STUDY OF ENERGY TRANSFER AND CONCENTRATION DEPOLARIZATION OF FLUORESCENCE IN MIXED DYE SOLUTIONS

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The concentration depolarization of fluorescence of mixed solutions with gradually increasing absorption spectral overlap was studied. The experimental results may be explained if one assumes energy transfer from higher vibrational levels before thermal relaxation is established.

1. Introduction

The study of the mechanism and rates of excitation energy migration is an important problem in biophysics and biochemistry, since it is responsible for the transfer of light energy in plants. The complex structure of the photosynthetic apparatus makes the study of energy migration "in vivo" complicated and the comparison of its results with existing theories almost impossible. There still exists therefore a need to investigate "in vitro" model systems of gradually increasing complexity.

This paper reports one of the first steps in such a model investigation and deals with energy migration between unlike organic dyes in mixed solutions. The possibility of energy migration from higher vibrational levels of the first electronic state as well as from their fully relaxed Boltzmann distribution is taken into consideration. The problem of "before-relaxation" energy transfer occuring before the Boltzmann distribution of vibrational energy is established has been studied by many authors both experimentally and theoretically [1–12].

A very general theory of fluorescence depolarization due to energy migration has been developed by Jabłoński [13]. This "active sphere" theory may be easily adapted to the case of multicomponent dye solutions and for different interaction mechanisms between dye molecules. Furthermore, not only "after-relaxation" energy transfer but also the possibility of "before-relaxation" energy migration can be taken into account. For the

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case of two different dyes in solution (dye I and dye II) this theory leads to the general expression

$$\frac{\langle r\rangle}{r_0} = \frac{\overline{F}_D}{\overline{F}_D+\overline{F}_A+\overline{F}'_A} \tag{1}$$

describing the dependence of the emission anisotropy (EA) of the fluorescence of dye I on concentration (concentration depolarization) [14], where r_0 is the limiting EA obtained by extrapolation of the experimental results to concentrations approaching zero. The parameters $\overline{F}_D$, $\overline{F}_A$ and $\overline{F}'_A$ denote the probability that the light is emitted in the solution by the donors of dye I, the acceptors of dye I and the acceptors of dye I excited by energy transfer from dye II donors, respectively, in the absence of any contribution of dye II emission. These quantities may be expressed as follows:

$$\begin{aligned}
\overline{F}_D &= \sum_{k=1}^{\infty}\sum_{l=0}^{\infty} P_{kl}\langle F_{Dkl}\rangle,\\
\overline{F}_A &= \sum_{k=1}^{\infty}\sum_{l=0}^{\infty} P_{kl}(k-1)\,\langle F_{Akl}\rangle,\\
\overline{F}'_A &= \sum_{k=0}^{\infty}\sum_{l=1}^{\infty} P'_{lk}l\langle F'_{Akl}\rangle.
\end{aligned} \tag{2}$$

P_{kl} and P'_{lk} denote the probabilities of the formation of centres[1] of kind I and kind II. Centres of kind I consist of one donor I, $k-1$ acceptors I and l acceptors of kind II (where $k = 1, 2, 3, \ldots$, $l = 0, 1, 2, 3, \ldots$) whereas centres of kind II consist of one donor II, $l-1$ acceptors II and k acceptors I (where $l = 1, 2, 3, \ldots$, $k = 0, 1, 2, 3, \ldots$). F_{Dkl} denotes the probability of photon emission by a directly excited donor of dye I in a centre consisting of $k+l$ molecules. The remaining quantities are defined accordingly.

From Eqs. (2) it follows that, to calculate $\frac{\langle r\rangle}{r_0}$ as a function of dye concentration (concentration depolarization curve), one has to evaluate the mean emission rates F_{Dkl}, F_{Akl} and F'_{Akl} for the appropriate number of dye molecules in the centre. To obtain these rates one has to average over all distances (within the limits of the centre diameter) as well as over all orientations of the dipole moments.

Using this method, with some simplifying assumptions in evaluating $\langle F_{Dkl}\rangle$ and $\langle F_{Akl}\rangle$ for $(k+l) > 3$, the theoretical concentration depolarization curve was calculated for a dye mixture with non-overlapping absorption curves [15]. The active sphere radius was taken to be $R = 3R_0$, where R_0 is the so-called "critical-distance". The agreement between the theoretical curve and experimental results was found to be very good in a broad range of concentration. This agreement was considered to be a proof that the assumption of a "very weak" interaction mechanism between unlike molecules is fully justified in the absence of interfering "before-relaxation" effects.

