ORIGINAL PAPER

How is Nitric Oxide (NO) Converted into Nitrosonium Cations (NO$^+$) in Living Organisms? (Based on the Results of Optical and EPR Analyses of Dinitrosyl Iron Complexes with Thiol-Containing Ligands)

Anatoly F. Vanin[1,2]

Abstract

The present work provides theoretical and experimental foundations for the ability of dinitrosyl iron complexes (DNICs) with thiol-containing ligands to be not only the donors of neutral NO molecules, but also the donors of nitrosonium cations (NO$^+$) in living organisms ensuring S-nitrosation of various proteins and low-molecular-weight compounds. It is proposed that the emergence of those cations in DNICs is related to disproportionation reaction of NO molecules, initiated by their binding with Fe^{2+} ions (two NO molecules per one ion). At the same time, possible hydrolysis of iron-bound nitrosonium cations is prevented by the electron density transition to nitrosonium cations from sulfur atoms of thiol-containing ligands, which are included in the coordination sphere of iron. It allows supposing that iron in iron–nitrosyl complexes of DNICs has a d^7 electronic configuration. This supposition is underpinned by experimental data revealing that a half of nitrosyl ligands are converted into S-nitrosothiols (RSNOs) when those complexes decompose, with the other half of those ligands released in the form of neutral NO molecules.

1 Part 1. Dinitrosyl Iron Complexes with Thiol-Containing Ligands as Sources of Nitrosonium Cations. Theoretical Justification

It is widely acknowledged that all living organisms, ranging from microorganisms and plants to animals and humans, continuously synthesize nitric oxide (NO), the simplest chemical compound, through enzymes. This compound is one of the main versatile regulators modulating various physiological and biochemical processes in

✉ Anatoly F. Vanin
vanin.dnic@gmail.com

1   Semenov Federal Research Center of Chemical Physics, Russian Academy of Sciences, Moscow, Russia

2   Institute for Regenerative Medicine, Sechenov University, Moscow, Russia

living organisms [1–3]. The biological effect of NO originates from its ability to easily penetrate the cell membranes and get into cells without being mediated by cell receptors, with a subsequent binding with heme groups of various heme-containing proteins, such as guanylate cyclase, thus initiating activation or inhibition of those proteins.

Biological activity of NO derivatives, nitrosonium cations (NO^+), which occur in cells and tissues due to the one-electron oxidation of neutral NO molecules, is also important. In terms of biological effects, nitrosonium cations replace protons in thiol groups of various thiol-containing compounds, leading to the respective S-nitrosothiols (RS-NO) being formed. This modification of thiol groups in proteins significantly increases the reactivity of those groups, both in terms of their reactions in redox processes and those of binding with various bioactive agents [4–6].

Researchers have assumed for a long time that one-electron oxidation of NO to NO^+ results from NO reaction with nitrogen dioxide (NO_2), which is produced through NO oxidation with a subsequent binding of NO and NO_2 molecules. In the resultant adduct, nitrogen trioxide ($NO–NO_2$), mutual oxidation–reduction of free radicals NO and NO_2, i.e., their disproportionation reaction, is to cause transformation of nitrogen trioxide into the adduct of a nitrosonium cation and a nitrite anion ($NO^+–NO_2^-$) [4–9]. If there are no thiols, the nitrosonium cation in the adduct should hydrolyse, i.e., convert (with a neutral pH being preserved) into the nitrite anion, due to a reaction with a hydroxyl ion. In the presence of thiols, which have a closer affinity to nitrosonium cations than the hydroxyl ion [7, 8], nitrosonium cations should convert into RS-NO, being thiol-bound. In the acidic environment, both $NO^+–NO_2^-$ adduct components should, in the absence of thiols, convert into two HNO_2 molecules via a hydrolysis reaction, while in the presence of thiols, they are to convert into two RS-NO molecules.

The assumption that oxygen is necessary for converting NO molecules into nitrosonium cations, providing for RS-NO production in living organisms, proved to be erroneous. Experiments on animal cell cultures and microorganisms producing nitric oxide showed that in those organisms production of RS-NO of low molecular weight and protein RS-NO was equally effective both in the presence and in the absence of oxygen. In other words, oxygen was not necessary for that process [10, 11].

How can nitric oxide convert into a nitrosonium cation ensuring S-nitrosation that is vital for living organisms? In my opinion, there is only one answer, taking into account the ability of NO free radicals to enter into the above-mentioned disproportionation reaction—mutual one-electron oxidation–reduction, due to which NO molecules convert into nitrosonium cations and nitroxyl anions (NO^-), as in reaction 1:

$$2NO \Longleftrightarrow NO^+ + NO^- \tag{1}$$

As shown below, this reaction is the basis for a mechanism of S-nitrosothiol formation in living organisms, which I assume in this work.

As I first demonstrated in [12], this very reaction determines the stoichiometric (in accordance with reaction 2) conversion of gaseous NO into nitrogen dioxide and nitrous oxide (N_2O), which occurs at gaseous NO pressure of several dozen atmospheres, that was first described by T. Melia:

$$3NO = NO_2 + N_2O \tag{2}$$

[13]

Obviously, comparing Reaction 1 and 2 equations shows that the nitrogen dioxide in Eq. 2 originated from nitrosonium cation, while the occurrence of nitrous oxide, which includes nitrogen with the lowest valency, is related to nitroxyl anion conversion into it. This can be easily verified by the following algebraic transformations of the equation describing reaction 1. By doubling both equation parts, and adding two water molecules to each of its parts (to provide for hydrolysis of the nitrosonium cation and the nitroxyl anion), we get Eq. 1:

$$4NO + 2H_2O \Longleftrightarrow 2NO^+ + 2OH^- + 2NO^- + 2H^+ \Longleftrightarrow 2HNO_2 + 2HNO \tag{1}$$
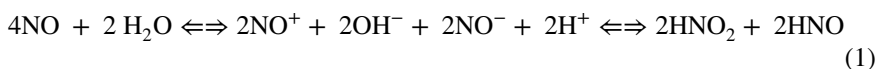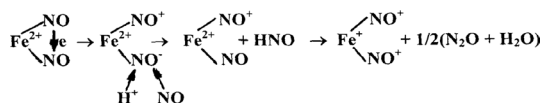
Binding of hydroxyl anions and protons with nitrosonium cations and nitroxyl anions, respectively, causes their conversion into two nitrous acid molecules and two nitroxyl (HNO) molecules, respectively, disproportionation of which (reactions 3 and 4)

$$2HNO_2 \Longleftrightarrow H_2O + NO + NO_2 \tag{3}$$

$$2HNO \Longleftrightarrow H_2O + N_2O \tag{4}$$
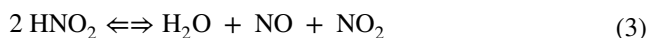
causes conversion of nitrous acid and nitroxyl molecules into nitrogen monoxide, dioxide and nitrous oxide, respectively, and water. If the right part of Eq. 1 is replaced by them, and water molecules and one nitric oxide molecule (from each part) are subsequently removed from both parts of the equation, Eq. 1 ultimately ends up with an equation for reaction 2.

Conversion of NO molecules into nitrosonium and nitroxyl ions, with the latter being subsequently converted into nitrogen dioxide and nitrous oxide, respectively, at high pressures of gaseous NO shows that in those conditions nitric oxide can act as an acid anhydride which ensures nitrous acid formation. This property is not detected at NO pressures of one atmosphere and lower, i.e., with the concentration of this agent equal to the one produced by living organisms. Obviously, in this case, steady-state concentration of NO^+–NO^- adducts does not reach the level necessary for forming a significant amount of nitrous acid in the process of nitrosonium cation hydrolysis, due to which NO cannot be considered as the acid anhydride in these conditions. Nevertheless, as I assumed in [12, 14], disproportionation of NO molecules in living organisms with the formation of NO^+ and NO^- in millimolar concentration is possible, if loosely bound Fe^{2+} ions occur in intracellular space; these ions are known to have a close affinity with NO molecules [15]. This property of divalent iron ions underlying their ability to effectively bind NO molecules—two molecules per iron ion with the formation of rather a stable dinitrosyl iron complexes $[Fe(NO)_2]$, or $[Fe(NO)_2]$ motifs—ensures NO molecule disproportionation in these motifs following reaction 1. This reaction makes these molecules being a part of $[Fe(NO)_2]$ motifs to convert into nitrosonium cations and nitroxyl anions (as shown in Scheme 1). The transfer of one electron from a NO molecule to another

$$Fe^{2+} \overset{NO}{\underset{NO}{\diagdown}} e \rightarrow Fe^{2+} \overset{NO^+}{\underset{NO^-}{\diagdown}} \rightarrow Fe^{2+} \overset{NO^+}{\underset{NO}{\diagdown}} + HNO \rightarrow Fe^+ \overset{NO^+}{\underset{NO^+}{\diagdown}} + 1/2(N_2O + H_2O)$$

$$\underset{H^+ \quad NO}{\uparrow}$$

Scheme 1. The proposed mechanism of $[Fe^+(NO^+)_2]^+$ motif formation in the reaction of NO molecules with a Fe^{2+} ion [16–20]

one, which is typical of this reaction, is provided by the formation of a common molecular orbital in a $[Fe(NO)_2]$ motif, including π-orbitals of NO molecules and *d*-orbitals of an iron ion [16–20].

Regarding the subsequent hydrolysis of nitrosonium cations and nitroxyl anions, i.e., their interaction with hydroxyl anions and protons, respectively, it is assumed that this reaction occurs only for nitroxyl anions. A nitroxyl molecule formed in this process (see Scheme 1) leaves the ligand environment, being replaced by a NO molecule (the third one). Therefore, iron in a $[Fe(NO)_2]$ motif gets a d^7 electronic configuration with a relevant resonance structure of this motif: $[Fe^+(NO^+)_2]^+$.
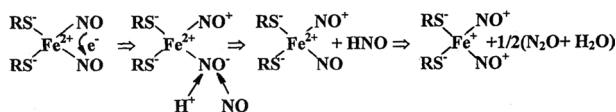
Obviously, the occurrence of the easily hydrolyzed NO^+ ligands in this motif makes the above-mentioned resonance structure unstable. The instability can be eliminated by introducing thiol-containing ligands (RS^-) in the iron coordination sphere. Since these ligands are efficient π-donors of electron density, they can ensure transfer of this density in $[Fe^+(NO^+)_2]^+$ motifs to iron and nitrosyl ligands. A resulting reduction in the positive charge of these ligands weakens their interaction with hydroxyl anions, preventing their hydrolysis, and therefore, ensuring the stability of paramagnetic mononuclear DNICs (M-DNICs) with thiol-containing ligands, formed in accordance with Scheme 2; one of their resonance structures may be presented as $[(RS^-)_2Fe^+(NO^+)_2]^+$. As a result, the lumped reaction of the mechanism for M-DNIC formation presented in Scheme 2 is described as reaction 5:

$$Fe^{2+} + 2RS^- + 3NO + H^+ \rightarrow \left[(RS^-)_2Fe^+(NO^+)_2\right]^+ + \tfrac{1}{2}(H_2O + N_2O) \tag{5}$$
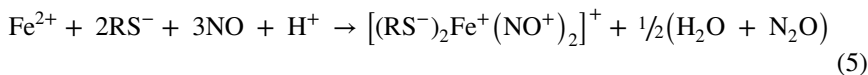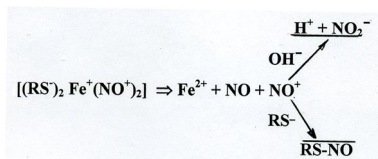
[16–20]

