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Proposed Mechanism of Nitrite-Induced Methemoglobinemia

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Abstract—A scheme of development of nitrite-induced oxyhemoglobin oxidation in erythrocytes based on the analysis of experimental data is proposed. It was found that, contrary to widespread opinion, direct oxidative-reductive interaction between hemoglobin and nitrite is absent or negligible under physiological conditions. The driving stage of this process is methemoglobin-catalyzed peroxidase oxidation of nitrite. The product of the oxidation (presumably $NO_2^{\bullet}$) directly oxidizes oxyhemoglobin to methemoglobin–peroxide complex without hydrogen peroxide release into the environment. The oxidant itself is reduced to nitrite or oxidized to nitrate as a result of interaction with another $NO_2^{\bullet}$ molecule. Thus, the stoichiometry of the process depends on the ratio of rates of these two reactions. Substances that are able to compete with nitrite for peroxidase and therefore to prevent the nitrite oxidation effectively protect hemoglobin from oxidation. Catalase is not able to destroy methemoglobin–peroxide complexes, but it can prevent their production in the course of interaction of methemoglobin and free peroxide by destroying the latter.

Key words: nitrite, oxyhemoglobin, methemoglobinemia

Determination of the mechanism of nitrite-induced hemoglobin oxidation remains one of the most topical lines in the investigation of physiological and toxic effects of nitrite. There are several aspects within this line to be studied: nitrite-induced hemoglobin oxidation as one of the principal factors of nitrite toxicity and as one of the ways of its metabolism in an organism, which is closely interconnected with the metabolism of nitrogen oxide, nitrogen dioxide, and nitrate [1-4].

Several schemes for the process have been proposed [5-10], but the following questions are still unclear: its stoichiometry, reason for sigmoid kinetics of hemoglobin oxidation under the effect of nitrite (which is observed in model systems), mechanisms of action of substances inhibiting and preventing the reaction, and also all the factors of the organism protecting it from this toxic effect of nitrite.

In this study we tried to develop a model system that would be in greatest similarity with the conditions that exist in erythrocytes *in vivo* in relation to nitrite content and the availability of compounds able to affect nitrite-induced hemoglobin oxidation. Based on our data on the kinetics of the process at various concentrations of nitrite and hemoglobin, the effect on the kinetics of a range of physiologically active compounds, enzyme and non-enzyme antioxidants, and also on earlier data on the effect of nitrite on the latter, we proposed a scheme of nitrite induced oxidation of oxyhemoglobin in human and animal erythrocytes. From this scheme we determined factors that are available in erythrocytes for preventing this process.

MATERIALS AND METHODS

Chemicals. NaH_2PO_4, NaCl, KBr, hydrogen peroxide, and ethanol were from Reakhim (Russia); KSCN, NaN_3, potassium nitrite, ascorbic acid, bovine liver catalase, methemoglobin, lactoperoxidase, and horseradish peroxidase (RZ 3.0) were from Sigma (USA); β-estradiol and testosterone were from Merck (Germany); serotonin-creatinine sulfate and benzidine were from Reanal (Hungary); mannitol, β-naphthol, and EDTA were from Chemapol (Czech Republic). Rabbit erythrocyte hemolysate was obtained by 20-fold dilution of erythrocyte mass (thrice washed with physiological solution) with 2.5 mM phosphate buffer, pH 7.2 [11].

Estimation of nitrite content in erythrocyte hemolysate. It is known that the determination of nitrite content by the widely used Griss method in medium containing hemoglobin and methemoglobin is related with some difficulties due to the necessity of protein precipita-

Abbreviations: $NO_2^{\bullet}$) nitrogen dioxide; HbO_2) oxyhemoglobin subunit; $(HbO_2)_4$) oxyhemoglobin tetramer.

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tion and the acidification of the medium. All these procedures can result in artifacts [12]. As we reported in the previous study [11], we showed that nitrite reversibly inhibits catalase in the presence of halide ions and thiocyanate, while in their absence the above effect is not recorded. No known catalase inhibitor has such ability. A quantitative relationship between the enzyme inhibiting degree and nitrite concentration did not depend on the presence of hemoglobin and methemoglobin in the range of concentrations used in this study. Nitrate as the stable end product of nitrite oxidation has no inhibiting properties in relation to catalase [11].

Therefore, comparison of the indices of hemolysate catalase activity in the presence and in the absence of exogenous chloride and also at various pH values gives us a very sensitive method for nitrite content estimation [13]. We already used this method for determination of nitrite content in milk. Its sensitivity is ~0.1 μM, i.e., higher than that of the Griss method. Also, this method allows determining nitrite concentration without previous preparation of a sample and at physiological pH, as the enzyme activity is determined calorimetrically using the method described earlier [14]. Therefore, in this case the initial color and turbidity of a sample do not interfere with the analysis.

First, we developed a calibration curve for quantitative determination of nitrite content through the addition of known concentrations of nitrite to hemolysate and the determination of catalase activity. As we have shown earlier, nitrite that is previously oxidized in phosphate buffer by lactoperoxidase and methemoglobin loses its ability to inhibit catalase. The data of catalase and Griss test coincided in this case [14]. Nitrite-induced oxidation of hemoglobin in erythrocyte hemolysates was accompanied by increasing catalase activity of the hemolysate up to its initial level that was recorded in the absence of nitrite [15].

Monitoring of hemoglobin oxidation. The accumulation of methemoglobin in a sample was recorded by the increase of its light absorbance at 630 nm. The kinetics of the increase in absorbance was recorded uninterruptedly while continuously agitating the reaction medium [15]. Quantitative content of methemoglobin was determined from the ratio between light absorbance maxima following Salvati et al. [16] and also from the comparison with the spectrum of the solution of a methemoglobin preparation from Sigma. Photometric determination of erythrocyte hemoglobin oxidation was carried using a SF-18 spectrophotometer equipped with an Ulbricht sphere. The length of optical path in all photometric experiments was 1 cm. Hemoglobin concentration in moles of heme group was determined using extinction coefficient $\varepsilon_{540} = 1.5 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [17].

The reaction medium in all the experiments contained 40 mM Na-phosphate buffer, pH 7.4, and erythrocyte hemolysate (100 μM heme) except for cases specified individually.

RESULTS

1. Kinetics of nitrite-induced oxyhemoglobin oxidation in erythrocyte hemolysates at different initial concentrations of nitrite. Two phases of the process. The addition of nitrite into reaction medium containing erythrocyte hemolysate resulted in an increase in absorbance at λ = 630 nm (Fig. 1). The assessment of light absorption spectrum of the product produced when kinetic curves reach the plateau (Fig. 1) indicate the total oxidation of hemoglobin to methemoglobin in accordance with the method of Salvati et al. [16], and also its comparison with the spectrum of the solution of standard methemoglobin preparation.

