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Journal

The Journal of Physical Chemistry, 93(10)

ISSN

0022-3654

Authors

Piston, David W
Bilash, Timothy
Gratton, Enrico

Publication Date

1989-05-01

DOI

10.1021/j100347a019

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temperature and above is associated with the immobility of the dimer species and the enhanced bonding in the excited state.

Acknowledgment. The research was supported in part by the U.S. Office of Naval Research (J.D.B. and B.D.) and by the NSF

(G.H. and J.I.Z.). We thank O. M. Stafsudd and A. Pechenik for many helpful discussions and D. Vivien and D. Gourier (Ecole Nationale Supérieure de Chimie, Paris) for ESR measurements.

Registry No. Na⁺-β''-alumina, 11138-49-1; Cu⁺, 17493-86-6.

Compartmental Analysis Approach to Fluorescence Anisotropy: Perylene in Viscous Solvents

David W. Piston, Timothy Bilash, and Enrico Gratton*

Department of Physics, Laboratory for Fluorescence Dynamics, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801 (Received: September 6, 1988; In Final Form: November 22, 1988)

The fluorescence and polarization anisotropy decays of perylene in viscous solvents are investigated at several temperatures between -20 and 35 °C by using the technique of multifrequency phase and modulation fluorometry. The anisotropy decay data are globally analyzed over all temperatures studied and fit directly to physical quantities by using a compartmental model. We present a generalized compartmental model that can be used to calculate anisotropy decay arising from any type of interconversion that introduces a change in the fluorescence polarization. This generalized model provides a simple method to calculate the anisotropy decay for systems undergoing different kinds of interconversions such as excited-state reactions, rotational diffusion, "jump" motions, or any combination which can be treated in a compartmental form. The basic idea is to consider a rotation as an interconversion between compartments which represent different geometries with respect to the laboratory frame. Compartments due to other processes can be added to these rotational compartments. A matrix is constructed by using the decay rates of each compartment and the rates of interconversion between these compartments. The polarization of each compartment and initial conditions are contained in vectors. Using this formalism, the fluorescence anisotropy decay can be represented as a standard eigenvector-eigenvalue problem which may be solved analytically or numerically. As a simple example, this compartmental model is applied to the perylene molecule undergoing only rotational diffusional motions, and is used to globally fit the anisotropy decay data at various temperatures and viscosities. We fit directly to the physical quantities needed to define the compartments, using only three fitting parameters, two rotational diffusion constants, and the average angle between the absorption and emission dipoles. In previous studies, four parameters were used to fit the anisotropy decay of perylene, that is, two rotational correlation times and two preexponentials. The results of this study are shown to be in agreement with previous measurements.

Introduction

Perylene has been considered to be an anisotropic rotator since steady-state polarization measurements first showed the existence of at least two distinct rotational motions.¹ More recently, studies of the time-resolved anisotropy decay as a function of temperature and/or viscosity have confirmed the observation of two rotational correlation times, which have been attributed to a disklike structure of the perylene molecule.²⁻⁵ However, these previous studies do not agree about the relative magnitude of the in-plane and out-of-plane rotational diffusion constants. In addition, previous analyses of the anisotropy decay data have been performed with more free parameters than the physical model for the perylene molecule strictly requires, and have not used a global analysis over many temperatures and/or viscosities to guarantee the best fit to all data simultaneously. By introducing these two constraints on our analysis, we can extract the fitting parameters of the system with a higher degree of confidence than by analyzing single experiments independently as sums of exponential components in the anisotropy decay.

For a nonspherical molecule, the observed fluorescence anisotropy decay may be complex, and this can lead to cases where, for a single fluorescence experiment, several different values of the fitting parameters may give nearly equally good fits to the data. This is a well-known effect due to the strong correlation between decay rates and preexponential factors. It is therefore important to find the set of fitting parameters which yields both sensible physical values and is internally consistent with all of the data sets. In the past, mathematical models used to analyze anisotropy data have been based on the diffusion equation.⁶⁻⁹

