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Pyrene Fluorescence Quenching by Aromatic Azides

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Abstract—Pyrene fluorescence quenching by phenylazide derivatives with donor and acceptor substituents has been studied by fluorescence spectroscopy and flash photolysis. The rate constants of quenching (k_q) in acetonitrile ($(0.2–1.2) \times 10^{10}$ l mol^{-1} s^{-1}) are found to be close to a diffusion limit; the rate constants were somewhat higher for perfluoro-substituted arylazides. It is found that k_q does not depend on solvent polarity; the formation of the pyrene cation in the course of pyrene fluorescence quenching by tolylazide was not detected. Pyrene fluorescence quenching occurred by an energy-transfer mechanism; this is supported by the coincidence of the quantum yields of the direct and sensitized photodecomposition of tolylazide. As estimated, energy transfer in rigid media occurs at characteristic distances of about 10 Å.

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Photoaffinity labeling is the most promising technique for studying the structure of nucleoproteins and the dynamics of processes in which they participate [1]. The photoactive group of an affinity reagent is covalently bound to a natural ligand of a biological macromolecule. The photolysis of a complex of a photoaffinity reagent with the test biological macromolecule results in the covalent binding of the ligand and the biomolecule. For example, oligonucleotides are the natural ligands of DNA and RNA. The affinity labeling of DNA and RNA with the use of reactive oligonucleotides is also referred to as sequence-specific modification.

Arylazides are widely used as the light-sensitive reactive groups of photoaffinity reagents [1–3]. An ideal reagent for photoaffinity labeling should exhibit considerable absorbance in the wavelength region above 300 nm, where amino acids and other chromophores of biopolymers do not absorb [3]. Phenylazide derivatives based on which commonly used photoaffinity reagents were developed do not exhibit absorption bands in the visible region of the spectrum and very weakly absorb in the near-UV region. Therefore, the use of affinity labeling photosensitized by dyes seems very promising.

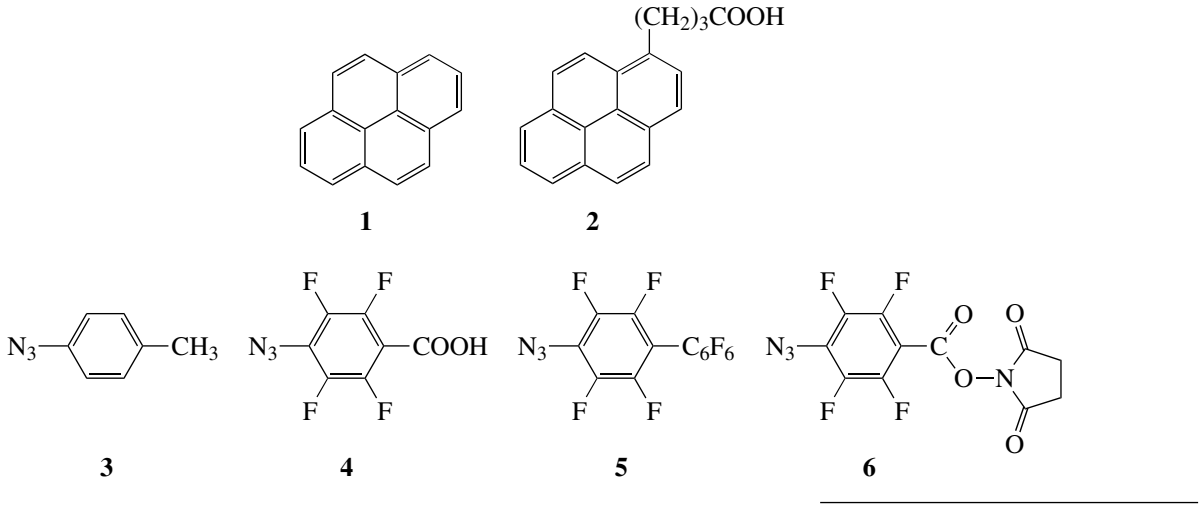
About a decade ago, Dobrikov and coauthors [4, 5] proposed a new approach to the sequence-specific photomodification of nucleic acids, which allowed them to improve considerably the efficiency and specificity of modification. This approach is based on the simultaneous use of two affinity reagents. In one of the reagents, an arylazide residue is attached to an oligonucleotide. In the other reagent, a sensitizer is bound to another nucleotide sequence. The use of these binary oligonucleotide reagents significantly increased the rate, specificity, and degree of modification of the DNA target. Anthracene, pyrene, and perylene derivatives were used as sensitizers in binary systems, and fluorine-substituted phenylazides were used as photoreagents [4–7]. The status of studies in this area was considered in a review [7]. However, the mechanism of sensitization remains unstudied experimentally.

