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MOLECULAR BIOPHYSICS

On the Mechanism of Oxygen Activation in Chemical and Biological Systems

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Abstract—It is shown that activation of oxygen in model chemical and biological systems associated with the formation of superoxide anion radicals ($O_2^{\bullet-}$) leads to a decrease in the content of molecular oxygen in the buffer. The following chemical and enzymatic reactions were considered: autooxidation of adrenaline in an alkaline medium, phenazine methosulfate/NADH, as well as the xanthine–xanthine oxidase reaction. It was found that when the chemical transformation of the substrate occurs in each of these reactions O_2 is consumed from the buffer, leading to temporary hypoxic conditions. Nitro blue tetrazolium and superoxide dismutase were used to determine the production of anion radicals $O_2^{\bullet-}$. It was discovered for the first time that $O_2^{\bullet-}$ is produced in the glucose–glucose oxidase reaction, which is known as a reaction that does not generate superoxide. Catalase in reactions reacted with hydrogen peroxide, the product of $O_2^{\bullet-}$ disproportionation, and molecular oxygen was detected under study buffer. Thus, the following oxygen conversions in the studied reactions are shown: $O_2 \rightarrow O_2^{\bullet-} \rightarrow H_2O_2 \rightarrow O_2$. They may also occur in intracellular systems with the involvement of appropriate oxidoreductases and antioxidant enzymes.

Keywords: superoxide, hydrogen peroxide, polarography, adrenaline, phenazine methosulfate/NADH, xanthine–xanthine oxidase, glucose–glucose oxidase, superoxide dismutase, catalase

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INTRODUCTION

In the family of reactive oxygen species, superoxide radical anions ($O_2^{\bullet-}$) play a special role, because this is the initially formed activated oxygen, which differs from the molecular oxygen present in the air or dissolved in water and biological fluids. In biological systems, at the cellular level, superoxide anions were previously considered exclusively as toxic substances [1–4]. It has been established that at certain concentrations $O_2^{\bullet-}$ radicals are essential components of the life activities of the cell and perform the most important functions in the organism: they can be priming compounds and signal molecules that trigger cascades of biochemical and, as a result, physiological processes [3–9]. Excess of $O_2^{\bullet-}$ and insufficiency of the antioxidant defense system cause oxidative stress, inflammation, and pathological processes [4–6]. The cell requires a balance between the generation of reactive oxygen species and the activity of the antioxidant defense. These processes are regulated at the molecular-genetic level by gene expression and subsequent synthesis of the corresponding antioxidant proteins. The balance at the metabolic level is also maintained by low-molecular-weight compounds with antioxidant properties (ascorbates, urates, etc.). The production of reactive oxygen species is associated with redox reactions in the cell that provide redox homeostasis. The "redox state" of a cell, tissue, or organ as a whole is a modern term. A scientific direction that investigates these processes has emerged [10], where the study of signaling pathways, namely, the redox-sensitive Nrf2/Keap1/ARE signaling system, which is involved in the regulation of cell proliferation, differentiation, and apoptosis (autophagy), plays an important role [4–10]. The study of the mechanism of signal transmission and the role of antioxidants in these processes is a relevant problem. Therefore, it is important to know what happens when molecular oxygen is activated. In this study, using model chemical and enzymatic reactions, we have shown the transition of molecular oxygen to a metabolically active state, the formation of superoxide and its subsequent conversions.

Abbreviations: SOD, superoxide dismutase; NBT, nitro blue tetrazolium; PMS, phenazine methosulfate; PBS, phosphate-buffered saline.

The main pathway of molecular oxygen activation is one-electron reduction (Eq. (1)):

$$O_2 + e^- \rightarrow O_2^{\bullet -}. \quad (1)$$

Electrons are supplied by chemical and enzymatic reactions, the so-called superoxide-generating systems, in which, during the substrate conversion, the electrons can be accepted by the oxygen dissolved in the medium to form $O_2^{\bullet -}$. As shown in our previous study [11], other radical compounds (carbonate anion radicals ($CO_3^{\bullet -}$) and carbon dioxide radicals ($CO_2^{\bullet -}$)) can arise in the same way. In the body, oxygen is activated with the involvement of enzymes oxidoreductases: electrons are supplied primarily by the mitochondrial electron transport chain and membrane NAD(P)H oxidases [4]. The level of $O_2^{\bullet -}$ in the cell is regulated by the enzyme superoxide dismutase (SOD), which, as a result of the disproportionation (dismutation) reaction, converts $O_2^{\bullet -}$ into hydrogen peroxide (H_2O_2) (Eq. (2)), thus being the primary component of the antioxidant defense system:

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2. \quad (2)$$

The purpose of this study was to experimentally show the "fate" of molecular oxygen dissolved in a buffer and involved in chemical and enzymatic reactions in which the conversion of substrate is coupled with the formation of $O_2^{\bullet -}$. The reactions used as superoxide-generating model systems for spectrophotometric methods for determination of SOD activity were performed in a polarographic cell [12–14]. In these reactions, superoxide anions are identified using a specific reagent, nitro blue tetrazolium (NBT), which under the influence of $O_2^{\bullet -}$ is reduced to colored diformazan and is recorded at a wavelength of 560 nm [12–15]. SOD inhibits this process and the percentage of formed diformazan serves as a measure of enzyme activity. This is a generally accepted approach that is used in various spectrophotometric methods for measuring enzyme activity [12–15]. We used the following model superoxide-generating systems: the adrenaline autooxidation reaction in an alkaline medium [11, 12, 16–19], the phenazine methosulfate/NADH (PMS/NADH) system [13], and the xanthine–xanthine oxidase reaction [14]. A similar experiment was performed with the glucose–glucose oxidase enzymatic reaction, which, however, is not called superoxide-generating and is usually used as a source of H_2O_2 [20]. It should be noted that since hydrogen peroxide is formed from $O_2^{\bullet -}$ as a result of the dismutation reaction it is likely that this oxidase reaction should also initially produce the superoxide anion. However, such data have not been found in the literature. Using a certain approach, we have also identified $O_2^{\bullet -}$ in this reaction for the first time.

MATERIALS AND METHODS

Polarography. The experiments were performed using a polarographic device with a closed platinum Clark electrode in a thermostated 1-mL cell at a fixed stirring rate. The system for automatic registration of oxygen consumption (ADC and software) was developed by V.A. Shlektarev (Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences) and V.S. Semenov (Institute of Cell Biophysics, Russian Academy of Sciences) [21]. The conditions of each model reaction described in the literature for spectrophotometric methods were reproduced in a polarographic cell with some correction (e.g., temperature, concentrations of substances, etc.), which is indicated in the captions to the figures.

Adrenaline autooxidation. The reaction of adrenaline autooxidation in an alkaline carbonate buffer was performed according to the protocol developed in our previous study [22]. The reaction was performed in 0.2 M carbonate buffer (pH 10.55) at a temperature of 37°C. The reaction was started by adding 0.23 mM adrenaline hydrochloride to the cell.

PMS/NADH reaction. The reaction conditions described in the spectrophotometric study [13] were also observed in the polarographic experiments, except that NBT was not added to the medium. The reaction was performed in potassium phosphate buffer (PBS) (150 mM KCl and 20 mM KH_2PO_4 (pH 7.4)) containing 60 μM PMS. The reaction was started by adding 150 μM NADH. The reaction temperature was 24°C.

The xanthine–xanthine oxidase enzymatic superoxide-generating reaction. In the polarographic study, we reproduced the reaction conditions described in [14]: 50 mM sodium carbonate buffer with 0.4 mM EDTA (pH 10.2). The content of xanthine oxidase was 11.0 μg protein/mL, NBT was absent. The reaction was started by adding 100 μM xanthine. The reaction temperature was 26°C.

The glucose–glucose oxidase enzymatic system. The reaction was performed in PBS buffer (pH 7.3) containing 5 mM glucose [20, 23]. The reaction was started by adding the enzyme. The amount of glucose oxidase in the cell was 7.5 μg protein/mL, the reaction temperature was 21°C.

Spectral measurements were carried out in a thermostated cell in a UV/VIS Uvikon-923 spectrophotometer (Italy) in the time driver mode at a wavelength of 560 nm (diformazan detection) or 347 nm (adrenochrome detection).

