MOLECULAR MOVEMENTS AND DYNAMICS IN SOLUTIONS STUDIED BY FLUORESCENCE DEPOLARIZATION MEASUREMENT

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Received August 13, 1992
Accepted November 17, 1992

Professor Jiří Dvořák was for a long time the Professor and the Head of the Department of Physical Chemistry at the Faculty of Science of the Charles University in Prague and the Editor of the Collection of Czechoslovak Chemical Communications. He died the 21. 1. 1992. We wish to express our gratitude for his scientific help during preparation of this manuscript and for his kind and sofisticated attitude to younger collegues at the Department. We wish to commemorate his outstanding personality to broader Czech and Slovak chemical society.

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Theories allowing interpretation of the results of time-resolved polarization spectrofluorimetry in solutions are reviewed and their applicability under various conditions is discussed. For the reorientation of rigid molecules in an isotropic medium, the most frequently employed models are presented, such as a rotational diffusion model, the Fokker–Planck–Langevin model, etc. Systems with internal rotation, systems in anisotropic media, systems with a complex electron relaxation and systems with energy transfer are discussed as examples of more complex systems. A special attention is devoted to the polarization fluorimetry of probes bound to/or sorbed at polymer and biopolymer chains. The review focuses on theoretical models of reorientational motion for interpretation of fluorescence anisotropy decays. Experimental studies and computer simulations are discussed only when it is necessary for comparison with theoretical predictions. Complicated models for simultaneous reorientational motion and energy transfer, solvent rela-

xation, etc., although very important for many applications, exceed the scope of this review and are mentioned only very briefly.

1. INTRODUCTION

Ultrafast time-resolved polarization spectroscopy enables the study of a number of dynamic phenomena on a molecular or submolecular level in a real time.

On irradiating the sample with a plane-polarized monochromatic light pulse of an appropriate wavelength, excitation of a certain fraction of molecules takes place. The probability that a photon is absorbed by a molecule depends not only on the square of the transition moment, $\vec{\mu}_{ij}$, (i.e. on optical selection rules):

$$|\vec{\mu}_{ij}| = \int \psi_{jc} \mathbf{P} \psi_{ic} \, dV \int \psi_{jv} \psi_{iv} \, dV \qquad (1)$$

but also on its orientation to the polarization of excitation radiation[1,2]. In Eq. (*1*), ψ_{jc} and ψ_{ic} are the electronic wave functions of the *j* and *i* states, respectively, ψ_{jv} and ψ_{iv} are the vibrational wave functions, **P** is the dipole moment operator and integration is performed over the entire space. The square of the second integral in the r.h.s. of Eq. (*1*) represents the Franck–Condon factor. Immediately upon a short and vertically polarized excitation pulse, orientation of excited molecules with an absorption transition moment parallel to the vertical axis, *z*, is dominant. This gives rise to non-equilibrium and non-isotropic distributions of molecules in both the excited and the ground states. The unstable distribution of excited molecules immediately starts to relax and the reequilibration is attained via several simultaneous processes[3]:

a) *Radiative and non-radiative energetic relaxation of excited molecules* (electronic, vibrational relaxation, energy transfer, etc.). The probability (or the rate) of the radiative electronic transition from the first excited singlet state S_1 to the ground state S_0 (fluorescence) is controlled by optical selection rules. The rates of possible competitive non-radiative processes (internal conversion, intersystem crossing etc.) are influenced by various factors (structure of the fluorophore, microenvironment polarity, viscosity, etc.). The absorption, $\vec{\mu}_a$, and emission, $\vec{\mu}_e$, transition dipole moments are in many fluorophores parallel to each other and the emission is strongly polarized parallel to the excitation polarization in early times and then depolarizes due to the overall relaxation of the system – see processes listed below in *b*) and *c*). (Dipole moments $\vec{\mu}_a$ and $\vec{\mu}_e$ may form a constant angle and the initial polarization may be smaller than that assuming the parallel orientations.)

b) *Rotational Brownian motion of molecules*. Processes falling in this category will be discussed in great detail.

c) *Reorientational relaxation of microenvironment.* Discussion of microenvironment relaxation (e.g. that of solvation shell) exceeds the scope of this review and will be omitted.

The aforementioned relaxation processes may be studied by the time-resolved optical spectroscopies: (i) either by means of a second delayed (probe) pulse (the transient absorption spectroscopy), or (ii) by studying the radiation emitted from the excited state (the time-resolved fluorescence spectroscopy).

The time-resolved fluorescence anisotropy measurement is an extremely suitable method for studying the reorientational motion of molecules. In this case, the time decay of fluorescence intensities polarized parallel, $I_{\parallel}(t)$, and perpendicular to the excitation polarization, $I_{\perp}(t)$, are measured. The time-resolved fluorescence anisotropy, $r(t)$, is defined by the following equation:

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

$S(t) = I_{\parallel}(t) + 2I_{\perp}(t)$ is proportional to the total population of molecules in the excited state and does not depend on the reorientational relaxation – i.e. $S(t)$ contains information on energy relaxation only[3]. Fluorescence decay $I(\gamma,t)$ polarized at an arbitrary angle γ to the excitation polarization may be expressed in the following form[4]:

$$I(\gamma,t) = \frac{1}{3} S(t) [1 + (3 \cos^2 \gamma - 1) r(t)] . \tag{3}$$

The principal advantage of Eq. (3) for experimental data analysis is that it allows one to analyze fluorescence depolarization decays of complex systems without major ambiguities (a global analysis method[5 - 7]). It is evident from Eq. (3) that in measurements with an analyzing polarizer oriented at the magic angle $\gamma_m = 54.7°$, ($3\cos^2\gamma_m - 1 = 0$), function $S(t)$ can be obtained directly:

$$I(\gamma_m,t) = S(t) . \tag{4}$$

The time-resolved fluorescence anisotropy, $r(t)$, is related to the time correlation function of: (i) the orientation of the emission transition moment at the time t, $\vec{\mu}_e(t)$, and (ii) the orientation of the absorption transition moment at the instant of excitation, $\vec{\mu}_a(t_0 = 0)$. Function $r(t)$ can be expressed in the form[8]:

$$r(t) = \frac{2}{5} \langle P_2 (\vec{\mu}_e(t) . \vec{\mu}_a(t_0 = 0)) \rangle , \tag{5}$$

where P_2 is the Legendre polynomial of the second order and the brackets < > denote ensemble averaging.

Time-resolved polarization fluorimetry monitors a relaxation of the emission dipole moment from a non-equilibrium state, which was created due to appropriately chosen initial conditions (a polarized excitation) to an equilibrium (random) state. As mentioned earlier, the anisotropy decay, $r(t)$, is influenced by the rotational movement of molecules and also by other types of relaxation (such as energy transfer[9 - 13], microenvironment relaxation[14]). A general theoretical model should consider all the above mentioned phenomena. In a number of systems, energy transfer does not take place, or may be suppressed by using low fluorophore concentrations. The reorientational relaxation of the microenvironment is usually faster than both the electron relaxation and the reorientational motion of common fluorophores in solutions, and it does not directly affect the shape of fluorescence anisotropy decays. The most frequent process occurring on the same time scale as electron relaxation is the rotational Brownian motion of molecules. Consequently, the theoretical analysis of the fluorescence anisotropy decays is most frequently based on so rotational diffusion model.

This model falls into the category of theories describing vector movements[15 - 32], some of which have quite general validity and may be applied to other experimental methods (relaxation of molecules by NMR, dipolar relaxation, electric and flow dichroism).

2. REORIENTATION OF RIGID MOLECULES IN AN ISOTROPIC MEDIUM

2.1. *Rotational Diffusion (RD) Model*

Historically the Debye hydrodynamic model[15] is the oldest one. Reorientation motion of molecules is described using a rotational diffusion equation with a rotational diffusion coefficient given by the Stokes–Einstein equation. The model has been improved and generalized by many authors[16 - 23] and at present the rotational diffusion (RD) model is the most widely used for interpretation of experimental data. A fluorophore is supposed to be a solid (in general asymmetrical) elipsoid immersed in a viscous liquid. Its orientation in the fixed laboratory coordinate system is characterized by Euler's angles Ω, which describe the instantaneous orientation of a coordinate system moving with the molecule with respect to the laboratory coordinate system. It is assumed that the molecule rotates by a very small angle between individual collisions (assumption of small diffusion angles). The diffusion equation has the following form:

$$\frac{\partial}{\partial t} f(\Omega,t) = - \mathbf{H} f(\Omega,t) \ , \qquad (6)$$

$\mathbf{H}$ is the Hamiltonian operator, $\mathbf{H} = \sum_i \sum_j \mathbf{L}_i D_{ij} \mathbf{L}_j$, where D_{ij} are the components of diffusion tensor, and $\mathbf{L}$ is the quantum mechanical operator of the angular momentum defined according to Rose[33]. Function $f(\Omega,t)$ stands for the probability, that at a given time t, the orientation of a molecule (regardless of the electronic state) is in a solid angle interval from Ω to $\Omega + d\Omega$. Provided that the moving coordinate system coincides with the diagonal components of the diffusion tensor, e.g. with the principal diffusion axes, the diffusion equation may be rewritten into a simplier form:

$$\frac{\partial}{\partial t} f(\Omega,t) = -\sum_{i=1}^{3} D_i (\mathbf{L})^2 f(\Omega,t) \qquad (7)$$

which is the quantum mechanical operator equation for an asymmetric solid rotor. The following classification of molecules is based on the properties of the diffusion tensor:

a) the asymmetric top – elements of diffusion tensor (principal diffusion coefficients) are mutually different,

b) the symmetric top – two main diffusion coefficients are identical, the third one is different,

c) the spherical top – all three coefficients are identical.

Probability density, $f(\Omega,t)$, is connected with the experimentally available quantities $I_{\parallel}(t)$ and $I_{\perp}(t)$ by means of the following equations:

$$I_{\parallel}(t) = \int d\Omega\, P_{\parallel}(\vec{\mu}_e,\Omega)\; K(t)\; f(\Omega,t) \qquad (8)$$

$$I_{\perp}(t) = \int d\Omega\, P_{\perp}(\vec{\mu}_e,\Omega)\; K(t)\; f(\Omega,t)\,, \qquad (9)$$

where $K(t)$, which is proportional to $S(t)$, is the probability that a molecule is in the excited state at the time t, and $P_{\parallel}(\vec{\mu}_e,\Omega)$ or $P_{\perp}(\vec{\mu}_e,\Omega)$ are the probabilities that a molecule with an emission transition dipole moment $\vec{\mu}_e$, whose orientation is determined by Euler's angles Ω, emits fluorescence radiation polarized parallel or perpendicular to the polarization of the excitation light, respectively.