In this work, we performed kinetic studies on pyrene fluorescence quenching by various arylazides in solution in order to determine the efficiency and mechanism of sensitization and to find the most promising pyrene–arylazide pairs.

EXPERIMENTAL

Materials

Pyrene (**1**) and 4-(5-pyrenyl)-*n*-butanoic acid (**2**) from Aldrich were purified by vacuum sublimation. The *para* derivatives of phenylazide, for example, tolylazide (**3**), were synthesized in accordance with a published procedure [8]. 4-Azidotetrafluorobenzoic acid (**4**) from Aldrich was used without additional purification. Perfluoro-4-biphenylazide (**5**) was synthesized as described previously [9]. *N*-Succinimidyl 4-azidotetrafluorobenzoate was kindly provided by Professor O.I. Lavrik (Institute of Chemical Biology and Fundamental Medicine, Siberian Division, Russian Academy of Sciences, Novosibirsk). Acetonitrile and hexane of chemically pure grade were distilled before use.



Fluorescence Quenching

A 365-nm line (DRSh-500 high-pressure mercury lamp), which was separated using a high-aperture monochromator and a combination of glass light filters, was used for fluorescence excitation. Luminescence emitted from a sample was focused on the entrance slit of an MDR-23 monochromator and scanned using a stepping motor. An FEU-119 photomultiplier with an optimum spectral sensitivity in the region 300–800 nm was used as a radiation detector. Luminescence spectra were measured in the photon count mode using a Nokia LP 4840 multichannel analyzer (800 channels) or in the analog mode using an 8-bit digital oscilloscope (ISA BUS CompuScope) from Gage Applied Sciences, which were directly interfaced to a personal computer. If necessary, the experimental luminescence spectra were corrected taking into account the spectral sensitivity of the system. The spectral sensitivity over the range 390–580 nm was measured with the use of quinine bisulfate as a luminescent standard [10].

The absorbance of a pyrene solution at the excitation wavelength (365 nm) was usually 0.05–0.1. The azide concentration in solution was varied over a range so that the observed fluorescence intensity of the dye changed by a factor of 5 or higher. None of the arylazides exhibited detectable absorption at the wavelength of pyrene fluorescence excitation. Acetonitrile and hexane were used as solvents. To remove oxygen, argon was bubbled through solutions for 20 min before measurements. In some experiments, freezing–pumping–thawing cycles (evacuation to a pressure of $\sim 10^{-4}$ Torr) were repeated five times in order to remove oxygen from solutions.

The kinetics of pyrene fluorescence decay was measured at a maximum of the fluorescence spectrum (392 nm) using laser flash photolysis [11] in the absence of probing radiation. The samples were excited using pulses from an excimer laser (Lambda Physik EMG 101; 308 nm; pulse duration of 15–20 ns). Some pulse experiments were performed using a system [12], in which the third harmonic of a neodymium laser (Spectra Physics LAB-150-10; 355 nm; pulse duration of 5 ns) was used for excitation.

Determination of the Quantum Yield of the Sensitized Decomposition of Azides

The quantum yield (φ_{sen}) was determined as the number of evolved nitrogen molecules per light quantum consumed for sensitization. The amount of evolved nitrogen was calculated from changes in pressure, which was measured with a liquid manometer. Light intensity was measured using the isomerization of 2-dialkylamino-1,4-naphthoquinones [13] as an actinometric photoreaction with a quantum yield of 0.1 for the photoisomerization of 2-dimethylamino-3-chloro-1,4-naphthoquinone in benzene. We took into account that a portion of absorbed quanta was emitted by the sensitizer.