[1] The luminescence centre is an initially excited fluorescent donor molecule surrounded by an active sphere in which various numbers of unexcited acceptor molecules may be present.

In solutions with two dyes having overlapping absorption spectra one excites both dyes and transfer can occur from dye II (with lower 0—0 transition frequency) to dye I, only before relaxation of vibrational energy to a Boltzmann distribution in the first excited electrinic state of dye II. In this case, if one wants to calculate numerically $\langle r \rangle$ from Eq. (1) one must take into account not only the retransfer itself but also its "before--relaxation" mechanism.

The population of higher vibrational levels of excited dye molecules decreases due to relaxation as a function of time elapsing from the excitation moment. Consequently, the rate of any "before-relaxation" energy migration will be time-dependent. Jabłoński introduced the simplifying assumption of two time-independent rates μ^b and μ^a for the migration before and after relaxation [6]. As a result of this assumption, the equations for the rates of emission from a molecule in a centre with two molecules, one donor and one acceptor of dye I, for instance, becomes quite simple [6]

$$F_{D20} = \frac{\eta}{2}\left[1 + \frac{1}{1+2\mu^a\eta\tau_0}\,\frac{\eta\tau_0+(1+2\mu^a\eta\tau_0)\Theta}{\eta\tau_0+(1+2\mu^b\eta\tau_0)\Theta}\right], \tag{3}$$

where η is the quantum efficiency, τ_0 — the natural lifetime and Θ is the vibrational relaxation time. This is, however, the most simple equation describing the rates in Eq. (2) and, even with the above mentioned simplifications, the numerical calculations of $\frac{\langle r \rangle}{r_0}$ according to Eq. (1) for a two component solution with possible "before-relaxation" energy migration becomes quite troublesome and computer-time consuming and has not yet been realized.

2. Experimental

The concentration depolarization of fluorescence of the three following equimolar two-component solutions was measured: 1. trypaflavine plus rhodamine B, 2. uranine plus rhodamine B, 3. rhodamine 6G plus rhodamine B.

The absorption spectral overlap of these dye pairs is the least in solution 1 and largest in solution 3. The donors in these solutions were trypaflavine, uranine and rhodamine 6G (dye I) while rhodamine B was the acceptor (dye II) in all cases. The solvent was glycerol to which ethyl or methyl alcohol and aqueous solutions of NaOH or CH_3COOH were added (2—4%) to ensure as low as possible a rate of dye molecule association. The viscosity of the solutions was high enough to allow one to neglect the depolarization due to Brownian rotations of the luminescent molecules.

Absorption and emission spectra of all the solution throughout the concentration range used were measured and an upper limit of dye molecule association of 2% was estimated. This estimate was confirmed by measurements of the optical densites of mixed solutions which were equal to the sum of optical densities of the appropriate solutions containing the pure components. The 0—0 transition wavelengths for the donors of the mixed solution 1, 2 and 3 were 476.5 nm, 510 nm, and 550 nm, respectively. The excitation of two-component solutions with light of the donor 0—0 transition wavelength eliminates before-relaxation effects in self-transfer.

The concentrations of the solutions studies covered a range of 10^{-5} M to 5×10^{-3} M. The mean decay times of rhodamine 6G solutions were measured as a function of concentration and found to be 4.2 ns regardless of concentration. The conclusion was therefore drawn that in the concentration range investigated, the quantum efficiency is constant, which means that concentration quenching and dye molecule association may be neglected.

The EA of the solutions studies, whose optical densities were kept below 0.1, was measured by means of a polarimeter (Fig. 1), which is a modified version of that described in [16]. The main change was the replacement of the Pockels cell (modulation frequency