The mathematical treatment[18,19] based on the theory outlined above leads to the following expression[23] for $r(t)$:

$$r(t) = \sum_{i=1}^{5} A_i \exp\left(\frac{-t}{\tau_i}\right). \qquad (10)$$

In the most general case there are five rotational correlation times, $\tau_i = f(D_1, D_2, D_3)$, which are functions of the principal diffusion coefficients, although only three of them

are independent. The pre-exponential factors $A_i = f(D_1, D_2, D_3, \alpha_j, \beta_j)$ are functions of the principal diffusion coefficients and angles describing the orientation of the absorption and emission transition moments with respect to the principal axes of diffusion. In many cases (e.g. a special symmetry of the molecule, or a special orientation of $\vec{\mu}_a$, $\vec{\mu}_e$ to each other, etc.), the analytical form of $r(t)$ is considerably simplified and the number of rotational correlation times is reduced – see Table I.

The model was later extended[8] also to the cases when the microscopic structure and molecular nature of the surrounding liquid cannot be neglected. The effect of the microscopic structure of the environment and of its interaction with a fluorophore on the rotational diffusion is caused especially by a capability of the studied fluorophore to bind solvent molecules (to form a solvation shell). Two limiting situations are considered: (i) stick condition, a situation when a layer of solvent molecules (first solvation shell) moves simultaneously with the fluorophore, and (ii) slip condition, a situation when the solvent molecules are not bound to the fluorophore and consequently the fluorophore rotation is free, nevertheless hindered by the necessity to dislocate the surrounding solvent molecules. This results in a hydrodynamic friction. In some cases

TABLE I
Special cases when the number of rotational correlation times, N_{CT}, is reduced

N_{CT}	Possible physical situations
3	*a*) $D_1 = D_\parallel \neq D_\perp = D_2 = D_3 \Leftrightarrow$ symmetric-top
	b) probe randomly attached to a rigid macromolecule e.g. all orientations of $\vec{\mu}_a$, $\vec{\mu}_e$ with respect to the diffusion axes are of equal probability, thus $(\mu_a^i)^2 = (\mu_e^i)^2 = 1/3$ for each i
	c) $\vec{\mu}_a$ or $\vec{\mu}_e$ is perpendicular to one of the main diffusion axes, but not parallel to any of the remaining ones
2	*a*) symmetric-top ($D_\parallel$, $D_\perp$) and simultaneously $\vec{\mu}_a$ or $\vec{\mu}_e$ is perpendicular to the axis of symmetry
	b) symmetric-top together with the slip conditions in theory they are three, but in practice two of them are indistinguishable
	c) $\vec{\mu}_a$ or $\vec{\mu}_e$ are parallel to some of the main diffusion axes
1	*a*) symmetric-top ($D_\parallel$, $D_\perp$) and simultaneously $\vec{\mu}_a$ or $\vec{\mu}_e$ are parallel to the axis of symmetry
	b) $D_1 = D_2 = D_3 = D \Leftrightarrow$ spherical-top
	c) for an oblate elipsoid in theory they are three, but in practice they are indistinguishable[17]

it is necessary to take into account not only this hydrodynamic friction, but also the dielectric friction[34], which is a consequence of dipolar interaction of a fluorophore with solvent molecules. Introduction of the stick and slip limiting conditions may affect drastically values of the rotational correlation times[35].

A very special theoretical anisotropy decay predicted by Eq. (*10*) for special orientations of $\vec{\mu}_a$, $\vec{\mu}_c$ (mutually perpendicular) and appropriate magnitudes of D_i (symmetric molecules with $D_\parallel > D_\perp$), when $r(t)$ may reach a maximum at intermediate times and later decreases again, has been obtained also experimentally for perylene[35,36].

In practice, only two or three correlation times can be resolved within experimental errors. The experimental rotational correlation times are therefore certain weighted averages of the theoretical rotational times. The fact that for many fluorophores the orientations of $\vec{\mu}_a$, $\vec{\mu}_c$ are not known may further complicate the evaluation of experimental data.

Further important characteristics are initial anisotropy $r_0 = r(t = 0) = \Sigma_i A_i$, and residual anisotropy $r_\infty = \lim_{t\to\infty} r(t)$. The initial anisotropy depends on the angle α between the absorption and emission dipole moments and for a fluid system of randomly oriented fluorophores (prior to excitation) it may be expressed as follows:

$$r_0 = 0.6 \cos \alpha - 0.2 . \qquad (11)$$

Experimental values r for various fluid systems should be in the interval <0.2; 0.4>. Coefficients A_i may depend on the excitation and emission wavelengths, but not on temperature, viscosity, and for symmetric-top on D_i. The residual anisotropy should attain zero value for a rigid molecule in an isotropic medium. If the experimental value differs from zero, it means that: (i) either the rotational movement is restricted (due to the bonding to the rigid system, as a result of anisotropy of the medium – see paragraph 4, etc.), (ii) or that the rotational correlation times are long as compared with the fluorescence lifetime and the true r_∞ cannot be reached experimentally. Information obtainable from fluorescence anisotropy measurements are summarized in Table II.

The rotational diffusion model has been employed by many authors for a successful description of experimental data of fluorescence depolarization and their relation to the rotation of molecules (see e.g. a survey of rotational correlation times of selected molecules on p. 137 in ref.[8]).

2.2. *Extended Diffusion (ED) Model*

The extended diffusion model (for linear[24] and for spherical[25] molecules, for symmetric[26,27] and for asymmetric[28] molecules) eliminates the assumption of small diffusion angles.

