

# AQUEOUS SOLUTIONS OF DINITROSYL IRON COMPLEXES WITH THIOL-CONTAINING LIGANDS: ISOTROPIC EXCITABLE AUTOWAVE-GENERATING MEDIA

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*To the memory of the outstanding Russian scientist Anatoly Markovich Zhabotinsky*

**Abbreviations:** DNIC, dinitrosyl iron complex; GS-NO, S-nitrosoglutathione, NO, nitric monoxide, RS-NO, S-nitrosothiol; RS, thiol-containing compound carrying an ionized thiol group.

## ABSTRACT

A drop (10  $\mu$ l) of the glutathione (0.5 mM) – bivalent iron (1 mM) mixture in 15 mM HEPES pH 7.7 applied to the surface of a 0.5 M solution of S-nitrosoglutathione in the same buffer during the synthesis of a dinitrosyl iron complex (DNIC) with glutathione in a thin (0.3 mm) layer generates autowaves thereby suggesting a space-time distribution of DNIC in the solution. The autowave pattern changes periodically every 0.4–0.6-s over a 3-s period following the addition of the drop to the solution after which the structured pattern of DNIC dissipates and the solution develops an even green colour as a result of uniform distribution of DNIC. Similar autowaves are recorded in a thin layer of 10 mM DNIC with glutathione after addition of 0.5 M S-nitrosoglutathione (10  $\mu$ l) suggesting that solutions of DNIC with thiol-containing ligands (e.g., glutathione) represent excitable media able to generate autowaves in the non-equilibrium (excited) state, e.g., in the course of DNIC synthesis by a reaction of S-nitrosothiols (or NO) with bivalent iron and thiols or after addition of the latter to DNIC in the state of a chemical equilibrium (quiescent state) with its constituent elements. The refractory state required for autowave generation in the given experimental system is characterized by a prolonged induction period characteristic of multistep synthesis of DNIC. Quite probably, the presence of such systems in cells and tissues of living organisms is a necessary prerequisite to effective space-time control over biological effects of NO and its endogenous derivatives.

**Key words:** nitric oxide, dinitrosyl iron complexes, autowaves.

## Introduction

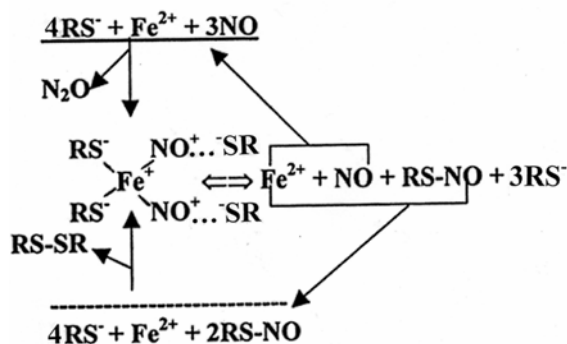
It has been established that virtually all living organisms, be them human beings, animals, plants or bacteria, possess an ability to perform continuous enzymatic synthesis of nitric monoxide (NO), one of the simplest chemical compounds and a universal regulator of an immense diversity

of physiological and biochemical processes [1-3]. Its regulatory function is not mediated by cell receptors as is the case with many other metabolic regulators, such as hormones, cytokins and so on. Nitric oxide and its ionized form, the nitrosonium ion ( $\text{NO}^+$ ), act directly on their specific targets, viz., heme (neutral NO molecules) or thiol (nitrosonium ions) groups of proteins, and

thus modulate their biological activities. A question arises: how do these low-molecular components interact with target proteins? Is their interaction a result of chaotic (Brownian) motion of NO and NO<sup>+</sup> molecules (the latter are delivered to target proteins within the composition of compounds preventing the hydrolysis of nitrosonium ions, e.g., S-nitrosothiols) or does their targeted delivery to intracellular proteins proceed in an orderly and timely mode, as autowave distribution of low-molecular agents in the intracellular space, in particular?