Reagents. In the study, we used Na_2CO_3, nitro blue tetrazolium, xanthine, xanthine oxidase (EC 1.1.3.22), catalase (EC 1.11.1.6), superoxide dismutase (EC 1.15.1.1), and glucose oxidase (EC 1.1.3.4) from Sigma (United States); $NaHCO_3$ from JT Baker (Netherlands); PMS and NADH from Sigma-Aldrich, Fluka (Germany); and 0.1% solution of adrenaline hydrochloride

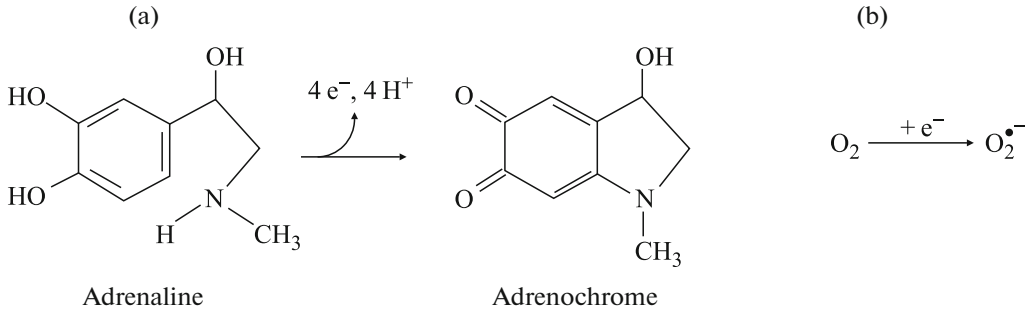

Fig. 1. A brief scheme of quinoid oxidation of adrenaline to adrenochrome (a) coupled with the formation of superoxide anion radicals (b). For details, see [17, 19].

(a Pharmaceutical form) from Moscow Endocrine Plant (Russia).

Statistics. The results were statistically processed using Student's *t* test in MS Excel: the mean value (M) and the standard deviation (sd) were determined. The plots are the specific experimental curves selected from independent measurements performed in four to six replicates in each experiment.

RESULTS AND DISCUSSION

Oxygen activation in the adrenaline autooxidation reaction. This system is known as a process of multistage chemical conversion of catecholamine adrenaline to the end product adrenochrome. It is coupled with the formation of superoxide [24–26]. A simplified scheme of the oxidation of adrenaline is shown in Fig. 1; this chemical reaction is described in detail in the literature [24, 25] and our previous studies [11, 12, 16, 17, 19].

This reaction was first used as a model system of generating superoxide for determine SOD activity [26]. Earlier, using the polarographic method, we determined what happens in the cell during the autooxidation of adrenaline in an alkaline carbonate buffer [22]. Later, we studied this reaction using NBT [12].

Figure 2 shows a record of the polarographic registration of adrenaline autooxidation in an alkaline buffer: a decrease in the oxygen concentration in the medium associated with its use in this chemical reaction is observed (curve *1*). Apparently, the transition of molecular oxygen to the radical form occurs according to Eq. (1), and the platinum electrode does not detect this oxygen species. The superoxide anion formed in the medium is detected when NBT is added to the polarographic cell: diformazan, a colored product of the NBT reduction with superoxide is recorded (qualitative assessment). SOD added in the course of the reaction inhibits oxygen consumption (Fig. 2, curve *2*), which also indicates the appearance of superoxide in the cell. If SOD is present in the buffer, the addition of adrenaline causes an increased lag period, and a significantly reduced rate of oxygen consumption is observed (Fig. 2, curve *3*). The presence of an induction period is determined by the initiation time of the chain reaction, which is typical for such coupled processes. In the presence of SOD, chain initiation is probably slower and, therefore, oxygen consumption is reduced. The following explanation is also possible: SOD catalyzes the dismutation reaction, the product of which, in addition to hydrogen peroxide, is molecular oxygen. The platinum electrode, which registers the molecular oxygen, reflects both the appearance of oxygen in the cell and, simultaneously, its decrease in the course of adrenaline autooxidation.

A lag period and a decrease in the oxygen consumption rate are also observed in the presence of various agents with antioxidant properties, which makes it possible to quantify their antioxidant activity [22].

