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PEROXIDASE ACTIVITY OF ERYTHROCYTES HEMOGLOBIN UNDER ACTION OF LOW-FREQUENCY VIBRATION

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Background. Hemoglobin is a hemoprotein which in the presence of oxidative equivalents, such as H_2O_2, can act as peroxidase with a very high oxidative potential. Hemoglobin oxidation is accompanied by generation of highly oxidized forms of iron and globin radicals that have high oxidative activity and are toxic to cells. In addition, peroxidase activity may indicate structural changes that occur in the hemoglobin molecule as a result of chemical modification.

Materials and Methods. Erythrocyte suspension was subjected to vibration for 3 h within the frequency range from 8 to 32 Hz with amplitudes of 0.5 ± 0.04 and 0.9 ± 0.08 mm. At certain intervals, hemoglobin peroxidase activity was determined together with the content of its ligand forms in the hemolysates of cells. Additionally, experiments were performed to investigate the mechanism and calculate the kinetic parameters of peroxidase reaction.

Results and Discussion. Experimental data on low-frequency vibrations effect on erythrocyte hemoglobin peroxidase activity were analyzed. The kinetics of the oxidation reaction of *p*-phenylenediamine by hemoglobin in erythrocytes was studied. It was found that peroxidase oxidation has a ping-pong mechanism. The kinetic parameters of the peroxidase reaction involving hemoglobin were determined. The change of kinetic parameters after two-hour exposure to the incubation medium and low-frequency vibration was studied. A possible mechanism of action of hemoglobin in oxidation reactions involving H_2O_2 was proposed.

Conclusion. Any effect that initiates the formation of methemoglobin leads to an increase in the peroxidase activity of hemoglobin due to the involvement of the latter in the pseudoperoxidase cycle and the formation of toxic reactive globin radicals. The high

content of oxyhemoglobin in the cell, observed under vibrations within the frequency range of 16–32 Hz with an amplitude of 0.9 ± 0.08 mm, can prevent its oxidation and involvement in the pseudoperoxidase cycle.

Keywords: ligand forms of hemoglobin, enzymatic kinetics, kinetic constants, pseudoperoxidase cycle, heme, hypoxia, *p*-phenylenediamine

INTRODUCTION

Hemoglobin is one of the most studied animal proteins, the function of which is related to oxygen transport. However, additional functions of hemoglobin are known as well (Paco *et al*., 2009; Kosmachevskaya & Topunov, 2018). Some of them have physiological significance and evolved to increase the adaptive potential of cells, others are the result of chemical and thermodynamic properties of the protein (Kosmachevskaya & Topunov, 2018).

Hemoproteins are of interest for researchers who study structure-activity relationship, firstly, because the active site, iron porphyrin, is the same for all hemoproteins and secondly, because additional activity could arise from the structural and conformational properties of this protein. Thus, peroxidase activity of hemoglobin can be used to test for any structural changes that may have occurred in the hemoglobin molecule as a result of chemical modifications. The consequence of these changes may be a change in the oxidative activity of modified hemoglobin (Everse *et al*., 1994). Although H_2O_2 binds directly to heme iron, the substrate can donate its electrons to the edge of the heme or in some cases even interact with the protein part of the enzyme (similar to peroxidases), because some substrates are too bulky to penetrate the heme pocket (Everse *et al*., 1994). According to the abovesaid, any changes that occur inside the chemical or structural heme environment can possibly lead to changes in oxidation rate of its substrate.

The dissociation of hemoglobin into monomers or the formation of crosslinks due to the oxidation of amino acid residues can lead to an increase in peroxidase activity of hemoglobin. Reeder *et al*. demonstrated (Reeder, 2010; Reeder, 2017) that the formation of a covalent bond between heme and protein fragment for Hb or Mb treated with hydrogen peroxide increases peroxidase activity and toxicity of the latter.

It is accepted that met- and oxyhemoglobin belong to pseudoperoxidases group, heme-containing proteins that perform essential functions in cells and were not originally intended to interact with H_2O_2, but could be converted into peroxidases as a result of environmental changes and development of oxidative stress (Vlasova, 2018). When interacting with H_2O_2, these compounds give hypervalent ferryl heme iron (FeIV = O) and globin tyrosyl radical (Svistunenko *et al*., 2002; Witting *et al*., 2002; Reeder, 2017; Huo et al., 2021), which is able to initiate further oxidation reactions (Dotsenko *et al*., 2018).