Notably, the disproportionation reaction typical of nitroxyl molecules, which causes their conversion into nitrous oxide and water, makes the formation of both dinitrosyl iron complexes (Scheme 1) and DNICs with thiol-containing ligands (Scheme 2) irreversible, which is determined by a weak chemical reactivity of nitrous oxide.
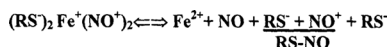
As DNICs with thiol-containing ligands in the mononuclear, as well as in the binuclear form (B-DNICs, formula $[(RS^-)_2Fe^+_2(NO^+)_4]^{4+}$) are spread widely in

$$\underset{RS^-}{\overset{RS^-}{\diagdown}}Fe^{2+}\overset{NO}{\underset{NO}{\diagdown}} e^- \Rightarrow \underset{RS^-}{\overset{RS^-}{\diagdown}}Fe^{2+}\overset{NO^+}{\underset{NO^-}{\diagdown}} \Rightarrow \underset{RS^-}{\overset{RS^-}{\diagdown}}Fe^{2+}\overset{NO^+}{\underset{NO}{\diagdown}} + HNO \Rightarrow \underset{RS^-}{\overset{RS^-}{\diagdown}}Fe^+\overset{NO^+}{\underset{NO^+}{\diagdown}} + 1/2(N_2O + H_2O)$$

$$\underset{H^+ \quad NO}{\uparrow}$$

Scheme 2. Proposed mechanism for the formation of M-DNICs with thiol-containing ligands in the reaction of divalent iron, thiols, and gaseous NO [16–20]

$$[(RS^-)_2 \, Fe^+(NO^+)_2] \Rightarrow Fe^{2+} + NO + NO^+ \underset{\substack{OH^- \\ RS^-}}{\overset{H^+ + NO_2^-}{\diagdown}} \begin{array}{c} RS\text{-}NO \end{array}$$

Scheme 3. The proposed mechanism for decomposition of M-DNICs with thiol-containing ligands, which, in the presence or absence (blockade) of thiols, causes accumulation of S-nitrosothiols or nitrite, respectively [14]

$$(RS^-)_2 \, Fe^+(NO^+)_2 \Longleftrightarrow Fe^{2+} + NO + \underset{RS\text{-}NO}{\underline{RS^- + NO^+}} + RS^-$$

Scheme 4. The chemical equilibrium between M-DNICs with thiol-containing ligands, and their components [16–20]

living organisms as one of dominating nitrogen oxide derivatives [19–22], it can be reasonably assumed that the inclusion of NO in DNICs with thiol-containing ligands ensures, as described above, its conversion into the nitrosonium cation necessary for S-nitrosation of thiols. Obviously, this may occur, for instance, in the conditions of DNIC decomposition to their components—iron ions, thiol-containing and nitrosyl ligands. In accordance with Scheme 3 illustrating this decomposition, the iron ion, transferring an electron to one of the nitrosyl ligands, is released from decomposing DNICs in the form of the Fe^{2+}ion, while the corresponding nitrosyl ligand has the form of neutral gaseous NO molecule. As regards another nitrosyl ligand, due to the release of thiol-containing ligands from the coordination sphere of iron, their impact on the condition of this nitrosyl ligand is lost. Therefore, one of the nitrosyl ligands is released in the form of a nitrosonium cation in the process of DNIC decomposition. Interacting with thiols, it forms respective S-nitrosothiols, and in their absence it is hydrolysed and, depending on the pH of the environment, is converted into either the nitrite anion (if the pH is neutral) or nitrous acid (if the pH is acidic).

It should be emphasized that, following Scheme 3, the number of NO molecules (gaseous NO) from decomposed DNICs, on the one hand, and nitrosonium cations (and, therefore, S-nitrosothiol or nitrite molecules formed from nitrosonium cations), on the other hand, must be the same. There may be an exception when nitrosonium cations can be reduced by various reductants to NO. As a result, the decomposition of DNICs with thiol-containing ligands will primarily entail the release of nitric oxide.

DNICs with thiol-containing ligands may be NO or NO^+ donors not only when they are decomposed, but also when the chemical equilibrium between these complexes and their components is established (as Scheme 4 shows):

Such a chemical equilibrium is possible only when the thiol-containing ligands released from DNICs are preserved. As Scheme 4 shows, thiol groups of these ligands or thiol-containing compounds which did not form a part of DNICs are bound with nitrosonium cations, forming the respective S-nitrosothiols, which are capable of generating DNICs in the absence of NO, and interacting with Fe^{2+} ions and thiols, in accordance with Scheme 5:
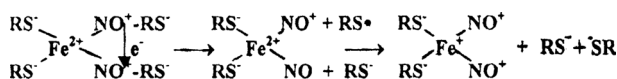
Scheme 5. The proposed mechanism for the formation of M-DNICs with thiol-containing ligands in the reaction of Fe^{2+}, thiols, and RS-NO [16–20, 23]

It is assumed that, when two different resonance RS-NO structures—$NO^{\bullet-}RS^{\bullet}$ and NO^+–RS^-, are bound with a Fe^{2+} ion, with subsequent disproportion (electron transfer from $NO^{\bullet}$ to NO^+) immediately after the decomposition of reacting RS-NO, M-DNICs are formed. At the same time they have the resonance structure $[(RS^-)_2Fe^+(NO^+)_2]$, which is similar to M-DNICs occurring in accordance with Scheme 2 in the reaction of divalent iron, thiols, and gaseous NO.

Notably, both processes of M-DNIC formation, presented on Schemes 2 and 5, are irreversible. For this process involving RS-NO, the fact of being irreversible is determined by a condensation of thiyl radicals emerging during RS-NO decomposition, characterized by the resonance structure—$NO^{\bullet}$–$RS^{\bullet}$. Disulphides (RS–SR) emerging as a result of such condensation could be converted into thiols interacting with M-DNICs only with exogenous reductants affecting the disulphides.

As far as the binuclear form of DNICs (B-DNICs) with thiol-containing ligands is concerned, it is assumed that it is formed as a result of condensation of two mononuclear forms of these complexes (irrespective of the ways of synthesizing the latter), when the content of thiol-containing ligands in a solution is reduced, in accordance with the scheme of reversible interconversion of M- and B-DNICs (Scheme 6):

As the interconversion of the mononuclear and binuclear forms of DNICs shown in Scheme 6 is determined by the amount of thiol-containing ligands ionized by thiol group, and when the pH is neutral, the degree of such ionization is small for most thiol-containing ligands, with such pH, DNICs with thiol-containing ligands are mainly presented by B-DNICs.

Taking into account the interconversion of M- and B-DNICs shown in Scheme 6, the mechanisms for these complexes to form as well as their ability to be NO and NO^+ donors (Schemes 2, 4 and 5) another overarching scheme (Scheme 7) could be proposed. This scheme shows that M-DNIC formation is possible through the two interchangeable mechanisms determined by reactions 1 and 2. With NO excess, reaction 1 is the dominant one, which entails RS-NO accumulation; therefore, due to this reaction, the NO level decreases, while the RS-NO content in this system increases. With this increase, reaction 2 gradually replaces reaction 1, which elevates the NO level, while decreasing the RS-NO amount, etc.

Therefore, the system of chemical reactions shown on Scheme 7 can be considered as a self-regulating chemical system, whose existence is determined by thiols and nitric oxide functioning as a "fuel" and an "oxidant", respectively. As a result of

$$\text{M-DNIC} \overset{\text{-RS}^-}{\underset{\text{+RS}^-}{\Longleftrightarrow}} \text{B-DNIC}$$
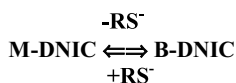
Scheme 6. Reversible interconversion of M- and B-DNICs with thiol-containing ligands [24]

$$\underline{2RS^{\cdot} + Fe^{2+} + 3NO}$$
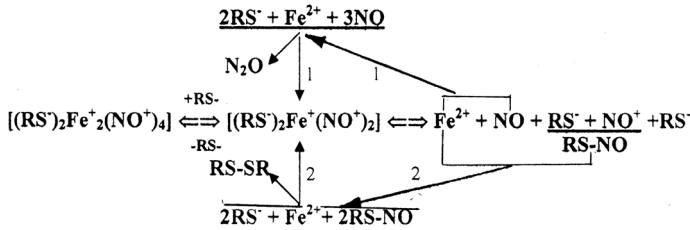
Scheme 7. Mechanisms for the formation of M- and B-DNICs with thiol-containing ligands, their proportion to components, and the ability of these complexes to release NO molecules and NO⁺ cations (general scheme)

their interaction, thiols and NO are converted into disulfide (RS–SR) and nitrous oxide (N_2O), respectively (reaction 6):

$$2RSH + 2NO \rightarrow RS - SR + N_2O + H_2O \qquad (6)$$
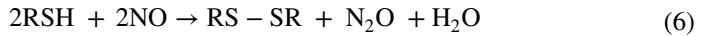
The occurrence of nitrous oxide and disulfide in reactions 1 and 2, respectively, makes these reactions irreversible.

Following Scheme 7, M-DNIC constitutes the main integral link in this system of chemical reactions. Its synthesis in both reactions 1 and 2 makes it possible for nitric oxide or RS-NO, respectively, to convert into nitrosonium cations in living organisms, which ensures RS-NO production in those organisms.

2 Part 2. Experimental Evidence of the Ability of DNICs with Thiol-Containing Ligands to be Donors of Nitrosonium Cations

Prior to discussing the experiment results that demonstrate the release of nitrosonium cations from DNICs, I will deal with some physical and chemical properties of these complexes.

The mononuclear form of these complexes, as opposed to the binuclear one, is paramagnetic and characterized by an EPR signal with $g_{av} = 2.03$ (2.03 signal). Registration of this signal allowed me to first discover M-DNICs in yeast cells, and then in animal tissues (Fig. 1) in collaboration with R. Nalbandyan 1964–1965 [25, 26], and later in collaboration with L. Blumenfel`d and A. Chetverikov in 1967 [27]. In 1964, a weak peak with $g = 2.03$ was registered by D. Mallard and M. Kent in chemically induced hepatocellular carcinoma [28], and in 1965, the 2.03 signal (more precisely, its part: a peak at $g_{\perp} = 2.035$) was recorded by B. Commoner and coauthors in a rat liver with the initiated hepatocarcinogenesis [29]. In the 1990s, when nitric oxide was recognized as one of the universal endogenous regulators of metabolic processes in living organisms, M-DNICs with thiol-containing ligands were found in many biological systems capable of producing nitric oxide [30–38, 65, 66].

