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Red-edge-excitation fluorescence spectroscopy of indole and tryptophan

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Abstract. Studies on the dependence of indole and tryptophan fluorescence emission spectra on excitation wavelength, λ^{ex}, show that the emission shifts to longer wavelengths for red-edge excitation in different solid and viscous solvents. In solid systems the spectral shifts for excitation in the range from 290 to 310 nm can reach tens of nm, and they are more significant than changes of λ^{ex}. In a viscous medium the magnitude of this effect is shown to be directly related to the dipole-reorientational relaxation of solvent molecules in the environment of the chromophore, which allows the relaxation times to be estimated. The method involves simple steady-state measurements of fluorescence spectra at the maximum and at the red edge of the absorption band. Since it is not necessary to obtain information on the fluorescence spectra of completely relaxed states, this method for the estimation of relaxation times may have advantages in studies of proteins compared with the conventional relaxation shift method, and may produce complementary information to that obtained by nanosecond time-resolved spectroscopy.

Key words: Dipole-reorientational relaxation, fluorescence spectroscopy, red-edge effects, indole, tryptophan, dynamics of chromophore environment

Introduction

The fluorescence spectroscopic properties of indole and tryptophan are of wide interest because the tryptophan residues contribute greatly to the intrinsic fluorescence of proteins. The analysis of protein spectroscopic data in terms of structure and dynamics of chromophore environment is usually based on the results of model studies of indole, tryptophan and their derivatives in isotropic media. Tryptophan fluorescence is known to be sensitive to the polarity of the environment (Konev 1967; Longworth 1971; Burstein 1976, 1977), formation of exciplexes (Lumry and Hershberger 1978; Burstein 1976, 1977) and to the rate of solvent and protein dipole-reorientational relaxation (Pikulik et al. 1967; Eisinger and Navon 1969; Lakowicz et al. 1980; Lakowicz and Balter 1982). Owing to these important factors, large variations of tryptophan fluorescence spectra (up to 45 nm in the position of the maximum emission) are observed. This is the basis for the high sensitivity of fluorescence spectra to structural changes and the reason why this method is so popular in studies of proteins. However, the analysis of protein spectra in terms of the contribution of these different mechanisms to observed spectral shifts is far from simple or unequivocal. For instance, when the dipole-reorientational equilibrium is not attained, one cannot estimate the polarity of chromophore environment by comparing the results obtained in experiments on proteins with those obtained on model liquid solvents. This is because an important component contributing to the fluorescence shift, that deriving from dipole-dipole interactions, is different. The recently developed methods of nanosecond time-resolved spectroscopy permit direct examination of spectral dynamics in time. Spectral relaxations on the nanosecond time scale may be revealed by time-dependent shifts of fluorescence spectra following pulse excitation (DeToma et al. 1976), by the dependence of fluorescence decay kinetics on observation wavelength (Grinvald and Steinberg 1974) as well as by using wavelength-dependent phase-sensitive detection of fluorescence (Veselova et al. 1965; Lakowicz and Balter 1982). However, the analysis of nanosecond kinetics data involves some new problems: (*i*) With many proteins, and even with tryptophan in solution, non-exponential or multi-exponential emission decay is often observed (Beechem and Brand 1985). (*ii*) In several proteins spectral shifts with time were not observed (Grinvald and Steinberg 1976). How high is the chromophore dipole-relaxational mobility in this case? (*iii*) Even if some dynamic property of the spec-

trum is detected, it may not necessarily be accounted for by dipole relaxation but could also be due to other excited state reactions, e.g., exciplex formation, proton transfer or changes in solvate shell composition (Demchenko 1986). A method is needed which is able to resolve the non-relaxed electronic excited states in proteins.

Recently it was suggested that dipole-relaxational mobility in proteins could be analysed using fluorescence spectroscopy with excitation at the red-edge of absorption band (Demchenko 1981, 1982, 1984). The red-edge effects in fluorescence spectra originate from distribution of chromophores within a population of microstates differing in interaction energy with the environment(Galley and Purkey 1970; Rubinov and Tomin 1970; Macgregor and Weber 1981). The exist a variety of specific red-edge effects, among which the shift of fluorescence emission spectra to longer wavelengths on red-edge-excitation is observed most easily. This effect is maximal when the chromophore environment is rigid on nanosecond 1 – ns time scale (emission from the non-relaxed state); it should decrease, if the fluorescence lifetime τ_F and dipole-reorientational relaxation time τ_R are of the same order of magnitude, and it should vanish, if the relaxation is rapid ($\tau_R \ll \tau_F$). Although this effect was extensively studied for different chromophores including phthalimides (Rubinov and Tomin 1970, 1984), rhodamines (Rubinov and Tomin 1984) and anilinonaphthalene sulfonates (Azumi et al. 1976; Demchenko 1982; Lakowicz and Keating-Nakamoto 1984), the data on indole and tryptophan derivatives are rather scarce and fragmentary (Galley and Purkey 1970; Demchenko 1981; Lami 1981).

In this report we present results of systematic studies concerning the effect of red-edge excitation on fluorescence spectra of indole and tryptophan in different solvent media: solid, highly viscous and liquid. We show that in polar solid media the red-edge shift effect may exceed 30 nm. The magnitude of the shift may exceed that which is observed on transition from a solid to a liquid solvent with normal mid-band excitation. These findings may serve as the basis for analyzing dipole-relaxational non-equilibrium excited states in proteins and allow direct evaluation of dipolar relaxation times.

Theory

General

Fluorescence spectroscopy allows direct observation of molecular motions occurring in the chromophore environment at nanosecond times. Absorption of a quantum of light leads to redistribution of electronic density in the chromophore, and then a new equilibrium is established between the excited chromophore and surrounding molecules (relaxation process). This brings about a time-dependent structural redistribution of the chromophore environment (solvate shell) resulting in a state which is in an intermolecular equilibrium with the excited chromophore. Such a state may be reached, or not, depending on the relative magnitude of the excited state lifetime, τ_F, and the dipole-reorientational relaxation time, τ_R.

The description of a real relaxation process by single values of τ_F and τ_R is first-order approximation, and a distribution of these values should be expected and is actually observed in many cases. However, the current models of relaxation operating with these single values provide a reasonably consistent description of experimental data (Lakowicz 1983).

