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Nitrite–Catalase Interaction as an Important Element of Nitrite Toxicity

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Abstract—It was established that nitrite in the presence of chloride, bromide, and thiocyanate decreases the rate of hydrogen peroxide decomposition by catalase. The decrease was recorded by the permanganatometric method and by a method of dynamic calorimetry. Nitrite was not destroyed in the course of the reaction and the total value of heat produced in the process was not changed by its presence. These facts suggest that nitrite induces inhibition of catalase with no change in the essence of the enzymatic process. Even micromolar nitrite concentrations induced a considerable decrease in catalase activity. However, in the absence of chloride, bromide, and thiocyanate inhibition was not observed. In contrast, fluoride protected catalase from nitrite inhibition in the presence of the above-mentioned halides and pseudohalide. As hydrogen peroxide is a necessary factor for triggering a number of important toxic effects of nitrite, the latter increases its toxicity by inhibiting catalase. This was shown by the example of nitrite-induced hemoglobin oxidation. The naturally existing gradient of chloride and other anion concentrations between intra- and extracellular media appears to be the most important mechanism of cell protection from inhibition of intracellular catalase by nitrite. Possible mechanisms of this inhibition are discussed.

Key words: nitrite, halide ions, catalase, calorimetry

The production of free radical nitrogen oxides and particularly of $NO_2^{\bullet}$ has been proved the basic element of the most toxic effects of nitrite [1-4]. And nitrite peroxidase oxidation is an important path for $NO_2^{\bullet}$ generation [1, 2, 5, 6]. Thus, the activity of peroxide metabolizing enzymes is an important factor determining nitrite toxicity.

However, in this connection an inverse factor, the effect of nitrite on the activity of peroxide metabolizing enzymes, assumes no less importance. Some data about such effects are available. Particularly, the ability of nitrite to reduce catalase compound I [7, 8]. Under some conditions, nitrite can be reduced to nitric oxide—an effective inhibitor of catalase [9]. The ability of nitrite to produce compounds with ferri-heme-containing enzymes, particularly with catalase, also was shown. But these compounds have been recorded at comparatively high and obviously non-physiological nitrite concentrations (10^{-3}-10^{-1} M) [10].

Some researchers have observed a decrease in velocity of peroxide destruction by erythrocytes and their hemolysates in the presence of nitrite [11, 12]. But mere estimation of the velocity of peroxide destruction is not enough to explain the mechanism of this nitrite effect, because nitrite can effect this parameter via a number of pathways (see above). Cohen et al. [11] supposed that the decrease is a result of competition between nitrite and hydrogen peroxide for catalase compound I. Nicholls [12] supposed the inhibition of catalase by a trace amount of nitric oxide.

So, we need a method to distinguish between catalase and peroxidase peroxide destruction pathways in biological materials to clarify the nature of this effect: a competition between nitrite (or its metabolites) and hydrogen peroxide for catalase compound I, or inhibition of the enzyme. Continuous recording of kinetics of H_2O_2 destruction is necessary for estimation of the stability of the effect. Also, this approach will reveal the agent inducing the effect: whether it is nitrite itself, or its metabolites produced in the course of the reaction.

We have developed a calorimetric method for monitoring of catalase and peroxidase reaction kinetics based on the recording of kinetics of heat production accompanying these processes. It has been shown that the latter in both cases completely coincided with peroxide decomposition kinetics and the quantity of heat produced was directly proportional to the quantity of decomposed H_2O_2 [13-15]. Heat efficiency of the catalase process, accord-

Abbreviations: NO_2) nitric dioxide; NED) N-(1-naphthyl)ethylenediamine dihydrochloride.

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ing to our data, was 3.42 times less than that of peroxidase catalysis and 2.89 times less than that of free radical catalysis following the pattern of the Fenton reaction [13]. Hence, this method can differentiate catalase, peroxidase, and free radical processes of H_2O_2 decomposition.

