Full Articles

Kinetics and mechanism of reactions of photoexcited kynurenine with molecules of some natural compounds

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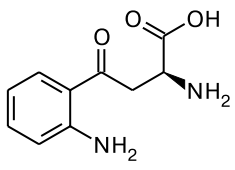
Photochemical reactions involving kynurenines, *viz.*, molecules present in the eye lens, can result in modifications of the lens proteins and cause a development of a cataract. The rate constants of the reactions of photoexcited kynurenine with several amino acids and antioxidants contained in the lens were measured. The most efficient quenchers of triplet kynurenine are amino acids tryptophan and tyrosine, as well as antioxidant ascorbate. In all cases, the quenching reaction proceeds by the electron transfer mechanism, except for the reaction with oxygen where transfer of the triplet energy to the oxygen molecule occurs.

Key words: kynurenine, laser flash photolysis, triplet excited kynurenine, antioxidants, amino acids, electron transfer.

Cataract is a progressive clouding of the natural eye lens preventing light transmission to the retina. Most frequently, the cataract develops in persons more than 60 years old under unfavorable environmental factors and because of poor genes or metabolic dysfunctions in the eye lens. One of the main risk factors for cataract development is the ultraviolet irradiation followed by the chemical transformations in the eye tissues.[1] The development of cataract is related to the oxidative processes in the eye lens involving tryptophan metabolites, *viz.*, kynurenines. Kynurenines (**1**) are structurally similar to natural amino acids and characterized by the absorption band at 300—400 nm and short fluorescence times.[2—9] Therefore, in the eye lens kynurenines act as light filters protecting the retina from UV radiation and improving visual acuity by decreasing the chromatic aberrations.[10] However, the concentration of UV filters in the eye lens decreases with aging.[6] The lenses of the human eye turn yellow,[11] and the UV spectra of the proteins of aged eye lenses are similar to the absorption spectra of the UV filter compounds.[8] Cataract develop-



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ment can be favored by the binding of kynurenines to the lens proteins (crystallines),[12,13] resulting in the protein modification and a change in the functionability of the UV filters.

The covalent addition of kynurenines to the proteins can proceed by different mechanisms. According to one of the hypotheses,[14—18] during the photochemical reaction photoexcited kynurenine reacts with amino acid residues of proteins. For a long time kynurenines were considered to be photochemically inert compounds. The only exception was *N*-formylkynurenine, whose photolysis generates a triplet excited molecule capable of reacting with a substrate either directly or through the formation of highly reactive singlet oxygen.[8,18,19] At the same time, the photolysis of oxygen-saturated solutions of **1** and 3-hydroxykynurenine exhibits very low yields of singlet oxygen and superoxide.[19] The singlet excited state of kynurenines relaxes to the ground state on the picosecond time scale.[8] These observations suggested that kynurenines present in mammalian eyes are poor photosensitizers and the absorbed light energy is consumed in processes that do not damage the tissue. However, as has been found later,[20] kynurenines can participate in the photoreduction of oxygen and nitromethane and sensitize the photooxidation of cysteine, NADH, and some other biologically important molecules including crystallines. The detailed mechanism of these reactions remains unknown.

As found in our earlier studies[21,22] of the photochemical properties of kynurenine **1**, the ultraviolet irradiation of aqueous solutions of **1** produces the short-lived kynurenine intermediate, namely, triplet excited state $^{T}\mathbf{1}$. Although in a neutral medium the quantum yield of this state is low[21] (Φ_T ~2%), in the mammalian eye lenses the formation of triplet **1** can lead to the formation of singlet oxygen and photochemically active reaction products and, eventually, to the irreversible chemical modification of crystallines. The amino acids and antioxidants present in the eye lens can react with the triplet kynurenine. It is important that in this case the amino acids can be potential targets for photooxidation. Assessment of the ability of antioxidants to act as protective substances due to their reactions with excited molecules is topical. This could prevent the interaction of triplet kynurenine with amino acid residues of proteins in the eye lens.