As regards the binuclear form of DNICs with thiol-containing ligands, it is diamagnetic, and therefore EPR silent. It is diamagnetic due to a spin–spin coupling of two dinitrosyl iron complexes—the so-called antiferromagnetic interaction of these
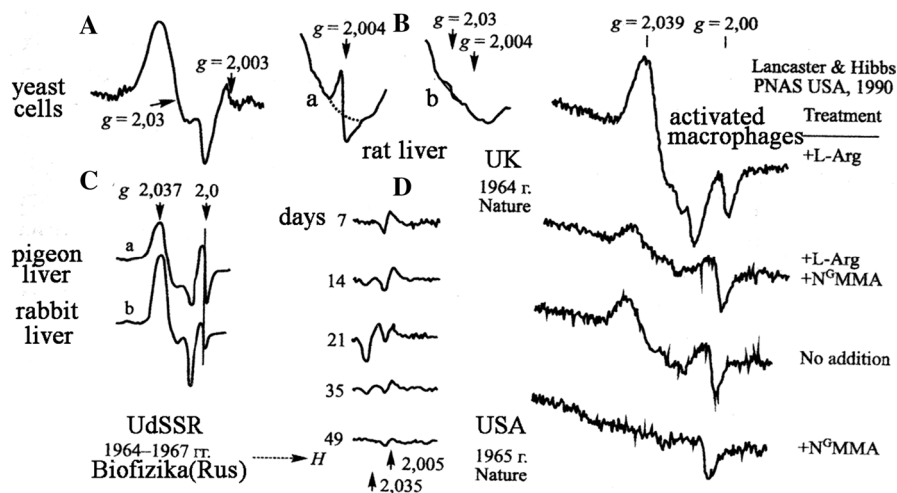
Fig. 1 The 2.03 signals recorded in the 1960th in **A** yeast cells [25, 26]; **B** chemically induced rat hepato-cellular carcinoma tissue [28]; **C, a, b** pigeon and rat liver [27]; **D** chemically induced rat hepatocellular carcinoma at different periods following the treatment of rats with hepatocarcinogenic agents [29]. Right panel: The 2.03 signals recorded in 1990 in activated rat macrophages in the presence of L-arginine, L-arginine + N-methyl-L-arginine, in the absence of L-arginine and N-methyl-L-arginine and in the presence of N-methyl-L-arginine alone [30]

complexes through bridging sulphur atoms of thiol-containing ligands. The latter bind dinitrosyl iron complexes into a single binuclear complex [24].

In 1965–1966, we studied dependence of the 2.03 signal on temperature in living organisms, and its microwave power saturation. The research showed that the signal is determined by paramagnetic centers of the same nature [39]. I managed to identify those centers as M-DNICs with thiol-containing ligands after I had discovered that frozen M-DNIC solutions with cysteine produce the EPR signal which is identical to the 2.03 signal in living organisms (Fig. 2). The same signal was registered for M-DNICs with other thiol-containing ligands, while replacing these ligands with other compounds registered a completely different EPR signal at a low temperature during registration [40].

The behavior of M-DNICs with thiol-containing ligands and the centers responsible for the 2.03 signal in living organisms was compared. The experiments where yeast cells and animal tissues were treated with gaseous NO and nitrite as the NO donor proved that M-DNICs with thiol-containing ligands do account for the 2.03 signal in living organisms. At the same time, the latter could be represented by both low- and high-molecular-weight (protein) compounds [27].

It should be noted that EPR signals of M-DNICs with thiol-containing ligands, for the first time registered in 1965 by C. McDonald, W. Philips and H. Mower, were recorded at room temperature, and those were relatively narrow symmetrical singlets with a center at $g = 2.03$ (Fig. 2). Symmetrization and small bandwidth typical of those signals (~0.7 mT) were obviously related to a high mobility of low-molecular-weight M-DNICs, which was sufficient for averaging anisotropy of

Fig. 2 The 2.03 signals recorded in wet preparations of rabbit liver (curve a) or yeasts (curve b) and the EPR spectra of aqueous solutions of DNIC with cysteine containing ^{14}NO (curves c and d) or ^{15}NO (curves e and f) or ^{57}Fe (curves g and h). The EPR spectra were recorded at ambient temperatures (curves a, b, d, f and h) or at 77 K (curves c, e and g). Right panel: identification of characteristics HFS of M-DNIC EPR spectra [18]
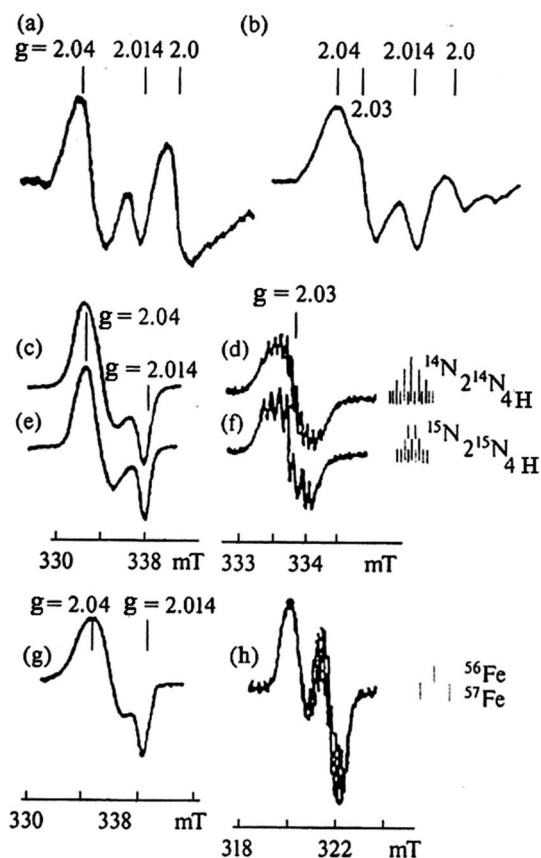
the g-factor and hyperfine structure (HFS) of those signals [41]. As our research showed most centers responsible for the 2.03 signal are protein-bound. So the low mobility of the latter at room temperature could determine the difference between the form of the 2.03 signal and EPR signals of M-DNICs with low-molecular-weight thiols, when they were registered at room temperature. To "immobilize" low-molecular-weight M-DNICs, I registered their EPR signal in an aqueous solution at 77 K. As a result, the form of EPR signals of those complexes, related to anisotropy of the g-factor and HFS, became absolutely identical to the 2.03 signal (Fig. 2) [40].

Currently, there are two viewpoints on the electronic and spatial structure of DNICs with thiol-containing ligands, described as part of the classification of DNICs with various anion ligands proposed by J. Enemark and R. Feltham as $[Fe(NO)_2]^7$ or $[Fe(NO)_2]^9$ (by a total number of electrons on higher orbitals of iron and nitrosyl ligands) [42], which correspond to a d^7 or d^9 electronic configuration of iron in those complexes. As it follows from the above, our group considers the d^7 configuration of an iron dinitrosyl group of DNICs to be valid. The validity of another viewpoint of our opponents [43–47] will be described below.

Our viewpoint on the electronic and spatial structure of DNICs with thiol-containing ligands seems to be in line with the above-mentioned mechanism for these complexes to be formed in the reaction of NO, Fe^{2+} and thiols in water (Scheme 2). Here, I will briefly discuss our interpretation of the 2.03 signal characteristics and largely focus on DNICs ability to be donors of nitrosonium cations.

Figure 2 shows that the 2.03 signal, like the EPR signal of low-molecular-weight M-DNICs with thiol-containing ligands (e.g., with cysteine), is characterized by the g-factor of $g_\perp = 2.04$, $g|| = 2.014$, $g_{av.} = 2.03$. The signal is registered at low and room temperature, which indicates a weak spin–lattice interaction for M-DNICs, characterized by the low-spin state with $S = 1/2$. The fact that there are only two different g-factor values—$g_\perp$ and $g||$—indicates axial symmetry of the ligand environment of those complexes, which makes the assumption about the planar-square spatial structure of M-DNICs rather reasonable. At the same time, it is assumed that the axial symmetry of the electron cloud of ligands is mainly determined by the impact of в polar water molecules, which are included in the coordination sphere of iron as a solvent.

For the EPR signal of M-DNIC with cysteine registered at room temperature the 13-component HFS is detected, which is related to interaction of an unpaired electron in these complexes with ^{14}N nitrogen nuclei with the nuclear spin $I = 1$ of two nitrosyl ligands, and four protons ($I = 1/2$) in methylene groups of two cysteine ligands (Fig. 2). If ^{14}N in nitrosyl ligands is replaced by the ^{15}N isotope ($I = 1/2$), the 13-component HFS in the signal is replaced by the nine-component one (Fig. 2). If the ^{57}Fe isotope with $I = 1/2$ is included in the complexes, the doublet HFS emerges (Fig. 2), which indicates that M-DNICS have only one atom of iron. Therefore, HFS analysis clearly shows that these complexes are really mononuclear and include two nitrosyl and thiol-containing ligands each.

As part of the planar-square spatial structure of M-DNICs with thiol-containing ligands proposed by us earlier [18, 48], the location of nitrosyl and thiol-containing ligands in the angles of a square, and the diagram of antibonding molecular orbitals (MO), which corresponds to this structure and a d^7 electronic configuration of iron, are justified. In accordance with it, the highest energy typical of MO($d_{x^2-y^2}$), is determined by a strong interaction of the $d_{x^2-y^2}$ orbital with the orbitals of nitrosyl and thiol-containing ligands in the xy plane. Moreover, the interaction of the same orbitals with the d_{z^2} electron density localized in the xy plane, increases the energy of MO (d_{z^2}), which is enhanced by the interaction of the d_{z^2} orbital with water molecules along the z axis. Therefore, MO(d_{z^2}) energy becomes higher than the energy of MO (d_{xy}), MO (d_{xz}) and MO (d_{zy}), which interact with NOπ orbitals; as a result, an unpaired electron in M-DNIC is localized on MO(d_{z^2}) (Fig. 3).

