ANALYTICAL APPLICATIONS OF TIME-DEPENDENT FLUORESCENCE DEPOLARIZATION*,**

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Time-dependent fluorescence depolarization is a novel technique for the determination of the rotational diffusion time of electronically excited molecules. With the help of a Boxcar integrator and dye laser excitation this method can be used in routine investigations with a time resolution of up to 100 ps. Its wide scope of applications is demostrated by examples in which the dependence of rotational diffusion time on the temperature, the solvent, the excitation wavelength, and the aggregation effects of organic dyes are determined.

1. Introduction

If an isotropic solution of fluorescent molecules is excited with polarized light, the emitted light is polarized to a certain degree which, according to Jabłoński, depends on the nature of the solute and solvent molecules and on the temperature [1]. The degree of polarization is time-dependent and decreases mainly because of the rotational diffusion during the lifetime of the excited molecule. This phenomenon can be observed as fluorescence depolarization and is due to Brownian molecular motions. Another way of determining the kinetics of molecules within the lifetime of electronically excited molecules is to measure the depolarization behaviour in absorption with repopulation of the ground state after excitation with laser [2, 3].

Recently, several attempts have been made to measure directly the time dependence of molecular polarization in the pico- and nasosecond ranges. However, picosecond pulse spectroscopy methods in absorption [2, 3] or emission [4, 5] are very laborious apart from operating in the single pulse regime and are confined to a limited number of excitation

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wavelengths; thus it has been hardly possible to use them on a general scale in analytical chemistry so far.

Phase fluorimetry using a cw argon laser also gave a time resolution of about 3 ps [6]. Data obtained by this method are more reliable, but it is again limited to a few excitation wavelengths if one wants to make use of such advantages as a high sensitivity which is obtained by excitation with laser.

For analytical purposes it therefore suggests itself to use pulse fluorimetry with flash lamps in combination with sampling technique [7] or single photon counting technique [8]. While these methods have the advantage of freely tunable excitation wavelengths in the UV and visible spectral regions, their applications are restricted because of the low intensity of the flash lamps and the fact that the flash duration limits time resolution to > 1 ns.

Provided the Boxcar integration method is used for detection, the low sensitivity due to the low flash lamp intensity can be avoided by using pulsed nitrogen and pulsed dye lasers [9, 10]. This method has been further improved by us and is now also applicable to routine studies of time-dependent fluorescence depolarization. With the arrangement described below we studied in rhodamine 6G and acridine orange the dependence of the rotational diffusion time on the viscosity and the temperature of the solvent, on the nature of the solvent, on the excitation wavelength and, in acridine orange, the interdependence of rotational diffusion time and aggregation effects.

2. *Mathematical formalism*

The total, time-dependent fluorescence intensity $I(t)$ is given by Eq. (1) as

$$I(t) = I_{\parallel}(t) + 2I_{\perp}(t). \tag{1}$$

For single exponential decay the fluorescence lifetime τ_{Fl} can be calculated from $I(t)$ by Eq. (2)

$$I(t) = I_0 \exp(-1/\tau_{Fl}). \tag{2}$$

The time-dependent emission anisotropy $r(t)$ is defined by Eq. (3) as

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} \tag{3}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities measured when the analyzer is parallel and perpendicular, respectively, to the polarizer after excitation.

For simplicity spherical and symmetrical molecules and hence only isotropic rotational processes are assumed in the following[1]. Further, other depolarization mechanisms, such as energy transfer, intramolecular torsional vibration and concentration depolarization, are only considered insofar as they have any bearing on our experiment.

[1] For a comprehensive theoretical treatment of the problem with reference to molecular anisotropy and potential libration processes compare [4, 8, 11].

Under the described simplified assumptions it holds Eq. (4)

$$r(t) = r_0 \exp(-t/\tau_D^*) \tag{4}$$

where τ_D^* is the rotational diffusion time in the excited state. According to the Stokes-Einstein hydrodynamical model τ_D^* as well as τ_D in the ground state can be expressed in terms of the diffusion coefficient D and the solvent viscosity η, respectively, and the effective molecular volume V_{eff}:

$$\tau_D = \frac{1}{6D} = \frac{\eta V_{eff}}{kT}, \tag{5}$$

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

3. Experimental

The laser spectrofluorimeter is shown schematically in figure 1. A nitrogen laser (IGL 300 of the Zentrum für Wissenschaftlichen Gerätebau der Akademie der Wissenschaften der DDR, Berlin — pulse width 2.0 ns, output 300 kW) pumps a broadband dye