Quantum-Chemical Calculations

The geometries of reagents and hypothetical cations and anions were optimized by the B3LYP method [14] in the 6-31+G* basis set [15]. Harmonic frequencies, which were calculated by the above method, were used for calculating zero-point vibrational energies. The solvent effect was taken into account in terms of the polarized continuum model (PCM) method of Tomasi [16–18]. All of the calculations were performed using the Gaussian 98 unit of programs [19].

RESULTS

Fluorescence Quenching

In this study, we used phenylazide derivatives with strong acceptor (4-cyanophenylazide) and donor (4-methoxyphenylazide) substituents, as well as *para*-tolylazide (**3**), which contains a weak donor group, as

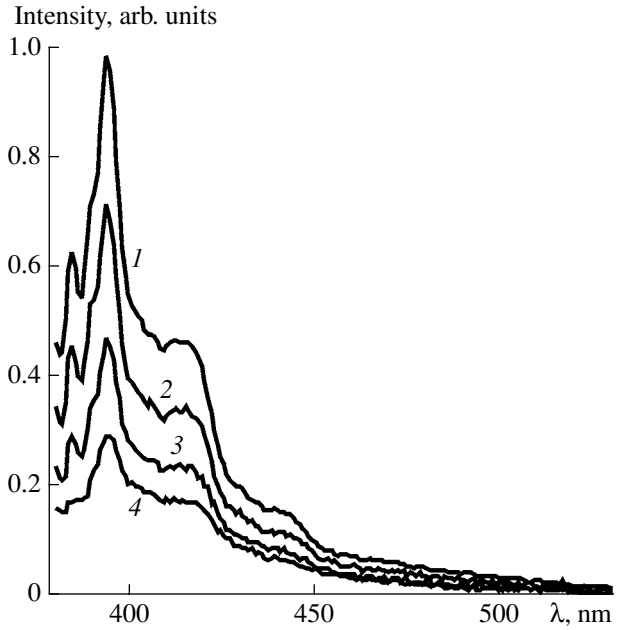


Fig. 1. Fluorescence spectra of pyrene in acetonitrile recorded upon excitation at 365 nm (*1*) in the absence of a quencher and in the presence of (*2*) 0.18, (*3*) 0.53, or (*4*) 1.06 mM 4-azidotetrafluorobenzoic acid. Pyrene concentration, 3.4×10^{-5} mol/l; correction for the spectral sensitivity of the instrument was not performed.

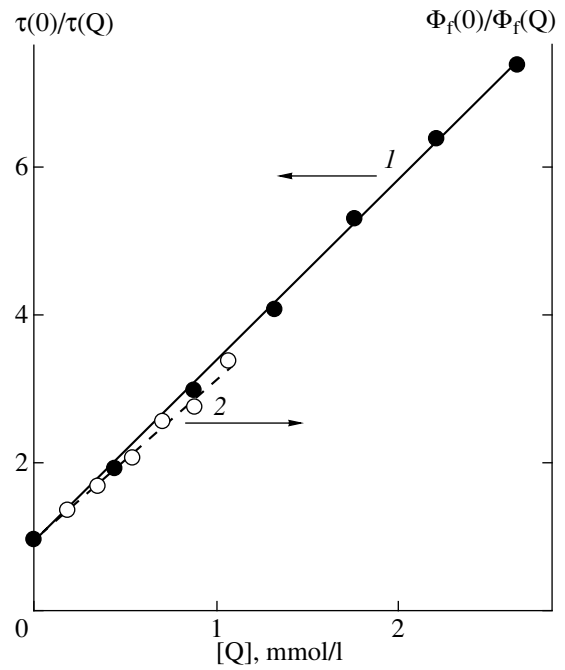


Fig. 2. Dependence of the (*1*) lifetime and (*2*) intensity of pyrene fluorescence on the concentration of 4-azidotetrafluorobenzoic acid in the Stern–Volmer coordinates. Pyrene concentration in acetonitrile, 3.2×10^{-5} mol/l.

quenchers of pyrene (**1**) and 4-(5-pyrenyl)-*n*-butanoic acid (**2**). It is well known that perfluoro-substituted arylazides are most promising for photoaffinity labeling [20–24] because the corresponding perfluoro-substituted singlet arylnitrenes have sufficiently long lifetimes [25, 26]. Therefore, we also used three perfluoro-substituted arylazides as quenchers.

