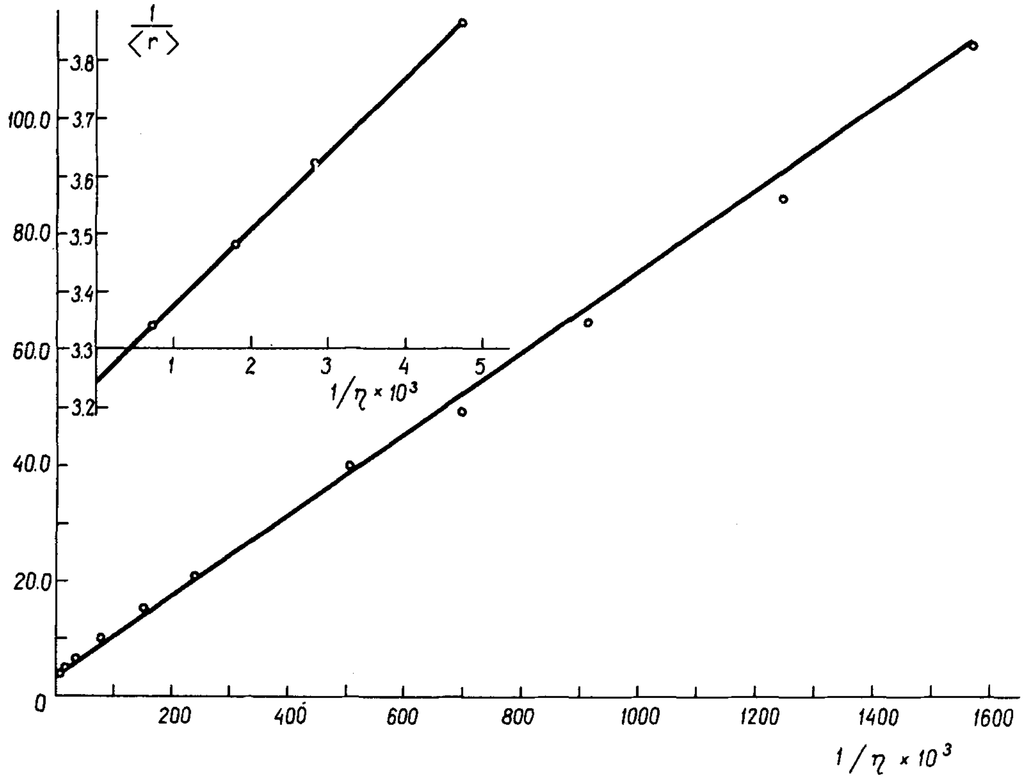

FIGURE 7. Plot of 1/⟨r⟩ vs. 1/η for uranine in solution in different mixtures of glycerol and methanol. (From Bauer, R. K.; Szczurek, T. *Acta Phys. Pol.* **1962,** *22,* 29. With permission.)

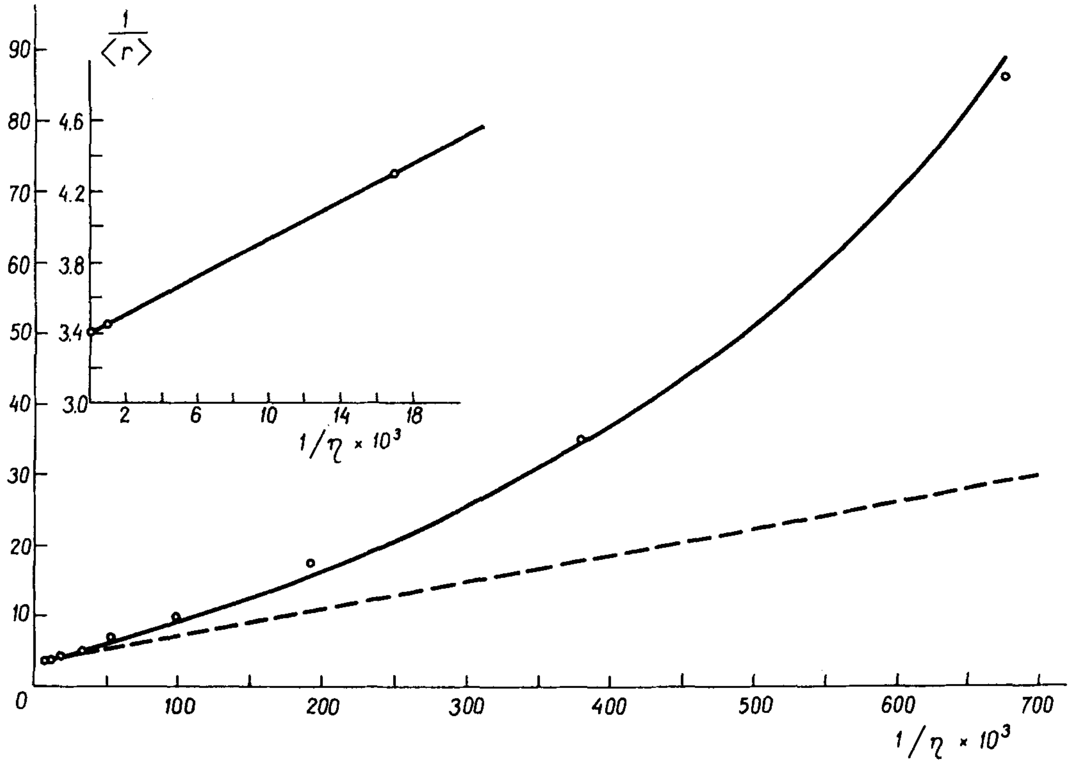

FIGURE 8. Plot of 1/⟨r⟩ vs. 1/η for uranine in different mixtures of glycerol and water. (From Bauer, R. K.; Szczurek, T. *Acta Phys. Pol.* **1962,** *22,* 29. With permission.)

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cence and that of its components in isotropic solutions excited by harmonically intensity-modulated primary light, was derived by Jabłoński.[99]

When the solution is excited at the moment t = 0 by an infinitely short light pulse, the fluorescence intensity, as well as that of any of its components, as a function of time t, for t ≥ 0, can be written as

$$I(t) = P(t) \cdot W \tag{67}$$

where

$$P(t) = \frac{I(t)}{\int_0^\infty I(t)\,dt} \tag{68}$$

is the so-called "decay function" and W is the total energy emitted from t = 0 to t = ∞. The function P(t) is normalized

$$\int_0^\infty P(t)\,dt = 1 \tag{69}$$

The mean lifetime of I(t) is defined as

$$\tau = \langle t \rangle = \int_0^\infty P(t)t\,dt \tag{70}$$

In the simplest case, when a purely exponential decay occurs

$$P(t) = \Gamma \cdot e^{-\Gamma \cdot t} \quad (\Gamma = \text{const.}) \tag{71}$$

is

$$\tau = \frac{1}{\Gamma} \tag{72}$$

In general:

$$I(t) = \sum_i a_i e^{-t/\tau_i} \tag{73}$$

(a_i and τ_i are constants) and the decay function becomes

$$P(t) = \frac{\sum_i a_i e^{-t/\tau_i}}{\sum_i a_i \tau_i} \tag{74}$$

The mean lifetime in this case:

$$\langle \tau \rangle = \frac{\sum_i a_i \tau_i^2}{\sum_i a_i \tau_i} \tag{75}$$

is not the arithmetical mean of τ_i.

The general expression for the fluorescence intensity, or that of its components, as a function of time, t, for all possible forms of f(t – s) is

$$I(t) = \int_0^\infty P(s)f(t-s)\,ds \tag{76}$$

where f(t – s) is proportional to the intensity of the primary light at $t' = t - s$. P(s) is normalized like P(t) (Equation 69).

If a solution is excited by intensity-modulated primary light, the intensity of the emitted fluorescence is also modulated with the same frequency but with the phase modulation shifted and the degree of modulation altered. The fluorescence of solution is excited with primary light, the intensity of which is harmonically modulated in time, i.e., when f(t) has the form:

$$f(t) = A + B \cdot \cos \omega t \tag{77}$$

where $A \geq B > 0$, and ω is the angular frequency of modulation. Substituting f(t – s) from Equation 77 into Equation 76 gives

$$\begin{aligned} I(t) &= \int_0^\infty P(s)[A + B \cdot \cos \omega(t-s)]\,ds \\ &= A + B[\langle \cos \omega s \rangle \cos \omega t + \langle \sin \omega s \rangle \sin \omega t] \end{aligned} \tag{78}$$

where

$$\langle \cos \omega s \rangle = \int_0^\infty P(s) \cos \omega s\,ds \tag{79}$$

and

$$\langle \sin \omega s\rangle = \int_0^\infty P(s)\sin \omega s \, ds \qquad (80)$$

are the mean values of cos ωs and sin ωs, respectively, averaged over the entire decay time.

Equation 78 can be written as

$$I(t) = A + b\cos(\omega t - \phi) \qquad (81)$$

where

$$\phi = \arctan \frac{\langle \sin \omega s\rangle}{\langle \cos \omega s\rangle} \qquad (82)$$

and

$$b = B\left(\langle \cos \omega s\rangle^2 + \langle \sin \omega s\rangle^2\right)^{1/2} \qquad (83)$$

Equation 81 has a form similar to that of Equation 77, but with the phase of modulation shifted by $-\phi$, and with the degree of modulation

$$M = \frac{b}{A} = \frac{B}{A}\left(\langle \cos \omega s\rangle^2 + \langle \sin \omega s\rangle^2\right)^{1/2} \qquad (84)$$

instead of B/A.

2. Applications to Particular Cases

We shall now consider the application of Equation 81 to the components of polarized fluorescence of isotropic solutions in which fluorescence depolarization is due to rotational motions (Equation 55). The intensities of the components of fluorescence parallel and perpendicular to the electric vector of the primary light are given by Equations 10 and 11. The intensity of component I_α polarized at an angle α with respect to $I_{\parallel}$ is

$$I_\alpha = I_{\parallel}\cos^2\alpha + I_{\perp}\sin^2\alpha = \frac{I}{3}\left(1 + L_\alpha r\right) \qquad (85)$$

where

$$L_\alpha = 3\cos^2\alpha - 1, \quad \left(2 \geq L_\alpha \geq -1\right)$$

The substitution of the decay laws (Equations 36 and 55) with $6D = 1/\tau_c$ into Equation 85 yields

$$I_\alpha(t) = \frac{I_0}{3}\left[1 + L_\alpha r_0 e^{-t/\tau_c}\right]e^{-t/\tau} \qquad (86)$$

Hence, the decay function (Equation 68), with Equation 85, becomes

$$P_\alpha(s) = \frac{I_\alpha(s)}{\int_0^\infty I_\alpha(s)\, ds} = e^{-s/\tau}\frac{\left[1 + L_\alpha r_0 e^{-t/\tau_c}\right]\cdot\left[1 + \frac{\tau}{\tau_c}\right]}{\tau\left(1 + \frac{\tau}{\tau_c} + L_\alpha r_0\right)} \qquad (87)$$

where $s = t - t'$, t is the time of observation of fluorescence emitted by groups of LM excited at various times $t' \leq t$, and τ is the mean lifetime of the total fluorescence $I = I_{\parallel} + 2I_{\perp}$.

Mean lifetime τ_α of component I_α is

$$\tau_\alpha = \int_0^\infty P_\alpha(s)\, ds = \tau\frac{\left[1 + \frac{\tau}{\tau_c}\right]^2 + L_\alpha r_0}{\left[1 + \frac{\tau}{\tau_c}\right]^2 + L_\alpha r_0\left[1 + \frac{\tau}{\tau_c}\right]} \qquad (88)$$

and should be independent of the measurement method. Obviously, $\tau_\alpha = \tau$ for $L_\alpha = 0$ ($\alpha = 54.7356°$). Based on Equations 79, 80, and 87, one obtains

$$\langle \cos \omega s\rangle = \frac{1 + \frac{\tau}{\tau_c}}{1 + \frac{\tau}{\tau_c} + L_\alpha r_0} \times \left[\frac{1}{1 + \omega^2\tau^2} + \frac{L_\alpha r_0\left[1 + \frac{\tau}{\tau_c}\right]}{\left[1 + \frac{\tau}{\tau_c}\right]^2 + \omega^2\tau^2}\right] \qquad (89)$$

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and

$$\langle \sin \omega s \rangle = \frac{\left[1+\frac{\tau}{\tau_c}\right]\cdot \omega\tau}{1+\frac{\tau}{\tau_c}+L_\alpha r_0}\left[\frac{1}{1+\omega^2\tau^2}+\frac{L_\alpha r_0}{\left[1+\frac{\tau}{\tau_c}\right]^2+\omega^2\tau^2}\right] \tag{90}$$

Hence,

$$\mathrm{tg}\phi_\alpha = \omega\tau \frac{\left[1+\frac{\tau}{\tau_c}\right]^2+\omega^2\tau^2+L_\alpha r_0\left[1+\omega^2\tau^2\right]}{\left[1+\frac{\tau}{\tau_c}\right]^2+\omega^2\tau^2+L_\alpha r_0\left[1+\frac{\tau}{\tau_c}\right]\left[1+\omega^2\tau^2\right]} \tag{91}$$

From Equation 63 it follows that:

$$1+\frac{\tau}{\tau_c}=\frac{r_0}{\langle r\rangle}$$

and Equations 88 to 91 may be written as

$$\tau_\alpha = \tau\frac{r_0+L_\alpha\cdot\langle r\rangle^2}{r_0+L_\alpha r_0\langle r\rangle} \tag{92}$$

$$\langle \cos \omega s\rangle = \frac{1}{1+L_\alpha\cdot\langle r\rangle}\left[\frac{1}{1+\omega^2\tau^2}+\frac{L_\alpha r_0^2\langle r\rangle}{r_0^2+\langle r\rangle^2\omega^2\tau^2}\right] \tag{93}$$

$$\langle \sin \omega s\rangle = \frac{\omega\tau}{1+L_\alpha\cdot\langle r\rangle}\left[\frac{1}{1+\omega^2\tau^2}+\frac{L_\alpha r_0\langle r\rangle^2}{r_0^2+\langle r^2\rangle\omega^2\tau^2}\right] \tag{94}$$

and

$$\mathrm{tg}\phi_\alpha = \omega\tau\frac{r_0^2+\langle r\rangle^2\omega^2\tau^2+L_\alpha r_0\langle r\rangle^2\left(1+\omega^2\tau^2\right)}{r_0^2+\langle r\rangle^2\omega^2\tau^2+L_\alpha r_0^2\langle r\rangle\left(1+\omega^2\tau^2\right)} \tag{95}$$

The degree of modulation of the corresponding "fluorescence curve" (see Equation 84) is

$$\begin{aligned} M &= \frac{b}{A} \\ &= \frac{B}{A\left[1+\omega^2\tau^2\right]^{1/2}} \\ &\quad\times\left[\frac{r_0^2+\omega^2\langle r\rangle^2\tau^2\left[1+L_\alpha r_0\right]^2\left[1+L_\alpha\langle r\rangle\right]^{-2}}{r_0^2+\omega^2\langle r\rangle^2\tau^2}\right]^{1/2} \end{aligned} \tag{96}$$

Similar expressions hold for the excitation by natural light, if $L_\alpha = 3\cos^2\alpha - 1$ is replaced with $L_\alpha = 3\cos^2\alpha - 2$, and r_0 and r with their values for the natural light excitation.

The problem of the decay of polarized fluorescence components was also treated by Spencer and Weber[100] and Merkelo et al.[101] Both their approach and that of Jabłoński[99] are restricted to the case of time-independent depolarization rate. This is the simplest one and in most cases constitutes a fairly good approximation. The application of general expressions derived by Jabłoński[99] (see General Considerations) is not restricted to the simplest case.

One can easily show that Equations 91, 95, and 96 can be obtained from expressions derived by Spencer and Weber,[100] if the notations of different quantities appearing in their equations are replaced with those used in this article. The two different treatments are fully equivalent, but the conclusions concerning the measured mean lifetime of fluorescence differ drastically. According to Spencer and Weber,[100] the difference in lifetime between the polarized components of the solution is a function of the ratio of the modulation frequency ω to the emission rate. Equations 87 to 91 show that such an assumption is not justified. Although ϕ and M depend *ceteris paribus* on frequency ω, τα (Equations 88 and 92) does not. In the particular case of a simple exponential decay, both treatments of Jabłoński[99] and Spencer and Weber[100] lead to identical, and frequency-independent, values of the mean lifetime.

The Jabłonski theory,[72–75] extended by Spencer and Weber[100] to embrace the case of $\omega\tau \geq 1$ in phase fluorometric measurements, gives an excellent fit between the measured and calculated values of $\tau_{\|}/\tau$ and $\tau_{\perp}/\tau$, the ratios of the decay times of the polarized components to that of the total fluorescence, for *N*-methylacridinium chloride in 1,2-propanediol solutions. This means that, in the simpler solvent, 1,2-propanediol, the r_0 values remain constant irrespective of the solution viscosity and temperature, similarly as for stilbene 3 investigated by Bauer et al.[82] *N*-Methylacridinium chloride as well as 9-aminoacridine in glycerol-water solutions were studied by Bauer et al.[102] In order to find the depolarization rate, $6D = kT/V\eta$ and the limiting fluorescence anisotropy, r_0, were calculated from Equation 91 as a function of temperature and water content in the glycerol solution, using the measured values of $\mathrm{tg}\,\phi_{90^\circ}$, $\mathrm{tg}\,\phi_{0^\circ}$, τ, ω, and $\langle r\rangle$. Based on the measured 6D value and assuming the Stokes-Einstein model, the correlation time τ_c can be calculated from Equation 64. The correlation times were also studied by the nuclear magnetic resonance (NMR) method. The NMR correlation times are practically characteristic of the solvent molecules only. Because of the low concentrations of dye molecules in the excited state, no influence of the dye molecules on the relaxation times was observed. The comparison of the inverse rotational diffusion coefficient, 6D, and the NMR correlation times shows very similar changes with the water content and temperature of the solutions. The conclusion is drawn that thermal motions of the dye molecules in the excited state are very similar to those of the solvent molecules themselves.

D. Superposition of the Effects Produced by Torsional Vibrations and Brownian Rotations of Luminescent Molecules

The time dependence of the fluorescence emission anisotropy in isotropic solutions excited by a linearly polarized pulse of monochromatic light was discussed by Jabłoński,[25] with the assumption that the overall effect produced by the motion of LM is a superposition of those due to torsional vibrations and Brownian rotations. As a result of the motion of LM, i.e., the torsional vibrations and Brownian rotations, the direction of the electronic transition moment in the emission of a particular LM differs from that in the absorption process. If β is the angle between the two directions of the transition moments, then, according to the Perrin Equation 20, the fluorescence emission anisotropy of a group of LM, emitting fluorescence at the moment $t \geq 0$, following excitation of the solution at $t = 0$, is expressed by:

$$r(t) = \frac{3}{5}\left\langle \cos^2 \beta(t)\right\rangle - \frac{1}{5} \tag{97}$$

where $\langle\cos^2 \beta(t)\rangle$ is the mean value of $\cos^2 \beta(t)$ averaged over all LM emitting fluorescence at the moment t. The value of $\langle\cos^2 \beta(t)\rangle$ depends on the probability distribution of the transition moment orientations of the LM at the moment of the absorption process and on their orientations at the instant of emission.

Let γ_a and γ_e denote the angles of deviation of the transition moments from the equilibrium orientation of a particular LM during absorption and emission, respectively. These deviations occur in different planes, making an angle φ. The above angles obey the relation:

$$\begin{aligned}\cos\beta(t) \equiv \cos\gamma_{ae}(t) &= \cos\gamma_a \cos\gamma_e(t)\\ &+ \sin\gamma_a \sin\gamma_e(t)\cos\phi\end{aligned} \tag{98}$$

All angles φ in the interval $o \leq \varphi \leq \pi$ occur with equal probability ($\langle\cos\varphi\rangle = 0$, $\langle\cos^2\varphi\rangle = 1/2$). Upon squaring 98 and averaging over angle φ, the following expression is obtained:

$$\begin{aligned}\left\langle\cos^2\gamma_{ae}(t)\right\rangle &= \left(\frac{3}{2}\left\langle\cos^2\gamma_a\right\rangle - \frac{1}{2}\right)\\ &\times\left(\left\langle\cos^2\gamma_e(t)\right\rangle - \frac{1}{3}\right) + \frac{1}{3}\end{aligned} \tag{99}$$

where $\langle\cos^2 \gamma_e(t)\rangle$ depends on the time elapsing from the moment $t = 0$ of excitation of the solu-

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tion, which is not the case for $\langle\cos^2\gamma_a\rangle$. The case in which $\langle\cos^2\gamma_a\rangle$ and $\langle\cos^2\gamma_e(t)\rangle$ depend only on torsional vibrations of LM in a rigid isotropic solution was also considered by Jabłoński.[50] In this case, Equation 97, with Equation 99, yields:

$$r(t)=\frac{2}{5}\left(\frac{3}{2}\left\langle\cos^2\gamma_a\right\rangle-\frac{1}{2}\right)\left(\left\langle\cos^2\gamma_e(t)\right\rangle-\frac{1}{2}\right) \tag{100}$$

When $u_a = \langle\sin^2\gamma_a\rangle$ is the dispersion of $\sin\gamma_a$ at $t = 0$ and $u_e(t) = \langle\sin^2\gamma_e(t)\rangle$ is that of $\sin\gamma_e(t)$ at $t \geq 0$, Equation 100 gives:

$$r(t)=\frac{2}{5}\left(1-\frac{3}{2}u_a\right)\left(1-\frac{3}{2}u_e(t)\right) \tag{101}$$

the form of Equation 30′ for $u_a = u_e$.

With an approximate assumption that the dependence of $u_e(t)$ on t is governed by

$$\frac{du_e(t)}{dt}=-\frac{1}{\theta'}\left(u_e(t)-u_e(\infty)\right) \tag{102}$$

where θ' is the relaxation time of $u_e(t)$, for the initial condition $u_e(t) = u_e(0)$ at $t = 0$ one obtains

$$u_e(t)=u_e(\infty)+\left(u_e(0)-u_e(\infty)\right)\cdot e^{-t/\theta'} \tag{103}$$

In Equation 103, $u_e(0)$ and $u_e(\infty)$ denote the values of $u_e(t)$ at $t = 0$ (prerelaxation value) and at $t \gg \theta'$ (postrelaxation value), respectively. Equations 101 and 103 imply that

$$r(t)=r(\infty)-\left(r(\infty)-r(0)\right)\cdot e^{-t/\theta'} \tag{104}$$

In the case of continuous illumination of the solution with polarized excitation light, the emission anisotropy of fluorescence, $\langle r\rangle$, is given by

$$\langle r\rangle=\frac{1}{\tau}\int_0^\infty r(t)e^{-t/\tau}\,dt=r(\infty)-\frac{r(\infty)-r(0)}{1+\dfrac{\tau}{\theta'}} \tag{105}$$

where $\langle r\rangle$, $r(\infty)$, $r(0)$, and τ are measurable and, hence, the value of relaxation time, θ', can be obtained.