The Bakhshiev-Mazurenko model of relaxation

Let us consider the four-level energy diagram which usually serves as a basis for the analysis of intermolecular interactions and relaxations in electronic spectra (Fig. 1). The absorption of a quantum of light, $h\nu_a$, by the system results in its transition to the excited Franck-Condon state, E_e^{FC}, which is not at equilibrium with its environment. Quanta emitted during the initial very short period of time (we approximate $t = 0$) are from this level, their energy is high, and the emission spectrum is centred at short wavelength. In such spectra the situation is analogous to the case of steady-state spectra in solid solutions, when $\tau_R \gg \tau_F$.

Quite different is the case when the observation time is very long ($t \to \infty$), the rearrangement of surrounding dipoles being complete, and the chromophore reaches energy equlibrium with the environ-

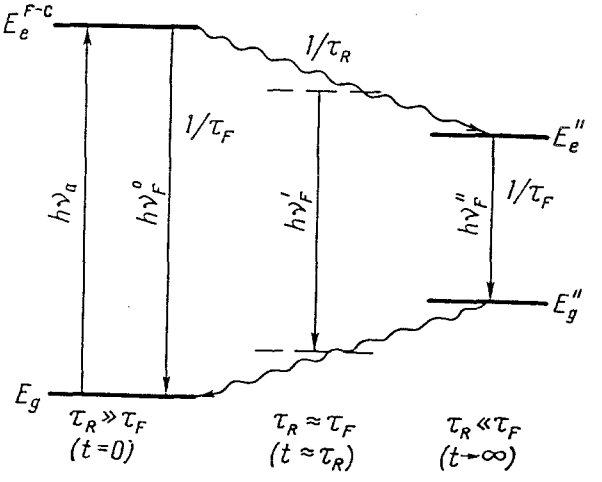

Fig. 1. Energy diagram of electronic levels for intermolecular interactions and relaxations (continuous relaxation model)

ment prior to emission. The situation is realized in the steady-state spectra of liquid solutions, when $\tau_F \gg \tau_R$.

The Bakhshiev-Mazurenko model (Bakhshiev et al. 1966; Mazurenko and Bakhshiev 1970; Bakhshiev 1972) considers the case where a decrease in the number of chromophores in the excited state occurs simultaneously with reorientational relaxation of their environment. In the following considerations we accept that the emission spectrum contour depends only slightly on time and may be expressed by a certain function $I(\nu - \xi)$, where ξ is a certain characteristic point of the spectrum. If N_0 of molecules was excited at the time $t = 0$, then in the time interval $[t, t + dt]$ and wavenumber range $[\nu, \nu + d\nu]$ the number of quanta emitted, dN, will be:

$$dN = N_0 \frac{\gamma}{\tau_F} I(\nu - \xi(t)) \exp\left(-\frac{t}{\tau_F}\right) d\nu\, dt, \tag{1}$$

where γ is a quantum yield of emission. The dependence $\xi(t)$ for the Debye model of dipole relaxation may be expressed in the form:

$$\xi(t) = \xi_{t\to\infty} + (\xi_{t=0} - \xi_{t\to\infty}) \exp\left(-\frac{t}{\tau_R}\right). \tag{2}$$

The function

$$I(\nu, t) = \frac{1}{\tau_F} I(\nu - \xi(t)) \exp\left(-\frac{t}{\tau_F}\right), \tag{3}$$

may be considered at a fixed short time as an "instantaneous" fluorescence spectrum, or for a fixed wavenumber as a law of the emission decay.

Since $I(\nu, t)$ describes the relative number of quanta emitted per unit time interval in a unit range of wavenumbers, the steady-state emission spectrum is:

$$I'(\nu) = \int_0^\infty I(\nu, t)\, dt. \tag{4}$$

If $\xi(t)$ is assumed to be equal to the "centre of gravity" of the distribution $I(\nu - \xi)$ for the wavenumber $\bar{\nu}$ describing the "gravity centre" of the integral spectrum $I'(\nu)$ from Eqs. (2), (3) and (4) we obtain (Mazurenko and Bakhshiev 1970; Bakhshiev 1972):

$$\bar{\nu} = \bar{\nu}_\infty + (\bar{\nu}_0 - \bar{\nu}_\infty) \frac{\tau_R}{\tau_R + \tau_F}, \tag{5}$$

where $\bar{\nu}_0$ and $\bar{\nu}_\infty$ are the centre of gravity wavenumbers that correspond to limiting cases of emission from non-relaxed and completely relaxed states. Equation (5) satisfactorily derscribes the relative shift of spectra for changes in τ_R and τ_F induced by fluorescence quenchers or changes of temperature. It allows τ_R to be estimated, provided τ_F is known.

In cases where the emission spectrum shape does not change significantly at different ratios of τ_R and τ_F or at different emission times, the position of emission maxima may be used instead of the "gravity centres" of spectra.

The maximal value of wavenumber shift $\bar{\nu}_0 - \bar{\nu}_\infty$ depends on the difference in dipole moments of the chromophore in the ground and excitet states $\mu_e - \mu_g$, static dielectric constant ε_0 and refractive index of the solvent n as described by the equation (Mazurenko and Bakhshiev 1970; Bakhshiev 1972):

$$\bar{\nu}_0 - \bar{\nu}_\infty = \frac{2(\mu_e - \mu_g)^2}{c\, h\, \varepsilon_0' a^3} \left(\frac{\varepsilon_0 - 1}{\varepsilon_0 + 2} - \frac{n^2 - 1}{n^2 + 2}\right), \tag{6}$$

where a is the chromophore Onsager radius, h is the Planck constant, ε_0' is the absolute dielectric constant in vacuum, and c is the velocity of light.

This model, which assumes no distribution in environments within the chromophore population in solution, is in agreement with much of the steady-state and time-resolved experimental data (DeToma et al. 1976). The model predicts and quantitatively describes the spectral shifts dependent on solvent and temperature which occur on the nanosecond timescale. However, there are observations which are not consistent with this model: (*i*) The temperature-dependent relaxation range actually observed is much wider than calculated by Eq. (5) (Pavlovich and Pikulik (1975) which may be due to a distribution in relaxation times. (*ii*) The spectra display substantial variation in bandwidth in the course of relaxation (DeToma et al. 1976). (*iii*) The model does not suggest any explanation for the dependence of fluorescence spectra on excitation wavelength which is observed experimentally and will be considered below.