In the present work, laser flash photolysis was used to measure the rate constants for quenching of the triplet excited state of kynurenine by amino acids (L-tryptophan, *N*-acetyl-L-tryptophan, L-cysteine, *N*-acetyl-L-tyrosine, *N*-acetyl-L-histidine, and *N*-acetyl-L-methylthionine) and antioxidants (L-ascorbate and reduced L-glutathione). The aim of the study is to determine the ability of the amino acids and antioxidants to deactivate the triplet state of kynurenine and to establish the mechanisms of the quenching reactions.

Experimental

D,L-Kynurenine (Fluka), L-ascorbate, reduced L-glutathione, L-tryptophan, *N*-acetyl-L-tryptophan, L-cysteine, *N*-acetyl-L-tyrosine, *N*-acetyl-L-histidine, and *N*-acetyl-L-methionine (Sigma) were used without additional purification. Doubly distilled water and sodium phosphate (reagent grade) were used to prepare buffer solutions.

Flash photolysis. The experimental equipment was described in detail earlier.[23,24] A solution placed in a rectangular quartz cell was irradiated with an Nd : YAG laser (Quanta-Ray LAB-130-10, (USA), 355 nm, energy up to 150 mJ pulse^{-1}, pulse duration 8 ns). The detecting system consisted of a DKsSh-150 short-arc xenon lamp (pulse duration 2 ms, probing beam cross section 2×3 mm), two synchronously controlled monochromators, a 9794B photomultiplier (Electron Tubes Ltd., United Kingdom), a digital two-channel oscilloscope with an 11-bit analog-to-digital converter (LeCroy 9310A (Switzerland), 400 MHz, time resolution 10 ns), and a system of filters and shutters. The technique is was controlled by an IBM PC 486 computer through an MP488CT IEEE 488 interface (USA). Since the detecting light concentrated in a rectangle 3 mm in height and 1 mm in width passes through the cell along the front (laser irradiated) window (width 8 mm, height 3 mm) of the cell, in all experiments the optical excitation path length was 1 mm and the detection optical length (l) was 8 mm. Correspondingly, the absorbances of the intermediates are given for $l = 0.8$ cm. To remove oxygen from the system, argon was bubbled through the solution for 15 min prior to and during the measurements. Actinometry was carried out using naphthalene solutions in cyclohexane. The energy of the incident laser radiation was determined from the absorption of the triplet state of naphthalene at 414 nm ($\varepsilon_T = 2.45 \cdot 10^4$ L mol^{-1} cm^{-1}, $\Phi_T = 0.75$).[25,26] The acidity of solutions was measured with a pH meter (Orion Research Inc., Germany) calibrated using a set of standard titres (GOST 8.135). Absorption spectra of the initial solutions were recorded on an Agilent 8453 spectrophotometer (Hewlett-Packard, USA).

Results and Discussion

A specific feature of the reactions in aqueous solutions is the dependence of structures of the reactants, intermediates, and products on the acidity of the solutions. The reactions of acid-base equilibrium for kynurenine in the ground and triplet states have previously been studied.[21] The photolysis of neutral solutions of **1** affords neutral $^{T}\mathbf{1}$ ($pK_a = 4.7$) characterized by the absorption spectrum with a maximum at 430 nm.[22]

$$\mathbf{1} \xrightarrow{h\nu} {}^{S}\mathbf{1}^{*} \xrightarrow{\text{ISC}} {}^{T}\mathbf{1},$$

where ISC is intersystem crossing, and $^{S}\mathbf{1}^{*}$ is the singlet excited state of kynurenine **1**.

In the absence of quenchers, the absorption decay kinetics of triplet **1** is described by a law of the bimolecular chemical reaction (triplet-triplet annihilation, $k_{T-T} = 4.1 \cdot 10^9$ L mol^{-1} s^{-1}).[21,22] In the presence of a quencher,

the kinetic curve of the triplet state decay becomes exponential with a pseudo-monomolecular rate constant k_{eff} proportional to the quencher concentration C_0: $k_{eff} = k_q C_0$, where k_q is the quenching rate constant. If the quenching reaction produces free radicals, then an increase in the signals with time was observed at the wavelengths corresponding to the absorption of these radicals, the rate of the signal increase being approximately equal to the fall-off rate of the signal of triplet kynurenine.