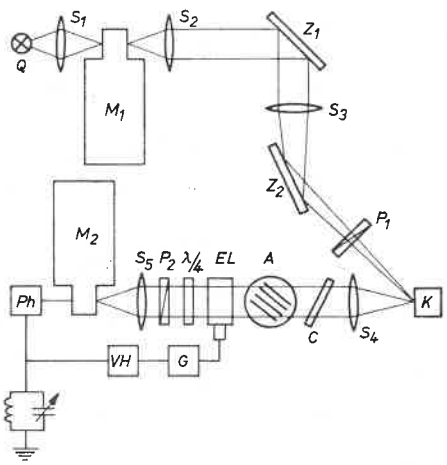

Fig. 1. Block diagram of the polarimeter: Q — xenon lamp, S — lenses, Z — mirrors, M_1 and M_2 — excitation and emission monochromators, P — polaroids, K — thin layer cuvette, C — compensation plate, A — Argo compensator, EL — ultrasonic standing wave modulator (54.7 kHz), Ph — photomultiplier (EMI-9558B), VH — phase sensitive voltmeter, G — generator (12 V, 0.2 W)

1 kHz) by an ultrasonic standing-wave modulator [17] with a modulation frequency of 54.7 kHz which resulted in increased sensitivity and signal-to-noise ratio. The ultrasonic modulator is very stable and conservative of electrical power (0.2 W). The change not only made possible EA measurements of solutions with optical densities below 0.1 but also allowed the use of monochromators in both the emission and excitation light paths.

To measure the EA of dye I in a mixed solution one has to isolate the fluorescence of dye I from the total emission. For dye combinations 1 and 2 this could be done simply by choosing an appropriately narrow bandwidth at approximately the wavelength of the donor fluorescence maximum, so that the acceptor fluorescence had no significant effect on the measurement. The excitation wavelengths were 476.5 nm for dye mixture 1 (0—0 transition) and 460 nm, 490 nm 510 nm for the mixture 2.

Since overlap of the emission spectra of the dyes in mixture 3 is quite strong, simple isolation with a monochromator is not sufficient to separate them. In the case of polarization measurements of these mixed solutions, one actually measures mixed emission at both wavelengths of maximum emission. To overcome this, we utilize a useful additive

property of the EA r defined by Jabłoński [18], which is more and more widely replacing the classical degree of polarization [19–21] and is defined as

$$r = \frac{I^{\parallel} - I^{\perp}}{I^{\parallel} + 2I^{\perp}}, \tag{4}$$

where $I^{\parallel}$ and $I^{\perp}$ are the intensities of the emission components polarized parallel and perpendicular to the electric vector of plane-polarized exciting light. It follows from this definition that, for a multicomponent emission

$$r = \frac{\sum_i I_i r_i}{\sum_i I_i}, \tag{5}$$

where I_i is proportional to total emission intensity ($I_i = I_i^{\parallel} + I_i^{\perp}$) and r_i is the EA of the i-th component of mixed fluorescence. The EA of the fluorescence of our mixed solution, observed at the wavelength of maximum emission of the donor, (λ_D), is therefore

$${}^{m}r_D = \frac{{}^{2}r_D\,{}^{2}I_D + b\,{}^{2}r_A\,{}^{2}I_A}{{}^{m}I_D}, \tag{6}$$

where ${}^{m}I_D$ is the intensity of fluorescence observed at λ_D, while ${}^{2}I_A$ and ${}^{2}I_D$ are the maximum intensities of fluorescence, of the acceptor and donor respectively, in a mixed

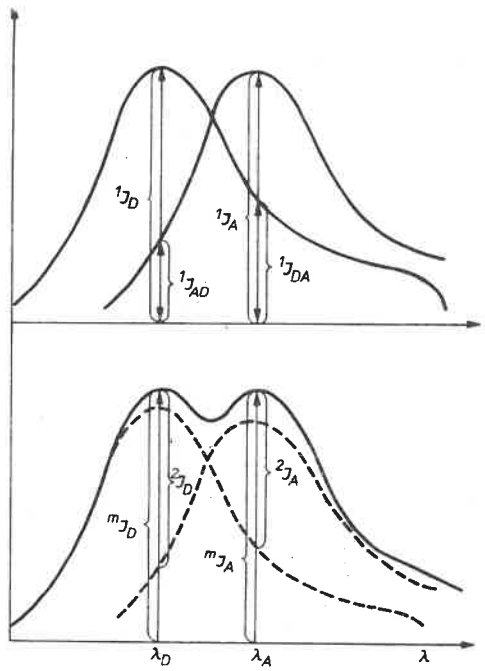

Fig. 2. Illustrations to the sub- and superscripts used in Eq. (5) to Eq. (15)