This study investigates the effect of nitrite on catalase activity with the use of the calorimetric method and from the data proposes a mechanism for the effect and possible physiological significance.

MATERIALS AND METHODS

Chemicals. NaH_2PO_4, NaCl, KBr, hydrogen peroxide, and potassium permanganate were from Reakhim (Russia); KSCN, sulfanilic acid, N-(1-naphthyl)ethylenediamine dihydrochloride (NED), potassium nitrite, bull liver catalase, and methemoglobin were from Sigma (USA). Cow erythrocyte hemolysate was obtained by dilution of erythromass thrice washed with physiological solution by 20 times with 2.5 mM phosphate buffer, pH 7.2 [3].

Estimation of hydrogen peroxide, methemoglobin, and nitrite concentrations. Hemoglobin oxidation has been recorded photometrically from the increase of optic density at 630 nm [6]. In all photometric analyses the length of the optical path was 1 cm.

Permanganatometric determination of hydrogen peroxide contents was used both for determination of peroxide concentration in basic solution and for recording of its decomposition by catalase. The determination procedure has been described before [13].

Nitrite concentration was determined from the reaction with sulfanilic acid and NED reagent [16].

Calorimetric assay. Catalase reaction kinetics were calorimetrically recorded using a unit developed on the basis of a device from Dithermanal (Hungary) [13, 14]. The device has two heat isolated cells: control and experimental. Each cell consisting of a Dewar flask with a plastic beaker inside with control and experimental solutions, accordingly. Magnetic stirrers under the cells ensured the continuous agitation of solutions. Highly sensitive thermistors (to 0.001°C) were immersed into the solutions from the top. Each was connected with a differential measuring bridge provided from a stabilized voltage source. The bridges were switched on towards each other through an interface block, and a signal equal to the voltage difference between the bridges was transmitted to an amplifier and then to a recording device.

The process was started by the addition of hydrogen peroxide (9 mM) into the experimental cell. The control cell contained a solution identical to the experimental one but no peroxide was added. Peroxide was used only in concentrations which did not result in enzyme inactivation in the course of reaction, a criterion for this being the identity of kinetic curves recorded after the first and subsequent H_2O_2 additions [13, 15].

Enzyme activity was determined from the slope of the initial rectilinear part of the kinetic curve that, as demonstrated, was in linear dependence with the concentration of active enzyme in the reaction medium [13, 14].

RESULTS

Catalase-induced hydrogen peroxide decomposition and heat production accompanying this process. Effect of nitrite. Kinetic curves of heat production accompanying hydrogen peroxide decomposition by catalase are presented on Fig. 1. The presence of nitrite and chloride