According to the ED model, reorientation of molecules proceeds via possibly long and mutually independent diffusion steps. Molecules rotate freely without any change in the angular momentum between collisions. The probability of diffusion steps of different lengths is given by the Poisson distribution. A collision is considered to be a very fast event as compared to the average time of the free rotation of a molecule. According to the character of changes in angular momentum, two limiting cases of the ED model can be distinguished. In the J-diffusion, changes in both the magnitude and the orientation of angular momentum take place. Changes in the angular momentum orientations are supposed to be random, and the probability of changes in the angular momentum magnitudes are given by the Boltzmann distribution. In the M-diffusion, the angular momentum magnitude does not change during a collision, only its orientation changes randomly.

Time-resolved anisotropy has a complex form of an infinite series. Gordon[24] has shown that for linear molecules the theoretical rotational correlation function may have in certain special cases a form of damped oscillations, which really has been observed experimentally, and the RD model could offer no explanation for this situation. The agreement of numerical values calculated using the ED model with experimental values has been verified for spherical molecules by McClung[37]. Calculations for asymmetric molecules[28] assuming the J-diffusion have agreed with experiments, whereas the assumption of M-diffusion has proved to be unrealistic.

TABLE II
Information obtainable from fluorescence anisotropy decay

Source	Information
N_{CT}	information on molecule symmetry
	information on mutual orientation of $\vec{\mu}_a$, $\vec{\mu}_e$ and principal axis of symmetry (see Table I)
Magnitude of rotational correlation times	magnitude of the principal diffusion coefficients D_i and/or D_i/D_j i.e. microviscosity of the medium, interaction with the medium, "shape" of a molecule in the excited state (in symmetric molecules e.g. the ratio of semi-axes
Initial anisotropy	specification of mutual orientation of $\vec{\mu}_a$ and $\vec{\mu}_e$
Residual anisotropy	criterion of equivalence of all directions of rotation (criterion of anisotropy of the medium)

2.3. *Fokker–Planck–Langevin (FPL) Model*

Another improvement of the hydrodynamic model eliminating the restriction to small diffusion angle steps is the Fokker–Planck–Langevin model (for linear[29], spherical[30] and symmetric[31] tops).

In the FPL model, the molecule is treated as a solid body of a convenient shape (elipsoid) immersed in a viscous liquid. Rotational movement is affected by: (i) the slowly changing frictional force given by the viscosity of the medium, and (ii) the quickly changing Brownian force, which results from the changes in instantaneous structure of the medium.

The FPL model is based upon a rotational Fokker–Planck equation for the conditional probability density of orientation and magnitude of angular velocity of the molecule. The derived correlation functions are expressed as an infinite series (whose terms are simple exponential functions of time), which converge sufficiently fast. Accurate numerical values for the correlation functions and spectral densities were obtained using only a small number of terms in the truncated series.

The physical assumptions of the FPL and ED models are quite different from each other although both are extensions of the rotational diffusion model and remove the restriction of small diffusion angles. The FPL and ED models represent two extreme approaches how to treat the influence of the microenvironment forces on the rotational diffusion of a molecule. In the ED model molecules perform unhindered rotations interrupted by strong collisions of short duration which result in great changes in the magnitude and orientation of the angular velocity of the molecule. In the FPL model both a slowly changing friction force and a more quickly changing Brownian force are involved. To create a great change in the FPL model, however, a large number of fluctuations of the Brownian force occurring in the statistical set of particles is needed.

A detailed comparison of calculations of the FPL and ED models for linear and spherical molecules with experimental values has been published by G. Lévi et al.[38]. Both models offer an almost indistinguishable macroscopic description of the rotational movement of molecules in liquids and it is difficult to decide which is more relevant. The authors recommend a great deal of caution in drawing conclusions concerning the microscopic details of movement of molecules in real liquids based on the agreement of experimental data with model predictions.

2.4. *Partially Relaxed Rotation (PRR) Model and Its Generalization, 2τ Model*

An interesting extension of the RD model for a symmetric-top fluorophore, which is similar to the ED model[32], is a partially relaxed rotation model and its generalization, a 2τ model[32].

These models are based on the experimentally verified fact that the rotation around the symmetry axis does not require any significant displacement of solvent molecules

and is quite free, while the tumbling motion connected with changes in spatial orientation of this axis is a complex relaxation process controlled by collisions with surrounding solvent molecules.

Non-correlated instantaneous binary collisions are supposed to perturb the angular velocity perpendicular to the symmetry axis (the tumbling motion) but leave the parallel component (the free rotation) unchanged. Distribution of angular velocities for the free rotation around the symmetry axis is given by the Maxwell–Boltzmann function and the relaxation of the tumbling motion is described by a characteristic time τ_1.

A more general model, which is called 2τ model, considers also the relaxation of the movement around the symmetry axis. This relaxation process is described by a characteristic time τ_2. The following limiting cases, may be distinguished:

1) if $\tau_2 \to \infty$, then for any τ_1, the model corresponds to the PRR model
2) if $\tau_1 \to \infty$, then for any τ_2, the model corresponds to the EDJ model
3) if $\tau_1 \to \infty$ and $\tau_2 \to \infty$, the model corresponds to the free rotor model.

3. SYSTEM WITH POSSIBLE INTERNAL ROTATION

Rigidity or flexibility of a molecule with possible internal rotation (mostly a synthetic polymer or a biological macromolecule) may be classified for the purposes of this review by the ratio of rotational correlation time of the individual part of the molecule to the rotational correlation time of the entire molecule. Fluorescence anisotropy in a flexible system monitors simultaneously several motions: (i) rotation of the system as a whole, (ii) rotation of the fluorophore around a single covalent bond and (iii) rotation of the fluorophore together with a part of the molecule. In some special cases it may monitor also a coiling or a bending of the polymer chain[39]. For fluorimetric studies the flexible systems may be divided into two large groups. The fluorophore may be: (i) either an inherent part of a macromolecule – an intrinsic probe, (ii) or it may be additionally chemically bonded to a macromolecule (fluorescent labelled polymers) or preferentially dispersed in a macromolecular system – an extrinsic probe. Dispersed fluorophores are not particularly suitable for study of possible internal rotations of a molecule or molecule flexibility and are used mainly for studies of anisotropic properties of a medium.

