Assessment of Oxidative and Antioxidant Capacity of Biological Substrates by Chemiluminescence Induced by Fenton Reaction

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The aim of the investigation was to analyze the association between the ability of the substrate to oxidation and antioxidant activity according to the results of calculating the kinetics of chemiluminescence induced by Fenton reaction.

Materials and Methods. The chemiluminescence induced by Fenton reaction was measured with the help of BKhL-07 biochemiluminometer (Russia) with 10–15 repetitions. The registration time was 30 s. The following reagents were used for Fenton reaction: solution of $FeSO_4$ (10^{-3} mol/L in the acid medium, pH=2), solution of hydrogen peroxide (10^{-3} and 10^{-1} mol/L).

The chemiluminescence of albumin, hemoglobin, mixture of albumin and hemoglobin, well-known antioxidants: phenol (C_6H_5OH), resorcinol ($C_6H_4(OH)_2$), pyrogallol ($C_6H_3(OH)_3$), and mixtures of antioxidants with hemoglobin and albumin induced by Fenton reaction was studied.

To calculate the kinetics of chemiluminescence processes, a scheme of reactions describing the process was worked out. On the basis of this scheme a system of differential equations was set up, where concentrations of the substances, participating in the process, were variables. The number of equations was equal to the quantity of the substances, participating in the reaction.

Results. The light sum of the chemiluminescence induced by Fenton reaction monotonically decreases at concentrations of $[H_2O_2]=10^{-1}$ mol/L with the reduction of substrate concentration (with dilution). Dependence of S/S0 on the concentration of the fragments [RH] being oxidized, inhibitor [InH] and oxidized substance [ROOH] was calculated. There is no linear dependence between the light sum S/S0 and concentrations of [RH], [ROOH] and [InH] at $[Fe^{2+}]<[H_2O_2]$, therefore the results obtained were of a qualitative character.

Conclusion. The chemiluminescence induced by Fenton reaction enables the investigators to observe the products of reaction rather than radicals. Fenton reaction runs in actually any substrate. The level of the chemiluminescence light sum, initiated by hydroxyl radicals — Fenton reaction — is determined by the rate constants of the initiating, continuing and chain breaking reaction. Increase or decrease of the chemiluminescence light sum speaks of various values of these constants, but does not show an antioxidant activity, i.e. inhibition of the chain reaction due to the formation of inactive products. Therefore, the ability of the substrate to be oxidized under the given conditions can be evaluated according to the level of the chemiluminescence induced by hydroxyl radicals generated in Fenton reaction.

Key words: Fenton reaction; chemiluminescence kinetics; ability of a substrate for oxidation; antioxidant activity.

Fenton reaction is often used in biomedical studies [1–3]. The main advantage of this method is the rapidity to control the changes in oxidizing capacity of biological substrates in pathological processes and physical and chemical effects. In 2010 a device — BKhL-07 — designed to measure chemiluminescence induced by Fenton reaction was developed and has been currently mass-produced in Russia. General properties of chemiluminescence kinetics have been previously considered by us [4, 5]. It has been estimated, that in Fenton reaction, initiated by hydrogen peroxide, a light sum S is proportional to the ability of a substrate to be oxidized by hydroxyl radicals.

Studying chemiluminescence induced by Fenton reaction two modes of action can be distinguished, when $[Fe^{2+}]\geqslant[H_2O_2]$ and $[Fe^{2+}]<[H_2O_2]$. In the first case ferrous iron is oxidized to ferric, ferrous iron is spent and the reaction time is determined by the initial concentration of iron. In the second case the oxidized ferric iron is regenerated into ferrous one. For this process hydrogen peroxide is consumed. The reaction time appears to be essentially longer than in the first case, and it is determined by the concentration of H_2O_2 [6, 7].

Case $[Fe^{2+}]\geqslant[H_2O_2]$ has been considered in work [4]. It was suggested to measure chemiluminescence successively diluting samples 10 times beginning with the

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initial concentration. Dependence of chemiluminescence light sum on the concentration (dilution) of the initial sample and the content of hydroperoxides and antioxidants in the sample at the concentration of reagents $[Fe^{2+}]=[H_2O_2]=10^{-3}$ mol/L has also been examined. The light sum reaches the maximum at a definite dilution. It has been shown, that the ability of the substrate to be oxidized is characterized by the light sum and substrate concentration, when the luminescence yield is maximal. The drawback of this mode is the necessity to measure chemiluminescence between serial sample dilutions, requiring much time, and therefore the method cannot be called an express test.

To study the characteristics of chemiluminescence light sum induced by Fenton reaction for $[Fe^{2+}]<[H_2O_2]$ case and to compare them with the characteristics at $[Fe^{2+}]\geqslant[H_2O_2]$ is of great interest.

The aim of the investigation was to analyze the association between the ability of the substrate to oxidation and antioxidant activity according to the results of calculating the kinetics of chemiluminescence induced by Fenton reaction.

Materials and Methods. Chemiluminescence induced by Fenton reaction was measured with the help of BKhL-07 biochemiluminometer (Russia) with 10–15 repetitions. The sensitivity of the device was ~200 photons/s. Directly before the work the device was calibrated using a reference light source [5]. The time of light sum S measurement was 30 s. A background (a noise current of photoelectronic multiplier) was calculated automatically. The device registered the signal amplitude (radiation intensity I) and calculated chemiluminescence light sum $S=\sum_j I_j$ over the whole period of measurement. The value of chemiluminescence, directly measured by the device, was a potential, therefore S and I were measured in millivolts (relative units).

