Antioxidative properties of nitroxyl radicals and hydroxyamines in reactions with triplet and deaminated kynurenine

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The reactions of triplet kynurenine and 4-(2-aminophenyl)-4-oxocrotonic acid, formed upon the thermal decomposition of kynurenine, with nitroxyl radicals and cyclic *N*-hydroxylamines were studied. Nitroxyl radicals were found to quench efficiently the triplet state of kynurenine (rate constant $3—6 \cdot 10^8$ L mol^{-1} s^{-1}). The quenching proceeds *via* the spin-exchange mechanism and affords no new products. Neither nitroxyl radicals, nor hydroxylamines react with 4-(2-aminophenyl)-4-oxocrotonic acid under conditions similar to physiological.

Key words: kynurenine, 4-(2-aminophenyl)-4-oxocrotonic acid, triplet excited state, quenching reactions, laser flash photolysis, nitroxyl radicals, hydroxylamines.

Cataract is progressing lenticular opacity resulting in worsening and, finally, in complete loss of vision. This disease develops in more than 50% population of the Earth who reached 65 years.[1] At present cataract can be cured only by surgery: the natural lens, which underwent opacity, is removed and an intraocular lens is implanted. Evidently, the properties of the implant are far from natural and, hence, vision cannot be reduced to a full extent. There is no drug capable of restoring lens transparency. Thus, struggling against this disease the main attention should be given to prophylactics remedies.

It is known that the main reason for the development of the age-related cataract is an oxidative stress in the lens nucleus leading to numerous post-translational modifications of lens proteins. However, the mechanism of these modifications remained unknown until recently. The works have appeared indicating that a series of low-molecular-weight compounds that act in the lens as ultraviolet filters play an important role in these processes.[2—10] These compounds, *viz.*, L-kynurenine (**1**) and its derivatives, are metabolic products of tryptophan; they absorb the major part of the UV radiation in the range 300—400 nm penetrating through the cornea, thus protecting the retina and the lens itself from photoinduced damages and improving visual ability due to reducing chromatic aberrations.

At the same time, it is assumed that the covalent addition of these substances to lens proteins can modify the proteins, namely, their coloration, cross-linking, a decrease in the solubility, and as a consequence, the development of cataract.[11—21] The lens proteins can be modified by UV filters due to both thermal and photochemical reactions. We have earlier found[22—24] that in photochemical reactions the key intermediate is kynurenine in the triplet state (^{T}**1**) capable of reacting with amino acid residues of proteins. It was shown[23,25] that kynurenine and its derivatives are thermally unstable under physiological conditions and undergo spontaneous deamination to form highly reactive compounds: 4-(2-aminophenyl)-4-oxocrotonic acid (**2**), being carboxyketoalkene, and its derivatives. Carboxyketoalkenes can add to nucleophilic amino acid residues of proteins (cysteine, histidine, and lysine)[15,16,26—28] distorting functionality of the proteins and enhancing their susceptibility to UV irradiation.

In the normal state the lens contains various substances that protect it from oxidation processes, including glutathione, glutathione peroxidase, NADPH, catalase, ascorbic acid, and superoxide dismutase. It has previously been found that the lens proteins are protected from photoexcited UV filters by ascorbate capable of quenching triplet molecules of kynurenine and its derivatives,[24] while

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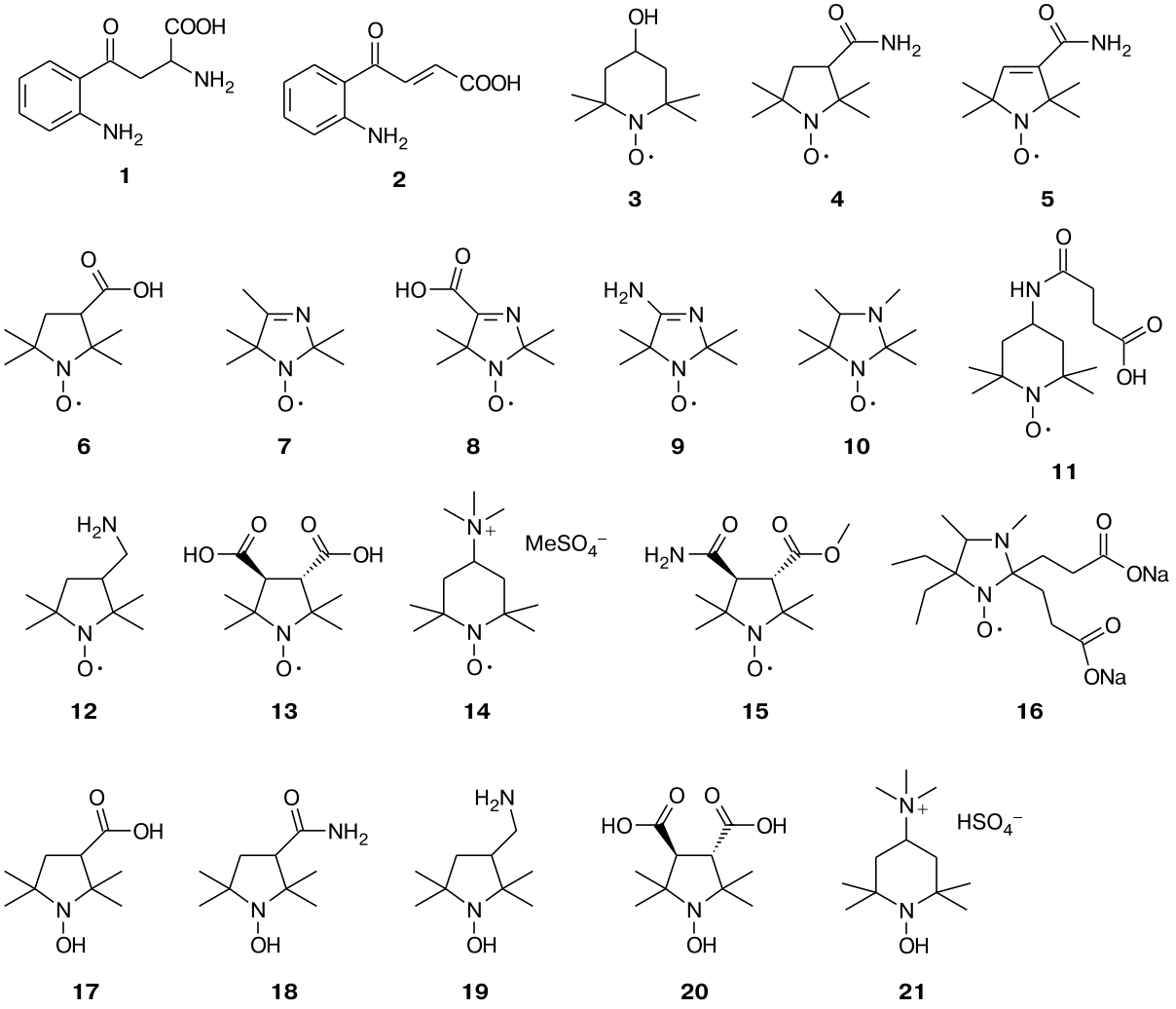
the protection from carboxyketoalkenes is performed by reduced glutathione, which can reduce the double bond in the side chain of acid **2** to form the adducts.[28—30] The concentration of these substances in human organism decreases with aging, which decreases the efficiency of the protective system. Therefore, the creation of new efficient drugs corresponding to modern requirements and capable of protecting the lens tissues from photochemical and thermal reactions involving UV filters is a topical problem.