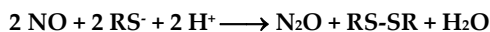
Our model study demonstrated that the role of one of such autowave-generating chemical systems can be played by a mixture of interconvertible endogenous derivatives of nitric oxide, namely, S-nitrosothiols and dinitrosyl iron complexes (DNIC) with thiol-containing ligands. Nitric oxide incorporated into these compounds is protected by them from damaging effects of the superoxide anion resulting in the formation of a highly toxic product, viz., peroxynitrite. In this way, NO storage in cells and tissues and effective transport of NO into cells and intercellular or interstitial space are realized [4].

The self-regulating self-sustained chemical system, which includes DNIC (chemical formula  $\{(RS)_2Fe^+(NO^+...SR)_2\}^+$  and S-nitrosothiols (chemical formula RS-NO), is depicted in Scheme 1:



Scheme 1.

Excess NO present in this system initiates the synthesis of DNIC and accumulation of S-nitrosothiols, while its depletion triggers early DNIC synthesis even in the S-nitrosothiol step and thus augments the NO pool. In total, this system effects NO reduction to N<sub>2</sub>O and oxidation of thiols to the corresponding disulfides (Scheme 2):

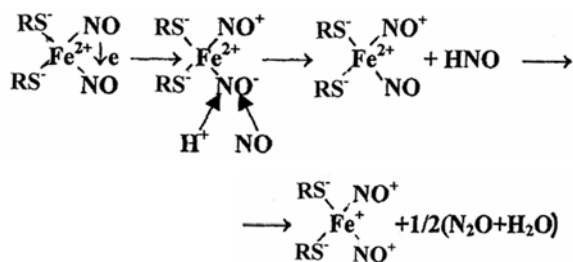


Scheme 2.

This system is decomposed after complete depletion of NO and thiols [5, 6].

The low-spin mononuclear paramagnetic DNIC with low-molecular thiols (Scheme 1) are characterized by an effective spin ( $S = 1/2$ ) and an EPR signal at  $g_{aver.} = 2.03$  and are easily formed upon treatment of aqueous solutions of bivalent iron and thiols with gaseous NO; noteworthy, thiols are usually taken in a 20-fold (with respect to iron) and more excess [5–10]. Obviously, the binding of two neutral (paramagnetic) NO molecules ( $S = 1/2$ ) and the Fe<sup>2+</sup> ion (its electronic configuration is d<sup>6</sup>) prerequisite to DNIC synthesis yields a complex with the electronic configuration d<sup>8</sup> whose low-spin variant is diamagnetic ( $S = 0$ ). What, then, is the mechanism whereby this DNIC passes into a low-spin paramagnetic state ( $S = 1/2$ )?

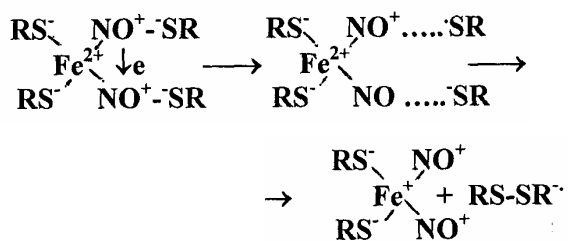
Earlier, we hypothesized [5,6,10] that this transformation is determined by the ability of neutral NO molecules for characteristic disproportionation, i.e., transfer of the unpaired electron from one NO molecule to another along the d-orbitals of iron (later, this hypothesis was verified in [11, 12]). As a consequence, NO molecules of DNIC are converted into the nitrosonium (NO<sup>+</sup>) or the nitroxyl (NO<sup>-</sup>) ion, respectively (Scheme 3):



Scheme 3.