The sample was composed of 0.1 ml of the examined substrate; 0.4 ml of solvent (water or Hanks' solution); 0.4 ml of Fe^{2+} solution. Immediately after the introduction of the last reagent — 0.2 ml of hydrogen peroxide — the registration of luminiscene (light sum S) was started. A blank sample — light sum S0 (Fenton reaction without the examined substrate) contained 0.5 ml of solvent (water or Hanks' solution); 0.4 ml of Fe^{2+} solution and 0.2 ml of hydrogen peroxide. In order to eliminate the influence of the equipment the value of relative light sum S/S0 was used in the result analysis.

2 ml cuvette with a diameter of 1 cm was used in the study. The following reagents were applied for Fenton reaction: solution of $FeSO_4$ (10^{-3} mol/L in an acid medium, pH=2), hydrogen peroxide solution (10^{-3} and 10^{-1} mol/L). As ferrous iron is unstable in the neutral medium, an acid medium was prepared by adding sulfuric acid to the distilled water.

Chemiluminescence of albumin, hemoglobin, mixture of albumin and hemoglobin, well-known antioxidants: phenol (C_6H_5OH), resorcinol ($C_6H_4(OH)_2$), pyrogallol ($C_6H_3(OH)_3$), and mixtures of antioxidants with hemoglobin and albumin induced by Fenton reaction were registered. Chemiluminescence of the mixture of antioxidants with hemoglobin and albumin was also investigated. Concentrations of albumin equal to 50 g/L ($7\cdot10^{-4}$ mol/L) and hemoglobin 70 g/L ($1.1\cdot10^{-3}$ mol/L) approached their content in the blood. Concentration of antioxidants was 50 g/L. Antioxidants (phenol, resorcinol, pyrogallol) for albumin and hemoglobin solutions were introduced directly to the sample before the addition of hydrogen peroxide in the quantity of 0.1 ml. Bovine serum albumin, fraction V, molecular mass 69 kDa, and oxidized, freeze-dried bovine hemoglobin with molecular mass of 64.5 kDa (BioWest, France) were used. In both preparations the mass fraction of albumin was not less than 95%, and that of fat (lipids) not more than 1%. Other reagents were chemically pure: twice-distilled water, pH=6.5; sterile Hanks' solution (BioloT, Russia).

Measurements of chemiluminescence were started with registration of the light sum S of the substance with the initial concentration, then followed serial tenfold dilution with Hanks' solution or water, the light sums were obtained at concentrations of 1, 10^{-1}, 10^{-2}, …, 10^{-10} of the initial concentration (dilutions 0, –1, –2, …, –10).

Calculation of the examined process kinetics was performed as follows [4].

A scheme of reactions describing the process was worked out. The scheme included all substances participating in the reaction. As the rate constants of all reactions are known, the kinetics of the process can be calculated. For this purpose a system of differential equations was set up on the basis of this scheme, where concentrations of the substances, participating in the process, were variables. Each equation included the rate of accumulation and spending of the substances whose concentration is considered as a variable. The number of equations was equal to the quantity of the substances, participating in the reaction. Initial conditions are set, i.e. concentrations of all substances at the initial time. The solution is concentrations of all substances participating in the reactions at specified time intervals after the beginning of the reaction. Duration of these intervals and total reaction time are set as the conditions. To solve the system of differential equations a MathCad 14 program package was applied.

Results. The dependence of the chemiluminescence light sum S/S0 in Fenton reaction on the dilution of albumin and hemoglobin by water (0, –1, –2, –3, –4) was calculated at hydrogen peroxide concentration of 10^{-3} mol/L (Figure 1 (a)) and 10^{-1} mol/L (Figure 1 (b)). It is seen on Figure 1 (a), that the light sum at $[H_2O_2]=10^{-3}$ mol/L reaches its maximum for both examined substances at a definite dilution: –1 for albumin and –2 for hemoglobin. At $[H_2O_2]=10^{-1}$ mol/L (Figure 1 (b)) the light sum monotonically decreases with the reduction of sample concentration.

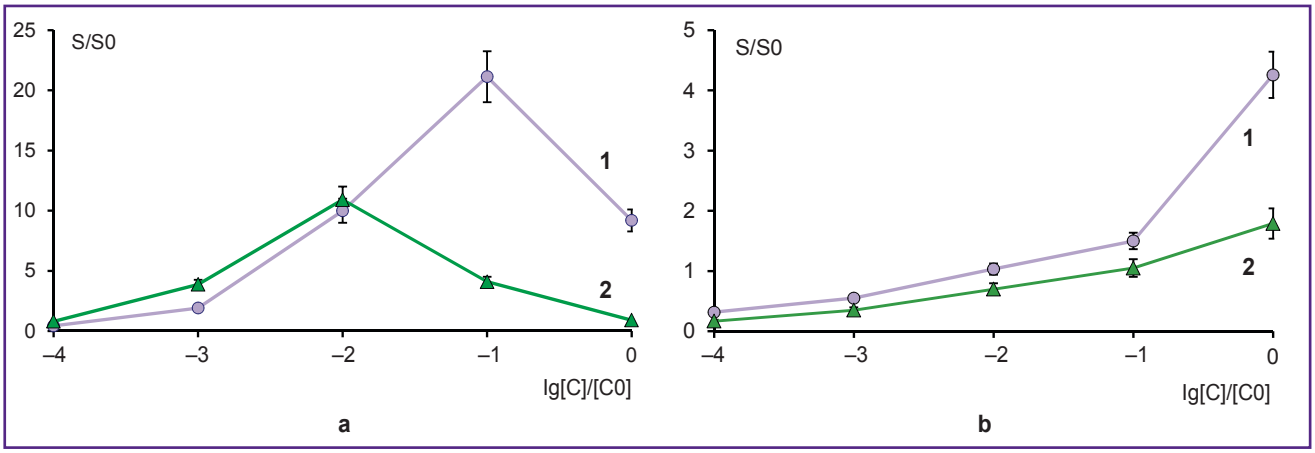

Figure 1. Dependence of hemiluminescence light sum S/S0 on the concentration (dilution) of the sample lg[C]/[C0] for 50 gL (*1*) water solution of albumin and 70 g/L (*2*) hemoglobin at concentration of $[Fe^{2+}]=10^{-3}$ mol/L and $[H_2O_2]=10^{-3}$ mol/L (a) and 10^{-1} mol/L (b). Experiment

