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Electrochemical Modeling of Antioxidants Action and Determination of Their Activity

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Abstract—The method of pulse voltammetry was applied to the investigation of the antioxidant activity of sulfur-containing biologically active substances. Three models were considered to characterize the substances in aqueous medium: First, the interaction of antioxidants with a ferrous ion to characterize the preventive activity on the initiation stage of free radical oxidation, second and third describing the interaction of an antioxidant with electrochemically generated hydroxy radicals and hydrogen peroxide, to characterize the inhibitory activity in the initial stages of propagation and branching of the chain of free radical oxidation. A synergistic effect of ascorbic acid and other studied compounds at their combined action on hydroxy radicals and hydrogen peroxide was revealed.

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Free radical processes occurring under the influence of unfavorable environmental conditions, chemicals, radiation, and many other actions on biological systems stipulate an increased number of studies [1–3] concerning the mechanism of action and effectiveness of known and potential antioxidants. Various pharmaceuticals and biologically active substances of synthetic and natural origin are used as antioxidants. The mechanism of their action is not studied sufficiently, and the antioxidant activity is not always adequate to their characteristics [1, 2]. The determination of the effectiveness of antioxidant *in vivo* requires overcoming considerable experimental difficulties related as a rule to the uncontrolled influence of many accompanying processes of vital activity.

Among the described methods for determining the activity of antioxidant *in vitro* it is often difficult to choose the most reliable, and to compare their data, since these methods use different model systems and consider differently the process of functioning of the antioxidants [1, 3].

Approaches to the determination of activity and choice of effective antioxidants *in vitro* should be based on the models, which ideally should be similar to the natural antioxidant protecting system [4, 5]. Now methods are widespread for estimation antixidant activity basing on determining the content of the final product of lipid reaction with peroxide, or the product of reaction with a stable radical [6, 7]. At the same time, the most significant role in biological systems plays the regulation of free radical processes in the steps of initiation and chain propagation. Although the reaction with peroxides develops as chain reactions in the lipid phase, the initial (and possibly intermediate) stage of this complex reaction system occurs in aqueous phase [8]. Therefore, of special interest is determining the effectiveness of the antioxidants in an aqueous physiological environment in the initial stages of lipid oxidation with peroxides.

To some extent, electrochemical methods allow us to estimate the effectiveness of an antioxidant from the value of its oxidation potential [9], which, however, have limitations. These methods allow us to fix the electron transfer reactions involving a biologically active substance and electrode, whereas in biosystems initial stages of free radical reactions include a biologicaly active substance and oxygen or its reactive species.

A described voltammetric method for determination of antioxidant activity of several drugs [10] by suppressing the two-electron wave of oxygen at the

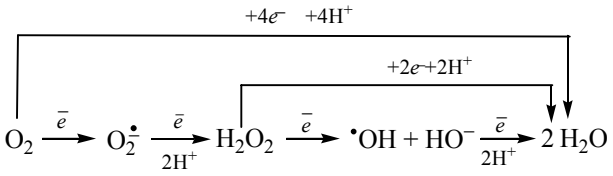
mercury film electrode, in our opinion does not allow a simulation of individual stages of the process.

The search for optimal models for developing the methods to study the mechanism and determine the effectiveness of the antioxidant, particularly in the initial stages of free radical oxidation, was and remains relevant, which determined the purpose and scope of this study.

As the object of the experiments we choose the water-soluble biologically active substances contributing to the antioxidant protection system of the body: cysteine, acetylcysteine, and glutathione, containing active sulfhydryl group, methionine, containing shielded sulfur, and, for comparison, L-ascorbic acid, one of the most powerful antioxidants, not containing a sulfur atom.

The procedure of the voltammetric studies of oxygen reduction on different electrodes in aqueous physiological media we described earlier [11].

Before choosing a model system to study the efficiency and the mechanism of action of antioxidants, it is necessary to consider briefly the scheme of reduction of oxygen in the oxidative metabolism in biological systems [12].



In the respiratory chain proceeds a four-electron reduction of oxygen to water safe for the body. Under the influence of various factors, including the unfavorable ones, stepwise one-electron reduction of oxygen proceeds resulting in the formation of active forms of oxygen that are capable to destroy biomembranes and cause the so-called oxygen stress. The latter is accompanied not only by the peroxide oxidation of lipids, but also by the peroxidative modification of protein macromolecules, which causes diseases of the cardiovascular, respiratory, nervous, and other systems, as well as premature aging [13].