Whereas the nitrosonium ion remains to be bound to iron, the nitroxyl ion enters into a reaction with the proton to be converted into nitroxyl, which leaves the complex. Its further dismutation by the second nitroxyl molecule yields water and nitrous monoxide. The vacancy is occupied by the neutral NO molecule effecting the transfer of the unpaired electron to the iron atom. Thus, the synthesis of one DNIC molecule is provided by three NO molecules; part of NO is expended for NO synthesis. The process is terminated by the synthesis of paramagnetic DNIC the iron atom in which has a  $d^7$  configuration. Its ligand environment includes two thiol molecules and two nitrosonium ions whose hydrolysis is prevented by the binding of ionized thiols. The chemical formula of the complex thus appears as  $\{(\text{RS}^-)_2\text{Fe}^+(\text{NO}^+ \dots \text{SR})_2\}$  [6-8].

Low-molecular DNIC with thiol-containing ligands can also be formed by a direct reaction between S-nitrosothiols and  $\text{Fe}^{2+}$  ions in the presence of thiols (Scheme 4) [5,6]:



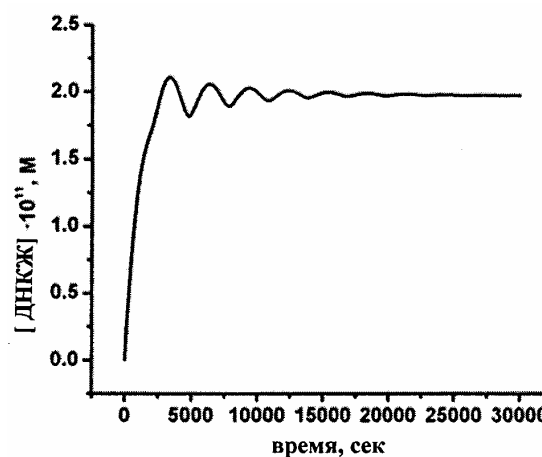
Scheme 4.

The dismutation of two S-nitrosothiol molecules within the composition of their complex with  $\text{Fe}^{2+}$  initiates single-electron oxidation-reduction of S-nitrosothiols

(Scheme 4), which, in its turn, results in their destabilization, release of the reduced form of the disulfide and, finally, DNIC synthesis. The disulfide radical is easily oxidized to a disulfide, e.g., by oxygen.

The dismutation mechanism in which one electron is transferred from one S-nitrosothiol molecule to another can be realized in the following way. Two paired electrons are transferred by the first S-nitrosothiol molecule to the vacant d-orbital of iron. However, because of a strong exchange interaction between electrons on the d-orbitals of iron the electron pair becomes unpaired as a result of which two unpaired electrons appear on the both upper d-orbitals of  $\text{Fe}^{2+}$ . These electrons are redistributed between two S-nitrosothiol molecules to be further transformed into unstable single electron-oxidized and -reduced forms, respectively.

A theoretical computer analysis of the kinetic equations of system 1 revealed time-dependent oscillations in the concentrations of DNIC [6] (Fig. 1), S-nitrosothiols and free (i.e., non-bound to DNIC) iron (A. Papina, personal communication). Similar oscillations in the DNIC level, damping with time, were seen in aqueous solutions obtained by mixing S-nitrosocysteine or a neutral NO donor with cysteine +  $\text{Fe}^{2+}$  (Fig. 1) [6].



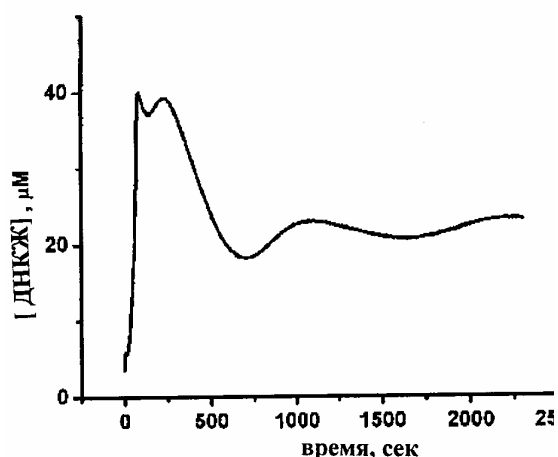
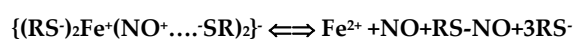