The two-state model

An alternative model of relaxation, the discontinuous two-state model, assumes that emission proceeds from two discrete states: initial and relaxed (Lakowicz and Balter 1982; Lakowicz 1983). The interplay of the two exponential processes is thought to be observed in time: fluorescence emission with rate constant $k_F = 1/\tau_F$ and transition to the relaxed state with a rate constant $k_R = 1/\tau_R$. The spectrum of the relaxed state is centred at $\bar{\nu}_R$ and is red-shifted with respect to the spectrum of the initial state, which is centred at $\bar{\nu}_F$. With no relaxation during the emission ($\tau_R \gg \tau_F$) the fluorescence spectrum is centred at $\bar{\nu}_F$ and when relaxation is complete ($\tau_R \ll \tau_F$) at $\bar{\nu}_R$.

This simple model is able, to a first-order approximation, to provide a description of emission heterogeneity. In the relaxation range the emission from both initial and relaxed states is seen in different proportions (these proportions are equal if $\tau_R = \tau_F$). Therefore at intermediate relaxation stages the fluorescence spectrum should be much broader than at the initial and final stages. The estimation of relaxation times requires knowledge of the basic fluorescence spectra of the initial and relaxed states.

The estimation of τ_R is simple on the basis of time-resolved information on fluorescence decay. If only non-relaxed chromophores are excited, the decay is exponential beyond the limits of the relaxation range and within this range is resolved into two components with emission-wavelength-independent apparent decay times τ_1 and τ_2. Then the longer component $\tau_1 = \tau_F$ and the shorter $\tau_2 = 1/(1/\tau_R + 1/\tau_F)$. This approach is difficult to apply when the emission is non-exponential beyond the relaxation range, as in the case of tryptophan (Beechem and Brand 1985).

The explanation of the red-edge effect on the basis of the two-state model is the following (Lakowicz and Keating-Nakamoto 1984). Red-edge excitation selects "relaxed" molecules, i.e., those which have the orientations that correspond to the relaxed state. The emission spectrum for red-edge excitation resembles the spectrum of the high-temperature relaxed state. This model does not explain the fact that the fluorescence spectrum for far red-edge excitation may be so far shifted as to be situated at lower energies than the spectrum of the completely relaxed state. At nanosecond times such spectra move to shorter wavelengths, this phenomenon is called "up-relaxation" (Nemkovich et al. 1980).

The difficulties with simple continuous and two-state models of relaxation requires the introduction of a more elaborate model. Two steps towards a realistic description of the relaxation process may be suggested: the introduction of a distribution in relaxation times and the introduction of a distribution in initial interaction energies for chromophores with their environment. The latter appears to be more promising since it suggests obtaining and evaluating new experimental data on the dependence of fluorescence spectra on excitation wavelength and the influence of relaxation on this dependence.

Site-selection model of relaxation

The systems with slow relaxation ($\tau_R \geqq \tau_F$) display specific "edge" effects in excitation, emission and excitation energy transfer (Demchenko 1986). Of these effects the shift of emission to longer wavelengths caused by red-edge excitation is the main one and is the easiest to observe (Galley and Purkey 1970; Rubinov and Tomin 1970; Macgregor and Weber 1981; Demchenko 1982). The analysis of these phenomena requires application of more complex models which take into account the statistical distribution of interaction energies for molecules with their environment, and photoselection of excited species according to the energy of absorbed or emitted quanta. The molecules interacting in solution may differ in their mutual orientation and interaction energy, which results in alteration of the energy of electronic transitions. Both the character of the energy distribution at the moment of excitation and its change with time (relaxation) determine the spectroscopic behaviour of the system.

Let us consider the energy-level diagram presented in Fig. 2. The diagram accounts for the distribution of interaction energy for molecules with their environment (Demchenko 1982). The energy, E, of each ground or excited electronic level may be represented as a sum of $E = E_0 + \Omega(W_{dd})$ where E_0 is the energy of the non-perturbed level, while the stabilization energy is represented by the distribution $\Omega(W_{dd})$, where W_{dd} is the energy of dipole interactions. These distributions are different in the ground and excited states, since the electronic chromophore structure changes considerably on excitation, and orientations of solvent molecules which are energetically advantageous in the ground state become disadvantageous in the excited state, and vice versa. If the excitation is provided at the longer wavelength edge by quanta with lower energy $h\nu_a^{\text{edge}}$, there occurs photoselection of only those chromophores whose interaction energy with the environment (the energy of solvation) is the lowest in the ground and the highest in the excited state. The photoselection is based on the fact that a quantum of light with lower energy may be absorbed only by those chromophores for which $h\nu_a^{\text{edge}} = E_e^{\text{edge}} - E_g^{\text{edge}}$, whereas for the rest which have a wider gap between the ground and excited electronic levels, the energy of the quantum is not sufficient for absorption. It is evident that if no dipole relaxation occurs during the time τ_F, the energy of the emitted quanta $h\nu_F^{\text{edge}}$ will be lower than $h\nu_F^{\text{mean}}$. This causes the red-edge-excitation shift of the fluorescence spectra to longer wavelengths.

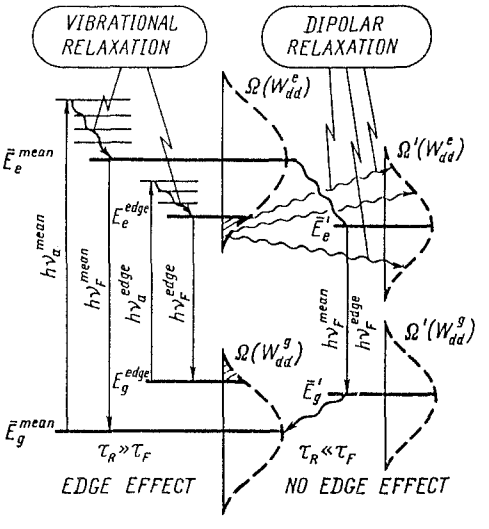

Fig. 2. Energy diagram of electronic levels including distribution of interaction energies in the ground and excited states and intermolecular relaxations (inhomogeneous broadening continuous relaxation model)

If, however, $\tau_R \ll \tau_F$, the rapid dipole relaxation leads to the rapid redistribution of the chromophore-environment interaction energies. As a result the chromophore, which is excited by a lower energy quantum at the red edge, "forgets this fact", and the fluorescence spectrum becomes independent of excitation wavelength.

To describe the red-edge-excitation effects as a function of the relaxation properties of the chromophore environment we made an attempt to generalize the Bakhshiev model by considering relaxation as a process of establishment of an equilibrium distribution in an ensemble of interacting particles.

