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CELL BIOPHYSICS

Investigation of the Level of Free-Radical Processes in Substrates and Biological Samples Using Induced Chemiluminescence

I. M. Piskarev[a], S. V. Trofimova[b, c], O. E. Burkhina[c], and I. P. Ivanova[b, c]

[a]*Skobeltsyn Scientific Research Institute of Nuclear Physics, Moscow State University, Moscow, 119234 Russia*
[b]*Nizhny Novgorod State Medical Academy, Ministry of Health of the Russian Federation, pl. Minina i Pozharskogo 10/1, Nizhny Novgorod, 603005 Russia*
[c]*Lobachevsky State University of Nizhny Novgorod, pr. Gagarina 23, Nizhny Novgorod, 603950 Russia*
e-mail: i.m.piskarev@gmail.com

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Abstract—The possibility of using induced chemiluminescence for estimation of the substrate capacity for peroxidation under the action of hydroxyl radicals and for determination of the level of free-radical processes that occur in biological objects based on the analysis of organic hydroperoxides in a sample was investigated. For this purpose the light sum of chemiluminescence was measured during the three stages: upon introduction of Fe^{2+} into the sample, during the Fenton reaction (introduction of Fe^{2+} and H_2O_2), and upon introduction of Fe^{2+} after the Fenton reaction. The dependence of the light sum on concentration (dilution) of the sample was elucidated. It was shown that the light sum reached its maximum value at a certain dilution of the investigated substrate. The position of the maximum was determined by the concentration of oxidizing RH fragments, while the chemiluminescence light sum value in the maximum was determined by the fraction of the inhibitor, [InH]/[RH], and the fraction of organic hydroperoxides in the sample, [ROOH]/[RH].

Keywords: free-radical processes, organic hydroperoxide, induced chemiluminescence, Fenton reaction, antioxidant

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The reactions in which free radicals participate are among the most important processes that occur in organisms. The rate of such a reaction is maintained at a certain level. The increase of the rate of free-radical processes can be the cause for the intensification of oxidative processes in the cell. Hence, it is suggested that a balance must exist between the peroxidants that support chain oxidation and the antioxidants that prevent the development of the chain reaction. Numerous techniques for the estimation of the balance between peroxidants and antioxidants has been developed and used [1]. These methods are based on induction of oxidative reactions in the sample or in the preparation with radicals of a certain type and recording the products that could be formed in the reaction or monitoring physicochemical effects that accompany the chain reaction. The quantitative evaluations are performed based on comparison with the calibration dependence that is obtained for a compound that is used as a standard under given conditions. According to the opinion in the review [1] there is no general approach for the evaluation of peroxidant and antioxidant capacities. The selection of a method is performed for each particular case.

One of the methods used for investigation is induced chemiluminescence that occurs in the reactions of hydroperoxides with two-valent iron. Application of activated chemiluminescence for the investigation of free-radical processes was considered in general terms in [2] and in more detail in [3, 4]. The major regularities of the process were considered analytically, which unavoidably led to introduction of simplifying assumptions that limit the applicability of the results. The method that was presented in [4] is based on measurement of the induction period, viz., the delay of luminescence after the introduction of two-valent iron in a sample containing hydroperoxide. The induction period increases with an increase in the antioxidant capacity of the sample. Following the construction of a calibration curve that connects the induction period with the concentration of a known antioxidant (such as Trolox) that is introduced in the sample, it is possible to determine the antioxidant capacity of the sample equivalent to a certain concentration of the standard antioxidant in this sample based on the calibration curve. Luminescence was observed immediately after the introduction of iron in [2–4], but the characteristics of this luminescence were not analyzed.

Recording of the luminescence that occurs as a result of the Fenton reaction immediately after the introduction of all of the reagents is used in medical research [5, 6]. The method allows one to estimate

protective forces, antioxidant capacity, and its changes in the process of undergoing physiotherapy based on the sample (a blood sample from the patient). The possibility of expeditious control of the state of the investigated object during the process of the application of certain physicochemical actions is the main advantage of the method, as the time that is required for recording one sample is 30 s. The BKhL-6 and BKhL-7 instruments (Nizhni Novgorod) have been developed for recording the luminescence. The mechanism of the luminescence initiation, the concentration of the reagents, and the procedure for conducting the measurements were described in [7–9]. It was shown that the recording of the chemiluminescence of the investigated sample during the Fenton reaction allows one to determine the capacity of the substrate to oxidation with the formation of luminous products. Nonetheless, the obtained information is incomplete because the formation of luminous products is only one of the channels of the chain of free-radical oxidation process, which is a drawback of this method.

Accumulation of hydroperoxides could be the consequence of free-radical processes that occur in the object unless they are not consumed in further transformations within the object (organism). Concentration of hydroperoxides allows one to estimate the intensity of processes that occur in the object that result in the formation of peroxides. If there are no hydroperoxides this provides additional information on the investigated process. It seems reasonable to investigate the possibility of using the chemiluminescence induced by two-valent iron and the Fenton reaction for the determination of total peroxidant and antioxidant capacity of a biological sample or biopreparation. Useful information could be derived from the estimation of the intensity of free-radical processes in the object from which the sample was taken, based on the detection of organic hydroperoxides that accumulated in the sample up to the moment it was taken and the capacity of the substrate to oxidation. This study is devoted to this issue.