Fig. 1. First panel: a calculated kinetic curve for the synthesis of DNIC with thiols in solutions of  $\text{Fe}^{2+}$  [ $10^{-4}$  M], S-nitrosothiol [ $5 \times 10^{-7}$  M] and thiol [ $5 \times 10^{-3}$  M]. Second panel: kinetics of synthesis of DNIC with cysteine in a solution of cysteine [ $10^{-2}$  M] to which a S-nitrosocysteine [ $4 \times 10^{-4}$  M] – ferrous sulphate [ $5 \times 10^{-5}$  M] mixture in 100 mM HEPES pH 7.4 was added (EPR data) [4]. Abscissa – time, s; ordinate – DNIC concentration.

The damping of the oscillations in DNIC concentration seems to be due to fast exhaustion of NO or thiols in these experimental systems.

The discovery of oscillations in DNIC concentration in the system depicted in Scheme 1 led one of us to hypothesize [4] that the autowave distribution pattern of DNIC is generated under far from equilibrium conditions determined by the ratio (Scheme 5):



Scheme 5.

A similar situation was observed in previous studies, namely during the analysis of an oscillating chemical reaction of bromomalonate, bromate and DNIC with 1,10-phenanthroline in an acidic medium where 1,10-phenanthroline was used as a catalyst. Under these conditions, the addition of a small dose of the catalyst to a thin (2 mm) layer of a two-dimensional oscillating

chemical system (bromomalonate + bromate) (without mixing) was followed by the appearance of ring autowaves, which reflected the space-time distribution of the stained oxidized and reduced forms of the iron complex with 1,10-phenanthroline [13,14]. In this study, the autowaves generated in response to addition of the glutathione– $\text{Fe}^{2+}$  mixture to a thin layer of S-nitrosoglutathione (without mixing) initiated the synthesis of DNIC with glutathione.

## Experimental

### Materials and methods

Reduced glutathione, sodium nitrite (Sigma, USA) and ferrous sulphate (Fluka, Switzerland) were used. Solutions of S-nitrosoglutathione (GS-NO) (500 mM) were prepared by adding a solution of 500 mM  $\text{NaNO}_2$  in distilled water to a solution of 500 mM glutathione in 10 mM HEPES buffer (pH 2,5) after which the mixture immediately turned pink due to formation of GS-NO [5, 6]. The pH of the solution was adjusted to 7.7 with concentrated NaOH. An aliquot of the test solution (0.2 ml) was pipetted carefully onto the bottom of a Petri dish and given the shape of a circle with the help of a glass rod. Its maximum size was determined from adhesion of the solution components to glass. The average thickness of the layer deposited on the bottom of the Petri dish was 0.3 mm. A drop (10  $\mu\text{l}$ ) of the aqueous mixture (1 mM  $\text{Fe}^{2+}$  and 500 mM glutathione in 10 mM HEPES pH 7.7) was coated carefully on the top of the layer with the help of a 20- $\mu\text{l}$  Eppendorf pipette. After the drop came into contact with the surface of the pink-coloured solution of GS-NO, it passed into the solution in the form of a pale circle after which dark-green rings and round spots with variable characteristics began to appear

in it. The course of events was recorded with the help of a digital videocamera. Subsequent computer analysis made it possible to obtain individual frames of time-dependent changes in the black-and-white patterns of the rings and round spots formed in the drop of the aqueous glutathione- $\text{Fe}^{2+}$  mixture after its application to the surface of the pink-coloured solution of GS-NO.