We shall now turn to the case of a liquid solution. In liquid solutions, the fluorescence emission anisotropy depends not only on torsional vibrations of LM, but also on their Brownian rotations. Instead of angle γ_e we have $\gamma_{e_1e_2}$ resulting from the superposition of the deviation caused by torsional vibrations, γ_{e_1}, and those due to Brownian rotations, γ_{e_2}. Jabłoński[25] assumed that γ_{e_1} and γ_{e_2} are independent of each other. This assumption leads to

$$\left\langle\cos^2\gamma_{e_1e_2}(t)\right\rangle=\left(\frac{3}{2}\left\langle\cos^2\gamma_{e_1}(t)\right\rangle-\frac{1}{2}\right)\times\left(\left\langle\cos^2\gamma_{e_2}(t)\right\rangle-\frac{1}{3}\right)+\frac{1}{3} \tag{106}$$

The substitution of $\langle\cos^2\gamma_{e_1e_2}(t)\rangle$ for $\langle\cos^2\gamma_e(t)\rangle$ in Equation 99 results in:

$$\left(\frac{3}{2}\left\langle\cos^2\gamma_{a,e_1e_2}(t)\right\rangle-\frac{1}{2}\right)\left(\frac{3}{2}\left\langle\cos^2\gamma_{e_1}(t)\right\rangle-\frac{1}{2}\right)\times\left(\left\langle\cos^2\gamma_{e_2}(t)\right\rangle-\frac{1}{3}\right)+\frac{1}{3} \tag{107}$$

The values of $\langle\cos^2\gamma_{e_1}\rangle = 1 - \langle\sin^2\gamma_{e_1}\rangle$ dependent on torsional vibrations of LM are given by Equation 103. Next we shall calculate $\cos^2\gamma_{e_2}(t)$ for the Brownian rotations of LM. It is assumed that

$$\frac{d\left\langle\cos^2\gamma_{e_2}(t)\right\rangle}{dt}=-\varphi\cdot\left(\left\langle\cos^2\gamma_{e_2}(t)\right\rangle-\frac{1}{3}\right) \tag{108}$$

where $^1/_3$ is the limiting value of $\cos^2\gamma_{e_2}(t)$ for $t = \infty$ and $\phi = 1/\tau_c = 6D$ (see Equation 64) is in general a function of t. The solution, fulfilling the initial condition that at the moment of excitation $t = 0$ and $\langle\cos^2\gamma_{e_2}(t)\rangle = 1$ (no depolarization by Brownian rotations of LM occurs), is

$$\left\langle\cos^2\gamma_{e_2}(t)\right\rangle=\frac{2}{3}\cdot e^{-\int_0^t\varphi(t)\,dt}+\frac{1}{3} \tag{109}$$

(For $\varphi = 6D = \text{const.}$, see Equation 48).

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From Equations 103, 107, and 109 for the superposition of the effects produced by torsional vibrations and Brownian rotations of LM, we obtain

$$\left\langle \cos^2 \gamma_{a,e_1e_2}(t) \right\rangle \equiv \left\langle \cos^2 \beta(t) \right\rangle = \frac{1}{3} + \left(\left\langle \cos^2 \Gamma(t) \right\rangle - \frac{1}{3} \right) \cdot e^{-\int_0^t \varphi(t)\,dt} \tag{110}$$

where

$$\left\langle \cos^2 \Gamma(t) \right\rangle = \left\langle \cos^2 \Gamma(\infty) \right\rangle + \left(\left\langle \cos^2 \Gamma(0) \right\rangle - \left\langle \cos^2 \Gamma(\infty) \right\rangle \right) \cdot e^{-t/\theta'}$$

in accordance with Equation 103.

The substitution of Equation 110 into Equation 97 leads to

$$r(t) = \left(\frac{3}{5} \left\langle \cos^2 \Gamma(t) \right\rangle - \frac{1}{5} \right) \cdot e^{-\int_0^t \varphi(t)\,dt} \tag{111}$$

$\langle \cos^2 \Gamma(0) \rangle$, $\langle \cos^2 \Gamma(\infty) \rangle$, and $\varphi(t)$ depend on the interaction of LM with the solvent molecules. This interaction depends on the vibronic state of the LM and, thus, on the frequency of the exciting light. The integral in Equation 111 can be replaced with $\varphi_0 \cdot t + \Delta(\nu)\theta\,(1-e^{-t/\theta})$, because of $\varphi(t) = \varphi_0 + \Delta(\nu)e^{-t/\theta}$, where $\Delta(\nu)$ depends on the frequency of the exciting light, and θ is the relaxation time of $\varphi(t)$. Instead of Equation 111, we have

$$r(t) = \left(\frac{3}{5} \left\langle \cos^2 \Gamma(t) \right\rangle - \frac{1}{5} \right) \cdot e^{-\varphi_0 t - \Delta(\nu)\theta\left(1-e^{-t/\theta}\right)} \tag{112}$$

In the case of excitation of the solution with $\nu_{0\text{–}0}$, $\langle \cos^2 \Gamma(0) \rangle = \langle \cos^2 \Gamma(\infty) \rangle$ and $\Delta(\nu) = 0$ and Equation 112 reduces to

$$r(t) = \left(\frac{3}{5} \left\langle \cos^2 \Gamma(\infty) \right\rangle - \frac{1}{5} \right) \cdot e^{-\varphi_0 t} \tag{113}$$

and for t = 0 one has

$$r(0) = \frac{3}{5} \left\langle \cos^2 \Gamma(\infty) \right\rangle - \frac{1}{5} \tag{114}$$

Equation 113 leads to the known Perrin Equation 55 for the time-dependent emission anisotropy of fluorescence.

E. Effect of Rotational Relaxation on the Anisotropy Caused by Sequential Two-Photon Excitation

It is interesting to note that when an ensemble of LM becomes doubly polarization-photoselected, the fundamental emission anisotropy, r_f, could fall outside the usual limits of $-1/5$ and $+2/5$. The extreme limits for r_f under these conditions are extended to the range from $-2/7$ to $4/7$.[103,104] Lin and Topp[104] noted that the observed polarization of the ultraviolet fluorescence of rhodamine B and rhodamine 6G caused by the sequential two-photon excitation in a liquid solution should depend, apart from the molecular rotation in the emitting state, on that associated with the first absorption event.

General formulae for the anisotropy of the S_m-fluorescence emission ($m \geq 2$) of LM excited by the sequential two-photon absorption process ($S_n \leftarrow S_1 \leftarrow S_0$, $n \geq 2$) were derived by Sato et al.[105] The S_m and S_n states may be the same or different, although the symmetry selection rules make the case $m \neq n$ the more probable one.

The sequential two-photon absorption is assumed to occur within a single exciting pulse. The vectors **p**, **q**, and **r** stand for the transition moments of $S_1 \leftarrow S_0$, $S_n \leftarrow S_1$, and $S_m \rightarrow S_0$ processes, respectively, of an arbitrarily chosen LM at the time of each absorption or emission event. The respective angles between the vectors **p** and **q**, and **q** and **r** are β_1 and β_2. The first photon ($S_1 \leftarrow S_0$) interacts randomly with LM in the S_0 state, whereas the second photon ($S_n \leftarrow S_0$) interacts with the collection of partially oriented S_1 LM when the rotational relaxation in the S_1 state is not completed before the absorption of the second photon.

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The most general case is when partial rotational relaxation occurs in the intermediate state (S_1) prior to the absorption of the second photon. In this case, β_1 includes the angle spanned by $S_1 \leftarrow S_0$ and $S_n \leftarrow S_1$ transition moments on the molecular frame, which is denoted by β_1^0, and the angle caused by the rotational relaxation, denoted by β_1'. β_1 is given by these two angles as in the case of Equation 13:

$$\cos\beta_1 = \cos\beta_1^0 \cos\beta_1' + \sin\beta_1^0 \sin\beta_1' \cos\vartheta \quad (115)$$

The angle ϑ is the one between the two planes (**E**,**P**) and (**E**,**q**) (see Figure 2, where **E** is the electric vector).

When $\beta_1' = 0$, i.e., with no rotational relaxation, Equation 115 implies $\beta_1 = \beta_1^0$. The equation for the general case is

$$r = \frac{1}{14} \frac{\left(11\langle\cos^2\beta_1\rangle + 1\right)\left(3\langle\cos^2\beta_2\rangle - 1\right)}{2\langle\cos^2\beta_1\rangle + 1} \quad (116)$$

If we put $\langle\cos^2\beta_1\rangle = 1/3$, Formula 116 reduces to Equation 20 ($\beta_2 = \beta$). For $\langle\cos^2\beta_1\rangle = 1$, i.e., no rotational relaxation occurs at all in the intermediate state (S_1) before the absorption of the second photon, Equation 116 gives

$$r = \frac{2}{7}\left(3\langle\cos^2\beta_2\rangle - 1\right) \quad (117)$$

According to Equation 117, the values of the fundamental emission anisotropy cover an interval of

$$-\frac{2}{7} \le r \le \frac{4}{7}$$

which is in agreement with the theory of the modified photoselection.[103] Sato et al.[105] also derived appropriate formulas for the time-dependent emission anisotropy for a collection of LM which absorb the second photon following an incomplete rotational relaxation in the S_1 state.

V. LOCAL TEMPERATURE OF LUMINESCENT CENTERS, THEIR RELAXATION AND EFFECT ON FLUORESCENCE ANISOTROPY

Equations 55 and 56 hold true under the assumption that the energy of thermal motions of the LM remains in equilibrium with the surrounding medium. The excitation of the luminescent centers (LC) with light of frequency ν different from $\nu_{0\text{-}0}$ of purely electronic transition results in a temporary loss of thermal equilibrium between the LM and their surroundings. Such "initial shock" causes an increase in the energy of nuclear motions in the LM, and, hence, the growth of the local temperature of the LC. In liquid solutions, excess thermal energy propagates very rapidly over the whole medium, which has been evidenced by the investigations of the intensity distribution in the fluorescence band[106–113] and of the Stepanov relation.[114,115]

However, theoretical considerations of Jabłoński[110] imply that the excess excitation energy affects the emission anisotropy via torsional vibrations dependent on the energy of thermal motions and the Brownian rotations of molecules. In complex polyatomic molecules, characteristic of which are continuous absorption and fluorescence spectra, there exists a very high probability of the excitation energy transfer between different vibrational degrees of freedom owing to marked anharmonicity of the potential energy. If, as a result of excitation ($\nu > \nu_{0\text{-}0}$), a molecule acquires excess vibrational energy $\Delta w = h\nu - h\nu_{0\text{-}0}$, then after a very short time (of the order of the vibration period, 10^{-13} s), vibrational energy distribution is established inside the molecule, corresponding to statistical equilibrium and characterized by the so-called vibrational temperature T^{vib}. As known, strong interactions between the LM and the solvent molecules of the nearest surroundings exist in solutions. In such a case we are dealing with "local temperature" of the LC.

Bauer et al.[20] assumed that the local temperature decreases exponentially in time (according to the Newton equation) due to the interaction with the environment:

$$\Delta T(t) = \Delta T(0) \cdot e^{-t/\theta_\ell} \quad (118)$$

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where $\Delta T = T^* - T$ is the difference between the local temperature and that of the medium surrounding the LM, t is the time from the instant of excitation, $\Delta T(0)$ is the above difference at the instant of excitation for $t = 0$, and θ_ℓ is the relaxation time of the local temperature of LC.

Since for one kind of LC the intensity distribution in the fluorescence band is independent of the excitation light frequency (which proves the establishment of thermal equilibrium between the LC and the surroundings prior to emission), it should be anticipated that the relaxation times, θ_ℓ, of the local temperature of LC will be substantially shorter than mean lifetime τ. The change in the local temperature is assumed to affect the depolarization factors (torsional vibrations and the Brownian rotations of molecules) similarly as does the change in the temperature of the solution as a whole. The excitation of the LC with light of frequency, ν, higher than that of purely electronic transition, $\nu_{0\text{-}0}$, results in the increase in the local temperature by ΔT, which in turn influences r_0 and $\varphi = 6D$ (Equation 43). Further, in the first approximation, the linear dependence of r_0 upon the temperature change is assumed:[20]

$$r_0\left(T_0 + \Delta T\right) = r_0\left(T_0\right) - a\,\Delta T \tag{119}$$

where $r_0(T_0)$ denotes the limiting value of the fluorescence anisotropy at temperature T_0, $r_0(T_0 + \Delta T)$ at $T_0 + \Delta T$, and

$$a = \frac{\Delta r_0}{\Delta T} = \frac{1 \cdot 2u - 1.8u^2}{T_0} \tag{120}$$

u can be calculated from Equation 30′, knowing r_o for T_0. u is usually very small since r_0 does not markedly differ from $r_p = 2/5$. We can therefore assume:

$$a \approx \frac{1,2u}{T_0}, \quad \text{and since } r_0 = 0.4 - 1.2u$$

we obtain

$$a = \frac{0.4 - r_0}{T_0} \tag{121}$$

According to the assumption accepted by Jabłoński[97] and Heldt,[58] φ is the following function of temperature:

$$\varphi(T) = b \cdot e^{-W/T} \tag{122}$$

where b is a constant value for the investigated solution, $W \cdot k$ is the activation energy for the Brownian rotation (k is the Boltzmann constant). For excitation with frequencies $\nu > \nu_{0\text{-}0}$, the increase in the local temperature and the drop in r_0 occur, according to Equation 119, and the factor of proportionality, a, is two times smaller since the local temperature only affects the emission oscillator and not the absorption one. Thus, for $\nu > \nu_{0\text{-}0}$, Equation 119 can be written as

$$r_0\left(T_0 + \Delta T\right) = r_0(T) - \frac{a}{2}\Delta T \tag{123}$$

where ΔT is the change in the local temperature of the LC. This increase in the local temperature by ΔT causes the increase in the probability, φ, of the depolarization by Brownian motion.

By expanding Function 122 in a Taylor series at point T_0 and including terms of the second order, we obtain:[21]

$$\varphi(T_0 + \Delta T) \approx \varphi(T_0) + b_1\,\Delta T + b_2\,\Delta T^2 \tag{124}$$

where

$$b_1 = \left.\frac{d\varphi}{dT}\right|_{T=T_0} = \varphi(T_0)\frac{W}{T_0^2} \tag{125}$$

$$b_2 = \left.\frac{1}{2}\frac{d^2\varphi}{dT^2}\right|_{T=T_0} = \frac{1}{2}\varphi(T_0)\frac{W}{T_0^3}\left(\frac{W}{T_0} - 2\right) \tag{126}$$

Since, according to Equation 118, the change in local temperature, ΔT, is a function of time, r_0 and φ are also functions of time. Therefore,

$$r_0(t) \approx r_0 - \frac{1}{2} \cdot a \cdot \Delta T(0) \cdot e^{-t/\theta_\ell} \tag{127}$$

$$\varphi(t) \approx \varphi(T_0) + b_1\, \Delta T(0) e^{-t/\theta_\ell} + b_2\, \Delta T^2(0) e^{-2t/\theta_\ell} \tag{128}$$

In general considerations, if r_0 and φ are functions of time, the emission anisotropy after pulse excitation changes in time in the following way:[21]

$$r(t) = r_0(t) e^{-\int_0^t \varphi(t)\,dt} \tag{129}$$

Substituting Equations 127 and 128 into Equation 129 and denoting the fluorescence anisotropies at excitation with frequency ν different from that of purely electronic transition, $\nu_{0\text{-}0}$, by r*, with the assumption that

$$b_1 \cdot \Delta T(0) \cdot \theta_\ell \ll 1, \quad b_2\, \Delta T^2(0) \frac{\theta_\ell}{2} \ll 1$$

and

$$\frac{1}{2} a \cdot \Delta T(0) \cdot b_1 \cdot \Delta T(0) \theta_\ell \ll r_0 \cdot b_1 \cdot \Delta T(0) \cdot \theta_\ell$$
$$\frac{1}{2} a \cdot \Delta T(0) \cdot b_2 \cdot \Delta T^2(0) \theta_\ell \ll r_0 \cdot b_2 \cdot \Delta T^2(0) \cdot \theta_\ell$$

we obtain the following expression for the fluorescence anisotropy decay in time:[21]

$$\begin{aligned} r^*(t) \approx{} & r_0(T_0) e^{-\varphi(T_0)t} - \frac{a}{2} \Delta T(0) e^{-[\varphi(T_0)+1/\theta_\ell]t} \\ & - r_0(T_0) \cdot b_1 \cdot \Delta T(0) \theta_\ell e^{-\varphi(T_0)t} \left(1 - e^{-t/\theta_\ell}\right) \\ & - r_0(T_0) \cdot b_2 \cdot \Delta T^2(0) \frac{\theta_\ell}{2} e^{-\varphi(T_0)t} \left(1 - e^{-2t/\theta_\ell}\right) \end{aligned} \tag{130}$$

The mean value of fluorescence emission anisotropy can be calculated based on Equation 130 in the following way:

$$\begin{aligned} \langle r^* \rangle &= \frac{1}{\tau} \int_0^\infty r^*(t) e^{-t/\tau}\, dt \\ &= \langle r \rangle \left[1 - \frac{a}{2\langle r \rangle} \frac{\Delta T(0)}{\frac{r_0}{\langle r \rangle} + \frac{\tau}{\theta_\ell}} - \frac{b_1 \tau\, \Delta T(0)}{\frac{r_0}{\langle r \rangle} + \frac{\tau}{\theta_\ell}} - \frac{b_2 \tau\, \Delta T^2(0)}{\frac{r_0}{\langle r \rangle} + 2\frac{\tau}{\theta_\ell}} \right] \end{aligned} \tag{131}$$

where

$$\langle r \rangle \equiv \langle r(T_0) \rangle = \frac{r_0(T_0)}{1 + \varphi(T_0)\tau}$$

expresses Perrin's Equation which is valid for continuous excitation of a solution with temperature T_0, by light frequency of $\nu_{0\text{-}0}$, and $r_0(T_0) \equiv r_0$. Denoting h/c* by σ (where c* is the thermal capacity of the LC), the change in the local temperature at the instant of excitation ($t = 0$) can be expressed as a function of frequency difference $\Delta\nu = \nu - \nu_{0\text{-}0}$

$$\Delta T(0) = \sigma\, \Delta\nu \tag{132}$$

and Equation 131 can be written as

$$\langle r^* \rangle(\Delta\nu) = \langle r \rangle - q_1\, \Delta\nu - q_2 \Delta\nu^2 \tag{133}$$

where

$$q_1 = \frac{\left(\langle r \rangle b_1 \tau + \frac{a}{2}\right)\sigma}{\frac{r_0}{\langle r \rangle} + \frac{\tau}{\theta_\ell}} \tag{134}$$

$$q_2 = \frac{\langle r \rangle b_2 \tau \sigma^2}{\frac{r_0}{\langle r \rangle} + \frac{2\tau}{\theta_\ell}} \tag{135}$$

Thus, ⟨r*⟩ is described by the parabola of the second order with respect to $\Delta\nu$. By measuring the emission anisotropy ⟨r*⟩ as a function of the excitation light frequency, ν, and drawing the

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second-order parabola through the experimental points by the least square method we find ⟨r⟩, q_1, and q_2. In order to determine θ_ℓ and σ, the knowledge of the values occurring in Equations 134 and 135 is indispensable. The variable a can be found from either Equation 119 or Equation 121, b_1 and b_2 from Equations 122, 125, and 126, and φ from the Perrin Equation for several temperatures.

Figures 9 and 10 show the results of measurements carried out by Grudziński,[21] concerning the dependence of the fluorescence anisotropy ⟨r*⟩ upon the difference between the excitation light frequency, ν, and the frequency of pure electronic transition, $\nu_{0\text{-}0}$, for fluorescein and acriflavine in glycerol-water mixture (9:1). The experimental points represent the values of ⟨r*⟩ measured for a given frequency of the excitation light, and the solid line is the parabola of the second order (according to Equation 133), plotted through the experimental points by the least squares method. The figures also show the absorption and fluorescence spectra for the same solutions. The dependence of $\varphi = kT/V\eta$ on temperature for the two fluorescence molecules investigated is pre-

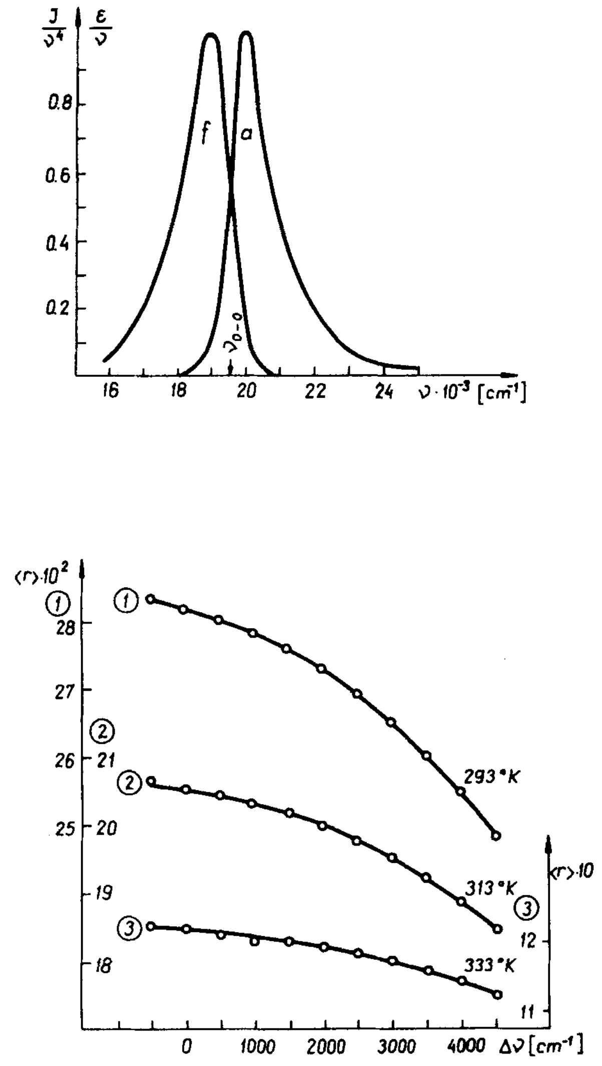

FIGURE 9. Absorption spectrum (a), fluorescence spectrum (f), and dependence of emission anisotropy ⟨r⟩, on $\Delta\nu = \nu_{exc} - \nu_{0\text{-}0}$ for fluorescein in 90% glycerol + 10% water. (From Grudziński, H. *Acta Phys. Pol. A* **1970,** *37,* 49. With permission.)