Exogenous antioxidants based on nitroxyl radicals often protect cells and living tissues from oxidation processes induced by the UV irradiation more efficiently that natural antioxidants.[31] The nitroxyl radical 2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl (TEMPOL) has earlier been used successfully for the protection of epithelial cells of the lens.[32] It was also shown that the application of the reduced form of this radical, *viz.*, cyclic *N*-hydroxylamine (TEMPOL-N), results in cataractogenesis inhibition *in vivo*.[33] The purpose of this work is to evaluate the ability of nitroxyl radicals **2**—**16** and hydroxylamines **17**—**21** of different structure to inhibit the key stages of thermal and photoinduced transformations of kynurenine resulting in the modification of lens proteins. In particular, the ability of nitroxyl radicals (RR´NO·) and their reduced forms, *viz.*, hydroxylamines (RR´NOH), to quench the triplet state of kynurenine **1** and reduce the double bond in the side chain of carboxyketoalkene **2** has been studied.

Experimental

D,L-Kynurenine (Fluka, Germany) and organic solvents for high-performance liquid chromatography (Kriokhrom, Russia) were used without additional purification. Double-distilled water and sodium phosphate (reagent grade) were used for the preparation of buffer solutions. Nitroxyl radicals **3**—**6** (see Ref. 34), **7**, **8** (see Ref. 35), and **9**—**13** (see Refs 36—40) were synthesized by procedures described in the indicated works.

2,2,6,6-Tetramethyl-4-trimethylammoniopiperidine-1-oxyl methylsulfate (14). Dimethyl sulfate (2.8 mL, 30 mol) and Na_2CO_3 (5 g, 0.047 mmol) were added to a solution of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-amino-TEMPO, ACROS Organics) (1 g, 5.8 mmol) in acetone (20 mL). The suspension



was vigorously stirred for 48 h. The precipitate was filtered off and washed with acetone, and the resulting mixture of salts and compound **14** was refluxed with MeOH, periodically separating the colored solution by decantation until the extraction of the radical ceased. The joined solutions were evaporated to dryness under reduced pressure. The residue was refluxed with a MeOH—$CHCl_3$ (1 : 3) mixture, and the obtained solution was filtered and again evaporated to dryness under reduced pressure. The residue was recrystallized from ethanol. The yield was 1.3 g (70%), m.p. 244—249 °C. Found (%): C, 47.67; H, 8.80; N, 8.29; S, 10.01. $C_{13}H_{29}N_2O_5S$. Calculated (%): C, 47.98; H, 8.98; N, 8.61; S, 9.85. IR (KBr), ν/cm^{-1}: 3047, 2980, 2945, 1502, 1487, 1469, 1228, 1183, 1061, 1016, 900, 747, 611, 579, 553.

3*R*(*S*),4*S*(*R*)-3-Carbamoyl-4-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (15) was isolated by chromatography on silica gel (eluent $CHCl_3$) as a by-product formed upon the treatment of dicarboxylic acid **13** containing the products of partial hydrolysis of *trans*-3,4-dicyano-2,2,5,5-tetramethylpyrrolidine-1-oxyl[40] with diazomethane in diethyl ether. M.p. 154—156 °C (from a hexane—ethyl acetate (1 : 1) mixture). Found (%): C, 54.40; H, 7.87; N, 11.63. $C_{11}H_{19}N_2O_4$. Calculated (%): C, 54.31; H, 7.87; N, 11.51. IR (KBr), ν/cm^{-1}: 3396, 3319, 3215, 2984, 2941, 1735, 1673, 1630, 1462, 1439, 1416, 1369, 1295, 1265, 1245, 1207, 1181, 1020, 998, 967, 784, 740, 637.

2,2-Bis(2-carboxyethyl)-5,5-diethyl-3,4-dimethylimidazolidine-1-oxyl disodium salt (16) was synthesized in three stages from 3-hydroxyamino-3-ethylpentan-2-one hydrochloride (**22**), which was prepared by an earlier described[41] procedure.