The spatial and electronic structure of M-DNICs shown in Fig. 3 proved to be identical to the same structures of the low-spin ($S = 1/2$) complexes of $^{57}Co^{2+}$ with phthalocyanines or porphyrins considered by B. Garvey [49]. Like M-DNICs, these complexes were characterized by a d^7 electronic configuration of the central atom, and the following proved to be similar: the ratio between the g-factor values ($g_\perp > g||$ > 2.0023) for M-DNICs and cobalt complexes, and the ratio between the modules of HFS tensor values for ^{57}Fe and ^{57}Co nuclei—$|A_\perp| > |A||$ [49]. B. Garvey's theoretical analysis of the electronic structure of cobalt complexes showed that with the

Fig. 3 The hypothetical electronic and spatial structures of M-DNIC with thiol-containing ligands in aqueous solutions [48]
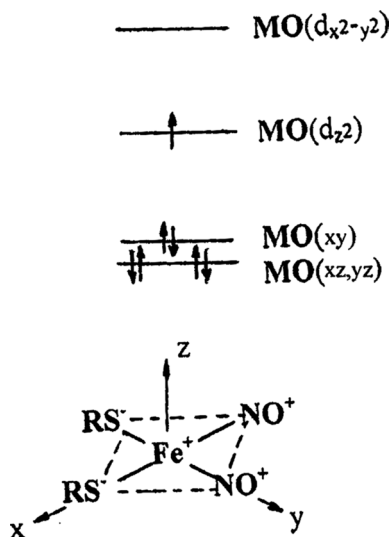
indicated ratios between the g-factor and HFS components from cobalt nucleus an unpaired electron in these complexes should be localized on MO (d_z2) of the central atom. Taking into account the similarity between many EPR characteristics of cobalt complexes, phthalocyanine (porphyrin) ligands and M-DNICs with thiol-containing ligands, the obtained theoretical result may also be applied to M-DNICs. Therefore, it can be convincingly argued that the latter, like the cobalt complexes, have a d^7 electronic configuration of iron, with the unpaired electron being mainly localized on the d_z2 orbital of iron. This is fully consistent with the spatial and electronic structure of M-DNICs shown in Fig. 3, and the mechanisms for these complexes formation (Schemes 2 and 5): one of their main resonance structures is presented as $[(RS^-)_2Fe^+(NO^+)_2]^+$ in accordance with these mechanisms, i.e., the structure with a d^7 electronic configuration of iron.

Currently, other experimental data have been obtained, which prove the mechanisms of M-DNIC synthesis shown on Schemes 2 and 5. In accordance with Scheme 2 and lumped reaction 5, characteristic of this Scheme, the formation of one M-DNIC requires three NO molecules, and one of those molecules subsequently participates in the formation of nitrous oxide, which is confirmed by findings of French and Serbian researchers, respectively [50, 51]. French researchers titrated solutions of Fe^{2+} and thiols with nitric oxide showed that the formation of one M-DNIC really requires about three NO molecules. Serbian researchers analyzed a gas mixture over the solution of formed M-DNICs and found nitrous oxide in it in the amount which is almost equimolar to the number of complexes formed.

Nevertheless, I assume that the experimental evidence for M- and B-DNICs with thiol-containing ligands, which are capable of being nitrosonium cation donors, given below is the most convincing fact which shows that the mechanisms for M-DNIC (and, therefore, B-DNIC) formation, given on Schemes 2 and 5, and determining a d^7 electronic configuration of iron in those complexes, are true.

As it follows from Schemes 3 and 4, in the process of mononuclear DNIC decomposition, or when the chemical equilibrium between those complexes and their components is established, nitrosyl ligands can be released from those complexes in an equal amount, and in the form of neutral (volatile) NO molecules and nitrosonium cations. The same applies to B-DNICs: their conversion into the mononuclear form is the first stage of their decomposition or establishing a chemical equilibrium.

In my research I mainly used B-DNICs with glutathione (GSH) or N-acetyl-L-cysteine (NAC), primarily synthesized with respective S-nitrosothiols (GS-NO or S-NO-NAC) as NO sources [12, 14, 52]. The relevant method implies gradually introducing sodium ferric sulphate and sodium nitrite with the molar ratio of 2:1:1 into GSH(NAC) buffer solution [53]. The following concentrations are given as an example: 20 mM GSH(NAC) + 10 mM ferrosulphate + 10 mM sodium nitrite. When the above-mentioned thiols are introduced into the solution, the pH is reduced to acidic (3–4), which allows full dissolution of ferrosulphate in the solution, and provides for the formation of respective S-nitrosothiols when sodium nitrite is subsequently added to the solution. Synthesis of the latter, completely depleting the nitrite in the solution, was controlled using the spectrophotometric method based on the absorption band intensity characteristic of RS-NO (334 nm; and the less intense absorption band at 543 nm) [54]. GS-NO synthesis in these conditions got finalized in 1–1.5 h, while the synthesis of S-NO-NAC took 20–30 min. After the end of that synthesis, the pH of the solution was increased to reach the neutral values by adding drops of concentrated alkali (100 mM of NaOH solution). The color of the solution changed from pink (typical of RS-NO) to orange-brown (characteristic of B-DNIC formation), with sediment consisting of iron hydroxide complexes. The process of B-DNIC-GSH or B-DNIC-NAC formation in those conditions took 6–10 h or one hour, respectively. After that, the sediment consisting of non-soluble iron hydroxide complexes, was removed using a paper filter from the B-DNIC solution.

The level of B-DNIC-GSH(NAC) in my experiments was controlled based on absorption band intensity at 360 and 310 nm with extinction coefficients (ε) equal to 3700 and 4600 M^{-1} cm^{-1} (per one iron atom in B-DNIC) [24] in the optical absorption spectrum of those complexes shown in Fig. 4. As far as the mononuclear form of those complexes is concerned, it was detected based on the 2.03 signal, its intensity in B-DNIC solutions with neutral pH values was equal to the inclusion of no more than 5% of total iron in the solution in M-DNICs (Fig. 4). If the pH in the solution increased to 10–11, the signal intensity was becoming 20–30 times higher; therefore, all iron which used to be in the solution was found in M-DNICs. At the same time, the absorption spectrum of B-DNICs was transformed into the spectrum characteristic of M-DNICs with the absorption band intensity of 390 num, with $\varepsilon = 4700$ M^{-1} cm^{-1} (Fig. 4) [24].

In accordance with Scheme 3, the release of NO^+ cations of DNICs, with a subsequent formation of RS-NO molecules involving them could occur only if thiol groups on the solution were preserved. In their absence, arising from SH complex oxidation or blockade by relevant reactants, hydrolysis of nitrosonium cations started. It converted NO^+ cations of DNICs into nitrite anions in case of pH values being neutral (neutral pH values).
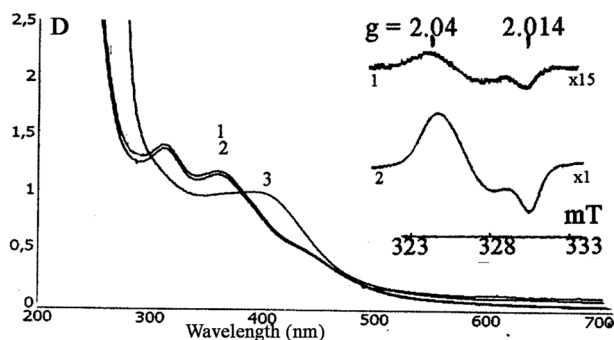
Fig. 4 On the left: B-DNIC-GSH optical absorption spectra registered in water solution with pH of 7.4 и 1.0 (curves 1 and 2, respectively). Curve 3—the absorption spectrum of M-DNIC-GSH registered in B-DNIC-GSH solution, in the presence of a threefold excess of free GSH (not included in B-DNICs), with pH = 11. On the right: the 2.03 signal (curves 1 and 2), registered at 77K in B-DNICs solution, characterized by absorption spectra 1 and 3 on the left [52]

As early as in the 1990s our group discovered interconversion of M-DNICs with cysteine, and S-nitrosocysteine (Cys-NO) [55]. In case of a sharp decrease in the pH of the 10 mM M-DNIC solution with cysteine synthesized involving gaseous NO, and the ratio of iron to cysteine was 1: 20, from pH = 7.4 to pH = 1–2, the green color of the solution characteristic of M-DNICs changed to pink, which is characteristic of Cys-NO. Optical measurement showed that M-DNICs were equimolarly converted into Cys-NO. If after that the solution pH was sharply increased to neutral values, M-DNICs emerged again in the concentration which was equal to a half of the initial one. Those results first demonstrated M-DNICs ability to be donors of nitrosonium cations, which comprised a half of nitrosyl ligands in those complexes, and therefore, to form RS-NO in the concentration equal to the concentration of M-DNICs. A reverse reaction of M-DNIC conversion from Cys-NO was caused by binding of two Cys-NO molecules with one Fe^{2+} ion, as shown on Scheme 5.

The following research by our group showed that DNICs with other thiol-containing ligands (both in the mononuclear and binuclear forms) are capable of producing respective RS-NO in the process of decomposition, after acidification to pH of 1–2; at the same time, the process was enhanced sharply when the solution was heated to 70–80 ℃ [12, 14, 52]. At room temperature, B-DNICs remained in acidified solutions for up to one hour, like GS-NO—one of the most stable representatives of low-molecular-weight RS-NO. As thiol molecules became smaller, the stability of their respective DNICs, as well as the time of RS-NO conversion in the acidic environment at room temperature, decreased.

It should be noted that M-DNICs in those experiments fully converted into B-DNICs which is in a complete agreement with Scheme 6 due to deionization of thiol groups in case of acidification.

Figure 5 shows the results of my first experiments with B-DNIC-GSH solutions heated to 80 ℃ after acidification to pH 1.0 [12]. Thirty seconds of heating on air was sufficient for those complexes to fully decompose. This was supported by their absorption spectrum completely disappearing. These complexes also were
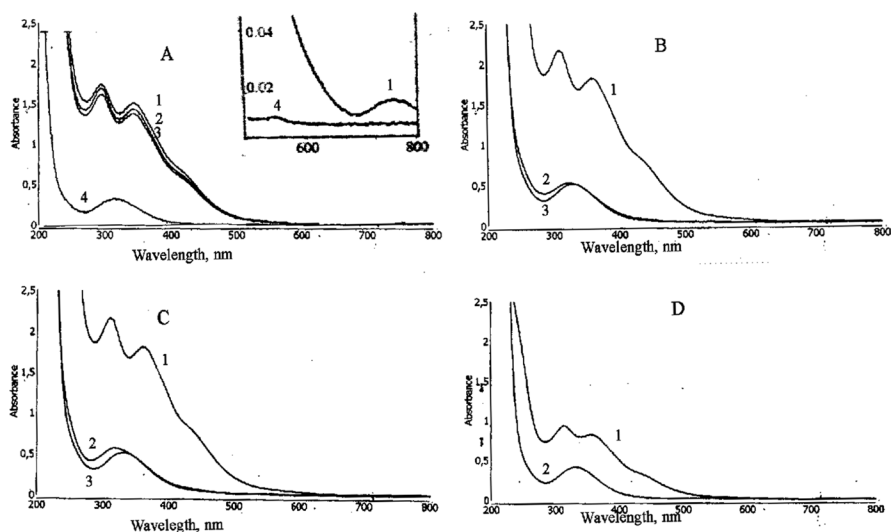
Fig. 5 The decomposition of B-DNIC-GSH and their conversion into GS-NO induced by heating acidified solutions. Panel A: the optical absorption spectra recorded 2 and 30 min after acidification of the initial 0.4 mM solution of B-DNIC-GSH (pH 7.4) (curve 1) to pH 1.0 (curves 2 and 3, respectively) and a subsequent 30-s increase in temperature from ambient to 80 ℃ (curve 4). Inset: the weak absorption bands of GS-NO and B-DNIC recorded at 543 and 768 nm (curves 4 and 1, respectively). Panels B and C: the optical absorption spectra recorded after acidification of a 0.5 mM solution of D-DNIC-GSH to pH 1.0 (curve 1) and 30-s incubation at 80 ℃ (Panel B) or 25 min incubation at 40 ℃ (Panel C) in the presence and in the absence of air (curves 2 and 3, respectively). Panel D: the absorption spectrum of a 9 mM B-DNIC-GSH acidified to pH 1.0 recorded after a 40-fold dilution with distilled water (curve 1) and 15-min air incubation at 80 ℃ followed by a 20-fold dilution of the final solution with distilled water (curve 2) [12]

equimolarly converted into GS-NO (detected based on the absorption band at 334 nm and the weak absorption band of 543 nm) (Fig. 5, Panel A). Comparison of those processes taking place in an aerobic and anaerobic environment (Fig. 5, Panels B and C) produced similar results, showing that oxygen had no impact on that process. It was also shown that conversion of B-DNIC-GSH into GS-NO was possible in more concentrated solutions (9 mM) of those complexes (Fig. 5, Panel D).