Acetonitrile was used as a solvent in studying the fluorescence quenching of compounds **1** and **2** by various arylazides. We found that the intensity of fluorescence significantly decreased in the presence of all of the test arylazides. For example, Fig. 1 shows changes in the fluorescence spectrum of compound **1** in the presence of 4-azidotetrafluorobenzoic acid (**4**). A comparison between the spectrum in Fig. 1 and the published spectrum of pyrene fluorescence demonstrates that the contribution of excimer emission ($\lambda_{max} \approx 475$ nm) was insignificant [27]. Note that the fluorescence spectra of compounds **1** and **2** were practically coincident.

Figure 2 shows the dependence of pyrene fluorescence intensity on the concentration of azide **4**. It can be seen that the experimental dependence obeys the Stern–Volmer equation [27]

$$\frac{\Phi_f(0)}{\Phi_f(Q)} = 1 + K_q[Q], \quad (1)$$

where $\Phi_f(0)$ is the intensity of fluorescence in the absence of a quencher, $\Phi_f(Q)$ is the intensity in the presence of a quencher, [Q] is the quencher concentration, and K_q is the quenching constant (Stern–Volmer constant). For all of the pyrene–arylazide pairs studied in this work, the dependence of fluorescence intensity on arylazide concentration was adequately described by Eq. (1).

The intensity of fluorescence was a linear function of quencher concentration in the Stern–Volmer coordinates in the cases of both dynamic (due to collisions) and static (complexation in the ground state) mechanisms of quenching [27]. In the case of dynamic quenching, the dependence of fluorescence decay time on quencher concentration is described by Eq. (2) with the same Stern–Volmer constant:

$$\frac{\tau_0}{\tau} = 1 + K_q[Q]. \quad (2)$$

To determine the character of quenching, we studied the effect of the concentrations of azides **3** and **4** on the kinetics of fluorescence decay of compound **1** in aceto-

Rate constants of pyrene fluorescence quenching (k_q) by tolylazide (**3**) and 4-azidotetrafluorobenzoic acid (**4**) at room temperature in different solvents

Azide	$k_q \times 10^{-9}$, l mol^{-1} s^{-1}	
	CH_3CN	C_6H_{12}
3	6.3 ± 0.6	7.4 ± 0.8
4	8.3 ± 0.8	9.1 ± 0.8

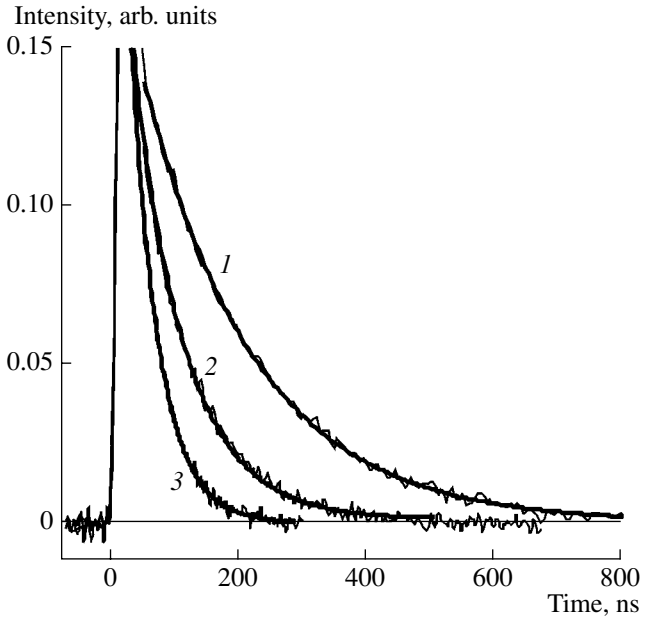


Fig. 3. Kinetics of pyrene fluorescence decay in acetonitrile measured at a spectral maximum (394 nm) upon excitation with the third harmonic of a Nd–YAG laser (355 nm) (*1*) in the absence and in the presence of (*2*) 0.77 or (*3*) 1.79 mM 4-azidotetrafluorobenzoic acid and their fitting to exponential functions of time.