Stage 1. **2,2-Bis(2-methoxycarbonylethyl)-5,5-diethyl-4-methyl-2,5-dihydroimidazol-1-oxyl (23).** 4-Ketoheptadioic (4-oxoheptanedicarboxylic) acid (4 g, 23 mmol) was dissolved in 25% aqueous ammonia (30 mL), and the resulting solution was evaporated to dryness under reduced pressure. Compound **22** (2 g, 11 mmol), ammonium acetate (4.5 g, 58 mmol), and MeOH (20 mL) were added to the solid crystalline substance that formed. The suspension was purged with argon for 30 min to remove oxygen, argon was passed through the reaction vessel, which was sealed, and the suspension was magnetically stirred for 6 h at 40—50 °C. The reaction mixture was stored for 60 days at this temperature, 25% aqueous ammonia (20 mL) was added, and the solution formed was purged with air for 72 h. The resulting orange solution was evaporated under reduced pressure, $CHCl_3$ (20 mL) and MeOH (20 mL) were added, the colorless precipitate formed was filtered off, and the solution was evaporated under reduced pressure. A 10% solution of sodium carbonate (20 mL) was added to the residue, which was extracted with $CHCl_3$. Diethyl ether (20 mL) was added to the remained aqueous solution, and the solution was carefully acidified with 10% HCl to pH 2—3, which resulted in the orange coloration of the organic layer. The ethereal solution was separated, the aqueous solution was extracted with ether, the joined ethereal extracts were dried with Na_2SO_4, and an excess of an ethereal solution of diazomethane, prepared according to an earlier described procedure,[42] until nitrogen evolution stopped. After 12 h, the resulting solution was evaporated under reduced pressure, and the residue was chromatographed on a column with silica gel using an *n*-C_6H_{14}—$CHCl_3$ (2 : 1) mixture as eluent. The yield was 0.33 g (9%), m.p. 94—96 °C (*n*-C_6H_{14}—C_6H_6, 2 : 1). Found (%): C, 58.74; H, 8.07; N, 8.47. $C_{16}H_{27}N_2O_5$. Calculated (%): C, 58.70; H, 8.31; N, 8.56. IR (KBr), ν/cm^{-1}: 2958, 2926, 2850 (CH, OMe); 1732 (C=O); 1641 (C=N); 1440, 1376, 1319, 1281, 1198, 1179, 1151, 1084, 1055, 985, 883, 807.

Stage 2. **2,2-Bis(2-methoxycarbonylethyl)-5,5-diethyl-3,4-dimethylimidazolidine-1-oxyl (24).** Dimethyl sulfate (0.2 mL, 2.1 mmol) was added to a solution of 2,2-bis(2-methoxycarbonylethyl)-5,5-diethyl-4-methyl-2,5-dihydroimidazole-1-oxyl (**23**) (0.33 g, 1 mmol) in anhydrous diethyl ether (5 mL). Diethyl ether was distilled off under reduced pressure, and the residue was stirred for 1 h at 40—50 °C under reduced pressure and then triturated with anhydrous diethyl ether. The residue that formed was washed with diethyl ether and dissolved in EtOH, and $NaBH_4$ (40 mg, 1.06 mmol) was added by portions to the obtained solution with stirring for 30 min. The reaction course was monitored by TLC (Sorbfil, *n*-C_6H_{14}—$CHCl_3$, 2 : 1). After the reaction ceased, EtOH was distilled off under reduced pressure, and the residue was diluted with water (10 mL) and extracted with diethyl ether. The extract was dried with Na_2SO_4, and the solvent was distilled off under reduced pressure. The residue was chromatographed on a column packed with silica gel using an *n*-C_6H_{14}—$CHCl_3$ (2 : 1) mixture as eluent. The yield was 0.25 g (75%), an orange oil. Found (%): C, 59.38; H, 9.31; N, 7.90. $C_{17}H_{31}N_2O_5$. Calculated (%): C, 59.45; H, 9.10; N, 8.16. IR (in thin layer), ν/cm^{-1}: 2973, 2882, 2800 (CH, NCH_3); 1736 (C=O); 1437, 1375, 1308, 1256, 1168, 1127, 987, 923, 888, 841.

Stage 3. **2,2-Bis(2-carboxyethyl)-5,5-diethyl-3,4-dimethylimidazolidine-1-oxyl disodium salt (16).** ***A.*** A solution of NaOH (80 mg, 2 mmol) in water (1 mL) was added to a solution of 2,2-bis(2-methoxycarbonylethyl)-5,5-diethyl-3,4-dimethylimidazolidine-1-oxyl (**24**) (165 mg, 0.5 mmol) in EtOH (3 mL). The reaction mixture was left to stay for 14 h at ~20 °C, then $NaHCO_3$ (200 mg) was added, and the suspension was stirred for 4 h. The residue was filtered off, the solution was evaporated to dryness under reduced pressure, the residue was dissolved in isopropyl alcohol and filtered, and nitroxyl radical **16** was precipitated with diethyl ether. The yield was 110 mg (60%). Found (%): C, 44.49; H, 7.59; N, 6.77; Na, 11.38. $C_{15}H_{25}N_2Na_2O_5 \cdot 2.5H_2O$. Calculated (%): C, 44.55; H, 7.48; N, 6.93; Na, 11.37. IR (KBr), ν/cm^{-1}: 2965, 2925, 2855, 2800 (CH, NMe); 1558 (br, C=O); 1423, 1357, 1337, 1277, 1242, 1233, 1193, 1086, 932, 872, 872.

B. The aqueous-alcohol solution of radical **16** obtained after hydrolysis was mixed with $NaHCO_3$ to remove alkali excess, the hydrocarbonate precipitate was filtered off, the solution was evaporated under reduced pressure, and the fine-crystalline residue that formed was triturated with an isopropyl alcohol—diethyl ether (1 : 2) mixture. Yellow crystals were obtained. Found (%): C, 38.44; H, 5.87; N, 5.44; Na, 17.81. $C_{15}H_{25}N_2Na_2O_5 \cdot 2H_2O \cdot Na_2CO_3$. Calculated (%): C, 38.33; H, 5.83; N, 5.59; Na, 18.34. These samples were used in the present study.