Among the active forms of oxygen shown in the scheme, the most reactive one is the hydroxy radical capable to interact with the molecules of unsaturated fatty acids and other organic RH compounds with the formation of the radical R$^{\bullet}$:

$$RH + OH^{\bullet} \rightarrow R^{\bullet} + H_2O.$$

Further, these radicals react with oxygen to form peroxy radicals which continue the chain reaction by interacting with the new molecules of unsaturated fatty acids with the formation of R$^{\bullet}$ and accumulation of lipid hydroperoxides (ROOH):

$$R^{\bullet} + O_2 \rightarrow RO_2^{\bullet},$$

$$RO_2^{\bullet} + RH \rightarrow R^{\bullet} + ROOH.$$

In the biological systems such a chain reaction becomes more extensive due to the splitting of the hydroperoxides with the formation of free radicals, which give rise to new chains of oxidation, that is, the process falls into the line with the general regularities of the chain oxidation [9, 14].

In the body, a substantial role in the redox processes belongs to the metal ions of variable valence, in particular, iron and copper. There is a relationship between an increased iron level and the intensity of lipid oxidation by peroxides induced by hydroxy radicals. A typical case is the participation of iron ions in the single-electron Fenton reaction such as decomposition of hydroperoxides with the formation of hydroxy radicals and other active forms of oxygen initiating the oxygen stress [15]:

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + HO^{-} + Fe^{3+}, \quad (1)$$

$$OH^{\bullet} + H_2O_2 \rightarrow H_2O + H^{+} + O_2^{\cdot-}, \quad (2)$$

$$O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH^{-} + OH^{\bullet}. \quad (3)$$

In biological systems the most stable active form of oxygen is hydrogen peroxide whose oxidation potential is relatively low, like that of superoxide anion [15]. Therefore it is believed that these active oxygen compounds are dangerous for the organism most likely because of the formation of hydroxy radical, the most powerful oxidizing agent appearing as a result of reactions (1) and (3). Therefore, as a model substance to determine the effectiveness of an antioxidant are used hydroxy radicals obtained in the Fenton reaction [7].

To the end of the last century the hydroxy radicals were generated in some studies by the electrochemical reduction of oxygen to hydrogen peroxide in the presence of bivalent iron ions, which interact with the peroxide:

$$O_2 + 2H^{+} + 2\bar{e} \rightarrow H_2O_2,$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}.$$

Under these conditions the electrochemical regeneration of ferrous ion, $Fe^{3+} + e^{-} \rightarrow Fe^{2+}$ and the

continuous reduction of the oxygen dissolved in the Fenton medium, that is, generation of hydrogen peroxide proceeds. This process was called *electro-Fenton*. In recent years, the process was widely studied and used to treat organic compounds in the environment polluting wastes of various industries [16].

The antioxidant protection system of the body is put into effect by reducing the level of active oxygen or the formed radicals, as well as through binding of metal ions of variable valence that initiate the formation of active forms of oxygen by protein functional groups. The antioxidants selection is based on the nearly the same scheme.

From our point of view, in the selection of a sufficiently correct model to determine the effecttiveness of antioxidant should be considered, first, the influence of the latter on the bivalent iron ions in order to characterize the ability of biologicaly active substance to act as preventive antioxidants; second, the effect on hydroxy radicals, which shows the ability of a biologicaly active substance to act as an antioxidant, terminating the chain at the start of the oxygen stress; and third, the influence of the biologicaly active substance on hydrogen peroxide participating in the Fenton and Haber–Weiss reactions. Thus, to determine the ability of a biologicaly active substance to act as a preventive antioxidant, we obtained the voltammograms of reduction of the bivalent iron ions at the platinum cathode in pulsed mode and studied the effect of the investigated biologicaly active substances on the process.

