Kinetic regularities of NO donation by binuclear dinitrosyl iron complexes with thiolate ligands based on thiophenol derivatives in the presence of red blood cells

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The kinetics of nitrogen oxide release by binuclear dinitrosyl iron complexes (B-DNICs) with thiolate ligands based on thiophenol and its several oxy, amino, and nitro derivatives was studied using a suspension of red blood cells simulating the internal medium of a blood vessel. The NO donating ability of the complexes was estimated by the kinetic parameters of first-order equation, which described the formation of intra-erythrocytic methemoglobin. Three typical kinetic profiles of NO donation were distinguished: pseudosaturation donation and donation with prolonged and explosive profiles. In the case of first-type NO donation typical of complexes with thiophenol and its nitro derivatives, the curves display a fast coming to saturation long before the complete release of all NO groups contained in the structure of the starting complex into a solution. Such a type of donation is likely due to the formation of long-lived nitrosyl intermediates in the system. The prolonged type of NO donation shown by the complex with hydroxyphenyl ligand is characterized by virtually constant rate of NO release into a solution without pronounced transition to saturation during experiment (10—12 min). In the case of explosive-type donation characteristic of the complex with aminophenyl ligands, a considerable portion of NO was fast released into a solution within 1—3 min. All complexes under study caused hemolysis of a 0.2% suspension of red blood cells. The complex with aminophenyl ligands exhibited the highest hemolytic activity.

Key words: red blood cells, methemoglobin, hemolysis, nitrogen oxide, binuclear dinitrosyl iron complexes.

Nitrogen oxide as a signaling molecule fulfills important biological function in a body associated, first of all, with blood pressure modulation, inhibition of thrombosis, nerve impulse transmission, and nonspecific immune defence.[1] The insufficiency of enzymatic synthesis of nitrogen oxide underlies the pathogenesis of many cardiovascular diseases, the pharmacological management of which can be carried out using exogenous donors of nitrogen oxide.[2]

Clinically widely used nitrogen oxide donors based on organic nitrates and nitrites have grave disadvantages associated with the development of tolerance and a number of endothelial dysfunctions.[3] For this reason, the search and study of bioactivity of new NO-donating compounds in order to design highly efficient medicines is one of important trends in modern medicinal chemistry.[4,5]

The long-term studies of A. F. Vanin summarized in the recently published monograph[6] provide a comprehensive insight into various manifestations of physiological activity of dinitrosyl iron complexes (DNICs) with thiol-containing ligands of endogenous nature. The observed regulatory effect of DNIC on different physiological processes was found to completely coincide with the physiological effect exerted by free nitrogen oxide.[6] It means that the bioactivity of DNICs with thiol-containing ligands is governed by their capability of acting as nitrogen oxide donors. This conclusion scientifically proves that DNICs are promising from the pharmacological point of view as exogenous sources of nitrogen oxide. Dinitrosyl iron complexes in question are not only complexes with endogenous ligands, such as cysteine and glutathione, but also those with any other organic thio derivatives. Such low-molecular-weight DNICs are currently considered as promising depot of nitrogen oxide to design new-generation pharmacological products for the treatment of socially significant diseases, first of all, cardiovascular and oncological diseases.[7,8] The development of synthesis methods that enabled preparation of various mono- and binuclear dinitrosyl complexes with thiol-containing ligands based on aromatic and heterocyclic thiols, as well as aliphatic sulfhydryl derivatives of amino acids and thiourea became a good basis for the progress of this research trend.[9—11]

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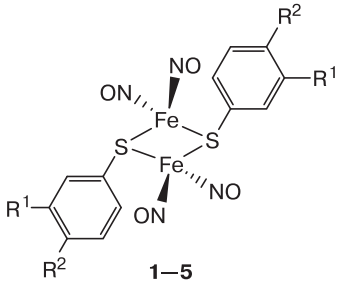
At the same time, the chemically stipulated NO-donating activity is realized under body conditions with certain specificity due to the features of interactions between exogenous NO donors and biosubstrates, which required special studies on the nature and mechanism of above-mentioned interactions. Nitrogen oxide is known to be physiologically active first of all in blood vessels filled with blood where erythrocytes are 90% of total blood cells. For this reason, a suspension of red blood cells was proposed as a model of the internal content of blood vessel, using which one can study the pharmacological potential of nitrogen oxide donors with regard to cardiovascular system.[12,13]

