ISSN 0018-1439, High Energy Chemistry, 2011, Vol. 45, No. 6, pp. 505–509. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © N.A. Aristova, I.P. Ivanova, S.V. Trofimova, D.I. Knyazev, I.M. Piskarev, 2011, published in Khimiya Vysokikh Energii, 2011, Vol. 45, No. 6, pp. 543–547.

PHOTOCHEMISTRY

Influence of Luminol on the Chemiluminescence Intensity in Fenton's Reaction

N. A. Aristova[a], I. P. Ivanova[b], S. V. Trofimova[b], D. I. Knyazev[b], and I. M. Piskarev[c]

[a] *Nizhni Tagil Technological Institute (branch), Ural Federal University, ul. Krasnogvardeiskaya 59, Nizhnii Tagil, Sverdlovsk oblast, 620031 Russia*

[b] *Nizhni Novgorod State Academy of Medicine, Federal Agency for Health and Social Development, pl. Minina i Pozharskogo 10/1, Nizhni Novgorod, 603005 Russia*

[c] *Skobeltsyn Research Institute of Nuclear Physics, Moscow State University, Moscow, 119992 Russia*

e-mail: i.m.piskarev@gmail.com

Received March 9, 2011; in final form April 11, 2011

Abstract—The kinetics of Fe^{+2} oxidation and buildup of luminol oxidation products during Fenton's reaction at pH 2 have been calculated. The characteristics of the process in neutral (pH 6) and alkaline (pH 12) media have been evaluated. The calculation results have been compared with experimental data on the yield of chemiluminescence induced by Fenton's reagent and luminol. It has been shown that trivalent iron ions suppress the luminol emission. The presence of iron or another transition metal in the sample can significantly reduce the chemiluminescence quantum yield after luminol introduction if .

DOI: 10.1134/S0018143911060038

The assessment of the rate of free radical reactions in multicomponent organic substrates (cells, tissues) is of interest for biomedical studies. The intrinsic luminescence of biological samples is extremely low, this is a super weak emission. That is why different activators of the free radical processes are currently used, which enhance the luminescence quantum yield (coumarin, Fenton's reagent, luminol, etc.). The physical and chemical aspects related to the luminescence have been surveyed presented in [1–3]. Methods employing the Fenton reaction, in which $OH^{\bullet}$. radicals are largely generated, are widely used for the analysis of prooxidant and antioxidant activities in biological samples. However, the results of studies in which Fenton's reagent is used are interpreted ambiguously. According to some sources, Fenton's reagent is introduced for simulating the oxidative stress or assessing the oxidizability of a substrate [4, 5]. In other sources, the level of the free radical reactions and antioxidant activity in biological samples are judged by the intensity of chemiluminescence induced by Fenton's reagent [6, 7]. It should also be noted that there is no information in the literature on the time taken by Fenton's reaction to go to completion; thus, the observation time is chosen arbitrary (from 30 seconds to several hours).

It is known that the most intensive luminescence of luminol is recorded in an alkaline medium [2]. Biological samples have pH ~ 6.8–7.8. Under these conditions, the introduction of luminol enhances emission to an insignificant (if any) extent. Varying the pH is unacceptable for biological samples, since it leads to irreversible changes in the cell and distorts investigation results. To investigate the products and the reaction time and to select optimal reagent concentrations for chemiluminescence observation using Fenton's reagent and luminol are of particular importance for biological and medical studies.

Thus, the aim of this work was to study the chemiluminescence intensity in water with Fenton's reagent and luminol at different pH values and different hydrogen peroxide, iron sulfate, and luminol concentrations. The objective of the study was to derive a kinetic model of Fenton's reaction; to calculate the reaction characteristics in acidic, neutral, and alkaline media; and to compare the calculation results with experimental data.

EXPERIMENTAL

Light emission was recorded with a BKhL-06 instrument (Nizhni Novgorod). The instrument was calibrated using a standard light source of a known intensity. The sample volume was 1 ml in the experiments without luminol: 0.4 ml of water, 0.4 ml of $FeSO_4$, 0.2 ml of H_2O_2, or 1.1 ml with luminol (volume of luminol 0.1 ml). The cell with the sample was placed immediately adjacent to the photocathode of a photomultiplier tube, an arrangement that ensured a high detection efficiency. The measurements were performed in acidic (pH 2), neutral (pH 6), and alkaline (pH 12) media. Hydrogen peroxide solutions of the required concentration were prepared in advance, and $FeSO_4$ solutions were prepared immediately before use in an aqueous medium with pH 2, 6, or