Relaxation of emission frequency of individual chromophores may be described by Eq. (2), $\xi_{t=0}$ is considered to be dependent on excitation wavelength. Since the equilibrium orientation of dipoles is reached at infinite times, $\xi_{t\to\infty}$ does not depend on which wavelength is used to excite the population of chromophores. Considerations similar to those mentioned above in the analysis of Eqs. (1), (3) and (4) may be applied for two distinct cases: excitation of the whole chromophore population (at the absorption maximum) and of that part with the highest interaction energy (at the red-edge).

Denoting emission frequencies for the mean and edge excitations $\bar{\nu}^{\text{mean}}$ and $\bar{\nu}^{\text{edge}}$, Eq. (5) may be rewritten for two cases. Subtraction with the condition that $\bar{\nu}_\infty^{\text{mean}} - \bar{\nu}_\infty^{\text{edge}} = 0$ (at infinite times fluorescence spectra should not depend on excitation wavelength) yields:

$$\bar{\nu}^{\text{mean}} - \bar{\nu}^{\text{edge}} = (\bar{\nu}_0^{\text{mean}} - \bar{\nu}_0^{\text{edge}}) \frac{\tau_R}{\tau_R + \tau_F}. \tag{7}$$

The condition $\tau_R = \tau_F$ is fulfilled for the transition midpoint, when

$$\bar{\nu}^{\text{mean}} - \bar{\nu}^{\text{edge}} = \tfrac{1}{2} (\bar{\nu}_0^{\text{mean}} - \bar{\nu}_0^{\text{edge}}). \tag{8}$$

The advantage of application of red-edge-excitation spectroscopy in the evaluation of τ_R using Eq. (7) over the analysis of τ_R by relaxational shifts of fluorescence spectral maxima using Eq. (5) follows from the fact that Eq. (7) does not contain $\bar{\nu}_\infty$. $\bar{\nu}_\infty$ is the parameter in molecular relaxation spectroscopy, the evaluation of which is associated with substantial difficulties and is not possible in many cases (e.g. in the studies of proteins, when the temperature rise causes denaturation). The application of the proposed method is simple: beside measuring τ_F it is sufficient to determine the shift between positions of fluorescence maxima at two excitation wavelengths (at the maximum and at the red-edge of the absorption band) and a limiting value of this shift. The latter is observed at short times after excitation (in time-resolved spectra) or in steady-state spectra on lowering the temperature (increasing τ_R) or introducing dynamic quenchers (decreasing τ_F).

Experimental

Indole and DL-tryptophan from Serva were used as well as indole from local suppliers that was further purified by sublimation.

The fluorescence spectra at different excitation wavelengths were measured using an Hitachi MPF-4 operated in the signal ratio mode. The light source was a 150 W xenon lamp. The spectral bandwidth of the exciting monochromator was 2 nm, that of emission one – 2 to 5 nm. In some experiments at 300 to 315 nm excitation, an interference filter (cut off at $\lambda > 320$ nm) was used. Correction of fluorescence spectra for the spectral sensitivity of the device was not performed because the measured spectra for indole and tryptophan corresponded to those described in literature with differences in emission maxima smaller than 1–1.5 nm.

Fluorescence of solid specimens was recorded with the front surface placed at an angle of 30° to the excitation beam. Glycerol solutions at temperatures from −15° to + 50 °C were studied using 1 cm rectangular cells which were thermostated either by circulating thermostat of by LKB cryothermostat. In experiments at liquid nitrogen temperatures round cells with 5 mm internal diameter and a vacuum Dewar flask was used.

Polyvinyl alcohol films of 0.2 – 0.5 mm thickness were prepared by slow evaporation of 5% solutions of polyvinyl alcohol to which indole or tryptophan had been added at the desired concentration. Glucose glasses were prepared by melting glucose on a glycerol bath, adding weighed amounts of indole or tryptophan and placing a thin layer of solution on a silica plate.

Absorption spectra were recorded on a Beckman model 25 spectrophotometer.

Concentrations of indole and tryptophan used were in the range 10^{-4} to 10^{-3} mol/l. Light reabsorption by the sample was negligible at these concentrations.

Nanosecond emission decay and the excited state lifetimes τ_F were determined using a PRA system 3000 (Photochemical Research Associates). Detection was provided by single-photon counting. Accumulation, signal analysis and decay curve deconvolution were under computer control, the finite width of the excitation pulse being taken into account. The single-exponent and double-exponent programs for data deconvolution are based on standard non-linear least-squares methods. The pulse curve was recorded by scattering light at the wavelength of excitation. In the studies of excitation wavelength dependence of decay, excitation and emission monochromator bandwidths were 4 and 8 nm. Studies on the temperature dependence of decay were performed with excitation at 270 nm (bandwidth 10 nm) and emission at 335 nm for tryptophan and 330 nm for indole (bandwidth 16 nm).

Results and discussion

Edge excitation fluorescence spectral shifts in solid systems and complexities of the electronic structure of the indole chromophore

Figure 3 shows the dependence of the position of the emission spectral maximum λ^F_{max} on excitation wavelength λ^{ex} for tryptophan in different solid solvents – polyvinyl alcohol film, glucose glass and a low-temperature glass of ethylene glycol. Similar data for indole and tryptophan in glycerol at different temperatures are shown in Figs. 4 and 5. The data on solid glycerol solutions were obtained at −196 °C. In these systems, notwithstanding significant differences in the positions of fluorescence spectral maxima, the following general regularities are apparent. (*i*) In the excitation range 260 – 280 nm there is no dependence on λ^{ex}. (*ii*) At 290 nm a small shift of 1 – 2 nm is observed, and significant shifts occur for the range 300 – 310 nm. Here the shifts of emission maxima reach several tens of nanometers (up to ca. 30nm) and significantly exceed the variation of chosen λ^{ex}. Numerous experiments were performed in liquid solvents (water, alcohols, esters, dioxane, etc.) without detecting edge-excitation effects. The effect gradually vanishes in glycerol as the temperature is increased from −196° to + 50 °C (Figs. 4 and 5). This shows that the shift effect under study depends on the mobility of solvent molecules, but not on the presence of impurities such as oxidized or modified derivatives of indole and tryptophan. The absence of any increase in the fluorescence bandwidth, and also of the slight decrease sometimes observed on transition to red-edge excitation, is additional evidence that the effect is due to the main aromatic component.