However, such analyses of the anisotropy decay of perylene, modeled as a disk, have been performed using four fitting parameters; that is, the rotational correlation times and preexponential factors have been fit independently, even though the theory gives only three independent parameters.^{2,4} In our analysis, we assume perylene to be a disk-shaped molecule. We also assume that both the absorption and emission dipoles lie in the plane of the disk. These two assumptions allow us to define one of the molecular axes to be collinear with the absorption dipole. To assure the correct relationship between the rotational correlation times and the preexponential factors in our analysis, we use a compartmental model to do a "target" analysis,¹⁰ that is, an analysis where we consider only solutions which have the correct mathematical form based on the physical model. We also know that, where the Stokes-Einstein relationship is valid, the rotational diffusion rates should vary by a factor of T/η , where T is the temperature and η is the viscosity of the medium. This relationship is well verified when the solvent molecules are small compared

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* To whom correspondence should be addressed.

with the rotating molecule, and it has been assumed in previous investigations of perylene in glycerol and glycols. In our study, we also assume the validity of the Stokes–Einstein relationship and use this relationship to link the rotational diffusion constants from different experiments. This linkage allows us to perform a global analysis of all the data at different temperatures and viscosities, thus recovering the rotational diffusion rates which (1) are consistent with a particular geometrical shape for the molecule, (2) agree with the Stokes–Einstein relationship, and (3) best describe all of the data simultaneously.

We have chosen to use the compartmental model since it allows us to separate species associated processes from decay associated processes from the beginning of our model. In the derivation of the anisotropy decay using the diffusional approach, this separation is not usually performed. Species associated processes involve the physical parameters of the system, but it is the decay associated processes that are observed. For example, some spatial compartments of a single molecular species may have different decay rates, yet this should not affect the rotational rates, since rotations are dependent only on the physical parameters of the molecular species and not on the decay rates. Also, for molecules undergoing excited-state reactions (which by their definition are in a compartmental form), the use of the diffusion equation becomes complicated,¹¹ whereas the compartmental model allows us to simply add an extra compartment for each excited-state product.¹² The compartmental model contains only intuitive, straightforward assumptions and can be used to fit data directly to physical parameters. For perylene, these parameters are two rotational diffusion constants corresponding to the in-plane and out-of-plane rotational motions and the average angle between the excitation and emission dipoles. The mathematical formalism of this model is a simple eigenvalue–eigenvector problem which allows us to obtain an analytical form for observable quantities, namely the rotational correlation times. This approach was first introduced by Weber,¹³ who later derived the equation for an anisotropic rotor with the excitation–emission dipole coincident with one of the symmetry axes of the molecule.¹⁴ Jump kinetic models have previously been used to describe rotations in the fields of dielectric relaxation and NMR.^{15,16} In the work presented here, Weber's approach is generalized to model the anisotropy change due to any type of interconversion (e.g., excited-state reactions) and not solely for rotations.

In this paper, we first present an outline of the model in the case where the absorption dipole is aligned with one of the principal axes of rotation and show how it can be used to calculate both time and frequency domain quantities. We then present the application of the model to the perylene system and finally use this application to fit differential phase and modulation data for perylene in viscous solvents.

Theory

As stated above, instead of using the diffusion equation to determine the relationship between the rotational diffusion constants, rotational correlation times, and preexponential factors, we use a compartmental analysis, which yields the rotational correlation times and preexponential factors as a function of the physical rotational parameters, decay rates and experimental conditions. In this section, we first present the general method used to calculate anisotropy decay with the compartmental model in the case where the absorption dipole is aligned with one of the principal axes of rotation of the fluorophore. This alignment assumption is valid for any disk-shaped molecule, such as perylene, where the absorption dipole is in the plane of the disk.

In general, to implement the compartmental model for n compartments, we use the following procedure. First we must define

a state vector, \mathbf{S} , which contains n entries, one for each compartment. These entries can represent different spatial orientations of the system, or possibly excited-state products. Next, we define a matrix of depopulation rates for each compartment

$$\mathbf{A} = \begin{pmatrix} -k_1 - \sum k_{i1} & k_{12} & \dots & k_{1n} \\ k_{21} & -k_2 - \sum k_{i2} & \dots & k_{2n} \\ \vdots & \vdots & \ddots & \vdots \\ k_{n1} & k_{n2} & \dots & -k_n - \sum k_{in} \end{pmatrix} \quad (1)$$