The objective of the study was an investigation of the kinetics of chemiluminescence induced by the introduction of two-valent iron and in the Fenton reaction in biopreparations that contain inhibitor and organic hydroperoxides. The chemiluminescence in organic preparations was recorded experimentally and the kinetics of the process were calculated using an accurate numerical solution of the system of differential equations that describe the accumulation and consumption of active particles. The numerical solution does not require any simplifying assumptions for the analysis of the process.

MATERIALS AND METHODS

Chemiluminescence in the Fenton reaction is due to emission from a singlet oxygen dimer that forms in the process of the reaction induced by hydroxyl radicals [7]. Aside from the singlet oxygen, hydroperoxides are formed in the Fenton reaction, which do not produce luminescence and cannot be recorded directly. Organic hydroperoxides can also be present in the initial sample. The organic hydroperoxides were determined in this work from the chemiluminescence that occurred after the introduction of a two-valent iron.

Chemiluminescence was recorded with a BKhL-7 biochemiluminometer (Nizhni Novgorod, Russia) according to the following scheme.

Prior to the determination of the organic hydroperoxide concentration the background emission of the sample, S_b, was evaluated before the introduction of the two-valent iron. The S_b emission occurs via the action of the radiation background in the apparatus [10]. It can change over a wide range (by several fold).

(1) A solution of two-valent iron was added to the investigated substrate (chemiluminescence in the reaction of iron with hydroperoxides that are present in the sample was evaluated as the light sum, S_1).

(2) A solution of two-valent iron with hydrogen peroxide was added to the investigated substrate (chemiluminescence from hydroxyl radicals that are generated in the Fenton reaction was evaluated; the yield of the luminous products was denoted as S_2).

(3) A solution of two-valent iron was added to the sample 290 s after the onset of the Fenton reaction (the chemiluminescence from the reaction of iron with the hydroperoxides that were in the sample initially and were accumulated during the Fenton reaction was evaluated and denoted as S_3). The two-valent iron that was introduced in sample 2 was completely consumed by this time [7]. As will be shown below, the mechanism of the luminescence generation remains the same at all three steps: the singlet oxygen dimer is the emitter, hence, the light sums S_b, S_1, and S_2 can be compared directly.

In all the cases the cuvette was transferred into an operating position by placing it in front of the photomultiplier immediately after the introduction of all reagents and shielded from light, which was followed by automatic recording of the luminescence. The time of recording was 30 s. The cuvette volume was 2 mL and the diameter was 1 cm. The following reagents were used: an $FeSO_4$ solution (10^{-3} mol/L) in an acidic medium at pH 2 and a hydrogen peroxide solution (10^{-3} mol/L). The acidic medium was prepared by the addition of sulfuric acid into distilled water; this was done due to the fact that the two-valent iron is unstable in a neutral medium. The selection of the concentration of the reagents and conditions for measurements was discussed in [7].

The sample volumes were:

(1) the background sample S_b (without the investigated substrate): 0.7 mL of the solvent (water or Hanks solution);

(2) sample 1: 0.1 mL of the investigated substrate, 0.6 mL of the solvent (water or Hanks solution), and 0.4 mL of Fe^{2+} solution added immediately prior to the measurement, i.e., recording of the S_1 luminescence;

(3) sample 2: 0.1 mL of the investigated substrate, 0.4 mL of the solvent, 0.4 mL of Fe^{2+}. Hydrogen peroxide (0.2 mL) was added the last followed by recording of the S_2 luminescence. The chemiluminescnce of a control (S_0, without the investigated substrate and containing only the Fenton reaction reagents, viz., 0.5 mL of the solvent, 0.4 mL of Fe^{2+} solution, and 0.2 mL of hydrogen peroxide) was measured in order to determine the background;

(4) Sample 3: 0.4 mL of Fe^{2+} solution was added to the same cuvette (sample 2) 90 s after the addition of hydrogen peroxide followed by the recording of S_3 luminescence. The background sample, S_b, was the same as in step 1.

The chemiluminescence levels of albumin, hemoglobin, a mixture of albumin with hemoglobin, glucose, oxalic acid, phenol, and blood induced by the Fenton reaction were investigated in the study. The chemiluminescence of a mixture of phenol with hemoglobin and albumin was also investigated. The concentrations of albumin (50 g/L), hemoglobin (70 g/L), and glucose (5 g/L) were selected to be close to the blood concentrations. The oxalic acid concentration was 50 g/L, while that of phenol was 50 g/L. The phenol (antioxidant) was introduced directly to the albumin and hemoglobin sample prior to the addition of 0.1 mL of hydrogen peroxide.