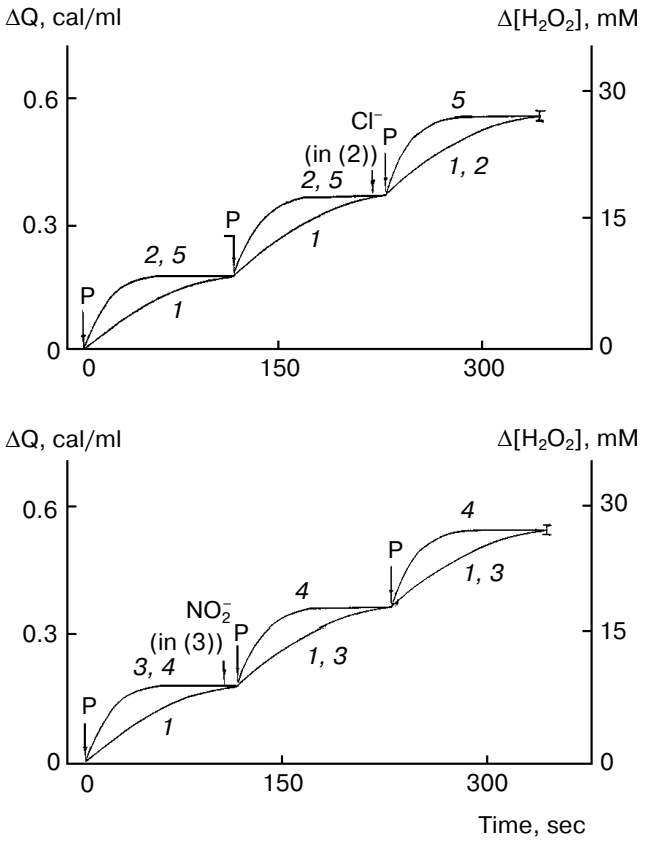

Fig. 1. Kinetic curves of heat production during hydrogen peroxide decomposition by catalase in the presence of nitrite and chloride: *1*) 5 μM KNO_2 and 150 mM NaCl; *2*) 5 μM KNO_2; *3*, *4*) 150 mM NaCl; *5*) without KNO_2 and NaCl. The reaction medium contained in all cases 40 mM sodium phosphate buffer, pH 6.5, 8 nM catalase, and also the above-mentioned additives. The points of addition of 9 mM H_2O_2, 150 mM NaCl (in (*2*)), 5 μM KNO_2 (in (*3*)) are marked by arrows "P", "Cl^-", and "NO_2^-", accordingly. Heat quantity per 1 ml of the reaction medium and the appropriate quantity of decomposed peroxide are presented on the ordinate axis.

induced significant decrease in the velocity of heat production. Therewith each element individually did not induce the effect. The quantity of heat produced at the moment when the kinetic curve reached the plateau was equal in all samples independently on these presence of the components. The kinetics of heat production, as demonstrated earlier, coincided with peroxide decomposition kinetics [13, 14]. Thus, nitrite in the presence of chloride decreases the velocity of hydrogen peroxide decomposition by catalase and did not decrease it if chloride had not been added. The last fact was supported by the results of permanganatometric test. According to data of this test, peroxide was destroyed by 55 ± 6% in samples containing both nitrite and chloride and completely destroyed in samples which not contain either one of these components.

The thermal effectiveness of the process was not changed in the presence of nitrite and chloride. This fact attested the inhibition of the catalase activity but not the change in peroxide decomposition pathway.

Inhibition degree was not changed upon subsequent H_2O_2 additions. The addition of a lacking component (nitrite or chloride) before the second or the third peroxide addition resulted in enzyme inhibition identical to the case when both components were initially present before the first peroxide addition (Fig. 1). These data indicate that the inhibitive substance is not accumulated nor does it disappear in the course of the reaction.

According to NED-test data, nitrite concentration was unchanging before and after three subsequent peroxide additions that are shown at Fig. 1.

The inhibition is reversible as follows from the data presented in Table 1. If the reaction medium containing catalase, nitrite, and chloride is diluted 10 times, the inhibition degree is the same as in samples initially containing the above components in the concentration before the dilution.

Inhibition degree was not changed significantly when the initial peroxide concentration varied from 5 to 30 mM. Further increase in H_2O_2 concentration can result in the enzyme inactivation by peroxide that makes it impossible to estimate its activity adequately [13].

Effect of halide ions and thiocyanate on catalase activity in the presence of nitrite. Basic features. The inhibitive effect remained when chloride was replaced by bromide or the pseudohalide thiocyanate. When halide anions were replaced by sulfate, phosphate, acetate, and citrate anions the inhibition ceased (Fig. 2). Dependences of the enzyme inhibition degree on chloride and bromide concentrations were practically identical (Fig. 2a). They achieved plateaus at NaCl and KBr concentrations about 80 mM independently of nitrite concentration and pH value at least in the range from 6.0 to 7.4. Also in the case with thiocyanate, the curves approached the plateau when thiocyanate concentration was about 20 μM, including with nitrite concentration of 37.5 and 250 μM, i.e., significantly higher than thiocyanate concentration (Fig. 2b).