As seen from Fig. 1a, in the differential voltammogram there is a wave of reduction of bivalent iron[1] in 0.1 M NaCl with a peak current at $E = -0.48$ V [17]. When acetylcysteine is added to the solution, the limiting current of this wave is reduced proportionally to the concentration of the added compound. Similar changes in the iron ion reduction wave occur under the influence of cysteine and glutathione (Fig. 1b). Furthermore, upon decrease in the limiting current of the wave of bivalent iron ions a new wave appears at more positive potential, which grows upon the increase in the concentration of biologically active substances containing sulfhydryl group (Fig. 1b). The possibility of appearance of such waves has been found previously at the electrochemical simulation of redox reactions of glutathione [18]. In the case of methionine whose sulfur atom is blocked much smaller decrease in the limiting current of iron reduction occurs (Fig. 2a).

The experimental results suggest that the studied biologically active substances containing a sulfhydryl group interact with the ferrous ion same as in biological systems.

Obviously, ascorbic acid acts similarly, exerting a similar influence on the wave of ferrous iron (Fig. 2b).

To determine the relative effectiveness of the compounds studied, we constructed a dependence on the concentration of the biologically active substance of the difference in the wave heights of Fe^{2+} ions in the absence and the presence of the biologicaly active substance divided by the height of the initial wave of iron ions. The slopes of the dependences obtained were used as the criterion of relative antioxidant activity of the biologicaly active substance (see the table).

The data obtained allow us to judge to some extent about the effect of structure of the studied biologically active substances on their activity as a preventive antioxidants. The compounds containing sulfhydryl group show a sufficiently high activity. The most active is acetylcysteine, which is consistent with its characteristics indicating its high efficiency as the antioxidant in biological systems [20].

At the same time, as stressed above, the most common methods of determining the effectiveness of antioxidants *in vitro* are the methods based on a model of interaction of the biologically active substances with the radicals, particularly hydroxy. The main types of reactions of these radicals are: splitting off a hydrogen atom from an organic molecule, adding to a molecule at the double bond, and electron transfer from an electron donor to form a hydroxy ion.

We believe that a sufficiently promising model for determining the effectiveness of an antioxidant is the earlier found possibility of generating by electrochemical reduction of molecular oxygen not only the hydroxy radical, but also the hydrogen peroxide. In this case, the antioxidant effectiveness can be estimated from the changes in the voltammetric curves under the influence of the studied biologically active substance [5, 11].

Thus, in the voltammetric curves obtained on a copper cathode in 0.1 M aqueous NaCl in a special

[1] It was found that the limiting current of the reduction wave of bivalent iron on the platinum cathode is proportional to its concentration both in the absence and the presence of oxygen.