During these studies it was found that the rate constants of oxidation of intra-erythrocytic oxyhemoglobin to methemoglobin can serve as a measure of the NO donating ability of exogenous NO donor. At the same time, the most of DNICs showed a regular decrease in the NO donating ability with an increase in the cell level in a suspension.[14] This effect was found to be due to partial adsorption of DNICs on the cell surface to result in the formation of additional equilibrium pool of the membrane-bound complex, the rate of hydrolytic dissociation of which decreased due to a limited contact with the aqueous medium. The NO donating ability of DNICs in the presence of red blood cells depends on the ratio of equilibrium concentrations of free and membrane-bound complexes.[13,15]

The biochemical transformation of nitrogen oxide to form peroxynitrite, which under certain conditions can induce the hemolysis of erythrocytes, is yet another important feature in the interaction of DNICs with red blood cells.[12,13,16]

Thus, the data obtained using the erythrocytic model and generalization made using these data provide a good basis for the study of new low-molecular-weight analogs of DNICs. In particular, fine relationships between the structural features of ligands belonging to one chemical family and the functional properties of corresponding DNICs should be studied.

The aim of the present work was to study the NO donating and hemolytic ability of binuclear dinitrosyl complexes **1—5** containing a $\{Fe(NO)_2\}$ moiety and thiophenol-derived thiolate ligands.



1—5

Compound	R^1	R^2	Compound	R^1	R^2
1	H	H	**4**	H	NO_2
2	OH	H	**5**	H	NH_2
3	NO_2	H			

Experimental

Nitrosyl complexes. The binuclear dinitrosyl iron complexes (B-DNICs) used in the present work had the composition $[Fe_2(SR)_2(NO)_4]$, where R is phenyl (**1**),[17] 3-hydroxylphenyl (**2**),[18] 3-nitrophenyl (**3**),[19] 4-nitrophenyl (**4**),[20] and 4-aminophenyl (**5**).[21] Compounds **1—5** were identified by elemental analysis and IR spectroscopy using equipment of the Center for Shared Use at the Institute of Problems of Chemical Physics of the Russian Academy of Sciences (IPCP RAS) and their physicochemical properties coincided with those obtained earlier.[17—21] The complexes were added to a suspension of red blood cells as the DMSO solutions which were freshly prepared prior to experiments. The concentration of DMSO in the samples did not exceed 3%.

Red blood cells. Blood was sampled from C 57 Bl/6f mice (3-month-old, the weight was 18—20 g) obtained from the nursery at the IPCP RAS. Red blood cells were isolated according to the described procedure[12] using pH 7.4 normal saline (145 m*M* NaCl, 3.88 m*M* Na_2HPO_4, and 1.12 m*M* NaH_2PO_4).

Total hemoglobin (the total content of all hemoglobin forms) was determined by the absorbance of hemolysate (taking into account corrections for light scattering) at a wavelength of 525 nm, which is an isosbestic point for the absorption spectra of deoxy-, oxy- and methemoglobin using an extinction coefficient of 7.5 L $mmol^{-1}$ cm^{-1} (per one heme).[22]

Intra-erythrocytic methemoglobin[12] ($HbFe^{3+}$]) was determined in preliminary hemolyzed aliquots of the red blood cell suspension by the absorbance at 630 nm. The concentration of methemoglobin was determined by the following equation

$$[HbFe^{3+}] = \frac{\Delta A_{630}}{\varepsilon_{met} - \varepsilon_{oxy}} \cdot d, \quad (1)$$

where ΔA_{630} is the absorbance increment of the sample at 630 nm compared to the control sample, ε_{met} and ε_{oxy} are the extinction coefficients of methemoglobin and oxyhemoglobin, respectively, and d is the dilution factor in a cuvette.