However, there is evidence that globins are true peroxidases with a specific biological role (Reeder, 2017). It was found that in the presence of peroxide and ascorbate or urate, oxyhemoglobin functions as a true enzymatic peroxidase (Cooper *et al*., 2008; Cooper *et al*., 2013). Peroxidase activity of oxyhemoglobin is low (this value is 5 times higher for methemoglobin compared to oxyhemoglobin). A high content of oxyhemoglobin promotes the consumption of hydrogen peroxide in large quantities, thereby preventing the oxidation of hemoglobin and the entry of H_2O_2 into the pseudoperoxidase cycle. The consequence of these processes is the inhibition of toxicity of ferryl forms of hemoglobin (Widmer *et al*., 2010; Huo *et al*., 2021).

Peroxidase activity of hemoglobin remains obscure, for example there is a lack of research data on physiological role of hemoglobins peroxidase activity and its role during hypoxia. So far, it has been accepted that mechanical stress could affect the properties of biomolecules and cells (Shatalov, 2012; Dotsenko & Troshchynskaya, 2014; Bunkin *et al*., 2020). Under conditions of vibration, it is convenient to simulate both oxidative stress and hypoxia by changing the parameters and duration of vibrations. In parallel, it is possible to study the effect of low-frequency vibrations on the state of human erythrocytes. Testing for hemoglobin peroxidase activity can be useful for understanding the state of hemoglobin in cells and their viability.

The aim of the study was to examine changes in peroxidase activity of human erythrocytes under the conditions of low-frequency vibrations, study the kinetics and discover the parameters of peroxidase reaction involving hemoglobin. We have proven that the erythrocyte peroxidase activity depends on the ratio of the hemoglobin ligand forms content in erythrocytes when the oxygen content in the cell surface changes.

MATERIALS AND METHODS

The protocol of the experimental part was in compliance with the principles of biological ethics and was agreed upon with the Local Ethics Committee of the Vasyl' Stus Donetsk National University, Faculty of Chemistry, Biology and Biotechnology (Vinnytsia, Ukraine). The meeting of Bioethics Commission No 2 of January 11, 2021.

Peripheral blood of healthy female donors of approximately the same age (45 ± 5.5 years) was used in the study. Erythrocytes were separated from plasma by centrifugation and washed three times with Na-phosphate buffer (0.015 mol, pH 7.4) (buffer solution 1) containing 0.15 mol NaCl. In the obtained erythrocyte paste, the total hemoglobin content was determined by the hemoglobin cyanide unified method using a standard kit.

The resulting erythrocyte paste was introduced into the medium of the same buffer solution. The hemoglobin content in the test suspensions was at the level of 1.4 ± 0.08 mg/mL. The suspensions were subjected to low-frequency vibrations for 3 h within the frequency range from 8 to 32 Hz at room temperature. The vibration frequency varied with step of 4 Hz. The vibration amplitude was maintained at 0.5 ± 0.04 and 0.9 ± 0.08 mm. The vibrations were generated using a vibrating stand which consisted of a sinusoidal low-frequency signal generator, an amplifier and a vibrator that oscillates in the vertical plane with a given frequency and amplitude. The experimental cuvette filled with the erythrocyte suspension was fixed vertically tightly on the moving part of the vibrator (in this case, mechanical vibrations are transmitted to the experimental cuvette with a slight loss of power).

The peroxidase activity was determined in the fresh lysates of cells. Erythrocyte lysate was obtained by lysis of cells in 0.01 M Na-phosphate buffer (pH 7.4) in cold for 10 min.

As a control, erythrocytes contained in buffer solution 1 and not exposed to vibration were used. The three-hour effect of the incubation medium on the peroxidase activity of hemoglobin was additionally tested.

Determination of hemoglobin peroxidase activity. All spectrophotometric studies were performed on SPEKOL® 1500 UV/Vis spectrophotometer at room temperature.

The concentration of hydrogen peroxide in the initial solution was measured using the value of the extinction coefficient ε_{240} = 43.6 $M^{-1}\times см^{-1}$. A stock solution of *p*-phenylenediamine (PDA) was prepared by dissolving the exact portion in buffer solution 1.

The level of PDA activity was determined spectrophotometrically, recording the oxidation of PDA at 485 nm for 3 min. To determine the activity, 0.1 mL of erythrocyte lysate was added to 2 mL of 20 mM *p*-phenylenediamine solution (pH 7.4). The reaction was started by the introduction of 0.1 mL of hydrogen peroxide. The concentration of hydrogen peroxide in the reaction mixture was 2 mM. All measurements were performed at 37 °C. The initial rate of reaction (v_0) was determined from the initial linear sections of the kinetic dependency. The enzymatic activity was calculated using a molar extinction coefficient 1.545×10^3 M^{-1} $см^{-1}$ and expressed in mM of phenylenediamine oxidized in 1 min.

