

LASER APPLICATIONS AND OTHER TOPICS IN QUANTUM ELECTRONICS

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Determination of photophysical parameters of tryptophan molecules by methods of laser fluorimetry

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***Abstract*. The photophysical parameters of tryptophan molecules at a low concentration in aqueous solution are measured by the methods of nanosecond laser fluorimetry upon excitation by 266-nm laser pulses. Two-step processes (reversible and irreversible photochemical transformations) taking place in this case are described quantitatively and it is shown that they can be neglected at the exciting photon flux density $F < 5 \times 10^{24}$ cm^{-2} s^{-1} in $\sim$ 10-ns pulses.**

Keywords: *tryptophan, nonlinear and kinetic fluorimetry, molecular photophysical parameters.*

1. Introduction

It is known that many proteins fluoresce in the near UV region due to the presence of tryptophan, tyrosine, and phenylalanine residues (aromatic amino acid residues) [1]. Because the parameters of fluorescence spectra (position, shape, intensity, etc.) of aromatic amino acids (fluorophores) characterise their interaction with the environment, they can be used to obtain information on the properties of protein molecules. To obtain more complete information, it is also important to know the molecular photophysical parameters of fluorophores such as absorption cross sections, singlet – triplet conversion constants, etc. Of special importance is studying the nature of fluorescence of tryptophan molecules because this fluorescence dominates in the so-called intrinsic fluorescence of proteins [1]. Although the fluorescence of tryptophan in solutions and proteins has been investigated in many papers (see review in [1]), the nature of the fluorescence band of this amino acid cannot be considered explained so far. The determination of the entire set of photophysical parameters of a tryptophan molecule and their dependence on the environment factors is promising for the development of the method of fluorescence diagnostics of proteins.

In this paper, we present the results of measuring the molecular photophysical parameters of tryptophan in aqueous solution by the method of nanosecond laser fluorimetry based on the simultaneous registration (by using one laser spectrometer) of the kinetic and fluorescence saturation curves. As shown in [2 – 4], this method can be used to determine (by processing the above-mentioned dependences) a set of photophysical parameters, whose measurements in classical spectroscopy require several methods and appropriate instruments. The method of nonlinear fluorimetry [2] can be used, in particular, to measure the absorption cross section of a fluorophore in the absence of information on its concentration and under condition when the absorption band of the fluorophore is overlapped by absorption bands of other molecules. The latter is important in studies of polyatomic organic compounds, in particular, proteins, as was convincingly demonstrated in paper [5].

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2. The object

We studied the solution of L-tryptophan (Ajinomoto, Japan) in mQ water (MilliPore, USA). Absorption spectra were recorded at the tryptophan concentration of 1.3×10^{-4} M, and fluorescence spectra were recorded and nonlinear fluorimetry was performed at the tryptophan concentration of 2×10^{-9} M. All the experiments were carried out at temperature $25 \pm 1\,^{\circ}$C.

Excitation of tryptophan molecules by UV radiation can induce a number of photophysical processes and photochemical reactions involving the excited state. Although the photophysical parameters of tryptophan molecules have been studied in many papers, the mechanisms of their phototransformations are not completely established so far. The values of photophysical parameters corresponding to these processes reported in the literature considerably differ [6 – 14] (see Table 1 below).

The following deactivation mechanisms of the first excited singlet state can be distinguished, which affect the fluorescence response of a low-concentration tryptophan solution excited by nanosecond laser pulses:

(i) Radiative and nonradiative transitions to the ground state.

(ii) Singlet – triplet conversion.

(iii) Spontaneous ionisation.

(iv) Photoionisation. This process was not considered in detail anywhere; it was also pointed out that it occurs and ionisation products are similar to products formed upon one-step ionisation [6 – 9]. This process, as process 3, is reversible, with recombination times at the micro-to-millisecond scale [7].

(v) Formation of a nonrecoverable photoproduct. The existence of such irreversible mechanism is pointed out in [6]; however, like process 4, it has not been quantitatively

studied. It seems that this process, as process 4, is caused by the absorption of a photon by a tryptophan molecule in the first excited singlet state (and, hence, is a two-step process as a whole). This is confirmed by the fact that the dependence of the product formation rate on the radiation intensity deviates from a linear dependence (inset in Fig. 1a), and the product formation is manifested in a gradual decrease in the fluorescence intensity (during UV irradiation) and optical density in the first and second singlet absorption bands of a sample (Fig. 1).