As in the experiments described above, a half of nitrosyl ligands in B-DNICs were involved in GS-NO formation, in accordance with Schemes 3 and 4, another half of those ligands should have been released from B-DNIC-GSH in the form of neutral (volatile) NO molecules. To verify that, B-DNIC-GSH solution (17 ml of 12 mM) at pH = 7.4 was placed in a modified Thunberg apparatus with a welded cylindrical cuvette with quartz sides (Fig. 6, apparatus B), where the absorption spectrum of gaseous NO was registered. The cuvette was set in the spectrophotometer along the light beam. After pumping air out of the apparatus, sulphuric acid, which had initially been put into the upper chamber of the apparatus, was added to the B-DNIC-GSH solution in its lower chamber.

Subsequent heating of the mixture up to 60 ℃ triggered an intense decomposition of the complex, which was manifested in NO bubbles, and increasing

Fig. 6 Left panel: the sum of optical absorption spectra of NO (four narrow equidistant bands) and NO$_2$ (the structureless spectrum) recorded in the gaseous phase. Right panel: the balloon-type Thunberg apparatuses used in this DNIC synthesis (A) or for optical measurements of gaseous NO (B)
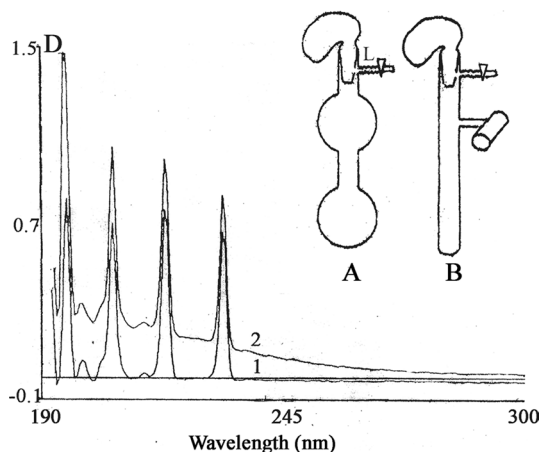
intensity of its optical absorption bands—four narrow equidistant absorption bands within the range of 230–190 nm (Fig. 7, Panel A).

In an hour, NO concentration in the gaseous phase (in 100 ml of the free volume of the Thunberg apparatus) reached 400 micromoles, implying the release of all nitrosyl ligands in the form of NO molecules from B-DNICs. That estimate was based on comparing the experimental and standard absorption spectra of NO shown in Fig. 7, Panels A and B, respectively.

In accordance with the data given in Fig. 5, Panel D, heating the B-DNIC-GSH solution (9 mM) on air at 80 ℃ for 15 min was sufficient for a full decomposition of those complexes and their replacement by an equimolar GS-NO amount. As shown in Fig. 5 (Panels B and C), similar conversion also took place in the absence of air; therefore, it was assumed that in the experiments shown in Fig. 7, GS-NO was also formed (in 20–30 min), but then it was decomposed with NO production, being influenced by, for instance, a catalytic amount of copper as impurity in ferric sulphate used [12].

Verifying that assumption by keeping the GS-NO solution in the acidic environment at 80 ℃ in the presence of ferrosulphate (12 mM) showed that there was no considerable reduction in RS-NO level. Therefore, I thoroughly replicated those experiments with heating acidified B-DNIC-GSH solutions in anaerobic conditions. I found out that the previously discovered effect was characteristic of B-DNIC-GSH in those conditions, but only in their solutions which had been kept in the air for 1 or 2 days. In fresh solutions of those complexes used for such experiments, the complexes were decomposed; however, no GS-NO was formed [52]. At the same time, optical measurements showed that there was no considerable decrease in B-DNIC-GSH level if those solutions were kept in the air.

It would be logical to assume that the expected GS-NO formation in fresh B-DNIC-GSH solutions was blocked since nitrosonium cations got reduced by free (not incorporated in B-DNICs) glutathione, as its content in the solution (after B-DNIC-GSH synthesis with the ratio of initial reactants being the following: GSH: ferrosulphate: nitrite = 20 mM: 10 mM: 10 mM) was 15 mM, i.e., three times as
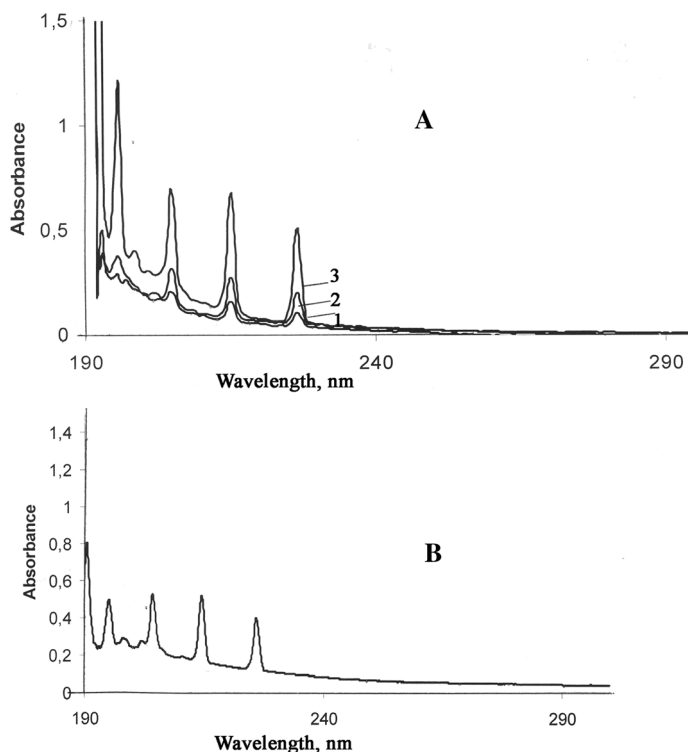
Fig. 7 Panel A: the optical absorption spectra of gaseous NO (four narrow equidistant bands) recorded after decomposition of B-DNIC-GSH (12 mM, 17 mL, pH 1.0) induced by heating at 80 ℃ for 3, 10, or 60 min (curves 1–3, respectively). Panel B: the absorption spectra of a standard sample of gaseous NO (200 μmol in 100 mL of the free volume of the Thunberg apparatus) [12]

large as the B-DNIC amount (per one atom of iron in those complexes). Fe^{2+} ions released from the complexes could play a significant role in that reduction as catalysts. As regards B-DNIC-GSH solutions kept in the air for 1–2 days, free glutathione content could decrease due to its oxidation; therefore, its amount was insufficient for the reduction of nitrosonium cations released from complexes to ensure that the latter, reacting with GSH, including those released from B-DNICs, formed GS-NO [52].

To test that assumption further studies were carried out. I tried to compare the ability of B-DNIC-GSH synthesized with the above-mentioned ratio between GSH, ferric sulphate and nitrite, and with their ratio of 15 mM: 10 mM: 10 mM. With this ratio, free glutathione content remaining in the solution after B-DNIC synthesis would be equal to the amount of complexes in molar terms.

Experiments with solutions of those complexes completely confirmed my assumption. In case of heating B-DNIC-GSH solutions with the same (5 mM) concentration, but with different free glutathione content at 80 ℃ in anaerobic conditions, a considerably higher content of GS-NO formed in the process of those

complexes decomposition was identified in the solutions of those complexes with a lower free glutathione content (Fig. 8, curves 3–5). GS-NO absorption band intensity (334 and 543 nm) indicates that concentration of B-DNIC-GSH decomposition product in those conditions was practically equal to the concentration of B-DNICs (per one atom of iron), i.e., GS-NO formation recruited precisely a half of nitrosyl ligands, which was obviously present there in the form of nitrosonium cations.

Notably, the GS-NO absorption band was shifted slightly by 334 nm, which was related to optical absorption of Fe^{3+} ions remaining in the B-DNIC solution after its synthesis.

The same result was obtained from similar experiments with B-DNIC solutions with N-acetyl-L-cysteine (NAC), synthesized with the ratio between the concentrations of NAC: ferric sulphate: nitrite equal to 30 mM: 10 mM: 10 mM or 15 mM: 10 mM: 10 mM (Fig. 9). In those experiments, during the process of B-DNIC decomposition with an elevated free glutathione concentration (which was five times higher than B-DNIC level), no S-nitroso-NAC (S-NO-NAC) was formed, even in trace amounts (Fig. 9, curves 3, 4): obviously, a fivefold excess of free NAC was sufficient for reduction of all nitrosonium cations to NO. As for S-NO-NAC formation in the process of B-DNIC-NAC decomposition with lower free-NAC concentration, S-NO-NAC concentration did not exceed 30% of the total amount of nitrosyl ligands forming part of B-DNIC-NAC (Fig. 9, curves 5, 6). It should not be ruled out that S-NO-NAC formation in the process of those complexes decomposition in anaerobic conditions was partially suppressed by the reducing effect of NAC on
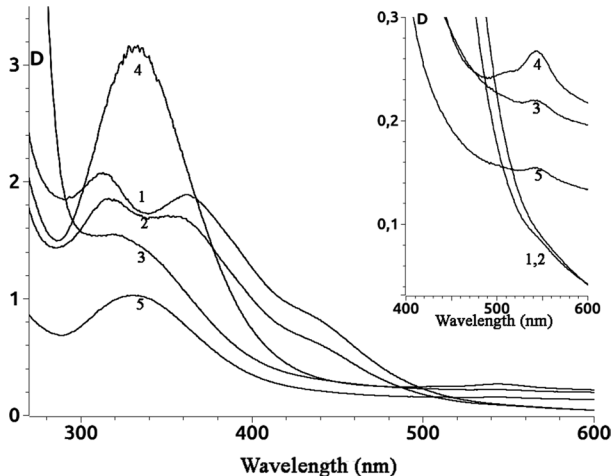