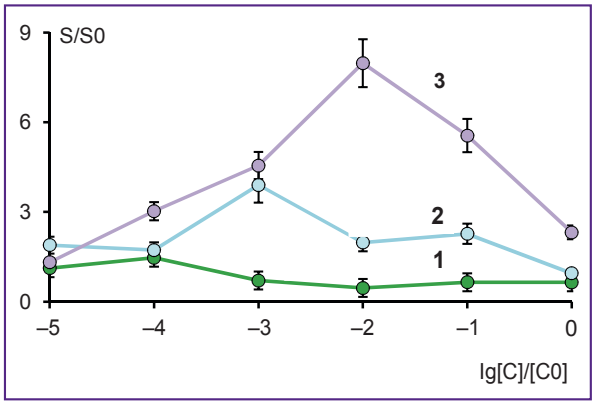

Figure 2. Dependence of the light sum S/S0 on the dilution of the initial solution lg[C]/[C0] for antioxidants: phenol (*1*), resorcinol (*2*), pyrogallol (*3*). The initial concentration of each substance is 50 g/L. $[Fe^{2+}]=[H_2O_2]=10^{-3}$ mol/L. Experiment

Table 1

Hemiluminescence S/S0 in Fenton reaction at $[Fe^{2+}]=[H_2O_2]=10^{-3}$ mol/L for different sample dilutions

Substance	Average light sum S/S0 at dilutions	
	from 0 to –4	from –5 to –10
Phenol	0.78±0.10	1.31±0.30
Resorcinol	2.16±0.25	
Pyrogallol	4.66±0.50	
Hemoglobin	4.0±0.5	1.03±0.20
Hemoglobin + phenol	1.0±0.2	0.97±0.20
Hemoglobin + resorcinol	1.53±0.30	0.75±0.20
Hemoglobin + pyrogallol	3.7±0.6	1.16±0.20
Albumin	12.4±2.1	1.08±0.20
Albumin + phenol	1.02±0.20	0.44±0.20
Albumin + resorcinol	1.22±0.20	0.84±0.20
Albumin + pyrogallol	7.2±1.3	1.05±0.20
Albumin + hemoglobin	4.4±0.7	1.2±0.2

The study of the dependence of chemiluminescence light sum on antioxidant concentration: phenol, resorcinol, pyrogallol at $[H_2O_2]=10^{-3}$ mol/L (Figure 2) showed, that phenol quenches chemiluminescence completely, S/S0<1. Two other substances, which are weaker antioxidants than phenol, are oxidized by hydroxyl radicals and maintain chain reaction for them in the maximum of chemiluminescence S/S0>1.

To improve statistical accuracy of the results mean values of the light sums in the region of chemiluminescence maximum (dilutions from 0 to –4) and below maximum (dilutions from –5 to –10) were analyzed. Mean values for concentrations of different ranges (Table 1) show, that for phenol at the dilutions from 0 to –4 S/S0=0.78, i.e. taking into account measurement errors, are notably less than 1. This means, that phenol does not allow the chain reaction of peroxidation, initiated by hydroxyl radicals generated in Fenton reaction, to develop. Resorcinol and pyrogallol are weaker antioxidants, a chain reaction in them develops, though rather poorly. At greater dilutions (from –5 to –10) an average light sum for all examined substances was near 1.

Table 2

The light sum in Fenton reaction for albumin (50 g/L), hemoglobin (70 g/L), and a mixture of albumin + hemoglobin with the same concentrations at $[Fe^{2+}]=10^{-3}$ mol/L and different concentrations of hydrogen peroxide

Substance	Light sum S/S0	
	$[H_2O_2]=10^{-3}$ mol/L	$[H_2O_2]=10^{-1}$ mol/L
Albumin	21.1±0.8 (–1)	4.25±0.30
Hemoglobin	10.8±0.5 (–2)	1.79±0.20
Albumin + hemoglobin	12.04±0.60 (–2)	3.02±0.30

Note. Dilutions, at which the maximum of hemiluminescence for $[H_2O_2]=10^{-3}$ mol/L is observed, are pointed out in brackets.

Hemoglobin and albumin are oxidized by hydroxyl radicals, generating chemiluminescence. Addition of phenol to the samples of hemoglobin or albumin quench chemiluminescence completely, S/S0=1. Addition of weaker antioxidants decreases chemiluminescence light sum, but does not stop chemiluminescence completely.

A curious case occurs, when two substances, albumin and hemoglobin, giving different light sum, are mixed. An average light sum of albumin solution — 12.4±2.1 — substantially exceeds the light sum of hemoglobin solution — 4.0±0.5. The light sum for their mixture appeared to be 4.4±0.7 (See Table 1).