Bovine serum albumin, fraction V (M = 69 kDa), and pig blood hemoglobin (M = 66.8 kDa) from Bio West were used. The protein mass fraction in both preparations was no less than 95% and the lipid mass fraction was no more than 1%. Chemically pure compounds (glucose, oxalic acid, and phenol) were used as well as bi-distilled water at pH 6.5. Heparinized whole blood from a healthy male Wistar rat was used as a biological substrate. The blood was hemolyzed by freezing at −20°C.

It was suggested in [7, 8] to investigate the dependence of the light sum on the concentration (dilution) of the sample. It was established experimentally that the light sum from the initial sample with a concentration that is typical of biological objects began to increase, reached a maximum, and then decreased with sample dilution. The occurrence of the luminescence peak at a certain dilution in both the Fenton reaction and in the reaction that proceeded after the introduction of the two-valent iron is due to the peculiarities of the kinetics of the process. The presence of the maximum at a certain dilution is related to competition of different channels in the reaction of the chain propagation.

This is the reason that in this work the measurements were started with the light sum of the sample at the initial concentration followed by serial 10-fold dilution of the sample with water producing the light sums at concentration of 10^0, 10^{-1}, 10^{-2}, ...10^{-10} of the initial concentration. The preliminary experiments showed that the full width at the half height of the luminescence maximum was ± one dilution, i.e., if the luminescence maximum was reached at the dilution of 10^{-2}, the light sum decreased approximately 2-fold at dilutions 10^{-3} and 10^{-1}. Hence, the selection of dilution steps of 1/10 seems reasonable. The chemiluminescence of the serially diluted samples was also measured after the introduction of the two-valent iron. It must be noted that in the case of very high concentrations of hydrocarbons RH in the sample no luminescence was generated in both the Fenton reaction and when only two-valent iron was introduced [8]. It occurred only following the dilution of the sample.

In order to decrease the effect of instrument instability on the results, the ratio S/S_0 was considered in the Fenton reaction, with S being the light sum of the sample and S_0 being the light sum of the empty sample without the investigated compound. If $S/S_0 > 1$, it means that the chain reaction propagates in the sample under the action of hydroxyl radicals and the compound in the sample is a pro-oxidant. The S/S_0 ratio increases with the increase of the pro-oxidant activity. The S/S_0 ratio is below or equals one in the case of the antioxidant compound (a compound that does not support the chain reaction) [8].

The calculations and measurements that were conducted earlier [7, 8] showed that the dependence of the S/S_0 light sum in the Fenton reaction on the dilution (concentration) of the investigated oxidizing compound reached the maximum in the dilution range from 0 (10^0, initial compound) to −4 (10^{-4}). The light sum decreased for the subsequent dilutions (10^{-5} to 10^{-10}) up to the values of $S/S_0 \sim 1$ and remained on the same level. The luminescence that occurred upon introduction of only the two-valent iron, which was due to the presence of organic hydroperoxides reached its maximum for a given sample at the same dilution as in the Fenton reaction, and the subsequent dilutions caused its decrease up to the radiation background S_b. Hence, both the average and the total light sum for the dilutions from 10^0 to 10^{-4} can be considered in order to improve the statistical accuracy.

THE CALCULATION OF CHEMILUMINESCENCE KINETICS

Calculation of the kinetics of the investigated processes was conducted as follows [7]. A scheme of the reactions was constructed that described the process. All the compounds that participate in the reaction were included in the scheme. Since the rate constants of all the reactions are known, it was possible to calculate the kinetics of the process. For this purpose a system of differential equations was compiled based on the reaction scheme, where the concentrations of the

compounds that participate in the process were the variables. The rates of accumulation and consumption of the compound that is considered as a variable are included in each of the equations. The number of equations equals to the number of compounds that participate in the reaction. The initial conditions, i.e., the concentrations of all the compounds at the zero time point, are set. The concentrations of all the compounds that participate in the reaction after the given time intervals following the onset of the reaction comprise the solution. The duration of these intervals and the total time of the reaction are preset as conditions. In order to calculate the kinetics of chemiluminescence and evaluate the effect of the inhibitor and hydroperoxides that accumulated in the sample prior to the test on the light sum in the Fenton reaction a system of 14 differential equation was solved [7]. The kinetics of the luminescence generation immediately after the introduction of only the two-valent iron in the sample was calculated by solving a system of eight differential equations. The MathCad 14 software package was used for solving the systems of differential equations.

