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A simple protocol for the synthesis of dinitrosyl iron complexes with glutathione: EPR, optical, chromatographic and biological characterization of reaction products ☆

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ABSTRACT

The diamagnetic binuclear form of dinitrosyl iron complexes (B-DNIC) with glutathione can be easily synthesized in the air at ambient temperature. The synthetic protocol includes consecutive addition to distilled water of glutathione, which decreases the pH of the test solution to 4.0, a bivalent iron salt (e.g., ferrous sulphate) and sodium nitrite at the molar ratio of 2:1:1, with a subsequent increase in pH to neutral values. Under these conditions, the amount of B-DNIC formed is limited by initial nitrite concentration. In the novel procedure, 20 mM glutathione, 10 mM ferrous sulfate and 10 mM sodium nitrite give 2.5 mM B-DNIC with glutathione, while 5 mM glutathione remains in the solution. Bivalent iron (5 mM) is precipitated in the form of hydroxide complexes, which can be removed from the solution by passage through a paper filter. After the increase in pH to 11 and addition of thiols at concentrations exceeding that of DNIC tenfold, B-DNIC are converted into a mononuclear EPR-active form of DNIC (M-DNIC) with glutathione. B-DNIC preparations synthesized by using new method contain negligible amount of nitrite or S-nitrosoglutathione as a contaminations.

All the steps of DNIC synthesis were characterized by using optical, EPR and HPLC methods. A long-lasting hypotensive action of DNIC formed was demonstrated.

Introduction

Recent studies have shown that mononuclear and binuclear water-soluble dinitrosyl iron complexes (M- and B-DNIC) with natural thiol-containing (RS) ligands, viz., glutathione or cysteine [formulae $(RS)_2Fe(NO)_2$ and $(RS)_2Fe_2(NO)_4$, respectively], possess a vast array of biological activities. They induce effective relaxation of blood vasculature and, as a consequence, stable hypotension [1–3], accelerate skin wound healing [4], inhibit platelet aggregation [5–6], increase red blood cell elasticity and thus improve microcirculation [7–8], suppress experimental endometriosis in rats [9], initiate persistent penile erection in experimental animals [10], etc. These capabilities reflect the ability of DNIC with thiol-containing ligands to act as donors of NO and nitrosonium ions (NO^+) [11], which fulfill the function of universal regulators for an immense diversity of metabolic processes occurring in living objects [12]. These findings prompt a conclusion that further investigations into biological activities of DNIC may culminate in the discovery of other remarkable properties of these compounds. Todays challenge is towards developing an efficient protocol for DNIC synthesis in order to make this procedure simple, uncomplicated and available for a broad range of practitioners in the field.

A simple version of a procedure for preparing DNIC with glutathione described herein is based on the ability of S-nitrosothiols (RS-NO), including S-nitrosoglutathione (GS-NO), to generate M-DNIC in a reaction between two molecules of RS-NO and a mixture of bivalent iron and thiols (RS^-) [13–15]. The hypothetical overall reaction of this synthesis is depicted in Scheme 1 [15]:

We conjectured that the formation of molecular orbitals including d-orbitals of iron and π-orbitals of NO and RS-NO favours the electron transfer from one S-nitrosothiol molecule to another and

Abbreviations: B- and M-DNIC, binuclear- and mononuclear dinitrosyl iron complexes; CTAC, cetyltrimethylammonium chloride; EPR, electron paramagnetic resonance; GS-NO, S-nitrosoglutathione; HPLC, high-performance liquid chromatography; MAP, mean arterial pressure; MNIC, mononitrosyl iron complexes; RS-NO, S-nitrosothiols.

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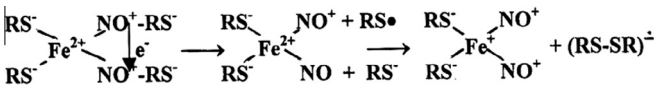


Scheme 1. Proposed mechanism of M-DNIC synthesis from S-nitrosothiols.

thus initiates their destabilization terminated by their decay. As a result, only one NO molecule and one nitrosonium ion remain bound to iron, which triggers the formation of the paramagnetic form of M-DNIC. The thiol groups released from GS-NO molecules generate reduced disulfide, which is easily oxidized to disulfide by, e.g., oxygen.