Reaction rate constants in Fenton's solution

No.	Reaction	Rate constant, l/(mol s), [8]
1	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$	$k_1 = 56$
2	$OH^{\bullet} + H_2O_2 \rightarrow HO_2^{\bullet} + H_2O$	$k_2 = 3 \times 10^7$
3	$HO_2^{\bullet} + HO_2^{\bullet} \rightarrow H_2O_2 + O_2$	$k_3 = 8.3 \times 10^5$
4	$Fe^{2+} + OH^{\bullet} \rightarrow Fe^{3+} + OH^-$	$k_4 = 3 \times 10^8$
5	$OH^{\bullet} + OH^{\bullet} \rightarrow H_2O + 1/2O_2$	$k_5 = 5.5 \times 10^9$
6	$OH^{\bullet} + HO_2^{\bullet} \rightarrow H_2O + O_2$	$k_6 = 7.1 \times 10^9$
7	$HO_2^{\bullet} \rightarrow H^+ + O_2^{\bullet-}$	$k_7 = 7.5 \times 10^6$
8	$H^+ + O_2^{\bullet-} \rightarrow HO_2^{\bullet}$	$k_8 = 1.2 \times 10^2$ $pK_a = 4.8$
9	$HO_2^{\bullet} + O_2^{\bullet-} \rightarrow HO_2^- + O_2$	$k_9 = 9.7 \times 10^7$
10	$HO_2^{\bullet} + OH^- \rightarrow O_2^{\bullet-} + H_2O$	$k_{10} = 10^{10}$
11	$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2$	$k_{11} = 1.9 \times 10^9$
12	$H_2O_2 \rightarrow HO_2^- + H^+$	$k_{12} = 2 \times 10^{-2}$
13	$HO_2^- + H^+ \rightarrow H_2O_2$	$k_{13} = 10^{10}$; $pK_a = 11.5$
14	$OH^{\bullet} + HO_2^- \rightarrow HO_2^{\bullet} + OH^-$	$k_{14} = 7.5 \times 10^9$
15	$Lum + OH^{\bullet} \rightarrow LOOH^{\bullet}$	$k_{15} = 8.7 \times 10^9$ [9]
16	$LOOH^{\bullet} + O_2^{\bullet-} \rightarrow P^*$	$k_{16} = 1$
17	$O_2^{\bullet-} + OH^{\bullet} + H^+ \rightarrow H_2O + O_2(s)$	$k_{17} = 10^{10}$
18	$Fe^{3+} + 3OH^- \rightarrow Fe(OH)_{3\downarrow}$	$k_{18} = 1$ (pH 6)
19	$Fe^{3+} + 3OH^- \rightarrow Fe(OH)_{3\downarrow}$	$k_{19} = 10^6$ (pH 12)

12 depending upon the experimental conditions. The pH was monitored with a pH-150M pH meter (Gomel). Water was brought to pH 2 or 12 by adding H_2SO_4 or NaOH, respectively. The reagents used were of the analytical grade, and water was doubly distilled (pH 6). All samples were measured in 10–15 replicates.

The recording of chemiluminescence was started after 0.5–1 s following the addition of peroxide and $FeSO_4$. This time was required for the peroxide addition and transfer of the cell to the chemiluminescence detection mode. Immediately before and after each measurement, the background signal was recorded, which was subtracted automatically. The acidic medium was used because the initial divalent iron is stable in it and the forming trivalent iron does not precipitate. This gives a possibility to correctly perform calculations on the process. The purity of the solutions and the product composition at different steps were monitored by following UV spectra recorded with a Fluorat-02 Panorama instrument (St. Petersburg). In particular, the trivalent iron concentration was measured by the absorption peak at $\lambda = 304$ nm, a molar absorption coefficient of $\varepsilon = 2200$ mol l^{-1} cm^{-1}. The solution to the set of chemical kinetic equations was found using the MathCad software [11].

RESULTS AND DISCUSSION

Kinetic Model of the Process

A model of the process in the acid medium includes reactions (1)–(17) (table). The concentrations of OH^- and H^+ ions (pH of the solution) were preset in the form of coefficients. The model suggests the interaction of divalent iron with hydrogen peroxide and the subsequent formation of $OH^{\bullet}$, $HO_2^{\bullet}$, and $O_2^{\bullet-}$ radicals and singlet oxygen; the dissociation of the hydrogen peroxide $H_2O_2 \leftrightarrow HO_2^- + H^+$, $pK_a = 11.5$ (reactions (12), (13)); and the equilibrium $HO_2^{\bullet} \leftrightarrow H^+ + O_2^{\bullet-}$,

$pK_a = 4.8$ (reactions (7), (8)). The values of the reaction rate constants are borrowed from [8].