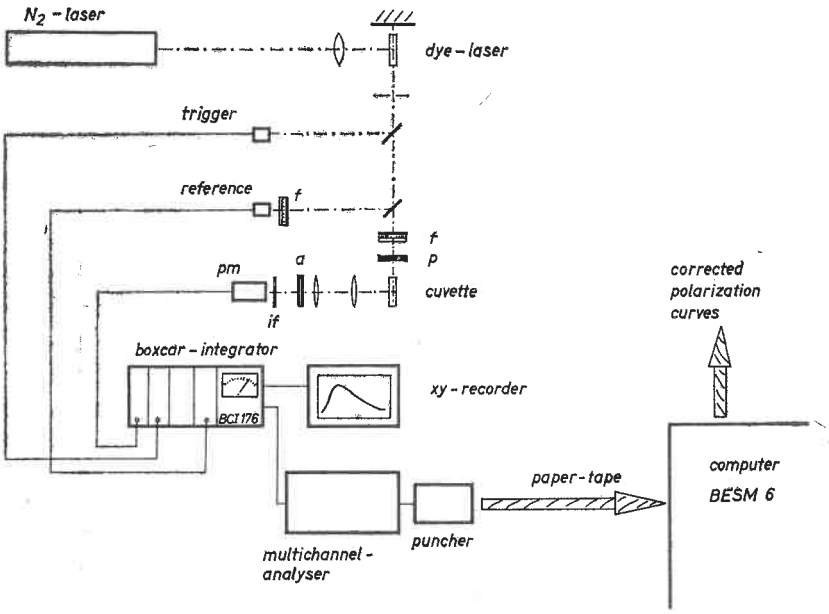

Fig. 1. Experimental set-up of laser spectrofluorimeter (for description see text)

laser whose light passes polarizer (p) and falls on the fluorescent sample in the cell. A neutral density filter is inserted to prevent saturation effects. After passing through analyzer (a) and an interference filter (if) the fluorescent light is taken at right angles to the exciting light and given on the photomultiplier (pm) (type 14 ELU FK, USSR). The spectral transmission of the filter (if) is matched to the maximum of the emission of the dye under study. The signals coming from the multiplier are processed by a Boxcar integrator

(BCI 176 of the Zentrum für Wissenschaftlichen Gerätebau der Akademie der Wissenschaften der DDR, Berlin — time resolution 250 ps); the Boxcar integrator is triggered by a rapid-acting opto-electronic trigger device [12]. Any variations in laser intensity are compensated by ratioing with the help of a reference channel. The pulse width of the dye laser was reduced to 700 ps after a method described by Mory et al. [13], and the time response of the multiplier was reduced from 1.7 ns to 600 ps by means of a compensating network [14].

Under these conditions the decay time of the measured excitation pulse was 0.7 ns. The measured data were recorded by a X–Y plotter and stored simultaneously in a multi-