Fig. 8 Optical absorption spectra of B-DNIC-GSH solutions (5 mM), synthesized with the ratio between GSH, ferrosulphate and nitrite of 20 mM:10 mM (curve 1) or 15 mM:10 mM:10 mM (curve 2). The solutions were diluted ten times. Curves 3 and 4, and the absorption spectra characteristic of GS-NO were registered in 5 mM solutions of the same B-DNICs heated at 80 ℃ with pH = 1.0 in anaerobic conditions for 8–9 min, without being diluted. The spectra are characterized by the absorption band at 334 nm slightly shifted to the short-wave region, and the weak band at 543 nm. Curve 5—the absorption spectrum of the solution, characterizing all those solutions by curve 4, after diluting that solution four times. The inset shows the spectra registered at higher spectrometer sensitivity in the band absorption region at 543 nm [52]
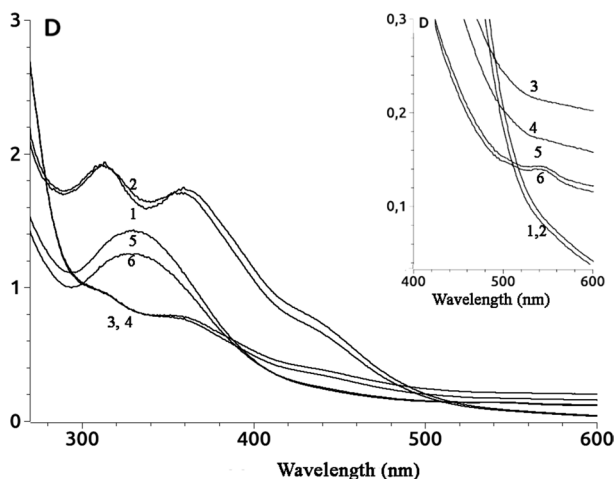
Fig. 9 The absorption spectra of B-DNIC-NAC solutions (5 mM), synthesized with the ratio between NAC, ferrosulphate and nitrite concentration of 30 mM: 10 mM: 10 mM (curve 1) or 15 mM: 10 mM: 10 mM (curve 2). The solutions were diluted ten times. Curves 3, 4 and 5, 6, respectively—the absorption spectra of solutions of the same complexes, heated after being diluted two times at 80 ℃ in anaerobic conditions for 7–9 and 2–3 min, respectively. Spectra 5 and 6 with absorption bands at 334 and 543 nm are related to S-NO-NAC. The inset shows the absorption spectra of all those solutions with higher spectrometer sensitivity in S-NO-NAC band absorption region at 543 nm [52]

nitrosonium cations even with a comparatively low content of that thiol-containing compound in the solution. Therefore, this effect was not identified in the process of similar B-DNIC-GSH complexes decomposition, which entailed the incorporation of a half of nitrosyl ligands of those complexes in GS-NO. It should not be ruled out that the difference was related to a higher reducing activity of NAC compared to that of glutathione.

Currently, one can only speculate on the possible reasons why thiols either reduce nitrosonium cations to NO or "prefer" forming respective RS-NO with them in the process of DNIC decomposition. Fe^{2+} ions released from DNICs can play an important role in their interaction. It should not be ruled out that these ions act as spin catalysts here, initiating the spin polarization in lone electron pairs of sulphur atoms in thiol-containing compounds. As a result, these atoms become capable of reducing nitrosonium cations to NO based on the one-electron mechanism (if the conservation law for resultant spins of reactants and products is preserved). The efficiency of this reaction should certainly increase when the concentration of reactants (thiol-containing compounds) grows, in accordance with the law of mass action.

Following Scheme 3 the decomposition of B-DNICs with thiol-containing ligands in the process of oxidation of the latter, or their irreversible blockade by specific reactants, should lead to nitrite accumulation in the solution with neutral pH values. Moreover, the concentration of the latter should be equal to a half of nitrosyl ligands in DNICs. This assumption was confirmed by experiments where B-DNIC-GSH decomposition in anaerobic conditions was initiated by potassium ferrocyanide (FeCN) being a very strong oxidant. To prevent acidification of the solution due

to hydrolysis of nitrosonium cations (which could cause conversion of nitrite into nitrous acid, with subsequent release of NO from it), I dissolved B-DNIC-GSH in strong 200 mM HEPES buffer (pH 7.4).

Immediately after adding FeCN (80 mM) to 16 ml of 8 mM B-DNIC-GSH solution at 60 ℃ in anaerobic conditions, effervescence of gaseous NO started; the level of gaseous NO in the Thunberg apparatus used for that experiment was estimated based on NO absorption spectrum registered in the cylindrical cuvette connected to the apparatus. In 20 min the level reached a plateau which corresponded to the release of a half of nitrosyl ligands in the form of NO, equal to the amount of B-DNIC-GSH—128 micromoles in 16 ml of the solution (Fig. 10) [52]. After that, gaseous NO was pumped out of the apparatus, and then several drops of sulphuric acid were put into its upper chamber (which were added to the solution after pumping again). It was assumed that nitrite, which could be formed as a result of hydrolysis of nitrosonium cations released from B-DNICs, should be converted into nitrous acid in the acidic environment, and that acid is capable of releasing NO as a result of the disproportionation reaction (as shown in Reaction 3 above).

Indeed, it was possible to register the first two long-wave bands (Fig. 10, curves 4 and 5), whose intensity reached a plateau in 20 min. At the same time, it was impossible to register the remaining two bands due to a shift of the base line position related to Prussian blue, formed as a product of FeCN and Fe^{2+} ions reaction, proceeding on the end walls of the cylindrical cuvette of the Thunberg apparatus [52].

The amount of NO, which was transformed into gas from acidified nitrite, was equal to 90 micromoles per 100 ml of the free volume of the Thunberg apparatus,
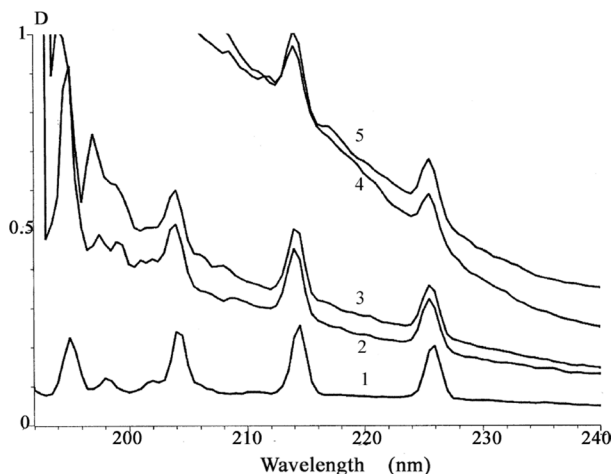

Fig. 10 Changes in the optical absorption spectrum of gaseous NO, registered in the Thunberg apparatus shown in Fig. 6b, in the process of decomposition of B-DNIC-GSH (8 mM, 16 ml) in 200 mM HEPES buffer, pH 7.4, due to adding 80 mM FeCN to the solution. Curves 2 and 3—NO absorption bands registered in 15 and 20 min after adding FeCN to B-DNIC-GSH solution (pH 7.4) heated at 60 ℃ in the degassed Thunberg apparatus. Curves 4 and 5—the first two (of four) NO absorption bands registered in the Thunberg apparatus degassed again, in 15 and 20 min after acidification of the solution with sulphuric acid. Curve 1—the absorption spectrum of a standard gaseous NO sample (125 micromoles in 100 ml of the free volume of the Thunberg apparatus [52]

which corresponded to conversion of 70% of nitrite, which was assumed to be formed in the process of B-DNIC-GSH decomposition caused by FeCN, into NO. Specific experiments for assessing such conversion of nitrite in an acidic environment confirmed that in those conditions 70% of nitrite really converts into NO. The remaining nitrite converted into nitric acid. It emerged due to the hydrolysis of NO_2^+-NO_2^-, adduct formed during the reaction of nitrogen dioxide molecules, which formed (like NO) as a result of the disproportionation reaction of nitrous acid molecules (as shown in Reaction 3 above).

Therefore, research was in full compliance with Scheme 3 and showed that in the process of decomposition of DNICs with thiol-containing ligands, a half of nitrosyl ligands are released from them in the form of neutral NO molecules, while another half, with another half released in the form of nitrosonium cations. If thiol groups are preserved (in an acidic environment), they can be fully included in RS-NO, while in the absence of thiols, nitrosonium cations convert (with neutral pH values) into nitrite anions.

The conversion of nitrosonium cations into nitrite anions was also detected in experiments with nitrosyl iron complexes with non-thiol ligands, for instance, water or phosphate. As our group already showed in the 1980s, water and phosphate, like citrate or ascorbate, form low-spin ($S = 1/2$) M-DNICs with divalent iron and gaseous NO, characterized by the EPR signal with the g-factor values of 2.05–2.014, and high-spin ($S = 3/2$) mononitrosyl iron complexes (MNICs), characterized by the EPR signal with the main component at $g = 4.0$, and the weak component at $g = 2.0$ [56]. The EPR signals of those complexes for M-DNICS and MNICs with water are shown in Fig. 11 (Panel A, curves a). EPR-active nitrosyl iron complexes with thiol-containing ligands are represented only by M-DNICs and give only the 2.03 signal shown in Fig. 11, Panel A, curve c, for M-DNIC-NAC. MNICs with ethylenediaminetetraacetate (EDTA), whose EPR signal and optical absorption spectrum are shown in Fig. 11 (Panel A, curve a, and Panel B), represent nitrosyl iron complexes existing only in the form of high-spin MNICs.

The complexes presented in Fig. 11 were synthesized using gaseous NO in the Thunberg apparatus shown in Fig. 6 (apparatus A), as described in [24]. As distinct from DNICs with thiol-containing ligands, M-DNICs and MNICs with water
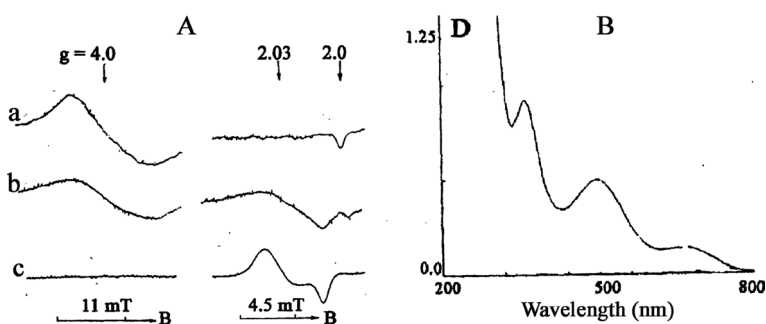

Fig. 11 a EPR spectra of nitrosyl iron complexes with EDTA (a), water (b) and NAC (c), registered at 77 K. b optical absorption spectrum of MNIC-EDTA or MNIC with water [54]

molecules as ligands (M-DNIC–H_2O and MNIC–H_2O) remained in the solution with neutral pH values only in the presence of gaseous NO in the Thunberg apparatus. When the latter was degassed and following 10–20 min those complexes got decomposed. Moreover, the released Fe^{2+} ions formed sediment consisting of insoluble hydroxide complexes thus sharply increasing the solution turbidity. The latter problem was eliminated by acidifying the solution to pH 1–2. In case thiols were added to those solutions after this procedure, for instance, glutathione (50 mM), bands at 334 and 543 nm emerged in the optical absorption spectrum of the solutions, which is characteristic of GS-NO (Fig. 12).