A question arises as to whether the effect of edge-excitation fluorescence shift may be attributed to peculiarities of the electronic transition properties of the indolic chromophore. Specifically, can it be associated with the possible presence in emission of two degenerate electronic transitions, ^1L_a and ^1L_b? It is generally accepted that ^1L_a is the only, or, at least, the principal emitting oscillator, while ^1L_b is revealed in absorption and excitation spectra as a narrow maximum at 285 nm for indole and 288 – 290 nm for tryptophan, and as a negative component in excitation polarization spectra at these wavelengths (Konev 1967; Valeur and Weber 1977). Quantum-mechanical calculations (Sun and Song 1977) and experimentally determined changes in the dipole moment on electronic excitation (Chang et al. 1974) demonstrate that if ^1L_b is present in emission it should display a very small Stokes shift. Therefore, if the transition to edge excitation resulted in the appearance and increase of the ^1L_b contribution to emission, there should be a significant shift to

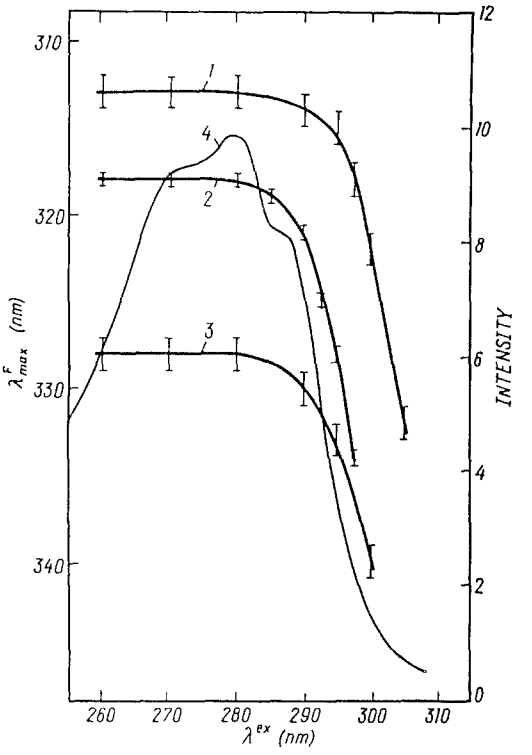

Fig. 3. Dependence of wavelength of fluorescence maxima on excitation wavelength for tryptophan in ethylene glycol at −196 °C (1), in polyvinyl alcohols film at + 25 °C (2) and in glucose glass at + 25 °C (3). (4) is the absorption spectrum of tryptophan in ethylene glycol

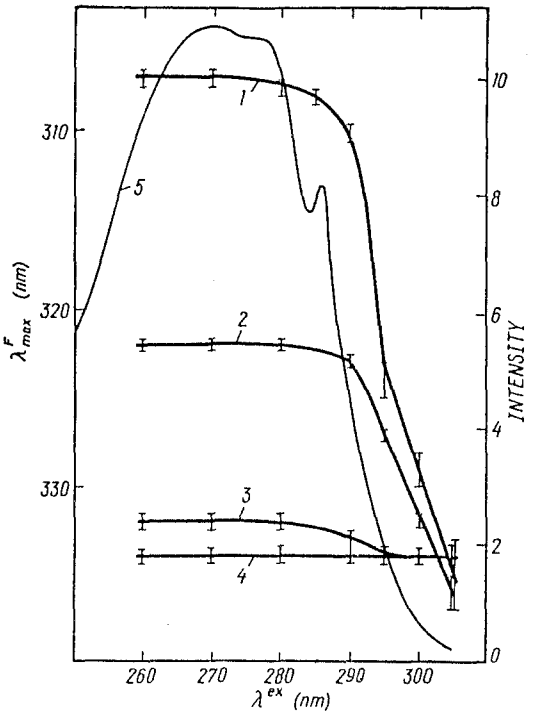

Fig. 4. Dependence of wavelength of fluorescence maxima on excitation wavelength for indole in glycerol at temperatures −196 °C (1), −10 °C (2), + 20 °C (3) and + 50 °C (4). (5) is the absorption spectrum of indole in glycerol at + 20 °C

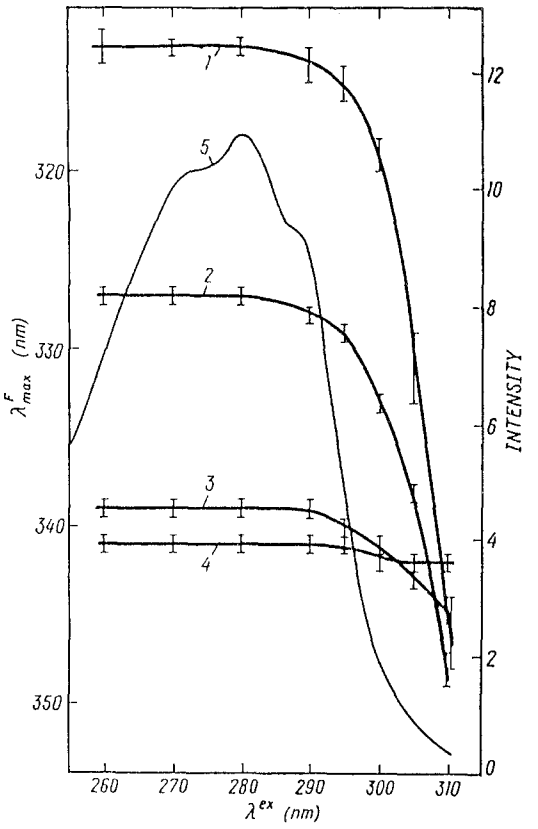

Fig. 5. Dependence of wavelength of fluorescence maxima on excitation wavelength for tryptophan in glycerol at temperatures −196 °C (1), −14 °C (2), + 20 °C (3) and + 50 °C (4). (5) is the absorption spectrum of tryptophan in glycerol at + 20 °C

shorter wavelengths, but not to longer ones, which is what is actually observed in experiment.

Thus, with it is probable that we may accept that the phemomenon under study is "bathochromic luminescence" (Rubinov and Tomin 1970) or an "edge-excitation fluorescence shift" (Azumi et al. 1976) which is well known in the spectroscopy of viscous and solid dye solutions (Macgregor and Weber 1981; Demchenko 1982; Rubinov and Tomin 1984; Lakowicz and Keating-Nakamoto 1984). The characteristic feature of this phenomenon is that, at sufficiently long excitation wavelength, the positions of fluorescence spectra in solid and viscous solvents reach and even exceed the positions recorded in corresponding liquid solvents. This is the range of "up-relaxation", an interesting spectroscopic phenomenon, in which relaxation results in increase of the energy of emitted quanta (Nemkovich et al. 1980). For indole in glycerol it is observed at excitation wavelengths longer than 303 to 304 nm (Fig. 4), and for tryptophan – longer than 307 – 309 nm (Fig. 5).