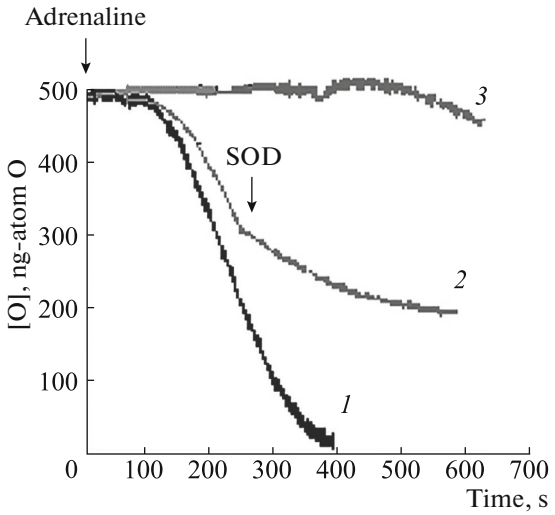

Fig. 2. Polarographic registration of oxygen consumption in the course of autooxidation of 0.23 mM adrenaline in 0.2 M carbonate buffer (pH 10.55): (*1*) adrenaline was added to the buffer; (*2*) the same, with the addition of SOD (5.5 μg/mL) during the reaction; (*3*) SOD was added to the buffer prior to adrenaline. Temperature 37°C.

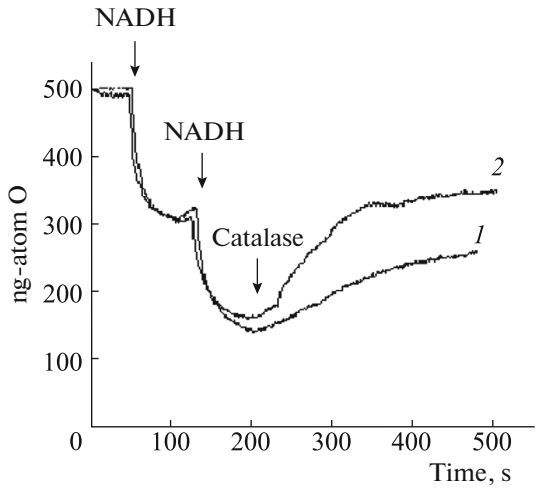

Fig. 3. Registration of oxygen consumption in the PMS/NADH chemical superoxide-generating system. Reaction conditions: PBS buffer (pH 7.4), 60 μM PMS in buffer. The arrow indicates the addition of NADH (twice, 150 μM): (*1*) spontaneous oxygen release; (*2*) effect of catalase addition (50 μg/mL). Temperature 24°C.

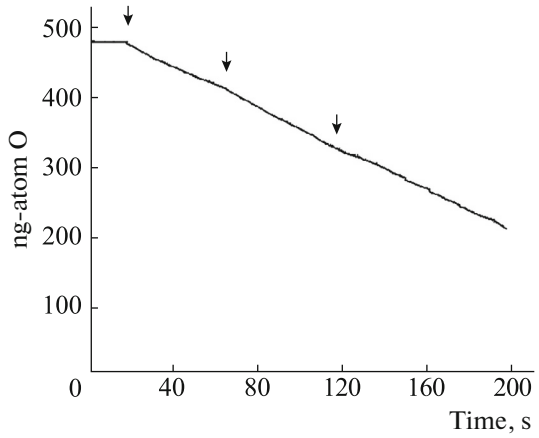

Fig. 4. Registration of oxygen consumption in the enzymatic superoxide-generating xanthine–xanthine oxidase reaction. Reaction conditions: 50 mM carbonate buffer (pH 10.2), 0.4 mM EDTA. The reaction was started by adding 100 μM xanthine; the arrows indicate repeated additions of the substrate. The enzyme concentration in the incubation medium is 11.0 μg/mL. Temperature 26°C.

In our previous methodical studies, the issue of oxygen activation in this reaction was not discussed [14, 18–22].

Oxygen activation in the PMS/NADH chemical reaction. This reaction is known as the superoxide-generating system. It is performed in the presence of NBT and underlies the spectrophotometric method developed for determining SOD activity [13]: the formation of diformazan, the product of NBT reduction with superoxide formed in this system, is measured at 560 nm. Polarographic studies of this reaction have not been found in the literature.