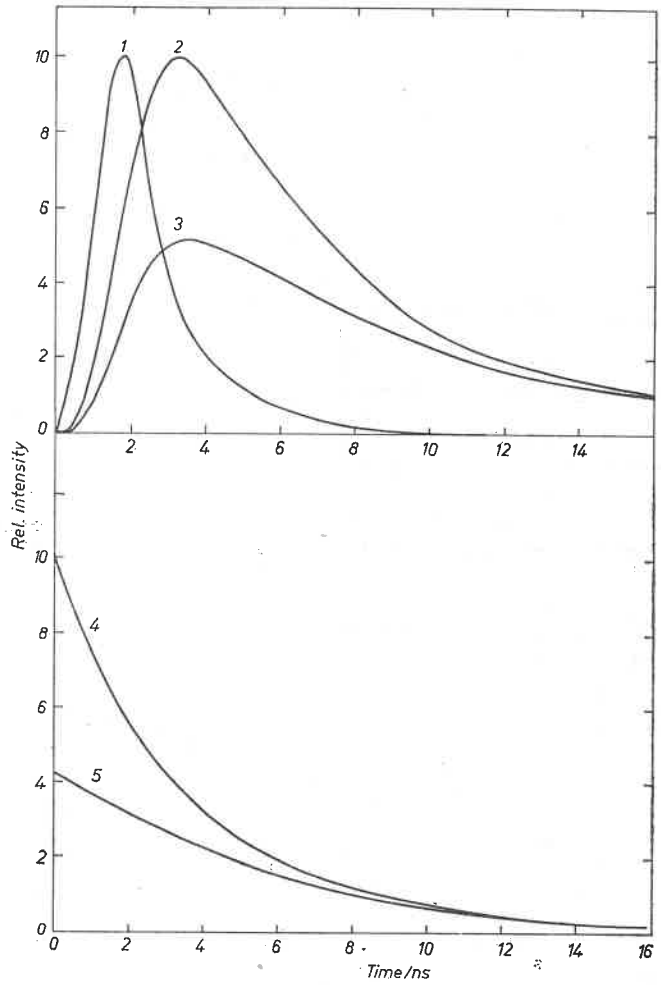

Fig. 2. Measured and calculated curves of time-dependent fluorescence depolarization of rhodamine 6G in undecanol. $T = 23°C$, $\eta = 16.6$ cP, $c = 2.0 \times 10^{-5}$ mole/l, excitation at 440 nm, measured wavelength of emitted light 600 nm. Above: experimental curves: *1* — dye laser, *2* — fluorescence intensity parallel $I_{||}$, *3* — fluorescence intensity perpendicular $I_{\perp}$; below: same curves after deconvolution by a computer program [15]: *4* — decay curve $I_{||}$, *5* — decay curve $I_{\perp}$

channel analyser (ICA-70, KFKI, Budapest). The data were then given to a BESM 6 computer (USSR) for evaluation in off-line regime on the basis of equations (6) and (7) which follow from Eqs (1) to (4):

$$I_{\parallel}(t)+2I_{\perp}(t) = I_0 \exp(-t/\tau_{Fl}), \tag{6}$$

$$I_{\parallel}(t)-I_{\perp}(t) = r_0 I_0 \exp -(1/\tau_{Fl}+1/\tau_D^*)t. \tag{7}$$

After deconvolution of the measured functions $I_{\parallel}(t)+2I_{\perp}(t)$ and $I_{\parallel}(t)-I_{\perp}(t)$ from the excitation pulse [15] the function $r(t)$ as well as the values of τ_{Fl}, τ_D^*, I_0 and r_0 could be determined by equations (6) and (7). With the laser spectrofluorimeter described above we obtained decay times down to 100 ps with an error of about ± 150 ps. An example which illustrates the great accuracy of the measurements is given in Fig. 2.

Rhodamine 6G (for microscopy, FERAK, Berlin-West) was not purified before use. The acridine orange (for microscopy, E. Merck, Darmstadt, FRG) is available as zinc double salt and was converted via the free base to the pure dye hydrochloride. All solvents were purified by repeated vacuum distillation and dried by known methods. Viscosity was checked experimentally at room temperature. For the temperature dependence of the viscosity we referred to tables [16]. The glycerol-water-mixtures are given in parts by weight.

4. *Results and discussion*

4.1. τ_D^* as a function of η/T

The dependence of the rotational diffusion time τ_D^* on the quotient from solvent viscosity η and temperature T is illustrated by Fig. 3 for acridine orange in glycerol--water mixtures.

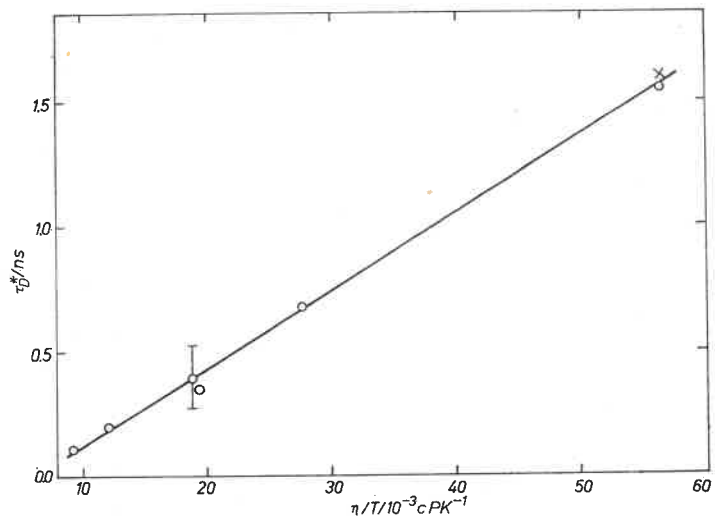

Fig. 3. Dependence of τ_D^* on η/T for acridine orange. Excitation at 450 nm; measurement at 550 nm. ○ — 1.1×10^{-3} mole/l in a glycerol-water mixture 50:50, × — 6.0×10^{-5} mole/l in undecanol

The concentration of the dye is relatively high so as to study the behaviour of the dye aggregates, as will be shown in Section 4.4. But the data recorded at lower concentrations (10^{-5}) lie on the same line so that an influence of the concentration on the rotational diffusion time can be precluded in this case.