Calculation of catalytic constants. The rate constants in the oxidation reaction of *p*-phenylenediamine, Michaelis constants for both substrates were determined based on stationary kinetics. For this purpose, the concentrations of both substrates were varied: in the range of 4.5 to 23 mM for *p*-phenylenediamine and 1.5 to 3.5 mM for H_2O_2. The concentration of H_2O_2 was maintained at 2 mM during the variation of *p*-phenylenediamine. The concentration of *p*-phenylenediamine was maintained at 20 mM during the variation of H_2O_2. The concentration of hemoglobin in the sample was 0.15 ± 0.013 mg/mL. The initial reaction rate was recorded as described above. The catalytic constant and Michaelis constants for substrates were calculated from the equation for the ping-pong type peroxidase mechanism defined by Dunford:

$$\frac{E_0}{\nu} = \frac{1}{k_{cat}} \cdot \frac{K_{m\mathrm{H_2O_2}}}{[\mathrm{H_2O_2}]} + \left(\frac{1}{k_{cat}} \cdot \left(1 + \frac{K_{m\mathrm{PDA}}}{\mathrm{PDA}} \right) \right),$$

where $[E]_0$ is the concentration of hemoglobin in the sample; k_{cat} – catalytic constant, corresponding to the monomolecular rate-limiting step; $K_{m\mathrm{H_2O_2}}$ – Michaelis constant for hydrogen peroxide; $K_{m\mathrm{PDA}}$ is the Michaelis constant for the *p*-phenylenediamine donor substrate. The kinetic parameters for the peroxidase reaction (k_{cat}, $K_{m\mathrm{H_2O_2}}$, $K_{m\mathrm{PDA}}$) were determined before the start of the experiment and after 2 h of exposure to buffer medium and vibration using the gradient descent method.

Determination of the hemoglobin ligand forms content. The resulting hemolysate was centrifuged to precipitate membrane-bound hemoglobin. The absorption spectra of the cytoplasmic fraction of erythrocyte hemoglobin were recorded in the wavelength range 500–700 nm in cuvettes with a thickness of 1 mm.

The total hemoglobin content in the cytoplasmic fraction of hemoglobin was determined by absorption at a wavelength of 523 nm using an extinction coefficient of 7120 M/cm (Ratanasopa *et al*., 2015).

To determine the content of ligand forms of hemoglobin, absorption at 540, 560, 576 and 630 nm (Dotsenko *et al*., 2020) was used. The content of ligand forms of hemoglobin (in M) was calculated using the equations given in (Dotsenko *et al*., 2020). The content of each form was expressed as a percentage of the total hemoglobin content in the cytoplasmic fraction.

All experiments were performed in triplicate (n =3), and their results were analyzed in Statistica 8.0 (StatSoft Inc., USA). Experimental data is presented as x ± m (x is the average, m is the relative error). Differences between kinetic parameters in the control

and experimental samples were determined using the Wilcoxon T-test. The critical level of significance in testing of statistical hypotheses was taken as equal to $p < 0.05$. Three-dimensional scattering plots were used to present the obtained experimental data and to identify the relationships between the studied parameters. Based on the network of starting points, the surface was built by the method of inversely weighted distances. To construct the distribution curves of the ligand forms of hemoglobin, we used data approximation by the method of least squares according to the polynomial regression equation of the 6th degree.

RESULTS AND DISCUSSION

Analysis of changes in erythrocyte hemoglobin peroxidase activity under vibration conditions. In fig. 1, 2 the change in the erythrocyte hemoglobin peroxidase activity depending on the frequency, amplitude and time of vibration are shown. At the beginning of the experiment, the activity was in the range of 0.22–0.4 mM/min×g Hb, an according to the figure is highly dependent on the ratio of oxyHb/metHb in cells. Under the action of vibrations within the frequency range of 8–32 Hz with an amplitude of 0.5 ± 0.04 mm peroxidase activity dose-dependently increased and after 3 hours of exposure exceeded the initial level by 1.5–1.8 times (**Fig. 1**). When vibrating in the frequency range of 16–28 Hz with an amplitude of 0.9 ± 0.08 mm peroxidase activity increased more than twice after 2 hours of exposure (**Fig. 2**).