Figure 3 shows the polarographic registration of the process in this reaction: PMS is in the buffer, the addition of NADH causes intensive consumption of oxygen and its decrease in the medium. Each subsequent addition of NADH at a sufficient concentration of PMS in the buffer once again causes depletion of molecular oxygen. This picture is also observed when PMS is added to the cell when NADH is in the buffer (data not shown). In the course of oxygen consumption in this reaction, $O_2^{\bullet-}$ is formed. In the original spectrophotometric method superoxide is identified with the aid of NBT in the medium [15]. In our experiment, when NBT is added to the polarographic cell, intense formation of violet-blue diformazan is observed during the reaction. Figure 3 (curve *2*) shows that the added catalase allows detection of hydrogen peroxide, which is formed in the cell as a result of the disproportionation reaction according to Eq. (2): oxygen appears in the buffer at 177 n-g atom O/mL. The spontaneous appearance of oxygen in the medium (115 n-g atom O/mL) is also observed (Fig. 3, curve *1*).

Thus, in the PMS/NADH model system, oxygen is activated, and $O_2^{\bullet-}$ appears, followed by the formation of hydrogen peroxide.

Oxygen activation in the xanthine–xanthine oxidase enzymatic reaction. As a superoxide-generating model, this reaction is often used to determine the activity of SOD by the known spectrophotometric method: in the presence of NBT, the produced superoxide is identified [14]. The enzyme xanthine oxidase is a well-studied flavoprotein that catalyzes the reaction represented by the following equation [27]:

$$\text{xanthine} + O_2 + 2H_2O \rightarrow \text{uric acid} + 2H_2O_2. \quad (3)$$

In reality, this reaction proceeds in two stages, at the first of which $O_2^{\bullet-}$ is identified with NBT:

$$\text{xanthine} + O_2 + 2H_2O \rightarrow \text{uric acid} + 2O_2^{\bullet-} + 4H^+. \quad (4)$$

Figure 4 shows a polarographic curve that illustrates the process of oxygen use during the functioning of the enzyme. Xanthine oxidase is present in the buffer; the addition of the substrate to the cell initiates the enzymatic reaction coupled with the generation of superoxide, which proceeds according to Eq. (1). The platinum electrode registers the loss of molecular oxygen from the medium. Repeated additions of xanthine in this experiment do not stimulate the reaction rate, which indicates that the substrate is sufficient for the given amount of the enzyme. At other enzyme-to-substrate ratios, the activation of oxygen consumption in response to the repeated additions of the substrate was also observed (data not shown). The formation of superoxide in this process was also detected using NBT, which was used in the original method [14]. We

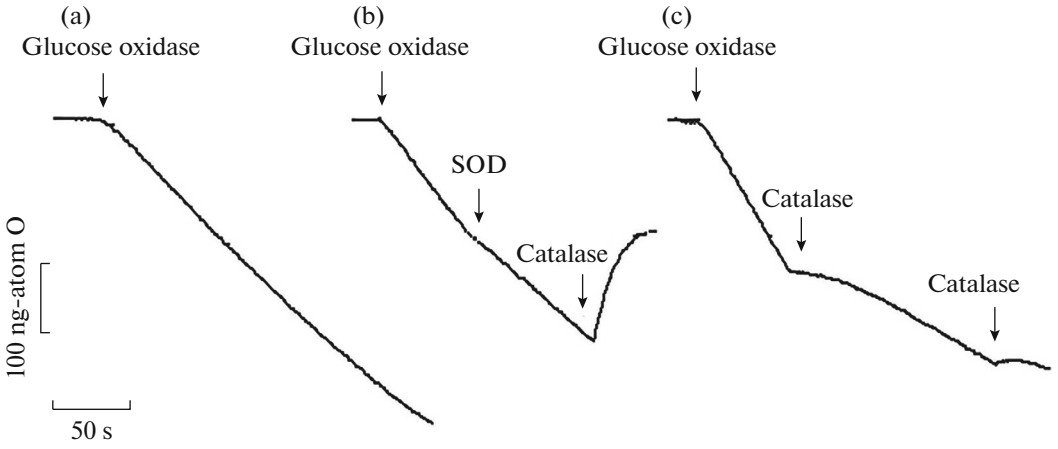

Fig. 5. (a) Registration of oxygen consumption during the functioning of glucose oxidase, the addition of enzymes hereinafter is indicated with an arrow; (b) under the same conditions, with the addition of SOD and then catalase; (c) catalase was added twice during the reaction. Reaction conditions: PBS buffer (pH 7.3), 5 mM glucose, 7.5 μg/mL glucose oxidase. Additions: SOD 5.5 μg/mL, catalase 50 μg/mL. Temperature 21°C.

initially used the spectrophotometric method to determine the enzyme activity and select the necessary ratios of the enzyme and the substrate and after that the polarographic experiments were performed.