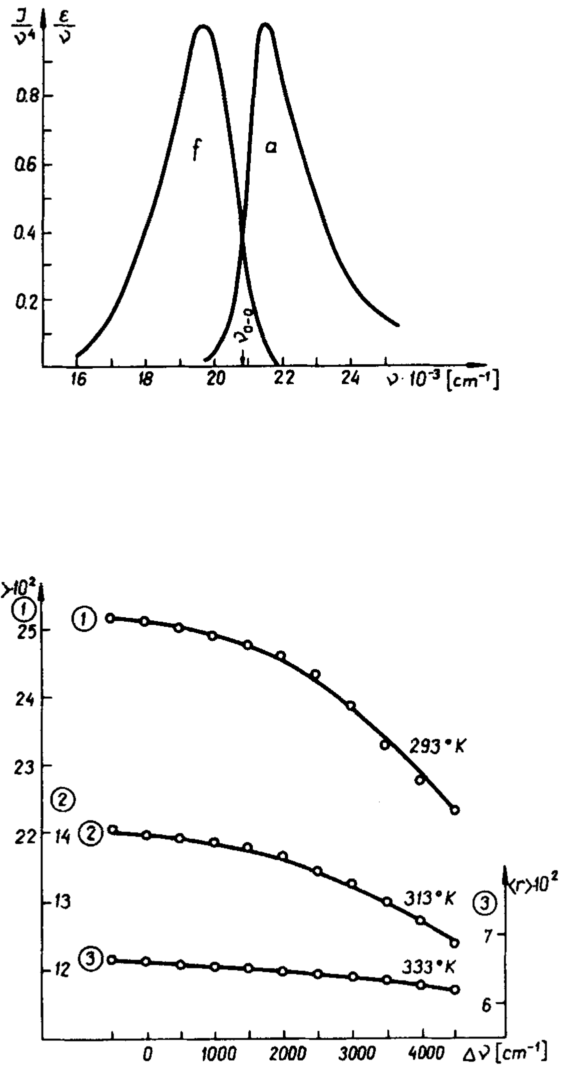

FIGURE 10. As in Figure 9, but for acriflavine in 90% glycerol + 10% water. (From Grudziński, H. *Acta Phys. Pol. A* **1970,** *37,* 49. With permission.)

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sented in Figure 11. The points denote the values of φ_{exp} calculated from experimental data, whereas the solid curve is the interpolation of $\varphi_{int}(T) = b \exp(-W/kT)$ fitted to the points by the least squares analysis. The activation energies of the Brownian rotations for fluorescein and acriflavine amount to 0.399 and 0.466 eV, respectively. It was found that the activation energy decreases, for a given kind of molecule, with the decrease of solvent viscosity. The activation energy of Brownian rotations decreases faster with viscosity for acriflavine than for fluorescein. It was also shown that, in a solvent with a similar glycerol and water content at the same temperature T (the same macrosopic viscosity), φ for acriflavine is always larger than for fluorescein. This indicates that the volume of acriflavine together with its solvation shell is smaller than for fluorescein. Table 2 summarizes the values of θ_ℓ and σ averaged over three temperatures: 293, 313, and 333 K (see Figures 9 and 10).

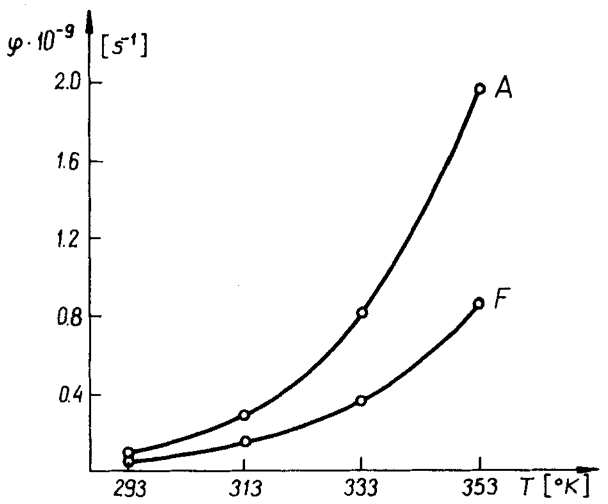

FIGURE 11. Temperature, T, dependence of the probability of depolarization by Brownian rotations per unit time, $\varphi = 1/\tau_c$ for fluorescein (F) and acriflavine (A) in 90% glycerol + 10% water. (From Grudziński, H. *Acta Phys. Pol. A* **1970,** *37,* 49. With permission.)

TABLE 2

Compound	$\langle\theta\rangle$	$\langle\sigma\rangle$
Fluorescein	1.65×10^{-11} s	5.0×10^{-2} deg cm^{-1}
Acriflavine	3.0×10^{-12} s	1.0×10^{-1} deg cm^{-1}

The determined relaxation times of the local temperature, θ_ℓ, for the molecules investigated by Grudziński, are much shorter than those obtained from Neporent's modified relation and Stepanov's relation.[116,117] The relaxation time of the local temperature for fluorescein is longer than that for acriflavine, which is in accordance with the considerations of de Groot.[118]

The measurements of the emission anisotropy of fluorescence of polar dyes in mixed solvents (with water) are doubtful, since such solutions contain LC of different kinds. It would be purposeful, therefore, to carry out similar measurements in unary, and not in mixed solvents.

When investigating the dissipation of the vibrational energy excess of excited LM in solution, the interfering factors should first of all be taken into account. The disregard of such factors as, for example, the effect of mixture of states, the overlap of absorption bands, and the inhomogeneous broadening of absorption spectra, has led in many cases to quite different conclusions.

The authors of References 20, 21, 119, and 120 assume a very fast relaxation of the vibrational energy excess and a sufficiently slow dissipation of this energy so that "local heating" of the LC during the lifetime of the LM in the excited state may occur. The influence of excess excitation energy on rotational relaxation of LM in solution was investigated by Pantke and Labhart.[121–123] They showed that the excess energy fed into the LM by excitation at short wavelengths is dissipated in such a short time that neither enhanced rotational diffusion nor a change in the "local temperature" can be observed.

In the work of Bauer and Balter,[124] in which phase-fluorometer and steady-state emission anisotropy were considered, no measurable influence of the vibrational energy excess on the Brownian rotations of LM with their solvation shells was detected. However, some evidence of possible coupling between vibrational modes and torsional vibrations (librations) of LM was found.[51,125] The dependence of the emission anisotropy decay of fluorescence of rhodamine 6G, fluorescein, and eosin dissolved in ethylene glycol and of rhodamine 6G in undecanol, vs. excitation wavelength was measured by Bauer and

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Balter[126–128] in order to study the relaxation of the vibrational energy excess. In Table 3, the results of pulse measurements of the dye fluorescence investigated in ethylene glycol as a function of temperature and excitation wavelength, λ_{exc}, are presented. The earlier phase fluorometric results[124] and the pulse fluorometric results[128] imply that, irrespective of the method employed, the values of rotational correlation times, τ_c (Equation 64), are independent of λ_{exc} within the limits of experimental error. The results obtained by Bauer and Balter[128] for rhodamine 6G in undecanol at 293 K as a function of λ_{exc} are presented in Table 4. It can be seen that the rotational correlation time, τ_c, is independent of λ_{exc}, contrary to the results of Heiss et al.[119] and Scholz et al.[120] It also brings into question the validity of the assumptions introduced by Bauer et al.[20] for these dyes. In other words, the rate of thermal rotational depolarization was found to be independent of vibrational energy excess. The observed dependence of the mean value of emission anisotropy $\langle r \rangle$ on λ_{exc} in Reference 124 leads to the conclusion that, besides the principal emission anisotropy, r_p, the torsional vibrations of the dye molecules also depend on the vibrational energy excess.[128] From Table 3 we can conclude that the rotational correlation time, τ_c, decreases with increase in temperature, i.e., with decrease in the solvent viscosity.

The effect of the local temperature on the fluorescence emission anisotropy with the mediation of the Brownian rotations might also be sought for in the case of LM for which the mean lifetime, τ, is comparable to the local temperature relaxation time, θ_ℓ (compare Tables 1 and 2). As yet, only LM with lifetimes of the order of several nanoseconds have been investigated. The inhomogeneous broadening of the electronic band is an important factor which, in the case of polar solutions of complex LM, play an essential role.[129,130] More detailed information on the investigations of inhomogeneous broadening in polar dye solutions by the use of high time-resolved spectroscopy can be found in a review article by Nemkovich et al.[131] The rotation rate of the LM has been found to depend on the configurational energy of the solute. This accounts for the change in the rate of the fluorescence depolarization within the wavelength interval excitation.[131]

VI. ROTATIONAL FLUORESCENCE DEPOLARIZATION OF ASYMMETRIC MOLECULES

A. Theory

In Section IV, we considered the most commonly used hydrodynamic Perrin's model in which

TABLE 3
Values of r_0, τ_c, and τ for Rhodamine 6G, Eosin, and Fluorescein in Ethylene Glycol at Different Temperatures and Two Different Exciting Wavelengths

		Rhodamine 6G			Eosin			Fluorescein		
λ_{exc} (nm)	T (K)	r_0	τ_c (nsec)	τ (nsec)	r_0	τ_c (nsec)	τ (nsec)	r_0	τ_c (nsec)	τ (nsec)
458	250	0.38	40.0	4.2	0.38	25.4	3.1	0.39	29.0	4.7
	273	0.39	15.2	4.2	0.39	9.9	3.0	0.38	8.6	4.4
	293	0.38	4.6	4.2	0.37	3.9	2.9	0.36	3.7	4.4
	313	0.35	2.4	4.1	0.36	2.0	2.8	0.33	2.0	4.3
515	250	0.41	41.7	4.3	0.40	26.2	3.1	0.37	26.8	4.4
	273	0.38	14.9	4.2	0.38	9.8	2.9	0.40	9.0	4.2
	293	0.36	4.4	4.2	0.36	4.1	2.9	0.37	3.3	4.3
	313	0.34	2.2	4.2	0.35	1.9	2.8	0.33	2.1	4.3

Data from References 126 and 128.

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TABLE 4
Values of r_0, τ_c, and τ for Rhodamine 6G in Undecanol at 293 K

	Phase method			Pulse method		
λ_{exc} (nm)	r_0	τ_c (nsec)	τ (nsec)	r_0	τ_c (nsec)	τ (nsec)
452	0.32	6.6	4.4			
458				0.32	5.9	4.5
477				0.36	5.5	4.5
497				0.36	5.5	4.6
501	0.35	6.4	4.4			
515				0.38	5.8	4.5
542	0.36	6.6	4.5			

Data from References 124 and 126.

the rotating LM is treated as a macroscopic solid body carrying in its motion the solvent molecules immediately adjacent to its surface (sticking boundary condition). The rotational diffusion of a spherical LM is described by the so-called diffusion tensor D, that depends on the solvent temperature and viscosity (Equation 43). This tensor has, generally, the character of an ellipsoid, i.e., it has three components D_1, D_2, and D_3, corresponding to the rotations of a molecule about three mutually perpendicular axes of the molecular coordination system, directed along the principal tensor axes.

The first attempts to generalize Perrin's theory[41,77,78] were undertaken by Perrin[132]* and Memming[133] to cover the case of an ellipsoidal molecule.

Later, Lombardi and Dafforn,[135] Tao,[136] Weber,[137] Chuang and Eisenthal,[138] Ehrenberg and Rigler,[139] and Belford et al.[140] considered the effect of thermal rotations of fully asymmetric LM on the fluorescence anisotropy. In 1972, three general theories were independently formulated.[138-140] A model very often used for optical experiments is that of Chuang and Eisenthal[138] and Belford et al.[140] A discussion on the general expression of Belford et al.[140] for the fluorescence decay r(t), given in a review by Fleming,[22] has shown that the complete expression contains corrected versions of the equations given by Lombardi and Dafforn[135] and Tao[136] as different special subcases. The Belford et al.[140] equation for r(t) was derived by a procedure presented by Lombardi and Dafforn,[135] who used Favro's theory[141] in their calculation. Tao's[136] derivation for r(t) is only correct if the absorption and emission transition directions in the body coincide.

In the following discussion we will consider the time-dependent and steady-state fluorescence emission anisotropy on the basis of the Chuang and Eisenthal[138] theory. Chuang and Eisenthal[138] found that after the excitation with a polarized δ-light pulse, up to six different time constants are required to describe the behavior of parallel and perpendicular components of the fluorescence:

$$\begin{aligned} I_{\parallel}(t) = P(t)\Big\{ & \frac{1}{9} + \frac{4}{15} q_1 q_2 \gamma_1 \gamma_2 \exp\left[-\left(3D_3 + D\right)\cdot t\right] \\ & + \frac{4}{15} q_2 q_3 \gamma_2 \gamma_3 \exp\left[-3\left(D_1 + D\right)\cdot t\right] \\ & + \frac{4}{15} q_3 q_1 \gamma_3 \gamma_1 \exp\left[-3\left(D_2 + D\right)\cdot t\right] \\ & + \frac{1}{15}(B + A)\exp[-(6D + 2\Delta)\cdot t] \\ & + \frac{1}{15}(B - A)\exp[-(6D - 2\Delta)\cdot t]\Big\} \end{aligned} \qquad (136)$$

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* An error affecting the calculations of the rotational relaxation times for ellipsoids of revolution has been corrected by Koenig.[134]

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and

$$I_{\perp}(t)=\frac{1}{6}P(t)-\frac{1}{2}I_{\parallel}(t) \qquad (137)$$

where P(t) is the probability that an initially excited LM remains excited at time t. If the LM has a single exponential decay with a lifetime τ then

$$P(t)=\exp\left(-\frac{t}{\tau}\right) \qquad (138)$$

The parameters are*

$$B=q_1^2\gamma_1^2+q_2^2\gamma_2^2+q_3^2\gamma_3^2-\frac{1}{3} \qquad (139)$$

$$\begin{aligned}A\cdot\Delta = {} & D_1\left(q_2^2\gamma_2^2+q_3^2\gamma_3^2-2q_1^2\gamma_1^2+\gamma_1^2+q_1^2\right)\\ & +D_2\left(q_3^2\gamma_3^2+q_1^2\gamma_1^2-2q_2^2\gamma_2^2+\gamma_2^2+q_2^2\right)\\ & +D_3\left(q_1^2\gamma_1^2+q_2^2\gamma_2^2-2q_3^2\gamma_3^2+\gamma_3^2+q_3^2\right)-2D\end{aligned} \qquad (140)$$

$$\Delta=\left(D_1^2+D_2^2+D_3^2-D_1D_2-D_2D_3-D_3D_1\right)^{1/2} \qquad (141)$$

$$D=\frac{1}{3}\left(D_1+D_2+D_3\right) \qquad (142)$$

γ_1, γ_2, and γ_3 are the projections of the unit vector of absorption dipole μ_{abs} along the molecular fixed axes, and q_1, q_2, and q_3 are the corresponding components of the emission dipole μ_{em}. These components have the property such that $\sum \gamma^2_i = 1 = \sum_i q^2_i$. The constant D is the average of the three principal diffusion constants and Δ is related to the anisotropy of the diffusion. 1, 2, and 3 are the principal axes for rotational diffusion.

The fluorescence emission anisotropy, defined in Equation 4, can be obtained from Equations 136, 137, and 138

$$\begin{aligned}r(t)=0.3\Big\{ & 4q_1q_2\gamma_1\gamma_2\exp\left[-3\left(D_3+D\right)t\right]\\ & +4q_2q_3\gamma_2\gamma_3\exp\left[-3\left(D_1+D\right)t\right]\\ & +4q_3q_1\gamma_3\gamma_1\exp\left[-3\left(D_2+D\right)t\right]\\ & +(B+A)\exp\left[-(6D+2\Delta)t\right]\\ & +(B-A)\exp\left[-(6D-2\Delta)t\right]\Big\}\end{aligned} \qquad (143)$$

That the fluorescence anisotropy decays as the sum of five exponentials follows also from the theories of Belford et al.[140] and Ehrenberg and Rigler.[139]

By assuming certain symmetry properties for the LM, Equation 143 can be simplified.

1. For a spherical rotor, for which $D_1 = D_2 = D$, Equation 143 reduces to Perrin Equation 55. If the absorption and emission dipole moments make an angle β then

$$r(t)=\frac{2}{5}P_2(\cos\beta)\exp(-6Dt) \qquad (144)$$

where $P_2(\cos\beta) = {}^3\!/_2 \cos^2\beta - {}^1\!/_2$ is the second Legendre polynomial. Thus, in this case the motion of LM is isotropic with a rotation correlation time $\tau_c = 1/6D$.

2. For a symmetrical ellipsoid of revolution (symmetric rotor) the LM possesses an axis of symmetry such that $D_1 = D_{\parallel}$ and $D_2 = D_3 = D_{\perp}$. In this case, Equation 143, similar to that of Belford et al.[140] and Ehrenberg and Rigler,[139] reduces to the sum of three exponentials:

$$\begin{aligned}r(t)=\frac{2}{5}\Big\{ & A_1\exp\left[-6D_{\perp}t\right]\\ & +A_2\exp\left[-\left(5D_{\perp}+D_{\parallel}\right)t\right]\\ & +A_3\exp\left[-\left(2D_{\perp}+4D_{\parallel}\right)t\right]\Big\}\end{aligned} \qquad (145)$$

---

* In the Belford et al.[140] expression a product of G · D occurred, which is equal to –A · Δ.

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where $D_{\parallel}$ and $D_{\perp}$ are the rotary diffusion coefficients for the rotation about the axis of symmetry and about either equatorial axis, respectively, and[139]

$$A_1 = \left(\frac{3}{2}\cos^2 \vartheta_1 - \frac{1}{2}\right)\left(\frac{3}{2}\cos^2 \vartheta_2 - \frac{1}{2}\right) \tag{146}$$

$$A_2 = \frac{3}{4}\sin 2\vartheta_1 \sin 2\vartheta_2 \cos \phi \tag{147}$$

$$A_3 = \frac{3}{4}\sin^2 \vartheta_1 \sin^2 \vartheta_2 \left(\cos^2 \phi - \sin^2 \phi\right) \tag{148}$$

where ϑ_1 and ϑ_2 are the angles formed by the absorption and emission transition moments, respectively, with the axis of symmetry of the ellipsoid. ϕ is the angle formed by the projections of the two moments in the plane perpendicular to the axis of symmetry.

When the transition moments of absorption and emission coincide, then $\vartheta_1 = \vartheta_2 = \vartheta$ and $\phi = 0$, and Equations 146 to 148 give the Tao[136] coefficients:

$$A_1 = \left(\frac{3}{2}\cos^2 \vartheta - \frac{1}{2}\right)^2 \tag{149}$$

$$A_2 = 3\cos^2 \vartheta \sin^2 \vartheta \tag{150}$$

$$A_1 = \frac{3}{4}\sin^4 \vartheta \tag{151}$$

The values of these coefficients have been plotted against the orientation angle, ϑ, in Figure 12.

When the transition dipole moment is parallel to the symmetry axis ($\vartheta = 0°$), from Equations 145 and 149 to 151 follows

$$r(t) = \frac{5}{2}\exp\left(-6D_{\perp}t\right) \tag{152}$$

This is the same form as Equation 144 but the diffusion constant is different. For the case when the transition dipole moment is perpendicular to the symmetry axis ($\vartheta = 90°$), r(t) has a double exponential form:

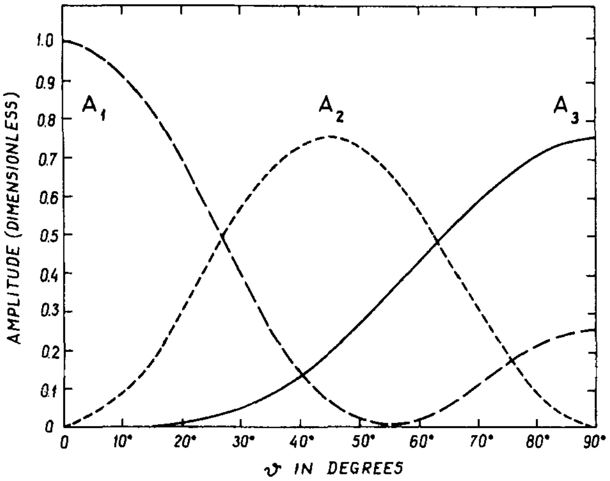

FIGURE 12. Plot of the three amplitudes appearing in Equation 145. ϑ is the angle between the transition dipole moment and the symmetry axis of the LM. The curves A_1, A_2, and A_3 were calculated from Equations 149 to 151, respectively.

$$r(t) = \frac{2}{5}\left\{\frac{1}{4}\exp\left[-6D_{\perp}t\right] + \frac{3}{4}\exp\left[-\left(2D_{\perp} + 4D_{\parallel}\right)t\right]\right\} \tag{153}$$

Using a numerical integration procedure, Small and Isenberg[142,143] have shown that in practical cases, at most three exponentials will be observed, and, of these, only two are independent.

Equations 145 and 149 to 151 were applied by Tao[136] to fit the experimental data according to the model of an equivalent hydrodynamic ellipsoid of revolution for the body. The principal diffusion coefficients are functions of the shape and size of the rotating LM and of the viscosity, η, and temperature, T, of the solvent. For a sphere, the well-known Debye-Stokes-Einstein Equation 43 for the rotational diffusion coefficient, D, holds true. General expressions for the three diffusion coefficients, D_i, of ellipsoids were derived in terms of the axial ratios, ρ, by Perrin,[132] Memming,[133] and others.[142,144,145] The diffusion coefficients for ellipsoids of revolution are given by

$$D_{\parallel} = \frac{3\rho(\rho - S)}{2(\rho^2 - 1)} D \tag{154}$$

and

$$D_{\perp} = \frac{3\rho\left[(2\rho^2 - 1)S - \rho\right]}{2(\rho^4 - 1)} D \tag{155}$$

where ρ is the axial ratio, less than unity for a disc-shaped LM (oblate rotor), greater than unity for a rod-shaped LM (prolate rotor), and $D = kT/6V\eta$ is the diffusion coefficient for a sphere with volume, V, equal to that of the ellipsoid. For an oblate ellipsoid:

$$S = (\rho^2 - 1)^{-1/2} \tan^{-1}\left[\frac{(1-\rho^2)^{1/2}}{\rho}\right] \quad \text{for } \rho < 1 \tag{156}$$

and for a prolate ellipsoid:

$$S = (\rho^2 - 1)^{-1/2} \ln\left[\rho + (\rho^2 - 1)^{1/2}\right] \quad \text{for } \rho > 1 \tag{157}$$

The three rotational correlation times, τ_i ($i = 1,2,3$), appearing in Equation 145 are functions of the rotational diffusion coefficients:

$$\tau_1 = \frac{1}{6D_{\perp}} \tag{158}$$

$$\tau_2 = \frac{1}{(5D_{\perp} + D_{\parallel})} \tag{159}$$

$$\tau_3 = \frac{1}{(2D_{\perp} + 4D_{\parallel})} \tag{160}$$

The values τ_1, τ_2, and τ_3 for various combinations of $D_{\parallel}$ and $D_{\perp}$ are tabulated in Table 5, as a function of axial ratios, ρ.[136,146] For prolate ellipsoids the three correlation times deviate from each other rapidly with increasing axial ratios. However, for oblate ellipsoids, although all three correlation times individually increase with ρ, the three hardly differ from each other. The calculation of r(t) (Equations 145, 152, and 153) thus resolves to the calculation of the equivalent volume sphere reorientation time and the application of the correction factors given in Table 5. The three correlation times (Equations 158 to 160) depend only on ρ (see Equations 154 and 155), whereas A_i ($i = 1,2,3$) depends only on ϑ. Therefore, a plot of $r(t) \cdot 5/2$ (Equation 145) vs. t/τ_c ($\tau_c = 1/6D$) depends only on ρ and ϑ. Such normalized fluorescence anisotropy plots are shown in a review by Yguerabide.[24]

The steady-state emission anisotropy for an exponential decay of fluorescence is expressed as

$$\begin{aligned}\langle r\rangle &= \frac{1}{\tau}\int_0^{\infty} r(t)\exp\left(-\frac{t}{\tau}\right)dt \\ &= 0.3\left\{\frac{4q_1q_2\gamma_1\gamma_2}{1+3(D_3+D)\tau} + \frac{4q_2q_3\gamma_2\gamma_3}{1+3(D_1+D)\tau}\right. \\ &\qquad + \frac{4q_3q_1\gamma_3\gamma_1}{1+3(D_2+D)\tau} + \frac{B+A}{1+(6D+2\Delta)\tau} \\ &\qquad \left. + \frac{B-A}{1+(6D-2\Delta)\tau}\right\}\end{aligned} \tag{161}$$

The formalism and results of the theories[138–140] apply to a wide variety of experiments, both to small LM as well as to macromolecules.