Hydroxylamines 17—21 (general procedure). Compounds **17—21** were synthesized according to an earlier described procedure[43] by the hydrogenation of the corresponding nitroxyl radicals with hydrogen under atmospheric pressure on the Pd/C catalyst. A solution or a suspension of the nitroxyl radical (10 mmol) in MeOH (50 mL) was placed in a conic flask equipped with an attachment for gas supply with a valve at the outlet, and the wet catalyst (Pd/C, 1%, 200 mg) was added. The flask was purged with nitrogen and then with hydrogen and connected to a gas meter. After the end of gas absorption (usually the amount of the gas somewhat exceeded the calculated value), the hydrogenation was stopped. The hydroxylamines formed are active reducing agents and can easily be oxidized in air to the starting

nitroxyl radicals and, therefore, after hydrogenation the reaction mixtures were acidified with HCl to pH 1—3. The catalyst was filtered off, the solvent was distilled off under reduced pressure, and the residue was recrystallized. The salts obtained are stable upon prolong storage in air.

1-Hydroxy-2,2,5,5-tetramethylpyrrolidine-3-carboxylic acid hydrochloride (17). This compound was incorrectly described previously,[44] and for the characteristics of the zwitterion, see Ref. 45. The yield was 70%, colorless crystals, m.p. 190—192 °C (decomp.) (from concentrated HCl). Found (%): C, 48.17; H, 8.12; N, 6.26; Cl, 15.78. $C_9H_{18}NClO_3$. Calculated (%): C, 48.32; H, 8.11; N, 6.26; Cl, 15.85. IR (KBr), ν/cm^{-1}: 1724, 1500, 1466, 1423, 1406, 1391, 1371, 1295, 1201, 1173, 1142, 853, 763, 721. ^{1}H NMR (200 MHz, D_2O), δ: 1.46 (br.s, 9 H, 3 Me); 1.56 (s, 3 H, Me); 2.26 (br.m, 2 H, CH_2); 3.31 (br.m, 1 H, CH); (200 MHz, $D_2O + N_2D_4$), δ: 1.18 (s, 3 H, Me); 1.22 (s, 3 H, Me); 1.34 (s, 3 H, Me); 1.42 (s, 3 H, Me); 2.08 (m, 2 H, CH_2); 2.83 (m, 1 H, CH). ^{13}C NMR (100 MHz, $CD_3OD + N_2D_4$), δ: 20.32, 27.46, 28.41, 28.85 (Me); 40.81 (CH_2); 53.34 (CH); 62.74, 66.91 (C(2), C(5)); 181.01 (COOH).

1-Hydroxy-3-carbomoyl-2,2,5,5-tetramethylpyrrolidine hydrochloride (18). The free base[43] and hydrobromide[46] were described earlier. The yield was 70%, m.p. 216—220 °C (from isopropyl alcohol). Found (%): C, 48.43; H, 8.66; N, 12.37; Cl, 15.99. $C_9H_{19}N_2ClO_2$. Calculated (%) C, 48.54; H, 8.60; N, 12.58; Cl, 15.92. IR (KBr), ν/cm^{-1}: 3308 br, 3138 br, 3018 br, 2813 br, 1659, 1622, 1500, 1482, 1435, 1387, 1372, 1307, 1158, 1054, 797, 718. ^{1}H NMR (400 MHz, $CD_3OD—D_2O$ (10 : 1)), δ: 1.44 (br.s, 3 H, Me); 1.53 (s, 3 H, Me); 1.58 (s, 3 H, Me); 1.60 (s, 3 H, Me); 2.36 (br.m, 2 H, CH_2); 3.19 (br.m, 1 H, CH).

3-Aminomethyl-1-hydroxy-2,2,5,5-tetramethylpyrrolidine dihydrohloride (19). The yield was 70%, m.p. 198—203 °C (from isopropyl alcohol). Found (%): C, 43.86; H, 9.16; N, 11.11; Cl, 28.42. $C_9H_{22}N_2Cl_2O$. Calculated (%): C, 44.09; H, 9.04; N, 11.43; Cl, 28.92. IR (KBr) ν/cm^{-1}: 3424 br, 2902 br, 2580 br, 2043 br, 1606, 1517, 1464, 1395, 1176, 1118, 979, 752. ^{1}H NMR (300 MHz; $CD_3OD—D_2O$ (10 : 1)), δ: 1.32 (br.s, 3 H, Me); 1.51 (br.s, 3 H, Me); 1.58 (br.s, 6 H, 2 Me); 2.12 (br.m, 1 H, H(4)); 2.35 (br.m, 1 H, H(4)); 2.53 (br.m, 1 H, C(3)H); 3.00 (1 H, ABd system, CH_2N, J_{AB} = 12.8 Hz, J_d = 10.3 Hz); 3.26 (1 H, ABd system, CH_2N, J_{AB} = 12.8 Hz, J_d = 3.3 Hz).

1-Hydroxy-2,2,5,5-tetramethylpyrrolidine-3*R*(*S*),4*S*(*R*)-dicarboxylic acid hydrochloride (20). The yield 70%, m.p. 260 °C (decomp.) (from concentrated HCl). Found (%): C, 44.65; H, 6.84; N, 5.38; Cl, 13.12. $C_{10}H_{18}NClO_5$. Calculated (%): C, 44.87; H, 6.78; N, 5.23; Cl, 13.24. IR (KBr), ν/cm^{-1}: 3059 br, 2942 br, 1721, 1498, 1458, 1426, 1404, 1385, 1274, 1195, 1166, 1133, 1053, 857, 785, 723. ^{1}H NMR (400 MHz, $(CD_3)_2SO$), δ: 1.21 (s, 6 H, 2 Me); 1.46 (s, 6 H, 2 Me); 3.19 (s, 2 H, 2 CH). ^{13}C NMR (100 MHz, $(CD_3)_2SO$), δ: 20.30, 24.93 (Me); 51.35 (CH); 70.45 (CMe_2); 170.51 (COOH).