where k_i is the decay rate of the i th compartment and k_{ij} is the rate of interconversion from the j th to the i th compartment. This matrix contains only constant rates, which can be interconversion rates or decay rates. An initial condition vector, \mathbf{B} , which contains the initial excited population of each compartment, is determined by the dipole selection law under excitation by polarized light and goes as the average of $\cos^2 \theta$ over the initial distribution of dipoles. Here, θ is the spherical coordinate of declination from the Z axis, and ϕ is the angle from the projection on the X – Y plane to the X axis. The initial conditions vector can be determined in this manner only for the case of the absorption dipole aligned with one of the principal rotational axes. Once we have defined these quantities, we can solve the eigenvalue problem $\mathbf{AS} = \lambda\mathbf{S}$ with boundary conditions, \mathbf{B} . This type of matrix always gives real and positive eigenvalues,¹⁷ although in practice it is difficult to obtain an analytical solution for $n > 6$. In the case of larger matrices, we can easily solve the eigenvalue problem numerically on a computer. The resulting λ_i 's give the decay associated rates and the eigenvectors, E_i , will lead to the preexponentials as shown below. The E_i 's each have j components, E_{ij} . Since we would like to use this method to calculate anisotropy decay, we must introduce our experimental geometries to the model. We define two polarization vectors, P_{\perp} and P_{\parallel} , such that the i th component of P_{\perp} is the relative perpendicular contribution to the fluorescence intensity of the i th compartment and the same for P_{\parallel} . These vectors are defined by the experimental geometry of the observation and are multiplied by the E_i 's, to obtain the preexponential factors. We can then use the results of our eigenvalue problem, along with the \mathbf{P} vectors, to calculate $I_{\parallel}(t)$, $I_{\perp}(t)$, and the total intensity, $I(t)$, with the following formulas

$$I_{\parallel}(t) = \sum_{i=1}^n e^{-\lambda_i t} \sum_{j=1}^n P_{\parallel j} E_{ij} \quad (2)$$

$$I_{\perp}(t) = \sum_{i=1}^n e^{-\lambda_i t} \sum_{j=1}^n P_{\perp j} E_{ij} \quad (3)$$

$$I(t) = I_{\parallel}(t) + 2I_{\perp}(t) \quad (4)$$

and of course the time-resolved anisotropy,

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} \quad (5)$$

This expression can be applied directly in the time domain or Fourier transformed, as follows, to fit the differential phase and modulation data obtained in our experiments. These results give the decay associated parameters, even though our model begins with only species associated parameters.

To do the Fourier transform, it is easiest to separately transform the parallel and perpendicular components. The real and imaginary parts of these transforms are proportional to

$$G_n = \int_0^{\infty} I_n \cos(\omega t) dt \quad (6)$$

$$S_n = \int_0^{\infty} I_n \sin(\omega t) dt \quad (7)$$

where n denotes either the parallel or perpendicular component.

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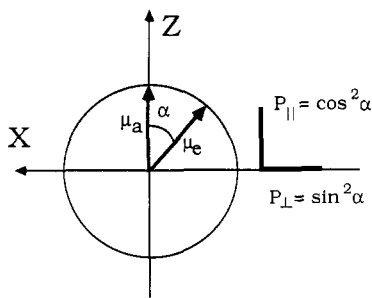


Figure 1. Model of perylene molecule as observed from + Y axis in the Z - X orientation. μ_a is the absorption dipole, μ_e is the emission dipole. P_{\perp} and P_{\parallel} are the projections of the intensity due to this emission dipole.

The differential phase, Δ , and differential modulation, Λ , can be derived in terms of the S 's and G 's¹⁴ as

$$\Delta = \tan^{-1} \frac{S_{\parallel}}{G_{\parallel}} - \tan^{-1} \frac{S_{\perp}}{G_{\perp}} \quad (8)$$

$$\Lambda = \left[\frac{S_{\perp}^2 + G_{\perp}^2}{S_{\parallel}^2 + G_{\parallel}^2} \right]^{1/2} \quad (9)$$

Using this method, we can calculate the anisotropy decay of many different fluorescent systems, and in particular, systems undergoing excited-state reactions.