The total rate of the process had no linear dependence upon the initial concentration of nitrite. The kinetics of methemoglobin accumulation is sigmoid, as had been earlier recorded by a number of researchers, and it has two phases: a phase of initiation and an autocatalytic one [6-10].

2. Effect of hydrogen peroxide, catalase, and heme-containing peroxidases on both phases of the process at high (1 mM) initial concentrations of nitrite. It is known that hemoglobin oxidation in the presence of oxygen is accompanied by the generation of reactive forms of oxygen including hydrogen peroxide [18, 19]. So it was reasonable to determine the effect of peroxide, peroxide metabolizing enzymes, and traps of oxygen free radicals on this process. From the data presented in Fig. 2, we can see that the phase of initiation was sensitive to the presence of hydrogen peroxide in the reaction medium. The

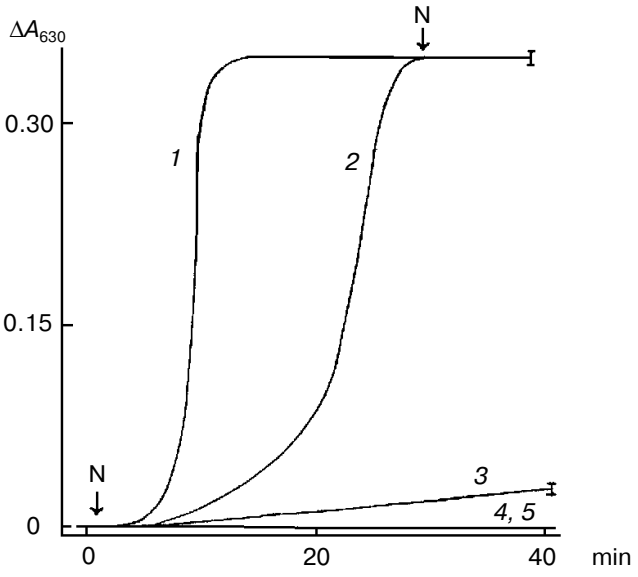

Fig. 1. Kinetics of oxyhemoglobin oxidation in erythrocyte hemolysate under the influence of nitrite (mM): *1*) 1.0; *2*) 0.5; *3*) 0.25; *4*) 0.1; *5*) 0. Points of nitrite addition are indicated by arrow N.

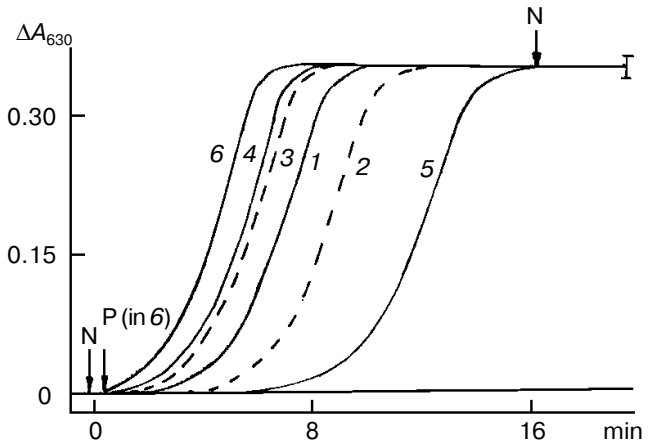

Fig. 2. Influence of the following additives on the kinetics of nitrite-induced oxidation of oxyhemoglobin in erythrocyte hemolysate: *1*) control; *2*) catalase (1.0 μM); *3*) methemoglobin (20 μM); *4*) lactoperoxidase (1.0 μM); *5*) NaN_3 (1.0 mM); *6*) hydrogen peroxide (70 μM). Components *2-5* were added before the introduction of 1 mM nitrite; the point of its addition is indicated by arrow N. The point of addition of peroxide in *6* is indicated by P.

addition of H_2O_2 resulted in its shortening to a minimum. The addition of an extra portion of catalase besides the quantity already present in the hemolysate, by contrast, resulted in the extension of the initiation phase. The initial presence of methemoglobin and lactoperoxidase contributed to its reduction. The presence of azide that is an inhibitor of ferriheme-containing enzymes resulted in the prolongation of the initiation phase. All these factors had no effect on the autocatalytic phase. Thus, there is a significant qualitative difference between these two phases.

3. Effect of substrates of methemoglobin-catalyzed peroxidase reaction on both phases of the process. The presence of ascorbate and other compounds oxidized in a peroxidase reaction catalyzed by methemoglobin (benzidine, 2-naphthol, serotonin, and β-estradiol) inhibited hemoglobin oxidation, and the effect was pronounced in both phases (Fig. 3). The efficiency of these substances as hemoglobin protectors against nitrite-induced oxidation is proportional to their efficiency as substrates of the methemoglobin-catalyzed peroxidase reaction, which we determined earlier [20, 21]. Substances that are not oxidized in methemoglobin-catalyzed peroxidase reaction (testosterone, EDTA, ethanol, mannitol, and nitrate [20, 21]) had no effect on the process, including traps of oxygen free radicals (ethanol, mannitol).

The most efficient peroxidase substrates, such as benzidine and 2-naphthol, virtually prevented or stopped the process that had been already started even if they were present in repeatedly lower concentrations than hemoglobin and nitrite (Fig. 3). This fact suggests that, contrary to widespread opinion [4-10], there is practically no direct oxidative–reductive interaction between nitrite and hemoglobin in neutral medium.

In the last case, the oxidation was induced by the addition of exogenous hydrogen peroxide (Fig. 4). However, depending on the concentration of substrate, the rate of hemoglobin oxidation either increased with time, remained the same, or decreased, so the introduction of additional H_2O_2 portions was required for maintaining the process (Fig. 4).

As it is evident from the data presented in Fig. 5, the protective effect of compounds preventing hemoglobin oxidation declined during the process, as was demonstrated by the disappearance of the protective effect with

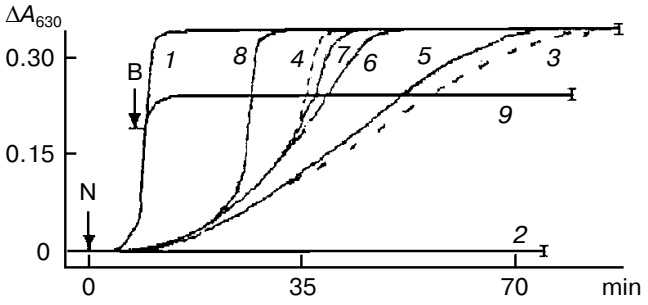

Fig. 3. Influence of a range of physiologically active substances on kinetics of nitrite-induced oxidation of oxyhemoglobin: *1*) control; *2-4*) benzidine (4, 0.2, and 0.1 μM, respectively); *5*) serotonin (5 μM); *6*) 2-naphthol (1 μM); *7*) β-estradiol (20 μM); *8*) ascorbate (10 μM); *9*) similar to *2* but benzidine was added at the point indicated by arrow B. (Mannitol (50 mM), ethanol (1.0 M), EDTA (10 mM), testosterone (100 μM), and KNO_3 (1.0 mM) had no significant effect on the kinetics.)