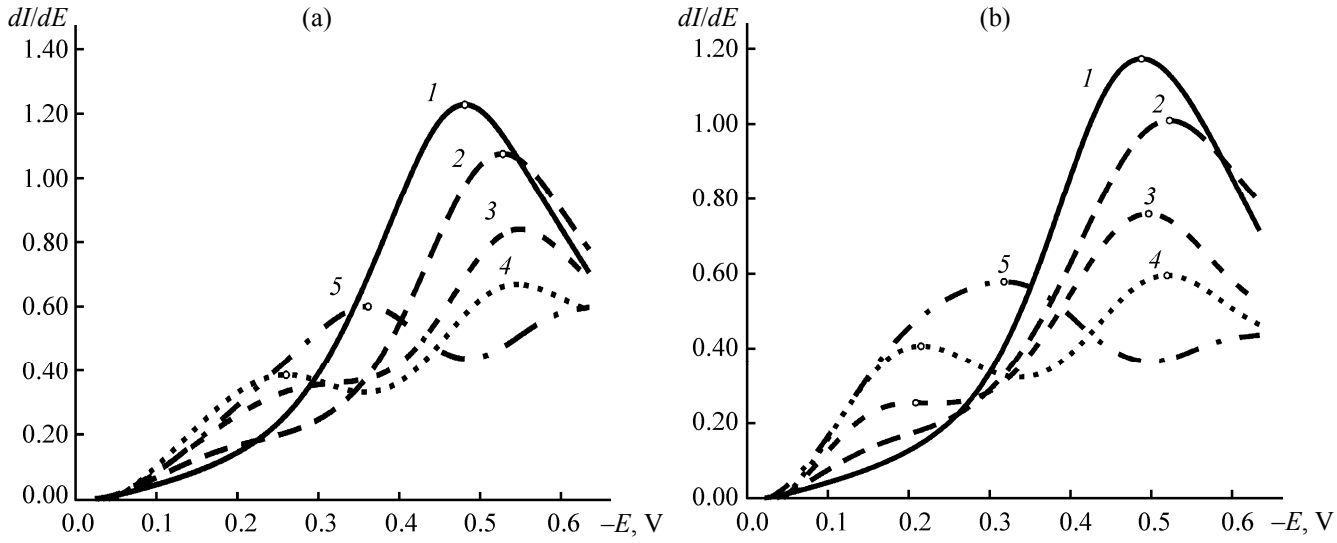


Fig. 1. (*1*) Differential voltammograms of reduction of Fe^{2+}, $C = 2.42\times10^{-3}$ mol l^{-1}, on a platinum cathode against 0.1 M NaCl solution in water in the presence of: (a) acetylcysteine at the concentrations: (*2*) 0.19, (*3*) 0.38, (*4*) 0.47, (*5*) 0.57×10^{-3} mol l^{-1}; (b) glutathione at the concentrations: (*2*) 0.19, (*3*) 0.38, (*4*) 0.56, (*5*) 0.74×10^{-3} mol l^{-1}.

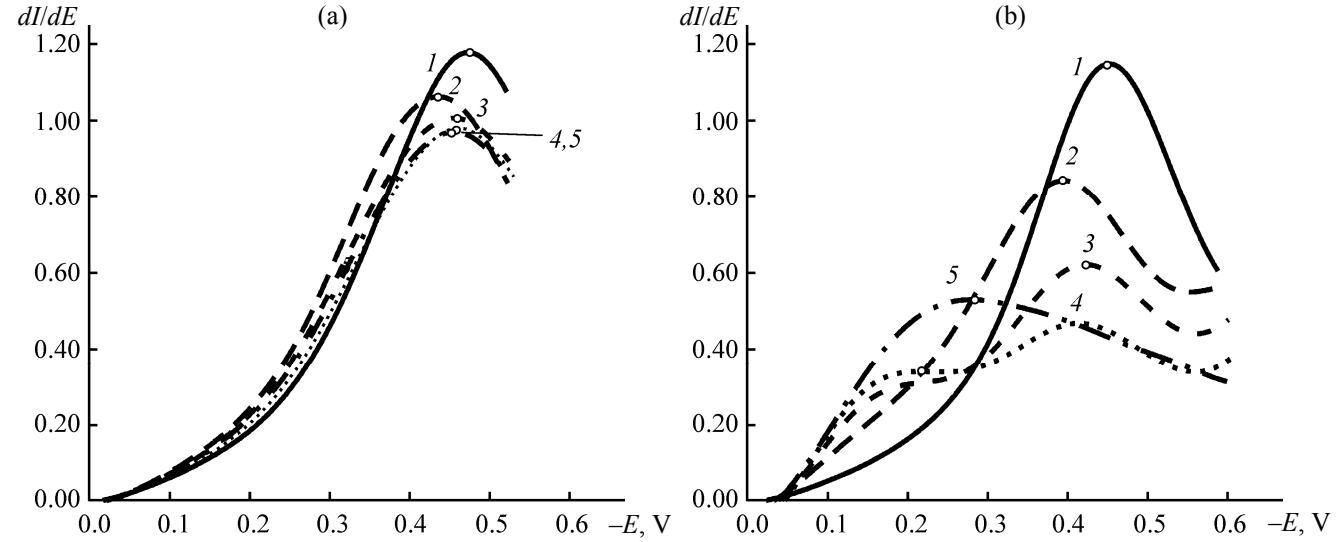


Fig. 2. (*1*) Differential voltammograms of reduction of Fe^{2+}, $C = 2.42\times10^{-3}$ mol l^{-1}, on a platinum cathode against 0.1 M. NaCl solution in water in the presence of different concentrations of (a) methionine and (b) ascorbic acid: (*2*) 0.19, (*3*) 0.38, (*4*) 0.56, (*5*) 0.74×10^{-3} mol l^{-1}.