B. Some Selected Experimental Results

The following techniques have been applied to investigate molecular rotations in the pico- and nanosecond time scale: kinetic fluorescence depolarization,[147–151] single-photon counting,[152,153] upconversion,[154] phase fluorimetry,[155–163] polarization spectroscopy,[164–166] absorption transient-dichroism,[167–172] and transient grating.[173,174]

From among numerous applications of the above theoretical considerations, we have performed the initial experiments of Tao[136,146] for a

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TABLE 5
Rotational Correlation Times as a Function of Axial Ratios, ρ

	Prolate ellipsoids				Oblate ellipsoids		
ρ	τ_1/τ_c	τ_2/τ_c	τ_3/τ_c	ρ^{-1}	τ_1/τ_c	τ_2/τ_c	τ_3/τ_c
1	1.0000	1.0000	1.0000	1	1.0000	1.0000	1.0000
1.5	1.1935	1.1236	0.9557	1.5	1.0132	1.0405	1.1324
2	1.5049	1.3152	0.9543	2	1.1316	1.1701	1.3032
2.5	1.8923	1.5230	0.9606	2.5	1.2888	1.3341	1.4914
3	2.3408	1.7276	0.9674	3	1.4645	1.5147	1.6885
3.5	2.8432	1.9206	0.9732	3.5	1.6506	1.7047	1.8906
4	3.3956	2.0984	0.9777	4	1.8431	1.9003	2.0955
4.5	3.9953	2.2601	0.9814	4.5	2.0399	2.0998	2.3023
5	4.6405	2.4060	0.9842	5	2.2398	2.3018	2.5104
5.5	5.3297	2.5372	0.9865	5.5	2.4418	2.5057	2.7194
6	6.0616	2.6548	0.9884	6	2.6455	2.7111	2.9290
7	7.6505	2.8549	0.9911	7	3.0564	3.1247	3.3495
8	9.4008	3.0162	0.9930	8	3.4705	3.5411	3.7711
9	11.3080	3.1472	0.9944	9	3.8869	3.9593	4.1934
10	13.3680	3.2545	0.9954	10	4.3049	4.3787	4.6162
15	25.8607	3.5774	0.9979	15	6.4073	6.4859	6.7338
20	41.8199	3.7280	0.9988	20	8.5193	8.6005	8.8538

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system of 1-anilino-8-naphthalene sulfonate (ANS) dye, complexed with the protein apomyoglobin. When choosing this system, the following conditions must be fulfilled. The macromolecule should have such a size that its rotational motion is neither too rapid nor too slow compared with the rate of the LM label fluorescence emission decay. The rotational correlation times of globular proteins can be estimated from the equation:[136,175]

$$\tau_c = \frac{1}{6D} = \left(\frac{M}{3.69}\right) \times 10^{-3} [\text{nsec}] \tag{162}$$

where M is the molecular weight of the macromolecule. Equation 162 implies that macromolecules with molecular weights of 10,000 to 80,000 g/mol have correlation times of from 3 to 20 nsec, a range that includes the singlet lifetimes of most organic fluorescent molecules. Myoglobin has a molecular weight of 17,000 g/mol and falls within this range of convenient molecular size. The properties of the ANS-apomyoglobin complex have been reported by Stryer.[176] Since the X-ray structure of intact myoglobin is known, one can readily compare the Tao measurements of the dimensions of the complex with those of the native protein.

Figure 13 shows the experimental intensity components $I_{\parallel}(t)$, $I_{\perp}(t)$, and the natural decay of I(t) for the ANS-apomyoglobin complex. Both $I_{\parallel}(t)$ and $I_{\perp}(t)$ intensity components deviate from the exponential decay as predicted by Jabłoński Equations 37 and 38. The natural decay of I(t) of the complexed dye is a good exponential for over three decades with a lifetime τ of 16.4 nsec. It can be inferred from Figure 14 that the $I_{\parallel}(t) - I_{\perp}(t)$ curve for ANS-apomyoglobin complex does not seem to deviate from a simple exponential decay. It was therefore assumed that the investigated macromolecule is spherical, and fitted the data with a single exponential according to the equation:

$$I_{\parallel}(t) - I_{\perp}(t) \sim \exp\left(-6D - \frac{1}{\tau}\right)t \tag{163}$$

From an independent measurement of τ, one can determine the diffusion coefficient D. The theoretically calculated plots of r(t) are shown in Figure 14 for prolate ellipsoids of revolution under the same conditions of volume, temperature, and viscosity. It was assumed that the transition di-

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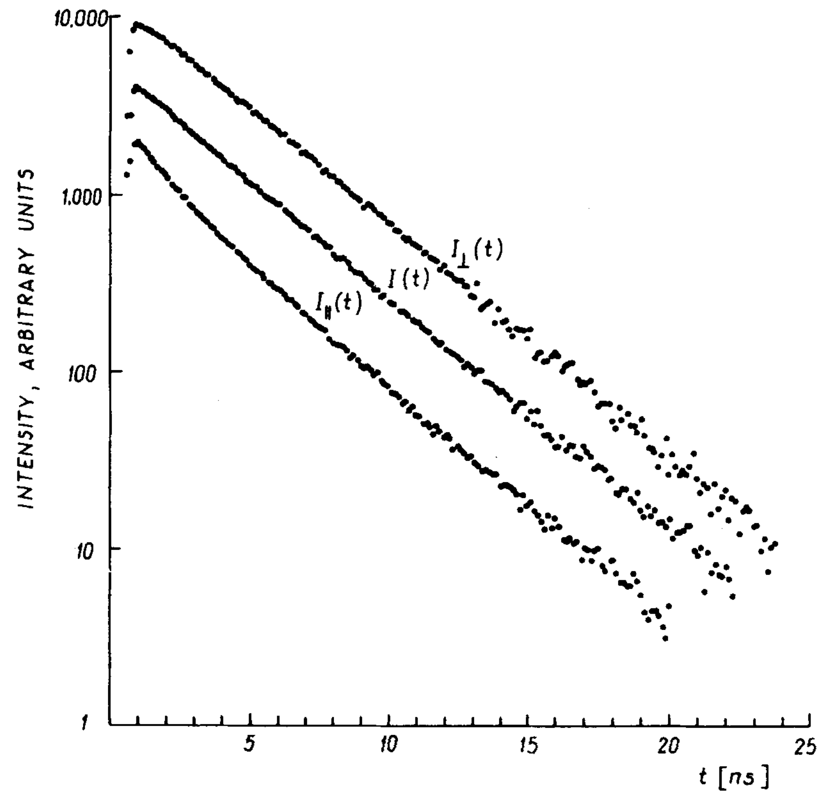

FIGURE 13. Decay of components $I_\parallel(t)$, $I_\perp(t)$ and the total intensity I(t) for the ANS-apomyoglobulin complex. (Data from Reference 136.)

pole moment of the dye label lies perpendicular to the longitudinal axis of the ellipsoid, so that Equation 153 can be used. As shown in Figure 14, the depolarization curve for a prolate ellipsoid with $\rho > 2$ evidently deviates from the experimental points.

Tao[136] measured the rotational diffusion coefficients of the ANS-apomyoglobin complex at different viscosities. From the linearity of the plot of 6D vs. T/η he obtained a value of 20.8 ± 0.6 Å for the radius of the ANS-apomyoglobin complex, which fairly well agrees with the value obtained from the X-ray structure measurements with regard to the correction for the solvent hydration (19.5 Å).

Cehelnik et al.[177] investigated the time dependence of the fluorescence intensities of the two components $I_\parallel(t)$ and $I_\perp(t)$ and the emission anisotropy, r(t), for all-*trans*-1,6-diphenylhexa-1,3,5-triene (DPH) in methylcyclohexane (viscosity $\eta = 0.744 \times 10^{-3}$ Pa·s at 293 K) and paraffin oil ($\eta = 134 \times 10^{-3}$ Pa·s at 298 K). In the former case, they obtained two identical curves for $I_\parallel(t)$ and $I_\perp(t)$. A complete rotational diffusion effectively occurs prior to emission, yielding $r(t) = 0$ over the whole measurable decay curve. In the second case, a time-dependent emission anisotropy effect was observed. r(t) has its maximum value, obtained by extrapolation, of 0.40 at time $t = 0$. Figure 15 shows the plots of $I_\parallel(t)$, $I_\perp(t)$, and r(t). Based on the data, the slope of the r(t) line gives 4.83 nsec for $1/6D_\perp$. The appropriate model for the Brownian rotational diffusion of DPH is that for a prolate ellipsoid. Using an axial ratio of $a/b = 5.96$, deduced from the molecular orbital data, and the above-mentioned value for $1/6D_\perp$, Equations 155 and 157 give 11.8 Å for 2a, i.e., the length of the DPH molecule along the long molecular axis. This value for the singlet excited state is in satisfactory agreement with a value of 14.0 Å estimated for the ground state.[178]

It should be noted that the Stokes-Einstein equation, $D = kT/6V\eta$, and the more general equations for ellipsoids are successful in predicting the rotational-diffusion coefficients of large mol-

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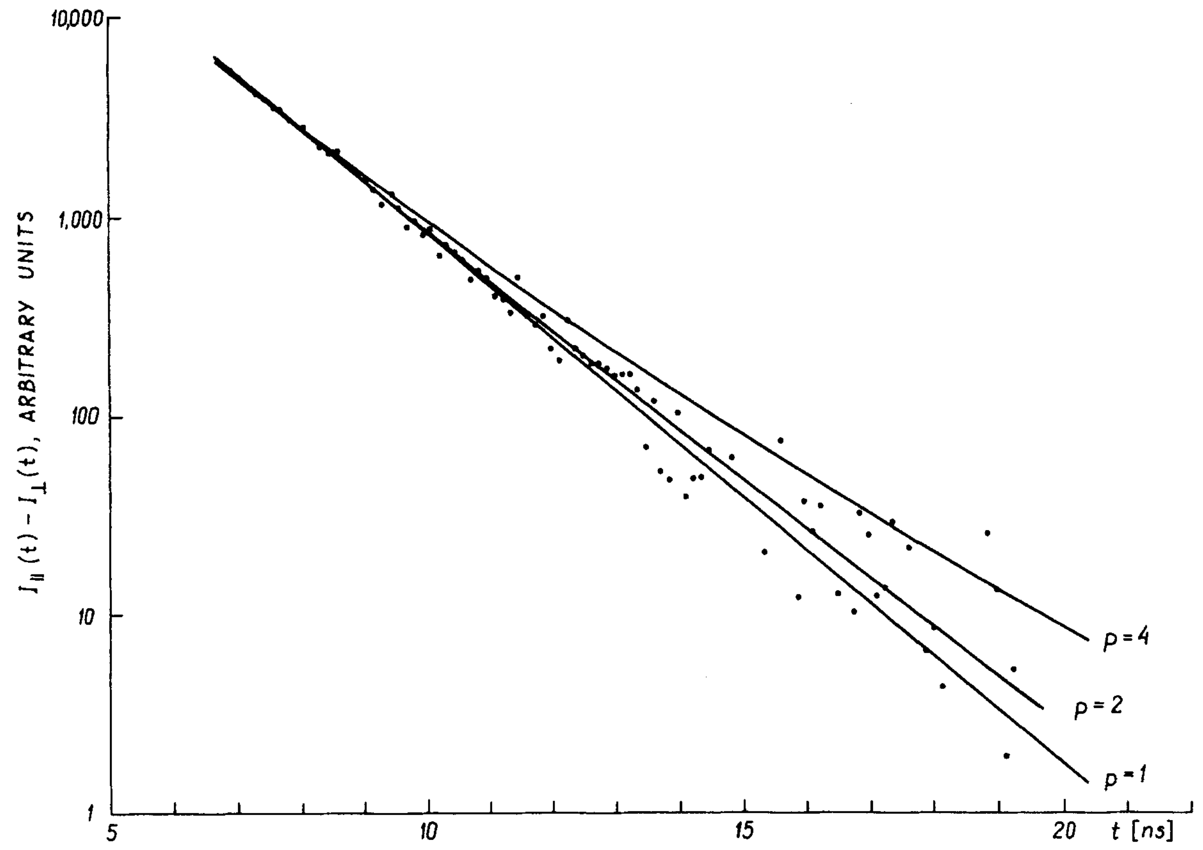

FIGURE 14. Plots of $I_{\parallel}(t) - I_{\perp}(t)$ for the ANS-apomyoglobulin complex: experimental points (•), theoretical curves (Equation 153) for the same quantity for prolate ellipsoids with various axial ratios. ρ. (Data from Reference 136.)

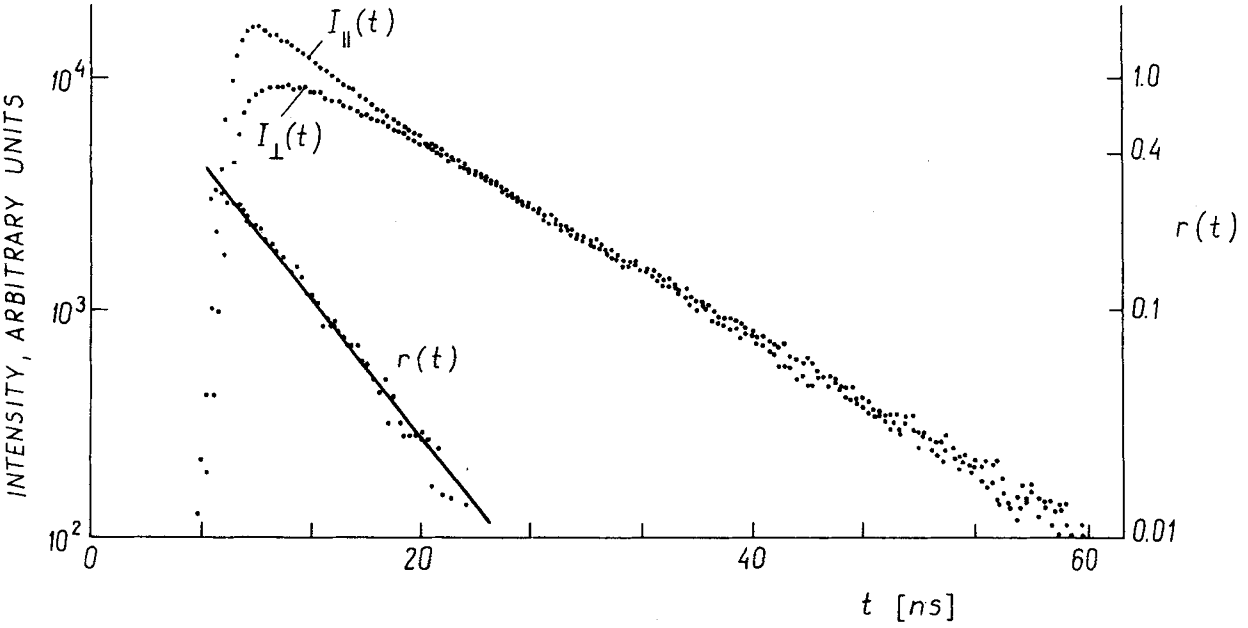

FIGURE 15. Time-dependent fluorescence intensities, $I_{\parallel}(t)$, $I_{\perp}(t)$, and emission anisotropy r(t) for DPH in paraffin oil. (Data from Reference 177.)

ecules, such as biological macromolecules (e.g., proteins or polymers). For small molecules in liquid solutions, the experimentally determined rotational-correlation times are often 5 or 10 times shorter than those calculated on the stick-boundary condition.[179–182]

According to Hu and Zwanzig,[182] the slip-boundary condition requires that the tangential component of the normal stress on the surface of the body vanishes. For rotational motion there is a large difference between slipping and sticking boundary conditions. Hu and Zwanzig[182] have tabulated the friction coefficients for the uniform rotation of prolate and oblate spheroids in a viscous fluid, with the slipping-boundary condition as a function of the axial ratio (Table 6). Similar calculations for the friction coefficients of ellipsoids devoid of axial symmetry were performed by Youngren and Acrivos.[183] An experimental test of the slipping theory[183] was reported by Harris and Newman[184] and the rotational correlation times for 9H-fluorene in chloroform-d_1 were deduced from carbon-13 relaxation data using bond lengths predicted from infrared stretching frequencies. The dimensionless friction coefficients for 9H-fluorene calculated on the basis of tabulated values[182] are qualitatively similar to the experimental data. The discrepancies were discussed in detail by Harris and Newman.[184]

A molecular theory for rotational diffusion of small molecules in noninteracting solvents was presented by Hynes et al.[185] The slip-boundary

TABLE 6
Friction Coefficients

	Prolate		Oblate	
χ[a]	ξ^*[b]	(slip)/(stick)[c]	ξ^*[b]	(slip)/(stick)[c]
1.00	0.0	0.0	0.0	0.0
0.95	0.0122	0.00168	0.0127	0.00169
0.90	0.0468	0.00702	0.0505	0.00712
0.85	0.100	0.0165	0.113	0.0169
0.80	0.169	0.0307	0.199	0.0316
0.75	0.250	0.0501	0.309	0.0520
0.70	0.341	0.0751	0.441	0.0787
0.65	0.436	0.106	0.596	0.112
0.60	0.534	0.144	0.771	0.154
0.55	0.631	0.189	0.967	0.203
0.50	0.723	0.240	1.18	0.261
0.45	0.807	0.299	1.41	0.327
0.40	0.880	0.359	1.66	0.402
0.35	0.937	0.433	1.92	0.484
0.30	0.976	0.508	2.18	0.572
0.25	0.991	0.584	2.45	0.664
0.20	0.980	0.660	2.71	0.755
0.15	0.936	0.732	2.95	0.842
0.10	0.853	0.797	3.16	0.918
0.05	0.715	0.855	3.32	0.976
0.01	0.503	0.905	3.39	0.999

[a] χ = (min)/(max) is the ratio of the shorter semiaxis (min) and the longer semiaxis (max) of the spheroid.

[b] Friction coefficients are in the dimensionless form, $\xi^* = -\text{torque}/(\text{max})^3 \pi\mu\omega$ (ω denotes the angular velocity of the spheroid and μ the fluid viscosity).

[c] The ratio (slip)/(stick) of the friction coefficient with slip to the friction coefficient of the same spheroid with stick.

Data from Reference 183.

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condition has been found, in this case, to give a satisfactory agreement with the experiment. The rotational dynamics of small LM in simple liquids,[125,186–191] associating solvents,[192,193] and simple glass-forming matrices[194] were investigated by the use of the Chuang and Eisenthal model. The experimental results of Sanders and Wirth[192] indicate that the structure of the solvation environment has a marked influence on the rotational diffusion behavior. The fluorescence anisotropy decay of small molecules such as perylene and 9-aminoacridine in glycerol at temperatures from 283 to 313 K were investigated by Barkley et al.[186] It was found that, under these conditions, the fluorescence lifetime of both compounds is constant and equal to 4.6 nsec for perylene and 12.8 nsec for 9-aminoacridine. The decay of the fluorescence emission anisotropy was analyzed in terms of a double exponential function (Equation 153) with rotational correlation times independent of excitation wavelength and dependent on temperature.

The principal diffusion coefficients of perylene and 9-aminoacridine in glycerol were calculated, according to Equations 158 and 160, based on the rotational correlation times τ_1 and τ_3, summarized in Table 7. The ratio of the diffusion coefficients for the in-plane and out-of-plane rotations of perylene $D_{\parallel}/D_{\perp}$ is 10 ± 1, which is in good agreement with a value of 10 ± 2 obtained by Zinsli[125] from the time-resolved fluorescence anisotropy in paraffin over the temperature range from 253 to 323 K. For 9-aminoacridine, the ratio of the diffusion coefficients $D_{\parallel}/D_{\perp}$ is 1.4 ± 0.1. This slightly anisotropic rotation was not observed by Mantulin and Weber[187] in a previous experiment by differential phase fluorimetry. It was found that the rotational dynamics of perylene is consistent with that of a disk (oblate ellipsoid) with the slipping boundary condition, but for 9-aminoacridine can be interpreted in terms of a hydrodynamic prolate ellipsoid of revolution. The results are in agreement with the previous findings,[125,187] suggesting that these molecules represent two extreme rotational behaviors, perylene being a highly anisotropic rotator with the slipping boundary condition, and 9-aminoacridine an isotropic rotator with the sticking boundary condition. However, according to Sasaki et al.,[195] the assumption of ideal slipping boundary condition for perylene seems to be unsatisfactory, because there is some weak interaction, such as hydrogen bonding, between perylene and solvent molecules.

Mention should be made that for a large bumpy cylinder, when the radius, R, exceeds 500 Å the two-boundary slip and stick conditions are identical.[196]

Recently, Wirth and Chou[191] measured the fluorescence anisotropy decays of tetracene in 1-butanol, 1-octanol, and 1-dodecanol at several temperatures using frequence domain spectroscopy. For tetracence, the long axis was denoted by 1, the short by 2, and the normal to the molecular plane by 3. The excitation to the O–O bond of

TABLE 7
Principal Diffusion Coefficients of Perylene and 9-Aminoacridine in Glycerol

	Perylene		9-Aminoacridine	
T (K)	$D_{\parallel} \times 10^{-7}$ (s^{-1})	$D_{\perp} \times 10^{-6}$ (s^{-1})	$D_{\parallel} \times 10^{-6}$ (s^{-1})	$D_{\perp} \times 10^{-6}$ (s^{-1})
283	1.5	1.5	2.7	2.1
288	2.5	2.4	4.2	3.2
293	3.7	3.7	7.2	4.8
298	5.8	6.0	12.0	7.6
303	8.8	9.8	19.0	12.0
308	14.0	13.0	27.0	19.0
313	22.0	19.0	38.0	28.0
Ratio $D_{\parallel}/D_{\perp}$	10 ± 1		1.4 ± 0.1	

From Barkley, M. D. et al. *J. Chem. Phys.* **1981,** *75,* 3581. With permission.