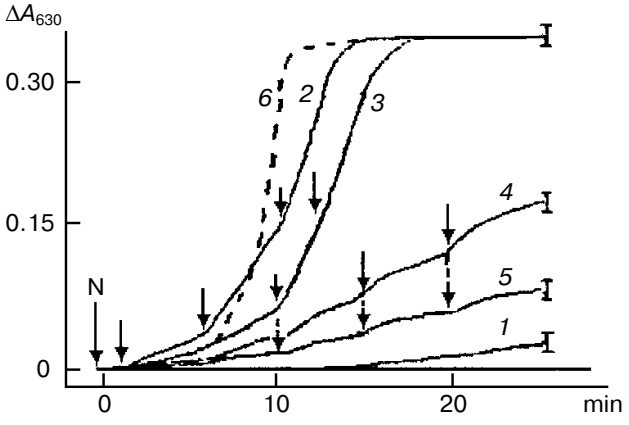

Fig. 4. Kinetics of nitrite-induced oxidation of oxyhemoglobin in the presence of different concentrations of 2-naphthol and exogenously added hydrogen peroxide. Initial concentration of 2-naphthol (μM): *1*, *2*) 2; *3*) 10; *4*) 25; *5*) 50; *6*) 0. The point of addition of nitrite (1.0 mM) is indicated by arrow N. At the points indicated by small arrows in *2-5*, 100 μM portions of H_2O_2 were added. In *1* and *6* peroxide was not added.

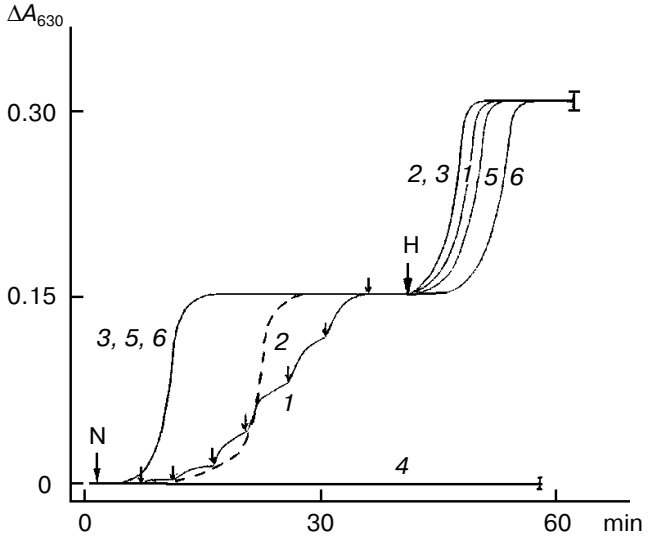

Fig. 5. Decrease in ascorbate protective effect in the course of nitrite-induced oxidation of hemoglobin. Initial concentration of ascorbate (μM): *1*, *4*) 40; *2*) 4; *3*, *5*, *6*) 0. At the point indicated by arrow N, 1.0 mM nitrite was added to the medium; at the point indicated by arrow H, hemolysate (50 μM heme) was added for the second time. Ten seconds before the second addition of hemolysate, 2 and 4 μM of ascorbate were added, respectively, to the samples *5* and *6*. In sample *1*, 100 μM portions of H_2O_2 were added at the points indicated by small arrows. Hemolysate containing 50 μM heme was initially present in the reaction medium.

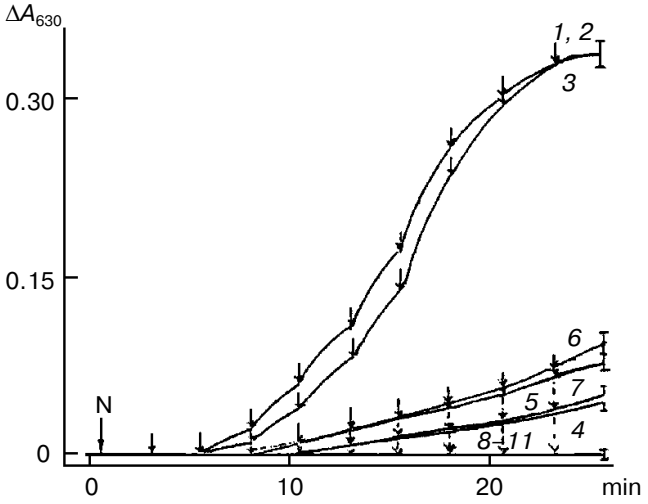

Fig. 6. Kinetics of oxidation of erythrocyte hemolysate oxyhemoglobin under the influence of low (200 μM) concentration of nitrite. Effects of catalase, chloride, bromide, and thiocyanate: *1*) 150 mM NaCl; *2*) 150 mM KBr; *3*) 50 μM KSCN; *4*) no additives; *5*) 150 mM NaCl + 0.2 μM catalase (in addition to endogenous catalase contained in hemolysate); *6*) 200 mM NaH_2PO_4; *7*) 120 mM Na_2SO_4. The point of addition of 200 μM KNO_2 is indicated by arrow N; 100 μM portions of H_2O_2 were added at the points indicated by small arrows; *8-11* correspond to *1-4* but with no KNO_2 added.

another addition of hemolysate. This is probably connected with the oxidation of these substances during the process.

The features presented in Figs. 4 and 5 were also recorded in experiments with β-naphthol, benzidine, and ascorbate [15].

4. Kinetics of the process at low (10^{-4}-10^{-5} M) initial nitrite concentrations. Effect of hydrogen peroxide, catalase, and heme-containing peroxidases. When nitrite was used at low (10^{-4} M and less) concentrations, there was a situation similar to that observed in the presence of highly efficient peroxidase substrate. Without the addition of exogenous peroxide, we observed practically no hemoglobin oxidation, at least within 20 min. The addition of exogenous H_2O_2 induced the oxidation of hemoglobin, but there were no sigmoid-shaped kinetics of the process. The rate of oxidation on the contrary decreased with time. With the addition of a subsequent portion of peroxide, the oxidation was reactivated. The slope of the kinetic curve increased as compared with the previous addition of H_2O_2, but the character of kinetics did not change (Fig. 6).