solution; ${}^2r_D$ and ${}^2r_A$ are the EA's of the fluorescence ${}^2I_D$ and ${}^2I_A$, respectively; and $b = \frac{{}^1I_{AD}}{{}^1I_A}$, is the ratio of fluorescence intensities of the acceptor, observed at λ_D and λ_A in pure solutions. Similarly, one can write an equation for the EA of the fluorescence of a mixed solution observed at wavelength λ_A

$$ {}^m r_A = \frac{{}^2r_A\,{}^2I_A + \beta\,{}^2r_D\,{}^2I_D}{{}^mI_A}, \tag{7} $$

where β is $\frac{{}^1I_{DA}}{{}^1I_D}$ (the assignments of the different super- and subscripts of the I-values are clarified in Fig. 2). Evaluating ${}^2r_A$ from Eq. (7) and putting it into Eq. (6), we get:

$$ {}^m r_D = \frac{{}^2r_D\,{}^2I_D(1-b\beta) + b\,{}^m r_A\,{}^mI_A}{{}^mI_D}. \tag{8} $$

From Eq. (8), it follows that

$$ {}^2r_D = \frac{{}^m r_D\,{}^mI_D - b\,{}^m r_A\,{}^mI_A}{{}^2I_D(1-\beta b)} \tag{9} $$

and symmetrically

$$ {}^2r_A = \frac{{}^m r_A\,{}^mI_A - \beta\,{}^m r_D\,{}^mI_D}{{}^2I_A(1-\beta b)}. \tag{10} $$

Eqs. (9) and (10) can be used to calculate the EA of the fluorescence of the donor or acceptor in a mixed solution with overlapping emission spectra, provided the intensities of fluorescence of pure donor and pure acceptor solutions at λ_D and λ_A, as well as those of mixed solutions, are measured under the same geometrical conditions. To find ${}^2I_A$ and ${}^2I_D$, we must take into account the fact that the intensities of fluorescence of mixed donor--acceptor solutions, measured at the wavelengths λ_D and λ_A, are

$$ {}^mI_D + {}^2I_D + b\,{}^2I_A \tag{11} $$

and

$$ {}^mI_A = {}^2I_A + \beta\,{}^2I_D. \tag{12} $$

Eqs. (11) and (12) contain two unknown factors, ${}^2I_A$ and ${}^2I_D$, which can be easily evaluated as

$$ {}^2I_D = ({}^mI_D - b\,{}^mI_A)/(1-b\beta) \tag{13} $$

and

$$ {}^2I_A = ({}^mI_A - \beta\,{}^mI_D)/(1-b\beta). \tag{14} $$

Finally

$$ {}^2r_D = \frac{({}^mI_D/{}^mI_A)\,{}^m r_D - ({}^1I_{AD}/{}^1I_A)\,{}^m r_A}{({}^mI_D/{}^mI_A) - ({}^1I_{AD}/{}^1I_A)}, $$

$$ {}^2r_A = \frac{({}^mI_A/{}^mI_D)\,{}^m r_A - ({}^1I_{DA}/{}^1I_D)\,{}^m r_D}{({}^mI_A/{}^mI_D) - ({}^1I_{DA}/{}^1I_D)}. \tag{15} $$

It is essential to maintain precisely the same optical geometry as well as gain in the detection circuitry during both the EA and fluorescence intensity measurements. To get reliable results for the EA as calculated from Eqs. (15), the quantities appearing in these equations must be measured contemporaneously without change in the apparatus (except for interchanging the samples with mixed and pure acceptor solution of equal concentration).

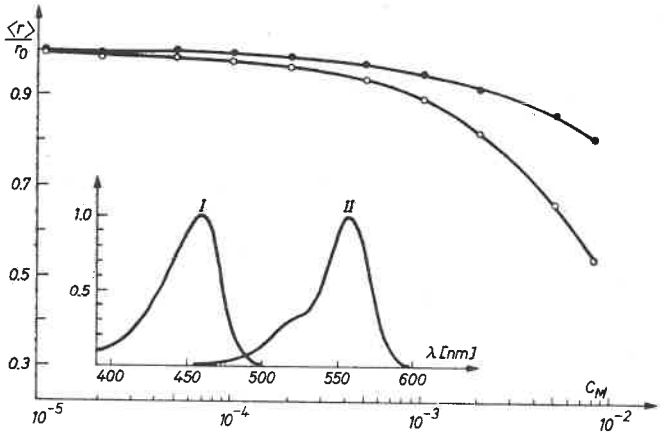

Fig. 3. The dependence of the EA on concentration of pure trypaflavine in its equimolar mixture with rhodamine B (inserted are their absorption spectra). Excitation wavelength $\lambda_{exc} = 476.5$ nm