pulsed mode (Fig. 3) the reduction waves can be isolated of molecular oxygen [Eq. (5)] with $E = -0.7$ V, of hydrogen peroxide [Eq. (6)] at $E = -1.1$ V, and of hydroxy radical [Eq. (4)] at $E = -0.2$ V, resulting from the one-electron reduction of hydrogen peroxide [Eq. (6a)].

$$^{\bullet}OH + \bar{e} \rightarrow OH^{-}, \quad (4)$$

$$O_2 + 2\bar{e} + 2H^{+} \rightarrow H_2O_2, \quad (5)$$

$$H_2O_2 + 2\bar{e} + 2H^{+} \rightarrow 2\,H_2O, \quad (6)$$

$$H_2O_2 + 1\bar{e} \rightarrow {}^{-}OH + {}^{\bullet}OH. \quad (6a)$$

Adding acetylcysteine to the solution causes the following changes in the voltammograms of oxygen reduction: reducing the limiting current of the first and third waves, a shift into the anodic region, and an increase in the current of the second wave (Fig. 3a). Similar changes in voltammograms also cause cysteine and glutathione. The differences are only in the degree of influence on each wave depending on the con-

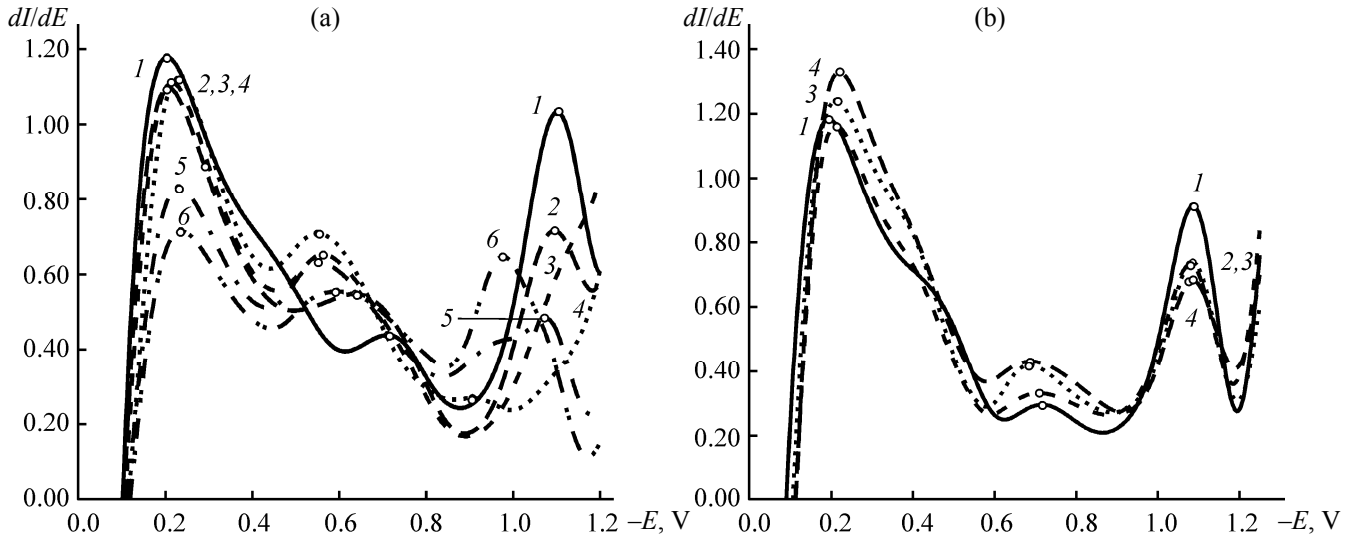


Fig. 3. Differential voltammograms of oxygen reduction on copper cathode against 0.1 M. (*1*) NaCl solution in water in the presence in various concentrations of: (a) acetylcysteine, (*2*) 0.19, (*3*) 0.38, (*4*) 0.56, (*5*) 0.74, (*6*) 0.91×10^{-3} mol l^{-1}, (b) methionine, (*2*) 0.29, (*3*) 0.48, (*4*) 0.91×10^{-3} mol l^{-1}.

centration of the added biologically active substances. The methionine causes similar changes in the second and third waves, while the limiting current of the first wave slightly increases with the concentration of this compound (Fig. 3b).