The same linear relationship was found with other dyes [6], hence the validity of the hydrodynamical model in describing rotational diffusion was confirmed in principle. The effective molecular volume according to Eq. (2) obviously is not dependent upon the temperature of the solvent. Since in the studied temperature range the function $\eta = f(T)$ can be roughly approximated by a straight line [16], there exists similarly a linear correlation between τ_D^* and η, as has been demonstrated in other systems [2, 5].

4.2. Dependence of τ_D^* on the solvent

The effect of solvents forming hydrogen bonds of differing strength on the rotational diffusion time is tabulated in Table I. After having shown above that the Stokes-Einstein relationship is valid, the effective molecular volume V could be calculated according to Eq. (5). The values are also included in Table I along with the theoretical molecular volume calculated from the Van der Waals radii.

TABLE I

Effect of solvent on τ_D^*. Glycerol-water = 66:34; Acridine orange: $\lambda_{exc} = 490$ nm, $\lambda_{fl} = 550$ nm; Rhodamine 6G: $\lambda_{exc} = 530$ nm, $\lambda_{fl} = 600$ nm

Substance	$c \times 10^{-5}$ [mole/l]	T [°C]	η/T [cP/K]	τ_D^* [ns]	V_{eff} [Å^3]	V_{theor} [Å^3]
AO in glycerol-water	7.1	21.5	0.054	1.48	378	445
AO in undecanol	6.0	23.0	0.056	1.60	394	445
Rh 6G in glycerol-water	2.8	20.0	0.056	3.87	954	730
Rh 6G in undecanol	2.0	23.0	0.056	10.97	2703	730

In acridine orange the rotational diffusion time — and hence the effective molecular volume — is not dependent on the nature of the solvent. V_{eff} is practically equal to V_{theor}, so that one could conclude that the solvent cage has practically no effect on τ_D^* whereas in rhodamine 6G we find a strong influence of the solvent on τ_D^* and hence also on V_{eff}. In glycerol-water mixtures V_{eff} is only slightly greater than the theoretical molecular volume, whereas it is greater by a factor of 4 in undecanol. In the literature, too, one finds contradictory statements as to the effect of the solvent on τ_D^*. For example τ_D^* in the excited state from fluorescence measurements of rhodamine 6G at room temperature in undecanol is 6.5 ns and in glycol 2.3 ns [7] or τ_D in the ground state from absorption measurements in undecanol is 2.8 ns and in ethylene glycol 2.13 ns [2] or 1.0 ns [3]. Other systems were studied in [2, 4–6]. These results cause one to question previous interpretations of V_{eff} according to Eq. (5). NMR spectroscopic findings have proved that dyes are highly solvated as a result of their high polarity. Even with nonpolar solvents like

benzene [17] solvation leads to weak complex formation and to a significant change in the electronic structure of the solute molecules [18].

On the other hand, it has been found that the effective molecular volume calculated for nonpolar aromatic hydrocarbons by Eq. (5) is smaller than the theoretical one by a factor of 5–10 [19, 20]. Similar results were obtained from the nuclear-spin-lattice relaxation times measured by NMR spectroscopy in the ground state of aromatic molecules [21]. The coincidence of V_{eff} with V_{theor} in some dyes therefore appears to be fortuitous. For this reason it would be premature to calculate from the estimated effective molecular volumes the size of the solvation cage, as done by other authors [6, 7]. Our result shows that this point needs to be thoroughly reconsidered. Intramolecular relaxation processes in the excited state may be involved here in addition, as it is also assumed for other molecules [22].

4.3. Dependence of τ_D^* on the wavelength of the excitation light

Since fluorescence in a molecule usually proceeds from the lowest electronically excited singlet state, the excess energy must be converted in a relatively short time to vibrational energy when the molecule is excited into higher states. According to Stepanov, this should cause local heating of the system [23]. In accordance with estimates made by Jabłoński and other authors with the help of static measurements of fluorescence depolarization as a function of the excitation energy [24] and, in addition, of the temperature [20], dissipation of the excess energy should take place within picoseconds and hence hardly affect fluorescence depolarization. Any differences that occurred at various wavelengths could be proved to be due to the anisotropy of the rotational diffusion in nonspherical molecules [25].

By contrast, Heiss and Dörr [7] found from measurements of the time-dependent fluorescence depolarization of rhodamine 6G with excitation by flash lamp that the rotational diffusion time of rhodamine 6G clearly decreases as one goes from the 0–0 transition to higher vibronic states of the S_1 state. Surprisingly, when exciting to the S_2 and S_3 states, τ_D^* started to steeply rise again [7, 26].