Thus, it has been shown that in this superoxide-generating system a decrease in oxygen concentration and the generation of superoxide in the medium are observed.

It should be noted that under normal conditions in the body xanthine oxidase exists as xanthine dehydrogenase; however, at an excess of hypoxanthine and xanthine, formed during the oxidative metabolism of purines [27], this enzyme acquires the function of an oxidase and utilizes both of these compounds as substrates, catalyzing the formation of uric acid and thus being a potent producer of superoxide anions [28].

Oxygen activation in the glucose–glucose oxidase reaction. The enzyme glucose oxidase, similarly to xanthine oxidase, belongs to the class of oxidoreductases and contains the cofactor flavin adenine dinucleotide; however, the reaction catalyzed with the participation of glucose oxidase is not discussed in the literature as superoxide-generating (i.e., this is not about the formation of $O_2^{\bullet-}$). The equation for this reaction is given below:

$$\beta\text{-D-glucose} + O_2 + 2H_2O \xrightarrow{\text{Glucose oxidase}} \beta\text{-D-glucono-1,5-lactone} + 2H_2O_2 \xrightarrow{+H_2O} \text{Gluconic acid} \quad (5)$$

The chemical formulas present the stages of oxygen reduction by flavins in detail, and the assumption is made that the formation of a superoxide anion radical instead of H_2O_2 cannot be ruled out [27]. This enzymatic system is used as a model for H_2O_2-induced damage [20]; i.e., it is H_2O_2, but not $O_2^{\bullet-}$, that is considered as a damaging agent, although initially, obviously, superoxide anion is generated and then hydrogen peroxide as a product of the disproportionation reaction appears. This is not discussed in the literature.

We reproduced this reaction in a polarographic cell. The data presented in Fig. 5 demonstrate the loss of oxygen from the medium, which indicates the use of molecular oxygen during the chemical transformation of glucose (Eq. (5)). In the cell, the oxidation of glucose to gluconic acid is coupled with the activation of molecular oxygen. Figure 5b shows that SOD slows oxygen consumption, which is associated with the conversion of $O_2^{\bullet-}$ into hydrogen peroxide according to Eq. (2), and catalase detects H_2O_2 in the cell. The sensitivity of this reaction to SOD, as well as the appearance of H_2O_2, indicate the presence of $O_2^{\bullet-}$ in this system. The observed inhibition of the reaction after the addition of SOD is probably associated with the appearance of molecular oxygen in the cell as a result of the dismutation reaction, which is visually perceived as a slower rate of oxygen consumption, which is recorded by the platinum electrode. However, NBT added to the polarografic cell where occurs glucose

oxidation does not detect $O_2^{\bullet-}$ as was observed in the reactions studied above. The conditions of this reaction are possibly not optimal for superoxide. We changed the composition of the buffer to make its pH alkaline, because it is known that superoxide anions require carbonate/bicarbonate ions [11, 29] and that the lifetime of $O_2^{\bullet-}$ increases in an alkaline medium [30, 31]. Table 1 shows the results of spectrophotometric measurement of the diformazan formation under various reaction conditions. Diformazan does not appear in the medium after the addition of the enzyme if the reaction is performed in PBS buffer with pH 7.3 in the presence of the substrate and NBT; i.e., $O_2^{\bullet-}$ is not detected. Under alkaline conditions (0.2 M carbonate buffer (pH 10.55)), in the presence of the substrate and NBT, the addition of glucose oxidase immediately reveals colored diformazan and the color intensity in the cell increases with time (see Table 1). Obviously, this is the reason that alkaline carbonate buffers are used in superoxide generating systems [14, 16, 26]. The confirmation of this can be found in [30], where it was shown that the formation of superoxide and the yield of adrenochrome during adrenaline autooxidation increase at higher pH values and that H^+ is required for the disproportionation reaction and this process takes place at low pH values. Under alkaline conditions and in the presence of carbonate ions, the lifetime of superoxide anion radicals probably increases, which allowed us to detect $O_2^{\bullet-}$ in the glucose–glucose oxidase reaction.