According to the obtained results, the studied biologically active substances containing sulfhydryl group interact with hydroxy radicals and hydrogen peroxide inhibiting the activity of the latter oxidants. They can be classified as the antiradical and antioxidant agents, capable, obviously, of affecting both the starting reactions of oxygen stress, and the reactions of chain propagation and chain transfer at the lipid oxidation with peroxides and the protein peroxide modification. Taking into account the effect of methionine on hydroxy radicals, we assume that it can exert the pro-oxidant action at the start of oxygen stress, and antioxidant action in the stage of lipid oxidation with peroxides and the protein peroxide modification.

Ascorbic acid in the investigated small concentrations increases the wave of hydroxy radicals, that is, shows a pro-oxidant effect (Fig. 4), which has been noted in biological systems [4]. However, at a concentration of 1.52×10^{-3} mol l^{-1} and above, it reduces the waves of hydroxy radicals and hydrogen peroxide, that is, exhibits antiradical, as well as a significant antioxidant effect.

As a criterion of relative antiradical and antioxidant activity of the investigated biologically active

Antioxidant activity of the studied biologicaly active substances

Compound	Preventive antioxidant activity	Antiradical activity	Antioxidant activity	Oxygen reduction potential shift, V	Total antioxidant activity of ascorbic acid with the test compounds	
					antiradical activity	antioxidant activity
Acetylcysteine	0.98	0.48	1.09	0.2	1.56	3.69
Cysteine	0.77	0.58	0.68	0.07	1.96	2.64
Glutathione	0.88	0.52	1.30	0.15	1.7	2.56
Methionine	0.35	–	0.56	0.03	1.44	1.22
Ascorbic acid	1.14	0.06	0.87	0.2	–	–

substances based on the proposed model can serve the differences in the wave heights of hydroxyl radical and hydrogen peroxide in the absence and the presence of the tested biologically active substance in a certain concentration, divided by the height of the wave of the baseline curve, $\Delta H/(H_0C)$ (see the table).

As can be seen from the table, the antiradical and antioxidant activities depend on the structure of the substance, and not always are the same for a given compound, apparently due to the difference in the mechanism of reactions with hydroxy radical and hydrogen peroxide.

Probably, the reaction of a biologicaly active substance with cysteine, acetylcysteine, and glutathione proceeds as the cleavage of the sulfhydryl group, and with ascorbic acid in the case of hydroxy radical as a cleavage of the hydroxy group to form water. Reaction with hydrogen peroxide most likely proceeds with the formation of peroxy radical $HO_2^{\bullet}$, as occurs in biological systems.

The table shows also the values of the anode shift of the molecular oxygen reduction potential in the presence of biologicaly active substance added to the cell. This shift indicates the facilitation of oxygen reduction typical for the complexation processes [21]. The complexation obviously contributes to the antioxidant effect of ascorbic acid and glutathione. The revealed possibility of complexation can apparently characterize to some extent the participation of a compound in the transport of oxygen in biological systems.

The developed electrochemical method for determining the effectiveness of antioxidant from the change in differential voltammograms of reduction of molecular oxygen we used to study the combined effect of ascorbic acid and the investigated sulfur antioxidant. The reason for this is the information that ascorbic acid actively cooperates with endogenous antioxidants in the body cells and interstitial fluid, in particular, with glutathione [4, 22].

As seen from Fig. 5, ascorbic acid at low concentrations (around 0.19×10^{-3} mol l^{-1}) increases the current of hydroxy radicals, that is, like in the biological systems [4, 22], has pro-oxidant effects. At the same time, ascorbic acid reacts actively with hydrogen peroxide, obviously similar to the action of peroxidase ascorbate occurring in biological systems.

$$2\text{Ascorbic acid} + 2H_2O_2 \rightarrow 2\text{Ascorbic acid}^{\bullet} + 2H_2O + O_2.$$