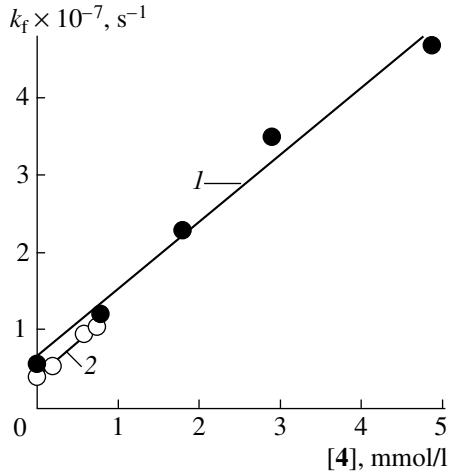


Fig. 4. Dependence of the rate constant of pyrene fluorescence decay on the concentration of azide **4** in (*1*) acetonitrile or (*2*) hexane at room temperature.

nitrile. The kinetic curves of fluorescence decay were described by exponential functions both in the absence and in the presence of azides (Fig. 3). Figure 2 shows the dependence of the fluorescence decay time of **1** on the concentration of azide **4**. It can be seen that experimental points lie in the straight line, and the slopes of two functions (intensity and lifetime) are coincident within the experimental error. Consequently, pyrene fluorescence quenching by arylazides is dynamic. The absence of any new bands in the absorption spectrum also suggests the absence of complexation between pyrene and azide **4**. Based on experimental data (Fig. 2), the rate constant of pyrene fluorescence quenching by azide **4** in acetonitrile was determined to be $(8.3 \pm 0.5) \times 10^9$ l mol^{-1} s^{-1}.

No additional bands were also observed in the absorption spectra of **1** and **2** in the presence of other test arylazides. Therefore, it is reasonable to assume that the quenching of fluorescence of compounds **1** and **2** by all of the test arylazides is dynamic. The rate constants of fluorescence quenching (k_q) of **2** in acetonitrile by various arylazides at room temperature, which were calculated based on the experimental Stern–Volmer constants and fluorescence decay times in the absence of a quencher, are given below.

Substituent	OCH_3	CH_3	CN	$C_6F_5^*$	COOSu*
$k_q \times 10^{-9}$, l mol^{-1} s^{-1}	2.2 ± 0.2	6.3 ± 0.5	7.4 ± 0.7	6.6 ± 0.7	12.9 ± 1.2

* Substituent in the *para* position of perfluorophenylazide.

Note that the rate constants of quenching of compounds **1** and **2** by the same arylazides were equal within the experimental error (±10%).

It can be seen that the rate constants of fluorescence quenching are high and close to a diffusion limit (~2×10^{10} l mol^{-1} s^{-1} in acetonitrile). The highest rate constant was measured for perfluoro-substituted arylazide **6**.

Pyrene fluorescence quenching by arylazides **3** and **4** was studied in both polar (acetonitrile) and nonpolar (hexane) solvents (Fig. 4, table). The results given in the table were obtained from an analysis of the fluorescence decay curves. It can be seen that the polarity of the solvent does not have a noticeable effect on the rate constant of quenching.

Flash Photolysis

We recorded transient absorption spectra the LFP of pyrene in hexane and acetonitrile. In hexane, the formation of pyrene in a triplet state (λ_{max} = 412 nm; Fig. 5, spectrum *1*) with a characteristic time equal to the fluorescence lifetime (~370 ns) was observed. Unlike hexane, both triplet pyrene and the pyrene cation (Fig. 5, spectrum *2*) were formed in acetonitrile even in the absence of a quencher. In this case, the pyrene cation was formed during a laser pulse (~5 ns), whereas triplet pyrene was formed during the fluorescence decay time (~270 ns). The absorption spectra of triplet pyrene and pyrene cation ($\lambda_{max} \approx 450$ nm) are well known [28]. As the energy of the laser pulse was decreased, the absorp-