An ideal fluorescence probe should meet the following requirements:

a) it should specifically monitor the behaviour of a selected part of the molecule,

b) it must not perturb the studied system (in biological molecules it should not influence the physiological function of the system),

c) it should have appropriate and simple spectral properties (single exponential fluorescence decay in an isotropic system with a convenient lifetime, i.e. somewhat longer than that of the reorientation motion).

The major ambiguity of the extrinsic probe techniques lies in the assumption that the rotational motion of the probe is a good indicator of that of the host system.

The fluorescence anisotropy is related in a relatively complicated way to the behaviour of the system. It is advantageous for interpretation of experimental data to begin with a hypothesis on the possible arrangement and processes occurring in the system. Owing to the complexity of these relations, there has been no general theory formulated so far, but since polarization fluorimetry is a promising source of interesting information on polymers and biopolymers, a number of papers has appeared analyzing simplified cases[40 - 57].

For a polymer containing a fluorophore as one of the internal chain segments (i.e. . . .M-M-F-M-M. . ., where M and F stands for monomeric unit and fluorophore, respectively), there exists a model, which considers a three-bond motion (a sort of the crankshaft motion) on a hypothetical tetrahedral lattice[43] . The derived fluorescence anisotropy may be written in the following form:

$$r(t) = r_0 \exp\left(\frac{t}{\rho}\right) \operatorname{erfc}\sqrt{\frac{t}{\rho}} , \qquad (12)$$

where ρ is the relaxation time which may contain parameters, describing the diffusion jump frequency and the conformational structure of the chain. In a more realistic model, when both valence angles and internal rotation angles are allowed to vary from those associated with an ideal lattice, the fluorescence anisotropy assumes the following form[44]:

$$r(t) = r_0 \exp\left(\frac{-t}{0}\right) \exp\left(\frac{t}{\rho}\right) \operatorname{erfc}\sqrt{\frac{t}{\rho}} , \qquad (13)$$

where 0 is the correction relaxation time reflecting the perturbation relaxation of the lattice points positions. The validity of Eq. (*12*) has been verified experimentally, e.g. in ref.[45] for anthracene labelled polystyrene (emission transition moment parallel to a polymer chain) and with 9,10-diphenyl anthracene labelled polystyrene (emission transition moment perpendicular to a polymer chain). The approximation has been found satisfactory with the exception of the short-time region.

Recently, the cooperative crankshaft motion of polymer chain has been discussed in ref.[46], which considers several (two, three or nine) simultaneous bond rotations.

A model for fluorescence anisotropy of a compact and rigid globular macromolecule with a mobile pendant fluorophore was presented by Gottlieb and Wahl[47]. The fluorescent group is supposed to be mobile around one of its rotational diffusion axes. This is a fairly frequent case of globular proteins where the axis of rotation represents a single covalent bond. A fixed and radial orientation of the axis of rotation with respect

to the carrier molecule is assumed in this model. Two limiting cases have been treated: (i) a non-hindered rotational diffusion of the fluorophore (which corresponds to a free internal rotation of the fluorophore) and (ii) a jump diffusion among discrete positions (which corresponds to an internal rotation with discrete positions).

The following analytical form has been derived for the free internal rotation:

$$r(t) = \exp\left(\frac{-t}{\tau_M}\right)\left\{\alpha_1 + \alpha_2 \exp\left(\frac{-t}{\tau_F}\right) + \alpha_3 \exp\left(\frac{-2t}{\tau_F}\right)\right\}, \qquad (14)$$

where τ_M is the rotational correlation time of a macromolecule, τ_F is the rotational correlation time of a fluorophore, and α_1, α_2, α_3 are constants depending on the angles between the transition moments and the axis of the rotation.

For a thermally activated jump diffusion[47], the time-resolved anisotropy has been derived in the form:

$$r(t) = \exp\left(\frac{-t}{\tau_m}\right)\left\{\alpha_1 + \alpha_2 \exp\left(-K w t\right)\right\}, \qquad (15)$$

where K is the normalization constant, w is the jump frequency and α_1, α_2 are constants reflecting the orientation of transition moments within a molecule.

A discontinuous jump model for large carriers with fluorophores which may have a finite number of positions has been also published by Weber[48].

A more general model for the time-resolved fluorescence anisotropy for a fluorophore undergoing rotational diffusion about a single axis while attached to a non-fluorescent rotationally diffusing, symmetrical carrier molecule, was presented by Burghardt[49]. In contrast to the previous work[47], the rotational axis of the fluorophore is assumed to have an arbitrary orientation relative to the carrier.

A system of compact spherical macromolecules, each containing a pendant fluorophore attached covalently to the macromolecule has been treated also by Tanaka et al.[50] for a more complex energy relaxation process (angularly dependent quenching of fluorescence). Numerical computations fitted well the experimentally observed fluorescence decays from single tryptophan in apocytochrome c.

An another approach was published in refs[51,52]. A general formulation was presented for the decay of the fluorescence polarization anisotropy of species that exhibit a local cylindrical symmetry. This formulation encompasses ordinary rotational diffusion of rigid species, and also admits various deformational Brownian motions that proceed under the influence of hookean restoring torques. These latter motions include the overdamped twisting deformations of an elastic filament, and overdamped local rotations of the fluorophore in a harmonic angular potential well fixed in the local macromolecular frame. Explicit formulas valid in particular cases have been presented.