Those most likely emerged due to S-nitrosation of glutathione with nitrous acid molecules, formed as a result of nitrite protonation. Following Scheme 3, nitrite
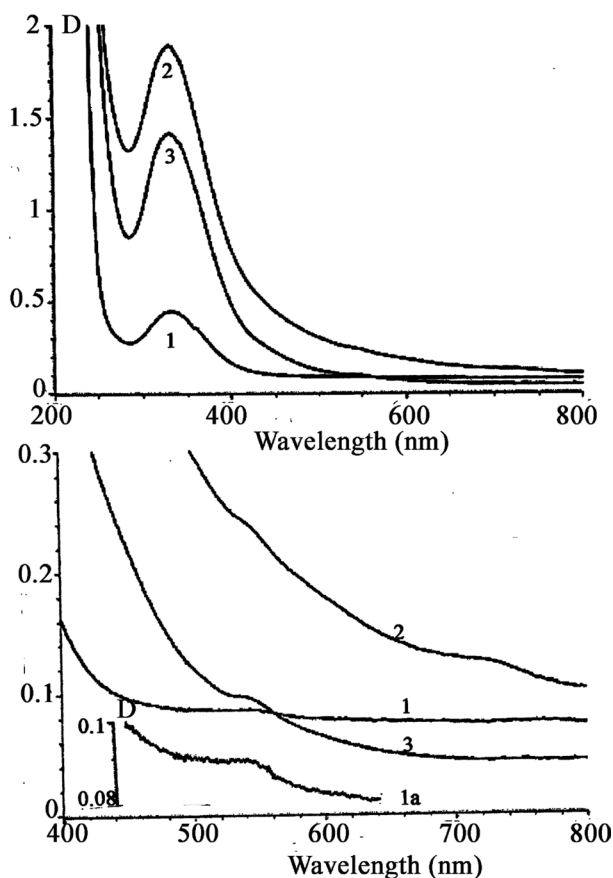

Fig. 12 The absorption spectra of GS-NO formed after adding glutathione to the solution of decomposed M-DNIC-H_2O. Curves 2 and 3—GS-NO spectra registered immediately, and in 5 minutes after adding glutathione (with pH = 1–2) to the solution of M-DNIC–H_2O decomposed in 20 min after pumping NO out of the Thunberg apparatus. Curves 1 and 1a—the absorption spectra of GS-NO formed in water due to the presence of some NO_2 in gaseous NO. Water was treated with NO without adding divalent iron (control experiments) [52]

should have emerged in the process of decomposition of M-DNICs with water molecules as non-thiol ligands. S-nitrosation of glutathione with high-spin MNIC–H_2O as the second form of nitrosyl iron complexes with water molecules (one of their resonance structures is described as Fe^+NO^+ and could be a donor of nitrosonium cations), it is impossible for such complexes. The fact that nitrosyl iron complexes with EDTA have no S-nitrosating activity (according to Fig. 11, such complexes exist only in the form of high-spin MNICs) provides solid evidence for this.

As I point out earlier in this review, gaseous NO also has nitrogen dioxide as an impurity, resulting from continuous disproportionation reaction of NO molecules in that gas. Interaction of nitrogen dioxide with NO causes the formation (in an acidic environment) of nitrous acid, which could cause S-nitrosation of glutathione introduced into the solution. Therefore, to determine the impact of that process on the level of GS-NO formed from decomposing M-DNIC–H_2O, control experiments were conducted, which involved treatment of water with gaseous NO without adding divalent iron to it. The level of GS-NO emerged in those experiments was considerably lower than that in experiments with M-DNIC–H_2O (Fig. 12, curves 1 and 1a).

3 Conclusion

The material provided in this work makes it reasonable to claim that DNICs with thiol-containing ligands can be the main source of nitrosonium cations in living organisms, ensuring S-nitrosation of thiols in those organisms. The fact that these cations emerge in DNICs and remain in them is related to the disproportionation reaction of NO molecules. It is established by a coordination of two NO molecules with a divalent iron ion, on the one hand, and, on the other hand, an effective transfer of π-electron density from sulphur atoms of thiol-containing ligands included in DNICs to nitrosonium cations, preventing hydrolysis of those cations, and thus essentially stabilizing these complexes.

This stabilization mechanism does not apply to DNICs with non-thiol ligands, which are unable to ensure the effective transfer of π-electron density to nitrosyl ligands (unlike thiols). Therefore, those complexes are quickly decomposed due to the hydrolysis of nitrosonium cations after eliminating exogenous NO from the environment, releasing nitric oxide and nitrite anions from it, as shown in Scheme 3.

The release of nitrosonium cations from DNICs with thiol-containing ligands, with their subsequent incorporation in S-nitrosothiols largely follows Scheme 3, as shown above. This process can be blocked through one-electron reduction of nitrosonium cations by thiol groups of low-molecular-weight thiols, with Fe^{2+} ions released from DNICs and involved in this reaction as spin catalysts. These ions may cause spin polarization in lone electron pairs of sulphur atoms (its transition to a triplet state) with a subsequent transfer of one electron to a nitrosonium cation and its release from the coordination sphere of iron in the form of a neutral (paramagnetic) NO molecule. Keeping up with the law of mass action, the efficiency of this reaction obviously should have increased or decreased as the concentration of free thiols was growing or falling, that was clearly revealed in our research.

Therefore, the effect of S-nitrosating activity of DNICs with thiol-containing ligands on thiol groups of thiol-containing cell components should lead to the following consequences. Based on the data provided in the work it should increase with a reduction in the level of intracellular thiols (for instance, in anaerobic conditions) and decrease with the growth of the level of intracellular thiols (for instance, in case of hypoxia).

Importantly, one-electron reduction of nitrosonium cations by thiol groups should have caused a conversion of those groups into free thiyl (RS·) radicals. According to the authors of a recently published work [57], those radicals could be bound with NO molecules, which, for instance, are released from DNICs with the formation of the corresponding S-nitrosothiols. In other words, excess of thiols could trigger a sequence of reactions shown on Scheme 8:

The data we discussed above support the interaction of thiols with nitrosonium cations ends with the latter converting into nitric oxide molecules, without their subsequent interaction with thiyl radicals. Such a reaction was able to cause the formation of respective nitrosothiols in that system. It should not be ruled out that such interaction could be blocked by quick recombination of thiyl radicals with the formation of respective disulphides, which could be supported by quick elimination of NO from the solution as a gaseous molecule not interacting with water.

I also do not rule out the possibility that the ability to initiate S-nitrosation of thiols, characteristic of DNICs with thiol-containing ligands, may determine toxic effect of these complexes on various cell cultures, microorganisms and animal [58–61]. For instance, experiments with malignant-tumor cell cultures showed that removal of an iron-nitrosyl (Fe^+NO^+) fragment from the iron-nitrosyl group of DNIC with thiosulphate by means of one of dithiocarbamate derivatives did not change the toxic effect of those complexes on cells [58]. This means that such DNIC impact was determined by nitrosonium cations released from DNICs during their decomposition caused by dithiocarbamate effect on them, rather than by NO molecules strongly bound by dithiocarbamate together with iron. It was assumed that the released nitrosonium cations caused S-nitrosation of cell-surface proteins, which initiated apoptosis. Experiments with rats in which DNICs with thiol-containing ligands suppressed the development of experimental endometriosis produced similar results. That effect was also preserved when animals received diethyldithiocarbamate [59].

The S-nitrosation effect of DNICs with cysteine on the protease of 2A Coxsackie-B viruses, demonstrated in the work [60], could, according to its authors, prove to be the main reason why those complexes blocked intracellular replication of the virus. That result is very important due to a search for antiviral treatment, especially given the current COVID-19 pandemic caused by the new coronavirus. It is natural to assume that DNICs with thiol-containing ligands initiating S-nitrosation may have a negative impact both on host cell proteases providing for the coronavirus RNA inclusion in those cells, and on virus proteins like 2A protease providing for intracellular replication of the virus. Similar activity of

$$RS^- + NO^+ \rightarrow RS^· + NO \rightarrow RS\text{-}NO$$

Scheme 8. The proposed sequence of reactions initiated by interaction of thiols and nitrosonium cations

DNICs with thiol-containing ligands can suppress the development of malignant tumors and bacteria. Treatment of bacteria with these complexes can reduce the ability of these microorganisms to develop protection from toxic effects of NO molecules, or, more precisely, peroxynitrite formed with their involvement. It was demonstrated that, in response to NO in bacteria, they express synthesis of heme-containing proteins oxidizing or reducing it to biologically inactive forms [61].

The material presented above, demonstrating the ability of DNICs with thiol-containing ligands to be donors of nitrosonium cations, is opposed to the opinion of many DNIC researchers who consider that a d^9 electronic configuration of iron in these complexes corresponds to their electronic and spatial structure most closely [43–47]. This opinion stems from the results of crystallographic studies of M- and B-DNICs with thiol-containing ligands. They imply that these complexes in crystal form are characterized by a tetrahedral spatial structure. In this case, paramagnetism characteristic of the M-form with $S = 1/2$ is related to localization of five electrons on upper t_{2g} (d_{xy}, d_{xz} и d_{yz}) orbitals (of a total of nine electrons localized on d-orbitals). At the same time, it is assumed that in case of dissolving, the tetrahedral spatial structure and the respective electronic structure characteristic of M-DNICs in crystal form remain unchanged.

This opinion is completely consistent with the results of P. Ford's group in the United States, who studied the mechanism for M-DNICs formation with thiol-containing ligands in a water solution in the process of the reaction between Fe^{2+}ions, thiols and gaseous NO [62]. These researchers came to a conclusion that paramagnetism of emerging M-DNICs at the first stage of their formation is due to one-electron reduction of the Fe^{2+}-NO mononitrosyl group by one of thiol-containing ligands bound with that group. As a result, the electronic configuration for iron in that group changes from d^7 to d^8, and with subsequently adding the second NO molecule to that group, which completes the formation of M-DNIC, the dinitrosyl iron group in those complexes starts having the d^9 electronic configuration of iron, or, according to Enemark-Feltham [42], the $[Fe(NO_2)]^9$ structure. To provide an experimental evidence to support the proposed mechanism, P. Ford et al. point to the fact that they found a free thiyl (RS) radical which emerged in the solution of synthesized complexes, which was formed due to one-electron oxidation of a thiol by the mononitrosyl iron group (Fe–NO).

Therefore, currently, there are two mutually exclusive opinions about the electronic structure of DNICs with thiol-containing ligands, characterized by the d^9, or d^7 electronic configuration of iron in dinitrosyl iron groups of those complexes. It is still an open question whether any of these opinions is true. The only thing which allows me to prefer the d^7 configuration is that it can easily explain the ability of M- and B-DNICs to be donors of not only NO molecules, but also nitrosonium cations, converted into either S-nitrosothiols or nitrite anions, based on Scheme 3.