As an application of this model, we now consider the case of perylene in a viscous solvent. We model the perylene molecule as a disk, with both the excitation and emission dipoles in the plane of the molecule. Since we have chosen 442 nm as the excitation wavelength, we can expect these two dipoles to be almost collinear; however, we include an average angle between them in the fitting routine to account for the observed initial anisotropy, r_0 , which near room temperature has been observed to be less than its theoretical limiting value of 0.4.²⁻⁵ We consider the perylene disk to have only two rotational rate constants R_{\parallel} and R_{\perp} . These rates correspond to twice the rotational diffusion constants, since each rate represents diffusion in both the forward and reverse directions. R_{\parallel} relates to the in-plane or parallel motion and R_{\perp} relates to the out-of-plane or perpendicular motion. Although the absorption and emission dipoles may be nonaligned, we assume that they are both in the plane of the disk. We first define our state vector to be the six independent spatial orientations of this system. These six states correspond to the two orientations of a disk along each of the three Cartesian axes.¹⁴ For the X axis, the first orientation is with the plane of the disk in the X - Y plane and the second one in the X - Z plane. These two orientations are distinguishable only if the absorption and emission dipoles are not aligned. Since there are six spatial states and no excited-state reactions, our eigenvalue problem will be based on a 6×6 matrix, and all vectors will contain six components. We define the S vector as

$$S = \begin{pmatrix} Z-X \\ X-Y \\ Y-Z \\ Z-Y \\ X-Z \\ Y-X \end{pmatrix} \quad (10)$$

where in each term of the vector, the first axis in an entry shows the location of the absorption dipole and the two together show the plane of orientation of the disk in the laboratory frame. For example, the first entry, Z - X , represents the disk in the Z - X plane so that the absorption dipole is aligned along the laboratory Z axis. Any other orientation of the disk is a linear combination of this basis set of states. If the laboratory axes are labeled so that the system is excited by light that is traveling in the negative X direction and is polarized along the Z axis with observation from the positive Y direction, the P vectors are determined by taking the intensity projections of the emission dipole in each com-

partment along the laboratory Z axis for the parallel direction and the X axis for the perpendicular direction (for the first entries in P_{\parallel} and P_{\perp} (see Figure 1). The P vectors are

$$P_{\parallel} = \begin{pmatrix} \cos^2 \alpha \\ 0 \\ \sin^2 \alpha \\ \cos^2 \alpha \\ \sin^2 \alpha \\ 0 \end{pmatrix} \quad (11)$$

$$P_{\perp} = \begin{pmatrix} \sin^2 \alpha \\ \cos^2 \alpha \\ 0 \\ 0 \\ \cos^2 \alpha \\ \sin^2 \alpha \end{pmatrix} \quad (12)$$

where α is the average angle between the absorption and emission oscillators. We can now construct our matrix by considering which states interconvert with a rate R_0 and which with a rate R_1 . Since perylene shows a single-exponential total intensity decay, we assume that the fluorescence from all compartments decays with the same rate, k . The matrix for this system is

$$A = \begin{pmatrix} -Q & 0 & 0 & R_0 & R_1 & R_0 \\ 0 & -Q & 0 & R_0 & R_0 & R_1 \\ 0 & 0 & -Q & R_1 & R_0 & R_0 \\ R_0 & R_0 & R_1 & -Q & 0 & 0 \\ R_1 & R_0 & R_0 & 0 & -Q & 0 \\ R_0 & R_1 & R_0 & 0 & 0 & -Q \end{pmatrix} \quad (13)$$

where $Q = k + 2R_0 + R_1$. To determine the initial conditions, we determine the contribution of all dipoles to each of the six spatial states. In this case we begin with an isotropic (nonaligned) sample, so we must integrate $\cos^2 \theta$ over all space with a uniform distribution of dipoles. For the initial population of a compartment with a dipole in the Z axis this integral is

$$B_z = \int_0^{2\pi} d\phi \int_0^{\pi} \sin \theta d\theta \cos^2 \theta (\hat{r} \cdot \hat{z})^2 \quad (14)$$

where the first term is the probability of exciting a dipole with angle θ from the excitation polarization (in our geometry this is the Z axis), and the second term, $(\hat{r} \cdot \hat{z})^2$, gives the intensity projection of the dipole on the Z axis. For B_z we use the identity $\hat{r} = \cos \theta \hat{z}$, and this integral reduces to

$$B_z = 2\pi \int_0^{\pi} \cos^4 \theta \sin \theta d\theta = \frac{4\pi}{5} \quad (15)$$

This calculation is the same for the initial populations along the X and Y axes by noting the two identities $\hat{r} = \sin \theta \sin \phi \hat{y}$ and $\hat{r} = \sin \theta \cos \phi \hat{x}$. The values are equal and are both $4\pi/15$. Since only the relative populations of each compartment are important to this calculation, we can normalize the initial conditions vector which now becomes

$$B = \begin{pmatrix} 0.3 \\ 0.1 \\ 0.1 \\ 0.3 \\ 0.1 \\ 0.1 \end{pmatrix} \quad (16)$$