THE KINETIC PECULIARITIES OF PEROXIDATIVE OXIDATION INDUCED IN THE REACTIONS OF TWO-VALENT IRON WITH HYDROGEN PEROXIDE AND HYDROPEROXIDE

Hydroxyl radicals are formed in the reaction of two-valent iron with hydrogen peroxide (Fenton reaction). The classic scheme of oxidation of RH hydrocarbons by hydroxyl radicals is described as follows:

$$RH + OH^{\cdot} \rightarrow R^{\cdot} + H_2O, \quad (1)$$

$$R^{\cdot} + O_2 + M \rightarrow ROO^{\cdot} + M, \quad (2)$$

$$ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}, \quad (3)$$

$$ROO^{\cdot} + ROO^{\cdot} \rightarrow ROOR + {}^1O_2. \quad (4)$$

Here, $^1O_2$ is singlet oxygen and M is a third particle that does not participate directly in the reaction but is needed to meet the requirements of the energy and momentum conservation law. Reaction (2) is impossible without the third particle, M, and the propagation of the chain reaction is also impossible without oxygen. The hydroperoxides, ROOH, stable compounds, ROOR, and singlet oxygen are the products of reactions (1)–(4). The yields of ROOR and of singlet oxygen are the same, because these products are formed in the same reaction. The singlet oxygen is recorded; hence, below only the singlet oxygen formation in the reaction (4) will be considered. The total yield of the free-radical oxidation equals the sum of the yields of hydroperoxides and of singlet oxygen. The singlet oxygen forms a dimer with its luminescence in the red region of the spectrum that is recorded by a biochemiluminometer [7].

Let us consider the effect of the antioxidant-inhibitor. Inhibition of the chain oxidation comprises the use of the reagent that is denoted as InH, which reacts with high speed with radicals such as $OH^{\cdot}$ and $ROO^{\cdot}$. The low-active secondary radical $In^{\cdot}$ is formed as a result of the reaction of InH with the primary radical (inducer $OH^{\cdot}$) or the secondary radical $ROO^{\cdot}$.

$$OH^{\cdot} + InH \rightarrow H_2O + In^{\cdot}, \quad (5)$$

$$ROO^{\cdot} + InH \rightarrow ROOH + In^{\cdot}. \quad (6)$$

The inhibitor intercepts the primary radical, $OH^{\cdot}$ in the reaction (5), thus preventing initiation of the chain reaction, and the inhibitor intercepts the secondary radical, $ROO^{\cdot}$ that forms after the initiation of the reaction by the hydroxyl radical in reaction (6) and prevents the chain propagation. If the InH inhibitor intercepts the primary radical, thus preventing initiation of the chain reaction, it can be said that it has antiradical activity. If the inhibitor intercepts the secondary radical and prevents the propagation of the chain reaction, it can be said that it demonstrates antioxidant activity. An inhibitor can only terminate the chain reaction incompletely, thus slowing it down. The reasons for this could be both the low activity of the inhibitor (a low reaction rate constant) and its low concentration.

In order to evaluate the properties of the preparation, it is introduced into the sample of interest for this particular case. If the tested compound (preparation) slows down the chain reaction that is initiated by an external action, it is reasonable to say that the given compound acts as an antioxidant (inhibitor). If the introduced compound accelerates the chain reaction, this compound acts as a pro-oxidant. The relative antioxidant capacity of the preparation can be evaluated by comparison with the action of another preparation, whose activity is taken as a standard.

If the organic hydroperoxide is present in the sample, it reacts with the two-valent iron.

$$ROOH + Fe^{2+} \rightarrow RO^{\cdot} + OH^{-} + Fe^{3+}. \quad (7)$$

The $RO^{\cdot}$ radicals initiate the peroxidation chain reaction of the organic compound RH provided it is present in the sample:

$$RO^{\cdot} + RH \rightarrow R^{\cdot} + ROH. \quad (8)$$

The $RO^{\cdot}$ radicals cease to exist when they interact with each other and they do not generate luminescence:

$$RO^{\cdot} + RO^{\cdot} + M \rightarrow ROOH + M. \quad (9)$$

CALCULATION OF THE CHEMILUMINESCENCE KINETICS IN THE FENTON REACTION

The calculation of the kinetics of chemiluminescence generation in the Fenton reaction in the presence of the InH inhibitor and ROOH hydroperoxides

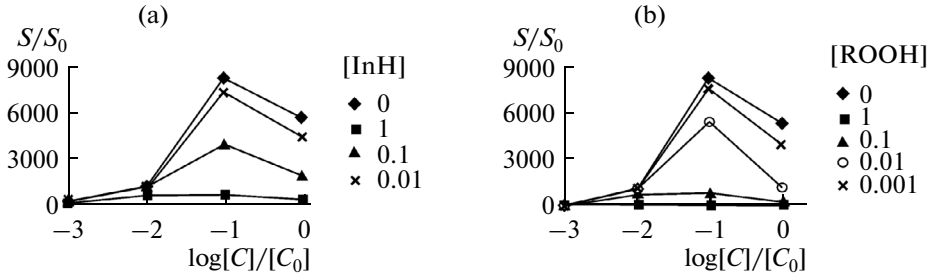

Fig. 1. The dependences of the light sums (S/S_0) in the Fenton reaction on the sample dilution ($\log[C]/[C_0]$) where [C] is the concentration of the diluted sample, [C_0] is the initial concentration of the sample) at a concentration of the oxidizing compound of [RH] = 1 mol/L for concentrations of inhibitor [InH] = 0, 0.01, 0.1, and 1 mol/L (a) and for concentrations of hydroperoxides [ROOH] = 0, 0.001, 0.01, 0.1, and 1 mol/L (b).

was conducted on the basis of the scheme from 21 reactions, in which reaction (5) was additionally included in the 20 reactions that were considered in [7]. A system of 14 differential equations was solved. The luminescence that accompanies the Fenton reaction is due to the decomposition of the singlet oxygen dimer. The singlet oxygen is formed in the reaction (4) [7].