Under photolysis of solutions of **1**, the triplet kynurenine can undergo photoionization through absorption of the second light quantum to form a kynurenine radical cation and a solvated electron[22]

$$^{T}\mathbf{1} \xrightarrow{h\nu} \mathbf{1}^{\bullet+} + e^{-}_{solv}.$$

To avoid the formation of electron adducts in possible reactions of the solvated electron with the initial kynurenine **1** and quencher molecules, all measurements were carried out in solvents with acetone (Ac) additives (0.01—0.02 mol L^{-1}). In this case, all solvated electrons that appeared under kynurenine photolysis disappear in the reaction with acetone ($k(e^{-}_{solv}—Ac) = 6 \cdot 10^9$ L mol^{-1} s^{-1}),[27] resulting in the electron adduct $Ac^{\bullet-}$, which is rapidly protonated in a neutral medium. The protonated acetone radical $AcH^{\bullet}$ is a weak chromophore and makes no contribution to the observed absorption of intermediates.

Ascorbate. The absorption spectra obtained under photolysis of a neutral solution of **1** ($1.9 \cdot 10^{-4}$ mol L^{-1}, pH 7.2) in the presence of ascorbate $AscH_2$ ($1.0 \cdot 10^{-3}$ mol L^{-1}) are presented in Fig. 1. Spectrum *1* observed immediately after the laser pulse corresponds to the absorption of the triplet **1**.[21,22] Spectrum *2* observed 4 μs after the laser pulse is characterized by an absorption maximum at 360 nm, being quite similar to the absorption spectrum of ascorbic acid radical anion $Asc^{\bullet-}$ recorded in pulse radiolysis of ascorbic acid solutions in a neutral medium.[28] These data suggest that triplet kynurenine is quenched by ascorbate by the electron transfer mechanism to give the kynurenine radical anion and ascorbate radical cation, which is rapidly deprotonated in a neutral solution (pH 7.2) to form the observed ascorbate radical anion ($pK_{a1} = 1.1$, $pK_{a2} = 4.25$)[28]

$$^{T}\mathbf{1} + AscH_2 \xrightarrow{k_q} \mathbf{1}^{\bullet-} + AscH_2^{\bullet+},$$

$$AscH_2^{\bullet+} \rightleftharpoons Asc^{\bullet-} + 2\,H^{+}.$$

The kinetic curves of the triplet kynurenine absorption decay observed at different concentrations of the quencher (ascorbate) are shown in Fig. 2. The signal decay rate increases as the $AscH_2$ concentration increases, which corresponds to the quenching of the triplet excited kynurenine by ascorbate. The triplet absorption decays show exponential patterns and the rate constant depends linearly on the quencher concentration (Fig. 3). The bimolecular rate constant for quenching by ascorbate was determined from the slope of the concentration plot presented in Fig. 3: $(8.5 \pm 1.2) \cdot 10^8$ L mol^{-1} s^{-1}.

Reduced glutathione. A transient absorption signal in the region 340—540 nm with a maximum at 420 nm was observed in the photolysis of solutions of **1** in a neutral medium in the presence of reduced glutathione GSH. The signal intensity and the rate of its formation increase with an increase in the GSH concentration and the

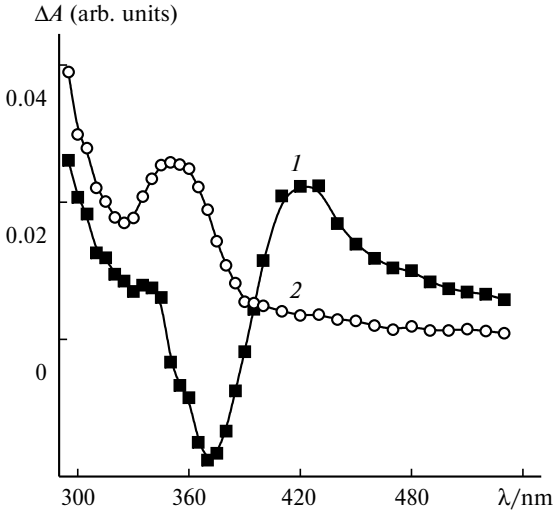

Fig. 1. Absorption spectra observed 80 ns (*1*) and 4 μs (*2*) after pulsed laser irradiation (λ =355 nm) of kynurenine solution in the presence of L-ascorbate ($1.0 \cdot 10^{-3}$ mol L^{-1}) at pH 7.2.