Hemolysis of red blood cells.[12] Hemolytic experiments were carried out using a 0.2% (v/v) suspension of red blood cells in normal saline at 37 °C with continuous slow stirring. The course of the red cell lysis was monitored by a change in the absorbance of the suspension at 700 nm. The degree of hemolysis was determined by Eq. (2)

$$\gamma = \frac{D_0 - D}{D_0 - D_{H_2O}}, \quad (2)$$

where D_0 and D are the absorbances of control and test samples, respectively, and D_{H_2O} is the absorbance of the sample under conditions when all red blood cells underwent complete lysis by distilled water. The hemolytic activity of complexes was quantitatively characterized by the induction period of hemolysis (I_{20}) determined graphically as a time when the degree of hemolysis reached 20%.

Results and Discussion

The presently studied compounds **1—5** relate to μ-S-type complexes. In their structures, two iron atoms each bearing two NO groups are linked through the bridging sulfur atoms of the corresponding thiol.

Processes occurring during decomposition of B-DNIC in a suspension of red blood cells can be represented as two successive reactions. First, B-DNIC undergoes hydrolytic dissociation outside of a red blood cell (reaction (3)):

$$[Fe_2(SR)_2(NO)_4] \longrightarrow$$

$$\longrightarrow 4\ NO + \text{Decomposition products of complex.} \quad (3)$$

Then, nitrogen oxide entering into a red blood cell through diffusion is rapidly oxidized as a result of the bimolecular reaction with oxyhemoglobin (reaction (4)):

$$[HbFe^{2+}]O_2 + NO \longrightarrow HbFe^{3+} + NO_3^-. \quad (4)$$

A high rate of the reaction between oxyhemoglobin and nitrogen oxide ($3—5 \cdot 10^7$ L mol^{-1} s^{-1}) (see Ref. 23) combined with a high concentration of oxyhemoglobin in a red blood cell (higher than 10^{-2} mol L^{-1} per heme) offer a higher priority to this channel of oxidative nitrogen oxide transformation, allowing one to consider a red blood cell suspension as an efficient trap of free nitrogen oxide.[24] According to the stoichiometry of reaction (4), the molar concentration of methemoglobin forming in the system should correspond to the number of moles of NO released from the complex into a free solution. In accordance with this, the course of formation of intra-erythrocytic methemoglobin gives us a kinetic profile of NO donation into a solution.

Figure 1 shows the kinetics of formation of intra-erythrocytic methemoglobin in the presence of complexes **1—5**. As it has been shown earlier,[12] the above-mentioned process is well described by the exponential dependence (5)

$$[HbFe^{3+}] = Ae^{-k_{eff}t} + [HbFe^{3+}]_\infty, \quad (5)$$

where $[HbFe^{3+}]$ is the actual concentration of methemoglobin, t is time, k_{eff} is the effective rate constant of methemoglobin formation, $[HbFe^{3+}]_\infty$ is the limit concentration of methemoglobin at $t \to \infty$, and A is the preexponential factor.

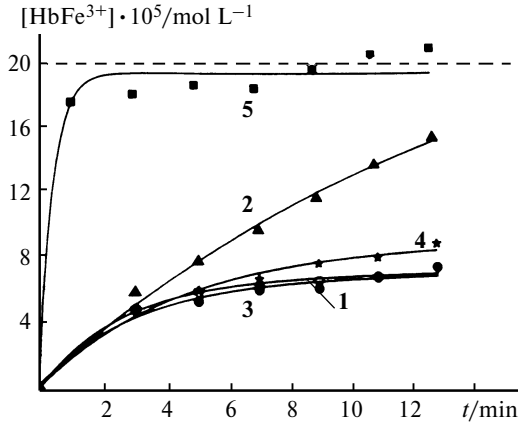


Fig. 1. Kinetic curve for the formation of methemoglobin ($HbFe^{3+}$) in the presence of complexes **1—5** at concentrations of $1.2 \cdot 10^{-4}$ mol L^{-1}. The concentration of hemoglobin was $2 \cdot 10^{-4}$ mol L^{-1}. The highest concentration of methemoglobin (corresponds to the starting concentration of hemoglobin) is shown as a dashed line.