The calculated dependencies of the light sum S/S_0 recorded in the Fenton reaction at different concentrations of the inhibitor InH and hydroperoxide ROOH are presented in Fig. 1. It was assumed during the calculations that the rate constant in the reaction (1) of the RH compound with hydroxyl radicals is 10^7 L mol^{-1} s^{-1}, and in the reaction (5) with an inhibitor it is 10^9 L mol^{-1} s^{-1}. The values for other constants are characteristic for the biological sample [8].

It can be seen from Fig. 1 that the position of the maximum for the chemiluminescence is independent on the concentrations of InH and ROOH. As was shown in calculation, the position of the maximum is determined by the RH concentration. However, the value of the light sum is determined by the ratio of concentrations [InH]/[RH] and [ROOH]/[RH], and does not depend on the [RH] value. The S/S_0 light sum in the Fenton reaction decreases with an increase of the concentration of both the inhibitor and the hydroperoxide.

CALCULATION OF THE KINETICS OF THE REACTION OF TWO-VALENT IRON WITH HYDROPEROXIDE

When two-valent iron is introduced into a sample that contains hydroperoxide, reactions (7) and (8) occur in which radicals are produced, thus initiating the chain reaction. The reactions (2)–(4) are the propagation of the chain reaction. Luminescence occurs following the formation of singlet oxygen in reaction (4). If the sample contains hydroperoxide ROOH and does not contain fragments of the organic compound, RH, the luminescence does not occur following the introduction of the two-valent iron because there will be no chain propagation (reactions (2)–(4)). In the case of large molecules such as protein molecules, any hydrocarbons that are capable of participating in the chain reaction are considered as RH. Their concentration is much higher than the concentration of the protein itself. If the concentration of the RH compound is too high, reaction (3) would dominate, which blocks the luminescence, and it would not occur. The role of reaction (3) decreases with the sample dilution, the yield of the reaction (4) becomes noticeable, and luminescence occurs. Hence, it is also necessary to measure the light sum at different dilutions in the process of the determination of the level of organic hydroperoxides, the same as in the Fenton reaction. Chemiluminescence recording at only one concentration of the sample may result in a significant error in the case where the positions of the maximum of the light sum for the two compared samples are at different dilutions.

The calculation of the luminescence generation kinetics following the addition of the two-valent iron in the sample was performed considering reactions (2)–(4) and (7)–(9). A system of eight differential equations was solved. The chemiluminescence of an arbitrary organic compound, RH, was calculated using the values of the constants that are characteristic for many hydrocarbons [7]. Two cases are considered: (1) [RH] = 10 mol/L at a concentration of hydroperoxides [ROOH] = 1, 10, and 100 mol/L, and (2) [RH] = 1 mol/L at [ROOH] = 0.1, 1, and 10 mol/L. The dependencies of the light sum, S, on dilution are presented in Fig. 2. It follows from the calculations that the value of the light sum is determined by the ratio of the concentrations [ROOH]/[RH]. The absolute value of the RH concentration determines the dilution at which the maximum of the light sum is observed. For the cases that are considered in Fig. 2 the same value of the light sum in the maximum (S = 1.6 arb. units) is observed for the concentrations [RH] = 1 and [RH] = 10 mol/L and if the [ROOH]/[RH] ratio is the same and equals 10. The position of the maximum is determined by the value of the RH concentration and it does not change with the

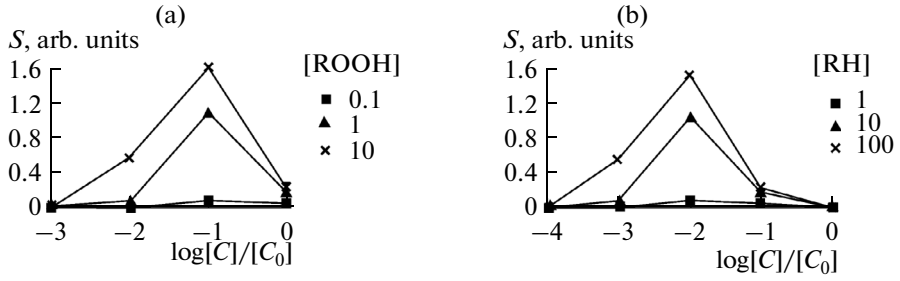

Fig. 2. The dependencies of the light sums S (arb. units) on the concentration ratio $\log[C]/[C_0]$ (sample dilution): (a, [RH] = 1 mol/L, [ROOH] = 0.1, 1, and 10 mol/L; (b), [RH] = 1, 10, and 100 mol/L.

change of the concentration of the ROOH hydroperoxides. In the case that is presented in Fig. 2 the maximum of the light sum for [RH] = 10 mol/L is reached at the dilution of 10^{-2}, and for [RH] = 1 mol/L, at the dilution of 10^{-1}.