Although nitrite inhibits catalase [11], our previous studies suggest that residual catalase activity completely prevents peroxide-induced destruction of hemoglobin at all tested concentrations of nitrite and peroxide [13, 19]. The addition of peroxide to samples that contained no

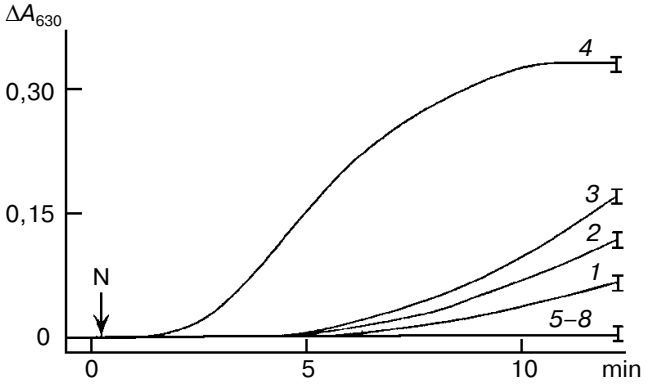

Fig. 7. Effect of heme-containing peroxidases on the kinetics of oxidation of erythrocyte hemolysate oxyhemoglobin in the presence of low (200 μM) nitrite concentration: *1*, *5*) controls; *2*, *6*) horseradish peroxidases (1.5 μM); *3*, *7*) methemoglobin (10.0 μM); *4*, *8*) lactoperoxidase (1.0 μM). KNO_2 was added to samples *1-4* at the point indicated by arrow N. In samples *5-8*, KNO_2 was not added. After the addition of nitrite, 100 μM portions of H_2O_2 were introduced in all the samples with 1-min interval.

nitrite did not result in the formation of methemoglobin (Fig. 6). Nevertheless, in the presence of 0.2 mM nitrite the rate of hemoglobin oxidation was much decreased with the addition of catalase in the medium making it

four times as active as that already present in the hemolysate. The presence of chloride and thiocyanate in plasma concentrations and also presence of bromide resulted in a significant intensification of hemoglobin oxidation (Fig. 6). In relation to the fact that chloride, bromide, and thiocyanate contribute to the inhibition of catalase in the presence of nitrite, there is reason to believe that this inhibition is responsible for the intensification. When higher concentrations of nitrite (1.0 mM) were used, halides and thiocyanate had no marked effect on the kinetics of the process [15].

The initial presence of methemoglobin and particularly of lactoperoxidase accelerated hemoglobin oxidation (Fig. 7). However, the presence of horseradish peroxidase at a concentration one and half times more than that of lactoperoxidase had no significant effect on the course of the process. As we showed earlier, of these three peroxidases lactoperoxidase has the highest affinity for nitrite as to a substrate, and horseradish peroxidase has the lowest [22].

It is noteworthy that with a high initial concentration of nitrite (1.0 mM) lactoperoxidase and methemoglobin slightly decreased the phase of initiation and had no effect on the autocatalytic phase (Fig. 2).

5. Stoichiometry of the process. The quantity of oxidized hemoglobin in the hemolysate–nitrite–exogenous peroxide system could significantly exceed the loss of nitrite in the case when the latter was initially present in quantity less than that of hemoglobin (table). With the total elimination of nitrite the hemoglobin oxidation reaction stopped [15].

6. Nitrite-induced oxidation of hemoglobin in erythrocytes. Erythrocytes, unlike their hemolysates, were much more resistant to nitrite-induced hemoglobin oxidation.

Disappearance of nitrite from the reaction medium in the course of nitrite induced hemoglobin oxidation

Initial ratio HbO_2/NO_2^-	Nitrite loss, %	
	with H_2O_2 addition	without H_2O_2 addition
1 : 1	55 ± 10	0 ± 7
1 : 0.5	80 ± 15	0 ± 8
1 : 0.25	85 ± 15	0 ± 6

Note: The content of reaction medium and the procedure of experiment are similar to those presented in Fig. 6 (curve *1*). The concentration of nitrite in samples was determined immediately after its addition and after the moment when the kinetic curve of hemoglobin oxidation reached the plateau with the total oxidation of hemoglobin. In samples in which peroxide was not added the concentration of nitrite was determined immediately and 1 h after its addition. Significant oxidation of hemoglobin within this period was not recorded.

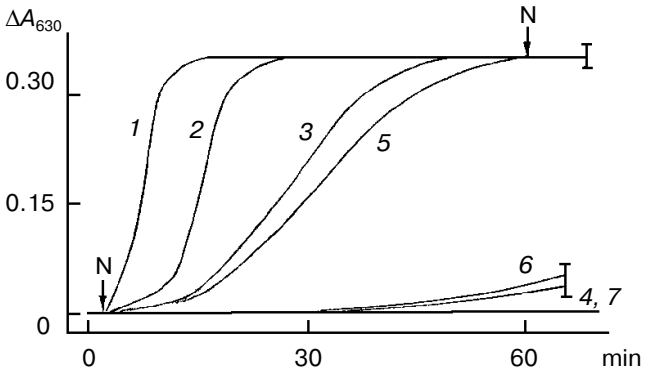

Fig. 8. Kinetics of oxidation of oxyhemoglobin in erythrocytes in the presence of nitrite (mM): *1*, *6*) 1.0; *2*) 0.1; *3*, *4*) 0.01; *5*) 0.005; *7*) 0. The point of addition of nitrite is indicated by arrow N. After the addition of nitrite, 70 μM portions of H_2O_2 were introduced in samples *1-5* and *7* with 1-min interval. Sample *4* was incubated for 3 min after the introduction of nitrite, and then the erythrocytes were washed four times with physiological solution and resuspended to achieve the previous concentration. Sample *6* was not supplemented with peroxide. The reaction medium in all samples contained 40 mM Na-phosphate buffer, 0.158 M NaCl, and erythrocyte suspension (total concentration of hemoglobin (heme) in the suspension was 100 μM), pH 7.4.

Nitrite at 1 mM concentration, which induced total oxidation of hemoglobin in hemolysate within 8 min, did not induce significant production of methemoglobin in a suspension of erythrocytes containing the same amount of hemoglobin within 60 min (Figs. 1 and 8). Repeated additions of peroxide induced oxidation and the quantity of oxidized hemoglobin could repeatedly exceed the initially added quantity of nitrite (Fig. 8).

In erythrocytes incubated for 3 min with 0.01 mM nitrite and then washed free of nitrite with physiological solution, methemoglobin was not produced even with systematic addition of peroxide. Therefore, nitrite forms no sustainable binding with erythrocyte structures.

DISCUSSION

1. Elucidation of the mechanism of the process. In practically all experiments on nitrite-induced oxidation of oxyhemoglobin on model systems, a sigmoid kinetic curve was recorded—a phase of slow oxidation (phase of initiation) was replaced with a phase of fast oxidation with accelerating kinetics (autocatalytic phase). It seems likely that some substances accelerating the process are produced in the course of the process.