In the works [63, 64], another mechanism for nitrosonium cation generation involving DNICs is proposed. It is suggested that this generation may be provided for by replacement of thiol-containing ligands in DNICs by various redox-active compounds which influence the redox condition of nitrosyl ligands. Consequently, DNICs can produce both nitrosonium cations and neutral NO molecules, as well as nitroxyl anions which are converted into nitrous oxide. It is proposed that such

replacement of ligands in DNICs may be provided for by various amino acid ligands in protein surroundings of those complexes. Yet, I consider it highly debatable.

My review article was included in a volume of *Applied Magnetic Resonance* journal dedicated to Professor Yakov S. Lebedev. My communication with Professor Lebedev started in early 1960s and proved to be particularly useful for me. His kindness and a "democratic" approach, and interest in the results of our group's research, as well as contact with him provided for quick identification of paramagnetic centers responsible for the 2.03 signal, which we first registered in yeast cells, and then in animal tissues.

References

1. L. Ignarro, *Nitric Oxide: Biology and Pharmacology* (Academic Press, San Diego, 2000)
2. M. Graziano, L. Lamattina, Trends Plant Sci. **10**, 4–8 (2005)
3. A.M. Stern, J. Zhu, Adv. Appl. Microbiol. **87**, 187–220 (2014)
4. B.M. Gaston, J. Carver, A. Doctor, L.A. Palmer, Mol. Intervent. **3**, 253–263 (2003)
5. Y. Zhang, N. Hogg, Free Rad. Biol. Med. **38**, 831–838 (2005)
6. D. Seth, D.T. Hess, A. Hausladen, L.W. Wang, J.S. Stamler, Mol. Cell **69**, 451–464.e6 (2018)
7. D.A. Wink, R.W. Nims, J.F. Darbyshir, D. Christodoulou, I. Hanbauer, G.W. Cox, F. Laval, J.A. Cook, M. Krishna, W.G. DeGraff, J.B. Mitchel, Chem. Res. Toxicol. **7**, 519–525 (1994)
8. V.G. Kharitonov, A.R. Sandquist, V.S. Sharma, J. Biol. Chem. **270**, 28158–28164 (1995)
9. N.S. Bryan, T. Rassaf, R.E. Maloney, S.M. Rodriguez, F. Saijo, J.R. Rodriguez, M. Feelisch, Proc. Natl. Acad. Sci. USA **101**, 4308–4313 (2004)
10. C.A. Bosworth, J.C. Toledo, J.W. Zmiewski, J.R. Lancaster, Proc. Natl. Acad. Sci. USA **106**, 4671–4676 (2009)
11. M.V. Foster, L. Liu, M. Zheng, D.T. Hess, J.S. Stamler, Biochem. **48**, 792–799 (2009)
12. A.F. Vanin, Austin J. Analyt. Pharmac. Chem. **5**(4), 1109–1119 (2018)
13. T.P. Melia, J. Inorg. Nucl. Chem. **27**, 95–98 (1965)
14. A.F. Vanin, Cell Biochem. Biophys. **77**, 279–292 (2019)
15. X. Xu, S.G. Chang, Chemisphere **67**, 1628–1636 (2007)
16. A.F. Vanin, I.V. Malenkova, V.A. Serezhenkov, Nitric Oxide Biol. Chem. **1**, 191–203 (1997)
17. A.F. Vanin, Nitric Oxide Biol. Chem. **21**, 1–13 (2009)
18. A.F. Vanin, D.Sh. Burbaev, Biophys. J. **14**, 818836 (2011)
19. A.F. Vanin, Nitric Oxide Biol. Chem. **54**, 15–29 (2016)
20. A.F. Vanin, *Dinitrosyl Iron Complexes as a "Working Form" of Nitric Oxide in Living Organisms* (Cambridge Scholars Publishing, Cambridge, 2019)
21. J.R. Hickok, S. Sahni, H. Shen, A. Arvindt, C. Antoniou, L.M.W. Fang, D.D. Thomas, Free Rad. Biol. Chem. **51**, 1558–1566 (2011)
22. V.D. Mikoyan, E.N. Burgova, R.R. Borodulin, A.F. Vanin, Nitric Oxide Biol. Chem. **62**, 1–10 (2017)
23. A.F. Vanin, A.A. Papina, V.A. Serezhenkov, W.H. Koppenol, Nitric Oxide Biol. Chem. **10**, 60–73 (2004)
24. A.F. Vanin, A.P. Poltorakov, V.D. Mikoyan, L.N. Kubrina, D.S. Burbaev, Nitric Oxide Biol. Chem. **23**, 136–149 (2011)
25. R.M. Nalbandyan, A.F.Vanin, L.A. Blumenfeld, in *Proceedings of the 1st All-Sovjet Union Meeting "Free Radical Processes in Biological Systems"* (Moscow, UdSSR, 3-6 June 1964) p.18
26. A.F. Vanin, R.M. Nalbandyan, Biofizika (Rus) **10**, 167–168 (1965)
27. A.F. Vanin, L.A. Blumenfeld, A.G. Chetverikov, Biofizika (Rus) **12**, 829–841 (1967)
28. J.R. Mallard, M. Kent, Nature **204**, 1192 (1964)

29. A.J. Vithaythil, B. Ternberg, B. Commoner, Nature **207**, 1246–1249 (1965)
30. J.R. Lancaster, J.B. Hibbs, Proc. Natl. Acad. Sci. USA **87**, 1223–1227 (1990)
31. C. Pellat, Y. Henry, J.-C. Drapier, Biochim. Biophys. Res. Comm. **166**, 119–125 (1990)
32. J.-C. Drapier, C. Pellat, Y. Henry, J. Biol. Chem. **266**, 10162–10167 (1991)
33. V. Lepoivre, J.-M. Flavan, Y. Henry, J. Biol. Chem. **267**, 22294–22300 (1992)
34. A.F. Vanin, P.I. Mordvintcev, S. Hauschildt, A. Mülsch, Biochem. Biophys. Acta **1177**, 37–42 (1993)
35. J. Stadler, H.A. Bergonia, M. DiSilvio, M.A. Sweetland, T.R. Billiar, R.L. Simmonsd, J.R. Lancaster, Arch. Biochim. Biophys. **302**, 4–11 (1993)
36. Y.-L. Geng, A.-S. Petersson, A. Wennmalm, G. Yannson, Exp. Cell Res. **214**, 418–424 (1994)
37. K. Odoi, T. Akaike, H. Horie, S. Fujii, N. Beppu, H. Ogawa, H. Maeda, Cancer **77**, 1598–1604 (1996)
38. R.N. Watts, C. Hawkins, P. Ponka, R.L. Richardson, Proc. Natl. Acad. Sci. USA **103**, 7670–7675 (2006)
39. A.F. Vanin, R.M. Nalbandyan, Biofizika (Rus) **11**, 178–179 (1966)
40. A.F. Vanin, Biokhimiya (Rus) **32**, 228–232 (1967)
41. C.C. McDonald, W.D. Philips, H.W. Mower, J. Am. Chem. Soc. **87**, 3319–3326 (1965)
42. J.H. Enemark, R.D. Feltham, Coord. Chem. Rev. **13**, 339–406 (1974)
43. T.T. Lu, C.C. Tsou, H.W. Huang, I.J. Hsu, J.M. Chen, T.S. Kuo, Y. Wang, W.F. Liaw, Inorg. Chem. **47**, 6050–6050 (2008)
44. C.E. Tinberg, Z.J. Tonzetich, H. Wang, L.H. Do, Y. Yoda, S.P. Cramer, S.J. Lippard, J. Am. Chem. Soc. **132**, 18168–18176 (2010)
45. T.T. Lu, S.Z. Lai, Y.W. Li, Inorg. Chem. **50**, 5396–5406 (2011)
46. A.F. Shestakov, Y.M. Shul`ga, N.S. Emel`yanova, N.A. Sanina, S.M. Aldoshin, Rus. Chem. Bull. **56**, 1289–1297 (2007)
47. D.C. Pestol, R.B. Chupik, S. Khan, M. Elsabahy, K.L. Wooley, M.Y. Darenbourg, S.-M. Lim, J. Am. Chem. Soc. **16**, 3178–3187 (2019)
48. D.Sh. Burbaev, A.F. Vanin, L.A. Blumenfeld, Khimii (Rus) **12**, 252–256 (1971)
49. B.R. McGarvey, Canad. J. Chem. **53**, 2498–2511 (1973)
50. B. D`Autreaux, O. Horner, J.L. Oddou, C. Jeandey, S. Gambarelli, C. Berthomieu, J.M. Latour, I. Michaud-Soret, J. Am. Chem. Soc. **126**, 6005–6016 (2004)
51. S. Stojanovic, D. Stanic, M. Nicolic, M. Spasic, V. Niketic, Nitric Oxide Biol. Chem. **11**, 256–262 (2004)
52. A.F. Vanin, Biofizika (Rus) **65**, 353–367 (2020)
53. R.R. Borodulin, L.N. Kubrina, V.O. Shvydkiy, V.L. Lakomkin, A.F. Vanin, Nitric Oxide Biol. Chem. **35**, 110–115 (2013)
54. D.L.H. Williams, *Nitrosation Reactions and the Chemistry of Nitric Oxide* (Elsevier, Amsterdam, 2004)
55. A.F. Vanin, I.V. Malenkova, P.I. Mordvintcev, A. Mülsch, Biokhimiya (Rus) **58**, 1094–1103 (1993)
56. A.F. Vanin, D.I. Aliev, Studia Biophysica **93**, 63–68 (1983)
57. D.R. Truzzi, S.V. Alves, L.E.S. Netto, O. Augusto, Antioxidants **9**, 276 (2020). https://doi.org/10.3390/antiox9040276
58. A.L. Kleschyov, S. Strand, S. Schmitt, D. Gottfried, M. Skatchkov, N. Sjakste, M. Deiber, V. Umansky, T. Münzel, Free Rad. Biol. Chem. **40**, 1340–1348 (2006)
59. A.F. Vanin, E.N. Burgova, L.V. Adamyan, Austin J. Reprod. Med. Infertil. **2**, 1019–1032 (2015)
60. C. Badorff, B. Fichtlscherer, A. Muelsch, A.M. Zeiher, S. Dimeller, Nitric Oxide Biol. Chem. **6**, 305–312 (2002)
61. J. Green, V.D. Rolfe, L.J. Smith, Virulence **5**, 1–33 (2013)
62. J.C.M. Pereira, A.V. Iveretskii, R.M. Han, P.C. Ford, J. Am. Chem. Soc. **1237**, 328–336 (2015)
63. T.-T. Lu, C.-H. Chen, W.-F. Liaw, Chem. Europ. J. **16**, 8088–8095 (2010)
64. C.-C. Tsou, W.-F. Liaw, Chem. Europ. J. **17**, 13358–13366 (2011)
65. N.R. Bastian, C.-Y. Yim, J.B. Hibbs, W.E. Samlowsky, J. Biol. Chem. **269**, 5127–5131 (1994)
66. D. Muller, A. Kleschyov, J.-C. Stoclet, Br. J. Pharmacol. **119**, 1281–1285 (1996)