1-Hydroxy-2,2,6,6-tetramethyl-4-trimethylammoniopiperidine hydrosulfate hydrochloride (21). The yield was 15%, m.p. 220—228 °C (from isopropyl alcohol). Found (%): C, 41.06; H, 8.79; N, 8.11; Cl 9.70; S, 9.32. $C_{12}H_{29}N_2ClO_5S$. Calculated (%): C, 41.31; H, 8.38; N, 8.03; Cl, 10.16; S, 9.19. IR (KBr) ν/cm^{-1}: 1483, 1419, 1532, 1288, 1260, 1226, 1176, 1147, 1037, 958, 907, 874, 743. ^{1}H NMR (300 MHz, CD_3OD), δ: 1.56 (s, 6 H, 2 Me); 1.59 (s, 6 H, 2 Me); 2.25 (2 H, ABd system, J_{AB} = 14 Hz, J_d = 12 Hz); 2.64 (br.s, 2 H, AB system, 2 CH_2, J = 14 Hz); 3.94 (m, 1 H, CH).

4-(2-Aminophenyl)-4-oxocrotonic acid (2) was synthesized by an earlier described procedure.[28] An aqueous solution of kynurenine **1** (5 mmol L^{-1}, 20 mL) was alkalized to pH 8.3 by the addition of NaOH monitoring with an Orion Research pH meter (USA). Argon was bubbled through the resulting solution for 10 min, after which the solution in the sealed ampule was incubated in a thermostat for 24 h at 70 °C.

The products formed were separated by chromatography on a ZORBAX Eclipse XBD-C18 semipreparative column 9.4×250 mm using an acetonitrile—aqueous solution of trifluoroacetic acid (0.05 wt.%) gradient. The content of acetonitrile in a gradient solution was 0—30% (0—2 min), 30—55% (2—32 min), 55—100% (32—34 min), and 100% (34—40 min). The flow rate was 0.9 mL min^{-1}, and the detection was performed at five wavelengths: λ = 254, 290, 315, 360, and 410 nm. The needed fractions were collected as the products were eluted. The isolated solution of acid **2** was neutralized to pH 7.2 and stored at −18 °C.

Laser flash photolysis (LFP). Rate constants of quenching of the kynurenine triplet state were measured by laser flash photolysis. The setup was described in detail in previous works.[47,48] A solution was placed in a rectangular quartz cell and irradiated with a Quanta-Ray LAB-130-10 Nd:YAG laser (USA) at 355 nm (pulse energy up to 150 mJ, pulse duration 8 ns). The detecting system consisted of a DKsSh-150 arc xenon lamp operating in the pulse mode with a pulse duration of 2 ms, a monochromator, an Electron Tubes Ltd 9794B photomultiplier (Great Britain), a digital two-channel oscillograph with a LeCroy 9310A 11-bit analog-to-digital converter (Switzerland) (400 MHz, time resolution 10 ns), and a system of filters and shutters. Since the detecting light concentrated in a rectangular of 3×1 mm passes through the cell along the front edge of laser radiation, in all experiments the optical excitation and detection path lengths were 1 mm and 0.8 cm, respectively; optical absorbance values of the intermediates are given for l = 0.8 cm. Argon was bubbled through the solution for 10 min before measurements and further during the whole experiment to remove oxygen from the system. The energy of the incident laser radiation was determined by a Coherent FM power meter (USA).

Absorption spectra of solutions were recorded on a Hewlett Packard Agilent 8453 spectrophotometer (Germany).

Reactions of 2-aminophenyl-4-oxocrotonic acid (2) with nitroxyl radicals and hydroxylamines (general procedure). Solutions of carboxyketoalkene **2** (3—4 mmol L^{-1}) and compounds **3**—**21** (1—2 · 10^{-2} mol L^{-1}) were prepared in a 0.1 *M* phosphate buffer (pH 7.2). The prepared solutions were placed in 0.5-mL ampules and purged with argon for 10 min. The ampules were sealed and incubated at 37 °C. An individual ampule was used for each experimental point. All ampules were simultaneously placed in a thermostat and taken from it after different time intervals. The samples taken from the thermostat were stored at −18 °C for several days.

Concentrations of acid **2** in incubated solutions were measured by reverse-phase HPLC on an Agilent LC 1200 chromatograph equipped with a gradient pump and a multiwave optical detector. Substances were separated on a ZORBAX Eclipse XBD-C18 analytical column 4.6×150 mm using an acetonitrile—aqueous solution of trifluoroacetic acid (0.05 wt.%) gradient. The acetonitrile content in the gradient solution was 0% (0—5 min), 0—50% (5—25 min), 50—100% (25—30 min), 100—0% (30—30.01 min), and 0% (30.01—35 min). The flow rate was 0.2 mL min^{-1}, and detection was carried out simultaneously at five wavelengths: λ = 254, 290, 315, 360, and 410 nm.