Rodkey [23] assumed that methemoglobin could serve as such accelerating agent, as its initial presence in the reaction medium shortened the phase of initiation. The presence of cyanide decreased the rate of hemoglobin oxidation but could not inhibit the process complete-

ly. The author supposed that in this case two independent reactions, cyanide-dependent and cyanide-independent, take place.

Other researchers showed that active forms of oxygen play a great role in the process.

Tomoda et al. [3, 9] presumed that a direct oxidizer of nitrite could be nitric oxide formed as the result of an interaction of nitrite with superoxide anion radical, which is produced in course of hemoglobin oxidation. They based their supposition on the fact that the presence of superoxide dismutase (SOD) lead to extension of the initiation phase if the reaction was carried out in Tris-buffer. The effect of SOD was more pronounced than that of catalase. However, neither SOD nor catalase influenced the autocatalytic phase [9]. Therefore, the sigmoid kinetics could not be explained by the accumulation of superoxides or of NO or hydrogen peroxide produced in the course of dismutation of superoxides (as supposed by the authors).

Also, other authors noted that the SOD effect occurs in Tris but not in phosphate buffer [8]. This is possibly related (see below) with a different efficiency of hemoglobin autooxidation depending on ionic composition of the medium. In the course of autooxidation, superoxide is released into the medium [18, 19]. In all our experiments (see "Results") the replacement of phosphate with Tris had no marked effect.

In experiments on purified hemoglobin, Kosaka et al. [8, 24] reported catalase and methemoglobin effects equivalent to those presented in Fig. 2. Besides, in course of the reaction they recorded the production of a compound that produces an ESR signal with parameters similar to those observed when methemoglobin interacts with peroxide. It also has been shown that the presence of amines reacting with nitrogen dioxide but not oxidized in methemoglobin-catalyzed peroxidase reaction slowed both phases of the process. This is an argument in support of the idea that during nitrite-induced hemoglobin oxidation formation of nitrogen dioxide occurs. The latter is an efficient oxidizer of hemoglobin [24].

By monitoring the accumulation of methemoglobin (spectrophotometrically), the loss of nitrite (by Griss method), and also the accumulation of nitrate (using an ion-selective electrode) the authors concluded that the quantity of methemoglobin produced at any point in time is equal to the quantity of produced nitrate and disappeared nitrite. Therefore, the stoichiometry of the process is 1 : 1.

On the basis of these data the following scheme of the process, which is still widely cited by many researchers [4-6, 25-27], has been proposed.

$$HbO_2(Hb^{3+}-O_2^-) + NO_2^- + 2H^+ \rightarrow$$

$$\rightarrow Hb^{3+} + NO_2^{\cdot} + H_2O_2; \quad (1)$$

$$Hb^{3+} + H_2O_2 \rightarrow {}^{\cdot}Hb^{4+} + H_2O; \quad (2)$$

$${}^{\cdot}Hb^{4+}({}^{\cdot}X\text{-}Fe^{4+}-OH^-) + NO_2^- + H^+ \rightarrow$$

$$\rightarrow Hb^{4+}(XH\text{-}Fe^{4+}-OH^-) + NO_2^{\cdot}; \quad (3)$$

$$Hb^{4+}(XH\text{-}Fe^{4+}-OH^-) + NO_2^- + H^+ \rightarrow$$

$$\rightarrow Hb^{3+}(XH\text{-}Fe^{3+}) + NO_2^{\cdot} + H_2O; \quad (4)$$

$$HbO_2 + NO_2^{\cdot} \rightarrow Hb^{3+} + O_2 + NO_2^-; \quad (5)$$

$$2\,NO_2^{\cdot} + H_2O \rightarrow NO_2^- + NO_3^- + 2\,H^+. \quad (6)$$

General stoichiometry of the process:

$$4HbO_2 + 4NO_2^- + 4H^+ \rightarrow$$

$$\rightarrow 4\,Hb^{3+} + 4NO_3^- + O_2 + 2H_2O.$$

Mechanism of nitrite-induced hemoglobin oxidation according to Kosaka et al. [8]

Scheme 1

(To avoid ambiguity all the designations are identical to those used by in [8]: HbO_2 is oxyhemoglobin (subunit), Hb^{3+} is methemoglobin (subunit), ${}^{\cdot}Hb^{4+}$ and Hb^{4+} are initial and partially reduced methemoglobin–peroxide complexes, respectively. Structural forms of corresponding compounds as they are conceived by the authors are presented in parentheses.)

Thus, Kosaka et al. consider the oxidation of individual heme groups irrespective of quaternary structure of the hemoglobin molecule. They suppose that the autocatalytic phase is provided by the reactions (3)-(5).

However there is an obvious inconsistency between their data and their scheme. Hydrogen peroxide that is necessary for reactions (2)-(4) is produced only in reaction (1). It is the compound that limits the rate of the process. But then sigmoid kinetics should not be observed. One may suppose that peroxide is accumulated in the medium. But the data with the use of catalase contradict this supposition.

Doyle et al. proposed a rather improved scheme [7]. They confirmed the effects of peroxide, catalase, and methemoglobin recorded by previous authors in the experiments on isolated pure hemoglobin. The stoichiometry of the process was determined by monitoring the amount of produced methemoglobin and the amount of produced nitrate. The amount of nitrate (determined

chromatographically) produced after the total oxidation of hemoglobin has been shown to depend upon the initial ratio $[NO_2^-]/[HbO_2]$, and the ratio $[NO_3^-]/[Hb^+]$ was proved to be maximally 1 : 1 at $[NO_2^-]/[HbO_2] > 8$.

From these data, the following mechanism of the process has been proposed.

$$HbO_2 + NO_2^- \rightarrow HbO_2^- + NO_2; \quad (1)$$

$$(Hb^+ + O_2^{2-} + NO_2);$$

$$Hb^+ + HOOH \rightarrow HbO_2H + H^+; \quad (2)$$

$$H^+ + HbO_2H + NO_2^- \rightarrow$$

$$\rightarrow [HbOH]^+ + NO_2 + OH^-; \quad (3)$$

$$H^+ + [HbOH]^+ + NO_2^- \rightarrow$$

$$\rightarrow Hb^+ + NO_2 + H_2O;$$

$$HbO_2 + NO_2 \rightarrow Hb^+ + O_2NOO^-; \quad (4)$$

$$2H^+ + O_2NOO^- + HbO_2 \rightarrow$$

$$\rightarrow Hb^+ + HOOH + NO_2 + O_2; \quad (5)$$

$$HbO_2 + O_2NOO^- \rightarrow$$

$$\rightarrow Hb^+ + O_2^- + NO_2^- + O_2; \quad (6)$$

$$2NO_2 \leftrightarrow N_2O_4 \xrightarrow{H_2O} NO_2^- + NO_3^- + 2H^+. \quad (7)$$

Mechanism of nitrite-induced hemoglobin oxidation according to Doyle et al. [7]

Scheme 2

(The designations correspond to those used in [7]: HbO_2 is oxyhemoglobin (subunit), Hb^+ is methemoglobin (subunit), HbO_2H and $[HbOH]^+$ are the initial and partially reduced methemoglobin–peroxide complexes, respectively). Doyle et al. also considered the oxidation of individual heme groups irrespective of quaternary structure of the hemoglobin molecule.