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the first excited singlet state creates a transition dipole oriented along axis 2 of tetracene.[6] This simplifies the Chuang and Eisenthal[138] Equation 143 for r(t) to a double exponential decay.[191]

$$r(t) = 0.3(B + A)\exp[-(6D + 2\Delta)t] + 0.3(B - A)\exp[-(6D - 2\Delta)t] \qquad (164)$$

For the excitation along axis 2 of the solute and when emission occurs at the angle ϑ with respect to axis 2, they obtained:[191]

$$A = \left(D - D_2 \cos^2 \vartheta - D_3 \sin^2 \vartheta\right)/\Delta \quad \text{and}$$

$$\beta = \cos^2 \vartheta - \frac{1}{3}$$

The knowledge of the preexponential factors and time constants allows calculation of the diffusion tensor components: D_1, D_2, and D_3. It was shown that the relative values of the rotational diffusion components vary with temperature, indicating nonhydrodynamic rotational diffusion. Subslip behavior was found to be associated with a value of D_2/D that is significantly larger than that predicted from hydrodynamics. Wirth and Chou[191] came to the conclusion that the subslip phenomenon can be interpreted as a consequence of the solvent structure.

Spears and Cramer[152] demonstrated for rose bengal dianion in different aprotic and protic solvents, that hydrogen bonding influences the rotational diffusion correlation time $\tau_c = 1/6D$ of the LM. The values of τ_c were determined from the time dependence of polarized fluorescence intensities. Figure 16 shows the results of τ_c measurements for rose bengal dianion vs. solvent viscosity. The observed rotational correlation time for this dye is two to three times longer in alcohol solution than in a polar but non-hydrogen bond donor solvent with comparable viscosity. Solvent attachment seems necessary to explain the slow rotation correlation times of these dye molecules (rose bengal, erythrosin) in alcohols.[22] Fleming et al.[147] showed that agreement with experimental values is obtained if the oblate molecule is padded out to form a sphere having the largest molecular dimension.

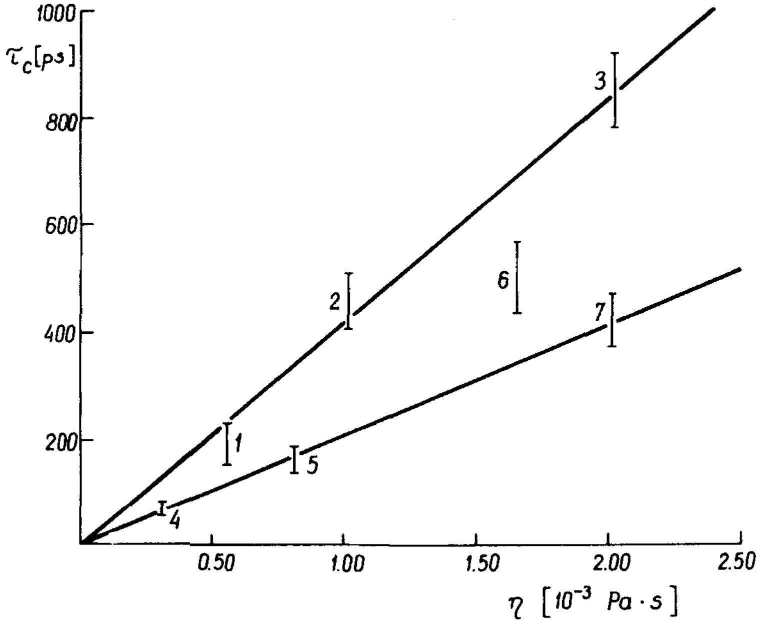

FIGURE 16. Plot of mean orientation correlation time, τ_c, in picoseconds observed for rose bengal dianion vs. solvent bulk viscosity, η. Solvents: methanol (1), ethanol (2), isopropanol (3), acetone (4), dimethylformamide (5), *N*-methylformamide (6), dimethylsulfoxide (7). (Data from Reference 152.)

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Three rotational correlation times were detected by Gryczyński et al.[197] for the rigid asymmetric Y_t-base molecule (Y-4,9-dihydro-4,6-dimethyl-9-oxo-1H-1-imidazol-1,2 *a*-purine) using frequency-domain fluorometry. For the excitation wavelengths of 290, 312, and 346 nm, fundamental (limiting) anisotropy values of 0.05, 0.19, and 0.32 were obtained, respectively. To vary the mean decay time from 9.1 to 4.2 nsec, CCl_4 was used as a collisional quencher. Nine sets of data were globally analyzed to recover the anisotropy decay law. For Y_t-base, the three correlation times, 0.8, 3.0, and 5.6 nsec, may originate from either its asymmetric structure or partial slipping about one or more axes.[197]

A number of investigators have carried out studies for different solvent types over wide viscosity ranges. In the review by Fleming,[22] one can find a comprehensive list of rotational diffusion times. Most of the LM deviate rather strongly from symmetric ellipsoids, and therefore an uncertainty arises in calculating the theoretical reorientation time from the equations given in Section VI.A. In the work of Rice and Kenney-Wallace,[198] a saturation of the retarding influence of viscosity was suggested. More recent results on rhodamine B reveal a linear dependence of τ_c on η/T up to 356×10^{-3} Pa s at room temperature.[174] However, for a given solvent series, a linear viscosity dependence has generally been observed, when the viscosity is varied both by the change of solvent and by temperature change. Waldeck and Fleming[164] investigated the monocation 3,3′-diethyloxadicarbocyanine iodide (DODCI) in different solvents (ethanol, 1-butanol, 1-decanol) and single solvents with varying temperature, and obtained a good linear dependence of τ_c vs. η/T. A typical data set with ethanol as the solvent is shown in Figure 17. The intercept of the linear dependence for the three solvents is very close to zero, as expected for simple Stokes hydrodynamics. The experimental dependence of τ_c on η/T can be compared with the empirical formula suggested by Bauer et al.[181]

$$\tau_c = c\eta + \tau_0 \tag{165}$$

where τ_0 is the zero viscosity intercept.

The free rotor reorientation time given by Bartoli and Litovitz and others:[199–201]

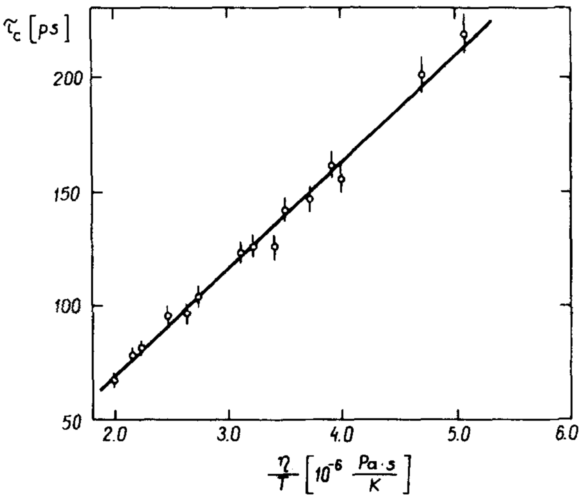

FIGURE 17. Plot of orientation time, τ_c, vs. the viscosity over temperature, η/T, for DODCI dissolved in ethanol. (Data from Reference 164.)

$$\tau_0 = \frac{2\pi}{9}\left(\frac{I}{kT}\right)^{1/2} \tag{166}$$

where I is the moment of inertia. The free rotor time for DODCI is 4 psec.[164] The factor (slope) C is determined by the shape of the LM and the hydrodynamic boundary conditions. For a sphere with stick boundary conditions C = V/kT and for an ellipsoidal molecule C must be derived from Equations 43 and 154 to 157.

The values of τ_c, determined experimentally from Equation 63, vs. viscosity, η, for 4-dimethylamino-ω-methylsulfonyl-*trans*-styrene (3a) in *n*-paraffins are shown in Figure 18. The data fit the straight line corresponding to Equation 165 fairly well. The values obtained for *3a* are C = 42.4 1/pa and $\tau_0 = 5.2$ psec. Hence, the volume and the moment of inertia can be determined: $V = 171 \times 10^{-30}$ m^3 and $I = 22.6 \times 10^{-44}$ kg m^2. The I_0 value calculated for a free molecule based on its geometry amounts to 4.96×10^{-44} kg m^2,[71] which is 4.9. times smaller than that determined from Equation 166.

It can be seen that the intercept τ_0 – s is nonzero as predicted by the Stokes-Einstein relation. The last example shows also that in the rotational fluorescence depolarization, both inertial and viscous effects contribute to orientational

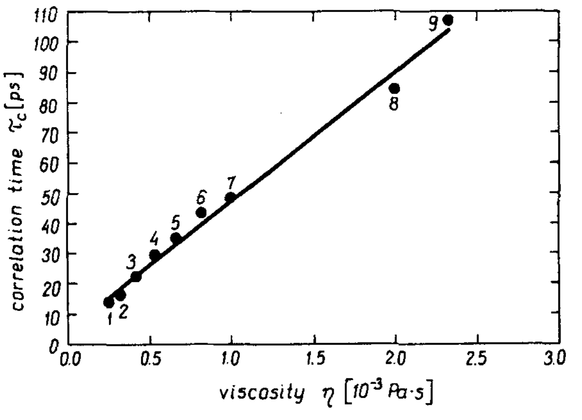

FIGURE 18. Plot of correlation time, τ_c, vs. viscosity, η, for *3a* in different *n*-paraffins (according to Table 11); correlation coefficient r = 0.996. (Data from Reference 71.)

relaxation of the solute in solvents, similarly as for depolarized Rayleigh scattering.[179,181] The influence of inertial effect on the rotational fluorescence depolarization will be considered in subsection C.

The spectroscopy of some LM, such as indole derivatives and tryptophan, is complicated by the existence of two overlapping electronic transitions, designated as ^1L_a and ^1L_b transitions.[202,203] The measured limiting fluorescence anisotropy for the excitation in the long-wave band for these LM is considerably smaller than 0.4, because the two transition moments are roughly oriented perpendicular to each other.[204,205] The important experimental value of the fluorescence anisotropy at a zero time, r(0), is the intrinsic anisotropy of the LM and is only a function of the relative orientations of the absorption and emission transition dipole moments.[7,27] Observations of experimental values of r(0) less than the theoretically predicted value $r_p = 0.4$ indicate the existence of relaxation processes (torsional vibrations, librational motions) occurring on a time scale shorter than the time resolution of the experiment. A discussion on the different explanations of the anisotropy deviation from 0.4 are given in the interesting work of Ruggiero and co-workers.[151] Using the time-resolved polarization spectroscopy technique, they most certainly observed an initial fluorescence anisotropy of 0.4 for tryptophan in water. In 1974, Cehelnik et al.[177] and later Fleming et al.[147,206,207] communicated that the theoretical value of 0.4 for the principal emission anisotropy, r_p, at zero time was observed for some dyes.

In their last work, Ruggiero et al.[151] used a frequency conversion optical gating to obtain an improvement in the time resolution by two orders of magnitude as compared to the previous measurements of tryptophan fluorescence emission anisotropy. The experimental data were interpreted with a generalized version of the model of Fleming[22] and Cross et al.,[208] with more than one electronic state, which also includes the effects of vibronic coupling.

Chuang and Eisenthal,[209] Lessing and Von Jena,[173,210] and Szubiakowski[211] have also considered some cases in transient absorption spectroscopy, involving level kinetics and rotational Brownian reorientation.

The fluorescence lifetime and anisotropy decays of indole in propylene glycol, and of the tryptophan emission of melittin monomer and tetramer in water solutions at 278 K were measured by Lakowicz et al.,[212] by the use of frequency-domain fluorescence spectroscopy. It was found that for both indole and melittin the anisotropy decays are heterogeneous and require two correlation times to fit the frequency-domain data. For indole in propylene glycol at 278 K they recovered correlation times of 0.59 and 4.10 nsec, which appear to be characteristic of the rigid and asymmetric indole molecule. For the melittin monomer, the correlation times obtained were 0.13 and 1.75 nsec, and for the melittin tetramer, 0.12 and 3.95 nsec. The shorter and longer correlation times of melittin were explained by the segmental motions and overall rotational diffusion of the polypeptide.

Further applications of fluorescence anisotropy decay of biopolymers labeled with an extrinsic fluorophore and of proteins displaying internal rotation can be found in the reviews published in recent years.[13,27,213,214] It must be mentioned that the basic theory of anisotropy decay was formulated by Jabłoński[38,74,75] and applied by Wahl et al.[215–219] and others[220–242] to the study of the rotational dynamics of biopolymers. Gottlieb and Wahl,[243] Wallach,[244] and Barkley and Zimm[245] expanded the basic theory to include the effects of internal rotations.

The development of the theory of the rotational Brownian motion of a particle coupled with

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an actively driving motor is also worth pointing out. Hoshikawa and Asai[246,247] developed such a theory and applied it to calculating the time-dependent fluorescence depolarization. Measurements of such depolarization provide a useful tool for determination of the rate of revolution of the rotating motor attached to cell bodies, such as bacteria under various solution conditions, if a fluorescent label is attached to the motor shaft. The employment of polarization spectroscopy has also played a key role in investigations of artificial polymers.[248–256]

C. Influence of Inertial Effect on Rotational Depolarization

It was shown that if the emission is polarized in the direction of the long axis (symmetrical axis) of the LM ($\vartheta = 0$), Equations 149 to 151 and 161 yield the Perrin Equation 56 which is valid for spherical LM only. Nevertheless, the experiments carried out in recent years for numerous LM, the emission transition moment of which lies in the direction of long molecular axis ($\vartheta = 0$), have shown distinct deviation of the experimental points from the linear dependence, in spite of the fact that all former theories predicted linear dependence for such a type of LM.[257–259] Figures 19 and 20 show, as an example, the results of the measurements of $1/\langle r \rangle$ as a function of $1/\eta$ for 4-dimethylamino-4′-nitrostilbene (DNS) and diphenylstilbene (DPS).[257,258] Strong discrepancy between the experimental results and linear Perrin's relation (Equation 63) predicted by the theory[41,133,138–140] can be seen readily in the range of low viscosities. A similar nonlinear relation was observed by Memming[133] for 3-amino-pyrene, by Griebel[260] for *p*-terphenyl and *trans*-stilbene, and by Salamon et al.[261] for methine dyes. No explanation, however, of this behavior of $1/\langle r \rangle$ as a function of $1/\eta$ has been given in these papers.

As will be shown below, the observed discrepancies between the experimental results and the theoretical considerations existing so far result from the disregard of the inertial effects in the theory of the influence of rotational diffusion of LM in solutions upon the fluorescence anisotropy. It has been found that the inertial effect substantially affects the fluorescence anisotropy decay for short lifetimes of an LM (picoseconds) following its excitation.

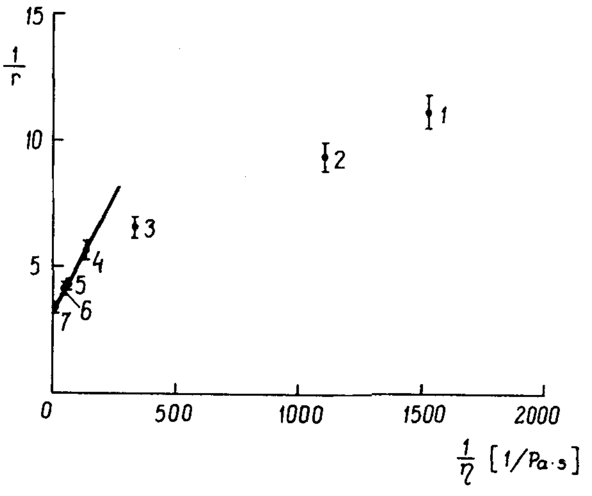

FIGURE 19. Dependence of 1/r of 4-dimethylamino-4′-nitrostilbene on 1/η at 293 K in benzene (1), chlorobenzene (2), *n*-butanol (3), *n*-heptanol (4), *n*-undecanol (5), ethylene glycol (6), and cyclohexanol (7). The continuous line is given by Perrin Equation 63. (Data from Reference 257.)

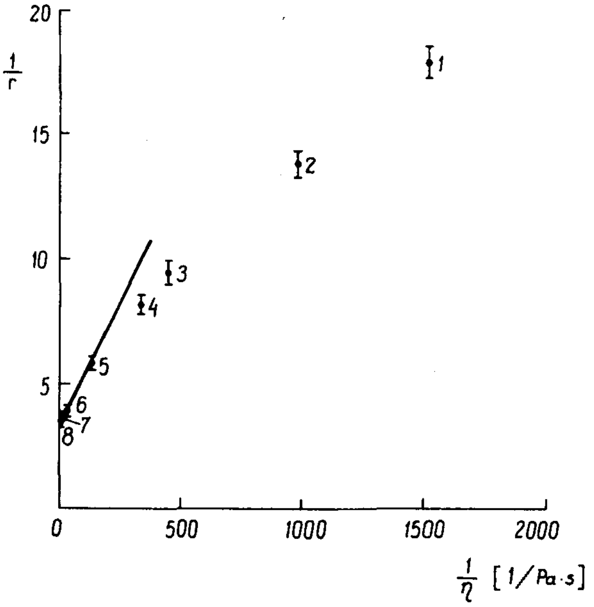

FIGURE 20. Dependence of 1/r of diphenylstilbene on 1/η at 293 K in benzene (1), cyclohexane (2), *n*-propanol (3), *n*-butanol (4), *n*-heptanol (5), triethyl-ester-glycerol (6), 1,2-propanediol (7), and cyclohexanol (8). The continuous line is given by Perrin Equation 63. (Data from Reference 258.)

The inertial effect upon the Brownian motions in Einstein's theory was taken into account

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by Uhlenbeck and Ornstein[262] in 1930. Since then, Gajsenok et al.[263] and Alicki et al.[264,265] have attempted to eliminate the inconsistency between the experimental results and the rotational depolarization theory by taking into account the inertial moments of the LM. In the theory of Gajsenok et al.,[263] a less precise equation of the rotational diffusion, which was obtained by Steele[266] and, before, by Kubo[267] was employed, whereas in the theory of Alicki et al.[264,265] the non-Markovian generalized diffusion equation was used. The effect of the moment of inertia on the rotational fluorescence depolarization was demonstrated in both theories.[263,264] Later, the theory[264] was generalized by Alicki and Alicka[265] to embrace also asymmetric LM, the directions of the emission and absorption transition moments of which were not coincident, making in general nonzero angles with the principal axes of the tensor of inertia.

In the generalized diffusion equation for the rotational motions of LM, employed by Alicki et al.[264,265] in the rotational fluorescence depolarization theory, the angular velocity autocorrelation function was introduced in relation to the molecular axes (in the case of a prolate LM, with respect to axes 1 and 2 perpendicular to the longitudinal axis 3). The diffusion equation employed therein describes the "memory" effect (convolution integral) related to the moment of inertia. An LM having a moment of inertia behaves during consecutive collisions in the solution as if it remembered the preceding collision and, hence, its motion is in part determined.

Let us first consider in detail the rotational motion of an ellipsoid of revolution (Figure 21). Axes 1, 2, and 3 are the principal axes of the friction tensor, ξ_{kl}, being simultaneously the principal axes of the tensor of inertia, I_{kl}. It is assumed, moreover, that $\xi_1 = \xi_2 = \xi$ and $I_1 = I_2 = I$, and that the direction of the emission transition moment is parallel to the long axis 3 of the LM. The electric vector, **E**, of the exciting light is directed along the z-axis.

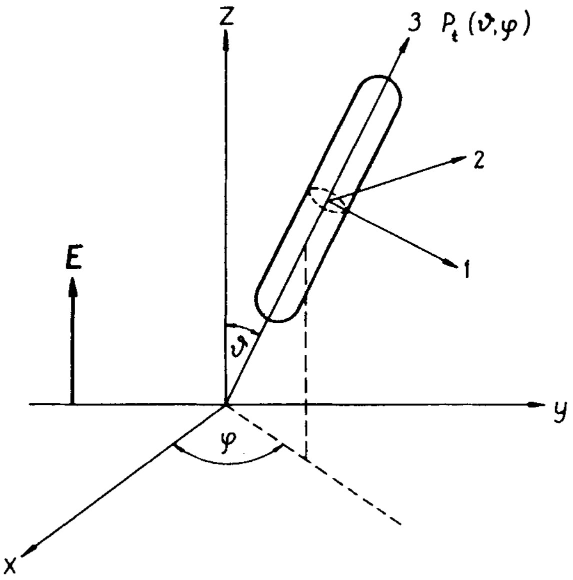

FIGURE 21. Geometry of the system.

As a result of excitation, an ensemble of LM emit anisotropic fluorescence radiation during the mean lifetime. The anisotropy of the excited LM is described at any instant t by the probability distribution $P_t(\vartheta,\varphi)$. The change of $P_t(\vartheta,\varphi)$ in time is given by the following generalized equation of rotational diffusion:[264]*

$$\frac{\partial P_t(\vartheta,\varphi)}{\partial t} = \int_0^\infty G(s)\Delta_\Omega P_{t-s}(\vartheta,\varphi)\,ds \quad (167)$$

where Δ_Ω is the angular part of the Laplace operator and

$$G(s) = \langle \omega_1(0)\omega_1(s)\rangle = \langle \omega_2(0)\omega_2(s)\rangle \quad (168)$$

is the autocorrelation function of the angular velocity with respect to axis 1 or 2. $\langle \ldots \rangle$ denotes the averaging process over the assembly of LM.

Equation 167 describes the "memory" effect related to the molecular inertia taken into account, i.e., the change of $P_t(\vartheta,\varphi)$ in time depends upon P_t also for $t' < t$. In order to calculate the autocorrelation function $G(s) = \langle \omega(0)\,\omega(s)\rangle$, we shall apply the Langevin equation:

---

* Derivation of the motion rule for a system with n degrees of freedom (e.g., a Brownian particle) interacting with a thermal bath with temperature T is given as an appendix in Reference 264.

$$I\frac{d\omega(t)}{dt} = -\xi\omega(t) + M(t) \qquad (169)$$

where I is the effective moment of inertia with respect to axes perpendicular to the long axis 3 of a LM, ξ is the rotational friction coefficient, M(t) the respective component of a stochastic force moment, $\omega = \omega_1$ or $\omega = \omega_2$. The mean value of M(t) over the assembly of LM is assumed to satisfy the condition $\langle M(t)\rangle = 0$.

The solution to Equation 169 has the form:

$$\omega(t) = \exp[-(\xi/I)t]\left\{\omega(0) + \int_0^t \exp[-(\xi/I)t']M(t')\,dt'\right\} \qquad (170)$$

Hence, we obtain:

$$\langle\omega(0)\cdot\omega(t)\rangle = \langle\omega^2(0)\rangle\exp[-(\xi/I)t] \qquad (171)$$

From the energy equipartition theorem:

$$\frac{1}{2}I\langle\omega^2(0)\rangle = \frac{1}{2}kT$$

and based on Equations 168 and 171, we obtain the following expression for the autocorrelation function:

$$G(s) = \frac{kT}{I}\exp[-(\xi/I)s] \qquad (172)$$

where the decay time of this function is $\tau_a = I/\xi = 1/(6V\eta)$, because $\xi = 8\,\pi a^3\eta = 6V\eta$. If $I \to 0$, then $G(s) \to D\cdot\supseteq\delta(s)$, where $D = kT/\xi = kT/6V\eta$ and $\delta(s)$ is the delta-function. Thus, the generalized diffusion Equation 167 becomes the ordinary diffusion Equation 42.