The Agilent ChemStation program for Windows was used for recording chromatograms and integrating peak areas. The change in the concentration of acid **2** was determined by a change in the peak area at λ = 410 nm (absorption maximum in the spectrum of compound **2**).[28]

Results and Discussion

Quenching of triplet kynurenine. The photolysis of kynurenine (**1**) in an aqueous solution results in the formation of the triplet state[22] in a ~2% quantum yield

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

where ${}^{S}\mathbf{1}^{*}$ is the excited singlet state, ${}^{T}\mathbf{1}$ is the triplet state, and ISC stands for the intersystem crossing. Triplet kynurenine formed upon intense laser irradiation can absorb the second photon and undergo photoionization to form the radical cation and solvated electron.[49] In turn, the solvated electron can add to molecule **1** in the ground state[49,50] yielding an electron adduct. To minimize two-quantum photoionization effects, all photochemical experiments were carried out using minimum acceptable energies of laser radiation (~60 mJ pulse^{-1}). In addition, acetone (10 mmol L^{-1}) was added to the solutions, because its molecules are efficient traps for solvated electrons. Solvated electrons formed upon the photolysis of compound **1** disappear in the reaction with acetone[51] ($k_e = 6 \cdot 10^9$ L mol^{-1} s^{-1}) with the formation of the electron adduct $Me_2CO^{\cdot -}$, which is rapidly protonated in a neutral medium. The protonated radical of acetone is a weak chromophore and does not contribute to the observed transient spectra.

The transient absorption spectrum observed 280 ns after the laser flash photolysis of an aqueous solution of kynurenine **1** ($1.6 \cdot 10^{-4}$ mol L^{-1}, buffer solution, pH 7.2) is shown in Fig. 1. Similar spectra were obtained upon the irradiation of solutions of compound **1** in the presence of the nitroxyl radicals and their reduced forms. The negative absorption with a maximum at λ = 360 nm corresponds to the pulse decrease in the concentration of kynurenine in the ground state, and the positive absorption is attributed to triplet kynurenine, whose optical spectrum[22,49] has maxima at λ = 280, 340, and 430 nm.

The decay kinetics of triplet kynurenine ${}^{T}\mathbf{1}$ was detected in its absorption maximum at λ = 430 nm. The decay of the signal of the triplet kynurenine in the absence of additives and in the presence of nitroxyl radical **14** in different concentrations is shown in Fig. 2. In the presence of radical **14**, the signal decay is well described by the exponential dependence. Thus, the decay kinetics of the kynurenine triplet state ${}^{T}\mathbf{1}$ can be described by the equation

$$[{}^{T}\mathbf{1}] = [{}^{T}\mathbf{1}]_0 \cdot \exp\{-(k_0 + k_q[RR´NO^{\cdot}])\},$$

where k_0 is the rate constant for the decay of the kynurenine triplet state in the absence of quenchers, k_q is the quenching rate constant, $[{}^{T}\mathbf{1}]$ is the concentration of triplet kynurenine, and $[RR´NO^{\cdot}]$ is the radical concentration. The values of the observed rate constant $k_{obs} = k_0 + k_q[RR´NO^{\cdot}]$ obtained in the experiment depend linearly on the concentration of nitroxyl radicals $[RR´NO^{\cdot}]$ in the range from 10^{-3} to 10^{-2} mol L^{-1}. The

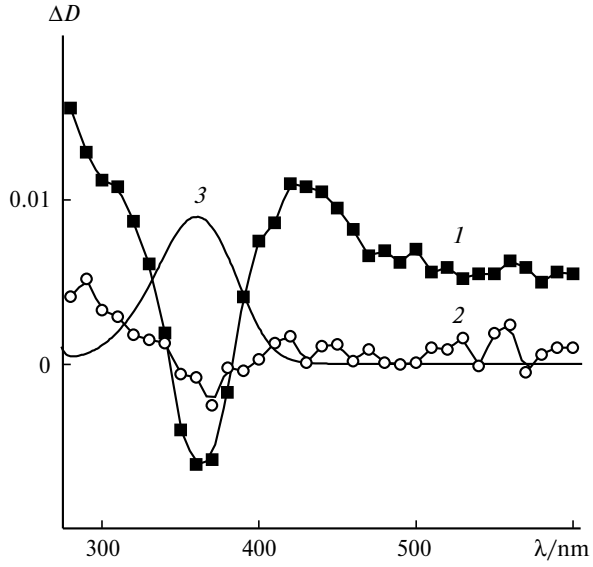


Fig. 1. Transient absorption spectra during the photolysis of an aqueous solution of compound **1** ($1.6 \cdot 10^{-4}$ mol L^{-1}) in the presence of radical **14** (1.3 mmol L^{-1}): *1*, 280 ns after the laser pulse; *2*, 8 μs after the laser pulse. Solid line *3* is the absorption spectrum of the initial compound **1**.

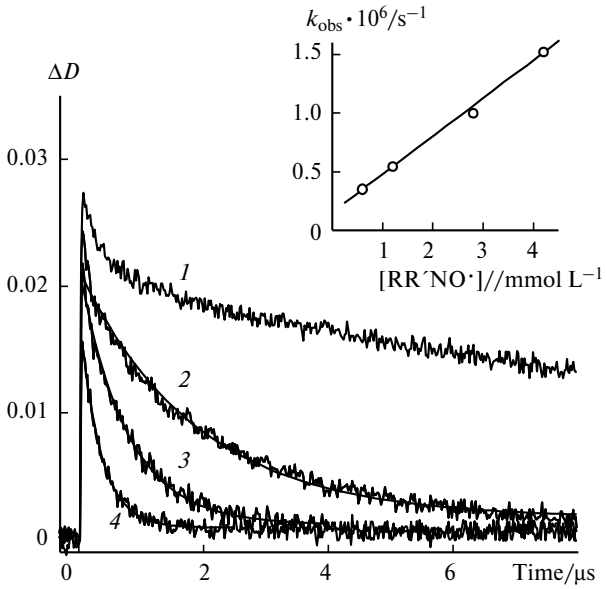


Fig. 2. Kinetic curves of the decay of the signal of the triplet state ${}^{T}\mathbf{1}$ in the presence of radical **14**. [**14**]/mmol L^{-1} = 0 (*1*), 0.53 (*2*), 1.6 (*3*), and 4.8 (*4*). Solid lines are calculated curves. Inset: dependences of the observed rate constant of ${}^{T}\mathbf{1}$ decay on the nitroxyl radical concentration.

values of the quenching rate constants k_q for different radicals determined from the slope of the dependence of the observed rate constant of triplet kynurenine decay on the concentration of the added radical are listed in Table 1.