Szabo[53] has formulated model-independent equations for fluorescence anisotropy, which can be used for any macroscopically isotropic system and which are particularly suitable for systems with an internal rotation:

$$r(t) = \frac{2}{5} \frac{\langle\langle k_f(t)\, \rho(\omega,t)\, P_2 [\, \vec{\mu}_e(t)\, \vec{\mu}_a(0)\,] \rangle\rangle}{\langle\langle k_f(t)\, \rho(\omega,t) \rangle\rangle} , \qquad (16)$$

where $\langle\langle k_f(t)\, \rho(\omega,t) \rangle\rangle$ is the overall fluorescence (i.e. $S(t)$), $k_f(t)$ is the electronic transition rate constant, and $\rho(\omega,t)$ describes the fluorescence spectrum, P_2 is the Legendre polynomial, and $\langle\langle\ \rangle\rangle$ denotes ansamble average with respect to possible orientations and energy states. Time-changes of the conditional probability density $p(i,\Omega;t\,|\,j,\Omega_0;0)$ must fulfill the following equation:

$$\frac{\partial}{\partial t}\ p(i,\Omega;t\,|\,j,\Omega_0;0) = \{\, \mathbf{L}(i,\Omega) - \mathbf{k}(i,\Omega) \,\}\ p(i,\Omega;t)\,|\,j,\Omega_0;0) , \qquad (17)$$

where parameter i indicates the energy state of a molecule, the Euler angles Ω describe its orientation, operator $\mathbf{L}(i,\Omega)$ comprises reversible energy and reorientation transitions, and $\mathbf{k}(i,\Omega)$ describes the complex rate constants of irreversible transitions of the system (both radiative and non-radiative). The form of the operator $\{\mathbf{L}(i,\Omega) - \mathbf{k}(i,\Omega)\}$ results from the chosen model. The set of Eqs (*17*) represents a matrix of integro-differential equations, which may take into account in a comprehensive and unified form a number of phenomena – energy transfer, heterogeneity of microenvironment (a location of the probe in conformations with different emission characteristics), interconversion of excited states with different emission characteristics, as well as both overall and internal rotation of a molecule and anisotropy of a medium. Energy transfer takes into account the mutual orientation of fluorophores and therefore the rate constants $\mathbf{k}(i,\Omega)$ depend explicitly on Ω. An analytical solution is possible only for some particular forms of operator $\mathbf{L}(i,\Omega)$ and rate constant $\mathbf{k}(i,\Omega)$, i.e. just for a few particular model systems. The solution is feasible provided that the energy and orientation relaxations are uncoupled, and the overall and internal rotations are not correlated. Paper[53] gives the fluorescence anisotropy behaviour for an internal rotation of a probe around one or several fixed axes or for a rotation around an axis which is not fixed and may move in an angularly symmetric potential field. The latter case can describe also the motion of a probe in a membrane (see also the Chapter 4). No particular assumptions on the nature of the internal motion of the probe were a priori made and jump motion, free motion and restricted motion of the probe were treated as special examples.

A realistic description of the behaviour of systems with possible internal rotations is so complex that all models had to introduce simplifying assumptions (often rather unrealistically oversimplifying assumptions), and a catholic use of the derived equa-

tions is impossible. The find fluorescence anisotropy formulas are usually so complicated that even superior quality data (with ultrashort excitation, deconvolution, a great number of counts in time-channels, etc.) do not permit to determine unambiguously all parameters with a sufficient accuracy. Despite a great number of theoretical studies[43 - 57] dealing with the problems of the internal rotation, analyses of experimental data are usually based on semiquantitative considerations[58 - 60].

For small and flexible molecules, such as biphenyl or butane, a more specific theoretical description of individual external and internal rotational modes is possible. For butane type molecules, there exists a strong coupling beween internal (recoil) and overall motions. Both rotational modes proceed on comparable time-scales and their coupling have to be taken into account to intepret experimental data correctly. A series of theoretical papers[61 - 65] have been published which treat the coupling effect from the point of view of the τ_1-NMR-relaxation times. Their applications to fluorimetry is fairly straightforward.

4. RIGID MOLECULE IN AN ANISOTROPIC MEDIUM

Fluorescence anisotropy of fluorophores embedded in a lipid bilayer, vesicle or membrane gives indirect information on the structure of the medium. The movement of a fluorophore is not equivalent in all three directions and the system usually does not relax completely – residual anisotropy is non-zero. Rotational relaxation is controlled by a potential field prefering certain orientations of the embedded probe. An entirely general theory does not exist; models for particular cases, however, do.

Most authors treat the behaviour of a symmetric-top probe. For a macroscopically isotropic suspension of plane lipid bilayers containing cylindrically symmetric fluorophore, a theory has been developed by Kinosita et al.[66]. The movement of lipid molecules as a whole is neglected and it is assumed that a fluorophore may rotate with respect to its symmetry axis, which can move. Movement of the symmetry axis is treated as a diffusion motion in an angularly symmetric potential. It has been shown quite generally (regardless of any model) that, the ratio of residual and initial anisotropies characterizes an effective anisotropy of the medium – which is called the degree of orientational constraint; and the time derivative of $r(t)$ at time $t = 0$ characterizes the average "velocity" with which the molecule wobbles within a confined angle.