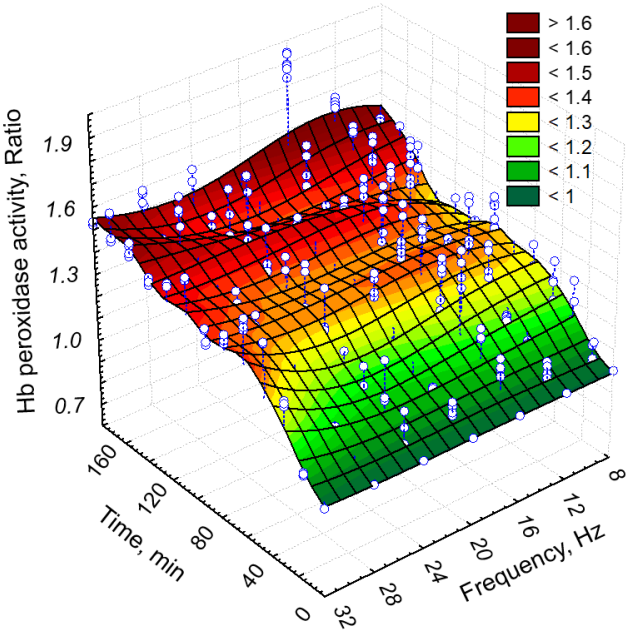

Fig. 1. Peroxidase activity of erythrocyte lysate under the conditions of vibration in the frequency range of 8–32 Hz with amplitudes of 0.5 ± 0.04 mm depending on the incubation time of cells. N (the number of points on the surface) = 108, n = 3

Рис. 1. Пероксидазна активність гемолізату еритроцитів в умовах вібраційного впливу в інтервалі частот 8–32 Гц з амплітудами 0,5 ± 0,04 мм залежно від часу інкубування клітин. N (кількість точок на поверхні) = 108, n = 3

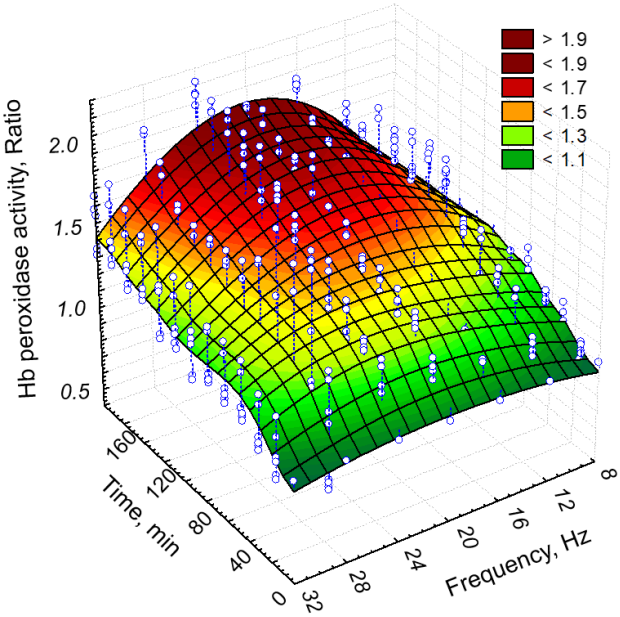

Fig. 2. Peroxidase activity of erythrocyte lysate under the conditions of vibration in the frequency range of 8–32 Hz with amplitudes of 0.9 ± 0.08 mm depending on the incubation time of cells. N (the number of points on the surface) = 108, n = 3

Рис. 2. Пероксидазна активність гемолізату еритроцитів в умовах вібраційного впливу в інтервалі частот 8–32 Гц з амплітудами 0,9 ± 0,08 мм залежно від часу інкубування клітин. N (кількість точок на поверхні) = 108, n = 3

Analysis of kinetic parameters of peroxidase reaction. To determine the kinetic constants of peroxidase reaction, graphs of the reaction rates dependency on the concentration of H_2O_2 and PDA in double inverse coordinates (Lineweaver–Burk plot) at a fixed concentration of another substrate were constructed. In these coordinates, the dependency is linear.

The obtained values of peroxidase reaction constants for hemoglobin of erythrocytes that were not exposed to vibrations are shown in **Table 1**. Given rather high values of Michaelis constants for substrates, it can be argued that, unlike heme peroxidases in plants, mammalian Hb peroxidase activity has not evolved to remove peroxide from biological solutions. For hemoglobin, the rate of H_2O_2 reduction is slower than PDA oxidation, which means that the reaction with H_2O_2 is a rate-limited step. Besides, a fairly wide range of changes in the values of the constants indicates that the peroxidase reaction involves both oxy- (oxyHb) and methemoglobin (metHb) and the found constants do not characterize a particular form of hemoglobin.