### *Results and Discussion*

The fundamental result of this study was the discovery of time-dependent changes in dark-green rings and round spots formed in the drop containing the glutathione- $\text{Fe}^{2+}$  mixture after its application to the surface of the pink-coloured solution of GS-NO. These structures were formed as a result of interaction of the drop with the GS-NO solution at the site of its exposure (without mixing), but not in the case of vigorous mixing of the drop with the solution. In the latter case, evenly coloured green round spots characteristic of mononuclear DNIC with glutathione were formed instead of the structurized pattern [5, 6]. Uniform staining was also observed for an initially structurized pattern obtained 5–7 s after addition of the glutathione- $\text{Fe}^{2+}$  mixture to the  $\text{Fe}^{2+}$  solution. The dark-green rings and round spots gradually lost their colour on going from the structurized image of the drop to the evenly stained green spot. In all probability, such a transition might be due to the decrease of the initially high local concentration of DNIC in the rings and circles and the uniform distribution of DNIC in the solution detectable by a characteristic EPR signal at  $g_{\text{aver.}}=2.03$  [6,9].

No structurized images of the drop were obtained in control experiments where a drop containing only glutathione (0.5 M) or bivalent iron (1 mM) was applied to the surface of the GS-NO solution. The only

important finding of this study was slight discoloration of the pink-coloured solution of GS-NO at the application site: the drop acquired the shape of a pale circle, but round and circular structures were not formed.

The temporal changes in the structurized distribution pattern of DNIC formed in the glutathione- $\text{Fe}^{2+}$  mixture after careful addition of the drop to the GS-NO solution are shown in Fig. 2. The appearance of a dark-green ring with a radius of the order of 6 mm was recorded as early as within 0.2 s after application as could be evidenced from the colour of the drop containing DNIC with glutathione. Its formation might be due to interaction of  $\text{Fe}^{2+}$  ions and glutathione with GS-NO on the periphery of the  $\text{Fe}^{2+}$  and glutathione-containing drop during its passage through the GS-NO solution and resulting synthesis of DNIC with glutathione (Scheme 1). However, further changes in the distribution pattern of DNIC, namely, the appearance in the drop of an intensely coloured dark-green round spot of a smaller radius in the next 0.2 s followed by the appearance, in this spot, of additional pale rings after 0.8 s and their complete disappearance 0.4 s thereafter fail to provide convincing evidence in favor of the interaction of the drop components with GS-NO molecules in the external (with respect to the drop) solution. Similar changes, viz., the appearance of a fine structure in the inner round spot and its subsequent dissipation, could be visualized within 2–3.2-s after application of the drop to the GS-NO solution. After more prolonged ( $\geq 3.2$  s) exposure, the fine structure of the inner round spot was stabilized, its colour became less intense, while the structurized pattern was replaced by an evenly stained round green spot.

