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METHODS AND TECHNIQUES OF PHYSICOCHEMICAL STUDIES

Analysis of the Oxidative Modification of Proteins by Means of Fluorescence and Elastic Scattering

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Abstract—The fluorescence of pure tryptophan and tryptophan residues in albumin is studied at an excitation wavelength of 288 nm. The range of wavelength registration is 280–380 nm. A broad fluorescence band at 350–355 nm and an elastic scattering line at 288 nm are observed in the spectrum measured at 90° relative to the primary beam. The fluorescence of pure tryptophan and tryptophan in albumin is greatly reduced under the impact of the plasma radiation of a spark discharge, while the elastic scattering peak remains unchanged within the limits of error. A comparison of the elastic scattering and fluorescence indicates that tryptophan loses its inherent property to fluoresce under an external influence. The structure of the other tryptophan levels remains unchanged.

Keywords: fluorescence, elastic scattering, tryptophan, albumin
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INTRODUCTION

Elastic photon scattering in the UV and visible ranges is a classical physical processes. There is Thomson scattering on free electrons [1] and Rayleigh scattering on inhomogeneities smaller than the wavelength that is used [2]. Fluorescence is widely used to study substances by optical means [3]. Elastic scattering on inhomogeneities (bubbles or solid fragments) affects fluorescence and deforms excitation and fluorescence spectra [4].

The fluorescence technique is based on the ability of some substances, referred to as fluorophores, to absorb light and UV radiation with a certain wavelength, referred to as excitation wavelength λ_{exc}, and then to emit a photon with longer wavelength λ_{reg}, referred to as the registration wavelength. Tryptophan, tyrosine, and phenylalanine have fluorescence properties, allowing us to determine their amount in proteins. Information on the content and changes in these amino acids in proteins is used to analyze different biochemical processes, since it allows us to determine changes in a protein's structure.

An excited state can be populated with the subsequent removal of excitation to emit photons. In addition, $\lambda_{exc} = \lambda_{reg}$; this is the elastic scattering of photons through the population of levels of the molecule being studied. At present, elastic scattering is not considered in analyzing the fluorescence of proteins [5]. The aim of this work was to study the role of elastic scattering and compare it to fluorescence in analyzing the oxidative modification of proteins.

EXPERIMENTAL

The fluorescence technique can be described as follows [3, 5]. Primary radiation with wavelength λ_{exc} falls on a test sample, and secondary radiation with wavelength λ_{reg} is recorded at 90° relative to the primary beam, exciting the test compound. The test solution is placed into a quartz glass cell 10 × 10 mm in size.

Optimum excitation and registration wavelength values λ_{exc} and λ_{reg} must be known prior to measurements. We initially used a priori (approximate) evaluations for the wavelengths of excitation and detection. The exact excitation and registration wavelengths for the fluorophore in water were obtained by measuring the fluorescence spectra of aqueous solutions of the test substances. Excitation wavelength λ_{exc} was first determined, and a fluorescence spectrum was acquired in the range of wavelengths that overlapped the expected wavelengths of registration. The wavelength at which maximum fluorescence was obtained was taken as the one of optimum registration, λ_{reg}. Once this registration wavelength was determined, fluorescence was then measured as a function of the excitation wavelength. The wavelength at which maximum fluorescence was obtained was taken as the one of optimal excitation, λ_{exc}. The measurements per-

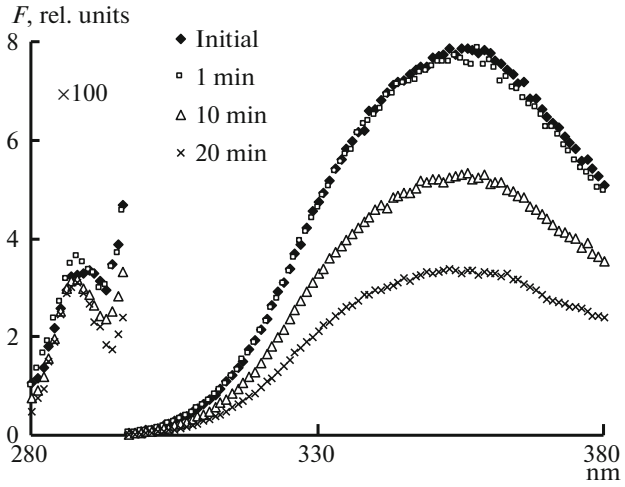

Fig. 1. Fluorescence and elastic scattering in our tryptophan aqueous solution (F) at an excitation wavelength of 288 nm, depending on wavelength of registration (in nm for the untreated solution) and after treatment with spark discharges of plasma radiation for 1, 10, and 20 min. The initial area is magnified by ×100.