Shifts of fluorescence spectra for excitation at 290 – 305 nm (indole) and 295 – 305 nm (tryptophan) substantially exceed similar effects observed earlier for red-edge excitation with other chromophores, e.g., anilinonaphthalene sulfonates (Demchenko 1982). Here we observe that a shift of λ^{ex} by 5 nm results in shifts of fluorescence emission maxima by 10 – 12 nm (Figs. 3 – 5). On the basis of the oscillatory model for the indole chromophore (Valeur and Weber 1977) this feature may be explained as follows. The wavelength range 260 – 280 nm is the range of the "main-band" excitation of the ^1L_a state, while 280 – 310 nm is its extended longer wavelength edge. But the sharp excitation maximum of ^1L_b (285 nm for indole and 288 – 290 nm for tryptophan) is superimposed on the excitation edge of ^1L_a band. Therefore, at these wavelengths the prevailing excitation of the ^1L_b state is selected with subsequent internal conversion to "main-band" ^1L_a state (with no "edge" effect). An unusually sharp increase in the red-edge-excitation fluorescence shift at longer excitation wavelengths is a result of several factors: presence of "red-edge" effect of ^1L_a, decrease in the contribution of ^1L_b and presence of "red-edge" effect of the latter. Therefore we suggest that the main factor which determines photoselection at 295 – 305 nm excitation is the selection based on chromophore-environment interaction energy, and that selection between ^1L_a and ^1L_b electronic excited states influences the "red-edge" effect, increasing its magnitude.

Analysis of solvent relaxation by the effect of red-edge-excitation fluorescence spectral shift of indole and tryptophan

The results presented in Figs. 4 and 5 demonstrate that in viscous glycerol solutions a temperature rise results in a shift of fluorescence emission spectra to longer wavelengths with a simultaneous decrease of the "red-edge" effect. Figure 6 illustrates the temperature dependence of wavelenghts of fluorescence maxima at different excitation wavelengths. Parallel with the maxima we present the parameter I_{310}/I_{350} (the ratio of intensities at 310 and 350 nm) which under conditions of our experiment is determined with greater accuracy and is a direct function of λ^F_{max} (Zyma et al. 1978). As we expected, the curves for "main-band" and "red-edge" excitation differ most significantly at low temperatures and converge on heating. Divergence from the usual sigmoid dependence of relaxation shift is due to a substantial drop of mean excited state lifetime τ_F in the temperature range + 20 – + 40 °C (from 4.0 to 3.1 ns for indole and from 5.2 to 3.5 ns for tryptophan) with virtually no changes at lower temperatures. This, along with changes of τ_R, results in spectral shifts (Eq. 5)).

The use of Eq. (7) to obtain information on dipolar relaxation times requires that the decay law for "main-band" and "red-edge" excitation conditions is identi-

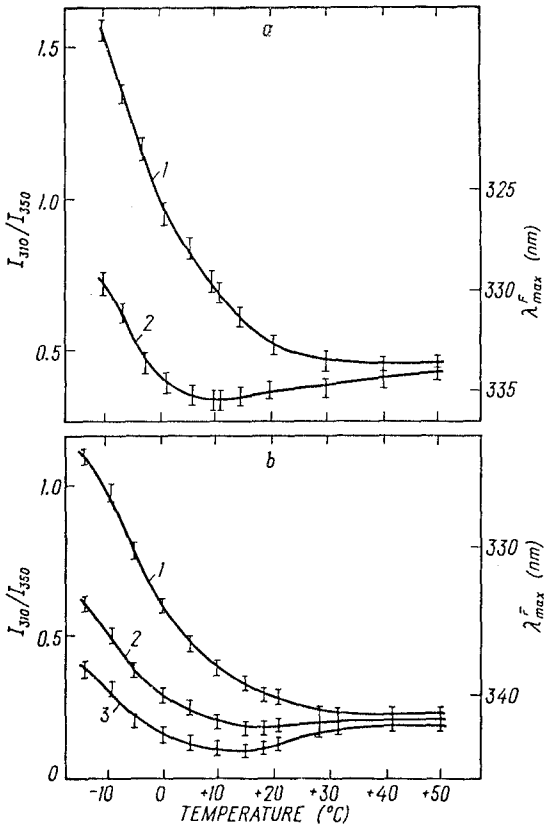

Fig. 6 a and b. Dependence of wavelength of fluorescence maxima and parameter I_{310}/I_{350} on temperature for indole (**a**) and tryptophan (**b**) in glycerol for different excitation wavelengths: 270 nm (1) and 295 nm (2) for indole and 280 nm (1), 300 nm (2) and 305 nm (3) for tryptophan

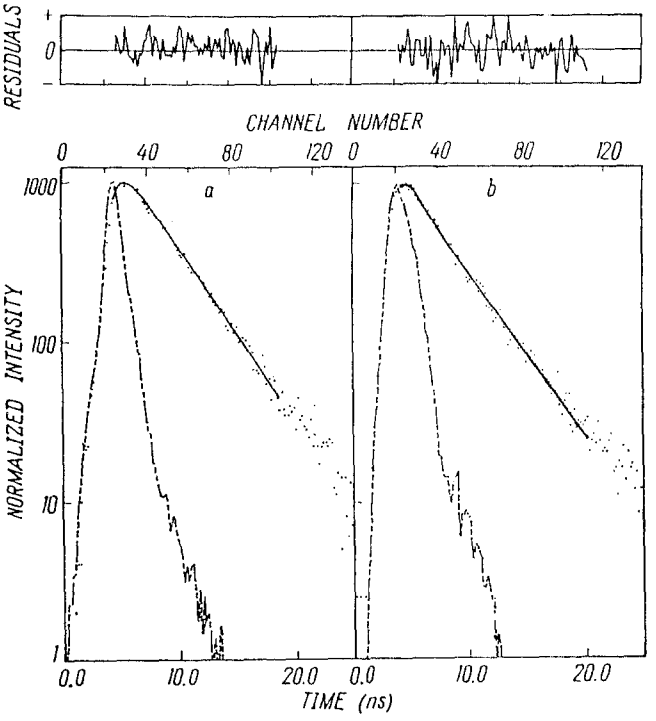

Fig. 7 a and b. Emission decay kinetics for tryptophan in polyvinyl alcohol films. **a** Excitation 270 nm, emission 340 nm, bandwidths 8 nm. The best-fit parameters for biexponential decay are: $\tau_1 = 4.30 \pm 0.03$; $A_1 = 0.10 \pm 0.01$; $\tau_2 = 0.09 \pm 0.12$; $A_2 = 0.25 \pm 0.34$, contribution of short-lived component to integral intensity is 5%, $\chi^2 = 0.60$. **b** Excitation 300 nm, emission 340 nm, bandwidth 4 nm. The best-fit parameters for bioexponential decay are: $\tau_1 = 4.53 \pm 0.06$; $A_1 = 0.10 \pm 0.02$, $\tau_2 = 0.06 \pm 0.11$; $A_2 = 0.66 \pm 1.23$; contribution of short-lived component to integral intensity is 8%, $\chi^2 = 1.24$