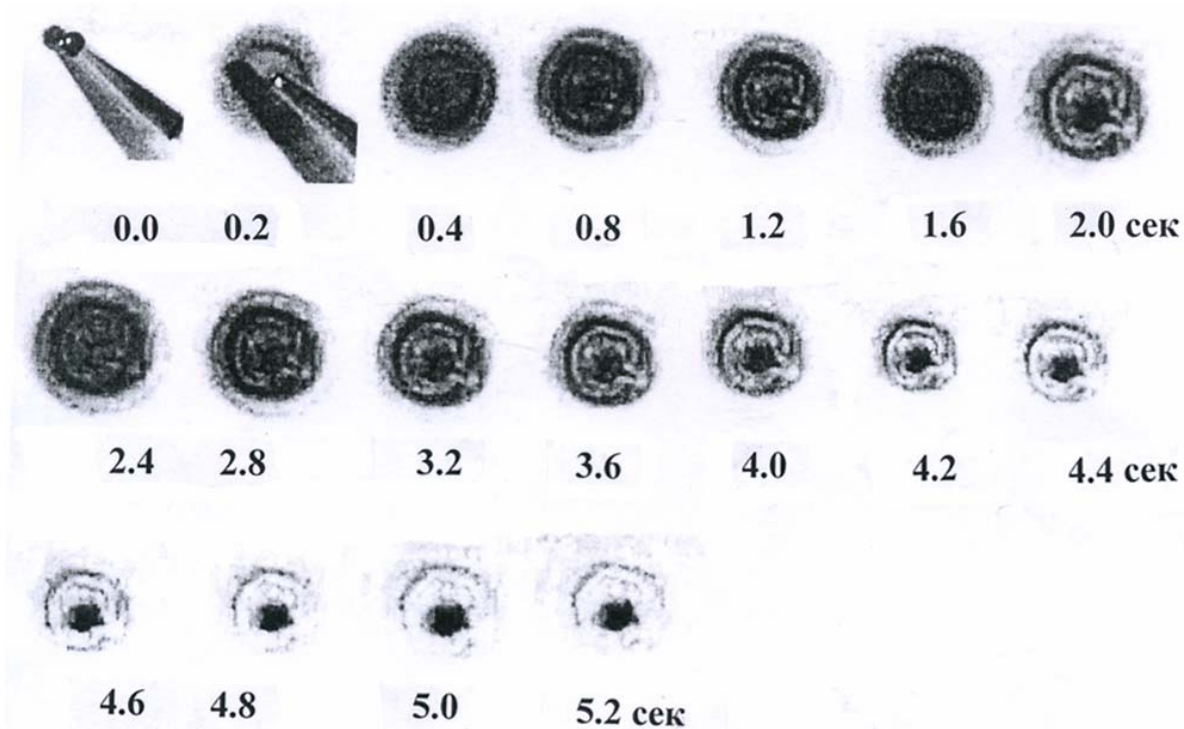


Fig. 2. Time-dependent changes in the drop containing the glutathione- $\text{Fe}^{2+}$  mixture after its addition to the GS-NO solution.

The complex changes in the distribution pattern of DNIC with glutathione formed upon interaction of the iron- and glutathione-containing drop with the GS-NO solution were stably reproduced in further experiments.

The complete identity of the distribution patterns of DNIC in the drop containing the  $\text{Fe}^{2+}$ -glutathione mixture after its exposure to the surface of the GS-NO solution visualized as interchanging dark and clear rings (Fig. 2) to the patterns (ring autowaves) recorded in the echo mode in purely chemical systems (two-dimensional isotropic excitable media) [ 13, 14 ] led us to conclude that we dealt with a similar phenomenon, i.e., generation of autowaves in the echo mode, and that dark green-coloured rings were actually produced by DNIC .

According to [14, 15], the dynamics of autowave processes recorded in the echo mode in two-dimensional isotropic excitable media can be either time-continuous or

followed, after a rather short period of time, by the quiescent state. In our study, the quiescent state was reached within 3 s after application of the drop to the surface of the GS-NO solution with subsequent dissipation of the autowaves and uniform distribution of DNIC in the solution (Fig. 2). In the preceding period, the autowave distribution of DNIC concentration was characterized by reversible dynamics manifested in structurization of the central round spot at regular (every 0.4–0.6 s) time intervals suggesting that in the system under study autowaves were generated in both dynamic regimes.

The reason for dissipation of autowaves in our experimental system may be dual. First, autowaves might be induced by hydrolysis of  $\text{Fe}^{2+}$  and a concomitant decline of DNIC concentration to a level insufficient for their generation. Our data suggest that the decrease (from 1.0 to 0.1 mM and even more) of  $\text{Fe}^{2+}$  concentration in the drop added to the GS-NO solution could hardly

initiate an autowave process characteristic of DNIC distribution. Obviously, autowaves were produced after the concentration of iron (and, as a consequence, of DNIC) in the drop exceeded a certain threshold level. Second, the NO content in the system (Scheme 1) might decrease due to irreversible conversion of NO into  $N_2O$  in the course of DNIC synthesis from NO,  $Fe^{2+}$  and thiol and the escape of NO from the solution. As a result, the rate of the reaction (Scheme 2) providing energy for the processes depicted in Scheme 1 might decrease due to depletion of the "fuel" required for maintaining the activity of the system (Scheme 1) that operates in a self-regulating self-sustained oscillating mode.