formed in this way for our tryptophan solution in water yielded excitation wavelengths $\lambda_{exc} = 288$ nm and registration wavelengths $\lambda_{reg} = 350–355$ nm. Similar measurements yielded identical values λ_{exc} and λ_{reg} for our albumin solution.

The relationship between fluorescence and fluorophore concentration was measured to determine the fluorophore concentration at which its quenching begins. When there is no quenching, fluorescence grows linearly along with concentration. When there is quenching, fluorescence is unchanged or falls with as the concentration of fluorophores rises. On the basis of these experiments, the tryptophan concentration was taken as 10^{-4} mol/L; that of albumin was taken as 7.2×10^{-6} mol/L.

The changes in fluorescence for pure tryptophan and tryptophan in albumin were studied under the action of an external influence. Weakly ionized plasma radiation from a spark discharge in air was used as our external action [6]. The main active factor of such plasma is its radiation. The pulse energy was 5.9×10^{-2} J, the duration of the leading edge was 50 ns, the total pulse duration was 100 μs, and the pulse repetition rate was 10 Hz. The density of the energy flow in the UV-C range during a pulse (at 100 μs and 10 Hz) at a distance of 1 cm from the electrodes was 2 J cm^{-2} s^{-1} (the energy in the pulse). The average density of the radiation's flow of energy at the same distance was $(2 \pm 0.3) \times 10^{-3}$ J cm^{-2} s^{-1}. The flow of photons in the UV-C range was $(1.26 \pm 0.3) \times 10^{-10}$ mol cm^{-2} s^{-1}. The luminosity over the entire range of wavelengths was 230 lx. The maximum of the emission spectrum was at a wavelength of 220 nm. The solutions were treated in a Petri dish 4 mL in volume, placed at a distance of 30 mm from the source of radiation (the discharge region) [7].

The fluorescence spectra were measured on a Fluorat-02 Panorama unit (St. Petersburg, Russia). To observe the elastic scattering and fluorescence, measurements were made at an excitation wavelength of 288 nm. The range of registration wavelengths was 280 to 380 nm. L-tryptophan (chemically pure), bovine serum albumin with $M = 69$ kDa (Biovest Company, Moscow), and distilled water with pH 5.5 were used in our experiments.

RESULTS AND DISCUSSION

The results from measuring fluorescence for tryptophan at $\lambda_{exc} = 288$ nm are presented in Fig. 1. In addition to the main fluorescence peak at $\lambda_{reg} = 350–355$ nm, there was a weak elastic scattering peak at $\lambda_{reg} = 288$ nm. After 20 min of treatment with plasma irradiation, tryptophan fluorescence was reduced by a factor of 3. Fluorescence yield F prior to treatment was 8.7 ± 0.9 rel. units. The changes in elastic scattering peak at 288 nm during treatment did not exceed 20%.

The results from measuring fluorescence for albumin are presented in Fig. 2. The fluorescence peak is in this case comparable to that of elastic scattering. Fluorescence yield F prior to treatment was 0.76 ± 0.09 rel. units. Albumin contains two tryptophan molecules [8]. The ratio of fluorescence yields for pure tryptophan and albumin conforms to two tryptophan molecules in the albumin, within the limits of error. Albumin fluorescence fell by a factor of around 10 after 20 min of treatment. As with pure tryptophan, the changes in the elastic scattering peak were no more than 20%.

To assess the contribution from elastic scattering on the solvent (water) and the cell, we measured the fluorescence of distilled water poured into the same cell as the test solution. The fluorescence yield of pure water did not exceed the spectrometer noise (0.005 rel. units), and we observed no features in the fluorescence spectrum.