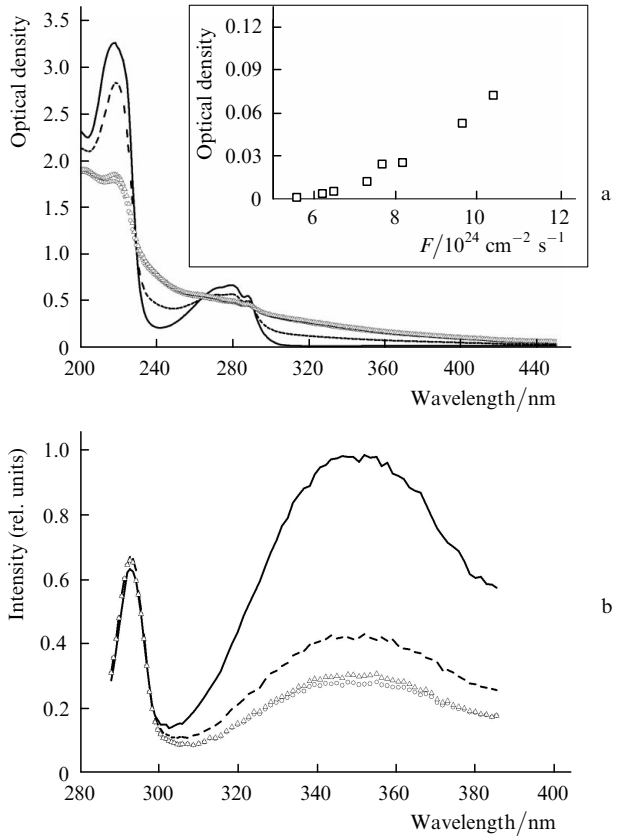

Figure 1. Irreversible changes in the absorption (a) and fluorescence (b) spectra of the tryptophan solution during its irradiation by UV light: before irradiation (solid curve), after irradiation for 15 min (dashed curve), after irradiation for 30 min (circles), and within six hours after the end of 30-min irradiation (triangles) for the photon flux density $F = 10^{25}$ cm^{-2} s^{-1}. The inset shows the dependence of the change in the optical density of the aqueous solution of tryptophan at 278 nm due to the photoproduct formation on the exciting photon flux density. The irradiation time is 30 min.

Two-step processes 4 and 5 are poorly studied since the experimental measurement of the quantum yield of ions and a photoproduct in the case of a two-photon reaction involves considerable difficulties because it is necessary to know the concentration of excited molecules absorbing the second photon and also their extinction coefficient at the excitation wavelength. Thus, it is difficult to describe these processes quantitatively within the framework of traditional classical approaches. The authors of most papers (for example, on flash photolysis [7–10]) deliberately selected the energy parameters of setups to minimise the contributions of two-step processes (specially specifying this fact) to the quantitative characteristics being determined. When molecular photophysical parameters are measured by the method of nonlinear fluorimetry upon excitation by high-power radiation, processes 4 and 5 can make a noticeable contribution to the fluorescence response, and therefore their neglect can lead to errors in the measurements of other parameters. Moreover, the consideration of these processes in the method of nonlinear fluorimetry allows one to obtain their quantitative parameters.

3. Experimental

We used a laser fluorimeter described in papers [1, 4, 16]. Fluorescence was excited by 10-ns, 0.7-mJ, 266-nm fourth-harmonic pulses from a Nd:YAG laser with a pulse repetition rate of 10 Hz. Fluorescence was recorded with an optical multichannel analyser consisting of a polychromator and a multichannel camera (DeltaTech, Stock of laboratory instruments, Moscow State University), which gave a two-dimensional optical image in the plane of the polychromator exit slit. The camera was coupled with a PC equipped with the software allowing operation both in the continuous (nonlinear fluorimetry) and gated (kinetic fluorimetry) regimes. The gate duration was 10 ns, the delay time step was 2.5 ns. The fluorescence intensity was measured by varying the laser photon flux density F from 2×10^{24} to 5×10^{25} s^{-1} cm^{-2}. For a fixed value of F (and a fixed delay time in the case of kinetic measurements [16]), the fluorescence signal was obtained by averaging over 100 pulses. The dynamics of photoproduct formation was studied by the methods of absorption and fluorescence spectroscopy by using a tryptophan solution of volume 3 mL. Nonlinear and kinetic fluorimetry was performed by using a continuous-flow system to exclude the photoproduct accumulation. Absorption spectra were recorded with a Lambda 25 PerkinElmer spectrophotometer.