When interacting with H_2O_2, OxyHb can form a compound II peroxidase cycle, in which heme iron is present in the oxoferryl form (Reeder, 2017; Vlasova, 2018):

$$P - Fe^{2+} + H_2O_2 \rightarrow \underset{\text{Compound II}}{P - [Fe^{4+} = O]} + H_2O. \quad (1)$$

OxyHb is easily oxidized to methemoglobin (Liu *et al*., 2020; Huo *et al*., 2021):

$$P - Fe^{2+} \cdot O_2 + H_2O_2 \rightarrow P - Fe^{3+} + O_2 + H_2O. \quad (2)$$

Interaction between hydrogen peroxide and methemoglobin leads to the formation of compound I, which is most likely an oxoferryl porphyrin-*p*-cation radical. Compound I immediately oxidizes aminoacid residues (Tyr, Trp, His), which are located near heme, with the formation of radicals based on protein and oxoferrylheme (Vlasova, 2018).

$$P - Fe^{3+} + H_2O_2 \rightarrow {}^{+}P^{\bullet} - [Fe^{4+} = O] + H_2O, \quad (3)$$

Compound I

$${}^{+}P^{\bullet} - [Fe^{4+} = O] \rightarrow P^{\bullet} - [Fe^{3+} = O] + P - [Fe^{4+} = O], \quad (4)$$

Compound II

$$P - [Fe^{4+} = O] + AH_2 \rightarrow P - Fe^{3+} + AH^{\bullet}, \quad (5)$$

$$P^{\bullet} - [Fe^{3+} = O] + AH_2 \rightarrow P - Fe^{3+} + AH^{\bullet}, \quad (6)$$

Peroxidase substrates can be oxidized in two ways: by direct electron transfer between the reducing agent and heme, if heme in hydrophobic pocket is available for the substrate (equation 5); or by a tyrosyl radical on the surface of the protein (equation 6), or in two ways simultaneously. Mediation of substrate oxidation by tyrosyl radicals accelerates heme recovery (Reeder, 2010; Vlasova, 2018). Obviously, these processes are present in erythrocytes with ascorbate as a natural substrate. The consequence of the formation of tyrosyl radicals is the formation of crosslinks and aggregation of protein, which reduces the oxidative potential of peroxidase (Vlasova, 2018).

Analysis of the obtained data showed that both incubation of cells in buffer solution and vibration creates conditions for non-competitive activation of hemoglobin peroxidase activity. Incubation of erythrocytes in Na-phosphate medium for 2 h does not change the catalytic constant of the reaction, but leads to a significant $K_{m\mathrm{H_2O_2}}$ increase and $K_{m\mathrm{PDA}}$ decrease (**Table 1**). Thus, the rate of the peroxidase process increases due to the increase in affinity for PDA. In this case, the high-affinity pathway involves electron transfer through the protein, utilizing a tyrosine as a redox-active center. Mediation of a substrate oxidation by tyrosyl radicals accelerates heme reduction but decreases the oxidative potential of peroxidase (Svistunenko *et al*., 2002; Vlasova, 2018).

Table 1. **The effect of incubation medium on the steady-state kinetic constants of peroxidase reaction involving hemoglobin**

Таблиця 1. **Вплив середовища інкубування на стаціонарні кінетичні константи пероксидазної реакції за участю гемоглобіну**

Fresh erythrocytes			Erythrocytes incubated in the medium for 2 hours		
k_{cat}, 1/min	$K_{m\mathrm{H_2O_2}}$, mM	$K_{m\mathrm{PDA}}$, mM	k_{cat}, 1/min	$K_{m\mathrm{H_2O_2}}$, mM	$K_{m\mathrm{PDA}}$, mM
33.7±5.01	105±12.7	83.74±11.8	30.8±4.8	136.9±10.6*	52.43±10.4*

Comment: * – the differences are significant at $p < 0.05$

Примітка: * – різниця достовірна при $p < 0,05$

Low-frequency vibration leads to k_{cat} growth, except for exposure with an 8 Hz frequency and 0.5 ± 0.04 mm amplitude (**Table 2**). Vibration exposure (frequency range 8–16 Hz, amplitude 0.5 ± 0.04 mm) causes a significant $K_{m\mathrm{H_2O_2}}$ increase, which indicates

certain structural changes associated with the constrained access of H_2O_2 to heme. Peroxidases are characterized by the specific location of amino acids in the active center for effective coordination and use of H_2O_2 (Vlasova, 2018). Binding of H_2O_2 causes immediate oxidation of protein amino acids that are close to the active center (most commonly tyrosine, tryptophan, or histidine). In addition, protein-based tyrosyl radicals are alternative reactive intermediates capable of initiating oxidation of hemoglobin itself.