The kinetic parameters of methemoglobin formation in the presence of compounds **1—5** are given in Table 1.

The analysis of kinetic curves (see Fig. 1) and corresponding quantitative parameters (see Table 1) allows us to distinguish three characteristic types of the kinetic profiles of NO donation. The kinetic curves for complexes **1**, **3**, and **4** almost coincide. These complexes are characterized by moderate rates of NO donation (k_{eff}) and low yields of NO. After a small portion of NO groups was released into a solution, the release of NO into a free solution is dramatically retarded followed by coming to saturation. It is important that saturation occurs long before the complete release of all NO groups contained in the complex into a solution. We will refer to such a profile

Table 1. Kinetic parameters for the formation of methemoglobin and erythrocyte hemolysis

Complex	$k_{eff} \cdot 10^3$ /s^{-1}	$[HbFe^{3+}]_\infty \cdot 10^5$ /mol L^{-1}	η(NO) (%)	I_{20}/min	
				$5 \cdot 10^{-6}$/mol L^{-1}	$2 \cdot 10^{-5}$/mol L^{-1}
1	6.7±0.9	6.4±0.2	13	–	33.0±1.5
2	1.1±0.3	25.4±5.1	52	63.8±1.2	7.2±0.4
3	5.9±1.3	6.2±0.3	13	130.5±2.1	25.1±0.8
4	3.9±0.6	8.2±0.4	17	–	23.5±1.0
5	38.6±11.5[a]	19.4±0.5[a]	40[a]	3.5±0.3	<1
	32.7±6.0[b]	17.1±0.4[b]	35[b]		

Note: k_{eff} is the effective rate constant of methemoglobin formation (characterizes the NO donating ability of the complex), $[HbFe^{3+}]_\infty$ is the limit concentration of methemoglobin (characterizes the depth of decomposition of the complex), η(NO) is the yield of NO determined as the ratio of $[HbFe^{3+}]_\infty$ to the total content of NO groups in the complex (4[B-DNIC]$_0$); and I_{20} is the induction period (the time for which the degree of hemolysis reaches 20%) at concentrations of the complex equal to $5 \cdot 10^{-6}$ and $2 \cdot 10^{-5}$ mol L^{-1}.

[a] According to the data of Fig. 1.

[b] According to the data of Fig. 3.

of NO donation as "donation with pseudosaturation". Note that the most of earlier studied B-DNICs with thiol-containing ligands of other chemical nature shows this type of NO donation.[12] It is likely that, in this case, NO groups are stabilized by some means being preserved in the system as long-lived nitrosyl-containing structures.

The second type of kinetics of NO donation is typical of complex **2**. In this case, the course of NO donation throughout experiment appears as a straight line, where a slight bend caused by a critical decrease in the content of oxyhemoglobin begins to take shape. Upon an increase in the concentration of oxyhemoglobin in the system, this effect is not observed and donation during the experiment proceeds at almost constant rate (Fig. 2, *a*). Qualitatively this type of kinetic profile can be described as "prolonged donation without pronounced coming out to saturation". Among its quantitative characteristics are a low rate of donation (k_{eff}) combined with a high $[HbFe^{3+}]_\infty$ value and a high yield of NO. For example, among all studied complexes complex **2** showed the lowest k_{eff} value and the highest values of $[HbFe^{3+}]_\infty$ and yield of NO (see Table 1). Earlier, we have observed such a type of NO donation in the case of B-DNIC with cysteamine ligands.[12]

Finally, the third type of NO donation kinetics observed in the case of complex **5** can be defined qualitatively as an explosive one. It features a fast release of NO into a solution, high values of k_{eff} and $[HbFe^{3}+]_\infty$, and a high yield of NO. The k_{eff} value characterizing the rate of NO release into a solution for complex **5** is by about one order of magnitude higher than that for remaining complexes (see Table 1). As a result, the system shows a fast depletion in oxyhemoglobin with the level of methemoglobin reaching the limit value. Importantly, this type of donation has been observed earlier for B-DNIC with penicillamine ligands.[14,15]