The temperature dependence of the quenching rate constant of triplet kynurenine was measured for radical **3** (Fig. 3) and was used to determine the Arrhenius parameters for this constant: pre-exponential factor $\log A = 12.0\pm0.7$ and activation energy $E_a = 18\pm3$ kJ mol^{-1}. The obtained E_a value is very close to the activation energy for water viscosity (19 kJ mol^{-1}). This indicates that the quenching reaction is diffusionally controlled.

The transient absorption spectrum detected 8 μs after the laser irradiation of a solution of compound **1** ($1.6\cdot10^{-4}$ mol L^{-1}) in the presence of radical **14** (3.1 mmol L^{-1}) is also shown in Fig. 1. It is seen that the quenching of triplet kynurenine ^{T}**1** results in the restoration of the starting compound in the ground state, but no new radicals or diamagnetic reaction products are formed. An insignificant residual absorption observed at $\lambda < 300$ nm increases with the laser energy increase quadratically and, hence, can be assigned to the kynurenine radical cation formed due to two-quantum photoionization.[49]

The channels of triplet kynurenine decay in the absence of quenchers are mainly conversion to the ground state and triplet-triplet annihilation occurring with the rate constant[22,24] $k_{T\text{-}T} = 4.1\cdot10^9$ L mol^{-1} s^{-1}. Under physiological conditions (*i.e.*, inside the lens), amino acid residues of proteins, antioxidants, and oxygen act as natural quenchers of triplet kynurenine. We have earlier shown[24] that among amino acids the most efficient quenchers of triplet kynurenine are tryptophan ($k_q = (2.6\pm0.7)\cdot10^8$ L mol^{-1} s^{-1}) and tyrosine ($k_q = (6.7\pm1.5)\cdot10^7$ L mol^{-1} s^{-1}), whereas among antioxidants this is ascorbate ($k_q = (8.5\pm1.2)\cdot10^8$ L mol^{-1} s^{-1}).

Table 1. Quenching rate constants of triplet kynurenine by nitroxyl radicals

Radical	Concentration of radical, $c\cdot10^{-3}$/mol L^{-1}	$k_q\cdot10^8$ /L mol^{-1} s^{-1}
3	1.8—10.8	4.3±1.1
4	0.9—8.2	4.0±1.0
5	1.4—8.5	4.8±1.3
6	1.2—10.8	5.3±1.3
7	1.5—9.3	4.5±1.3
8	1.1—6.8	3.9±1.2
9	1.8—11.0	4.4±1.2
10	1.6—11.0	4.5±1.4
11	1.0—6.7	3.7±0.9
12	1.1—9.8	3.7±1.1
13	0.5—4.8	5.7±1.4
14	0.5—4.5	3.5±1.0
15	1.1—7.8	4.2±1.0
16	0.7—1.7	3.2±0.8

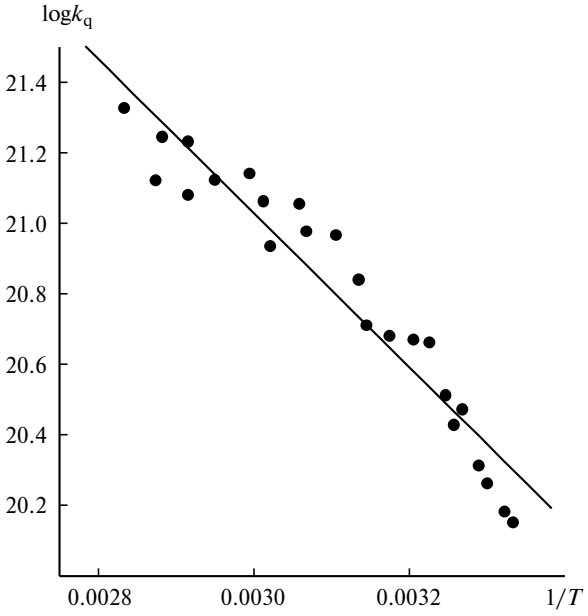


Fig. 3. Temperature dependence of the quenching rate constant of the triplet state ^{T}**1** by nitroxyl radical **3** (circles). Solid line is the calculation with the Arrhenius parameters $A = 9.2\cdot10^{11}$ L mol^{-1} s^{-1} and $E_a = 18.0$ kJ mol^{-1}.

Oxygen also quenches triplet kynurenine with a high rate ($k_q = (2.1\pm0.5)\cdot10^9$ L mol^{-1} s^{-1}). However, since the free oxygen concentration in the eye lens is very low,[52] this reaction does not play a noticeable role in the evolution of UV filters. Triplet kynurenine is quenched by amino acids and antioxidants *via* the electron transfer mechanism to form the kynurenine radical anion and the radical cation of amino acid or antioxidant.[24]

The question about the mechanism of quenching of singlet and triplet states of organic molecules (M*) by radicals was repeatedly discussed in literature. In the general case,[53] three mechanisms of quenching of photoexcited molecules by nitroxyl radicals RR´NO$^\cdot$ are possible: (1) energy transfer[54,55] with the formation of the excited state of a doublet RR´NO$^\cdot$*, (2) charge transfer,[56,57] and (3) electron spin exchange.[54—58] The first mechanism assumes that the nitroxyl radical has an absorption band with a lower energy than the energy of the excited state of molecule M*. The quenching rate constant should depend on both the energy of the excited state of molecule M* and the position of the absorption band of the nitroxyl radical, and the excited radical RR´NO$^\cdot$* should be the reaction product. In the case of charge transfer, the quenching reaction affords the radical cation (radical anion) of the initial molecule. Finally, the quenching *via* the mechanism of electron spin exchange does not result in the appearance of new products.