The chemiluminescence light sums of albumin, hemoglobin and their mixtures for various concentrations of hydrogen peroxide, 10^{-3} and 10^{-1} mol/L (Table 2), are oxidized by hydroxyl radicals in the chain reaction. It should be noted, that the light sum in the maximum of chemiluminescence at $[H_2O_2]=10^{-3}$ mol/L is substantially greater than the maximal light sum at $[H_2O_2]=10^{-1}$ mol/L. The light sum, registered during hemoglobin oxidation for both concentrations of hydrogen peroxide, appears to be smaller than during oxidation of albumin. In the mixture of hemoglobin and albumin the light sum is greater than for hemoglobin, but is smaller than for albumin.

Discussion

The mechanism of chain oxidation in Fenton reaction in the presence of antioxidants and hydroperoxides. The scheme of reactions, going on during the chain oxidation of organic substance RH in Fenton reaction, is presented in Table 3. The constants of reaction rate were analyzed in work [8].

In the reaction with hydrogen peroxide (reaction *1*, Table 3) a hydroxyl radical is formed, which has the greatest oxidizing potential $E_{ox}(OH^{\bullet})=2.8$ V. This potential is referred to $OH^{\bullet}+H^{+}+e^{-}\rightarrow H_2O$ reaction, in which the concentration of hydrogen ions is supposed to be equal to 1 mol/L, which corresponds to an acidic medium, pH=0. The oxidizing potential in the neutral medium is less according to Nernst equation: it decreases by 0.059 V with the increase of pH by 1 unity. Potentials of other oxidants, pertaining to the active forms of oxygen are much lower in the acidic medium: $E_{ox}(O^{\bullet})=2.42$ V; $E_{ox}(O_3)=2.07$ V; $E_{ox}(H_2O_2)=1.76$ V; $E_{ox}(HO_2^{\bullet})=1.5$ V; singlet oxygen $E_{ox}(^1O_2)=0.94$ V [9].

In the reaction with hydroperoxide (reaction *2*, Table 3) an essentially less active organic radical $RO^{\bullet}$ is formed, whose oxidizing potential is determined by the nature of R radical and is about 0.7 V. $OH^{\bullet}$ and $RO^{\bullet}$ radicals can initiate the chain process, which is triggered by reactions *3*, *4*. In both cases the chain process develops in the presence of oxidizing substance RH.

Chemiluminescence induced by Fenton reaction is caused by the luminescence of a singlet oxygen dimer, generated in the process of reactions, initiated by hydroxyl radicals [4]. Apart from singlet oxygen, hydroperoxides are also formed in Fenton reaction, but as they do not emit any light, they cannot be registered directly.

The radicals themselves, formed during the initial interaction of peroxides with ferrous iron, may be destroyed while interacting with each other, forming inactive products (reactions *5*, *6*). Reactions *7*, *8*, *9* are the continuation of the chain. The rate of the chain process is determined by the rate constant of reaction *8* with an oxidative substance RH, whose value is small and amounts to 1–60 $L(mol\cdot s)^{-1}$ in the majority of cases. In the course of the chain reaction hydroperoxides (reaction *8*) and singlet oxygen (reaction *9*) are formed [5]. The luminescence of a singlet oxygen dimer is registered by biochemiluminometer, with the yield of reaction *9* being also measured. In spite of a small rate constant of reaction *8*, it can make a decisive contribution, if concentration of $[RH]>>[ROO^{\bullet}]$. At a high concentration of [RH] there may be no luminescence in reaction *9*, as all $ROO^{\bullet}$ radicals will be spent in reaction *8*. On the basis of the yield of reaction *9* one can judge about the rate of chain oxidation by hydroxyl radicals

Table 3

The scheme of initiating chain oxidation in Fenton reaction

Reaction No.	Reaction	Rate constant $L(mol\cdot s)^{-1}$ [8]
1	$HOOH+Fe^{2+}\rightarrow OH^{\bullet}+Fe^{3+}+OH^{-}$	56
2	$ROOH+Fe^{2+}\rightarrow RO^{\bullet}+Fe^{3+}+OH^{-}$	50
3	$RH+OH^{\bullet}\rightarrow R^{\bullet}+H_2O$	10^7
4	$RH+RO^{\bullet}\rightarrow R^{\bullet}+ROH$	10^3–10^4
5	$RO^{\bullet}+RO^{\bullet}+M\rightarrow ROOR+M$	10^4–10^5
6	$OH^{\bullet}+OH^{\bullet}\rightarrow H_2O+½O_2$	10^9
7	$R^{\bullet}+O_2+M\rightarrow ROO^{\bullet}+M$	10^5
8	$ROO^{\bullet}+RH\rightarrow ROOH+R^{\bullet}$	1–60
9	$ROO^{\bullet}+ROO^{\bullet}\rightarrow ROOR+{}^1O_2$	10^5
10	$OH^{\bullet}+InH\rightarrow H_2O+In^{\bullet}$	10^9
11	$ROO^{\bullet}+InH\rightarrow ROOH+In^{\bullet}$	10^8
12	$ROOH+OH^{\bullet}\rightarrow ROO^{\bullet}+H_2O$	10^7

with the formation of only luminous products. The total rate of the chain oxidation is determined by the yield of reactions *8* and *9*, in which hydroperoxide and singlet oxygen are formed.

Prooxidants (substances, which maintain chain reaction) and antioxidants (substances, which inhibit chain reaction) are usually considered in the schemes of oxidation-reduction processes. Under the action of antioxidants free radicals are transferred to inactive form, resulting in slowing down the initiation of chain reaction or stopping it completely. Chain reactions are not restricted only by the first act of initiation. Following the law of conservation of free valence, which acts in the majority of cases, one radical cannot be destroyed, as they are generated and destroyed in pairs. As a result of primary reaction with a radical, a secondary radical is created, which can again participate in reactions, if its activity is great enough. A free radical can be inactivated only interacting with another radical, or a chain reaction can slow down or stop, if a secondary radical will turn out to possess low activity.