In general, the proposed typology of the kinetic profiles of NO donation derived from the kinetic regularities of formation of intra-erythrocytic methemoglobin in a simple *in vitro* model system can be quite useful for the primary estimation of pharmacological properties of exogenous nitrogen oxide donors. For example, the studies of complexes **1—5** on the models of cell and bacterial cultures showed that complex **2** assigned to the prolonged profile of NO donation exhibited the highest cytotoxicity (according to the data of MTT test on Vero cells) and the highest antibacterial activity (inhibition of the growth of *E. coli*).[25] The earlier studied B-DNIC with cysteamine ligands, relating to the prolonged type of NO donation, demonstrated high indices of the anticancer activity in the comprehensive *in vitro* and *in vivo* studies of tumor growth inhibition and was recommended for preclinical trials.[7]

Let us consider in more detail the properties of complexes **2** and **5** relating to the prolonged and explosive types of NO donation, respectively.

Figure 2, *a* shows the kinetic curves for the formation of methemoglobin in the presence of complex **2**. It is seen that the rate of methemoglobin formation characterizing the NO-donating ability of the complex considerably decreases with an increase in the concentration of hemoglobin in the suspension. Figure 2, *b* shows the effective rate constant of methemoglobin formation k_{eff} as a function of the concentration of red blood cells in the suspension. A drift in the effective rate constant observed in this case has been studied in detail in our earlier works.[13,15] This phenomenon was explained by the fact that a portion of the complex molecules adsorb on the cell surface to form an additional equilibrium pool of the bound complex, which possesses a lower rate of hydrolytic dissociation due to a decreased content of water in the cell surface contact area. As a result, the effective rate constant observed in certain experiment is found to be a weighted mean value depending on the volume ratio of the aqueous and membrane-bound pools of the complex.

The analysis of this issue in terms of the Langmuir monomolecular adsorption theory resulted in a hyperbolic

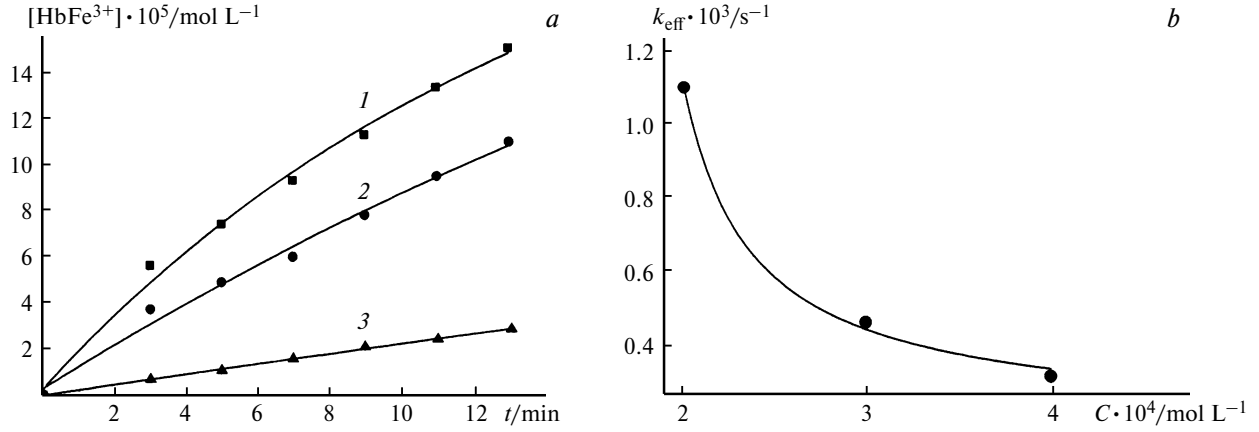


Fig. 2. (*a*) Kinetic curves for the formation of methemoglobin ($HbFe^{3+}$) in the presence of complex **2** at a concentration of $1.2 \cdot 10^{-4}$ mol L^{-1}. The concentration of hemoglobin was $2 \cdot 10^{-4}$ (*1*), $3 \cdot 10^{-4}$ (*2*), and $4 \cdot 10^{-4}$ mol L^{-1} (*3*). (*b*) Effective rate constant of methemoglobin formation (k_{eff}) as a function of the red cell concentration in the suspension (*C*).