The solution to the eigenvalue problem, $AS = \lambda S$, gives six eigenvalues. Two pairs of these are repeated roots, and one of the eigenvalues is not observable. In fact, the eigenvector corresponding to this nonobservable eigenvalue is always zero after the boundary conditions for this particular experiment are applied. The three observable eigenvalues are

$$\lambda_1 = k \quad (17)$$

$$\lambda_2 = k + 3R_0 \quad (18)$$

$$\lambda_3 = k + R_0 + 2R_1 \quad (19)$$

λ_1 is the fluorescence decay rate, and the other two eigenvalues are a combination of rotational correlation rates plus the decay rate. The lifetime terms all cancel out of the calculation of the anisotropy decay. For the case of a disk-shaped molecule with both the absorption and emission dipoles in the plane of the disk, this model yields the same rotational correlation rates and their corresponding preexponential factors as the results of the diffusion equation solution presented by Chaung and Eisinger.⁷

We have developed a program to utilize this model to fit differential phase and modulation data. In this program the eigenvectors are not determined analytically, but numerically using the IMSL (Houston, TX) eigenvector routine with the initial conditions applied. The value for the decay rate is determined separately by a magic-angle excitation experiment. The resulting I_{\parallel} and I_{\perp} are Fourier transformed as described above and then used to fit the differential phase and modulation data from the perylene experiments directly to the three physical parameters of this model, α , R_1 and R_0 .

Materials and Methods

Perylene was purchased from Aldrich and used without further purification. The solvents, propylene glycol from Baker and glycerol from Aldrich, were both spectroscopic grade. Samples were prepared by adding 25 μ L of saturated perylene in ETOH to 1.5 mL of the solvent. Both the fluorescence lifetime and anisotropy decay measurements were performed on the instrument previously described by Gratton and Limkeman,¹⁸ using a HeCd laser at 442 nm as the excitation source. The laser light passes through a beam-splitting polarizer and then a Pockel's cell which has a sinusoidally varying electric field applied to it. The resulting output from this setup is a polarized beam of sinusoidally intensity modulated laser light, which is used as the excitation beam for our experiment. The fluorescence emission is observed at a right angle from the excitation. Anisotropy decay measurements are performed using vertically polarized light for excitation and observing the differences in both phase and modulation between the emission observed in the vertical (parallel) and horizontal (perpendicular) directions of polarization. Total fluorescence intensity decay measurements are done using magic angle (54.7° from vertical) excitation and collecting the total emission. To eliminate interference of scattered light, all emissions were observed through a 470-nm band-pass filter (Corion P470-10). The temperature of the sample was monitored throughout the experiment by measuring the temperature of alcohol in the reference cuvette. Lifetime measurements were performed at the same time as the anisotropy experiments. These measurements were done by using a glycogen scatter as a reference. This scatter is in the same solvent as the sample.

The anisotropy decay data analysis is performed using a global analysis program based on a Marquardt-Levenberg nonlinear least-squares algorithm.¹⁹ The compartmental anisotropy model described above has been incorporated into the global analysis program as a subroutine which is called iteratively throughout the minimization. This subroutine calculates the expected differential phase and modulation values for each temperature. In order to profit from a global analysis, one needs to be able to link specific parameters between different experiments. For the perylene case, the angle α was assumed to be constant over the temperature range studied and was linked across all experiments. However, while the rotational rates cannot be directly linked between these experiments (because, as mentioned above, they change with temperature and viscosity), they can be linked between experiments using the Stokes-Einstein relationship. In this case, the moments of inertia of the perylene molecule remain constant, and we can map these values to the rotational rates as a linear

TABLE I: Temperature and Viscosity Values for Glycerol and Propylene Glycol^{20,21}

temp, K	glycerol		propylene glycol	
	η , cP	T/η	η , cP	T/η
253			1750	0.1446
258			979	0.2635
263			585	0.4496
268			364	0.7363
273	9420	0.0290	243	1.123
278	5180	0.0537	162	1.716
283	3090	0.0916	111	2.550
288	1700	0.1694	78.5	3.669
293	1150	0.2548	56.0	5.232
298	715	0.4168		
303	500	0.6060		
308	338	0.9112		