A substance, which fully prevents both initiation and development of a chain reaction is called an inhibitor InH. Inhibition of the chain oxidation occurs when there is a substance, which rapidly interacts with the radical. Interacting with the primary radical (initiator, e.g. $OH^{\bullet}$) or with the secondary radical, created in the process of the chain reaction $ROO^{\bullet}$, the inhibitor InH produces a low-active secondary radical $In^{\bullet}$ (reaction *10*, *11*, Table 3).

In reaction *10* the inhibitor intercepts the initial $OH^{\bullet}$ radical, preventing the beginning of the chain reaction, while in reaction *11* it intercepts a secondary $ROO^{\bullet}$ radical, generated after the initiation of the reaction by a hydroxyl radical, and prevents the chain from continuation. The chain oxidation of the examined substance RH is initiated and continues in reactions *3*–*9*.

The analysis of the chain reaction with an inhibitor (reactions *3*–*11*) allows us to draw a conclusion about the existence of two ways of inhibiting chain reaction: to use a trap for primary radicals, initiating reaction (reaction *10*) or a trap for secondary radicals, formed at the stage of chain continuation (reaction *11*). As the properties of primary and secondary radicals are different (in described example $OH^{\bullet}$ radical is primary, and $ROO^{\bullet}$ radical is secondary), the traps for them may be different in a general case. Thus, we may say, that the traps for primary radicals, initiating the chain reaction, possess antiradical activity, while the traps for secondary radicals, generated at the stage of chain continuation, possess antioxidant activity.

However, the scheme of inhibition, based on reactions *10* and *11*, is strongly simplified. Each inhibitor (antioxidant) has its own scheme of conversion to an inactive product after the primary interaction with radicals. An example is a scheme of ascorbic acid oxidation, considered in work [10]. Besides, inhibitors can interact only with the definite radicals.

The processes of inhibiting chain reactions were studied in detail for protection of polymeric materials from thermal-oxidative destruction [11]. In this case a single definite compound is protected from the radicals of a known nature. In biology the task is essentially more complicated [12, 13]. For example, various substances are present in the blood samples, whose composition is unknown, therefore the nature of the primary radicals initiating oxidation can also be different.

When several substances are detected in the sample, the results can be interpreted in two ways. If one substance is an oxidant and does not maintain chain reaction, all or the majority of hydroxyl radicals are spent for oxidation of this substance, and a chain reaction of oxidizing other substances is strongly inhibited. If some definite material is added to the sample, practically full inhibition of chemiluminescence may denote, that it possesses a strong antiradical activity and consumes all hydroxyl radicals.

If all substances can participate in chain reaction, then primary hydroxyl radicals initiate oxidation of all matters. The yield ratio of the substance oxidation products is determined by the concentration of these substances and the relation of the rate constants of the reactions of initiation, continuation and chain breaking.

It may turn out, that preferably one of these substances will be oxidized. Then we may speak, that this material possesses antioxidant effect relative to others. The effect may be considered to be established, if we will observe the products of chain reaction. It is impossible to draw an unambiguous conclusion on the antioxidant properties of some substance on the basis of the light sum value of the multicomponent substrate only. Such situation is illustrated by the data obtained for the mixture of albumin and hemoglobin (See Table 2). Light sum of the mixture appeared to be larger than that of hemoglobin, but smaller than that hemoglobin. The conclusion, that hemoglobin possesses antioxidant properties relative to albumin cannot be drawn from here. The analysis of oxidation products and consumption of the reference substances shows, that it is albumin that possesses antioxidant properties in relation to other components of blood [14].

Specific features of Fenton reaction at different concentrations of hydrogen peroxide

Case 1: $[Fe^{2+}] \geqslant [H_2O_2]$, $[Fe^{2+}]=10^{-3}$ mol/L, hydrogen peroxide concentration is 10^{-3} mol/L. Dependence of chemiluminescence light sums at serial tenfold dilutions of the sample is studied experimentally. This dependence for albumin (lg[C]/[C0]=0, –1, –2, –3, –4 dilutions) is presented on Figure 1, curve *1*. The light sum is seen to reach its maximum at –1 dilution, the light sum for the initial concentration (0 concentration) is smaller, while at more strong dilutions (–2, –3, –4) the light sum also decreases. For hemoglobin the situation is similar (Figure 1, curve *2*), but maximal chemiluminescence is observed at –2 dilution. The effect of chemiluminescence

decrease at a large concentration of [RH] in both cases is connected with spending $ROO^{\cdot}$ in reaction *8*, so reaction *9* appears to be inhibited [4]. Molecular mass of albumin is 69 kDa, that of hemoglobin 64.5 kDa, their content in the initial solution is 50 and 70 g/L, which corresponds to concentration of ~10^{-3} mol/L. The calculations showed, that inhibition of the chemiluminescence in reaction *8* is possible at [RH] concentration of ~1 mol/L and more [5]. Observation of chemiluminescence inhibition effect may denote, that not all albumin as a whole participates in reaction *3* and *8* (Table 3) but rather its RH fragments. At the concentration of albumin molecules of ~10^{-3} mol/L the concentration of the fragment being oxidized can be much stronger and amount to 1–10 mol/L, therefore inhibition of luminescence is observed at the concentration of albumin molecules of ~10^{-3} mol/L.