A model of diffusion-in-a-cone assumes the simplest angularly dependent potential: $W(0) = 0$ for $0 \leq 0_{max}$ and $W(0) = \infty$ for $0 > 0_{max}$, 0 being the angle between the symmetry axis of fluorophore and the axis which is perpendicular to the bilayer. This model has been applied to three types of fluorescent molecules:

a) rod-shaped molecules with emission transition moment parallel to the long symmetry axis (a wobbling-in-a-cone model),

b) rod-shaped molecules with emission transition moment perpendicular to the long symmetry axis (a spinning-in-an-equatorial-band model),

c) disk-shaped molecules with emission and absorption transition moments in the plane of a disk, which undergo free in-plane rotation and restricted out-of-plane rotation.

Model predictions for the $r(t)/r(0)$ and r_∞/r_0 ratios has been presented in the form of infinite series, as well as truncated approximate formulas.

In later work Kinosita et al.[67] introduced the Gaussian model (the steady-state distribution of probe orientations prior to excitation is approximated by a Gaussian curve) and compared the results of the Gaussian model with those of the diffusion-in-a-cone model. The authors concluded that if only two parameters are used, the details of the model have no decisive impact on the data analysis provided that the symmetry of the rotational constraint is properly respected.

A similar model of fluorophores embedded in a membrane was developed by Lipari et al.[68]. He generalized Kinosita's model[66] by also considering the overall segmental motion under the assumption that the overall and internal motions are independent. In this work[68], a general and model-independent equation for residual anisotropy of rod-shaped and disk-shaped molecules has been derived:

$$r_\infty = \frac{2}{5} P_2(\cos(0_a))\ P_2(\cos(0_c))\ S^2 , \tag{18}$$

$$S = \langle P_2(\cos 0) \rangle , \tag{19}$$

where S is an order parameter, 0_a and 0_c are the angles between $\vec{\mu}_a$, $\vec{\mu}_c$ and the symmetry axis of molecule, $\langle\ \rangle$ denotes an ensemble average, P_2 is the second degree Legendre polynomial and 0 is a variable angle between the symmetry axis of the fluorophore and that of the lipid bilayer.

A promising model-independent approach to fluorescence depolarization of cylindrically symmetric fluorophore in the most compact form has been introduced by Heyn[69]. Orientation of the fluorophore is described by the probability density function, $f(0)$, where 0 is the angle between the symmetry axis of the fluorophore and that of the lipid bilayer. Function $f(0)$ is expanded in a series of Legendre polynomials:

$$f(0) = \frac{1}{2} \sum_{n=0}^{\infty} (2n + 1) \langle P_n \rangle P_n(\cos 0) , \tag{20}$$

where the n-rank orientational order parameters S_n are defined:

$$S_n = \langle P_n \rangle = \int_{-1}^{+1} P_n(\cos 0)\ f(0)\ \mathrm{d}(\cos 0) . \tag{21}$$

Symmetry properties of Legendre polynomials for odd n's lead to $S_n = 0$. For an isotropic medium, $f(0)$ is independent of the angle 0, and it follows from Eq. (*21*) that $S_n = 0$ for all $n \neq 0$ and $S_0 = 1/2$. For a system with perfectly ordered (aligned) fluorophores (i.e. $f(0) \neq 0$ only for $0 = 0$) are all $S_n = 1$. In a general case, S_n may attain values in the interval <0,1> depending on the directional constraint of the fluorophore movement.

This model-independent approach has been further improved by van der Meer et al.[70]. In agreement with the conclusions in ref.[67] it has been shown that if only two parameters are used, the choice of a model is unimportant. The applicability of the approach presented in ref.[70] has been verified experimentally by Ameloot et al.[71].

Later works[72] have proved that more than two parameters cannot be unambiguously determined from fluorescence anisotropy measurements in vesicles.

A model-independent determination of more parameters was successfully achieved for macroscopically oriented lipid multilayer systems[73,74].

A solution for an asymmetric probe reorienting in a membrane bilayer was presented by Tarroni and Zannoni[75]. They gave general expressions for the rotational diffusion matrix elements and performed numerical computations for some special cases.

Some older experimental fluorimetry works together with theoretical aspects of membrane studies are reviewed in ref.[76].

5. MORE COMPLEX ELECTRON RELAXATIONS

As soon as two or more electron transitions may take place simultaneously, expressions for fluorescence anisotropy become much more complicated.

In measurements of fluorescence decay kinetics of two or more different (mutually noninteracting) labels in the same medium ($i = 1, N$) or of one label in several distinct microenvironments ($i = 1, N$), the following equation holds for $r(t)$:

$$r(t) = \frac{\Sigma_i I^i_{\parallel}(t) - \Sigma_i I^i_{\perp}(t)}{\Sigma_i I^i_{\parallel}(t) + 2\Sigma_i I^i_{\perp}(t)} = \frac{\Sigma_i D^i(t)}{\Sigma_i S^i(t)} =$$

$$= \frac{\Sigma_i S^i(t)\, r^i(t)}{\Sigma_i S^i(t)} = \Sigma_i f^i(t)\, r^i(t)\,, \qquad (22)$$

$$\text{where } f^i(t) = \frac{S^i(t)}{\Sigma_i S^i(t)} \qquad (23)$$

are the fractional intensities. The necessary prerequisite for such a situation is that all the radiative electronic transitions must contribute additionally to the same emission

wavelength. For the simplest case of this type – i.e. two fluorophores each of which exhibits only one fluorescence lifetime and one rotational correlation time (the two lifetimes differ from each other and the same do the two rotational correlation times), the fluorescence anisotropy is the sum of two fluorescence anisotropies describing the respective fluorophores. The pre-exponential factors are time dependent, but can be obtained by measuring the fluorescence lifetimes, which facilitates the analysis of fluorescence anisotropy.