Table 2. **Steady-state kinetic constants for peroxidase activity of hemoglobin**

Таблиця 2. **Стаціонарні кінетичні константи пероксидазної активності гемоглобіну**

Frequency, Hz	Vibration amplitude, mm					
	0.5 ± 0.04			0.9 ± 0.08		
	k_{cat}, 1/min	$K_{m H_2O_2}$, mM	$K_{m PDA}$, mM	k_{cat}, 1/min	$K_{m H_2O_2}$, mM	$K_{m PDA}$, mM
8	25±5.5	130±9.2*	40±8.6*	42.5±5.5	151.1±9.6*	80.7±4.5
16	50.4±6.2*	151.2±10.1*	80.8±6.3	41.9±4.6	123.6±8.1	62.3±5.5*
24	45.9±5.5*	128.7±6.7*	65.8±5.5	43.2±6.4	121.6±6.1	60.5±5.1*
32	39.8±6.5	119.5±7.1	75.0±6.4	40.2±6.6	120.5±7.3	62±5.0*

Comment: * – the differences are significant at $p < 0.05$

Примітка: * – різниця достовірна при $p < 0.05$

Exposure in the frequency range of 24–32 Hz (amplitude 0.5 ± 0.04 mm) and 16–32 Hz amplitude 0.9 ± 0.08 mm) leads to a smaller $K_{m H_2O_2}$ increase and $K_{m PDA}$ decrease. Under these conditions, these kinetic parameters remain at approximately the same level.

The erythrocyte incubation medium contains some amount of dissolved oxygen contained in nanobubbles stabilized by adsorbed Na^+ ions (Bunkin & Bunkin, 2016; Uchida *et al*., 2016). It is assumed that the target of vibration is nanobubbles of dissolved air, which are retained on the surface of erythrocytes due to Coulomb interactions and are able to coagulate and increase in size under the action of vibration (Bunkin *et al*., 2011). It is thought that oxygen bubbles are involved in the processes of oxygenation – deoxygenation with the participation of hemoglobin (Shatalov *et al*., 2012). Shaking of the solutions leads to the collapse of the bubbles and release of energy. However, the consequence of further growth of microbubbles is degassing of the liquid as a result of the multiplied force of Archimedes that pushes the microbubbles to the surface of the solution (Shatalov *et al*., 2012). Thus, depending on the parameters and duration of vibration, it is possible to simulate both an increase in the concentration of oxygen in the surface of erythrocytes and a decrease as a result of degassing of the liquid.

Given the mechanism of action of vibration described above, we hypothesized that the described changes in kinetic parameters are associated with a different ratio of oxyHb/metHb hemoglobin in the cytoplasmic fraction of cells. **Fig. 3–6** show the change of ligand forms of hemoglobin in erythrocytes incubated for 3 h in Na-phosphate buffer and under the conditions of vibration.

In the absence of vibration, the level of oxyHb decreased rapidly during 20 min of the experiment and turned into met- and deoxy-form (**Fig. 3**). Under the conditions of

low vibration intensity, the decrease in oxyHb content occurred 60–80 min after the beginning of the experiment (**Fig. 4**). Under the action of vibration in the frequency range of 16–32 Hz with an amplitude of 0.9 ± 0.08 mm, oxyHb content remained at the initial level for 90 min of the experiment and then decreased by 4–5 % (**Figs. 5**, **6**). The content of metHb in erythrocytes not exposed to vibration increased and reached the level of 15.7 ± 1.26 % (**Fig. 3**), when vibrating with a 16 Hz frequency, the content of methemoglobin in erythrocytes was lower (**Fig. 4**, **5**), and when vibrating with a frequency of 32 Hz, the amplitude of 0.9 ± 0.08 mm was at the level of 11.6 ± 0.7 % (**Fig. 6**).

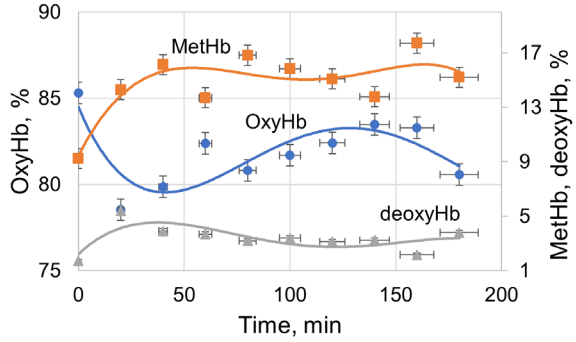

Fig. 3. The distribution of ligand forms in the composition of cytoplasmic fractions of erythrocyte hemoglobin incubated in the medium of Na^+-phosphate buffer for 3 h. Each point represents x ± m for n = 5

Рис. 3. Розподіл лігандних форм гемоглобіну в цитоплазматичній фракції еритроцитів, що інкубували впродовж 3-х годин у середовищі Na^+-фосфатного буфера. Кожна точка представлена як x ± m для n = 5