When Fenton's reaction proceeds in a neutral or alkaline medium, trivalent iron precipitates, following the reaction $Fe^{3+} + 3OH^- \rightarrow Fe(OH)_{3\downarrow}$ (reactions (18), (19)). The rate constants of these reactions were estimated based on observation of the precipitate formation time.

Fe^{+2} Oxidation Kinetics

The number of oxidation events $N(Fe^{2+} \rightarrow Fe^{3+})$ in acidic medium (pH 2) for the case of $[Fe^{2+}] > [H_2O_2]$ at $[H_2O_2] = 10^{-6}$ mol/l was calculated depending on the CL recording time. Both initially oxidized Fe^{2+} ions introduced with the reagent and the Fe^{2+} ions produced from Fe^{3+} ions in the reduction reaction (reaction (11)) were considered. The value of $N(Fe^{2+} \rightarrow Fe^{3+})$ reaches a limit of 2×10^{-6} mol/l, i.e., $2 \times [H_2O_2]$, for all Fe^{2+} concentrations beginning from 10^{-2} to 10^{-5} mol/l. The limit is determined by the fact that hydrogen peroxide has a normality of two. The oxidation process ceases when the hydrogen peroxide is fully consumed.

The divalent iron introduced into the sample is consumed rapidly at $[Fe^{2+}] < [H_2O_2]$ and pH 2. Trivalent iron will be reduced into the divalent species (reaction (11)), and the process will proceed until the total peroxide consumption; its rate is determined by the Fe^{3+} reduction rate. It was found that the number of oxidation events $N(Fe^{2+} \rightarrow Fe^{3+})$ significantly exceeds the amount of $[Fe^{2+}]$ ions initially introduced into the solution. This is caused by the hydrogen peroxide-maintained chain reactions; that is, by the "regeneration" of the divalent iron ions formed in reaction (11).

The calculated kinetics is supported by the spectrophotometric measurements of the absorption peak at $\lambda = 304$ nm related to the trivalent iron formation. The reaction time (time of a 100-fold decrease in $[Fe^{2+}]$) at $[Fe^{2+}] > [H_2O_2]$ was 6, 60, 600, or 6000 s for $[Fe^{2+}] = [Fe^{2+}] = 10^{-2}$, 10^{-3}, 10^{-4}, or 10^{-5} mol/l, respectively.

The principal agent reducing divalent to trivalent iron in an acidic medium is hydrogen peroxide and $OH^\bullet$ radicals (reactions (1) and (4)). The concentrations of reactive species formed in the Fenton's solution at $[Fe^{2+}] = 10^{-3}$ and $[H_2O_2] = 10^{-4}$ mol/l, pH 2 for the time of 60 s from the onset of the reaction are presented in Fig. 1. It is clear that the major reactive species are hydroxyl radicals; however, the concentration of superoxide radicals $O_2^{\bullet-}$ is significantly higher at the beginning of the reaction when Fe^{3+} ions have not been produced in a sufficient amount. Trivalent iron ions consume superoxide radicals, and the buildup of $O_2^{\bullet-}$ radicals is fast when Fe^{3+} is still absent.

In a neutral medium (pH 6), the interaction of divalent iron with hydroxide ions (reaction (18)) plays

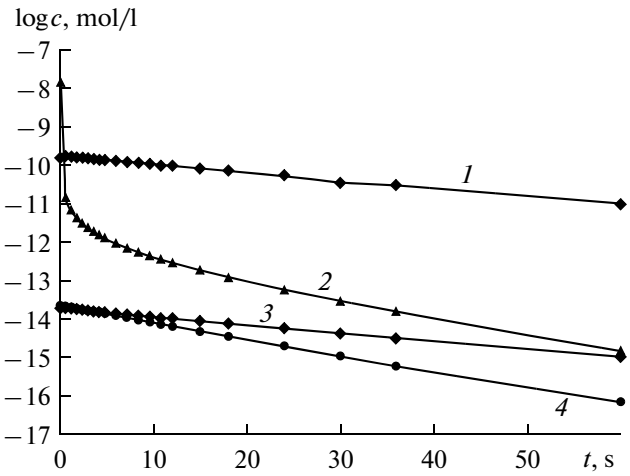

Fig. 1. Dependence of the logarithmic of concentration of reactive species log*c* (mol/l) at $[Fe^{2+}] = 10^{-3}$ mol/l, $[H_2O_2] = 10^{-4}$ mol/l, and pH 2 on time *t* (s): (*1*) $OH^\bullet$, (*2*) $O_2^{\bullet-}$; (*3*) HO_2^-; and (*4*) $HO_2^\bullet$.