Fluoride acted otherwise. Its presence resulted in a decrease in catalase activity. This effect was not influenced by chloride. The addition of nitrite (250 μM) did not increase the enzyme activity drop (Table 2, experiments 1-4). Nevertheless, fluoride availability in nitrite–chloride–catalase system protected the enzyme from inhibition. Increasing the chloride concentration contributed to a decline in the protective effect (Table 2, experiments 5-8). The effect remained when chloride was replaced by bromide or thiocyanate.

At high (millimolar) nitrite concentrations, it induced catalase inhibition even in the absence of chloride. In this case the protective effect of fluoride was not observed (Table 2, experiments 9-12).

Figure 3 demonstrates the dependence of catalase inhibition degree on nitrite concentration in the presence

Table 1. Reversibility of catalase inhibition by nitrite at the presence of chloride ions

Experiment number	Basic components			Catalase activity, %
	KNO_2, μM	NaCl, mM	catalase, nM	
1	7.5	160	8	30 ± 3
2	7.5	16	8	58 ± 3
3	75	160	8	4 ± 1
4*	75	160	80	59 ± 3
5*	75	160	80	30 ± 3
6	—	—	8	100.0 ± 4

Note: All samples contained 40 mM sodium phosphate buffer, pH 6.5, and also correspondent additives.

* Sample 4 has been diluted tenfold with 40 mM sodium phosphate buffer, pH 6.5, before the measurement after incubation for 30 min at 25°C. Sample 5 corresponds to 4, but after it had been diluted, NaCl concentration was adjusted to 160 mM.

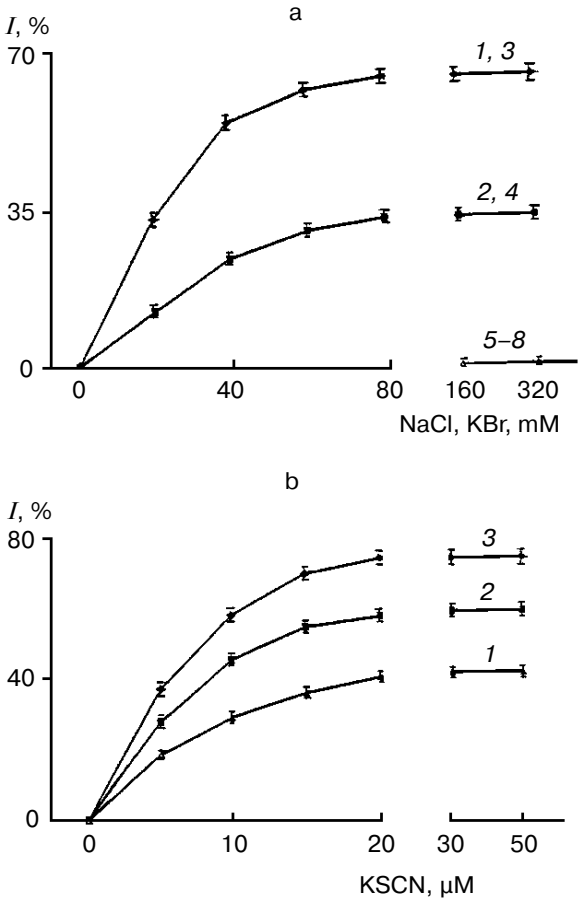

Fig. 2. Dependence of catalase inhibition degree (*I*) on halide-ion (a) and thiocyanate (b) concentrations at constant nitrite content. a: *1*) 250 μM KNO_2 + NaCl; *2*) 12.5 μM KNO_2 + NaCl; *3*) 250 μM KNO_2 + KBr; *4*) 12.5 μM KNO_2 + KBr; *5-8*) 250 μM KNO_2 + KH_2PO_4, Na_2SO_4, CH_3COOK, or $C_6O_7H_5K_3$, accordingly. b) KSCN + KNO_2. KNO_2 concentration (μM): *1*) 12.5; *2*) 37.5; *3*) 250. All samples initially contained 40 mM sodium phosphate buffer, pH 7.4, 8.0 nM catalase, and also nitrite and other additives in the concentrations marked on the abscissa axis. $I = A_0 - A/A_0 \cdot 100\%$, where *A* is catalase activity of a given sample; A_0 is activity of a control sample without nitrite.