It should be noted that glucose oxidase is widely used in industrial technologies (in food, chemical, and pharmaceutical industries, in clinical chemistry, biotechnology and other industries) for the disinfection and preservation of products, because it "removes" oxygen from the medium using endogenous glucose as a substrate and molecular oxygen in as an electron acceptor, with the simultaneous release of H_2O_2, as is known from the literature [32]. However, according to our data, it is superoxide that forms first, and only then does hydrogen peroxide form. Both of these products have a bactericidal effect and in practice it is probably not important which of them is the "killer" of bacteria, superoxide radical $O_2^{\bullet-}$, which is initially formed in this reaction, or H_2O_2, which is produced further from $O_2^{\bullet-}$. In scientific experiments it should be considered that the primary reaction product in the reactions catalyzed by glucose oxidase is the superoxide anion. Immobilized preparations of this enzyme are used as a biosensor in the operation of glucometers.

Table 1. Formation of diformazan during the functioning of glucose oxidase

Time, min	Optical density, $E_{560\,nm}$	
	pH 7.3*	pH 10.55**
1	0.022 ± 0.003	0.0433 ± 0.003
5	0.022 ± 0.003	0.0838 ± 0.003

Reaction conditions: * PBS buffer (pH 7.3), 7.5 mM glucose, 125 μM NBT, 6.0 μg/mL glucose oxidase; ** 0.2 M carbonate buffer (pH 10.55), 7.5 mM glucose, 125 μM NBT, 6.0 μg/mL glucose oxidase.

Thus, in this study it was experimentally shown that in chemical and enzymatic systems $O_2^{\bullet-}$ forms first. This is activated oxygen, which is then converted to hydrogen peroxide either spontaneously or with the involvement of SOD, and, most importantly, the molecular oxygen enters the medium again. The appearance of molecular oxygen in the medium, in the cells of the body, is provided by catalase and peroxidase. Thus, the oxygen homeostasis is maintained in the body. The oxidase-mediated process of the conversion of molecular oxygen into superoxide is accompanied by a decrease in oxygen content in the medium; i.e., the hypoxic conditions onset. The appearance of oxygen with the involvement of catalase and/or SOD can restore its balance in the cell. These conversions of oxygen are shown in the present study:

$$O_2 \rightarrow O_2^{\bullet-} \rightarrow H_2O_2 \rightarrow O_2.$$

It should be noted article by Timochko et al. [33]. These authors formulated a hypothesis about the presence of a reserve depot of oxygen in the body in the form of hydrogen peroxide and lipid hydroperoxides, which is functionally associated with the peroxidase–catalase system and SOD. This system can release molecular oxygen and thus maintain an oxygen gradient in organs and tissues [33]. The authors introduced the concept of "endogenous oxygen." Initially, it is depleted from the medium and transits to an activated state, after which a "reserve depot" of oxygen is created, with subsequent release of oxygen from hydrogen peroxide and lipid hydroperoxides under the influence of the corresponding enzymes [33].

It should also be noted that much earlier the term "activated oxygen" or "metabolically active oxygen" was first introduced by A.N. Bach [34, 35]. However, in that works, were studied solely hydrogen peroxide, from which the "activated oxygen" is formed under the influence of catalase or peroxidase. According to the modern concept, the emphasis is somewhat different: initially, superoxide is formed in chemical and enzymatic (oxidase) reactions in the cell and in the body, and only then is hydrogen peroxide formed, whose level is regulated by peroxidase and catalase. Also A.N. Bach was the first who to determine the important positive role of hydrogen peroxide in the life of living organisms [35].

CONCLUSIONS

In the studied model systems, it was experimentally shown that in chemical reactions and in the course of functioning of enzymes of the oxidoreductase class (oxidases), superoxide anions, which are metabolic activated oxygen, are initially formed. Oxygen conversions ($O_2 \rightarrow O_2^{\bullet-} \rightarrow H_2O_2 \rightarrow O_2$) may take place in intracellular systems with the involvement of the corresponding oxidoreductases and antioxidant enzymes.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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