The autocatalytic phase is provided by reactions (2)-(5). The higher the concentration of nitrite, the more intensive will be its oxidation in reaction (3) and so the greater the production of $NO_2^{\bullet}$. The intensification of its production by n times in principle will result in increase in the rate of the reaction (4) also by n times (with the maintenance of the previous concentration of HbO_2) and of the reaction (7) by n^2 times and, consequently, in the increase in the ratio $[NO_3^-]/[Hb^+]$. It seems logical. The authors affirm that with the initial ratio $[NO_2^-]/[HbO_2] > 8$, the final ratio $[NO_3^-]/[Hb^+]$ reaches the maximum 1 : 1. However this may be achieved if produced molecules of $NO_2^{\bullet}$ chiefly react as in reaction (7) and not as in reaction (4). A quantitative estimation shows that it is necessary that 14 out of 15 $NO_2^{\bullet}$ molecules react by reaction (7). But in this case most of the methemoglobin will be produced in the course of reaction (1) and sigmoid kinetics will not be observed. But the data obtained by the same authors show the contrary: with the ratio $[NO_2^-]/[HbO_2]$ even equal to 20 there are pronounced initiation and autocatalytic phases, and most of he-moglobin (about 90%) is oxidized in the autocatalytic phase.

It is noteworthy that the scheme does not explain why neither catalase nor methemoglobin influence the autocatalytic phase.

The oxidation of hemoglobin in erythrocytes under the influence of nitrite was at the least an order of magnitude slower than that of hemoglobin of erythrocyte hemolysates under the same conditions, and the kinetics were not always accelerating [28]. There is no clear reason for this difference.

Several schemes have been proposed which develop earlier schemes without fundamentally contradicting them [6, 10].

In our opinion all previous studies of the mechanism of nitrite-induced oxidation of hemoglobin suffered from the following shortcomings. First, the process was considered as a direct interaction of nitrite and hemoglobin. Such factors as the initial concentration of peroxide resulting from spontaneous autooxidation of hemoglobin, the contribution of peroxide from the outside, and substances normally contained in erythrocytes that can influence the process were excluded. We would like to note that these substances can include not only ascorbate, steroid hormones and catecholamines, but also several amino acids and polypeptides that are also peroxidase substrates [29]. Without regard for these factors the reasons for the two-phase process and the differences between the phases are difficult to understand.

Second, in view of a low rate of the process in case of physiological concentrations of nitrite (see Figs. 1 and 8) all model experiments were carried out at obviously non-physiological nitrite concentrations (of the order of mM) and under conditions of significant predominance of nitrite concentration over that of hemoglobin. It is difficult to determine the stoichiometry of the process under these conditions.

In the majority of studies [4-6, 25-27], the authors have worked from the stoichiometry 1 : 1 proposed by

Kosaka et al. [8]. Thus, considering the concentration of hemoglobin in erythrocytes, the fact that in erythrocytes the reaction goes much less efficiently than in hemolysates and in pure hemoglobin ([28] and also Figs. 1 and 8) and also the circumstance that nitrite is efficiently removed from blood by the kidneys [4], one could determine what concentration of nitrite is required for achieving even 10% methemoglobinemia (the minimal concentration recorded visually). This concentration should be no lower than 1-2 mM, and, moreover, it should be maintained for a rather long time—at least for half an hour (see Fig. 8). But *in vivo* a significantly higher concentration of methemoglobin (50% and more) was achieved with the presence in blood of nitrite in concentrations more than an order of magnitude lower, where nitrite was either introduced once exogenously or produced endogenously as a consequence of inflammatory reactions and disbacteriosis [4].

Therefore, no model of nitrite-induced oxidation of hemoglobin available at present explains the real reaction taking place both in model systems and *in vivo*. In particular, the role of peroxide and peroxide-metabolizing enzymes remains unclear: what can explain their different effect on the initiation and autocatalytic phases? The stoichiometry of the process is also unclear.

2. Initiation of the process and its driving stage. We can see from the data presented in Fig. 2 that the process is peroxide- and methemoglobin-dependent. The data of experiments using lactoperoxidase, methemoglobin, horseradish peroxidase (Figs. 2 and 7), and peroxidase substrates (Figs. 3 and 4) show that the process is peroxidase-dependent.

Compounds that can be oxidized in the methemoglobin-catalyzed peroxidase reaction prevented the development of the process, affecting both its phases over the whole duration of kinetics curve (Figs. 3-5). Therefore, we can suppose that the driving stage of the process is peroxidase oxidation of nitrite catalyzed by methemoglobin acting as peroxidase. The product of this oxidation (apparently nitrogen dioxide [24]) is a direct oxidizer of hemoglobin. Compounds competing with nitrite for peroxidase protect nitrite from oxidation and prevent the production of a direct oxidant.

The fact that the most efficient peroxidase substrates prevent the process completely and stop it at any stage, even if they are present in concentrations much lower than these of nitrite and hemoglobin (Fig. 3), indicates that a direct oxidative–reductive interaction between nitrite and hemoglobin in a neutral medium is virtually absent, contrary to widespread opinion [5-10, 23, 25-27]. The rate constant of reaction (1) from the schemes of Kosaka and Doyle, if this reaction really takes place, is much lower than that of following reactions. On the basis of our data using peroxidase substrates, competitors of nitrite, this constant is less than 0.1 $M^{-1}\cdot sec^{-1}$ [15]. Therefore, this reaction is not likely to play a significant role in the presence of nitrite under physiological concentrations (10^{-7}-10^{-5} M [11]).

But how can the process be initiated?

It is known that in the presence of oxygen hemoglobin is subject to autooxidation with the production of active forms of oxygen and hydrogen peroxide. Some anions can enhance this process [18]. Possibly, nitrite also has such ability. Produced peroxide and some quantity of methemoglobin (its concentration in erythrocytes normally is up to 2% [17, 18]) are apparently the factors determining the development of methemoglobin–peroxide complexes and initial oxidation of nitrite by these complexes. *In vivo* peroxide also comes into erythrocytes from the outside due to the activity of immunocompetent cells and various oxidases [17, 18]. This source seems to be of considerable if not of first importance.