the crucial role in the oxidation of divalent iron and the precipitation of trivalent iron. However, the influence of these processes is not significant during the recording of chemiluminescence over 600 s and the estimates made for the acidic medium are valid. In a strongly alkaline medium (pH 12), these processes accelerate (reaction (19)), but the proposed model can be used for the assessment of the kinetic features on the initial stage of the process (up to 30 s).

Emission with Luminol

Luminol was introduced to enhance the chemiluminescence quantum yield of the process. The mechanism of the formation of excited states that emit photons is considered in [2, 3, 9]. In the first stage of the process, luminol reacts with hydroxyl radicals (reaction (15)) yielding $LOOH^\bullet$ radicals. The subsequent transformation of $LOOH^\bullet$ radicals could lead to the formation of the photon-emitting products per se. The mechanisms of radiative reactions can differ; therefore, let us first consider the $LOOH^\bullet$ formation kinetics. If emission occurs instantaneously, the chemiluminescence intensity will be proportional to the $LOOH^\bullet$ formation rate and the chemiluminescence yield will be proportional to the amount of the $LOOH^\bullet$ radicals generated.

Let us consider the case of $[Fe^{2+}] > [H_2O_2]$, pH 2 and estimate iron and luminol concentrations that are convenient for the experimenter. The time dependence of the $LOOH^\bullet$ formation rate during 1000 s for $[Fe^{2+}] = 10^{-3}$, 10^{-4} , and 10^{-5} mol/l and [Lum] = 10^{-4} mol/l is presented in Fig. 2. Since the emission should be recorded during the entire reaction time, there is no problem to determine the chemiluminescence yield at $[Fe^{2+}] = 10^{-3}$ mol/l during 60 s. At

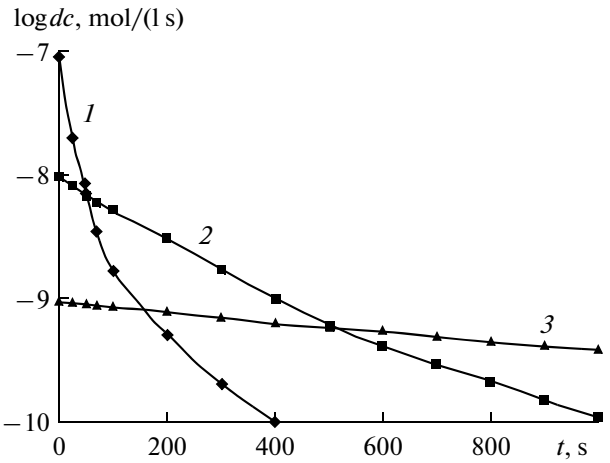

Fig. 2. The rate of luminol radical $LOOH^{\bullet}$ formation dc, mol l^{-1} s^{-1} at different concentrations of divalent iron $[Fe^{2+}]$ of (*1*) 10^{-3}, (*2*) 10^{-4}, and (*3*) 10^{-5} mol/l; t is the observation time, s. The concentration of H_2O_2 is 10^{-5} mol/l, and that of luminol is 10^{-4} mol/l, pH 2.

$[Fe^{2+}] = 10^{-4}$ mol/l, it is also possible to record the emission over the reaction time of 600 s; however, the emission intensity will become tenfold lower and the measurement time will increase by a factor of 10; that is, the signal/noise ratio for the obtained chemiluminescence yield will be much smaller. It is difficult to record the emission for 6000 s at $[Fe^{2+}] = 10^{-5}$ mol/l, since the signal to noise ratio will decrease even more.

One of the possible ways of the further transformation of $LOOH^{\bullet}$ is interaction with the superoxide radical $O_2^{\bullet-}$ (reaction (16)). The excited molecule P* is formed. The lifetime of the excited molecule P* is reportedly [9] much shorter than 1 s. Impurity molecules can increase this time, but there are no such molecules in the Fenton's solution. Therefore, in further consideration we assume that the emission of P* proceeds instantaneously and take the rate constant of reaction (16) to be 1.