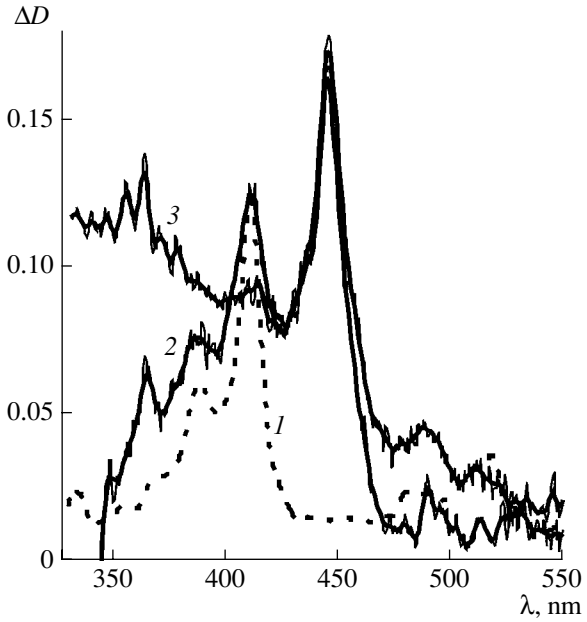


Fig. 5. Absorption spectra recorded 1 μs after excitation with the third harmonic of a Nd–YAG laser (355 nm; 5 ns; 50 mJ) in a pyrene solution (0.1 mM) in (*1*) hexane or (*2*) acetonitrile and (*3*) 80 ns after the excitation of a pyrene (0.1 mM) and tolylazide (20 mM) solution in acetonitrile.

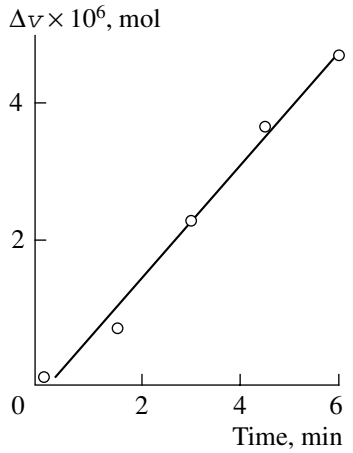


Fig. 6. The time dependence of the amount of gas released in the course of irradiation of a pyrene (0.2 mM) and tolylazide (1.9 mM) solution with light (365 nm) at room temperature.

tion of the cation decreased much greater than that of triplet pyrene. Consequently, the pyrene cation was formed because of a two-photon process. The formation of the pyrene cation can be completely avoided only at a low pulse energy (2–3 mJ) when the absorbance at the absorption maximum of triplet pyrene is no higher than 0.005.

The addition of azide **3** to a pyrene solution in acetonitrile significantly decreased the signal of triplet pyrene and had almost no effect on the formation of the pyrene cation. Moreover, in the presence of azide **3**, a broad absorption band in the region 300–400 nm was observed (Fig. 5). This absorption is characteristic of didehydroazepines, which are formed as intermediates in the isomerization of singlet arylnitrenes [26]. With the use of laser pulses of very low energy (~2–3 mJ), the formation of the pyrene cation in the presence of an azide was also not detected.

Efficiency of the Sensitized Decomposition of Arylazides

The efficiency of sensitization (i.e., the number of decomposed azide molecules per light quantum consumed for sensitization) was experimentally evaluated for the pyrene–tolylazide pair. The quantum yield was estimated as the ratio of the amount of gas evolved (presumably nitrogen, $\Delta(N_2)$) to the number of absorbed quanta (ΔI_{abs}) taking into account the fraction consumed for sensitization (χ):

$$\varphi = \Delta(N_2)/(\Delta I_{abs}\chi),$$
$$\chi = K_q[Q]/(1 + K_q[Q]), \quad (3)$$

where K_q is the Stern–Volmer constant determined above.

Figure 6 exemplifies the time dependence of gas evolution in the pyrene-photosensitized decomposition of tolylazide. The quantum yield of sensitization was 0.7 ± 0.2. Consequently, pyrene fluorescence quenching in reality resulted in azide decomposition with the release of molecular nitrogen with an efficiency close to unity. Note that the above experimental value is close to the quantum yield of tolylazide photodissociation (0.59 [29]).

DISCUSSION

The quenching of the fluorescence of condensed hydrocarbons by arylazides was reported previously [30–32]. It was assumed [30, 32] that quenching occurred by an energy transfer mechanism, whereas Shields *et al.* [31] drew the conclusion of quenching by an electron transfer mechanism. If quenching occurs by an energy transfer mechanism, an arylazide in the singlet excited state is formed, and its subsequent transformations are identical to those in direct photolysis, whose mechanism is currently well known [26].