Modern versions of the methods of nonlinear and kinetic fluorimetry of complex organic compounds are described in detail in [2–4]. Here, we point out only their basic features and take into account the specific features of a low-concentration aqueous solution of tryptophan.

The model of a fluorescence response, which is used in the methods of nonlinear and kinetic fluorimetry and takes into account processes pointed out in section 1, is described by the system of kinetic equations for populations of the energy states of an organic molecule (Fig. 2):

$$\begin{aligned}
\frac{\partial n_3(t,\boldsymbol{r})}{\partial t} &= F(t,\boldsymbol{r})\sigma n_1(t,\boldsymbol{r}) - \frac{n_3(t,\boldsymbol{r})}{\tau_{31}} - (K_{32} + K_{3\mathrm{i}})n_3(t,\boldsymbol{r}) \\
&\quad - F(t,\boldsymbol{r})(\sigma_{3\mathrm{i}} + \sigma_{\mathrm{ph}})n_3(t,\boldsymbol{r}), \\
\frac{\partial n_2(t,\boldsymbol{r})}{\partial t} &= K_{32}\, n_3(t,\boldsymbol{r}), \\
\frac{\partial n_{\mathrm{i}}(t,\boldsymbol{r})}{\partial t} &= K_{3\mathrm{i}}\, n_3(t,\boldsymbol{r}) + F(t,\boldsymbol{r})\sigma_{3\mathrm{i}} n_3(t,\boldsymbol{r}), \\
\frac{\partial n_{\mathrm{ph}}(t,\boldsymbol{r})}{\partial t} &= F(t,\boldsymbol{r})\sigma_{\mathrm{ph}} n_3(t,\boldsymbol{r}), \\
n_0 &= n_1 + n_2 + n_3 + n_{\mathrm{i}} + n_{\mathrm{ph}},
\end{aligned} \tag{1}$$

where n_0 is the sum of concentrations of initial organic molecules and their photoproducts; n_3, n_2, and n_1 are

concentrations of molecules in the first excited S_1 singlet (level 3), the first excited T_1 triplet (level 2), and the S_0 ground (level 1) states, respectively; n_i the total concentration of reversibly ionised and photoionised molecules; n_{ph} is the concentration of reversibly photoionised and irreversibly photodegraded molecules; $F(t, \boldsymbol{r})$ is the photon flux density of exciting radiation at the instant t at the point with coordinate $\boldsymbol{r} = \{x, y\}$ in the plane perpendicular to the laser beam direction (the dependence of parameters on the coordinate z along the beam direction is neglected, i.e. the optically thin layer approximation is used); σ is the absorption cross section of the fluorophore; $\tau_{31} = 1/(K_{31} + K'_{31})$; K_{31} and K'_{31} are the rate constants of radiative and nonradiative transitions from the excited S_1 singlet state to the S_0 ground state (level 1); K_{32} is the rate constant of singlet–triplet conversion; K_{3i} the rate constant of spontaneous ionisation from the S_1 state; σ_{3i} and σ_{ph} are the cross sections for photoionisation and photodegradation of molecules from the S_1 state induced by irradiation. The total lifetime of a molecule in the S_1 state (fluorescence decay time) in this model is described by the expression

$$\tau_3^{-1} \equiv K_3 = \tau_{31}^{-1} + K_{32} + K_{3i} + F(\sigma_{3i} + \sigma_{ph}), \tag{2}$$

which at low excitation intensities (when the absorption of radiation by molecules in the S_1 state can be neglected) has the form

$$\tau_3^{-1} \equiv K_3 = \tau_{31}^{-1} + K_{32} + K_{3i}. \tag{3}$$

The lifetimes of the $T_1 - S_0$ transition and recombination of ions can lie in the micro- and millisecond ranges, which greatly exceeds the laser pulse duration but is smaller than the time between laser pulses (0.1 s). This allows us to neglect these processes in system (1) [6–9, 12].