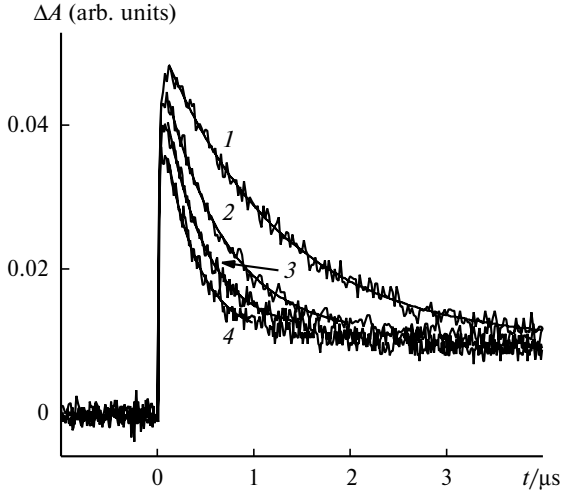

Fig. 2. Kinetic curves observed at λ = 430 nm under photolysis of kynurenine solution (pH 7.2) in the presence of L-ascorbate. Ascorbate concentration: $7.6 \cdot 10^{-4}$ (*1*), $1.5 \cdot 10^{-3}$ (*2*), $2.3 \cdot 10^{-3}$ (*3*), and $3.0 \cdot 10^{-3}$ (*4*) mol L^{-1}. Results of calculation are shown by solid lines.

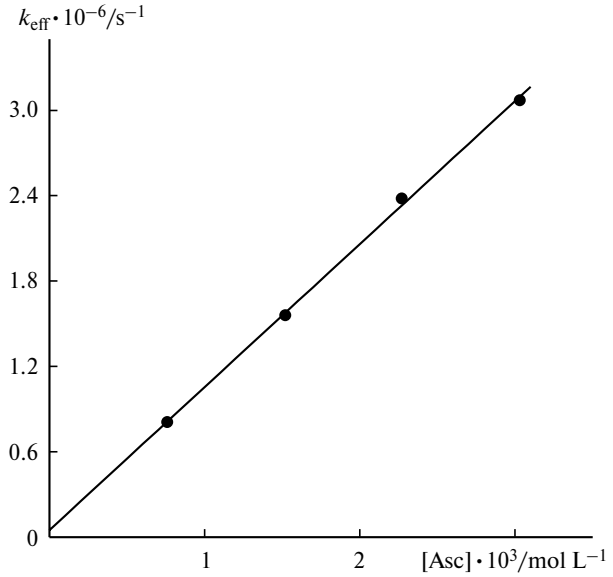

Fig. 3. Plot of the pseudo-monomolecular rate constant for triplet kynurenine decay *vs.* concentration of L-ascorbate.

pH value of the solution. This signal was assigned to glutathione disulfide radical anion $GSSG^{\bullet -}$ (maximum at ~420 nm, $\varepsilon_{420} \approx 1.6 \cdot 10^5$ L mol^{-1} cm^{-1})[29] formed upon addition of the thiyl radical $GS^{\bullet}$ to the GS^- anion with the rate constant[30,31] $k(GS^{\bullet}—GS^-) = 6.6 \cdot 10^8$ L mol^{-1} s^{-1}.

$$GS^{\bullet} + GS^- \longrightarrow GSSG^{\bullet -}.$$

The concentration of the GS^- anions is determined by the acid-base equilibrium constant for aqueous glutathione solutions ($pK_a = 9.2$)[32]