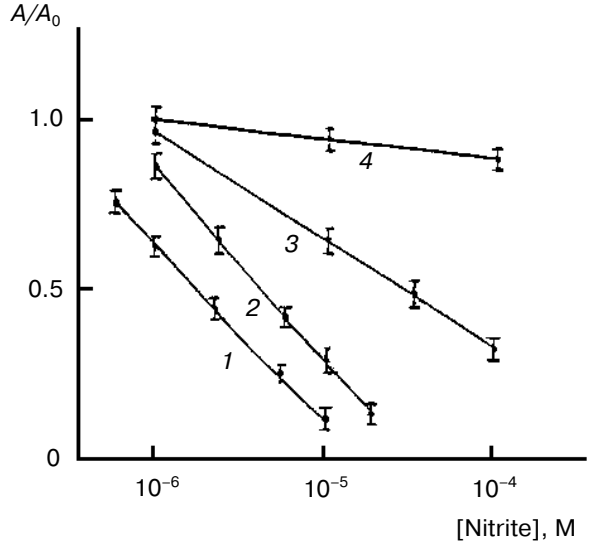

Fig. 3. Dependence of nitrite inhibitive effect on catalase on its concentration at pH: *1*) 6.0; *2*) 6.5; *3*) 7.4; *4*) 8.5. *A* and A_0 are activities of the enzyme in the presence and in the absence of nitrite, accordingly. Composition of the reaction medium: 40 mM sodium phosphate buffer, 150 mM NaCl, 8 nM catalase, and corresponding concentration of KNO_2.

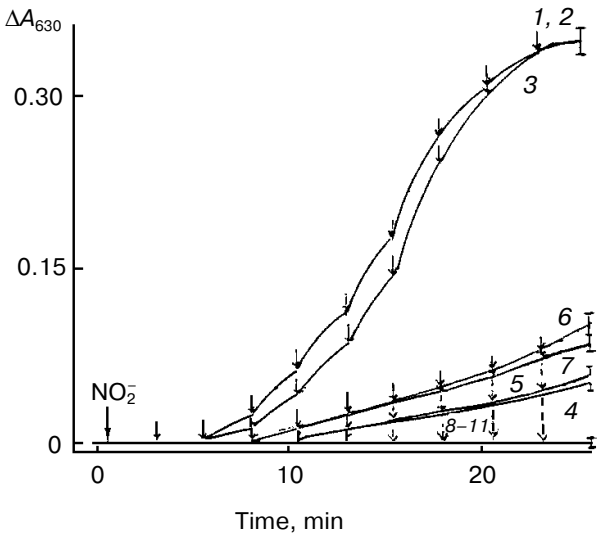

Fig. 4. Kinetics of nitrite-induced oxidation of erythrocyte hemolysate hemoglobin. Influence of catalase, chloride, bromide, and thiocyanate: *1*) 150 mM NaCl; *2*) 150 mM KBr; *3*) 50 μM KSCN; *4*) without additives; *5*) 150 mM NaCl + 0.2 μM catalase (in addition to endogenous enzyme contained in hemolysate); *6*) 200 mM NaH_2PO_4; *7*) 120 mM Na_2SO_4. The point of KNO_2 (200 μM) addition is indicated by the large arrow "NO_2^-". The points of H_2O_2 (100 μM) addition are indicated by small arrows. Samples *8-11* correspond to *1-4* except that KNO_2 was not added. All samples initially contained 40 mM sodium phosphate buffer, pH 7.4, erythrocyte hemolysate (100 μM hemoglobin).

of 150 mM NaCl at different pH values. Inhibition efficiency increased with increasing acidity of the medium. The observed relation was practically identical both for enzyme isolated from bull liver and for catalase activity of biological materials used in our research: hemolysates of rabbit, chicken, cow, and human erythrocytes and chicken liver homogenates [17].