The absorption spectrum of the solution with an albumin concentration of 7.2×10^{-6} mol/L is shown in Fig. 3. An absorption peak at 280 nm is visible in the spectrum. The position of the absorption peak is unchanged after treatment with plasma radiation. The shift of the absorption maximum (280 nm) relative to the excitation peak wavelength (288 nm) is due to features of the radiation spectrum of the lamp used in the fluorimeter [5]. The lamp's emission spectrum is strongly heterogeneous, so the result was normalized to the intensity of the primary radiation when measuring the optical density. No normalization was used when measuring fluorescence. We may therefore say that we observe the same population of the state of the tryptophan molecules both in the absorption spectrum and during fluorescence. The optical density of

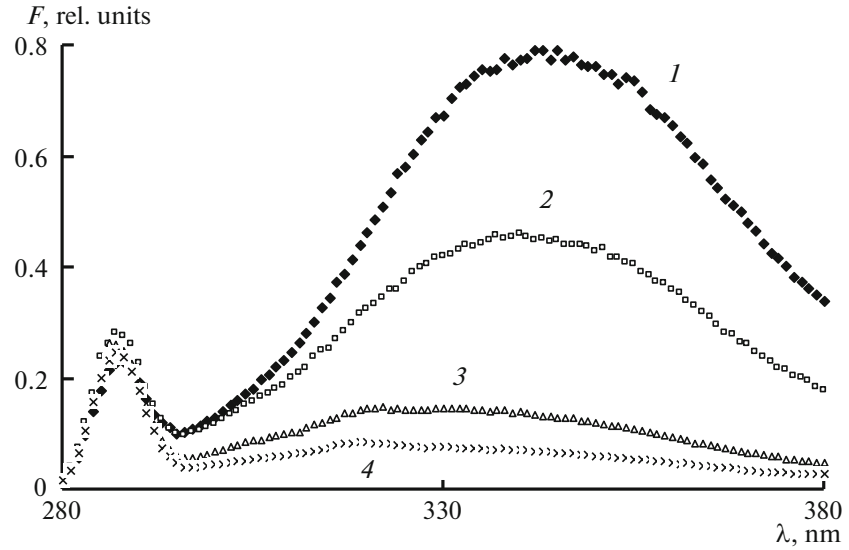

Fig. 2. Fluorescence and elastic scattering in albumin aqueous solution at an excitation wavelength of 288 nm, depending on the wavelength of registration, in nm: (*1*) untreated solution; (*2–4*) after treatment with spark discharges of plasma radiation for 1, 10, and 20 min, respectively.

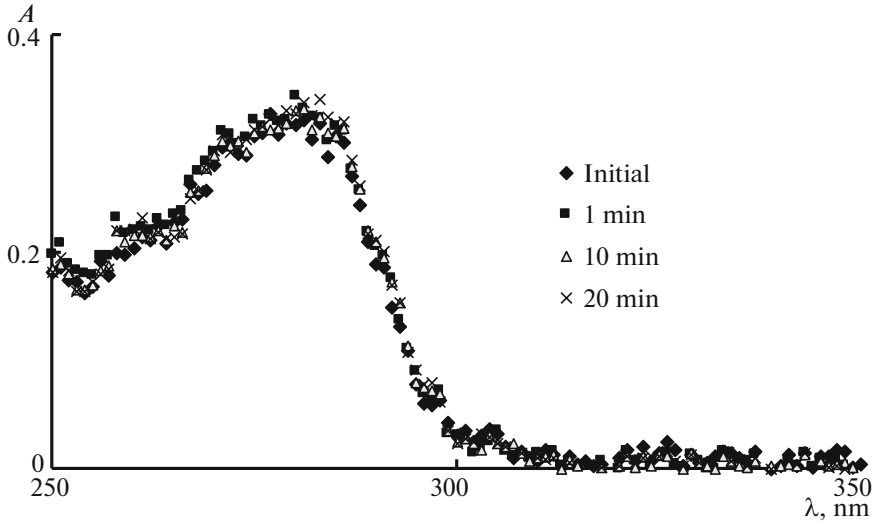

Fig. 3. Absorption spectrum of albumin solution in the wavelength range of 250–350 nm. *A* is the optical density (absorbance) for the initial solution and after treatment with spark discharges of plasma radiation for 1, 10, and 20 min.

this state and the intensity of elastic scattering were unchanged during treatment with plasma radiation, while the fluorescence was greatly reduced.