Spontaneous dimerization of M-DNIC culminates in the formation of B-DNIC by a reversible reaction shown in Scheme 2:

Quite naturally, the addition of free thiols to the test system (Scheme 2) causes a left-hand shift of the reaction equilibrium. Nevertheless, in the case of DNIC with glutathione this shift is insignificant at neutral pH even at a 10-fold excess of free thiols over DNIC. Under these conditions, about 93–95% of iron remains bound to B-DNIC [11,16].

Materials and methods

Materials

Ferrosulfate (Fluka, Buchs, Switzerland), reduced glutathione, and sodium nitrite (Sigma, St. Louis, USA) were used.

HPLC assay

The reaction products were identified by high-performance liquid chromatography (HPLC) as described in [11]. HPLC included reversed phase chromatography [17] on a 150-mm silica gel column C-18 (Ø 7 μM, length 5.5 cm) modified with an anionic detergent (cetyltrimethylammonium chloride, CTAC).

EPR and optical measurements of DNIC preparations

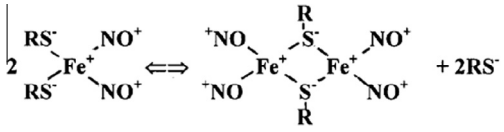
EPR spectra of DNIC preparations were measured at ambient temperature or at 77 K using a modified Radiopan EPR spectrophotometer (Poland) (microwave power 5 mW, modulation amplitudes 0.05–2 mT). The concentrations of DNIC were determined by double integration of the EPR signals. A solution of M-DNIC with glutathione of known concentration synthesized at the Fe^{2+}: glutathione molar ratio of 1:100 in 10 mM HEPES (pH 11.0) was used as a reference sample.

Optical measurements were carried out on a UV-2501PC spectrophotometer (Shimadzu Europa GmbH, Germany) using a flat quartz cuvette with an optical path of 10 mm. All the measurements were performed at ambient temperature.

Animal studies

The investigations were carried out in conscious male Wistar rats weighing 200–250 g. A day prior to onset of experiment, catheters were implanted into carotid artery and jugular vein under ketamine narcosis (100 mg/kg). The main arterial pressure (MAP) was continuously monitored through electromanometer Gould Statham P23 on Gould Brush 2400S polygraph (USA).

To study of the hypotensive effect of B-DNIC with glutathione in rats, 0.2 mL of 5 mM solution of the complex (as calculated per one iron atom in B-DNIC) containing 0.9% NaCl was injected into the vein and MAP was measured.



Scheme 2. Chemical equilibrium between M- and B-DNIC with thiol-containing ligands.

Statistical analysis

The data obtained in animal experiments were presented as mean SEM for five experimental animals.

Results

Synthesis of B-DNIC with glutathione

The novel procedure for the synthesis of the binuclear form of DNIC with glutathione includes three steps, viz., (i) synthesis of GS-NO in acidic media containing glutathione and bivalent iron; (ii) increase of pH of the test solutions to neutral values favouring the formation of DNIC, and, (iii) filtration of the final solution to remove residual hydroxyl iron complexes. The molar ratio of added glutathione, iron and nitrite is 2:1:1. All synthetic procedures were carried out in the air at ambient temperature.

The protocol for the synthesis of a 2.5 mM solution of B-DNIC with glutathione in 10 ml of distilled water reads as follows.

Step 1: includes preparation of soluble GS-NO. To achieve this, 62 mg of glutathione (GSH, 20 mM) is added in the air to 10 ml of distilled water placed into a 15-mm long glass test tube (Ø 1.5 cm); as a result, the pH of the test solution diminishes to 4.0 ± 0.1 due to the acidifying effect of glutathione. An addition of 28 mg of $FeSO_4 \times 7H_2O$ (10 mM) causes a further decrease of pH to 3.8 ± 0.1 after which 6.9 mg $NaNO_2$ (10 mM) is added to the solution with its subsequent shaking for 1 min. Sodium nitrite undergoes protonation to nitrous acid (HNO_2) in an acidic medium with subsequent S-nitrosation of glutathione and formation of 10 mM GS-NO by Reaction 1 [18]:

$$HNO_2 + GSH = GS - NO + H_2O \qquad \text{(Reaction1)}$$

As a result, the solution acquires a pink colour characteristic of GS-NO. The formation of GS-NO is complete within 1.5–2 h, as can be judged from the increase in the optical absorption of GS-NO at 334 nm (Fig. 1, top panel, spectrum 3). Under these conditions, the whole of nitrite is utilized for the synthesis of GS-NO, while 10 mM glutathione remains in the solution in the free state.