equation of type (6) describing the relationship between the effective rate constant and the concentration of free binding sites [*P*] in the system[13]

$$k_{\text{eff}} = \frac{k_w - k_m}{K_a[P]+1} + k_m, \qquad (6)$$

where k_w and k_m are the limit k_{eff} values for the aqueous and membrane-bond pools of complex, respectively, and K_a is the equilibrium constant.

As Fig. 2, *b* shows, the behavior of complex **2** completely agrees with these perceptions. Namely, with an increase in the concentration of red blood cells in the suspension the concentration of free binding sites [*P*] increases, which leads to a decrease in k_{eff} in accordance with Eq. (6).

The dependence of the NO-donating ability of complex **2** on the concentration of red blood cells in the medium should be taken into account upon its possible pharmacological application as an NO donor. A low rate of NO donation interferes with achievement of a fast vasorelaxation under conditions of hypertensive crisis. However, the lifetime of a NO-donating substance in a body will increase upon a low rate of donation, which will provide a long-term pharmacological effect upon different forms of cardiac failures.

The concentration of complex **5** in the experiment shown in Fig. 1 was $1.2 \cdot 10^{-4}$ mol L^{-1}. It is seen that oxyhemoglobin was consumed rapidly at this concentration. This means that the limit of methemoglobin formation and the yield of NO can depend on the initial concentration of oxyhemoglobin. To clarify this issue, we repeated the experiment with the three-fold deceased concentration of complex **5** at three different concentrations of red blood cells. The obtained data are shown in Fig. 3. No statistically significant differences in the k_{eff} values for three different concentrations of cells were detected. This means that, in this case, there is no drift in the k_{eff} constant typical of **2**. Table 1 gives parameters for complex **5** determined using the data from Figs 1 and 3 at identical concentrations of red blood cells in the suspension. It is seen that a three-fold decrease in the concentration of complex **5** did not result in a statistically significant change in the limit level of methemoglobin formation and the yield of NO. This means that, in this case, there is no stoichiometrically complete release of nitrogen oxide into a solution, which we have observed earlier for the complex with penicillamine ligands.[15]

All studied complexes possessed hemolytic activity being evident on diluted suspensions of red blood cells. Figure 4, *a* shows the kinetics of erythrocyte lysis in the presence of complex **2** at different concentrations. As the quantitative characteristic of the hemolytic activity of the complexes we used the induction period of hemolysis I_{20} (the time for which the degree of hemolysis reaches 20%), which was determined graphically. As Fig. 4, *b* shows, the

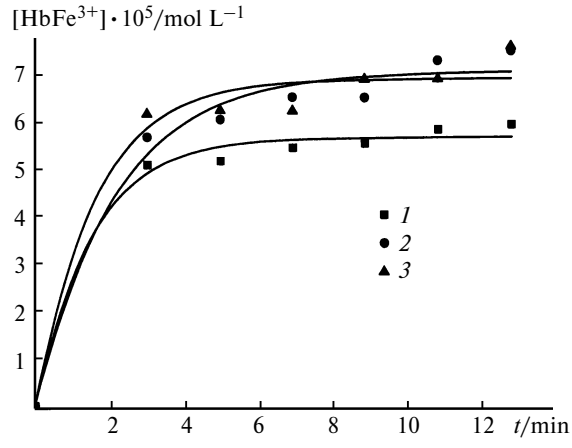


Fig. 3. Kinetic curves for the formation of methemoglobin ($HbFe^{3+}$) in the presence of complex **5** at a concentration of $4 \cdot 10^{-5}$ mol L^{-1}. The concentration of hemoglobin in the suspension was $2 \cdot 10^{-4}$ (*1*), $3 \cdot 10^{-4}$ (*2*), and $4 \cdot 10^{-4}$ mol L^{-1} (*3*).