Electron transfer should result in an arylazide cation or anion, the subsequent transformations of which are practically unknown. To determine the possibility and direction of electron transfer, we estimated the free energies (ΔG) of reactions (I) and (II) based on the electronic energies of pyrene, tolylazide, and correspond-

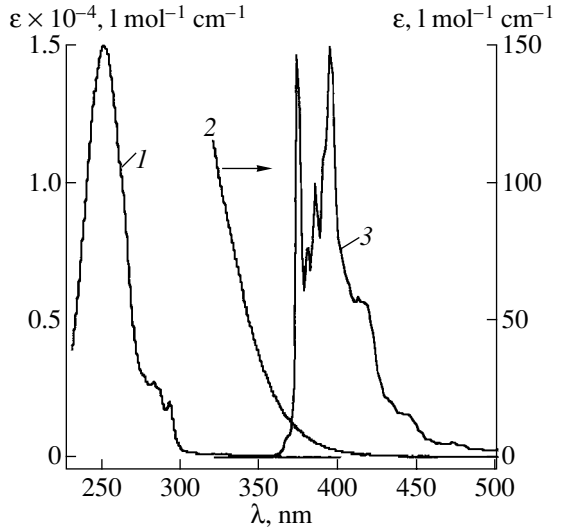


Fig. 7. Absorption spectra of (*1*, *2*) *para*-tolylazide and (*3*) the fluorescence spectrum of pyrene in acetonitrile corrected for spectral sensitivity.

ing cations and anions calculated by the DFT method using the hybrid B3LYP method.

$$\mathbf{1}^* + \mathbf{3} \longrightarrow {}^2\mathbf{1}^+ + {}^2\mathbf{3}^-, \quad \text{(I)}$$

$$\mathbf{1}^* + \mathbf{3} \longrightarrow {}^2\mathbf{1}^- + {}^2\mathbf{3}^+. \quad \text{(II)}$$

According to calculated data, the free energy of reaction (II) is positive and high in absolute value (0.51 eV). Consequently, reaction (II) is thermodynamically impossible. In turn, the calculated free energy of reaction (I) is negative (–0.85 eV). At these values of ΔG, the rate constant of electron transfer should be sufficiently high (~10^{10} l mol^{-1} s^{-1} [31]).

However, according to our experimental data, the pyrene cation ($^2\mathbf{1}^+$) was not formed in the course of the quenching of **1** by tolylazide. The following two alternative explanations can be given for this fact:

(1) Electron transfer does not make a detectable contribution, and quenching occurs because of energy transfer. The calculated value of ΔG_1 is underestimated (by no less than 0.5 eV).

(2) Quenching mainly occurs due to electron transfer. However, the reverse electron transfer occurs very rapidly during the lifetime of a geminate pair, and free $^2\mathbf{1}^+$ is not formed. Because the quantum yield of the sensitized decomposition of **3** is high (0.7 ± 0.2), the very rapid decomposition of anion $^2\mathbf{3}^-$ with the release of a nitrogen molecule and the formation of the tolylnitrene anion ($^2\mathbf{N}^-$) should be assumed. According to calculations, this process is thermodynamically favorable (ΔG = –5.6 kcal/mol). The nitrene anion ($^2\mathbf{N}^-$) should give an electron to the pyrene cation. In this case, the formation of pyrene in the ground state and a nitrene in a singlet (excited) state should be expected:

$$^2\mathbf{1}^+ + {}^2\mathbf{N}^- \longrightarrow \mathbf{1} + {}^1\mathbf{N}. \quad \text{(III)}$$

The free energy of this reaction was calculated as ΔG_3 = –1.6 or –2.1 eV for the formation of tolylnitrene in a singlet state with a closed electron configuration or in a lower singlet state with an open electron configuration, respectively. At these free energy values of reverse electron transfer, the yield of radical ions that have escaped geminate recombination should be detectable (~0.02–0.04 [33]).

Although the absence of the free pyrene cation does not definitely demonstrate the insignificance of electron transfer for fluorescence quenching, it strongly supports this hypothesis. This is additionally supported by the fact that the quenching rate constant is independent of solvent polarity (see the table) [28].