The physical processes in a fluorophore molecule can be illustrated using a Jablonski diagram (Fig. 4). A molecule in ground singlet state S_0 absorbs photons with wavelength λ_{exc}. As a result, singlet-excited states S_i are populated: $S_1 - S_n$, where n is the number of singlet-excited states. Excited states $S_2 - S_n$ remove excitation in a cascade via the emission of photons, and the lowest excited singlet state S_1 is then populated. The radiation that removes the excitation from the S_1 level and conforms to the transition $S_1 \rightarrow S_0$ is registered on a fluorimeter. Wavelength λ_{reg} of the photons that remove the excitation from the S_1 state is always higher than λ_{exc}, which populates states $S_2 - S_n$; the energy of the transitions between levels S_0 and S_1 is less than that between levels $S_0 - S_n$, where $n > 1$. The ability to accumulate excitation in low singlet state S_1 is a property of a specific fluorophore molecule.

We can populate excited state S_i with subsequent removal of the excitation from this state via the emission of photons: $S_0 \rightarrow S_i$. In addition, $\lambda_{exc} = \lambda_{reg}$; this is

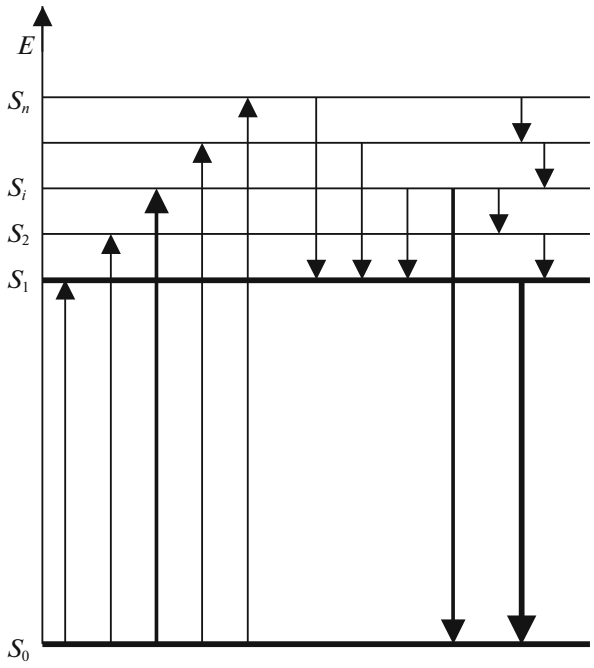

Fig. 4. Jablonski scheme of energy transitions between the levels of a fluorescent molecule.

the elastic scattering of photons through the population of the excited states of a fluorophore molecule. The angular distribution of dipole radiation has its maximum at an angle of 90° relative to the primary beam [2]. Earlier, elastic scattering was not allowed for when considering protein fluorescence.

The results obtained by comparing the yields of fluorescence and elastic scattering also allow us to draw conclusions on the effects radiation has on a fluorophore. Our fluorescence measurements indicate that tryptophan decays under the action of an external influence. Observations of the elastic scattering show that the structure of the singlet excited states of tryptophan molecules remains unchanged, but the molecule loses its inherent property of fluorescence and the S_1 level decays.

Analysis of structure of tryptophan levels shows that the S_1 level, through which excitation is removed from a tryptophan molecule, forms in the benzene and pyrrole rings of tryptophan [9–11]. The loss of fluorescence properties indicates destruction of these rings under an external influence.

The $HO_2^\bullet$ radical is the main active particle formed in an aqueous solution under the action of the plasma radiation from the source used in this work. The energy (368 kJ/mol) needed to split a hydrogen atom from an RH molecule is released in the reaction between this radical and the organic RH substance [12]. If the energy of hydrogen is greater than this value, oxidation with $HO_2^\bullet$ radicals is impossible. The energy of hydrogen bonds in the amine and the carboxyl groups of amino acid is more than 368 kJ/mol [13], so these groups in amino acids do not decay under the action of plasma radiation. The R radicals in side chains decompose; in tryptophan, these are the phenolic and indole rings. Our observations of the elastic scattering of photons that accompanies fluorescence enable us to specify the mechanism of the processes that occur in the amino acid under the action of an external influence.

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