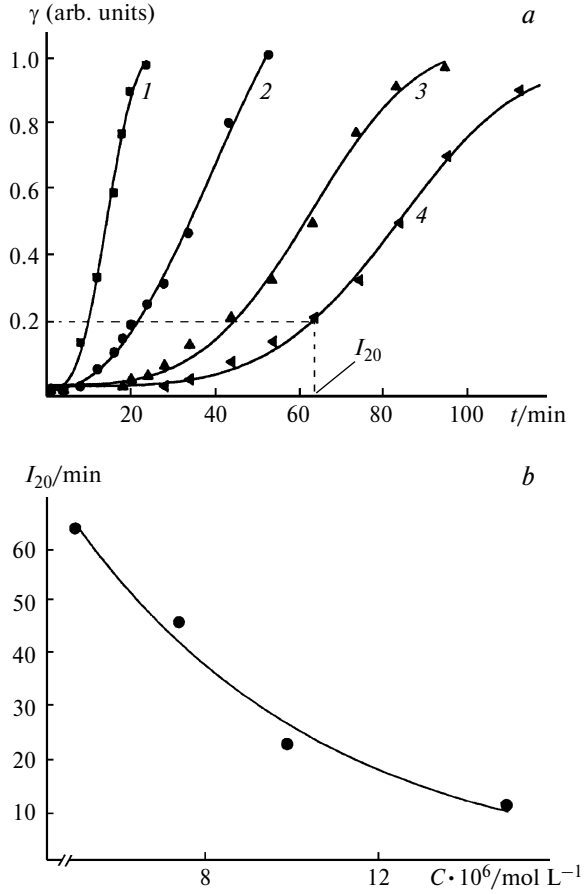


Fig. 4. (*a*) Kinetics of hemolysis of the 0.2% red cell suspension in the presence of complex **2** at concentrations of $15 \cdot 10^{-6}$ (*1*), $10 \cdot 10^{-6}$ (*2*), $7.5 \cdot 10^{-6}$ (*3*), and $5 \cdot 10^{-6}$ mol L^{-1} (*4*) at 37 °C; γ is the degree of hemolysis. (*b*) Induction period (I_{20}) of erythrolysis as a function of the concentration of complex **2** (*C*).

hemolytic effect of complex **2** depends on its concentration. Complexes **1** and **3**—**5** were studied in a similar manner. Due to a wide range of hemolytic characteristics, the hemolytic activities of the complexes were compared by the I_{20} value at concentrations of $5 \cdot 10^{-6}$ and $2 \cdot 10^{-5}$ mol L^{-1} (see Table 1). It follows from the obtained data that complex **5** is the most efficient hemolytic agent, its hemolytic activity is by one or more orders of magnitude higher than that of the other complexes. The hemolytic activity of complex **5** directly correlates with its NO-donating ability estimated by the kinetic parameters of methemoglobin formation.

In general, the obtained data show that the NO-donating activity of B-DNICs can significantly differ for complexes not only with *S*-ligands of different chemical nature, but also with ligands belonging to the same chemical family. The typology of kinetic profiles of NO donation proposed in the present work can be used to analyze the pharmacological potential of representatives of the entire class of NO donors based on DNICs with thiol-containing ligands. A considerable portion of DNICs studied by us (both bi- and mononuclear ones) are characterized by the profile of NO donation with pseudosaturation. This is likely explained by the fact that NO groups are stabilized by some means being preserved in the system as long-lived nitrosyl-containing structures. Adsorption of complexes on the surface of biosubstrates, which can decrease the rate of hydrolytic dissociation, can be one among the mechanisms of such stabilization.[13—15] At the same time, our recent studies showed that long-lived nitrosyl intermediates can form in a solution during transformation of the starting bi-[18,26,27] and mononuclear[28,29] DNICs even in the absence of biosubstrates. Regardless of certain mechanism, the capability of forming long-lived nitrosyl-containing structures is an important feature of DNICs with thiol-containing ligands exerting a significant effect on their pharmacological potential.

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