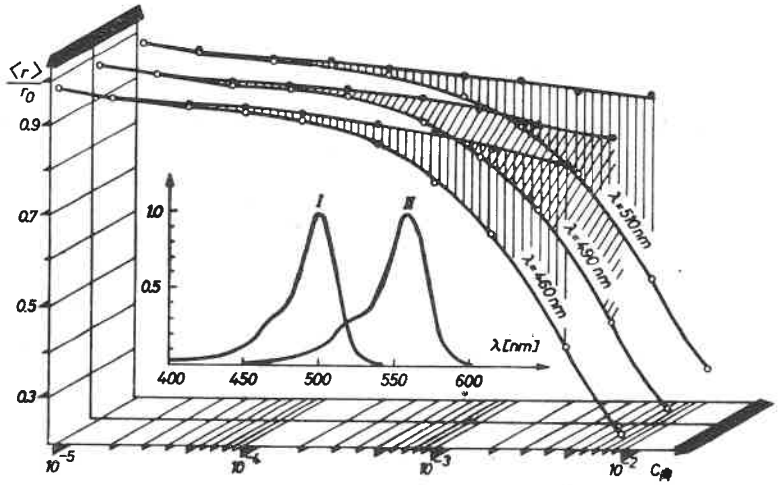

Fig. 4. The dependence of the EA on concentration of pure uranine in its equimolar mixture with rhodamine B (inserted are the two absorption spectra). Excitation wavelengths: 510 nm, 490 nm and 460 nm

The results of measurements of the polarization as a function of concentration of the dye and of the excitation wavelength are plotted in figures 3—5, together with the absorption spectra of the two dyes forming the mixtures 1, 2 and 3. From these results, the general conclusion may be drawn that the concentration depolarization for the mixed solutions

(upper curves) is always less pronounced than that for pure solutions. This effect is expected since the quantum efficiency and the decay time of the donor fluorescence are decreased by transfer to unlike acceptors making the donor concentration depolarization less pronounced.

Solutions 1 have a negligible absorption spectral overlap and the mutual position of the two curves in Fig. 3 does not depend on the excitation wavelength. The dependence of the EA on concentration of pure uranine or of the mixed dye combination 2 is illustrated

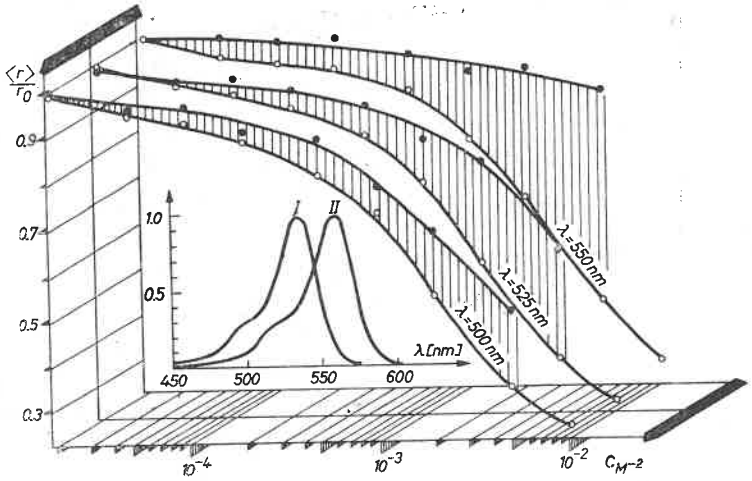

Fig. 5. The dependence of the EA on concentration of pure rhodamine 6G in its equimolar mixture with rhodamine B (inserted are their absorption spectra). Excitation wavelengths: 550 nm, 525 nm and 500 nm

in Fig. 4. Here, although again the polarization of the donor in mixed solutions is always higher as compared with that of the pure solution, this difference decreases with decreasing excitation wavelength (λ_{exc} equal 510 nm, 490 nm and 460 nm, respectively). As we see from figure 5, which represents the experimental results for the dye combination 3 (with greater overlap of absorption curves), there is also a difference between the upper and lower curves as a function of excitation wavelength. This λ_{exc}-dependent difference is in this case even more pronounced that for dye combination 2.