The chemiluminescence light sum that is recorded during the introduction of the two-valent iron increases with an increase of the concentration of the [ROOH] hydroperoxides. It is proportional to the level of [ROOH] hydroperoxides in the sample. If the light sum maximum is not observed during the serial dilution, and the light sum only decreases with the decrease of the sample concentration (upon dilution), this indicates that the concentration of hydrocarbon fragments (RH groups) in the initial sample is low, lower than the value at which the maximum could be observed under the given conditions.

Let us emphasize for comparison that according to the calculations (see Fig. 1) the chemiluminescence light sum always decreases with the increase of the ROOH concentration during the Fenton reaction in the investigated sample with ROOH organic hydroperoxide, and the position of the maximum of luminescence also remains at the same spot. A similar pattern is observed during the introduction of the InH antioxidant into the investigated sample: the chemiluminescence light sum always decreases in the Fenton reaction with the increase of the InH concentration and the position of the maximum is determined by the RH concentration. If the concentration of RH groups remains the same, the position of the maximum does not change.

If the position of the chemiluminescence maximum shifts to the right (towards lower dilutions) in the process of any physicochemical action on the investigated substrate after the Fenton reaction, as well as after the introduction of the two-valent iron in the sample, this indicates that the concentration of RH fragments in the sample decreases following physicochemical action. If the position of the maximum shifts to the left toward larger dilutions, this means that the initial high-molecular-weight compound (such as a protein or lipid) decomposes under the action, releasing many RH fragments, which did not exhibit any chemical activity when they were in the composition of the protein or another complex compound.

We showed that the chemiluminescence light sum, S, in the region of the maximum luminescence in both the Fenton reaction and upon the introduction of the two-valent iron was independent on the RH concentration (the position of the luminescence maximum) and was determined by the ratio of the inhibitor concentration and the concentration of accumulated organic hydroperoxides: InH/RH, ROOH/RH. Hence, in order to increase the statistical accuracy during the analysis of the experimental results both the average S value at different dilutions in the region of the luminescence maximum (dilutions from 10^0 to 10^{-4}) and the sum of S values for all the dilutions in the region of the maximum can be considered.

EXPERIMENTAL RESULTS AND DISCUSSION: THE FENTON REACTION

If a chain reaction that results in the formation of luminous products is initiated and propagated during the Fenton reaction, then the light sum S that is recorded in the process is higher than the light sum of the empty sample S_0, $S/S_0 > 1$. The S/S_0 ratio characterizes the capacity of the substrate to oxidation with

Table 1. The average light sum S/S_0 upon dilutions from 10^0 (initial sample) to 10^{-4}

Substrate in the sample	Average light sum S/S_0
Phenol	0.78 ± 0.1
Hemoglobin	4.0 ± 0.5
Hemoglobin + phenol	1.0 ± 0.2
Albumin	12.4 ± 2.1
Albumin + phenol	1.02 ± 0.2
Oxalic acid	1.6 ± 0.4
Glucose	1.63 ± 0.4

Table 2. The light sum (mV, arb. units) that is recorded upon the introduction of two-valent iron in the sample (S_1 and S_3) and in the process of the Fenton reaction (S_2). S_1 is the chemiluminescence of the sample after introduction of Fe^{2+} (1st step); S_2 is the chemiluminescence of the sample after the introduction of $Fe^{2+} + H_2O_2$ (2nd step); S_3 is the chemiluminescence of the sample 90 s after the chemiluminescence induction in the 2nd step and after introduction of Fe^{2+} (3rd step); and S_b is the radiation background in the apparatus. In all cases $S = \sum S_i$, where S_i is the light sum upon dilutions from 10^0 to 10^{-4}

Substrate in the sample	Background S_b	S_1	S_2	S_3	$(S_1 + S_2 + S_3) - 2S_b$
Albumin	450 ± 100	440 ± 100	14780 ± 500	12870 ± 550	27190 ± 1000
Hemoglobin	430 ± 100	460 ± 100	6930 ± 400	7640 ± 400	13670 ± 800
Albumin + hemoglobin	510 ± 100	700 ± 100	8850 ± 400	9170 ± 420	17720 ± 850
Blood hemolyzate	550 ± 100	1360 ± 150	7650 ± 400	8600 ± 390	16310 ± 900

the formation of these luminous products. The experimental results for the series of investigated compounds, viz., the average values of the S/S_0 ratios at dilutions from 10^0 to 10^{-4}, are presented in Table 1.