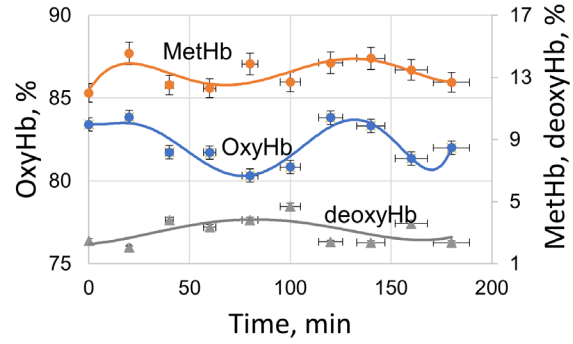

Fig. 4. The distribution of ligand forms in the composition of cytoplasmic fractions of erythrocyte hemoglobin under the conditions of vibration with the frequency of 16 Hz and amplitude of 0.5 ± 0.04 mm. Each point represents x ± m for n = 5

Рис. 4. Розподіл лігандних форм гемоглобіну в цитоплазматичній фракції еритроцитів, що перебували в умовах вібраційного впливу з частотою 16 Гц, амплітудою 0,5 ± 0,04 mm. Кожна точка представлена як x ± m для n = 5

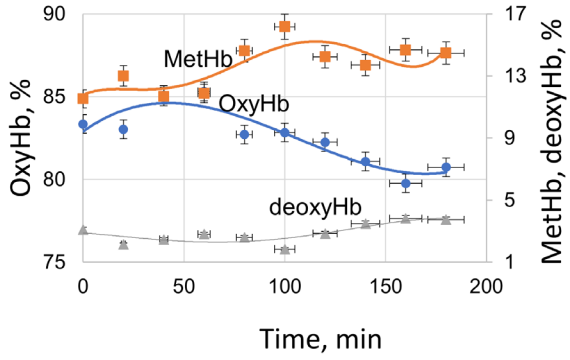

Fig. 5. The distribution of ligand forms in the composition of cytoplasmic fractions of erythrocyte hemoglobin under the conditions of vibration with the frequency of 16 Hz and amplitude of 0.9 ± 0.08 mm. Each point represents x ± m for n = 5

Рис. 5. Розподіл лігандних форм гемоглобіну в цитоплазматичній фракції еритроцитів, що перебували в умовах вібраційного впливу з частотою 16 Гц, амплітудою 0,9 ± 0,08 mm. Кожна точка представлена як x ± m для n = 5

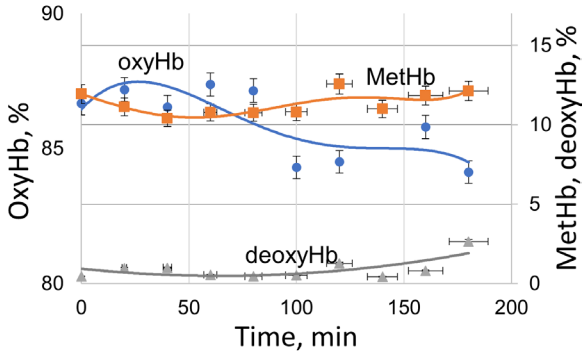

Fig. 6. The distribution of ligand forms in the composition of cytoplasmic fractions of erythrocyte hemoglobin under the conditions of vibration with the frequency 32 Hz, and amplitude of 0.9 ± 0.08 mm. Each point represents x ± m for n = 5

Рис. 6. Розподіл лігандних форм гемоглобіну в цитоплазматичній фракції еритроцитів, що перебували в умовах вібраційного впливу з частотою 32 Гц, амплітудою 0,9 ± 0,08 mm. Кожна точка представлена як x ± m для n = 5

In this study, we measured kinetic parameters after 2 h of exposure, hence, in our opinion, it is very important to understand the nature of changes in oxyHb/metHb that occur in cells. Huo *et al*. (2021), who used tyrosine as a substrate, showed that under the conditions of high content of oxyhemoglobin, the latter retains peroxidase properties and oxidation of the substrate occurs mainly in the pocket of heme (by reaction 6). In addition, the authors argue that P – Fe^{2+} participation in the peroxidase cycle inhibits the pseudoperoxidase cycle. A high concentration of oxyHb causes a fairly high rate of peroxidase process. The appearance of metHb, or its addition to the reaction mixture (Huo *et al*., 2021) increases the speed of the peroxidase process by connecting a pseudoperoxidase cycle, which attracts radical forms of protein in the processes of electron transfer from substrate to heme (Reeder, 2010). Our data on the change in peroxidase activity over time (**Fig. 2**) correlate well with the obtained dependence of the change in metHb content in cells (**Fig. 5**). The high level of oxygenation of hemoglobin for 100 min in the conditions of vibration in the frequency range of 16–32 Hz with an amplitude of 0.9 ± 0.08 mm allows to keep hemoglobin in a functional state avoiding its damage.

Due to the tendency of oxyHb to autooxidation, the formation of metHb is unavoidable. However, reducing the intensity of this process or increasing the level of oxygenation will reduce the content of highly reactive compounds that occur in the pseudoperoxidase cycle.