The fluorescence anisotropy at time t following an instantaneous light pulse of excitation is given by

$$r(t) = \int d\Omega\, P_t(\vartheta,\varphi)\left[\frac{3}{2}\cos^2\vartheta - \frac{1}{2}\right] \qquad (173)$$

where $d\Omega = \sin\theta\, d\theta\, d\varphi$.

On differentiating Equation 173 and employing Equation 167, we obtain:

$$\frac{dr(t)}{dt} = \int_0^t d(s)G(s)\int d\Omega\left[\Delta_\Omega P_{t-s}(\vartheta,\varphi)\right] \times\left[\frac{3}{2}\cos^2\vartheta - \frac{1}{2}\right] \qquad (174)$$

To find an equation describing the function r(t) we must take advantage of the following facts:

1. The Laplace operator Δ_Ω is self-adjoint

$$\int d\Omega(\Delta_\Omega P)f = \int d\Omega\, P(\Delta_\Omega f)$$

2. Spherical functions $Y_{\ell m}(\vartheta,\varphi)$ are the eigenfunctions of the Laplace operator Δ_Ω,

$$\Delta_\Omega Y_{\ell m} = -\ell(\ell+1)Y_{\ell m}, \qquad \begin{array}{l}\ell = 0,1,2,\dots\\ m = -\ell,\dots,+\ell\end{array}$$

3. $^3/_2\cos^2\vartheta - {}^1/_2 = CY_{20}$, C – constant.

Applying these properties to Equation 174, we obtain:

$$\frac{dr(t)}{dt} = -6\int_0^t G(s)r(t-s)\,ds \qquad (175)$$

Function r(t) possesses the following qualitative properties which meet Equation 175:

$$\left.\frac{dr(t)}{dt}\right|_{t=0} = 0 \qquad (176)$$

Second, if the decay time, $\tau_a = I/\xi$, of the autocorrelation function G(s) is markedly shorter than that of function r(t), and $t > \tau_a$, then the following approximated equation may be substituted for Equation 175:

$$\frac{dr(t)}{dt} = -6Dr(t) \qquad (177)$$

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where

$$D = \int_0^\infty G(s)\, ds$$

Equation 177 implies that for the properties mentioned above, function r(t) is proportional to exp(–6Dt) (see Perrin Equation 55).

The qualitative properties of function r(t) given by Equation 175 are shown schematically in Figure 22. An interesting topic in picosecond spectroscopic studies is the initial behavior of the fluorescence emission anisotropy r(t).

For continuous excitation of an isotropic fluorescent solution, the mean emission anisotropy, ⟨r⟩, can be calculated from the following equation:

$$\langle r \rangle = \frac{1}{\tau}\int_0^\infty \exp\left(-\frac{t}{\tau}\right) r(t)\, dt \tag{178}$$

Knowledge of the solution to Equation 175 is not indispensable to calculate ⟨r⟩ according to Formula 178. Applying the Laplace transform to both sides of Equation 175, in which function G(s) is given by Equation 172, after simple transformations we obtain:[264]

$$\langle r \rangle \equiv r = r_f \frac{1 + A\dfrac{\tau}{\tau_c}}{1 + (1 + A)\dfrac{\tau}{\tau_c}} \tag{179}$$

where

$$A = \frac{I}{6\,kT\,\tau^2} \tag{180}$$

$$\tau_c = \frac{\xi}{6\,kT} = \frac{V\eta}{kT} \tag{181}$$

and

$$\gamma_f = \frac{3}{5}\cos^2\beta - \frac{1}{5}$$

Equation 181 includes the effective volume of the LM, $V = 4/3\,\pi{\cdot}a^3{\cdot}\psi$, where a is its radius and ψ is the fitting parameter dependent on the molecular shape.

Figure 23 shows a family of curves calculated from Equation 179 for different parameters A. When A = 0 (i.e., I = 0 or τ is very high) or when the solvent viscosity, η, increases, i.e., T/η → 0, the well-known Perrin Equation 63 is obtained

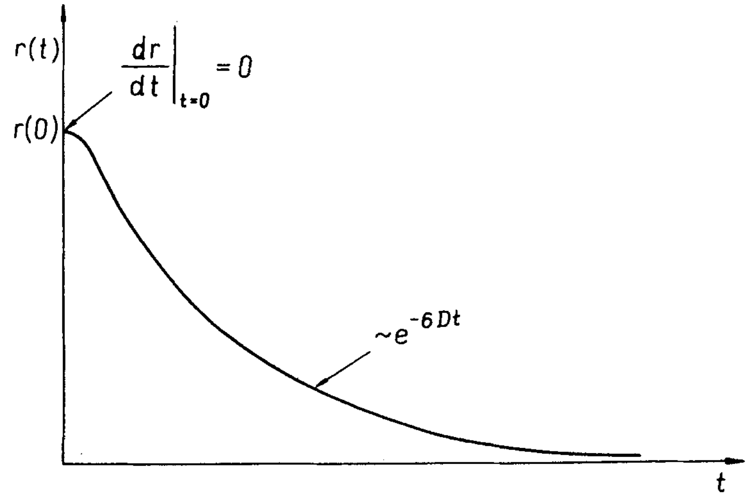

FIGURE 22. Qualitative properties of function r(t) given by Equation 175.

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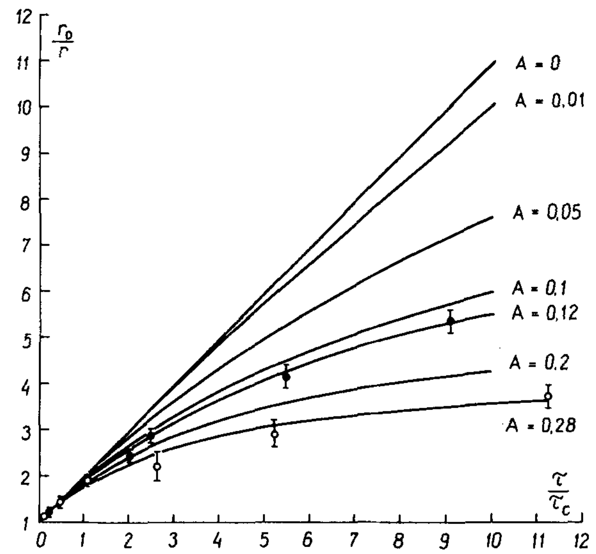


FIGURE 23. Theoretical curves of r_0/r as a function of τ/τ_c (Equation 179) for different values of A. Experimental values for diphenylstilbene (●) and 4-amino-4′-nitrostilbene (o). (From Alicki, R. et al. *Z. Naturforsch. Teil A* **1981** *36,* 1158. With permission.)

which yields the linear dependence of $1/\langle r\rangle$ upon T/η.

Based on the comparison of the experimental results ($1/r$ vs. T/η, see Figure 23) for r_0 and V_{eff} determined previously from the Perrin Equation, with the theoretical values obtained according to Formula 179 within the whole viscosity range examined, A can be determined and then, from Equation 180, the effective moment of inertia, I, of the investigated LM, assuming that the mean value τ is known and constant.[268,269] However, if τ changes strongly in different solvents, the following form of Equation 179 should be employed:[270]

$$\frac{\tau^2}{\frac{r_0}{r}-1} = \frac{V\eta\tau}{kT} + \frac{I}{6\,kT}, \quad (r_f = r_0) \tag{182}$$

Plotting the left-hand side of Equation 182 vs. $\eta\tau$ one obtains a straight line, from the slope of which the volume V, and from its intersection with the ordinate axis, the moment of inertia I can be found. Figures 24 to 27 show the comparison of the experimental results obtained for diphenylstilbene and *trans*-stilbene[270] in different solvents

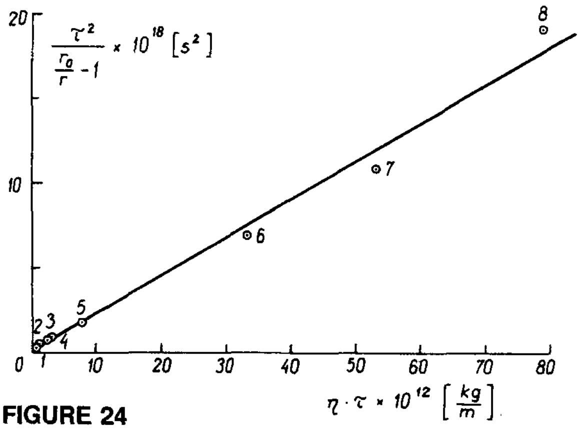
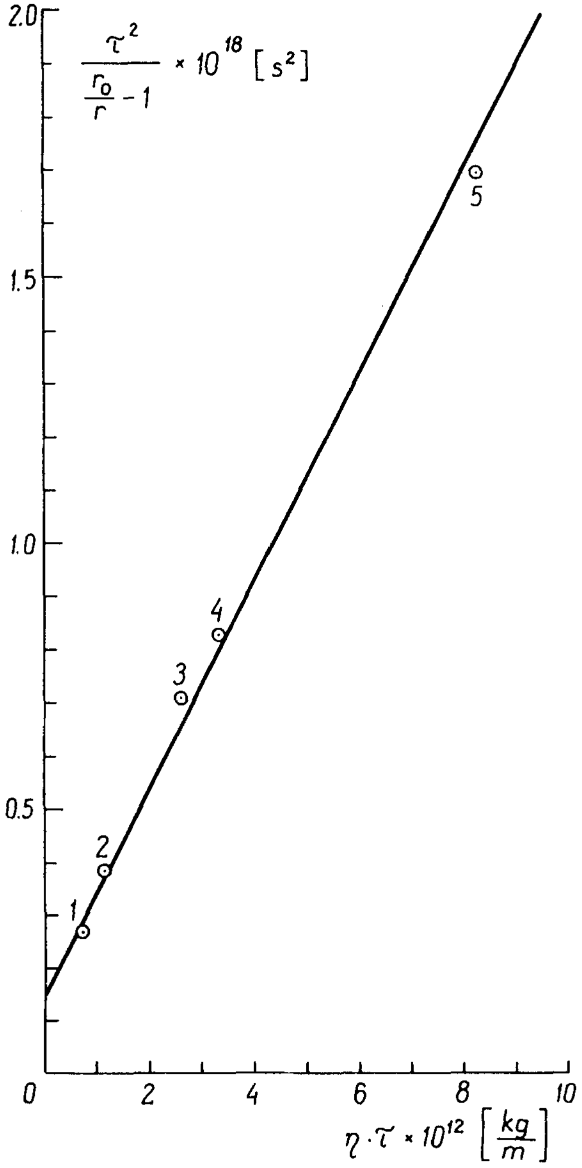


FIGURE 24



FIGURES 24 and 25. Dependence of $\tau^2/(r_0/r - 1)$ on $\eta\tau$ for diphenylstilbene at 293 K in benzene (1), cyclohexane (2), *n*-propanol (3), *n*-butanol (4), *n*-heptanol (5), triethyl-ester-glycerol (6), 1,2-propanediol (7), and cyclohexanol (8). (Data from Reference 258.)

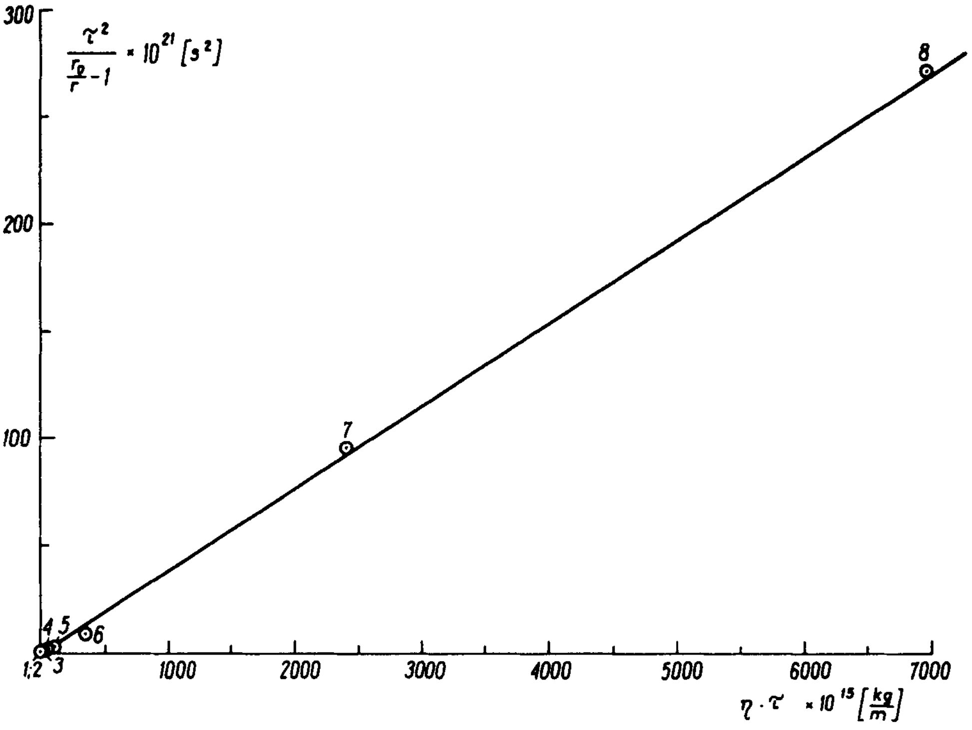
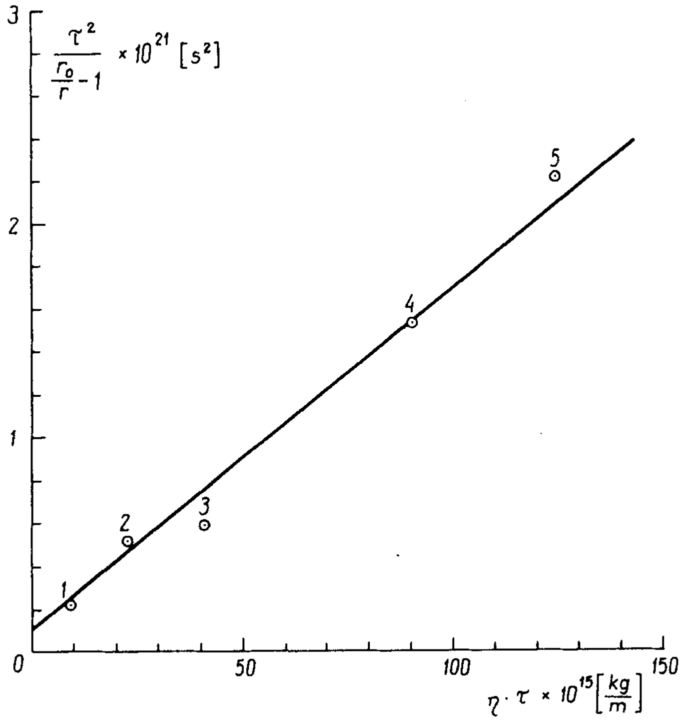


FIGURE 26



FIGURES 26 and 27. Dependence of $\tau^2/(r_0/r - 1)$ on $\eta\tau$ for *trans*-stilbene in different solvents at 293 K; *n*-hexane (1), benzene (2), ethanol (3), *n*-propanol (4), *n*-butanol (5), *n*-heptanol (6), ethylene glycol (7), 1,2-propanediol (8). (Data from Reference 94.)

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with Equation 182. The fluorescence lifetime of *trans*-stilbene in the viscosity range from 0.321 to 45.88 × 10^{-3} Pa · s, varies between 30 and 152 psec.[94] The effective volumes, V_{eff} and the moments of inertia, I_{eff} thus determined for some selected LM have been summarized in Table 8.

The above theory of the rotational depolarization of fluorescence, taking into account the inertial effect, was also verified experimentally for some selected elongated LM in one solvent, cyclohexane, by the method of quenching with nitrobenzene.[60] For example, Figure 28 shows the experimental results for 2,2′-*p*-phenylenebis(-5-phenyloxazole) (POPOP) of the five substances investigated. In all five experiments, a linear dependence according to Equation 182 has been observed. The moments of inertia obtained are lower (slightly more than by one order of magnitude) but still higher than those calculated based on the geometry of the LM. The volume, V, and the inertial moment, I, for POPOP molecules determined in benzene (a solvent of high polarizability) by the quenching method gives slightly higher values than those obtained in cyclohexane. The volume of the POPOP molecule together with its shell varies between $V_{eff} = 782 \times 10^{-30}$ m^3 ($I_{eff} = 1.8 \times 10^{-40}$ kg m^2) in cyclohexane to $V_{eff} = 853 \times 10^{-30}$ m^3 ($I_{eff} = 2.72 \times 10^{-40}$ kg m^2) obtained in benzene.

A year after the report[264] had appeared, a paper[271] was published in which the Brownian rotational motions of nonspherical molecules were investigated in terms of the theory of dielectric relaxation. In order to calculate the angular velocity autocorrelation function for a symmetrical molecule (axis 3 being the symmetry axis) Morita[271] employed a more general Langevin equation differing from that used by Alicki et al.[264] in the terms of the order of ($\omega_k \omega_i$) (see Reference 265).

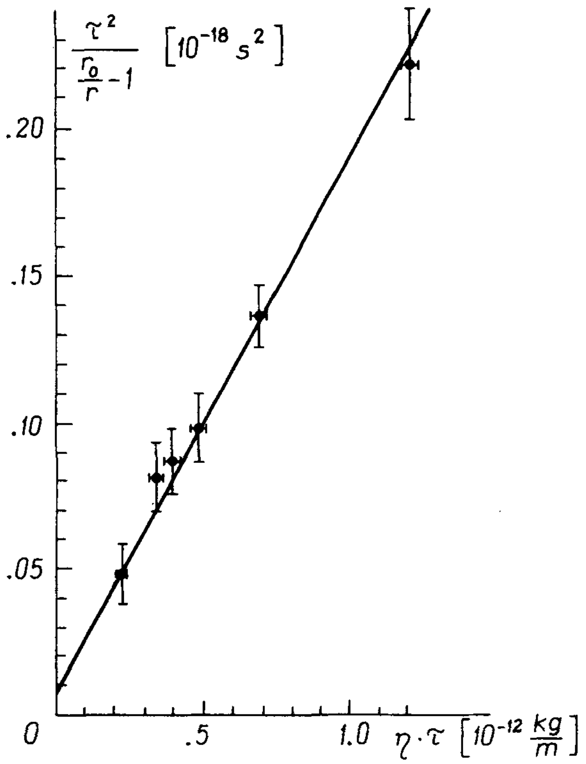

FIGURE 28. Dependence of $\tau^2/(r_0/r - 1)$ on $\eta\tau$ for POPOP in cyclohexane quenched by nitrobenzene. (From Kawski, A. et al. *Z. Naturforsch. Teil A* **1985**, *40*, 313. With permission; in this paper, in Figures 2 and 3, the quantity $\eta\tau$ [10^{-12} kg/m] should be on the abscissae.)

TABLE 8
Effective Volumes, V_{eff}, and Effective Moments of Inertia, I_{eff}, of Some Luminescent Molecules

Compound	V_{eff} (10^{-30} m^3)	I_{eff} (10^{-40} kg · m^2)	Ref.
trans-Stilbene	65	0.0245	270
4-Dimethylamino-ω-methylsulfonyl-*trans*-styrene	170	0.024	71
p-Terphenyl	205	2.45 (2.95)	270 (268)
Diphenylstilbene	898	2.42	60
1,6-Diphenyl-hexa-1,3,5-triene	319	3.15	60

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When the angular velocity autocorrelation function obtained by Morita[271] and derived from the generalized Langevin Equation is taken into account in the theory,[264] the equation for the rotational fluorescence depolarization assumes the form:[61]

$$\frac{\tau^2}{\frac{r_0}{r}-1}=\frac{V\eta\tau}{kT}+\frac{I}{6\cdot kT}+\frac{1}{6}\left\{\frac{\tau^2 b(1-b)^2}{1+\frac{6V\eta\tau}{I}\left(\frac{1+b}{b}\right)}\right\} \qquad (183)$$

where $b = I_3/I$ is the ratio of the moments of inertia ($I_1 = I_2 = I$), V the effective volume of the LM, and τ its mean lifetime in the excited state.

For $I_3 = 0$ and $I_1 = I_2 = I \neq 0$ (the case of a long molecule), $b = 0$; and for $I_1 = I_2 = I_3$ (a sphere), $b = 1$. Hence, the third term in Equation 183 disappears and the Equation obtained by Alicki et al.[264] holds true. For $\eta \to 0$, Equation 183 reduces to:

$$\frac{\tau^2}{\frac{r_0}{r}-1}\to\frac{I}{6\,kT}+\frac{1}{6}\tau^2 b(1-b)^2 \qquad (184)$$

As the viscosity grows, the third term in Equation 183 becomes negligible. It is therefore essential that the validity of Formula 183 be verified for extremely low viscosities and short lifetimes.

According to Equation 183, the knowledge of b, describing the ratio of the minimal moment of inertia ($I_{min} \equiv I_3$) with respect to the long axis of the LM to the maximum moment of inertia ($I = I^{av}_{max}$) perpendicular to this axis, is indispensable. Table 9 summarizes the relevant moments of inertia of three free LM, 2,5-diphenyloxazole (PPO), 2,2′-*p*-phenylene-bis(-5-phenyloxazole) (POPOP) and *p*-bis[2-(5-α-naphthyloxazolyl)]-benzene (α-NOPON) (Figure 29), calculated from the lengths and directions of the interatomic bonds. The fluorescence anisotropy of these LM was investigated in liquid *n*-paraffins (from n = 5 to n = 11) at 304.5 K. The mean lifetimes at 304.5 K in *n*-heptane amount to 1.34, 1.18, and 1.26 nsec for PPO, POPOP, and α-NOPON, respectively, and do not differ essentially from the lifetimes in

TABLE 9
Calculated Mean Moments of Inertia I (in 10^{-44} kg m^2) for Free Molecules

Compound	$I^{\parallel}_{max}$	$I^{\perp}_{max}$	$I^{av}_{max} = (I^{\parallel}_{max} + I^{\perp}_{max})/2$	I_{min}	$b = I_{min}/I^{av}_{max}$
PPO	4.3	4.6	4.45	0.39	0.088
POPOP	18.3	19.7	19.0	1.43	0.075
α-NOPON	32.6	34.1	33.35	1.73	0.052

Denotations:	$I^{\parallel}_{max}$,	the moment of inertia with respect to the axis in the plane of the molecule perpendicular to the long axis of the molecule and passing through the center of mass.
	$I^{\perp}_{max}$,	the moment of inertia with respect to the axis perpendicular to the plane of the molecule and passing through its center of mass.
	I_{min},	the moment of inertia with respect to the long axis of the molecule.