The measurements showed that the chemiluminescence maximum for proteins was observed at dilutions of 10^{-1} (albumin) and 10^{-2} (hemoglobin). The calculation showed that the maximum at these dilutions can be at the concentrations RH = 1 and 10 M, respectively. The existence of chemiluminescence maxima at these dilutions confirms the suggestion that many protein fragments, RH, participate in the process of peroxidative oxidation, because the molar concentration of the proteins themselves is low, below 10^{-3} M. The possible presence of a lipid admixture in these preparations (lower than 1%) also cannot explain the occurrence of a chemiluminescence maxima at the 10^{-1} and 10^{-2} dilutions.

It can be seen from Table 1 that the light sum for the antioxidant (phenol) does not exceed the light sum of the empty sample, $S/S_0 < 1$. When the antioxidant (phenol) is added to the solutions of hemoglobin and albumin, the chemiluminescence of these compounds in the Fenton reaction ceases to occur ($S/S_0 \sim 1$) even though $S/S_0 > 1$ in the initial solutions. Oxalic acid, which does not contain an RH fragment does not support the chain oxidation with formation of luminous products, there is no maximum of the chemiluminescence for oxalic acid. However, a slight increase of the light sum, S, in comparison with the empty sample is observed. This could be related to formation of the products affecting chemiluminescence of the Fenton reaction itself. In particular, hydrogen peroxide is formed in the process of oxalic acid oxidation by hydroxyl radicals [11] and an increase in its concentration results in an increase in chemiluminescence.

Direct oxidation by oxygen with the release of energy is characteristic for carbohydrates, including glucose. Glucose is easily oxidized to carbonic acid without the formation of hydroperoxide. Hence, no peculiarities that are characteristic for the peroxidation of hydrocarbons (lipids) are observed. The increase of the light sum, S, over the background value, S_0, is most likely related to the peculiarities of the reaction mechanism. The average value of the light sum at large dilutions (from 10^{-5} to 10^{-10}) for all the investigated compounds ($S/S_0 = 1.09 \pm 0.2$) is close to one within the limits of experimental error.

EXPERIMENTAL EVALUATION OF THE SUBSTRATE OXIDATION CAPACITY

In order to determine the substrate oxidation capacity and the level of free-radical processes that occurred in the object before the sample was taken, i.e., the availability of organic hydroperoxides in it, the following parameters were determined: (1) the chemiluminescence light sum (S_1) after introduction of iron ions; (2) the light sum in the Fenton reaction (S_2); and (3) the chemiluminescence light sum after the Fenton reaction and additional introduction of iron ions, viz., the evaluation of the initial hydroperoxides and those that accumulated during the Fenton reaction (S_3); (4) the light sum due to the radiation background in the facility S_b. We have shown earlier that the emission mechanism is the same in all cases; the luminescence due to the singlet oxygen dimer is recorded, a single oxygen is formed in the reaction (4), hence, the light sums S_1, S_2, S_3, and S_b can be compared directly. The total light sum values for all the dilutions from 10^0 to 10^{-4} for albumin, hemoglobin, their mixture, and hemolyzed blood are presented in Table 2. The background light sum in the Fenton reaction (S_0) is significantly lower than the S_2 light sum, it is just within the limits of experimental error, hence, the S_0 is not taken into consideration. The light sums are presented in arbitrary units (millivolts). The millivolt units were selected due to the fact that the signal from the biochemiluminometer is measured in millivolts. The total light sums that were obtained for all three steps with

account taken for the radiation background of the apparatus, $(S_1 + S_2 + S_3) - 2S_b$, are also presented there. It can be seen from Table 2 that there are no organic hydroperoxides in the initial compounds (albumin, hemoglobin, or their mixture); the S_1 value does not exceed S_b. The hemolyzed blood of the male rat contains organic hydroperoxides ($S_1 > S_b$). Organic hydroperoxides occur in albumin, hemoglobin, and their mixture following the Fenton reaction ($S_3 \gg S_b$). The organic hydroperoxide content in hemolyzed blood increases after the Fenton reaction ($S_3 > S_1$). The ratio of the light sums in steps 2 and 3 (S_2/S_3) characterizes the ratio of the probabilities of the channels in the Fenton reaction, in which singlet oxygen (S_2) and organic hydroperoxide (S_3) are formed. It can be seen from Table 2 that the S_2/S_3 ratio changes for different compounds, hence it is incorrect to characterize the oxidation capacity of the sample only by the yield of luminous products in the Fenton reaction. The light sum S_3 characterizes the oxidation capacity of the substrate under the action of hydroxyl radicals with the formation of hydroperoxides. The $S_2 + S_3$ sum characterizes the total oxidation capacity of the substrate both with the formation of luminous products and with the formation of hydroperoxides.