Effect of halide ions and thiocyanate on erythrocyte hemoglobin oxidation induced by nitrite. The presence of chloride, bromide, and thiocyanate in the reaction medium containing erythrocyte hemolysate, nitrite, and hydrogen peroxide contributed to a multiple intensification of hemoglobin oxidation compared with samples

Table 2. Influence of fluoride on catalase activity and catalase inhibition by nitrite

Experiment number	Components			Catalase activity, %
	KNO_2, mM	NaCl, mM	KF, mM	
1	—	320	—	96 ± 4
2	—	—	100	74 ± 4
3	—	320	100	72 ± 3
4	0.25	—	100	73 ± 4
5	0.25	160	—	33 ± 4
6	0.25	320	—	32 ± 4
7	0.25	160	100	62 ± 4
8	0.25	320	100	42 ± 3
9	1.5	—	—	48 ± 3
10	1.5	—	100	45 ± 3
11	5	—	—	29 ± 3
12	5	—	100	27 ± 3

Note: All samples contained 40 mM sodium phosphate buffer, pH 7.4, 8 nM catalase, and also corresponding additives. The catalase activity in 40 mM sodium phosphate buffer was taken as 100%.

containing none of these halides and pseudohalide and also containing sulfate or phosphate instead. Addition of exogenous catalase that was 4 times more active than endogenous one (the activity was determine by the calorimetric method) eliminated the effect of intensification of oxidation (Fig. 4).

DISCUSSION

Based on the data presented in Figs. 1 and 2 and also in Table 1, we can suppose that nitrite reversibly inhibits catalase in the presence of chloride, bromide, and thiocyanate. In their absence a significant inhibition was observed only at high (millimolar) nitrite concentrations (Table 2), while in their presence the inhibition occurred even at micromolar (physiological) concentrations of nitrite.

But what is the nature of the inhibitor? Why is chloride, bromide, or thiocyanate required for inhibition? The dependences of inhibition degree on nitrite concentration at different pH values, presented at Fig. 3, were practically identical for isolated enzyme preparation and for catalase activity of erythrocytes hemolysates and liver homogenates [17]. This fact does not suggest that the direct inhibitor is nitric oxide that can be present in solution of nitrite, otherwise oxyhemoglobin would inevitably effect the inhibition effectiveness.

The data presented at Fig. 1 suggest that the inhibitor neither accumulated no disappeared in the course of the reaction and nitrite concentration did not change. So, we suppose that the inhibitor was not a metabolite produced in this process.

Several possible mechanisms can be supposed: 1) immediate inhibitors are halide or thiocyanate derivatives of nitrite produced in reaction medium as a result of a reversible interaction of these components; 2) a direct inhibitor is a short-lived compound produced in the active center of the enzyme (or near it) in the presence of nitrite and halide ions or thiocyanate and quickly dissociated to initial substances; 3) halides modify catalase in such a way that it gains increased ability to bind nitrite, being a direct inhibitor of the enzyme.

Let's consider the first possibility. Halide derivatives of nitrite are known. They are formed in the reversible interaction of halide ions with nitrite (exactly with NO^+-cation) occurring principally in acid medium [18, 19]. It can be supposed that direct inhibitors are compounds of NOCl or NOSCN type. But the following facts contradict this supposition. Curves of dependence of inhibition degree on thiocyanate concentration achieved plateau at its concentration about 20 μM independently of the used nitrite concentration including the cases of 37.5 and 250 μM, i.e., substantially higher the thiocyanate concentration (Fig. 2b). Inhibition degree did not increase on the addition of additional quantities of thiocyanate (over 20 μM). It increased only with increasing nitrite concentration. So, getting into the plateau of the curves cannot be explained by transferring of all nitrite to NOSCN compounds. But what is the reason of the curves reaching a

plateau and why was the plateau achieved at the same thiocyanate concentration independently of nitrite concentration? The last peculiarity was observed when thiocyanate was changed to chloride or bromide (Fig. 2a). It could be noted that curves of dependence of inhibition degree on chloride and bromide concentrations achieved plateau at the same concentration of these anions in the pH range from 6.0 to 7.4. This fact also contradicts the first supposition because acidification of the medium favors the production of halide derivatives of nitrite [18, 19].