Step 2: includes pure synthesis of DNIC with glutathione. A 10 mM solution of NaOH in distilled water is added dropwise to the final solution of GS-NO upon continuous monitoring of pH with a UP-5 pH meter (Denver Instrument, USA). At neutral values of pH, the solution acquires a dark-brown colour due to formation of first M-DNIC (Scheme 1) and then of B-DNIC with glutathione (Scheme 2). The optimum value of pH at which the whole bulk of GS-NO is expended for DNIC synthesis is 7.2 ± 0.1. The formation of DNIC is complete after incubation of the solution at ambient temperature for several hours or overnight. The slow increase in pH during titration of the solution with NaOH is provided by the buffering capacity of glutathione.

Step 3: includes removal of non-incorporated iron precipitated from the DNIC solution in the form of water-insoluble hydroxide iron complexes by passage through a common paper filter (Khimreaktivkomplekt, Russia) in the air without evacuation of the headspace gas from the solution.

The identification of DNIC thus synthesized as B-DNIC with glutathione is followed from a virtually complete coincidence of the

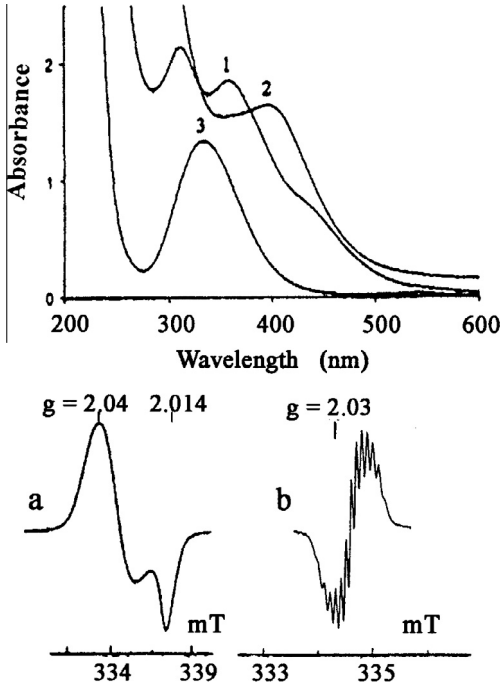


Fig. 1. Top panel – the absorption spectra of a 0.25 mM solution of B-DNIC with glutathione pH 7.2 (spectrum 1), a 0.4 mM solution of M-DNIC with glutathione synthesized in response to the increase of pH of the B-DNIC solution to 11.0 after addition of 2.5 mM glutathione (spectrum 2) and a 1.5 mM solution of GS-NO (spectrum 3). Bottom panel – the EPR signals of solutions of M-DNIC with glutathione recorded at 77 K (a) and at ambient temperature (b). B-DNIC with glutathione were synthesized in accordance with the novel protocol with subsequent 10-fold dilution of B-DNIC with distilled water.

shape of their optical absorption spectrum with main bands at 310 and 360 nm (Fig. 1, top panel, spectrum 1) with that of .B-DNIC with glutathione obtained by other methods [16]. The concentration of B-DNIC prepared according to the novel protocol calculated from the values of the extinction coefficients of the optical absorption bands at 310 and 360 nm (9200 and 7400 $M^{-1}cm^{-1}$, respectively) [16] was found to be equal to ~2.5 mM (5 mM as calculated per one iron atom in B-DNIC).

Based on these data and taking the ratio between glutathione, iron and nitrosyl ligands equal to 1:1:2 for B-DNIC with glutathione, we hypothesized that 5 mM (out of initial 10 mM) iron, 5 mM glutathione (out of 10 mM glutathione remaining in the solution after the formation of 10 mM GS-NO) and 10 mM nitrosyl ligands were incorporated into B-DNIC, i.e., the whole amount of nitric oxide released from GS-NO appeared to be bound to B-DNIC. Under these conditions, 5 mM glutathione and 5 mM free iron remained non-incorporated into B-DNIC (the latter precipitated from the solution in the form of a hydroxide complex); therefore, the final solution contained 2.5 mM B-DNIC, 5 mM free glutathione and 5 mM oxidized glutathione. The latter was formed in the reaction of M-DNIC synthesis in accordance with Scheme 1.