The P* formation rate is defined by the equation $d[P^*]/dt = k_{16}[LOOH^{\bullet}][O_2^{\bullet-}]$.

The time dependence of the P* formation rate at $[Fe^{2+}] = 10^{-3}$ mol/l, $[H_2O_2] = 10^{-4}$ mol/l, and [Lum] = 10^{-4} mol/l at pH 2 during the first 12 s of the reaction is presented in Fig. 3 (curve *1*). The decline in the reaction rate with time is due to the decrease in $[O_2^{\bullet-}]$ concentration (Fig. 1). It can be seen in Fig. 3 (curve *1*) that the rate of P* formation decreases by more than 1000-fold during the first 0.7 s. This is due to the decrease in the superoxide radical concentration, which is at the maximum at the beginning of the reaction. The total concentration of the P* species produced during the first 0.7 s is 5.15×10^{-15} mol/l versus 5.39×10^{-15} mol/l during 12 s. If the emission proceeds instantaneously, the information on almost all of the emitted photons is lost when recording starts after 0.5–1 s. Moreover, varying recording start time results in scatter of the light yield, which significantly exceeds the statistical error.

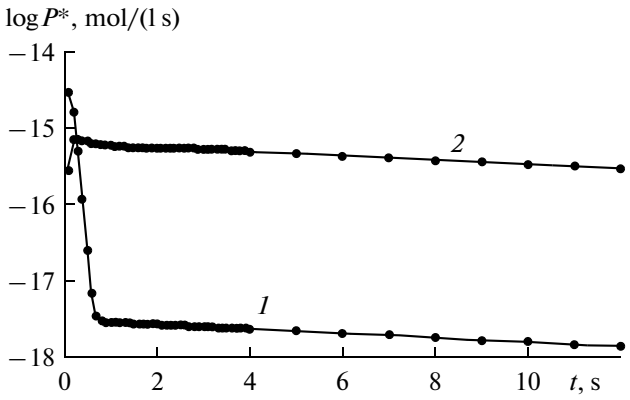

Fig. 3. Rate of P* formation, mol/(l s), see text. pH 2 (*1*) and pH 12 (*2*).

Qualitatively this situation in the case of pH 2 is confirmed by the experimental chemiluminescence yield presented in Fig. 4. The chemiluminescence intensity is at the maximum during the first seconds after peroxide addition and rapidly decreases. Evidently, the initial spike of chemiluminescence during the first second of the reaction remains undetected, since the recording starts 0.5–1.0 s after peroxide addition. Hence, the CL values that we managed to measure are all that left from the powerful burst during the first milliseconds. Luminescence was measured at pH 2 for the samples with (Fig. 4, curve *1*) and without luminol (curve *2*). The chemiluminescence yield over 30 s was 1.7 10^6 photons (2.3×10^{-15} mol/l) in the sample without luminol and 4.1 10^6 photons (5.6×10^{-15} mol/l) with luminol. That is, luminol increases the luminescence yield by a factor of ~2.5 in the acidic medium. The calculated $[LOOH^{\bullet}]$ concentration is $\sim 10^{-5}$ mol/l. Hence, it follows that the chemiluminescence quantum yield is $\sim 10^{-10}$. The effect of luminol in the neutral medium is similar.

Another situation is in the case of pH 12 (Fig. 3, curve *2*) when the trivalent iron formed in the reaction precipitates almost immediately (reaction (19)). In this case, the concentration of trivalent iron ions decreases to such an extent that the consumption of superoxide radicals (reaction (11)) becomes insignificant, and, correspondingly, the $[O_2^{\bullet-}]$ concentration does not significantly decrease and $[P^*]$ increases. The experimentally measured chemiluminescence quantum yield in the alkaline medium at pH 12, $[Fe^{2+}] = 10^{-3}$ mol/l, and $[H_2O_2] = 10^{-4}$ mol/l increases by a factor of $\sim 10^5$ in comparison with the yield at pH 2. Thus, trivalent iron ions suppress the luminol emission.