$$GSH \rightleftharpoons GS^- + H^+.$$

A question about the origin of thiyl radical $GS^{\bullet}$ arises. It can be assumed that this radical is formed due to electron transfer from GSH to triplet **1** followed by fast deprotonation of the $GSH^{\bullet +}$ radical. In this case, an increasing transient absorption signal at 290 nm corresponding[22] to the absorption band of radical $\mathbf{1}^{\bullet -}$ should be observed. However, this signal was not observed in experiment. To establish the mechanism of $GS^{\bullet}$ formation in solution, the dependence of the yield of these radicals on the laser radiation intensity was studied. We showed that the yield of the experimentally observed $GSSG^{\bullet -}$ radicals exhibits a quadratic dependence on the laser pulse energy. Based on these data, we concluded that the $GS^{\bullet}$ radicals are formed by the reduction of the $AscH^{\bullet}$ radicals with glutathione. In turn, the $AscH^{\bullet}$ radicals were formed due to the two-photon ionization of **1** followed by the addition of a solvated electron to acetone (electron trap).

To minimize the contribution of the two-photon processes, the further experiments were carried out at lowest acceptable laser radiation energies (~50 mJ pulse^{-1}) at pH 5.5. At these pH values, the equilibrium concentration of the GS^- anions in solution is only 0.02%, and no strongly absorbing $GSSG^{\bullet -}$ radicals are formed even at high GSH concentrations. A minor amount of the $GS^{\bullet}$ radicals that could be present in solution gave no noticeable contribution to the observed absorption because of the low absorption coefficients of this radical.[33,34] Under these conditions, the spectrum showed no appreciable changes up to a concentration of GSH added of 0.2 mol L^{-1}. Thus, the rate constant for quenching of triplet kynurenine by glutathione does not exceed 10^6 L mol^{-1} s^{-1}.

Tryptophan. Photolysis of an aqueous solution of **1** ($2.0 \cdot 10^{-4}$ mol L^{-1}) in the presence of *N*-acetyl-L-tryptophan (TrpH) gives rise to two increasing transient absorption signals, at 470—540 nm (Fig. 4) with a maximum at 510 nm and in the short-wavelength region at 300—340 nm. These signals were attributed to the neutral tryptophan radical $Trp^{\bullet}$ characterized by absorption maxima at 320 and 510 nm.[35—38] The increase in the signal amplitude is well described by a single exponent. The rate constants k_{eff} for the increase in the signals detected at 320 and 510 nm were similar to each other and proportional to the tryptophan concentration

$$k_{eff} = k_q(\text{TrpH})[\text{TrpH}],$$

where k_q(TrpH) is the rate constant for quenching of $^T\mathbf{1}$ by tryptophan.

The rate constant for the triplet kynurenine quenching by tryptophan calculated from the plot of k_{eff} *vs.* tryp-

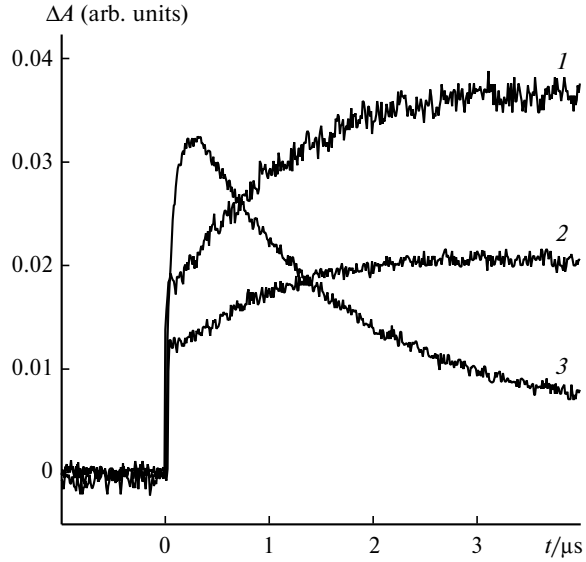

Fig. 4. Transient absorption kinetics of intermediates formed under photolysis of an aqueous kynurenine solution in the presence of *N*-acetyl-L-tryptophan (0.02 mol L^{-1}) in the buffer solution (detection at 320 (*1*) and 510 nm (*2*)) and in the non-buffer solution (detection at 570 nm (*3*)).