Thus, pyrene fluorescence quenching by arylazides was most likely due to energy transfer. This is consistent with the fact that the quantum yields of direct and sensitized photodecomposition of compound **3** are equal within the limits of experimental error.

Singlet–singlet energy transfer can occur through a dipole–dipole mechanism (Foerster mechanism) [27]; however, an exchange mechanism (Dexter mechanism [34]) cannot be excluded either. The probability of energy transfer via the dipole–dipole mechanism ($k_{dd}(r)$) depends on distance (r) in the pair [27]:

$$k_{dd}(r) = \frac{1}{\tau_0}\left(\frac{R_0}{r}\right)^6. \quad (4)$$

The critical distance of energy transfer (R_0) is determined by the equation

$$R_0^6 = \frac{9000 \ln 10 \theta^2 \varphi_f}{128\pi^5 n^4 N_A} \int F(\nu)\varepsilon(\nu)\frac{d\nu}{\nu^4}, \quad (5)$$

where θ^2 is the orientation factor, which is taken equal to 2/3 in nonviscous liquids; n is the refractive index of the solvent; φ_f is the quantum yield of fluorescence; $F(\nu)$ is the normalized corrected fluorescence intensity; $\varepsilon(\nu)$ is the extinction coefficient of azide. It can be seen in Fig. 7 that the overlap of the fluorescence spectrum of **1** and the absorption spectrum of **3** is small; therefore, the critical distance of energy transfer is only 8.8 Å and quenching is weak. Note that tolylazide was purified by chromatography immediately before the measurement of the electronic absorption spectrum.

The rate constant of quenching via the dipole–dipole mechanism in the case when diffusion does not limit the reaction is calculated from the equation [35]

$$k_{q,dd} = 4/3\pi R^3 k_{dd}(R), \quad (6)$$

where R is the sum of the characteristic radii of the energy donor and acceptor (~5 Å). As estimated from Eqs. (4)–(6), $k_{q,dd}$ for the fluorescence quenching of **1** by tolylazide was found to be equal to 2.9×10^7 l mol^{-1} s^{-1},

which is much lower than the experimentally measured value ($(6.3 \pm 0.6) \times 10^9$ l mol^{-1} s^{-1}).

Thus, energy transfer should occur via the exchange mechanism with a probability that exponentially decreases with distance [34]:

$$k_{exp}(r) = (1/\tau_0)\exp(-2(r - r_0)/L), \quad (7)$$

where $L \approx 1$ Å is the characteristic parameter of exponential probability decay.

In this case, the rate constant of quenching has the form [35]

$$k_{q,\,exp} = 2\pi L R^2 k_{exp}(R). \quad (8)$$

With the use of the experimentally measured rate constant of quenching, we can evaluate the probability of quenching in contact between **1*** and **3** (~6×10^{10} s^{-1}). The characteristic distance of energy transfer in this pair (r_0) is ~10 Å.

Thus, the fluorescence of pyrene, which is widely used in binary reagents for the photoaffinity labeling of biopolymers, is quenched in the presence of arylazides with either donor or acceptor substituents. The rate constants of quenching in solution fall within the range $(0.2–1.2) \times 10^{10}$ l mol^{-1} s^{-1}; the rate constants are higher for perfluoro-substituted arylazides. Because the rate constant of quenching is independent of the polarity of the solvent and the formation of the pyrene cation was not detected, we can conclude that quenching occurs via the energy transfer mechanism. The coincidence of the quantum yields of the direct and sensitized photodegradation of tolylazide is consistent with the energy transfer mechanism. As estimated, energy transfer will occur in rigid and organized systems such as a complex of binary reagents with DNA with a characteristic transfer distance of about 10 Å.

In the case of quenching via the energy transfer mechanism, as well as in the direct photolysis of arylazides, the dissociation of the azido group occurs in the primary process with the formation of molecular nitrogen and arylnitrene in a singlet state with an open electron configuration [26]. As noted previously, perfluoro-substituted singlet arylnitrenes exhibit the longest lifetimes and can participate in bimolecular reactions, for example, with DNA bases or the amino acids of protein complexes. Therefore, the use of perfluoro-substituted arylazides in binary reagents is most promising.

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