In the framework of present-day theories, autowaves are generated in systems that can exist in quiescent, excited and refractory states [ 15 ]. The quiescent state of DNIC with thiol-containing ligands (Scheme 5) is characterized by an equilibrium between DNIC and its constituent elements. Disturbances in this equilibrium (e.g., upon excitation of the system) can be generated, in particular, by addition of a RS-NO or NO excess to the solution, which, in its turn, initiates interaction of these compounds with bivalent iron and thiols and formation of DNIC according to Scheme 1. Taking into account the multistep character of this process, the formation of DNIC by this reaction should be characterized by a prolonged induction period sufficient for decomposition of original DNIC and release of  $Fe^{2+}$  ions able to interact with RS-NO or NO. In the far from equilibrium state, such systems usually generate autowaves, which have the appearance of ring-shaped structures and are characterized by space-time distribution of DNIC. Indeed, in our study the addition of a 10- $\mu$ l drop of a 0.5 mM GS-NO solution to 10 mM DNIC with glutathione was followed by the appearance of autowaves structurally similar to those

depicted in Fig. 2. After several seconds, the autowave pattern disappeared and the solution developed an even green colour due to formation of DNIC with glutathione in the quiescent state, i.e., the chemical equilibrium of DNIC with its constituent elements was reached in this case.

The appearance of autowaves in response to addition of the  $Fe^{2+}$ -glutathione mixture to the GS-NO solution (Fig. 2) could also be induced by the non-equilibrium excited state of the system under study in the course of DNIC synthesis. The autowaves disappeared during the transition of DNIC to the equilibrium (quiescent) state.

What is the reason for the appearance of the refractory state responsible for generation of autowaves in this particular system? In the system described by A.N. Zaikin and A.M.Zhabotinsky [13,14] (bromomalonate, bromate + DNIC with 1,10-phenanthroline), the transition of the complex to the refractory state was induced by the appearance of an inhibitor in the course of the reaction. In nerve fibers whose excitation is associated with generation of autowaves, the transition to the refractory state is determined by a considerable expenditure of macroergs. Their recovery in the course of metabolic processes occurring in nerve fibers is followed by their transition to the quiescent state with subsequent excitation [15]. Presumably, the refractory state of DNIC with thiol-containing ligands as an excitable system is characterized by a prolonged induction period characteristic of the non-equilibrium synthesis of DNIC in the reaction:  $Fe^{2+} + \text{thiol} + \text{RS-NO}$  (or NO).

The discovery of autowave oscillations in DNIC concentration, which reflect the ability of a system for space-time self-organization, adds additional intrigue to the hypothesis according to which this system is responsible for the space-time

control over biological effects of nitric oxide (NO), one of the most universal regulators of metabolic processes in human, animal, plant and bacterial organisms [1-3]. (It is not excluded that these changes are also characteristic of other components of the experimental system, see Scheme 1). The concentric ring autowave distribution patterns of NO and its derivatives observed in our study are not unique, since autowaves may acquire different shapes. What is really important, autowaves determine non-chaotic space-time molecular distribution of NO and its derivatives in cells and tissues and thus effect space-time control over their biological activities.

The "highly-ordered" space-time distribution of NO molecules in various cells and tissues produced chaotically by NO synthases is reached during their interaction with the excitable equilibrium system of DNIC with thiol-containing ligands. Thus, nitric oxide initiates the transition of the system to the non-equilibrium state concomitantly with generation of autowaves by the aforesaid hypothetical mechanism and space-time-organized distribution of DNIC. The latter can exert direct action by acting as signalling agents or play the role of NO and NO<sup>+</sup> donors that influence the corresponding targets.

From the above-said it follows that DNIC, being integral components of the system depicted in Scheme 1, have every reason to be regarded not only as NO and NO<sup>+</sup> donors, but also as key regulators of biological effects of these compounds and RS-NO.

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