3. Development of the process and the nature of the two phases. Based on the effects of methemoglobin, lactoperoxidase, catalase, azide (Fig. 2), and cyanide [8, 23] there is a qualitative difference between the initiation and autocatalytic phases. The first depends on the above factors and the second does not. However substances that can be oxidized in the methemoglobin-catalyzed peroxidase reaction effect both phases and depending on their concentration contribute to the delay of autocatalytic phase or to its elimination. We noticed that the ratio of these phases in the quantity of hemoglobin oxidized during each depends both on nitrite concentration (Fig. 1) and on the presence of the above substances as far as a clear delimitation between the two phases disappears (Fig. 3). The data presented in Figs. 3 and 5 suggest that the presence of nitrite competitors for peroxidase determines not only the character of kinetics but the ratio between the phases as well: the autocatalytic phase develops only when these compounds are predominantly oxidized.

A similar situation in the case of low concentrations of nitrite apparently resulted from the presence of competitive substrates as an admixture in the hemolysate. They can also be present in a solution of pure hemoglobin as several amino acids and apoenzyme-adsorbed low-molecular-weight compounds [29]. If we supposed that these substrates on average have a higher affinity to methemoglobin as a peroxidase as compared with nitrite, a significant oxidation of the latter is possible only after the oxidation of the former.

The matter of the ratio of oxidation phases is connected with the dependence of the process on exogenously added hydrogen peroxide. Both in the case of high concentrations of competitive substrates and in the case of low concentrations of nitrite (Figs. 4 and 6) the process depends on exogenously added hydrogen peroxide: it does not take place without the addition of peroxide. It is remarkable that in the latter case the rate of the process is dependent to a greater extent both on catalase concentration and on factors influencing its activity as

opposed to the case of higher nitrite concentrations (Figs. 1 and 6).

Conceivably, there may be two processes taking place at the same time: one requires free peroxide accessible for catalase and another that does not require free peroxide. In this connection, the latter does not depend either on exogenously added catalase, peroxidases, and their inhibitors (cyanide-dependent and cyanide-independent reactions according to Rodkey [23]). In both processes hemoglobin oxidation takes place and peroxidase oxidation of nitrite is the driving stage. The product of this oxidation (obviously $NO_2^{\bullet}$ [24]) is a direct oxidizer of hemoglobin. In this reaction, hydrogen peroxide is produced, which is necessary for maintaining the process. In the presence of any competitive substrates, a part of the peroxide is spent for their oxidation that does not result in the production of new H_2O_2 molecules, and the exogenous addition of peroxide is required to maintain the process.

Why is the autocatalytic phase not sensitive to the presence of catalase? Earlier we supposed that in response to the oxidation of oxyhemoglobin subunit by the products of nitrite oxidation a methemoglobin–peroxide complex is immediately formed [15]. This complex is similar to that in the schemes of Kosaka and Doyle but without emergence of peroxide out of the globule, and hence peroxide is not accessible for catalase. This complex as a biequivalent oxidizer can oxidize two molecules of nitrite to $NO_2^{\bullet}$, if there are no other substrates. These two $NO_2^{\bullet}$ molecules will provide for the production of the two new complexes and, therefore, the rate of the process accelerates with time. In the presence of substrates, competitors of nitrite that can also be oxidized by these complexes, but without production of new ones, the character of the kinetics will depend on the number of new complexes that on average generate the previous complex: one, or more or less than one.

4. Mechanism of direct hemoglobin oxidation. How does the oxidation of hemoglobin occur where a methemoglobin–peroxide complex is immediately formed without the liberation of peroxide into the medium? Unfortunately, we cannot propose a concrete mechanism here. Earlier we presumed that $NO_2^{\bullet}$ induces the autooxidation of a hemoglobin subunit and also of a neighboring subunit (according to Tomoda [9]) by oxidizing a structure of the active center or close to it. $NO_2^{\bullet}$ itself is reduced to nitrite [15]. Two O_2^- produced in the course of the autooxidation of two neighboring subunits can dismutate into peroxide, which is bound with the active center of one subunit without emerging from the globule. It is also possible that one O_2^- penetrates into a neighboring subunit, taking one electron from its active center, which results again in the production of methemoglobin–peroxide complex, as proposed in the scheme of Fridovich [30]. The oxidation via the stage of peroxinitrate production as suggested by Doyle [7] is also possible with the difference that methemoglobin–peroxide complex and nitrite are produced and not $NO_2^{\bullet}$. Otherwise, competitors of nitrite for peroxidase could not inhibit the process fully (see Scheme 2). It is obvious that in any case two electrons are required: for reduction of $NO_2^{\bullet}$ to nitrite and of superoxide to peroxide. It is conceivable that one electron is taken from heme and the other from the apoenzyme.

In this relation we consider it necessary to examine the scheme of Lissi [5], who in 1998, a year after our study [15], also suggested a direct production of methemoglobin–peroxide complex. The author criticized the scheme of Kosaka as unable to explain the accelerated kinetics, indicating the same reasons that we had advanced. In this connection (only in the effort to explain the kinetics without their own experimental data), Lissi suggested that stage (5) in the scheme of Kosaka et al. is as follows:

$$HbO_2 + NO_2^{\bullet} + H_2O \rightarrow Hb^{3+}HOOH + NO_3^-.$$

Therefore, the oxidation of oxyhemoglobin by nitrogen dioxide results in the production of methemoglobin–peroxide complex and nitrate. An electron required for reduction of superoxide to peroxide is taken from nitrogen dioxide, which acts hence as a reducer. Methemoglobin–peroxide complex can oxidize two molecules of nitrite with the production of two $NO_2^{\bullet}$, which, in turn, will oxidize two new subunits with the formation of two new complexes. This is the reason for the acceleration of the rate of the process with time. The existence of two phases in the process is explained by the fact that reactions (1) and (2) (see Scheme 1) prevail at the initial stage and then reactions (4)-(6). This also explains a different effect of catalase on the first and second phases.

However, according to this scheme the stoichiometry could not exceed $\Delta HbO_2/\Delta NO_2^- = 1:1$ or $2:1$, based on the hypothesis that the oxidation of one subunit in a hemoglobin tetramer immediately induce the oxidation of a neighboring one, as Tomoda [9] and Huisman [31] have shown. Moreover, the above values will be lower because part of the $NO_2^{\bullet}$ will react between them with the production of nitrate (reaction (6) according to Kosaka [8]). But this is inconsistent with our data (table and Fig. 8).