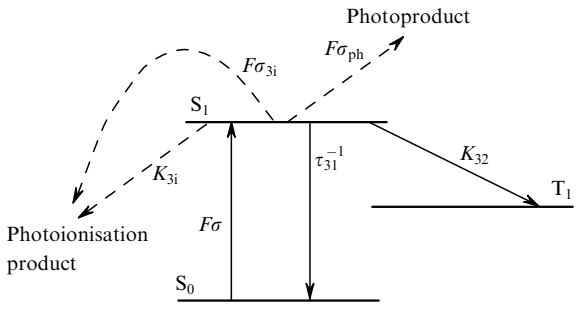

Figure 2. Energy level diagram of an organic molecule, corresponding to the system of kinetic (balance) equations (1). The arrows indicate processes considered in section 1 ($F\sigma$ is the probability of photon absorption from the S_0 state; τ_{31}^{-1} is the total rate of radiative and nonradiative transitions from the excited S_1 single state to the S_0 ground state; K_{32} and K_{3i} are the rate constants of singlet–triplet conversion and spontaneous ionisation, respectively; $F\sigma_{3i}$ and $F\sigma_{ph}$ are the probabilities of induced photoionisation and product formation, respectively).

Knowing variations in molecular concentrations $n_3(t, \boldsymbol{r})$, we can calculate the number N_{fl} of fluorescence photons emitted upon excitation by a laser pulse. For an axially symmetric beam, we have

$$N_{fl} = K_{31} l \int_0^{+\infty} 2\pi r \mathrm{d}r \int_{-\infty}^{+\infty} n_3(t, \boldsymbol{r}) \mathrm{d}t, \tag{4}$$

where l is the thickness of a medium layer from which fluorescence is detected.

The dependence of the number of emitted photons on F for low excitation intensities F is linear ($N_{fl} \sim F$); however, this dependence begins to deviate from a linear one with increasing incident radiation intensity and the saturation of fluorescence occurs (the fluorescence saturation for most of the organic molecules appears for $F > 10^{23}$ cm^{-2} s^{-1}). The fluorescence saturation in the model under study is caused by a finite $S_1 - S_0$ fluorescence decay time, the singlet–triplet conversion, mechanisms of ionisation and photoionisation and the formation of a photoproduct.

The method of nonlinear fluorimetry involves the measurement (by solving the inverse problem) of molecular photophysical parameters of organic compounds from the saturation curve $N_{fl}(F)$. It is convenient to normalise the number N_{fl} of detected fluorescence photons to the reference signal N_{ref}, which can be a Raman scattering line of water (or other solvent), and to study the dependence $[\Phi(F)]^{-1} = N_{rep}/N_{fl}$ instead of $N_{fl}(F)$, where $\Phi(F)$ is a fluorescence parameter.

In principle, all photophysical parameters of model (1) can be determined from the fluorescence saturation curve. However, the practical stability of the solution of the corresponding inverse problem allows one at present to determine no more than three parameters [2]. Therefore, along with the measurement of the saturation curve, we recorded the fluorescence kinetics by using the same laser spectrometer. This kinetics represents the dependence of the number $N_{fl}(t_{del})$ of fluorescence photons in the detector gate on the gate delay time t_{del} with respect to the laser pulse. The gate position at which its centre coincided with the laser pulse maximum was taken as the zero delay. This was detected by the maximum of the Raman line of water [16].

The parameter τ_3 was determined from the fluorescence kinetic curve and the inverse problem of nonlinear fluorimetry was solved by using the variation algorithm [17] in which the discrepancy between experimental curves and dependences calculated from model (1) was minimised by varying parameters.

The lifetime τ_3 found from the kinetic curve $N_{fl}(t_{del})$ recorded at low-intensity laser pulses ($F < 10^{23}$ cm^{-2} s^{-1}) was a fixed parameter in the model describing the saturation curve. The combined use of kinetic and nonlinear fluorimetry allows us to solve the four-parameter inverse problem with a sufficient practical stability. However, as follows from (2), by using the method of kinetic fluorimetry, two parameters $\alpha \equiv \tau_3^{-1} = \tau_{31}^{-1} + K_{32} + K_{3i}$ and $\beta \equiv \sigma_{3i} + \sigma_{ph}$ can be determined as well by recording several (minimum, two) kinetic curves for different values of F. By complicating further the measurement procedure based on the simultaneous use of nonlinear and kinetic fluorimetry, the inverse problems of even greater dimensionalities can be solved [4].