cal. We have tested this conditions experimentally by studying tryptophan emission in rigid media ($\tau_R \gg \tau_F$). These are the conditions where the population differences in chromophore-environment interaction are substantial and independent of time. The fluorescence decay curves for tryptophan in polyvinyl alcohol film for "main-band" and "red-edge" excitation are presented in Fig. 7. They demonstrate the similarity of emission kinetics with minor differences in excited state lifetimes.

Figure 8 shows Arrhenius plots ($\lg \tau_R$ vs. $1/T$) for the temperature dependence of τ_R for indole, determined from the results on temperature-dependent shifts of fluorescence spectra (for excitation at the maximum of the absorption band, $\lambda^{ex} = 270$ nm). For each temperature the results were calculated with Eq. (5). $\bar{\nu}_0$ and ν_∞ were taken as the low-temperature ($-196\,°C$) and high-temperature ($+50\,°C$) limits respectively. Similar data were obtained for tryptophan with $\lambda^{ex} = 280$ nm. The calculation of τ_R from the "red-edge" effect by Eq. (7) was performed using the data on the shift of fluorescence spectra for the two excitation conditions:

$\lambda^{ex}_{(mean)} = 270$ nm and $\lambda^{ex}_{(edge)} = 295$ nm for indole

and

$\lambda^{ex}_{(mean)} = 280$ nm and $\lambda^{ex}_{(edge)} = 305$ nm for tryptophan

and limiting values of "red-edge" effects were obtained at these wavelengths at $-196\,°C$. These results are compared with the temperature function of glycerol dipolar relaxation time τ_R obtained from experimental data on dielectric relaxation time τ_D (McDuffie and Litovitz 1962) using the correction factor suggested by Mazurenko and Bakhshiev (1970):

$$\tau_R = \tau_D \frac{n^2 2}{\varepsilon_0 + 2}, \tag{9}$$

where n is the refractive index and ε_0 is the static dielectric constant, its dependence on temperature being accounted for (McDuffie and Litovitz 1962). This correction factor is necessary when spectroscopic and dielectric dispersion data are compared: the relaxation time in spectroscopy is not of the solvent dipole motions but is the relaxation of the reactive field created by their action on the chromophore (Mazurenko and Bakhshiev 1970; Bakhshiev 1972).

The values of τ_R calculated by Eq. (5) from relaxation shifts of fluorescence spectra for indole and tryp-

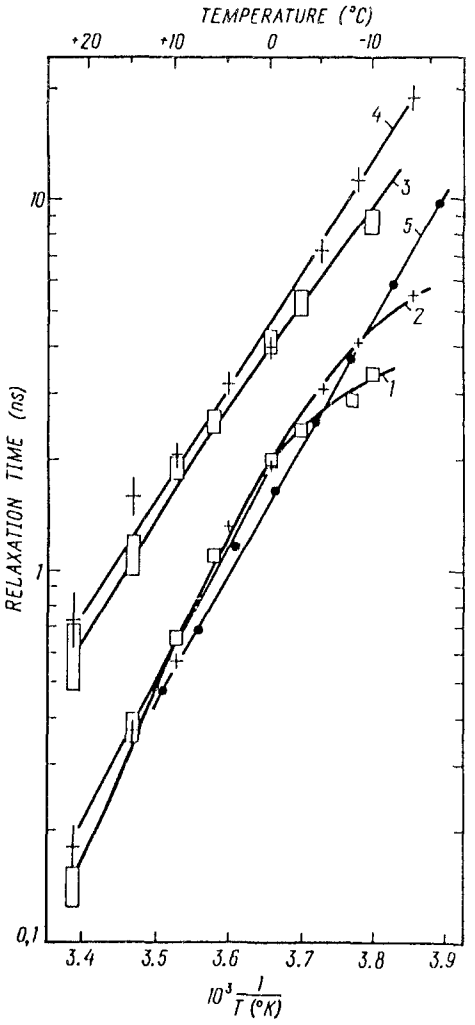

Fig. 8. Calculated dependence of dipole-reorientational relaxation time on temperature for indole and tryptophan in glycerol. Indole at $\lambda^{ex} = 270$ nm (1) and tryptophan at $\lambda^{ex} = 280$ nm (2) calculated with Eq. (5). Indole at 270 and 295 nm (3) and tryptophan at 280 and 305 nm (4) calculated with Eq. (7). Temperature dependence of dipole relaxation time in glycerol (5) was obtained from the data of dielectric studies (McDuffie and Litovitz 1962) and recalculated by Eq. (9)

tophan are in good agreement with the values obtained from dielectric dispersion data recalculated by Eq. (9). Such a correspondence is surprising in view of the limitations of the Debye model in the description of the dielectric properties of glycerol and of the Onsager model applied in the derivation of Eq. (9). Divergences from linearity of relaxation shift curves in the lower temperature range are, probably, of similar origin to those observed by Pavlovich and Pikulik (1975) for tryptophan in isopropanol. They were explained by the existence of a distribution of activation energy within the population of chromophores.

For the data on red-edge-excitation fluorescence shifts the slope of the plot of lg τ_R vs. $1/T$ is similar to that obtained for the relaxation shift. However, the absolute values of τ_R are three times as high for tryptophan and 2.5 times as high for indole. The probable explanation of this divergence is the following: in solvates with increased interaction energy in the excited state which are photoexcited under "red-edge" conditions, the relaxation rates are expected to be slower. This suggestion for photoselection of states with higher τ_R should be verified on other chromophore-solvent systems. Generally, the results obtained demonstrate that the data on the red-edge-excitation fluorescence spectral shift allow the determination of the order of τ_R and its temperature dependence.