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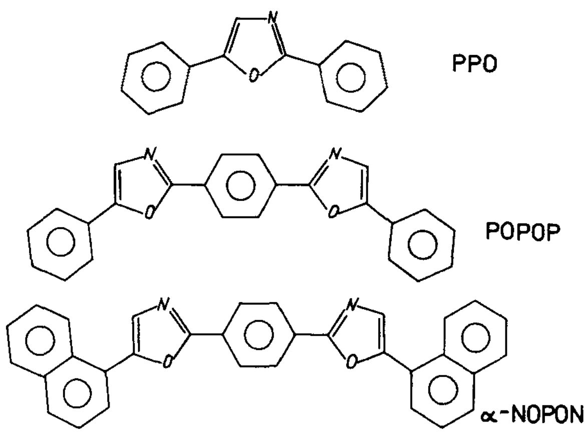

FIGURE 29. Structural formulas of PPO, POPOP, and α-NOPON.

the remaining solvents. The values of the limiting anisotropy, r_0, determined in rigid medium (PVA) amount to 0.324, 0.339, and 0.315 for PPO, POPOP, and α-NOPON, respectively, differing significantly from the value of principal anisotropy $r_p = 0.4$.[61]

Figure 30 shows straight lines plotted through the experimental points by the least squares method, corresponding to Equation 183 with the third term on the right-hand side omitted (i.e., Equation 182). In this way, the effective moments of inertia, I_{eff}, and effective volumes, V_{eff}, listed in Table 10 were determined. For I_{eff}, V_{eff}, and b thus obtained, the theoretical dependences described by Equation 183 are represented by dots in Figure 30. As is readily seen, for all LM investigated, both equations (with and without the third term in Equation 183) correspond very well to the experimental points in a broad range of $\eta\tau$. However, the theoretical curves (dotted lines obtained for Equation 183 embracing the third term) differ only in the range of the lowest $\eta\tau$ values, i.e., when $\eta \rightarrow 0$ or τ becomes very small. This range of $\eta\tau$ is, however, beyond the experimental capabilities of verification for PPO, POPOP, and α-NOPON molecules. It is worth pointing out that the differences for the initial lowest values of $\eta\tau$ between the curves (solid and dotted curves) for α-NOPON is smaller than those observed with POPOP and PPO, i.e., for the lowest ratio, b, of the moments of inertia. This is in agreement with Equation 183, since for $b \rightarrow 0$, the Formula 182 is obtained.

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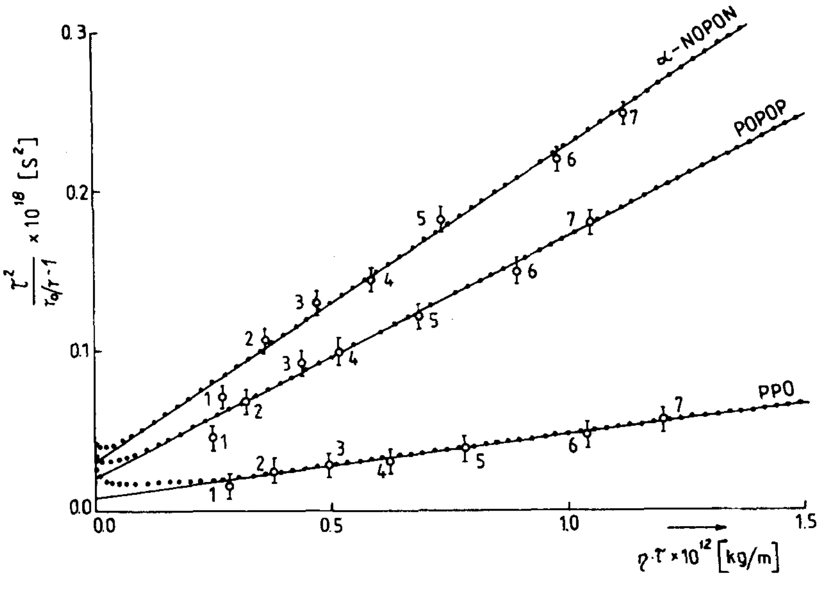

FIGURE 30. Dependence of $\tau^2/(r_0/r - 1)$ on $\eta\tau$ for PPO, POPOP and α-NOPON in different paraffins at 304.5 K: *n*-pentane (1), *n*-hexane (2), *n*-heptane (3), *n*-octane (4), *n*-nonane (5), *n*-decane (6), and *n*-undecane (7). Dots: according to Equation 180. (From Kawski, A. et al. *Z. Naturforsch. Teil A* **1990,** *45,* 1357. With permission.)

TABLE 10
Effective Moments of Inertia, I_{eff}, and Effective Volumes, V_{eff}, of Luminescent Molecules

	I_{eff} (10^{-40} kg · m^2)		
Compound	**Equation 182**	**Equation 183**	V_{eff} (10^{-30} m^3)
PPO	1.5	1.5	171
POPOP	5.1	5.1	624
α-NOPON	7.5	7.5	823

From Kawski, A. et al. *Z. Naturforsch. Teil A* **1967**, *22*, 1507. With permission.

For linear prolate LM with lifetimes of about 1 nsec, and viscosities ranging from 0.22 × 10^{-3} Pa s to 1 × 10^{-3} Pa s, the third term in Equation 183 is of minor importance. In order to have access to the range of $\eta\tau$ in which the difference between the curves considered occurs, it is advisable to examine also LM with mean lifetimes of the order of picoseconds, with parameter $b \neq 0$. The slight deviation of the two curves at low values of $\eta\tau$ does not affect the magnitude of the determined moments of inertia. The values of I_{eff} obtained with Equation 184, valid for $\eta \rightarrow 0$, do not differ from those calculated according to Equation 182. For comparison, the values obtained from the approximation (Equation 183) have also been given in Table 10.

From among the three ω-substituted acceptors in 4-dimethylamino-*trans*-styrenes examined,[71] distinct rotational depolarization was observed for 4-dimethylamino-ω-methylsulphonyl-*trans*-styrene (3a), since the mean lifetime of this compound in *n*-paraffins (see Table 11) amounts to 18 and 29 psec for pentane and hexadecane, respectively, being still sufficiently high for the rotational depolarization to be observed.

For 3a in the whole viscosity range the linear relation (Equation 182) was observed. For this LM, the following values were obtained: $I_{eff} = 2.4 \times 10^{-42}$ kg m^2 and $V_{eff} = 170 \times 10^{-30}$ m^3 (see Table 8). The determined moment of inertia, in this way, is one order greater than the value obtained by Equation 166, which results from the solvent effect.

TABLE 11
Fluorescence Anisotropies, r, Mean Lifetimes, τ, and Correlation Times $\tau_c = V\eta/kT$ of *3a* in different *n*-paraffins at 293 K

Solvent	η (10^{-3} Pa · s)	τ (psec)	**r**	τ_c (psec)
Pentane	0.247	18	0.1644	14.1
Hexane	0.326	19	0.1725	16.2
Heptane	0.420	23	0.1842	22.2
Octane	0.532	25	0.2029	29.5
Nonane	0.665	25	0.2187	35.0
Decane	0.818	26	0.2345	43.4
Undecane	0.993	26	0.2441	48.5
Pentadecane	1.996	27	0.2840	84.3
Hexadecane	2.330	29	0.2950	106.9

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Recently, Alicki and Alicka[265] have extended the previous results[264] to embrace the case of anisotropic diffusion and derived a general expression for a steady-state fluorescence depolarization. The problem was solved by reducing it to the case of Markovian diffusion and using the final results of Chuang and Eisenthal.[138] Applying the method presented in Reference 264, they derived the following approximate integrodifferential equation (generalized diffusion equation) for the probability density p(Ω,t) of a given orientation Ω of LM at time t:

$$\frac{\partial}{\partial t}P(W,t) = -\int_0^t \varkappa[G(s)]P(W,t-s)\,ds \qquad (185)$$

where the operator $\varkappa[\mathbf{D}]$, $\mathbf{D} = (D_1, D_2, D_3)$, is defined as

$$\varkappa[\mathbf{D}] = \sum_{k=1}^{3} D_k L_k^2 \qquad (186)$$

and **L** is the quantum mechanical angular momentum operator. $\{D_k\}$ are real numbers. The functions $G_k(s)$, k = 1, 2, 3, are the angular velocity autocorrelation functions:

$$G_k(s) = \left\langle \omega_k(s)\omega_k(0)\right\rangle, \quad s \geq 0 \qquad (187)$$

The fluorescence anisotropy at time t after an instantaneous light pulse of excitation is

$$r(t) = \int d\mathbf{\Omega}\,P(\mathbf{\Omega},t)R(\mathbf{\Omega}) \qquad (188)$$

where the functions p(Ω,0) and R(Ω) are determined by orientation of the absorption and emission transition dipole moments, respectively. The steady-state fluorescence anisotropy for an exponential decay of fluorescence is expressed by Equation 178.

Introducing the Laplace transformation

$$f(t) \to \hat{f}(\lambda) = \lambda\int_0^\infty e^{-\lambda t} f(t)\,dt \qquad (189)$$

and using Equations 185 to 188 and the properties of transformation (Equation 189), they obtained an expression

$$r = \hat{r}\left(\frac{1}{\tau}\right) = \int d\mathbf{\Omega}\left\{\left[1 + \tau^2 \varkappa\left[\hat{\mathbf{G}}\left(\frac{\mathbf{1}}{\tau}\right)\right]\right]^{-1} P(\mathbf{\Omega},0)\right\} R(\mathbf{\Omega}) \qquad (190)$$

which can also be obtained from the corresponding expression for a Markovian diffusion equation used by Chuang and Eisenthal:[138]

$$\frac{\partial}{\partial t}P(W,t) = -\varkappa[\mathbf{D}]P(W,t) \qquad (191)$$

if D_k is replaced with $\hat{D} \equiv \tau\,\hat{G}_k\,(1/\tau)$.

Hence, one can use the results of Chuang and Eisenthal[138] for the final expression given by Equation 161. In Equation 161 D_k should be replaced with $\hat{D}_k$, D with $\hat{D}$, A with $\hat{A}$, B with $\hat{B}$, and Δ with $\hat{\Delta}$. Similar substitutions should be made in Equations 140, 141, and 142. Coefficient $\hat{D}_k$ can be obtained from the formula:

$$\hat{D}_k = \int_0^\infty e^{-t/\tau}\left\langle \omega_k(t)\omega_k(0)\right\rangle dt \qquad (192)$$

In order to calculate $\hat{D}_k$, the results of Morita[271] were used, which are exact in the case of a symmetrical body and approximate for an asymmetrical one.

It is very often sufficient to use an exponential approximation for $\langle\omega_k(t)\,\omega_k(0)\rangle$ which may be obtained from the approximate Langevin Equation 169, which differs from the exact one used by Morita[271] by terms of order $(\omega_k\omega_l)$. In this case, the Langevin equation may be written as

$$I_k\frac{d\omega_k}{dt} = -\xi_k\omega_k + M_k(t) \qquad (193)$$

Here $\{I_k\}$, $\{\xi_k\}$ are the diagonal elements of the inertial and friction tensor, respectively; $M_k(t)$ is the random torque.

Within this approximation, Alicki and Alicka[265] obtained

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$$\hat{D}_k = D_k \frac{1}{1+6A_k D_k \tau}$$

$$D_k = \frac{kT}{\xi_k}, \quad A_k = \frac{I_k}{6\,kT\,\tau^2} \qquad (194)$$

For the special case considered above, if $I_1 = I_2 = I$, $\xi_1 = \xi_2 = \xi$, and $\mathbf{q}$ and γ are parallel to the axis 3, the well-known Equation 179 is obtained.

Thus, the generalized Equation 161, taking into account the effect of inertia, describes the behavior of the fluorescence emission anisotropy of molecules with any shape and optional position of the transition dipole moment relative to the principal molecular axes. The equation is applicable in the investigations of rotational motions of biopolymer macromolecules, frequently having distinct asymmetrical shape. As has already been mentioned in the previous chapters, it is important to observe the behavior of the emission anisotropy during the decay of fluorescence of the LM investigated. To this end, the formula given by Chuang and Eisenthal[138] for the time-dependent emission anisotropy (Equation 143) can be employed, taking also into account Formula 192 and replacing D_k with $\hat{D}_k$, D with $\hat{D}$, A with $\hat{A}$, B with $\hat{B}$, and Δ with $\hat{\Delta}$.

It should be added that in the investigations of the rotational fluorescence depolarization, with the inertial effect taken into account, an important problem is the proper choice of the autocorrelation function. The autocorrelation functions employed by Alicki et al.[264,265] and Morita[271] are only the approximations of true autocorrelation functions. Lynden-Bell and Steele[272] have assumed that a molecule can temporarily be located in a certain limited cavity in liquid, performing harmonic librations (restricted rotations) with different frequencies. They obtained a function similar, but not identical, to that of Morita, which for the case when the degree of fluctuations of cavities in liquid tends to zero ($\gamma \rightarrow 0$) is equal to the autocorrelation function used by Grzywacz and Trumpakaj.[273] Molecules such as Br_2, CF, and CS_2, which are small compared to luminescent molecules, are considered in References 272 to 274.

The search for a reasonable shape of the angular velocity correlation functions is fully justified and should contribute to further explanation of the nature of rotational motions of fluorescent molecules in liquids.

VII. LIMITED ROTATIONAL MOTIONS IN HETEROGENEOUS MEDIA

If the luminescent molecules, the so-called fluorescent probes, are in an anisotropic environment, particularly in biomembranes or liquid crystals, then following excitation by a polarized light pulse, the anisotropy of emitted fluorescence decays from a maximum value, r(0), to a stationary value, r(∞), after a certain period of time.[275] This suggests that LM in an anisotropic medium perform wobbling motions rather than free rotations.[276,277]

These investigations have important applications in biology, particularly in connection with the dynamic organization of lipids in natural and model membranes.[278,279] Fluorescence analysis of dynamic systems can be performed either with decay or steady-state measurements. As fluorescence probes, such compounds as 1-anilino-8-naphthalene sulfonic acid, perylene, 2-methylanthracene, *N*-phenyl-1-naphthylamine, and 1,6-diphenyl-1,3,5-hexatriene (DPH)[280] are very often used. DPH is the most widely used probe for estimating membrane microviscosity. It has an all-*trans*-polyene structure and a rod-like shape. When the DPH molecule is excited in the long-wave absorption band, the absorption and fluorescence transition dipole moments lie along the major axis of the molecule.[43] Data on the location and interaction of fluorescent probes in natural membranes can be found in articles by Radda and Vanderkooi[281] and Sackmann[282] and also in the book by Lakowicz.[205]

From the fluorescence emission anisotropy measured under constant illumination, Shinitzky and Barenholz[278,283] estimated the "microviscosity" of membranes. This interpretation assumed the rotational motion of DPH (or perylene) to be isotropic. The addition of cholesterol to these phospholipid membranes increased the magnitude of their steady-state fluorescence anisotropy. Hence, it was inferred that cholesterol increased the microviscosity of the membrane interior.

Veatch and Stryer[275] carried out nanosecond fluorescence anisotropy studies to gain a deeper understanding of the effect of cholesterol on membrane dynamics. Sample solutions were excited by light pulses and the time courses of fluorescence decay were measured by the single photon counting technique. The nanosecond fluorescence emission anisotropy, r(t), measurements carried out by Veatch and Stryer[275] for DPH in liposomes, consisting of di-(dihydrosterculoyl) phosphatidyl choline and varying amounts of cholesterol, are shown in Figure 31. A striking change in emission anisotropy kinetics was observed as the cholesterol content was increased from 0 to 33 mol%, whereas there was virtually no change in the excited-state lifetime (7.0 nsec). Twenty nanoseconds after the light pulse, the magnitude of the emission anisotropy becomes markedly higher with the increasing cholesterol content, showing that cholesterol restricts the rotational mobility of DPH. These observed r(t) data were satisfactorily fitted to a two-component decay

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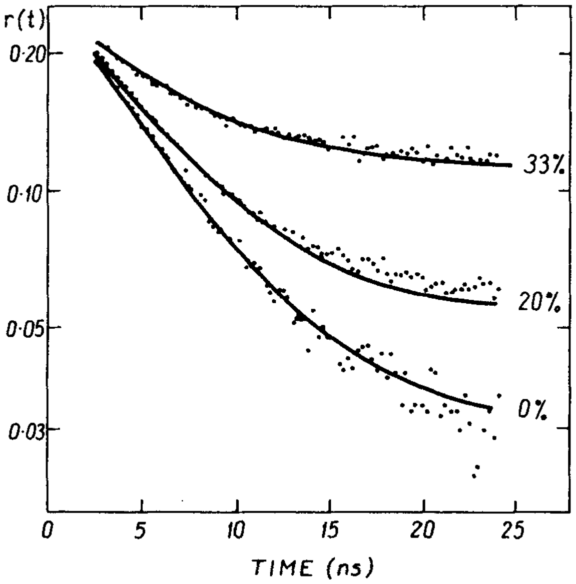

FIGURE 31. Nanosecond emission anisotropy kinetics of DPH in liposomes consisting of di-(dihydrosterculoyl) choline and varying amounts of cholesterol (20 to 33 mol %). The filled circles represent the observed emission anisotropy. The curves calculated from Equation 209 (Table 12) are shown as solid lines. (Experimental data from Reference 275.)

$$r(t) = A_1 + A_2 e^{-t/\tau_c} \tag{195}$$

in which A_1 corresponds to a constant component during the observed interval and τ_c is the rotational correlation time.

These nanosecond emission anisotropy studies demonstrate that DPH undergoes more than one kind of rotational motion in the investigated membrane interior. Similar behavior of DPH was observed in dimyristoyl and dipalmitoyl phosphatidyl choline liposomes.[230,277] The fluidity of the membrane interior for probes such as DPH cannot be satisfactorily characterized by a "microviscosity". The concept of microviscosity is only valid for such an environment that is both homogeneous and isotropic. According to Chen et al.,[230] cholesterol renders the membrane interior anisotropic. Consequently, the emission anisotropy curves exhibit two components since the rotational motion of the transition dipole moment of DPH is not isotropic and cholesterol enhances this rotational anisotropy.

In order to express the time dependence of emission anisotropy, r(t), a theory of fluorescence polarization decay in membranes was worked out by Kinosita et al.[276] Two cases have been discussed: a rod-shaped molecule with the emission transition moment parallel and perpendicular to the long axis. Since the general expressions obtained are very complex, the resultant expression for r(t) was compared with an approximate expression, which was later justified.[284–286]

We assume that the absorption and transition dipole moments of a prolate ellipsoid molecule are incident along the major axis of the LM. We further assume that OY is the direction of the excitation propagation and OZ is the direction of the electric vector, E, of the exciting light (Figure 32). $I_{\parallel}$ and $I_{\perp}$ designate the components of the fluorescence intensity parallel and perpendicular to E as observed along the x-axis, respectively. The emission anisotropy is given by Equation 4. The fluorescent solution is excited with a very short light pulse (δ-function-shaped) at time t = 0. Various excited LM will emit fluorescence at

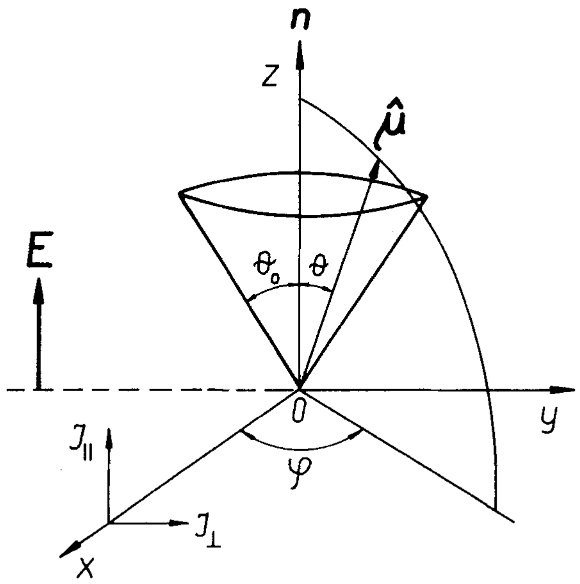

FIGURE 32. Wobbling-in-cone model.

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different times t >> 0. At the initial moment, $^3/_5$ (= $I_{\parallel}(0)/I(0)$) of LM (at first isotropically distributed), as a result of photoselection, belong to the z-axis, and 1/5 (= $I_{\perp}(0)/I(0)$), to each of the remaining axes (Figure 33).

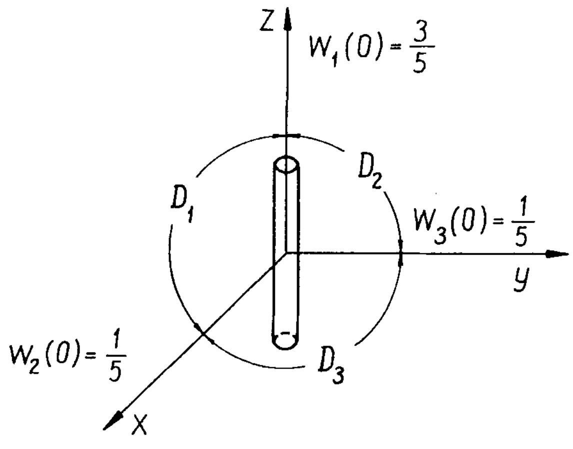

FIGURE 33. Geometry of the system.

If molecular rotation occurs, these fractions are time dependent. For the three components:

$$\left[\frac{I_{\parallel}(t)}{I(t)}\right]_z = W_1(t), \quad \left[\frac{I_{\perp}(t)}{I(t)}\right]_x = W_2(t),$$
$$\left[\frac{I_{\perp}(t)}{I(t)}\right]_y = W_3(t) \qquad (196)$$

we have the following differential equations:[257,286]

$$\frac{dW_1}{dt} = -2(D_1 + D_2)W_1 + 2D_1W_2 + 2D_2W_3 \quad (197)$$

$$\frac{dW_2}{dt} = +2D_1W_1 - 2(D_1 + D_3)W_2 + 2D_3W_3 \quad (198)$$

$$\frac{dW_3}{dt} = +2D_2W_1 + 2D_3W_2 - 2(D_2 + D_3)W_3 \quad (199)$$

valid for time $t > t_0$, t_0 being the terminal moment of excitation. D_1, D_2, and D_3 are the rotational coefficients of the LM diffusion towards the three axes.