4. Experimental results and discussion

It was shown in papers [11, 14, 15] that the fluorescence decay of the aqueous solution of tryptophan excited by picosecond laser pulses was described by two exponentials: the long-lived (~ 3 ns) component making a dominant contribution (85 %–95 %) and the short-lived (~ 0.5 ns) component. So far no convincing mechanisms were proposed that would predict the nonexponential fluorescence decay of tryptophan. For this reason and also taking

into account the nanosecond duration of the exciting pulse and the nanosecond time resolution of a detector, we solved the inverse problem of kinetic fluorimetry by using a model with one excited-state lifetime $\tau_3 = (\tau_{31}^{-1} + K_{32} + K_{3i})^{-1}$.

Figure 3 presents one of the kinetic curves that we obtained. The processing of such curves by the variation method gave $\tau_3 = 2.8 \pm 1$ ns.

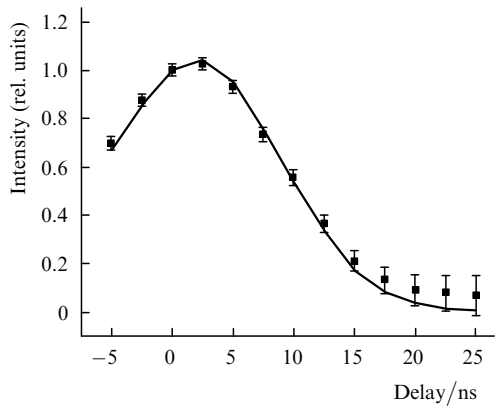

Figure 3. Fluorescence kinetic curve of tryptophan excited by a 10-ns laser pulse with $F = 10^{23}$ cm^{-2} s^{-1}; squares are experimental data, the solid curve is the theoretical dependence.

Figure 4 shows one of the fluorescence saturation curves. The solution of the three-parametric inverse problem with the fixed parameter $\tau_3 = 2.8$ ns gave the following values of the other model parameters: $\sigma = (1.6 \pm 0.3) \times 10^{-17}$ cm^2, $\sigma_{3i} + \sigma_{ph} = (2.2 \pm 0.7) \times 10^{-18}$ cm^2, and $K_{32} + K_{3i} = (6 \pm 2) \times 10^7$ s^{-1}. These values give the sum of triplet and ionisation quantum yields $\eta_T + \eta_i = 0.17 \pm 0.05$.

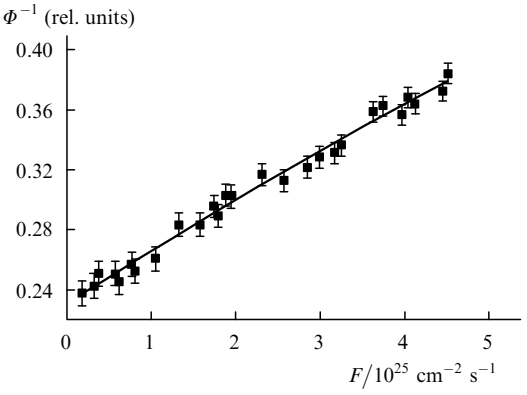

Figure 4. Fluorescence saturation curve for tryptophan [squares are experimental data, the solid curve is calculated by expressions (1)–(4)] for $\tau_3 = 2.8$ ns, $\sigma = 1.6 \times 10^{-17}$ cm^2, $\sigma_{3i} + \sigma_{ph} = 2.2 \times 10^{-18}$ cm^2, $K_{32} + K_{3i} = 6 \times 10^7$ s^{-1}.

The parameter $\beta \equiv \sigma_{3i} + \sigma_{ph}$ was also determined from kinetic measurements by measuring two kinetic curves for low and high values of F. For $F = 10^{23}$ cm^{-2} s^{-1}, the calculated lifetime τ_3 was 2.8 ± 1 ns, while for $F = 10^{26}$ cm^{-2} s^{-1}, this time was 2 ± 1 ns. Then, by using (2), we determined $\sigma_{3i} + \sigma_{ph} = (1.2 \pm 0.4) \times 10^{-18}$cm^2, which agrees (taking the confidence interval into account) with the value obtained from the saturation curve.