5. Proposed scheme of the process. Based on the above analysis of data obtained by us and by other researchers we propose the following scheme of nitrite-induced oxidation of hemoglobin. This scheme examines the reactions taking place in the four-subunit molecule of hemoglobin.

$$Hb^+ + H_2O_2 \rightarrow HbOOH + H^+; \quad (1)$$

$$H^+ + HbOOH + NO_2^- \rightarrow$$

$$\rightarrow [HbOH]^+ + NO_2^{\bullet} + OH^-; \quad (2)$$

$$[HbOH]^+ + NO_2^- \rightarrow Hb^+ + NO_2^{\bullet} + OH^-;$$

$$H^+ + HbOOH + H_2S \rightarrow$$

$$\rightarrow S^{\bullet} + 2H_2O + Hb^+; \quad (3)$$

$$NO_2^{\bullet} + (HbO_2)_4 + H^+ \rightarrow$$

$$\rightarrow (HbO_2)_2\,(Hb^+)(HbOOH) + \quad (+)$$

$$+ NO_2^- + O_2; \quad (4)$$

$$2NO_2^{\bullet} \overset{H_2O}{\leftrightarrow} N_2O_4 \rightarrow NO_2^- + NO_3^- + 2\,H^+. \quad (5)$$

Our proposed mechanism of nitrite-induced oxidation of hemoglobin

Scheme 3

Here, S are substrates, competitors of nitrite; $(HbO_2)_4$ is oxyhemoglobin (tetramer); Hb^+ is methemoglobin subunit as part of completely oxidized tetramer or semi-oxidized hybrid; HbOOH and $[HbOH]^+$ are initial and partially reduced methemoglobin–peroxide complexes, respectively. They can be constituents of a semi-oxidized hybrid, $(HbO_2)_2\,(Hb^+)(HbOOH)$, and also of a completely oxidized tetramer.

From the above considerations, we regard reaction (1) from the scheme of Kosaka and Doyle as unimportant under physiological conditions, methemoglobin and hydrogen peroxide being always present in erythrocytes [17, 18] producing HbOOH complexes (reaction (1)). These complexes oxidize nitrite to $NO_2^{\bullet}$ (reaction (2)), which is a direct oxidizer of hemoglobin. From the oxidation of one subunit, a methemoglobin–peroxide complex is produced and $NO_2^{\bullet}$ is reduced to nitrite. One electron required for the reaction is presumably taken from apoenzyme (symbol "+" above in the reaction (4)). The oxidation of one subunit induces the oxidation of a neighboring one [9, 31]. The produced complex, in turn, oxidizes either nitrite or a competitive substrate (reaction (3)).

Semi-oxidized tetramers of hemoglobin are oxidized according to the same scheme.

According to the data of some researchers, the rate constant of reaction (5) is about 300 sec^{-1} [32]. That is a relatively slow reaction. Constant (1) according to our data [22] and also data of other researchers [33] is of the order of $10^4\ M^{-1}\cdot sec^{-1}$. The overall constant of nitrite loss as the result of methemoglobin-catalyzed peroxidase oxidation (reaction (2)) is about 300 $M^{-1}\cdot sec^{-1}$ [22, 27]. Hence nitrite is relatively inefficient as a substrate of the methemoglobin-catalyzed peroxidase reaction, and there are good grounds to believe that admixture substrates in erythrocytes or their hemolysate should be more efficient on the average. From our estimation based on the data of stoichiometry and the total rate of the process, constant (4) is within 10^4-$10^5\ M^{-1}\cdot sec^{-1}$.

The stoichiometry of the process is thus dependent on the ratio of rates of reactions (4) and (5), if there are no other substances in the medium able to react with $NO_2^{\bullet}$ without reduction the latter to nitrite. Hence, the stoichiometry can be greater than ΔHbO_2 (subunit): $\Delta NO_2^- = 2 : 1$, and lower in the case when the rate of nitrogen dioxide production is rather high, the concentration of oxyhemoglobin being relatively low (conditions for predominance of reaction (5)) and an external source of continuous hydrogen peroxide addition being available. In fact, in the case with lactoperoxidase (see Fig. 7) the stoichiometry of the process was about 1 : 2 [22].

The autocatalytic phase can develop only when all substrates, competitors of nitrite (S), are oxidized to an extent that every methemoglobin–peroxide complex should produce more than one such compound on average. This is apparently the reaction that takes place during the initiation phase, the duration of which depends on the availability of these substrates (Fig. 3) and also on the presence of free peroxide and peroxidases in the medium (Fig. 2). Catalase, by destroying free peroxide, and azide and cyanide, by binding methemoglobin, oppose the oxidation of competitive substrates and extend the initiation phase.

6. Principal mechanisms of protection of erythrocytes against nitrite toxicity. It follows from our data that nitrite even in millimolar concentrations does not induce hemoglobin oxidation if systems oxidizing it to a direct oxidizer of hemoglobin do not operate (Fig. 3). On the contrary, even micromolar concentrations of nitrite are able to provide practically total oxidation of hemoglobin in erythrocytes when there is a source of peroxide and methemoglobin (Fig. 8). Thus, the availability of a pool of compounds competing with nitrite for methemoglobin as peroxidase is obviously this main factor that prevents erythrocyte hemoglobin from nitrite-induced oxidation. Catalase cannot completely stop the process that has been already started, as it is not able to destroy methemoglobin–peroxide complexes. But it can delay the process and even prevent it with a relatively low concentration of nitrite and a high concentration of competitive substrates by destroying free peroxide required for the production of methemoglobin–peroxide complexes. The situation taking place in the experiments presented in Figs. 6 and 8 is apparently similar to that which exists *in vivo*: physiological concentrations of nitrite or close to these and continuous inflow of peroxide from the outside.

The considerably higher resistance of erythrocytes to nitrite-induced methemoglobin production as com-

pared with hemolysates is evidently due to the following factors.

1) The efficient ascorbate reductase system, which maintains a stable concentration of ascorbate. Ascorbate, in turn, maintains the concentration of other peroxidase substrates unchanged [20, 21, 34]. The ascorbate reductase system in hemolysates operates with much lower efficiency due to the decline of local concentrations of glutathione and ascorbate [35].

2) The low concentration of anions including halide anions inside erythrocytes as compared with the plasma. It is known that normally the concentration of anions in a cell is many times lower than in the extracellular medium. The concentration of chloride in a cell is about 5 mM according to some evidence [36]. Under these conditions, the inhibition of catalase in the presence of nitrite at concentrations below 0.2 mM is not significant [11, 13]. However, according to our data peroxidase activity of methemoglobin and lactoperoxidase does not change in relation to nitrite in the presence of halides [22]. The low concentration of anions inside cells can be considered as an important protective mechanism against nitrite toxicity.

Production of NO in the course of the interaction of nitrite with hemoglobin recorded by Tomoda [3] probably results from side reactions of nitrite, which occur independently of the presence of oxygen. The production of NO has been detected by other researchers during the interaction of nitrite with deoxyhemoglobin [37, 38]. Judging from its rate and stoichiometry [37, 38], this process is unlikely to play a significant role in the development of methemoglobinemia under physiological conditions, but it may be responsible for a vascular response observed with exogenous input of large doses of nitrite into an organism [39].

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