In contrast, fluoride protected catalase from the inhibition by nitrite when chloride was present in the reaction medium. Protection degree decreased as chloride concentration increased. However, at high (millimolar) concentrations of nitrite, when it induced a marked catalase inhibition with no chloride, bromide or thiocyanate present, fluoride had no protective effect (Table 2). It can be supposed that the fluoride protective effect is realized not in competition with nitrite but in that with chloride.

It can be supposed that halides and thiocyanate can bind with the enzyme independently of nitrite and catalase can be inhibited if both components are bound. The halide effect may be expressed in the enzyme modification resulting in its increased ability for binding of nitrite that is the direct inhibitor. We cannot exclude that the direct inhibitor is a short-lived compound produced in the interaction of nitrite with halide ions and thiocyanate inside the enzyme globule and quickly dissociated to initial substances.

It is worth noting that for other well-known catalase inhibitors such as azide, sulfide, and fluoride inhibitive effect did not depend on the presence of chloride [17]. We reported earlier about the enhancement of inhibitive effect of hydroxylamine in the presence of Cl^-, Br^-, SCN^- and also a F^- protective effect. But in this case the efficiency of inhibition had a pH dependence different from that of nitrite [17].

Not only the decrease in catalase activity in the presence of nitrite, but also change in ratio between activities of catalase and heme-containing peroxidases has physiological significance. It was shown that peroxidase oxidation of nitrite catalyzed by methemoglobin is a key stage of hemoglobin oxidation in erythrocytes induced by nitrite. This process is dependent on hydrogen peroxide concentration in the reaction medium [3].

We reported that nitrite at least at concentrations up to 100 μM does not inhibit peroxidase activity of methemoglobin at physiological pH independently of the presence of halide ions and thiocyanate [5, 6]. But catalase was effectively inhibited by nitrite in such concentrations in the presence of Cl^-, Br^-, and SCN^- (Figs. 1 and 2). So, the presence of nitrite should lead to an increase in the rate of peroxide metabolized by methemoglobin. The latter circumstance should contribute to intensification of nitrite oxidation to nitrogen dioxide and, as consequence, to intensification of hemoglobin oxidation.

As we can see, the presence of the above-mentioned halides or thiocyanate contributed to a multiple intensification of hemoglobin oxidation in medium containing erythrocyte hemolysate, nitrite and hydrogen peroxide. The substitution of chloride, bromide, and thiocyanate by sulfate or phosphate and also the addition of exogenous catalase over that initially contained in the hemolysate removed the effect of the intensification of oxidation (Fig. 4). So, the inhibition of the hemolysate catalase by nitrite is probably the reason for the observed phenomenon.

The blood of healthy humans contains normally from 0.5 to 3.6 μM nitrite [20]. But its concentration can approach the level of dozens of micromoles per liter in response to some diseases. Thus, serum nitrite concentration in patients with pneumonia resulting from AIDS virus infection was as high as 36 μM [21]. Nitrite in such concentrations can inhibit catalase activity at more than 50% in the presence of chloride or thiocyanate in plasmatic concentrations at physiological pH value. In must be noted that acidification that take place in inflammation zones increase the ability of nitrite to inhibit catalase (Fig. 3).

It is known that normally intracellular anion concentration is manifold less than the extracellular one. So, intracellular chloride concentration is about 5 mM according to data of some researchers [22]. At that chloride concentration nitrite at least up to 250 μM practically does not induce catalase inhibition at physiological pH values (Fig. 2a). We suppose that the gradient of anion concentration between the intra- and extracellular medium is one of the most important elements of cell protection mechanisms from toxic action of nitrite.

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