The data presented above are adequately described by application to the mid-band and red-edge-excited populations of chromophores of the Bakhshiev-Mazurenko model, which is an approximation operating with a single value of τ_R. Glycerol and other associated liquids are known to possess a wide range of dielectric dispersion covering several orders (Davidson and Cole 1951). However, since the fluorescence method is of low structural resolution, it is difficult to gain more detailed information on characteristics of molecular motions in chromophore environment. Therefore the values of τ_R obtained should be considered as an effective parameter which describes averaged properties of environment and allows its variation for different experimental conditions to be observed.

Calculation of the width of the inhomogeneous broadening function

The inhomogeneous broadening function is a distribution function for the electronic transition wavenumbers of chromophores, which is a consequence of the distribution of interaction energies in the ground and excited states. Assuming a Boltzmann distribution of interaction energies and applying the Onsager model, Rubinov and Tomin (1984) suggested the following expression for the width of the distribution function $\Delta\nu$, which was assumed to have a Gaussian shape:

$$\Delta\nu = \frac{2(\mu_e - \mu_g)}{hc\sqrt{\varepsilon_0' a^3}} \sqrt{\frac{\varepsilon_0 - 1}{2\varepsilon_0' + 2}} kT, \tag{10}$$

where h is Boltzmann's constant.

From the results of our experiments on temperature-dependent spectral shifts for indole in glycerol we obtain using Eq. (10), $\Delta\nu = 1040\ \mathrm{cm}^{-1}$ for the temperature range -15 to $+20$ °C. This amounts to a dispersion in wavelengths by 11 nm. Such a width for the distribution is substantial, it is greater than the differences in positions of absorption spectra of indole and tryptophan which are observed on varying the chromophore environment in different solvents, which is usually in the range of 1 – 3 nm (Demchenko 1986). In

energy units the width of the distribution is about 12.5 kJ/mol. It roughly corresponds to a distribution in energy of chromophores participating and not participating in Van-der-Waals interactions or hydrogen bonds. In a given solvent there are chromophores whose individual spectra vary to a greater extent than do the averaged spectra in various solvents.

Red-edge-excitation spectroscopy as a tool in the study of molecular dynamics

The establishment of an equilibrium distribution in the ensemble of chromophore microstates having different interaction energies with the environment is probably the most characteristic feature of dipole-reorientational relaxation. This randomization of chromophore-environment interactions may occur simultaneously with emission. It is this process which results in easily observable "red-edge" effects which include not only spectral shifts, but also the effects on polarization (with and without time resolution) and on excitation energy transfer (Demchenko 1986).

Our data for the indole chromophore as well as numerous results for other aromatic molecules demonstrate that the dependence of fluorescence spectra on excitation wavelength suggests a new dimension in fluorescence spectroscopy. Red-edge excitation effects vary with the mobility of solvent molecules and this phenomenon may be used for obtaining information on such motion. The evaluation of solvent dipole-reorientational relaxation times by the suggested method, as well as in the case of other spectroscopic methods (including time-resolved spectroscopy) is dependent on the model of the relaxation process. We show that the Bakhshiev-Mazurenko model, which is one of the most advanced models applied in steady-state and time-resolved spectroscopy, may be used in a modified form for the treatment of red-edge effects. We modified this model by introducing the distribution of chromophores with different chromophore-solvent interaction energy and photoselection of chromophore subpopulations within this distribution. The mean and photoselected chromophores are thought to relax according to the Bakhshiev-Mazurenko model. The suggested model appears to produce an adequate discription of the data of steady-state experiments and may perhaps be extended to time-resolved spectroscopic data.

The close relation of red-edge-excitation and time-resolved spectroscopy in the analysis of solvent dipolar relaxation is evident from the data of Nemkovich et al. (1980). In time-resolved experiments with phthalimides these authors demonstrated that while the magnitude of motion of spectra in time varies and even changes sign on red-edge excitation, the rate of motion of spectra is independent of excitation wavelength.

Thus for homogeneous media the edge-excitation and time-resolved approaches are near-equivalent in relation to model-dependent information on relaxation times. But since the primary data are different, each method has its own strong and weak points. The disadvantages of red-edge-excitation spectroscopy are probably the following:

1. The time-dependent events are observed without direct resolution in time. One operates with the display of relaxation in time-integrated spectra.
2. The ground-state heterogeneity of chromophores (e.g., of those which participate and those which do not participate in specific interactions) and not only the distribution in dipolar interactions may be the cause of photoselection.

The important advantages of red-edge-excitation spectroscopy should also be mentioned.

1. Since only the effective fluorescence lifetimes are necessary for the determination of τ_R from red-edge experiments, the frequently observed non-exponentiality of decay does not produce such a substantial complication as in the case of the commonly applied time-resolved spectroscopic method. Our data demonstrate that identical values of τ_R are obtained in glycerol with indole and tryptophan, which differ in complexity of emission decay kinetics.
2. Red-edge excitation effects are thought to be specific for solvent dipolar relaxation, and this phenomenon may be distinguished from other reactions producing temporal shifts in spectra (e.g. isomerization, proton-transfer, solvent-substitution effects in multicomponent solvents). The latter reactions are usually slower than dipolar-reorientational relaxation, they occur with already randomized chromophore populations and the spectral shifts in time associated with them should not depend on excitation wavlength.
3. Red-edge excitation spectroscopy readily recognizes the unrelaxed states even in the absence of time-resolved shifts of spectra, when $\tau_R \gg \tau_F$. In this case the red-edge effects reach their maximum values.

These peculiarities of red-edge-excitation spectroscopy may be of value in the studies of chromophores in heterogeneous media, specifically, in protein molecules. In these cases complementary information may be obtained about the distribution of chromophore microstates and the dipolar relaxation times (Demchenko 1986). The direct combination of time-resolved and red-edge-excitation spectroscopy is possible by observing the excitation wavelength dependence of emission decay and of time-resolved spectra. Such a multidimensional approach has been suggested (Demchenko 1986) and applied to the analysis of molecular dynamics in proteins and membranes by fluorescence probes (Demchenko 1985; Demchenko and Shcherbatska 1985).

Thus, red-edge-excitation spectroscopy is a new method for analyzing the establishment of dynamic equilibrium in condensed media, which may be of interest in studies of molecular dynamics in proteins and other objects of biological origin. It may produce information complementary to that obtained by conventional methods of steady-state and time-resolved spectroscopy.

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