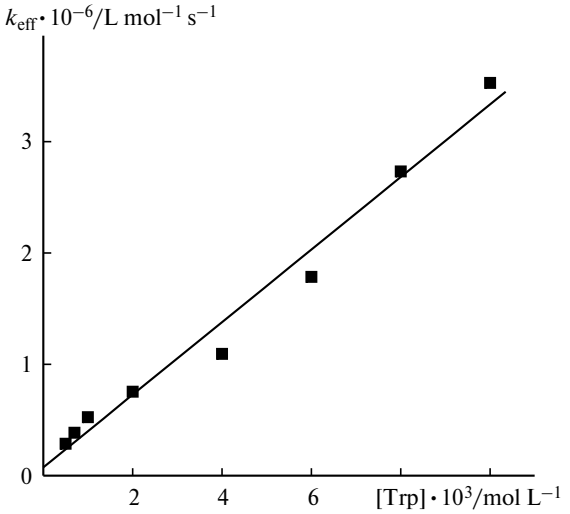

Fig. 5. Plot of the pseudo-monomolecular rate constant for Trp$^\bullet$ radical formation *vs.* tryptophan concentration.

tophan concentration (Fig. 5) was $(3.2\pm0.6)\cdot10^8$ L mol^{-1} s^{-1}.

The Trp$^\bullet$ radical can be formed either upon hydrogen atom abstraction from tryptophan by photoexcited kynurenine or due to electron transfer followed by deprotonation of the TrpH$^{\bullet+}$ radical cation. The latter is characterized by pK_a = 4.3 (see Refs 36, 39, and 40). In a buffer solution at the neutral pH values, the deprotonation occurs very rapidly due to the interaction with the buffer components

$$^T\mathbf{1} + \mathrm{TrpH} \xrightarrow{k_q} \mathbf{1}^{\bullet-} + \mathrm{TrpH}^{\bullet+},$$

$$\mathrm{TrpH}^{\bullet+} \rightleftharpoons \mathrm{Trp}^\bullet + \mathrm{H}^+.$$

To establish the quenching mechanism, an experiment in the non-buffer aqueous solution containing **1** ($2.1\cdot10^{-4}$ mol L^{-1}) and TrpH (0.02 mol L^{-1}) was carried out. The value pH 7.0 was achieved by adding small amounts of NaOH dropwise to the solution. The transient absorption signal was detected at a wavelength of 570 nm corresponding[36,40] to the absorption maximum of TrpH$^{\bullet+}$ radical. It is important that other intermediates (^{T}**1**, Trp$^\bullet$ radical, and kynurenine radicals), which can be formed during the reaction, almost do not absorb at this wavelength. Pulsed irradiation of the sample gives rise to a signal that first increases and then decreases on the microsecond time scale, which corresponds to the quenching of ^{T}**1** causing the formation of TrpH$^{\bullet+}$ radical followed by its deprotonation (Fig. 4). This unambiguously indicates that the quenching of triplet kynurenine with tryptophan proceeds by the electron transfer mechanism.

Similar measurements were carried out for L-tryptophan. The quenching rate constant is given in Table 1.

***N*-Acetyl-L-tyrosine.** Under photolysis of **1** in the presence of *N*-acetyl-L-tyrosine (TyrOH), an increasing signal was observed in the 290—350 nm region with a maximum at 320 nm, which corresponds to the absorption of the tyrosine radical TyrO$^\bullet$ formed in the electron transfer reaction followed by fast deprotonation of the radical cation[41]

Table 1. Rate constants (k_q) for triplet kynurenine quenching by amino acids and antioxidants

Compound	k_q/L mol^{-1} s^{-1}
L-Ascorbate	$(8.5\pm1.2)\cdot10^8$
L-Glutathione, reduced	$<10^6$
N-Acetyl-L-tryptophan	$(3.3\pm0.6)\cdot10^8$
L-Tryptophan	$(2.6\pm0.7)\cdot10^8$
N-Acetyl-L-tyrosine	$(6.7\pm1.5)\cdot10^7$
L-Cysteine	$<10^6$
N-Acetyl-L-histidine	$<10^6$
N-Acetyl-L-methionine	$<10^6$
Oxygen	$(2.1\pm0.5)\cdot10^9$

$$^T\mathbf{1} + \mathrm{TyrOH} \xrightarrow{k_q} \mathbf{1}^{\bullet-} + \mathrm{TyrOH}^{\bullet+},$$

$$\mathrm{TyrOH}^{\bullet+} \rightleftharpoons \mathrm{TyrO}^\bullet + \mathrm{H}^+.$$

The pseudomonomolecular rate constant k_{eff} for the increase in the signal observed at 320 nm depends linearly on the tyrosine concentration. The bimolecular rate constant for quenching k_q(TyrOH) determined from the slope of the plot of k_{eff} *vs.* TyrOH concentration is $(6.7\pm1.5)\cdot10^7$ L mol^{-1} s^{-1}.