Because the influence of the singlet–triplet conversion (the rate K_{32}) and spontaneous ionisation (the rate K_{3i}) on the fluorescence kinetics and saturation curves is indistinguishable, we can determine within the framework of our method only the total rate of these processes. A similar situation takes place for cross sections for reversible photoionisation (σ_{3i}) and formation of a stable photoproduct (σ_{ph}). These parameters can be determined separately by supplementing our method by classical methods of flash photolysis and kinetic spectrophotometry.

Table 1 compares the results obtained in our paper with the data reported in the literature. To estimate the consequences of the neglect of two-step processes, we approximated the experimental saturation curves by the curves calculated numerically by model (1) with $\sigma_{3i} = \sigma_{ph} = 0$. As a result, the values of parameters σ and $K_{32} + K_{3i}$ at which the discrepancy achieved a minimum were found to be 1.9×10^{-17} cm^2 and 7×10^7 s^{-1}, respectively [i.e. they differ approximately by 17% from the values obtained for model (1) with $\sigma_{3i} + \sigma_{ph} \neq 0$]. The minimal discrepancy itself increased by a factor of 1.2, which suggests that the model taking two-step processes into account more adequately describes the formation of the fluorescence response of tryptophan molecules excited by laser pulses with parameters presented above.

Table 1. Photophysical parameters of tryptophan molecules in an aqueous solution.

Parameter	This paper	Data from the literature
τ_3/ns	2.8 ± 1	3.32 [14], 3.13 [6]
$\sigma/10^{-17}$ cm^2, nonlinear fluorimetry	1.6 ± 0.3	–
$\sigma/10^{-17}$ cm^2, photometry	1.7	1.9 [17]
$\eta_T + \eta_i$	0.17 ± 0.05	$\eta_T = 0.08$ [10], $\eta_T = 0.12$ [6], $\eta_i = 0.25$ [7], $\eta_i = 0.08$ [6, 8, 10], $\eta_i = 0.21$ [9]
$K_{32} + K_{3i}/10^7$ s^{-1}	6 ± 2	–
$\sigma_{3i} + \sigma_{ph}/10^{-18}$ cm^2	2.2 ± 0.7	–

5. Conclusions

We have demonstrated the possibilities of the method of laser fluorimetry, which is so far little used, for studying complex organic compounds in which photophysical processes are described by many parameters (in our case, by four parameters). We plan in the future to use the so-called matrix method [4] based on the combination of kinetic and nonlinear fluorimetry, which will allow us to investigate in more detail photophysical processes in two- and three-fluorophore proteins taking into account the intermolecular energy transfer between fluorophores.

By applying the methods of nanosecond kinetic and nonlinear fluorimetry based on the use of a laser spectrometer, we have measured the photophysical parameters of tryptophan molecules at low-concentration aqueous solutions excited by 266-nm laser pulses: the excited-state lifetime (in the monoexponential approximation and at a low excitation level) $\tau_3 = 2.8 \pm 1$ ns; the absorption cross section $\sigma = (1.6 \pm 0.3) \times 10^{-17}$ cm^2; the total rate of singlet–triplet conversion and ionisation from the first excited S_1 singlet state $K_{32} + K_{3i} = (6 \pm 2) \times 10^7$ s^{-1}; the sum of the

triplet and ionisation quantum yields $\eta_T + \eta_i = 0.17 \pm 0.05$; the total cross section for reversible and irreversible phototransformations after absorption of the 266-nm radiation by molecules in the first excited state $\sigma_{3i} + \sigma_{ph} = (2.2 \pm 0.7) \times 10^{-18}$ cm^2 (obtained from the saturation curve); and $\sigma_{3i} + \sigma_{ph} = (1.2 \pm 0.4) \times 10^{-18}$cm^2 (kinetic measurements).

The values of parameters τ_3, σ, and $\eta_T + \eta_i$ coincide within the experimental error with the values measured by other methods [6, 11, 14, 15, 18]. The value of the parameter $\sigma_{3i} + \sigma_{ph}$ is obtained for the first time and suggests that processes described by this parameter can be neglected for exciting photon flux densities $F < 5 \times 10^{24}$ cm^{-2} s^{-1} in $\sim$ 10-ns pulses. The neglect of these processes in measurements of photophysical parameters by the method of nonlinear fluorimetry can lead to measurement errors for σ and $\eta_T + \eta_i$ of about 17 %.

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