Thus, hemoglobin cannot avoid certain reactivity to peroxide if it wants to retain its ability to bind oxygen. Further interaction with ascorbate becomes a requirement to prevent damage after peroxide has inevitably reacted with heme (Cooper *et al*., 2008). Completion of the peroxidase cycle is a protective mechanism that sacrifices ascorbate or urate to prevent damage to heme or other biological targets such as lipids. During hypoxia, the oxidized form of P – Fe^{2+} participates in the pseudoperoxidase cycle, which leads to lipid peroxidation, protein modification and accelerates apoptotic processes.

CONCLUSION

Any effect that initiates the formation of methemoglobin leads to an increase in peroxidase activity of hemoglobin due to the involvement of the latter in the pseudoperoxidase cycle and the formation of toxic reactive globin radicals. The high content of

oxyhemoglobin in the cell, that is observed in the conditions of vibration in the frequency range of 16–32 Hz with an amplitude of 0.9 ± 0.08 mm, can prevent its oxidation and entry into the pseudoperoxidase cycle.

COMPLIANCE WITH ETHICAL STANDARDS

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Human Rights: This article does not contain any studies with human subjects performed by any of the authors.

AUTHOR CONTRIBUTIONS

Conceptualization, [O.I.D.]; methodology, [O.I.D.]; validation, [O.I.D.; G.V.T.]; formal analysis, [O.I.D; G.V.T.].; investigation, [O.I.D.; G.V.T.; A.M.M.]; resources, [O.I.D.; G.V.T.; A.M.M.]; data curation, [O.I.D.; G.V.T.; A.M.M.]; writing – original draft preparation, [O.I.D.]; writing – review and editing, [O.I.D.; G.V.T.; A.M.M.]; visualization, [O.I.D.] supervision, [O.I.D.]; project administration, [O.I.D.]; funding acquisition, [-].

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ПЕРОКСИДАЗНА АКТИВНІСТЬ ГЕМОГЛОБІНУ ЕРИТРОЦИТІВ ЗА ДІЇ НИЗЬКОЧАСТОТНОЇ ВІБРАЦІЇ

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Вступ. Як гемопротеїн, гемоглобін за наявності окислювальних еквівалентів, таких як H_2O_2, може діяти як пероксидаза з дуже високим окислювальним потенціалом. У ході цих реакцій гемоглобін утворює форми з високоокисненим залізом (Fe^{IV}) та радикали глобіну, що мають високу окисну активність і є токсичними для клітин. Крім того, пероксидазна активність може свідчити про будь-які структурні зміни, які могли статися в молекулі гемоглобіну внаслідок хімічних модифікацій. У роботі проаналізовано експериментальні дані щодо впливу низькочастотної вібрації на пероксидазну активність гемоглобіну еритроцитів, вивчення кінетики та пошуку кінетичних параметрів пероксидазної реакції за участю гемоглобіну.

Матеріали та методи. Суспензію еритроцитів піддавали дії вібрації протягом 3-х годин в інтервалі частот від 8 до 32 Гц, амплітудами 0,5 ± 0,04 та 0,9±0,08 мм. Через певні проміжки часу в лізатах клітин визначали пероксидазну активність гемоглобіну та вміст його лігандних форм. Окремо проведено експерименти, що дають змогу дослідити механізм і розрахувати кінетичні параметри пероксидазної реакції.

Результати. Проаналізовано експериментальні дані щодо впливу низькочастотної вібрації на пероксидазну активність гемоглобіну еритроцитів. Досліджено кінетику реакції окиснення *п*-фенілендіаміну гемоглобіном еритроцитів. З'ясовано, що пероксидазне окиснення проходить за механізмом типу пінг-понг. Знайдено кінетичні параметри пероксидазної реакції за участю гемоглобіну. Досліджено зміну кінетичних параметрів після двогодинного впливу середовища інкубування і низькочастотної вібрації. Запропоновано можливий механізм дії гемоглобіну в реакціях окиснення за участю H_2O_2.

Висновки. Будь-який вплив, що ініціює утворення метгемоглобіну, призводить до росту пероксидазної активності гемоглобіну завдяки залучення останнього до псевдопероксидазного циклу й утворення токсичних високореакційних глобінових радикалів. Високий вміст оксигемоглобіну в клітині, що спостерігають за вібрації в інтервалі частот 16–32 Гц, амплітудою 0,9 ± 0,08 мм, може запобігти його окисленню і вступу до псевдопероксидазного циклу.

Ключові слова: лігандні форми гемоглобіну, ензиматична кінетика, кінетичні константи, псевдопероксидазний цикл, гем, гіпоксія, *п*-фенілендіамін

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