The ions of iron and other transition metals can occur in the biological sample itself. If the sample con-

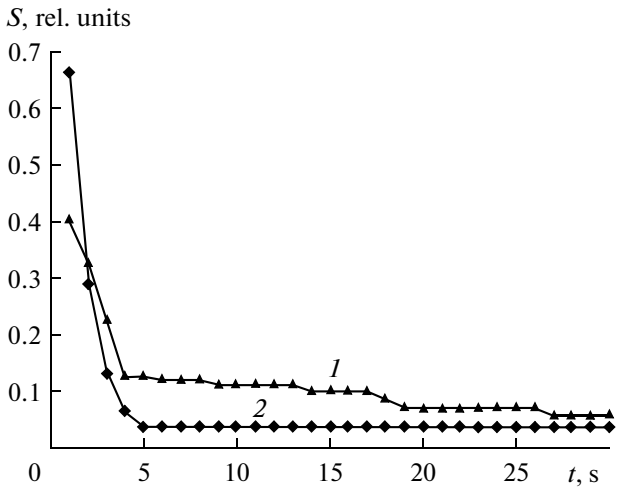

Fig. 4. Experimentally measured chemiluminescence intensity of the sample (S, rel. units) in the (*1*) presence and (*2*) absence of luminal; t is the time, s; $[Fe^{2+}] = 10^{-3}$, $[H_2O_2] = 10^{-4}$, and [Lum] = 10^{-4} mol/l.

tains peroxides, the Fenton reaction will proceed without the additional introduction of the reagent and it can be accompanied by detectable emission. However, along with the peroxide and transition metals, inhibitors of radical reactions can be present [10]. The Fenton reaction rate increases with an increase in $[Fe^{2+}]$, and the background emission becomes more intense. In this case, the introduction of luminol could enhance chemiluminescence; however, the concentration of Fe^{2+} increases with the increase in the Fe^{3+} concentration and the quantum yield of luminol chemiluminescence decreases in the presence of Fe^{3+}. Therefore, the quantum yield of luminol chemiluminescence in the presence of Fenton's reagent can distort the information on the level of free radical reactions in biological samples.

CONCLUSIONS

(1) The time of Fenton's reaction (100-fold decrease of the iron concentration) at $[Fe^{2+}] > [H_2O_2]$ is 6, 60, 600, and 6000 s for $[Fe^{2+}] = 10^{-2}$, 10^{-3}, 10^{-4}, and 10^{-5} mol/l, respectively, and does not depend on the peroxide concentration.

(2) The luminescence of the Fenton's solution in acidic and neutral media after luminol addition can exceed the background emission, but a part of information on the number of photons can be lost because of the short flash duration of ~5 s if recording starts after this period of time.

(3). Trivalent iron ions suppress the light emission from luminol; therefore, it is unreasonable to use it in the cases when iron is an initiator of free radical processes.

REFERENCES

1. *Fizika i tekhnika spektral'nogo analiza. Lyuminestsentnyi analiz* (Physics and Equipment of Spectral Analysis: Luminescent Analysis) Konstantinova-Shlezinger, M.A., Ed., Moscow: Izd. Fiziko-Matematicheskoi Literatury, 1961.
2. Vasil'ev, R.F., *Usp. Fiz. Nauk*, 1966, vol. 89, no. 3, p. 409.
3. Vladimirov, Yu.A. and Proskurina, E.V., *Usp. Biol. Khim.,* 2009, vol. 49, p. 341.
4. Kazakova, V.V. and Elkina, N.M, *Ukr. Biokhim. Zh.*, 2007, vol. 79, no. 4, p. 34.
5. Elkina, N.M., Kazakova, V.V., and Konoshenko, S.V., *Uch. Zap. Tavricheskogo Natl. Univ., Ser. Biol. Khim.,* 2009, vol. 22(61), no. 3, p. 35.
6. Ivanova, I.P., *Nizhegorodskii Med. Zh.*, 2006, no. 2, p. 183.
7. Kuz'mina, E.I., Nelyubin, A.S., and Shchennikov, M.K., in *Mezhvuzovskii sbornik biokhimii i biofiziki mikroorganizmov* (Interuniversity Collection of Articles on Biochemistry and Biophysics of Microorganisms), Gorky, 1983, p. 179.
8. Pikaev, A.K. and Kabakchi, S.A., *Reaktsionnaya sposobnost' pervichnykh produktov radioliza vody* (Reactivity of Primary Products of Water Radiolysis), Moscow: Energoizdat, 1982.
9. Vladimirov, Yu.A. and Potapenko, A.Ya., *Fiziko-khimicheskie osnovy fotobiologicheskikh protsessov* (Physicochemical Principles of Photobiological Processes), Moscow: Vysshaya Shkola, 1989.
10. Burlakova, E.B., *Ross. Khim. Zh.,* 2007, vol. 11, no. 1, p. 3.