TABLE II: Recovered Average Angles between Absorption and Emission Dipole, α , and In-Plane, R_1 , and Out-of-Plane, R_0 , Rotational Rates for Perylene from a Global Analysis across Temperature and Viscosity^a

solvent	α , deg	R_0 , ns ⁻¹	R_1 , ns ⁻¹
glycerol ($k = 0.217$)	15.9 \pm 0.8	0.013 \pm 0.015	0.293 \pm 0.015
propylene glycol ($k = 0.192$)	21.3 \pm 1.0	0.024 \pm 0.002	0.309 \pm 0.018
both solvents	15.9 \pm 0.6 (Gly) 22.3 \pm 0.7 (PG)	0.023 \pm 0.003	0.283 \pm 0.013

^a For both solvents α is allowed to vary with solvent. R_0 and R_1 values are given for $T/\eta = 1$.

function of T/η . The simultaneous analysis of data at different temperatures is thus performed by analyzing the data in terms of a constant α and a single pair of rotational rates which are linked at the multiple temperatures through the Stokes-Einstein relationship. The error of each fitting parameter is determined by performing an absolute confidence interval analysis.¹⁹ In this routine, we choose one parameter to step along and observe the change in the minimum χ^2 as a function of that parameter. This minimum value is determined for each step by fixing the parameter and performing a complete least-squares fit. We then plot the minimum χ^2 value along each fitting parameter axis, and we can statistically determine one and two standard deviations in the χ^2 value which correspond to the areas of 66% and 99% confidence in our recovered fitting parameters. The errors reported in the fitting parameters correspond to the area of 66% confidence in our fitting parameters.

For the magic-angle measurements, the lifetimes are determined for each temperature by using a single-exponential decay fit provided by ISS software (ISS, Champaign, IL).

Experimental Results

In both solvents the fluorescence lifetime varies by less than 0.1 ns throughout the temperature range. We obtain average lifetimes of 4.7 ns in glycerol and 5.2 ns in propylene glycol. For glycerol, this is in agreement with previously reported values of 4.6–4.8 ns.^{2–5} Since changing the lifetime by 0.1 ns had no effect on the anisotropy decay data fits, we chose to use the average values for all fits throughout the temperature range. The viscosity as a function of temperature for both solvents was taken from the literature.^{20,21} The values of T , η , and T/η used for the global fits are shown in Table I. Recovered values for α , R_0 , and R_1 from the global analyses for each solvent and for both solvents simultaneously are listed in Table II. The R values reported in this table are for $T/\eta = 1$ and must be multiplied by 2 times the relative T/η for each experiment to obtain the rotational diffusion constant for each temperature. The fitting errors are determined by the absolute confidence interval method described in the Materials and Methods section and are relatively symmetric.

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TABLE III: Values for Preexponentials, β_1 and β_2 , Initial Anisotropy, r_0 , and Rotational Constants, R_1/R_0 , for This and Previous Studies

study (excitation λ)	β_1	β_2	r_0	R_1/R_0
Barkley et al. ² (450 nm)	0.10	0.24	0.34	10 ± 1
Lakowicz et al. ⁸ (442 nm)	0.12	0.19	0.31	8.8
this study (442 nm)				
glycerol	0.10	0.25	0.35	22 ± 10
propylene glycol	0.10	0.22	0.32	12.8 ± 1.5
both solvents	0.10	<i>a</i>	<i>a</i>	11.8 ± 1.3

^a β_2 and r_0 are dependent on α which is allowed to be independent in each solvent.

Therefore, we report only one error value for both the plus and minus values. This error corresponds to the area of 66% confidence in our fitting parameters.

Discussion

We have developed a technique for analyzing in a simple way time-resolved anisotropy decay data, which yields the relevant physical parameters of the system. In this section, we discuss the results of the specific application of the technique to perylene and how these results compare with previous investigations of this system. It is not the purpose of this paper to discuss the particular physical situations that justify the use of large jump motions, such as the 90° rotations used in this work, in contrast to the infinitely small steps assumed by the continuous diffusion equation. However, we only utilize the mathematical equivalence of the two approaches for the case of a disk-shaped molecule with both the absorption and emission dipoles in the plane of the disk.