The calculation of chemiluminescence/reagent concentration relationship is performed in work [15]. The concentration of [RH] fragments being oxidized is shown to determine the position of chemiluminescence maximum (sample dilution). Inhibitor InH and hydroperoxide ROOH, which may be in the sample, decrease the light sum of chemiluminescence, and the value of the light sum in the maximum is determined by the relation of [InH]/[RH] and [ROOH]/[RH].

A specific feature of this mode consists in the fact, that chemiluminescence is defined only by the yield of the luminescence products in reaction *9*. Hydroperoxides, which are also the products of the chain reaction, do not contribute to chemiluminescence and the information obtained is not complete.

Case 2: $[Fe^{2+}]<[H_2O_2]$, $[Fe^{2+}]=10^{-3}$ mol/L, hydrogen peroxide concentration is 10^{-1} mol/L. When concentration of peroxide is more than concentration of iron, ferrous iron, oxidized to ferric iron in Fenton reaction, regenerates to ferrous iron. In this case hydrogen peroxide is spent, and the process will continue till a full consumption of peroxide [7]. The duration of the reaction is determined by the concentration of hydrogen peroxide, and it will exceed 30 s (the selected time of chemiluminescence measurement). But the basic quantity of reaction acts occurs in the first fraction of a second, thereafter the hemiluminescence decreases [4]. Accumulation of organic hydroperoxides does not take place, as they are immediately spent in the reaction with ferrous iron (reaction *2*, Table 3). Luminescence arising is proportional to the yields of reactions *8* and *9*. Concentration of ferrous iron does not fall so rapidly as in case 1, but is kept at almost the same level due to regeneration [7]. Since the speed of the registering equipment is limited, a part of information (part of the light sum, displayed during the front edge of the hemiluminescence pulse) is lost, but, on the other hand, the registered light sum is proportional to the sum of the yields of reactions *8* and *9*, i.e. a total yield of the chain reaction. If the conditions of the experiment are highly reproducible in different trials, the light sum will be proportional to a total oxidative capacity of the sample.

Calculation of hemiluminescence kinetics for case $[Fe^{2+}]<[H_2O_2]$. Within the frame of the scheme consisting of 25 reactions, described previously [4], in which reactions with an inhibitor and a reaction of hydroperoxide with hydroxyl radical (reactions *10–12*, Table 3) were additionally included, the dependence of a relative light sum S/S0 on concentration of oxidized [RH] fragments in the sample is calculated. To calculate the kinetics, a system of 15 differential equations was solved. It was assumed, that the rate constant of the reaction of hydroxyl radicals with the inhibitor was 10^9 $L(mol\cdot s)^{-1}$, while with RH substance it amounted to 10^7 $L(mol\cdot s)^{-1}$. The rate constants of other reactions were selected as the most characteristic for the biological substrates [8]. [RH] concentration in case of highly molecular compound (albumin) was assumed to be the concentration of fragments of this compound, capable of independent oxidation. The concentration of albumins in the blood similar to the presented above case 1 ($[Fe^{2+}]\geqslant[H_2O_2]$) is in the order of 10^{-3} mol/L, while the concentration of oxidizing [RH] groups may reach 1 mol/L and more. The results of calculation are presented on Figure 3. The light sum S/S0 is seen to grow monotonically with the growth of [RH] concentration. It should be underlined, that this dependence is nonlinear. It is seen both from calculations and experimental data (Figure 1 (b)).

At the concentration of the [RH] fragments being oxidized equal to 10 mol/L, dependence of S/S0 on inhibitor [InH] concentrations and oxidized substance [ROOH] was counted (Figure 4). The light sum diminishes with the growth of inhibitor and hydroperoxide concentration. It is seen, that hydroperoxide decreases the light sum greater than the inhibitor at equal

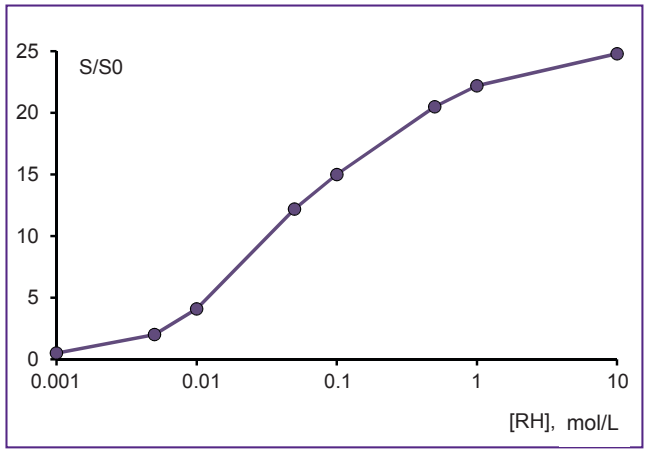

Figure 3. Dependence of the hemiluminescence light sum S/S0 in Fenton reaction at $[Fe^{2+}]=10^{-3}$ mol/L and $[H_2O_2]=0.1$ mol/L on concentration of the oxidized substance [RH]. Calculation