EXPERIMENTAL EVALUATION OF THE INTENSITY OF FREE-RADICAL PROCESSES IN AN OBJECT

Oxidation-reduction reactions occur constantly in living organisms, including the peroxidation of lipids and RH hydrocarbon fragments that are the components of more complex molecules, such as proteins. The peroxidation capacity of the substrate that was considered above characterizes the protective reaction of an object, which is determined based on the sample. Hydroperoxides that can accumulate in these reactions present evidence of free-radical reactions that occur in an object (organism). Hence, it can be expected that the normal and pathological states of an organism can be distinguished by determining and comparing the concentrations of hydroperoxides in both states. As was shown above, the light sums S_1 and S_3 are determined by the concentration of hydroperoxides in the sample. Let us consider how one could estimate the intensity of free-radical processes in an object from which a sample was taken based on the light sums.

The light sum of the initial sample, S_1, characterizes the intensity of free-radical processes in an organism (object) that occurred prior to the moment the sample was taken and resulted in the accumulation of hydroperoxides. The comparison of the light sums S_1 and S_3 for a particular sample allows one to determine the number of free-radical processes that occurred in the object. The essence of the approach is as follows. The number of hydroxyl radicals, N_f, that are produced and consumed in the Fenton reaction is known: $N_f = 10^{-3}$ mol/L (the concentration of the introduced two-valent iron ions). These radicals are consumed almost completely in the reaction with the RH sample because the concentration of the sample is much higher than the concentrations of any of the intermediate products of the Fenton reaction [7]. This number of hydroxyl radicals leads to a light-sum increase:

$$N_f \rightarrow (S_3 - S_1). \quad (10)$$

The light sum $S_3 - S_1$ was formed under the action of N_f hydroxyl radicals in the Fenton reaction on the sample. Various reactions occurred in the object, which were initiated by different radicals; however, their result during peroxidation was the formation of the ROOH hydroperoxide. Let us denote the amount (concentration) of radicals in the sample with an activity that is equivalent to the action of the hydroxyl radical by N_{OH}. These radicals produced organic hydroperoxide in the object, which were taken for analysis in the sample. The generated chemiluminesence produced a light sum of $S_1 - S_b$. The light sum $S_1 - S_b$ was generated via the action of N_{OH} radicals in the object:

$$N_{OH} \rightarrow S_1 - S_b. \quad (11)$$

By setting up the proportion we obtain:

$$N_{OH} = N_f \frac{S_1 - S_b}{S_3 - S_1}. \quad (12)$$

Let us analyze this situation for hemolyzed blood using the data that are presented in Table 2. By substituting the numerical values from Table 2 into equation (12) we obtain:

$$N_{OH} = N \frac{1360 - 550}{8600 - 1360} = 10^{-3} \cdot \frac{810}{7240} = 1.1 \cdot 10^{-4} \text{ mol/L}. \quad (13)$$

Hence, it can be stated that a number of acts of peroxidation that resulted in the accumulation of hydroperoxides occurred in the hemolyzed blood prior to the analysis, which was equivalent to the action of hydroxyl radicals at a total concentration of $N_{OH} = 1.1 \cdot 10^{-4}$ mol/L. It can be seen that the analysis of the samples using the Fenton reaction and its modification (reaction with only two-valent iron) allows one to determine the activities of free-radical processes in the object from the content of hydroperoxides in the sample that was taken from this object. The total peroxidation capacity of the substrate is also determined in the process as $S_2 - S_0 + S_3 - S_b$. In order to evaluate the level of the oxidation capacity of the sample it can be compared with the oxidation capacity of the standard, which would be worthwhile for this particular task.

REFERENCES

1. V. V. Khasanov, G. L. Ryzhova, and E. V. Mal'tseva, Khim. Rast. Syr'ya **3**, 63 (2004).
2. Yu. A. Vladimirov and E. V. Proskurina, Usp. Biol. Khim. **49**, 341 (2009).
3. Yu. A. Vladimirov, E. V. Proskurina, and D. Yu. Izmailov, Bull. Eksp. Biol. Med. **144** (Suppl. 2), 390 (2007).
4. Yu. A. Vladimirov, E. V. Proskurnina, and D. Yu. Izmajlov, Biophysics (Moscow) **56** (6), 1055 (2011).
5. O. V. Zanozina, N. N. Brovkova, and T. G. Shcherbatyuk, Sovrem. Tekhnol. Med. 3, 104 (2010).
6. A. V. Alyasova, K. N. Kontorshchikova, I. G. Terent'ev, et al., Sovrem. Tekhnol. Med. 4, 27 (2010).
7. I. P. Ivanova, S. V. Trofimova, I. M. Piskarev, et al., J. Biophys. Chem. **3** (1), 88 ().
8. I. P. Ivanova, S. V. Trofimova, and I. M. Piskarev, Sovrem. Tekhnol. Med. 4, 14 (2014).
9. I. P. Ivanova, S. V. Trofimova, and I. M. Piskarev, Biophysics (Moscow) **58** (4), 453 (2013).
10. S. V. Ermolin, I. P. Ivanova, D. I. Knyazev, S. V. Trofimova, and I. M. Piskarev, Russ. J. Phys. Chem. A **86** (6), 1029 (2012).
11. N. A. Aristova, T. S. Mokina, and I. M. Piskarev, Russ. J. Gen. Chem. **72** (5), 715 (2003).

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