The ascorbate radical suffers fast disproportion into

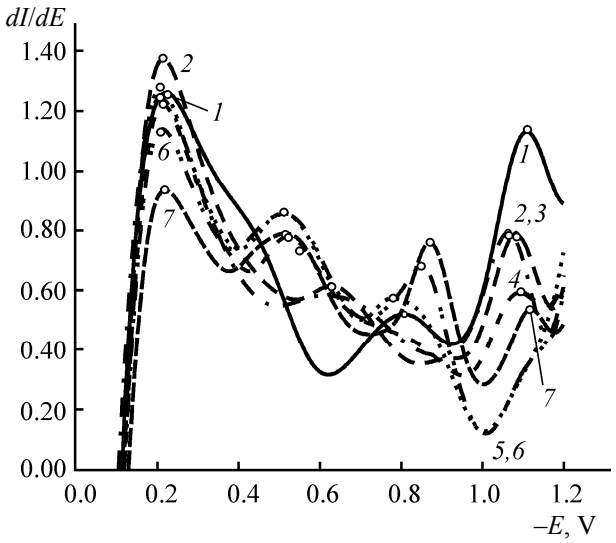


Fig. 4. Differential voltammograms of oxygen reduction on copper cathode against 0.1 M. (*1*) NaCl solution in water in the presence of ascorbic acid at the concentration: (*2*) 0.38, (*3*) 0.56, (*4*) 0.91, (*5*) 1.23, (*6*) 1.52, (*7*) 1.80×10^{-3} mol l^{-1}.

ascorbic and dihydroascorbic acids. At low concentrations, both glutathione and ascorbic acid exhibit pro-oxidant action toward the hydroxy radicals, while the combined presence in the solution of ascorbic acid and glutathione results in a strong decrease in wave heights of both the hydroxy radical and the hydrogen peroxide.

To show the combined action of ascorbic acid with glutathione, as well as with other studied compounds, we plotted the relative values of peak current of the waves of hydroxy radicals and hydrogen peroxide versus the concentration of ascorbic acid alone and together with the tested biologicaly active substance. On the basis of these relationships we determined the values of antiradical and antioxidant activity listed in the table. As seen from the table, at the combined presence of ascorbic acid and investigated biologicaly active substance a pronounced synergistic effect is observed in the interaction with both hydroxy radicals and hydrogen peroxide. It is interesting to note that the synergistic effect of methionine is the most pronounced with respect to hydroxy radicals. The maximum synergistic effect occurs at the ratio ascorbic acid : antioxidant = 1:2.

The mechanism of such combined action is not entirely clear, however, in biological systems in the conditions of oxygen stress, when the resource of enzymes is exhausted, a rise of mutual influence on the redox processes occurs of ascorbic acid and low

molecular endogenous antioxidants [4, 23]. In addition, it was shown in [23] that under strong oxidative stress ascorbic acid can be directly reduced by glutathione through both enzymatic and non-enzymatic pathways:

2GSH + 2Ascorbic acid → GSSG + 2Ascorbic acidN.

In the same study it was suggested that reduced glutathione interacts with the active form of oxygen, increases the acting concentration of ascorbic acid in a biological system, and thus enhances its antioxidant action [23]. The latter indicates that the approach to determination of combined action of antioxidants by voltammetry method proposed in this paper can greatly simplify such measurements reducing the labor consumption.

Thus, the presented approach to the voltammetric determination of the effectiveness of antioxidant based on the proposed mechanism of their interaction with the hydroxy radical, hydrogen peroxide, and ferrous ion, is, in our view, quite promising. Our hope is based on the correspondence between the obtained results and those described in the literature for biological systems [12–15].

EXPERIMENTAL

All investigated biologicaly active substances purchased from Merck (Germany) were used without further purification. Solutions of biologicaly active substance and $FeCl_2$ in 0.1 M aqueous NaCl were prepared just prior to measurements.

Background electrolyte (0.1 M NaCl, saline solution) was prepared from NaCl of chemically pure grade twice recrystallized from distilled water.

The oxygen concentration in the test solution corresponded to an equilibrium at atmospheric pressure and temperature of 20°C [5].

Studies were performed using polarograph IP-1 connected with a computer, in a three-electrode cell. The differential current–voltage curves of oxygen reduction in 0.1 M NaCl in water were registered in a special pulse mode using a copper cathode [11]. In the same mode were registered the waves of ferrous ion on the platinum cathode. The potential of working electrode was set relatively to the silver chloride reference electrode. Auxiliary electrode was a platinum spiral.

The antioxidant activity was estimated from the changes in the current–voltage curves observed after adding a biologicaly active substance in the background solution.

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