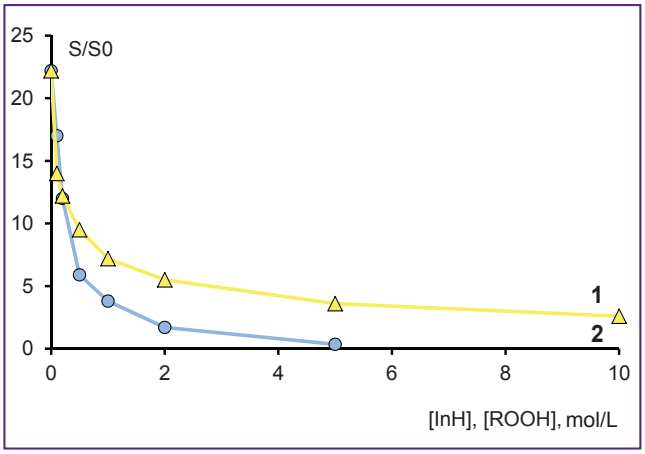

Figure 4. Dependence of the light sum S/S0 on concentration (mol/L) of ROOH hydroperoxide (*1*) and InH inhibitor (*2*) at concentration of the oxidized substance [RH] equal to 10 mol/L; $[Fe^{2+}]=10^{-3}$ mol/L; $[H_2O_2]=10^{-1}$ mol/L. Calculation

concentrations of the inhibitor and hydroperoxide. It is connected with the fact, that hydroperoxide interacts with iron (reaction *2*, Table 3), spends it, decreasing the yield of radicals in Fenton reaction. Concentration of iron in reaction *2* is higher than the concentration of $ROO^{\bullet}$ radicals, with which the inhibitor is interacting (reaction *11*). Therefore, the effect of hydroperoxides on the value of the light sum appears to be greater, despite a substantial difference of constant values of reactions *2* and *11*.

Thus, if there is hydroperoxide in the sample, it will consume ferrous iron, diminishing the yield of hydroxyl radicals and the light sum of hemiluminescence induced by Fenton reaction. It should be emphasized, that hydroperoxide is not an antioxidant. Interaction of hydroperoxide with hydroxyl radicals (reaction *12*) maintains the chain reaction. However, hydroperoxide decreases the number of primary hydroxyl radicals by diminishing reagent concentrations, and thereby decreases the light sum of hemiluminescence. If there are many hydroperoxides in the initial sample, it is strongly oxidized, and hemiluminescence will be inhibited. Decrease of hemiluminescence of the oxidized sample relative to the sample of the same composition but not oxidized, will not mean that there are antioxidants in the sample. It will only mean, that an oxidizing capacity of the preoxidized sample is less than that of nonoxidized. And this is a specific feature of Fenton reaction rather than a property of a substrate.

Case 2 ($[Fe^{2+}]<[H_2O_2]$) allows a more operative assessment of the substrate ability for oxidation, but case 1 ($[Fe^{2+}]\geqslant[H_2O_2]$) makes it possible to obtain additional information on sample composition [15].

There does not exist any relation between some characteristic of the examined substance and the registered light sum hemiluminescence. It is seen from the experimental data (See Figure 1) and the calculated results as well (See Figures 3, 4). In all cases at $[Fe^{2+}]<[H_2O_2]$ the light sum decreases with the growth of [InH] antioxidant and [ROOH] hydroperoxide concentration and increases with the growth of [RH]. A linear dependence exists only at $[Fe^{2+}]=[H_2O_2]=10^{-3}$ mol/L (case 1) between the concentration of [RH] and the dilution, at which the maximum of hemiluminescence is observed. Thus, at $[Fe^{2+}]<[H_2O_2]$ there is no linear dependence between the light sum S/SO and concentrations of [RH], [ROOH], and [InH], therefore, the results obtained are of qualitative character.

Association of the light sum in Fenton reaction with antioxidant activity of the sample. Reaction of peroxidation of the RH organic substance is a chain reaction, and a slight primary effect results in serious final damages in the object. The task of an oxidant is to interrupt or prevent its occurrence. To slow-down chain oxidation development is also of great importance for maintaining the balance in the system, the balance of oxidants and prooxidants.

An “antioxidant” in biology, and an “inhibitor” in the chemistry of polymers is a substance, which after the interaction with a radical forms a low-activity radical, which cannot participate in the continuation of the chain. After the introduction to the system, an antioxidant is consumed, and once it has been consumed, the chain reaction will continue.

With reference to biology the term “antioxidant” may be defined as a substance, which when present in low concentrations relative to the oxidizing substrate, significantly slows-down or prevents oxidation of the substrate. Oxidizers are active forms of oxygen and nitrogen, being radicals in the majority of cases. But there exist active particles of various types. They may not be radicals. Peroxynitrite, nitrous and peroxynitrous acids are referred to such particles. Antioxidant can intercept an active particle (interact primarily with it), or prevent its creation. Thus, antioxidant should be oriented to the object, which it must protect.

Various terms are used to characterize antioxidant ability [16–18]: total antioxidant capacity, total antioxidant response. Since antioxidants are oriented to a definite type of radicals, terms oriented to the type of absorbed radicals are used, e.g. oxygen radical absorbance capacity. The characteristics of the examined substances are linked with the characteristics of the known antioxidants, e.g. Trolox-equivalent antioxidant capacity. The most common is the parameter, characterizing the ability of antioxidants to absorb all radicals (total radical-trapping antioxidant parameter (TRAP)). The value of TRAP is equal to the sum of all antioxidant concentrations, multiplied by the stoichiometric coefficient of each reaction. But the content of each

antioxidant is determined separately. Characteristics of radicals generating in biological objects and their reactions are given in works [19, 20].

For biological substrates prooxidant–antioxidant balance is important. The works have been performed, in which this balance was estimated in a single experiment [21, 22]. To evaluate the balance, the ability of TMB (3,3'.5,5'-tetramethylbenzidine. 2HCl, T-3405) reagent to change its color in the water solution was used: when oxidized TMB solution becomes colored, the optical density is measured at λ=450 nm relative to the optical density of 620 or 570 nm lines. When regenerated, the solution becomes colorless. The method does not work, if regenerative capability of the sample exceeds the oxidizing capability, the solution will be always colorless.