B-DNIC with glutathione represent thioesters of glutathione with a red Roussin's salt [16]. These complexes are diamagnetic and do not generate EPR signals characteristic of M-DNIC. Their diamagnetism is provided by pairing of the spins (so-called "antiferromagnetic interactions") of two $Fe(NO)_2$ groups through sulfur-containing bridging ligands within the composition of two thiol molecules of B-DNIC.

In the paradigm of the equilibrium shown in Scheme 2, the presence of free glutathione in the final preparation of B-DNIC with glutathione (5 mM against 2.5 mM) indicates to the presence of M-DNIC in the test solution. The latter were indeed detected in the test solution by a characteristic EPR signal with $g_{aver.} = 2.03$ [19] (Fig. 1, bottom panel); however, its intensity was far too low and corresponded to incorporation of no more than 5% of iron present in M-DNIC. Notwithstanding, the intensity of this EPR signal increased after addition of glutathione at a concentration exceeding that of B-DNIC tenfold. Under these conditions, no more than 10% of iron present in the solution was incorporated into M-DNIC. In all probability, glutathione could react with B-DNIC only in the ionized (at the thiol group) state, while at neutral values of pH of B-DNIC solutions the degree of ionization was too low.

Judging from ionization of the thiol group of glutathione (pK~10), the rate of B-DNIC conversion into M-DNIC in alkaline media might be drastically increased in the presence in the test solution of a tenfold excess of glutathione. Indeed, in our study the intensity of the EPR signal of M-DNIC was increased 8-fold, while the absorption spectrum of the test solution contained an absorption band at 390 nm (Fig. 1, top panel, spectrum 2) characteristic of M-DNIC with glutathione prepared by other methods [16]. Judging from the value of the extinction coefficient for this band (4700 M^{-1} cm^{-1}), the concentration of M-DNIC in the test solution was equal to 4 mM suggesting the incorporation of approx. 80% of $Fe(NO)_2$ groups of B-DNIC into M-DNIC.

The EPR signals of M-DNIC recorded at 77K and at ambient temperature are shown in Fig. 1, bottom panel (a and b). At 77 K, the EPR signal had an anisotropic shape, which reflected the anisotropy of the g-factor ($g_{\perp} = 2.04$, $g_{||} = 2.014$, $g_{aver.} = 2.03$). At ambient temperature, where the high mobility of low-molecular DNIC caused the averaging of the g-factor anisotropy, the EPR signal had the shape of a symmetric singlet with a peak at $g = 2.03$; its 13-component hyperfine structure (HFS) reflected the interaction of the unpaired electron in M-DNIC with the nitrogen nuclei of two nitrosyl ligands and four protons of the methylene groups in the vicinity of the thiol atoms of sulfur in two glutathione ligands [16].

Thus, judging from the optical and EPR characteristics of DNIC synthesized in accordance with the novel protocol, these DNIC can be used for the synthesis of both B- and M-DNIC with glutathione. As regards the biological activity of DNIC with glutathione synthesized by the novel method, our experiments on rats established that these DNIC produce the same long-lasting hypotensive effect as DNIC with glutathione synthesized by other methods [1,2] (Fig. 2). An injection of DNIC with glutathione in dose of 4 μmol/kg to conscious Wistar resulted in prompt hypotensive effects seeing after 20–30 s followed by gradual recovery of MAP to the initial level in 2 h.