For a prolate ellipsoid of revolution: $D_1 = D_2 = D$. Besides, the relation

$$W_1(t) + W_2(t) + W_3(t) = 1 \qquad (200)$$

holds. The emission anisotropy in our case is determined solely by $W_1(t)$:

$$r(t) = \frac{3}{2}W_1(t) - \frac{1}{2} \qquad (201)$$

Thus we need only the solution of Equation 197. Equation 197 with 200 and $dW_1(\infty)/dt = 0$ can be written as

$$\frac{dW_1(t)}{dt} = -6[W_1(t) - W_1(\infty)] \qquad (202)$$

with the general solution ($W_1(\infty) \neq 0$):

$$W_1(t) = [W_1(0) - W_1(\infty)]e^{-6Dt} + W_1(\infty) \qquad (203)$$

with the conditions $W_1(0) = {}^1/_3 + {}^2/_3\, r(0)$ and $W_1(\infty) = {}^1/_3 + {}^2/_3\, r(\infty)$, according to Equation 201, we have

$$W_1(t) = \frac{1}{3}[1 + 2r(\infty)] + \frac{2}{3}[r(0) - r(\infty)]e^{-6Dt} \quad (204)$$

This equation holds, if the motions are restricted so that the emission anisotropy cannot drop below the value of r(∞). In a similar way we obtain the expression for

$$W_2(t) = \frac{1}{3}[1 - r(\infty)] - \frac{1}{3}[r(0) - r(\infty)]e^{-6Dt} \quad (205)$$

Provided the decay of total (emitted in all directions) fluorescence intensity follows the simple exponential law $I(t) = I_0 \exp(-\gamma t)$, from Equations 204 and 205 we obtain the following equations for the parallel and perpendicular components of fluorescence intensity, if the motions are restricted:

$$I_{\parallel}(t) = \frac{I_0}{3}[1 + 2r(\infty)]e^{-\gamma t} + \frac{2}{3}I_0[r(0) - r(\infty)]e^{-(\gamma + 6D)t} \quad (206)$$

$$I_{\perp}(t) = \frac{I_0}{3}[1 - r(\infty)]e^{-\gamma t} - \frac{I_0}{3}[r(0) - r(\infty)]e^{-(\gamma + 6D)t} \quad (207)$$

where $\gamma = 1/\tau$. Equations 206 and 207 were published by Weber.[287]

For free rotational motion of LM $r(\infty) = 0$, and Equations 206 and 207 result in the well-known Jabłoński Equations 37 and 38.[72–75] From Equations 201, 204, and 205 (also from Equations 206, 207, and 3) for the time-dependent emission anisotropy we obtain:[257,286]

$$r(t) = r(\infty) + [r(0) - r(\infty)]e^{-6Dt} \quad (208)$$

or

$$\frac{r(t)}{r(0)} = A + (1 - A)e^{-6Dt} \quad (209)$$

where

$$A = \frac{r(\infty)}{r(0)} \quad (210)$$

Equation 209 is a good approximation of the general expression by Kinosita et al.[276] for the "wobbling-in-cone" model. The orientation of the emission transition dipole moment $\hat{\mu}$ (the long axis) is confined within the cone around the normal **n** to the membrane (see Figure 32) and fluctuates within this cone with a wobbling diffusion constant D_w, which is constant throughout the cone. In this case, Equation 210 has the form:[276]

$$A = \frac{r(\infty)}{r(0)} = \left[\frac{1}{2}\cos\theta_0(1 + \cos\theta_0)\right]^2 \quad (211)$$

For $\theta_0 = \pi/2$ parameter A is equal to zero $[r(\infty) = 0]$ and the decay of the emission anisotropy follows the exponential law.

Lakowicz et al.[284,285] pointed out that the above interpretation of r(∞)/r(0) is model-dependent. The difficulty in this "wobbling-in-cone" model is that the calculation of the θ_0 value from r(∞)/r(0) depends upon the existence of a square-well potential in the membrane. A more reasonable interpretation of the r(∞) value seems the fact of r(∞) being related to the order parameters, S, of the fluorescent probe.

The model-independent interpretation of the limiting fluorescence emission anisotropy, r(∞), of probes embedded in membrane suspensions given by Lipari and Szabo[288] is adequate.

If either the absorption transition dipole moment μ_a, or the emission transition dipole moment, μ_e, of the probe lies along its unique symmetry axis (the long molecular axis, $\hat{\mu}$), the time-dependent emission anisotropy, r(t), can be expressed in terms of the correlation function as:[136,288]

$$r(t) = \frac{2}{5}P_2(\cos\beta)\langle P_2[\hat{\mu}(0)\cdot\hat{\mu}(t)]\rangle \quad (212)$$

where β is the angle between μ_a and μ_e and $P_2(x) = {}^3/_2\, x^2 - {}^1/_2$ is the second Legendre polynomial. The unit vector $\hat{\mu}(t)$ specifies the orientation of the probe at time t in a coordinate system attached to the membrane (X_M, Y_M, Z_M in Figure 34). The

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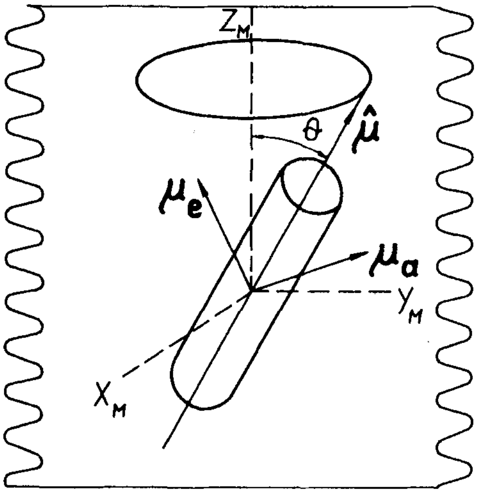

FIGURE 34. Schematic representation of a prolonged fluorescence probe embedded in a membrane.

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angular brackets indicate an equilibrium average, defined as

$$\langle(\cdots)\rangle = \int_0^{2\pi} d\varphi \sin\theta \, d\theta(\cdots)P_{eq}(\theta) \qquad (213)$$

where $P_{eq}(\theta)$ is the normalized equilibrium orientation distribution function (see Equation 41), i.e.:

$$2\pi\int_0^{\pi} \sin\theta \, d\theta \, P_{eq}(\theta) = 1 \qquad (41')$$

To obtain an expression for the explicit time dependence of r(t) from Equation 212 it is necessary to assume a model for the dynamics of the probe. However, r(0) and r(∞) can be computed independently of any model. The emission anisotropy, r(0), can be found as

$$r(0) = \frac{2}{5}P_2(\cos\beta)\langle P_2[\hat{\mu}(0)\cdot\hat{\mu}(0)]\rangle = \frac{2}{5}P_2(\cos\beta) \qquad (214)$$

The general expression for r(∞), where neither μ_a nor μ_e lie along the unique symmetry axis of the probe is[288–290]

$$r(\infty) = \frac{2}{5}P_2(\cos\theta_e)P_2(\cos\theta_a)\langle P_2(\cos\theta)\rangle^2 \qquad (215)$$

θ_a and θ_e are the angles between μ_a and μ_e and the unique symmetry axis of the probe, respectively. Equation 215 is valid not only for a "rod-shaped" probe (Figure 34), but also for a "disk-shaped" probe. As pointed out by Lipari and Szabo,[288] the most probable orientation of a rod-shaped probe is such that $\hat{\mu}$ is perpendicular to the plane of the membrane, while for a disk-shaped probe it is parallel to this plane. In a simple case, instead of Equation 215, it follows that:

$$r(\infty) = \frac{2}{5}P_2(\cos\beta)\langle P_2(\cos\theta)\rangle^2 \qquad (216)$$

a result that is consistent with Equation 25 in the work of Kinosita et al.[276]

Equations 215 and 216 relate the limiting behavior of the fluorescence anisotropy to the order parameter, S, of the probe, defined by Saupe[291] as

$$S = \langle P_2(\cos\theta)\rangle \qquad (217)$$

The Saupe order parameter plays an important role in NMR, electron spin resonance, and fluorescence probe studies of membranes.[292–300] The order parameter determines the first nontrivial term in the series expansion of the orientational distribution function in terms of Legendre polynomials:[288]

$$\begin{aligned} P_{eq}(\theta) &= \sum_{\ell=0}^{\infty} \frac{4\ell+1}{4\pi}\langle P_{2\ell}(\cos\theta)\rangle P_{2\ell}(\cos\theta) \\ &= \frac{1}{4\pi} + \frac{5}{4\pi}\langle P_2(\cos\theta)\rangle P_2(\cos\theta) + \cdots \end{aligned} \qquad (218)$$

Thus, the order parameter provides model-independent information about the orientational distribution function at equilibrium, or equivalently, the potential in which the fluorescent probe moves.

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Kinosita et al.,[276,277] as mentioned above, have developed alternative interpretation in terms of a model in which $\hat{\mu}$ can undergo free rotational diffusion within a cone of semiangle θ_0 (Figure 32). This corresponds to the normalized equilibrium distribution:

$$P_{eq}(\theta)=\begin{cases} {}^{1}\!/_{2}\pi(1-\cos\theta) & 0\le\theta\le\theta_0 \\ 0 & \theta>\theta_0 \end{cases} \qquad (219)$$

Using this equilibrium distribution gives:

$$S=\left\langle P_2(\cos\theta)\right\rangle=\frac{1}{2}\cos\theta_0\left(1+\cos\theta_0\right) \qquad (220)$$

If either μ_a or μ_e is parallel to $\hat{\mu}$, then by combining this result with Equations 214 and 216, one finds

$$\frac{r(\infty)}{r(0)}=\left\langle P_2(\cos\theta)\right\rangle^2=\left[\frac{1}{2}\cos\theta_0\left(1+\cos\theta_0\right)\right]^2 \qquad (211')$$

which is in agreement with Equation 211.

The first nontrivial expansion of the orientational distribution in a series of Legendre polynomials (Equation 218) is the most unique information about $P_{eq}(\theta)$ that can be extracted from the measurements of $r(\infty)/r(0)$.[288]

To obtain the time dependence of r(t) according to Equation 212, it is necessary to evaluate the correlation function $\langle P_2[\hat{\mu}(0)\hat{\mu}(t)]\rangle$, which requires the choice of a dynamic model of the probe. For the model corresponding to diffusion in a cone, Kinosita et al.[276] showed that

$$\frac{r(t)}{r(0)}=\left\langle P_2\left[\hat{\mu}(0)\cdot\hat{\mu}(t)\right]\right\rangle=\sum_i A_i e^{-D_w t/\sigma_i} \qquad (221)$$

where D_w is the "wobbling" diffusion constant and A_i and σ_i are constants which depend on θ_0.

Kinosita et al.[276] presented a convenient approximate expression for r(t)/r(0), similar to Equation 209:

$$\frac{r(t)}{r(0)}=A_\infty+\left(1+A_\infty\right)e^{-D_w t/\langle\sigma\rangle} \qquad (222)$$

where $A_\infty = r(\infty)/r(0)$ is given by Equation 211′ and

$$\langle\sigma\rangle=\sum_{i\neq\infty} A_i\sigma_i \qquad (223)$$

The simple Expression 222 for r(t)/r(0) agrees to a good approximation with the theoretical Expression 221, as was shown by Kinosita et al.[276] Quantity $\langle\sigma\rangle/D_w$ is the time in which the initially photoselected distribution of orientations approaches the stationary distribution. It was also shown that the relaxation time is proportional to $[r(0) - r(\infty)]/r(0)$.

Figure 31 shows the fluorescence anisotropy decay curves calculated from Equation 209 for different parameters A. The best fits of the observed data[275] to Expression 209, which is identical with Equation 195, are given in Table 12. It can be seen that the magnitude of $A = r(\infty)/r(0)$ markedly increases as the cholesterol content in the membrane is enhanced. Indeed, the sum $A_1 + A_2 = r(0) = 0.28$ (Table 12) is constant. The rotational correlation time $\tau_c = 1/6D$ of the rapid motion appears to be virtually unaffected by cholesterol.[286]

Many experiments using polarized fluorescence for anisotropic systems have been performed with a steady-state method.[207,280,284,293,301] In this case, the emission anisotropy is given by averaging r(t) (Equation 209) over the decay process and may be expressed in the form:[257,286]

$$\frac{r}{r(0)}=\frac{1}{\tau}\int_0^t\frac{r(t)}{r(0)}e^{-t/\tau}\,d\tau=A+\frac{1-A}{1+6D\cdot\tau} \qquad (224)$$

which is equivalent to:[284,285]

$$r(\infty)=r+\frac{r-r(0)}{6D\cdot\tau} \qquad (225)$$

The curves from Equation 224 computed for different parameters A have identical course as in Figure 23. In a special case, when $A(\infty) = 0$ $[r(\infty) = 0]$ the Perrin Formula 63 is obtained. The deviation from the Perrin linear dependence may be explained by the fact that the rod-shaped LM in heterogeneous complex media and biological

TABLE 12
Effect of Cholesterol on Emission Anisotropy of DPH in Liposomes

Cholesterol (mol %)	$\tau_c = 1/6D$ (nsec)	$A_1 = r(0) - r(\infty)$	$A_2 = r(\infty)$	$A_1 + A_2 = r(0)$	$A = r(\infty)/r(0)$	θ_0
0	6 ± 1	0.27 ± 0.03	0.01 ± 0.01	0.28 ± 0.03	0.036	72.94°
20	6 ± 1	0.24 ± 0.02	0.04 ± 0.01	0.28 ± 0.03	0.143	59.79°
33	6 ± 1	0.16 ± 0.01	0.11 ± 0.01	0.26 ± 0.02	0.423	41.82°

membranes do not freely rotate but perform irregular librations within a definite solid angle. The measured emission anisotropy (steady state) depend on both molecular ordering and molecular libration of the chromophore in this case.

Equation 222 was generalized to embrace the case when fluorescent probes are attached to macromolecules by simply multiplying them by $\exp(-6D_M t)$, where D_M is the rotational diffusion coefficient of the macromolecule.[288] The equation obtained was compared with the empirical expression used by Munro et al.[233] to analyze their data on the fluorescence emission anisotropy of tryptophans in a variety of proteins. The Munro et al. expression follows from the Lipari and Szabo[288] equation if one assumes that the wobbling motion is much faster than the overall rotational motion of the macromolecule (i.e., $\tau_M = 1/6D_M \gg \tau_{eff} = 1/6D_w$).

The last years witnessed a progressive refinement in the technique of time-domain measurements of emission anisotropy decay and the application of this method to a wide variety of biopolymer and membrane systems. The report on the current status of the technique and some selected applications can be found in the review of Steiner.[214]

A theory of time-dependent fluorescence depolarization in liquid crystals and membranes has been developed by Zannoni[302,303] and recently by Fisz.[304] In general, it is assumed that the orientational distribution of the fluorescent probe molecules in their ground and excited states are the same. However, from the experimental results of Johansson,[298] it follows that the orientational distribution of DPH probe in the ground and excited states are not the same, while for perylene both distribution functions are equivalent.

VIII. FLUORESCENCE EMISSION ANISOTROPY OF SOLUTIONS IN AN ELECTRIC FIELD

If a solution of polar LM is placed in an external electric field of strength **E**, directed parallel to the OZ-direction (see Figures 1 and 2), then, as a result of additional orientation of the electric dipole moments in the direction of the field applied, the emission anisotropy increases compared to that observed in the absence of the field. This effect was first detected by Czekalla[14,15] and employed for the determination of electric dipole moments, μ_e, in excited singlet (S_1) state. In simplified Czekalla's theory[14] the mean lifetime, τ, of the excited LM is assumed to exceed markedly the rotational relaxation time, τ_R, which results in a complete depolarization of fluorescence ($r \approx 0$) in the absence of the external electric field. It is assumed, moreover, that the mean lifetime of the excited LM is high enough, compared to the dielectric relaxation time of the solvent molecules, for the fluorescence to occur from the state of statistical equilibrium between the excited LM and their surroundings (solvent).[129] Thus, the electric dipole moment, μ_e, in the excited state is responsible for the increase in the fluorescence emission anisotropy caused by the external electric field.

Under these assumptions Czekalla[14] derived the following equation:

$$r_E = \frac{1}{15}\left(\frac{3}{2}\cos^2\alpha - \frac{1}{2}\right)\left(\frac{\mu_e}{kT}\right)^2\left(\frac{\varepsilon+2}{3}\right)^2 E^2 \qquad (226)$$

where ε is the electric permittivity of the solvent, α is the angle between the electric dipole moment, μ_e, and the direction of the transition moment μ ($S_1 \leftarrow S_0$). In most cases it can be assumed

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(e.g., for stilbene and styrene derivatives, a = 0), that the directions of μ_e, μ_g, and μ are the same.

When the observed emission anisotropy in the absence of electric field is different from zero and amounts to r_i, then in the electric field the total emission anisotropy is

$$r = r_i + r_E \tag{227}$$

Expression 227 is only valid for small values of r_i.

In the cases when the initial emission anisotropy, r_i, is of a magnitude comparable to the limiting emission anisotropy, r_0, in the absence of the Brownian rotational motions, it is indispensable to take into account the rotational diffusion of LM under the influence of the electric field (which is the case for short-lived molecules, such as stilbene and styrene derivatives).

The theory of the rotational diffusion of dipoles in an electric field was worked out by Benoit.[305] Employing Benoit's theory under conditions of isotropic depolarization, Weber[18] derived an equation which allows the calculation of the excited dipole moment when the equilibrium distribution is not attained during the excited state. Such calculations yielded the equation for the fluorescence emission anisotropy in the electric field:

$$r_E = r_i + \frac{1}{10}\left(\frac{\mu_g}{kT}\right)^2 E_{eff}^2 \left\{ \frac{9}{7} r_i + \frac{\left(\frac{r_0}{r_i}-1\right)^2 n^2}{\frac{r_0}{r_i}\left(2+\frac{r_0}{r_i}\right)} + \frac{\frac{27}{5}\cos\beta\left(\frac{r_0}{r_i}-1\right)n}{\frac{r_0}{r_i}\left(2+\frac{r_0}{r_i}\right)} \right\} \tag{228}$$

where $r_0/r_i = 1 + 6D\tau$ is the Perrin Equation 56 and $n = \mu_e/\mu_g$.

From this equation the following conclusions can be drawn:

1. The field-dependent emission anisotropy is additive to that observed in the absence of the field;
2. The emission anisotropy in the field depends upon both μ_g and μ_e; when r_i approaches r_0, the contribution of μ_g becomes particularly important;
3. If r_0/r_i and $n = \mu_e/\mu_g$ are not too large, which is the usual case in practice, the third term in Equation 228 makes an appreciable contribution.

Calculations carried out by Weber[18] on the basis of Equation 228 give results qualitatively similar to those of Czekalla and other authors (see Table 13 for three selected compounds). In view of experimental difficulties, few reports have appeared up to now.[14,15,17,19,308–310]

The most general theory was developed by Liptay,[16] who took into account the absorption and fluorescence band shifts due to the change in the electric dipole moments ($\mu_e \rangle \mu_g$), and the fact that the lifetime of LM might be comparable to the dielectric relaxation time. The general expression for the increment in the emission anisotropy, $\Delta r = r_E - r$ (where r_E is the degree of emission anisotropy when the electric field is applied and r is the degree of emission anisotropy in the absence of the field), was obtained by Liptay[16] as a function of squared electric field intensity, which involves: (1) dipole moments and polarizabilities of LM examined in the ground and excited singlet states, (2) characteristics of the absorption and fluorescence spectra, (3) the product of lifetimes and the probability of reorientation, $k_A\tau$. In the case of LM with sufficiently high dipole moments in the ground and excited states, the terms incorporating the polarizabilities may be neglected. In this case, the complicated equation for Δr reduced to a simple one.

The magnitude of $k_A\tau$ can be obtained from the Perrin Equation , generalized by Liptay,[16] for the case of a liquid solution:

$$r = \frac{3\cdot\left(\mu_{abs}\cdot\mu_{em}\right)^2 - 1}{5\cdot\left(1 + k_A\tau\right)} \tag{229}$$

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TABLE 13
Dipole Moments[a] of Some *trans*-Stilbene Derivatives in the First Excited, S_1, State

Compound	$n = \mu_e/\mu_g$	μ_g	μ_e^b	μ_e^c	Dichroism μ_e^d	Solvent effect μ_e^e	μ_e^f
					10^{-30} C·m (Debye)		
Dimethylamino-nitrostilbene	3.25	23.8 (7.15)	76.7 (23)	73.1 (21.9)	76.7 (23)	77.0 (23.1)	70.7 (21.2)
Amino-nitro-stilbene	3.30	21.7 (6.5)	70.0 (21)	70.0 (21)	— —	69.0 (20.7)	83.6 (25.0)
Dimethylamino-cyanstilbene	3.72	23.2 (6.95)	63.4 (19)	40.0 (12)	68.4 (20.5)	74.4 (22.3)	67.2 (20.1)

[a] The conversion factor for the dipole moment: $[\mu]_{SI}/C \cdot m = 3.33564 \times 10^{-30} [\mu]_{cgs}/D$, where D is the symbol for debye and 1D = 10^{-18} esu cm.
[b] Equation 228.
[c] Equation 226.
[d] Data from Reference 306.
[e] Data from Reference 307.
[f] Data from Reference 19.

(μ_{abs} and μ_{em} are the unit vectors for absorption and emission transition moments), by measuring the emission anisotropy, r, of LM in a rigid solution (i.e., in a frozen solution). For $k_A\tau \approx 0$, the magnitude of $(\mu_{abs} \cdot \mu_{em})^2$ can be determined from Equation 229, and then from the same expression for fluid solution, $k_A\tau$.

The electric dipole moments in the first excited singlet state, μ_e, were measured by Czekalla et al.[17] and recently by Kawski and Kubicki,[19] (Table 13) using the generalized Liptay's Equations.

It seems worthwhile mentioning a model of Weill and co-workers[308,309] pertaining to the emission anisotropy in an external electric field. This model is an interesting example of the application of some of Czekalla's and Weber's ideas in the biophysics of macromolecules. It was evidenced that by measuring the emission anisotropy in a solution placed in an external electric field one obtains valuable information on the structure and properties of biologically active macromolecules (DNA and its complexes, globular and fibrous proteins).

IX. MENTION OF MEASUREMENT TECHNIQUES

The methods used in the fluorescence measurements are primarily divided into three groups:

1. Statical (steady-state), when the excitation light intensity is constant, I(t) = const
2. Pulse excitation, when I(t) is a pulse of the order of pico- or nanoseconds
3. Modulation, when the excitation light intensity is modulated (Formula (77)

The principles of the emission anisotropy measurements by the use of the steady-state and time-resolved techniques were discussed in detail in many review articles.[8,9,24,26–33,205] The time-resolved emission can be determined using time-correlated single-photon counting, streak cameras or from the frequency response of the emission to the intensity-modulated light. The frequency-domain fluorometers described by Lakowicz and Gryczyński[311,312] provide good resolution of multiexponential intensity[313,314] and emission anisotropy decays,[197,234,235,315] enabling time-dependent rate constants[316,317] and conformational distributions[317,318] to be obtained. More complex emission anisotropy decays are expected for hindred probes in membranes or for macromolecules with segmental flexibility.

The present state-of-the-art instrumentation for frequency-domain measurements and the future development have also been presented in the review of Lakowicz and Gryczyński.[312] Frequency-domain fluorometers are commercially available and are used in many laboratories as standard equipment.

X. FINAL COMMENTS

The intention of this review is the presentation of the principal problems concerning the fluorescence emission anisotropy in rigid and liquid isotropic solutions, as well as in partially ordered fluid systems, and to indicate the possibilities of the practical use of these phenomena in physicochemical and biophysical investigations.

The dependence of the initial course of the emission anisotropy r(t), as well as the dependence of its stationary value of r on the moment of inertia of a luminescent molecule, seems to be an important, recently discovered, effect.[263–265,319] This effect is related to the fact that the change of the stage of rotating luminescent molecules is conditioned not only by the value of acting instantaneous moment of a force but also by its inertia (it depends on its moment of inertia). This problem requires further investigation, especially the kinetics of the decay of fluorescence depolarization in initial stages. Until now, no singularities of initial stage have been reported in the picosecond range. The initial stage, in which nonexponential time dependence of fluorescence emission anisotropy should be seen, is difficult to observe inasmuch as the excitation pulse always has a finite width comparable to that of the autocorrelation function decay $\tau_a = I/\xi$ (see Equation 172).

We do hope that the fast development of pulsed experimental methods will also enable this problem to be solved.

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