When antioxidant activity is studied, an artificial source of free radicals (oxidation initiator), which acts on the examined substance, is usually used. Then intermediate products of the oxidation process or the decrease of the initial substance are registered. The process of oxidation is also characterized by oxygen consumption, which can be measured. For quantitative characteristics of antioxidant activity of the examined sample, antioxidant sample indices are compared with the antioxidant indices taken as a reference. Different initiators of oxidation generate different free radicals. Therefore, it is important to know for each specific task, antioxidant activity of which radicals should be investigated.

One of the express methods of assessing antioxidant capacity uses observation of hemiluminescence, registered by biochemiluminometer, after the introduction of ferrous iron to the sample [23, 24]. If hydroperoxide is available in the sample, ferrous iron allows generation of $ROO^{\bullet}$ radicals for a long time. These radicals can be identified by the formation of singlet oxygen.

As a source of free $ROO^{\bullet}$ radicals 2,2'-asobis (2-amidinopropane)dihydrochloride or 2,2'-asobis(2-amidinopropane) (ABAP) were used in work [24], and radiation, generated during preparation heating, was intensified by luminol. At 37°C these substances are decomposed with formation of two $R^{\bullet}$ radicals and gaseous nitrogen. The period of half-decay for ABAP is 195 h, therefore, once a steady-state conditions have been established, it becomes a source of radicals of constant intensity. Combination with the air oxygen ($R^{\bullet}+O_2$) results in generation of $ROO^{\bullet}$ radicals, which initiate luminescence of luminol. If a substance possessing antioxidant properties is added, it intercepts $ROO^{\bullet}$ radicals and influences the luminescence: decreases it, if the oxidation chain is broken, but can also increase the luminescence, if $ROO^{\bullet}$ radicals initiate continuation of the chain reaction. In the reaction with radicals antioxidant is being spent. Antioxidant capacity is proportional to the duration of the induction period: the longer the period, the greater is the antioxidant capacity. Dependence of the induction period on the concentration of the introduced antioxidant appears to be linear [23–25]. The method of induction period observation does not work, if the oxidizing capacity exceeds the regenerative, as the luminescence will appear immediately after iron addition.

Let us consider the possibilities of applying Fenton reaction for the assessment of antioxidant activity. Fenton reaction is the source of hydroxyl radicals capable of oxidizing actually any substances, including all antioxidants. Hemiluminescence is caused by the products of interaction of hydroxyl radicals with substances. Only strong antioxidants, such as phenol, completely quench hemiluminescence and do not maintain the chain reaction. Therefore, in Fenton reaction one can speak of the ability of a substrate to oxidation by hydroxyl radicals. And the results obtained are qualitative, since there is no strictly linear dependence between sample characteristics (concentrations of [RH], [InH], [ROOH]).

Traditionally applied methods of identifying antioxidant activity are based on determining the number of radicals, produced by a reagent, generating the radicals. Then decrease of their concentration after antioxidant introduction is registered, connected with the absorption of the radicals. If the antioxidant is strong and immediately absorbs all radicals, radicals disappear and a period of induction is observed, i.e. the time required for the antioxidant to be spent, and the radicals to appear again. In these methods absorption of radicals by antioxidants takes place, which is a direct evidence of antioxidant availability and its activity.

In contrast to this, products of the reaction rather than radicals themselves are observed in Fenton reaction. Besides, the reaction occurs actually with any substance. The yield of reaction products is determined by the rate constants of reactions of initiation, continuation and breaking the chain. The increase or decrease of the yield speaks of different values of these constants, but does not indicate to the inhibition of the chain reaction by generating inactive products. That is why it is impossible to prove how the capacity of the substrate to oxidation by hydroxyl radicals is connected with the antioxidant capacity of the sample.

It is possible to identify antioxidant activity using reaction of ferrous iron with hydroperoxide (reaction *2*, Table 3). After reactions *4* and *7* $ROO^{\bullet}$ radical is generated. This radical is identified in reaction *9* by the luminescence of a singlet oxygen dimer. When an examined substance, being an oxidant and consuming $ROO^{\bullet}$ radicals, is introduced to the sample, the luminescence originating in reaction *9* will decrease. If the substance is prooxidant and maintains the chain reaction, the luminescence will increase. Hydroperoxide used in this reaction must be a strong oxidizer. For example, tret-butil hydroperoxide may be used. Therefore, investigation of hemiluminescence, occurring

in the reactions of hydroperoxides with ferrous iron, may be of interest for further researches.

Conclusion. Hydroxyl radicals, generated in Fenton reaction, initiate chain reaction in the substrate. The product of the chain reaction are hydroperoxide and singlet oxygen. Characteristics of hemiluminescence, originating in Fenton reaction, are determined by the proportion of concentrations of ferrous iron and hydrogen peroxide.

At $[Fe^{2+}] \geqslant [H_2O_2]$ ferrous iron is consumed, the light sum of hemiluminescence is identified by the yield of the luminescence products (singlet oxygen). The time of reaction is determined by the concentration of ferrous iron. Hydroperoxides, generated in the chain reaction, remain unidentified.

At $[Fe^{2+}] < [H_2O_2]$ oxidized to trivalent condition, iron regenerates to a bivalent one. Reaction time is determined by the concentration of hydrogen peroxide. The light sum of hemiluminescence is proportional to the total yield of hydroperoxide and singlet oxygen. The light sum increases with the growth of concentration of [RH] groups and diminishes with the growth of concentration of [InH] inhibitor and [ROOH] hydroperoxide, accumulated in the sample. There is no linear dependence between the light sum and sample characteristics, therefore, the obtained results are qualitative.

The light sum in Fenton reaction characterizes the ability of the substrate to oxidation, and in a general case is not connected with the antioxidant capacity of the sample.

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