The results of our analysis have been shown above in Table II. Although we fit directly to physical parameters, we can calculate the initial anisotropy, r_0 , and the individual preexponential factors, β , from our fitting parameters. These are shown in comparison with previous studies in Table III. The values for the ratio of R_0 to R_1 from this and previous studies are also listed in the table; however, the values of the rotational diffusion constants in previous investigations have been calculated by the theoretical relationship between the rotational correlation times (their fitting parameters) and the rotational diffusion constants (as given above). In our analysis, we fit directly to these rotational diffusion rates. A previous study of the perylene system using multifrequency differential phase and modulation fluorometry⁵ was severely limited in resolution since only one temperature was investigated and thus no global analysis could be performed. In the previous time domain study which investigated perylene in the 450-nm excitation range,² the results are very consistent with our frequency domain studies, as can be seen in Table III. While the r_0 and β 's change as a function of excitation wavelength, the ratio R_0/R_1 should be the same for all excitations. Additional studies have been performed using excitation near 250 nm, and they report values of R_0/R_1 from 6.5 to 28. A thorough list of these studies has been reported by Christiansen et al.⁴ The value of R_1 remains approximately constant between the two solvents, with a small uncertainty in both solvents. The value of the out-of-plane rotational rate, R_0 , changes considerably between the two solvents investigated. Due to the high viscosity of glycerol at the lower temperatures it is difficult to accurately determine the value of R_0 , since it is very slow as compared with the fluorescence lifetime. In fact, there is a large uncertainty associated with the value of this parameter when we perform the absolute confidence interval analysis. When the two solvents are considered together in a global

analysis, however, the value of R_0 is recovered quite accurately. In this analysis of the results from both solvents simultaneously, the values of the rotational rates are linked directly between both solvents via the Stokes-Einstein relationship, thus assuming that the shape of the molecule is the same in each solvent. However, the angle α is allowed to vary between the two solvents.

The recovered values for α presents two problems. First, it is greater in the less viscous solvent (propylene glycol), and second, since α is not zero, r_0 is less than its theoretical limit of 0.4. The difference between solvents may be due to the difference in dielectric constant. Since we know that there is a lifetime change between solvents, it is not unreasonable to assume that the fluorescence process is slightly different in each solvent. Indeed, the excitation spectrum of perylene in glycerol is slightly blue-shifted from that in propylene glycol. At low temperatures (−60 °C), the excitation spectra of perylene in both solvents narrow, and also, the initial anisotropy at 442 nm becomes 0.4. This may indicate that there is some overlap between the S_1 and S_n transitions, which decreases with temperature, and is different between the two solvents at the temperatures studied (see Table I). At 442 nm, we are exciting the S_1 transition, which has a limiting anisotropy near 0.4; however, the S_n transition has a limiting anisotropy near −0.2, so any overlap would result in an apparent drop in the limiting anisotropy of the S_1 band. This difference will not affect the rotational rates, since they depend only on the structural properties of the molecule. If there is a difference between the overlap in the two solvents, this could explain the change in α . This change in α is also seen in the simultaneous global fit between the two solvents which allows for a different value of the angle in each solvent. In this fit, any polarization change between the two solvents which is not due to the in-plane or out-of-plane rotations will manifest itself as a change in the angle α . Since we have measured r_0 to be 0.4 at low temperatures in both solvents (data not shown), this effect would have to be temperature dependent, which is consistent with the narrowing of the excitation bands at lower temperatures. A second possible reason for a lower observed r_0 is the presence of a fast ($\ll \tau$) depolarizing motion which is manifested in a lower r_0 rather than by a rotational rate in our fit. However, the steady-state polarization shows a smooth increase as a function of temperature, which is inconsistent with the change in slope that would be observed due to an independent fast motion. We are currently further investigating this effect.

In this paper, we have demonstrated the applicability of the compartmental model to calculate the fluorescence anisotropy decay due to anisotropic rotations and its ability to recover the physical parameters of this motion. We have also described the general outline of this model to demonstrate its possible applications to more complicated systems. The advantages of this model are that it is simple to understand and easy to apply in a computer program. It is a model in which, from the beginning, one needs only to include the physical situation under investigation, rather than first solving the general case, and then making the simplifications for the particular experiment of interest. Most importantly, the results of this method are shown to be in agreement with those of the diffusion equation approach to anisotropic rotations, and in many cases the compartmental model is easier to use.

Acknowledgment. The experiments and analysis described here have been made possible through the support of grants from NSF (PCM 84-03107) and NIH (PHS 1P41-RR-03155).

Registry No. Perylene, 198-55-0.