Figure 1 shows that no new paramagnetic or diamagnetic products are formed in solution upon the quenching of triplet kynurenine by nitroxyl radicals (the spectrum

observed 8 μs after the laser pulse). Note that the molar absorption coefficients of the kynurenine radicals are close to those of the triplet and the lifetimes of these radicals are tens of microseconds.[49] If the quenching proceeded *via* the charge transfer mechanism, noticeable absorption signals of these radicals would be observed. Therefore, we concluded that triplet kynurenine is quenched by nitroxyl radicals *via* the electron spin exchange mechanism

$$^{T}\mathbf{1}(\uparrow\uparrow) + RR'NO^{\cdot}(\downarrow) \longrightarrow \mathbf{1}(\uparrow\downarrow) + RR'NO^{\cdot}(\uparrow).$$

Thus, nitroxyl radicals can protect the lens tissues from reactions with photoexcited UV filters even better than natural antioxidant ascorbate: the quenching of triplet kynurenine by ascorbate produces free radicals that are dangerous for biological tissues. It was shown that the products of ascorbate photooxidation results in the glycosylation of lens proteins.[59] In the case of nitroxyl radicals, the quenching proceeds *via* the physical mechanism without the formation of chemical reaction products.

For all nitroxyl radicals studied in this work, the rate constants are within $3—6 \cdot 10^8$ L mol^{-1} s^{-1}. The lowest quenching rate constant is detected for radical **16** containing the bulky substituents in the region of the radical center. Probably, this indicates that the value of the quenching rate constant is primarily determined by the diffusion coefficient and steric factors, whereas the donor-acceptor properties exert no noticeable effect on the quenching rate. This additionally confirms that the quenching proceeds *via* the physical mechanism of electron spin exchange.

We also studied the ability of hydroxylamines **17**—**21** to quench the triplet state of kynurenine $^{T}\mathbf{1}$. It turned out that even at the highest concentrations of hydroxylamines (up to 10^{-2} mol L^{-1}) the decay kinetics of triplet kynurenine remains unchanged. This means that, unlike the nitroxyl radicals themselves, their reduced forms do not react with the triplet state of kynurenine.

Incubation with carboxyketoalkene 2

To elucidate the influence of nitroxyl radicals RR´NO$^{\cdot}$ and hydroxylamines RR´NOH on the reduction of the side chain of carboxyketoalkene **2**, we incubated aqueous solutions of acid **2** at 37 °C (pH 7.2) in the presence of nitroxyl radicals and their reduced forms and measured the change in the concentration of compound **2** during incubation by HPLC. The kinetics of thermal decomposition of acid **2** in the absence of additives and in the presence of hydroxylamine **17** ($1.5 \cdot 10^{-2}$ mol L^{-1}) is illustrated in Fig. 4. No formation of any additional products was observed in the chromatograms obtained after incubation with the addition of RR´NO$^{\cdot}$ or RR´NOH to the solution. The kinetic curves for decay of acid **2** shown in Fig. 4

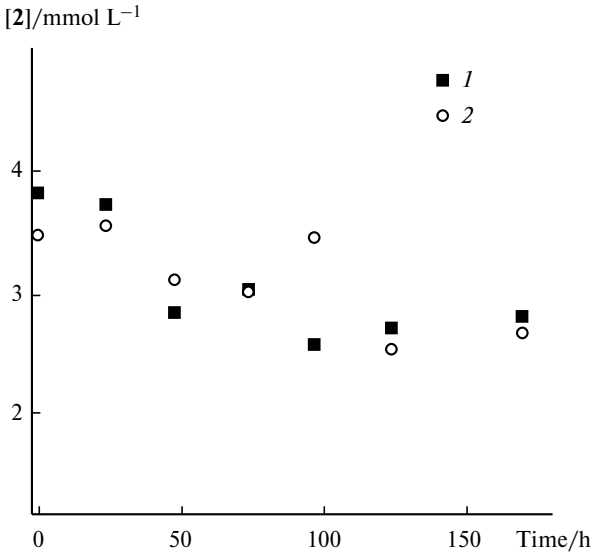


Fig. 4. Kinetic curves of the decay of carboxyketoalkene **2** during incubation at 37 °C: *1*, without additives; *2*, in the presence of hydroxylamine **17** ($1.5 \cdot 10^{-2}$ mol L^{-1}).

also indicate that the nitroxyl radicals and hydroxylamines exert no effect of the decay rate of molecules **2**.

The results on the incubation of carboxyketoalkene **2** in the presence of nitroxyl radicals and hydroxylamines show that the rate constants of the reactions of acid **2** with these compounds are low compared to those for the reactions of this acid with nucleophilic amino acids and antioxidants. The rate constants for the addition of carboxyketoalkene **2** to lysine and histidine at 37 °C (physiological temperature) lie in a range of $1—2 \cdot 10^{-4}$ L mol^{-1} s^{-1}, whereas for thiols (glutathione and cysteine) these constants are 2.1 and 36 L mol^{-1} s^{-1}, respectively.[28] According to our measurements, the rate constants of the reaction of acid **2** with the nitroxyl radicals and hydroxylamines do not exceed 10^{-4} L mol^{-1} s^{-1}. Thus, these compounds cannot serve as an efficient antioxidant protection of lens proteins from the addition of deaminated kynurenines.

Thus, we established that the nitroxyl radicals and their reduced forms are inert toward reactive molecules of carboxyalkenes **2** formed due to the deamination of natural UV filters. At the same time, the nitroxyl radicals are efficient quenchers of triplet states of kynurenine **1**, and the quenching proceeds *via* the electron spin exchange mechanism that affords no additional products. Due to this property and the well known ability of nitroxides to bind free radicals, there are wide prospects to use these compounds in the development of solar-protective drugs.

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