TABLE II

Effect of excitation wavelength on τ_D^*. Glycerol-water = 66:34; Acridine orange: $\lambda_{fl} = 550$ nm, $C = 7.1 \times 10^{-5}$ mole/l; Rhodamine 6G: $\lambda_{fl} = 600$ nm, $C = 2.8 \times 10^{-5}$ mole/l. In the table the wavelength of the excitation light is given in brackets after τ_D^*

Substance	η/T [cP/K]	τ_D^* (490 nm)/[ns]	η/T [cP/K]	τ_D^* (450 nm)/[ns]
AO in glycerol-water	0.054	1.48	0.054	1.5
AO in undecanol	0.056	1.60		[a]
		τ_D^* (530 nm)/[ns]		τ_D^* (440 nm)/[ns]
Rh 6G in glycerol-water	0.056	3.87	0.050	1.34
Rh 6G in undecanol	0.056	10.97	0.056	4.58

[a] fluorescence intensity was too weak.

We repeated these measurements using dye laser excitation and can confirm the decrease of τ_D^* in rhodamine 6G on excitation with shorter wavelengths: Table II. Here, too, acridine orange shows a different behaviour. Its rotational diffusion time within the longest wavelength vibronic band is not dependent on the wavelength of the exciting light (Table II).

To preclude the possibility of this effect being merely due to an error of measurement, we have studied the dependence of τ_D^* on the intensity of the laser excitation. With intensive excitation of the dye at higher vibronic levels one would have expected saturation to occur much earlier as absolute absorption is much lower than when exciting the intensive 0–0 transition. No changes, however, were found to occur in τ_D^* when the intensity of excitation was varied between 200 kW/cm² and 30 MW/cm², so that we are dealing here with an typical effect of the dye. If one were to explain it simply by local heating, rhodamine 6G and acridine orange should behave in the same way. Therefore, here too, specific dye effects have to be taken into account, maybe the same internal relaxation processes [22] which has been supposed in the context of explaining the different solvation behaviour of the two dyes.

4.4. Dependence of τ_D^* on dye aggregation

In contrast to normal concentration quenching of the fluorescence in organic molecules in solutions of concentrations of $> 10^{-4}$ mole/l the fluorescence lifetime of acridine orange becomes longer as concentration increases which is due to the formation of dye aggregates [27, 28]. Hence it was of interest to carry out a similar study of time-dependent concentration depolarization. If this produces no effect in oriented aggregates, it should be possible to measure the effect of aggregation on the rotational diffusion time.

We could, in fact, prove this effect in a solution of 10^{-3} mole/l acridine orange when the temperature was varied between 16 and 22°C. The aggregation is strongly dependent on the temperature in this range [29]. The measured τ_D^* values are given in Table III.

At 16°C the τ_D^* of the aggregates is 1.6 times greater than the τ_D^* of the monomers. This is obviously attributable to the greater effective volume of the aggregates.

With rising temperature τ_D^* of the aggregates decreases markedly by a factor of about 3 because of the reduction in size of the aggregates, as is evident from the shortening of the

TABLE III

Effect of dye aggregation on τ_D^*. Acridine orange in glycerol-water = 50:50; Monomeric molecules: $\lambda_{exc} = 490$ nm, $\lambda_{fl} = 550$ nm; Aggregates: $\lambda_{exc} = 450$ nm, $\lambda_{fl} = 630$ nm

C [mole/l]	Aggregates				Monomeric molecules			
	T [°C]	r_0	τ_{fl} [ns]	τ_D^* [ns]	T [°C]	r_0	τ_{fl} [ns]	τ_D^* [ns]
1.1×10^{-3}	16	0.23	5.53	0.8	13.2	0.34	2.90	0.68
1.1×10^{-3}	22	0.18	4.50	0.25	21.5	0.38	2.75	0.35
1.0×10^{-2}	22	—	12.85	—	—	—	—	—
0.9×10^{-1}	22	—	30.0	—	—	—	—	—

fluorescence lifetime of the aggregates [28]. By comparison, τ_D^* of monomeric molecules is reduced over the same range by only a factor of 1.4 in keeping with the normal temperature dependence shown in Fig. 3.

Unfortunately it is not possible to study rotational diffusion times of aggregates at higher concentrations. r_0 decreases rapidly at concentrations of $> 10^{-3}$ mole/l, either because of a normal concentration depolarization due to intermolecular energy transfer [30–32], or as a result of a depolarization within the aggregates whose intramolecular mobility increases maybe with the size of the aggregates.

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