3. *Conclusion*

The dependence of the EA of fluorescence in the solutions investigated on the frequency of exciting light, indicates the existence of "before-relaxation" energy transfer. In Fig. 6, three level diagrams illustrating possible excitation energy paths for pairs of molecules with different 0—0 transitions are drawn. Diagram (a) illustrates the case of 0—0 excitation of the donor. The radiationless energy transfer from molecule I to molecule II causes a decrease in efficiency and decay time of the fluorescence of molecule I. In case (b) molecule I is excited with a frequency higher than ν_{0-0}; however, the vibrational energy relaxation is so fast, that excitation energy transfer occurs only after the Boltzmann distribution

is established. No dependence of the EA of fluorescence of molecule I on λ_{exc} is observed. In diagram (c), the excitation energy transfer paths for "before-relaxation" transfer are illustrated. Excitation energy transfer to molecule I may be a retransfer (changing only the efficiency) or a normal transfer of energy from the primarly excited molecule II (this means that molecule I emits highly depolarized sensitized fluorescence). Both effects decrease the polarization of the donor fluorescence. The efficiency of "before-relaxation"

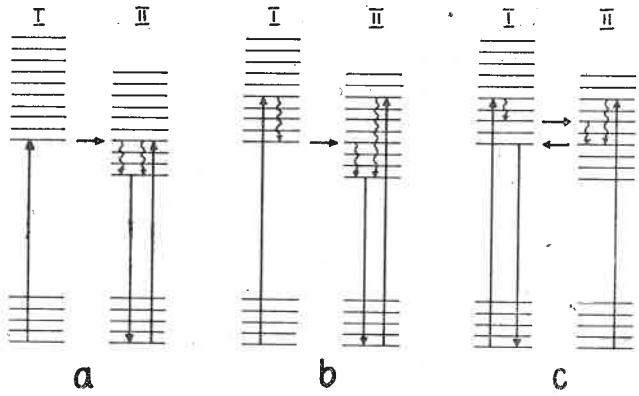

Fig. 6. Level diagrams for two molecules with different 0–0 transitions: (a) excitation to the 0–0 level of I, (b) excitation to higher vibrational levels. Energy transfer is of the "after-relaxation" kind, (c) excitation to higher vibrational levels in both molecules; "before-relaxation" transfer and retransfer is possible

energy transfer depends on the value of the overlap integral of the virtual donor's emission[2] spectrum and the acceptor's absorption spectrum. Thus it depends on the frequency of the exciting light and on the overlap of the absorption spectra of molecules I and II.

The experimental results for systems 2 and 3 may be fully explained on the grounds of the scheme presented in Fig. 6, i.e. under the assumption that the excitation energy transfer occurs from higher vibrational levels of the electronic excited state.

Energy transfer from one molecule to another depends on an interaction between them. The determination of the character of this intermolecular coupling would make possible an explanation of the mechanism of "before-relaxation" energy transfer. However, the question of whether "before-relaxation" energy transfer is possible in the case of a "very weak" interaction, or whether in order to explain it one must necessarily assume that the interaction between molecules is "weak", has no definite answer as yet. Some authors [22–24] assume that intermediate interactions are also possible. In this case the rate of transfer between two isolated molecules need not be strictly R^{-6} (very weak interaction) or R^{-3} (weak interaction) distance-dependent.

More exact information about the mechanism responsible for "before-relaxation" energy transfer may be obtained by comparison of the experimental and theoretical concentration depolarization curves.

[2] The virtual emission is defined as emission that would occur from higher vibrational levels of the excited electronic state if thermal relaxation or energy transfer did not occur first.

Up to now numerical calculations have been performed under the assumption that the exciting light does not disturb the thermal equilibrium between the excited molecules and environment, i.e. for the case of excitation in the region of the 0—0 transition. An attempt has been made to extend these calculations to the case of "before-relaxation" energy transfer for the pure and mixed solution, on the basis of Jabłoński's active sphere theory. The results obtained have given no definite answer as to whether the mechanism invoked correct or not. It seems, however, that measurements on the picosecond time--scale of the vibrational energy relaxation and on the quantum efficiency of fluorescence from higher vibrational levels will allow to use Jabłoński's concentration depolarization theory for the more precise interpretation of the "before-relaxation" energy transfer mechanism.

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