Expansion of the RD model to fluorescence anisotropy of a single fluorophore with several possible electron transitions is treated in refs[77,78]. Molecular movement is described in terms of the rotational diffusion of an asymmetric rotor. It is assumed that electronic transitions may be described by first-order rate constants unaffected by molecule orientation. Electronic transitions proceed very quickly so that no change in orientation takes place during the transitions. The number of energy levels is not limited, and the diffusive motion of fluorophores at individual energy levels may be generally different. To simplify the problem, a restricting assumption of the identical orientation of the principal diffusion axes for all electron states is employed, although the magnitudes of diffusion coefficients for individual excited states may differ. Authors used the following set of differential equations for probability densities of molecule orientations of individual electron levels, $f^i(\Omega,t)$:

$$\frac{\partial}{\partial t} f^i(\Omega,t) = -\sum_{m=1}^{3} D_m^i (\mathbf{L}_m)^2 f^i(\Omega,t) + \sum_{j=0}^{n-1} K_{ij} f^i(\Omega,t) , \qquad (24)$$

$$i = 0, 1, \ldots, n-1 ,$$

where D_m^i (m = 1, 2, 3) are diagonal elements of diffusion tensor for an i-th level, $\mathbf{L}$ is operator of the orbital angular momentum defined according to Rose[33], K_{ij} are elements of the matrix of rate constants in an n-level system. For a general case of an n-level system, the set of Eqs (*24*) cannot be solved directly, but it can be solved formally using diagonalization. For n = 1 the equations reduce to the RD model. The study[77,78] includes application of the method for five actual cases:

a) two-level system with different diffusion tensors,

b) four-level system, in which the transition from the excited state does not go back to the ground state, but in which chemical isomerization takes place,

c) three-level system, in which the main contribution to the signal results from birefringence,

d) system in which a fast relaxation into several other electron levels may take place,

e) system in which an absorption into two mutually overlapping energy bands may occur.

The same fluorescence anisotropy decay of a multilevel system without internal rotation can be obtained as a special case of the more general Szabo's[53] approach.

The theory of multilevel fluorescence depolarization was in the most complex and model-independent form discussed by Fisz[79].

6. FLUORESCENCE ANISOTROPY DECAYS FOR SYSTEMS WITH MORE COMPLEX ENERGETIC INTERACTIONS

Depolarization of fluorescence may be also influenced by some processes in which excited fluorophores take part. Processes depending on the chemical composition of a solution may be roughly divided into two groups: (i) processes depending on fluorophore concentration (such as energy transfer or migration, exciplex or excimer formation and other photochemical reactions), and (ii) processes depending on the chemical nature of the solvent (such as reorientational relaxation of solvation shell).

Energy transfer or migration are strongly concentration-dependent, and therefore their influence is often called concentration depolarization. These effects have been treated in a number of experimental and theoretical studies (e.g. refs[12,13,80 - 82]). A theoretical description of energy transfer or migration (see e.g. refs[83,84]) is most frequently based on the Forster mechanism of energy transfer[85]. The first theoretical studies presumed that orientational relaxation did not affect fluorescence depolarization (steady-state measurements[86], frozen solutions[9,11,87 - 90]). The influence of the concentration fluorescence quenching on the fluorescence anisotropy decay was also studied[91]. Later, it was assumed that orientational relaxation did proceed, but was uncoupled to energy transfer[92,93]. Recently, with the latest achievements of ultrafast spectrofluorimetry, newer studies[94 - 96] have appeared which try to treat both coupled phenomena.

Although it is obvious that the concentration depolarization must be taken into account in many systems, we will not deal with this effect in more details, as it exceeds the scope and extent of this review.

For a more general case, when it is necessary to take into account an influence of photochemical reactions on fluorescence anisotropy, it is possible to use the Fisz theory[79].

An interesting anisotropy decay was predicted theoretically for a system in which rotational and translational diffusion take place simultaneously with a reversible complex formation[97]. Description of such molecular behaviour is based on the rotational difffusion model and the Smoluchowski theory of diffusion-controlled reactions.

In the case that the solvent relaxation is slow and proceeds on the same time-scale as the rotational diffusion (e.g. in polar organic solvents containing inorganic ions), the solvation shell of the excited fluorophore may change during anisotropy decay measurements. This may provoke changes in the effective rotor shape and modify the decay

curve. Such behaviour has been observed experimentally[98]. This problem will not be treated here in more detail.

7. CONCLUSION

The paper presents a survey of principal theoretical models describing a reorientational motion of molecules in solutions and summarizes theories suitable for interpretation of anisotropy decays. The prerequisites, scope of applicability and resulting equation for experimentally measurable quantities are analyzed. Although a general theory applicable to any system is lacking, for a great majority of the studied systems a fairly suitable model may be found. So far a description of systems in which reorientation is coupled with a complex energy relaxation (by means of energy transfer or migration, dynamic equilibrium of formation and decay of excimer or exciplex, etc.) has not yet been satisfactorily solved, but these problems have been intensively studied in the recent time.

The authors wish to express their gratitude to Professor D. Phillips from Department of Chemistry, Imperial College of Science, Technology and Medicine, London, U.K. and to Professor S. E. Webber from Department of Chemistry and Biochemistry, and Center for Polymer Research, University of Texas at Austin, Texas, U.S.A. for helpful discussion.

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Translated by the author (Z. L.).