***N*-Acetyl-L-histidine, *N*-acetyl-L-methionine, and L-cysteine.** The addition of up to 0.1 mol L^{-1} of *N*-acetyl-L-histidine and *N*-acetyl-L-methionine to the solution produced no noticeable changes in the observed spectra and the kinetic curves of transient absorption. In the presence of L-cysteine (RSH) at pH 7.0, the formation of RSSR$^{\bullet-}$ radical anions characterized by the absorption maximum at 420 nm was observed. However, as for the case with glutathione (see above), it was shown that these radicals are formed due to the two-photon ionization of **1** followed by the addition of the solvated electron to acetone rather than by the quenching of triplet **1** by cysteine. Then the AcH$^\bullet$ radicals are reduced with cysteine and the RS$^\bullet$ radicals formed in this reaction add to the RS$^-$ anions. Thus, we concluded that the rate constants for quenching of triplet **1** by *N*-acetyl-L-histidine, *N*-acetyl-L-methionine, and L-cysteine are at most 10^6 L mol^{-1} s^{-1}.

Oxygen. In the presence of oxygen ($1.4\cdot10^{-3}$ mol L^{-1}), an exponential decay of the triplet kynurenine with a rate constant of $2.9\cdot10^6$ s^{-1} was observed; this corresponds to the bimolecular rate constant for quenching $k_q(O_2)$ = $2.1\cdot10^9$ L mol^{-1} s^{-1}. No transient absorption signals of radicals of **1** were observed in the spectrum recorded 2 μs after laser irradiation of the kynurenine solution. Therefore, in this case the quenching proceeds by the mecha-

nism of triplet energy transfer from photoexcited **1** to oxygen.

Thus, among the amino acids, the most efficient quenchers of the triplet kynurenine are the aromatic amino acids tryptophan and tyrosine. Therefore, the tryptophan and tyrosine residues in crystallines are the most susceptible sites for the modification of these proteins with photoexcited kynurenine. Among the main antioxidants present in the eye lens, ascorbate is characterized by the high rate constant for quenching of triplet kynurenine. Probably, it is this antioxidant that protects the eye lens proteins from the damages induced by the photoexcited molecules of kynurenine and its derivatives. From this point of view, it seems interesting to compare the concentrations of possible quenchers of the triplet kynurenine in the human eye lens. The concentrations of glutathione and ascorbic acid vary considerably from induvidual to individual; however, they can be estimated[6,42,43] on the average as several mmoles per liter. The concentrations of other antioxidants, *viz.*, carotinoids, are several orders of magnitude lower.[44] The oxygen concentration in the eye lens tissue decreases sharply on going from the external to internal layers of the lens,[45] and the oxygen pressure in the eye lens nucleus is less than 2 Torr. Crystallines account for 50% of the total weight of the eye lens; therefore, the concentration of individual amino acid residues (tryptophan, tyrosine) can be estimated as several tens of mmoles per liter. However, it should be taken into account that the majority of amino acid residues in protein molecules are buried inside the protein globule, being inaccessible to the attack by triplet kynurenine. Unfortunately, quantitative estimation of the accessibility of various amino acid residues containing in the crystallines is presently impossible.

It has previously been shown[46,47] that kynurenines can undergo thermal deamination under physiological conditions. Highly reactive carboxyketoalkenes formed in the reaction can also add to crystallines. Glutathione is the most efficient scavenger of these compounds.[48] It can be assumed that in the human eye lens proteins are protected from photochemical reactions of kynurenine by ascorbate and from the thermal reactions by glutathione. Deficiency of any of these antioxidants may cause the development of cataract.

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