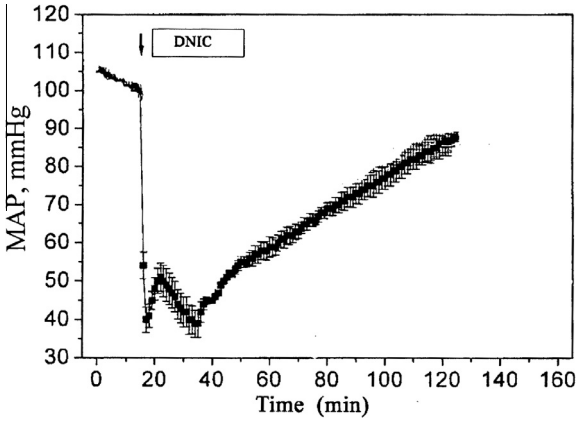


Fig. 2. Relative time course of MAP after DNIC with glutathione introduction (4 μmol/kg of the body weight) to normotensive conscious Wistar rats ($n = 5$). Values are expressed as m ± SEM. The common time scale is in minutes.

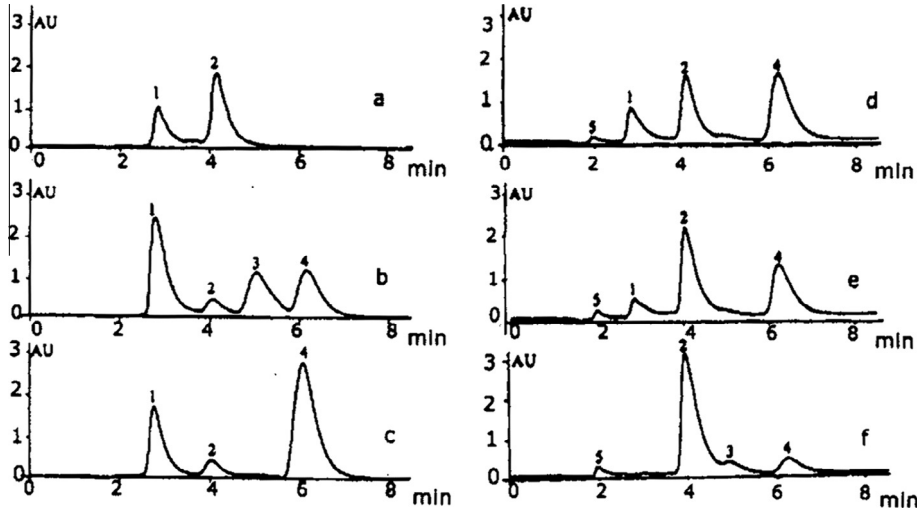


Fig. 3. Left: the HPLC chromatogram of standard (0.5 mM) solutions of reduced and oxidized glutathione in distilled water (pH 4.2) (panel a); panels b and c, respectively – 10 min and 2 h after addition of 0.5 mM $NaNO_2$ to a 2 mM solution of reduced glutathione + 1 mM ferrosulfate in distilled water (pH 4.0). 1 – reduced glutathione; 2 – oxidized glutathione; 3 – nitrite; 4 – S-nitrosoglutathione (GS-NO). Right: time-dependent change in the HPLC chromatogram of the test solution (panel c) 20, 80 and 180 min after the increase in pH from 3.8 to 8.0 (panels d, e and f, respectively).

HPLC analysis of products formed in GS-NO and DNIC synthesis

The HPLC chromatograms illustrating GS-NO synthesis in distilled water (pH 3.8) after mixing of reduced glutathione and nitrite in the presence of ferrosulfate are shown in Fig. 3 (left, panels b and c). Panel (a) depicts a chromatogram of standard (0.5 mM) solutions of reduced and oxidized glutathione (elution bands 1 and 2, respectively) with the indication of the positions of the bands with the retention times of 2.8 and 4.2 min, respectively.

Ten minutes after addition of a 1.0 mM solution of sodium nitrite (panel b, band 3 with a retention time of 5.1 min) to a 2 mM solution of reduced glutathione + 1.0 mM ferrosulfate, there appeared a significant amount of GS-NO (band 4 with a retention time of 6.2 min) as a result of which the concentrations of glutathione and nitrite decreased. Two hours thereafter, the GS-NO synthesis was virtually complete (Fig. 3c). As a consequence, the concentration of reduced glutathione decreased to 1.0 mM against the background of a complete disappearance of nitrite from the solution, which testified to the formation of 1.0 mM GS-NO (band 4). The relatedness of band 4 to GS-NO was established by optical detection at 340 nm characteristic of GS-NO absorption. Noteworthy, band 4 remained on the chromatogram, while other bands were undetectable (data not shown, see Ref. [11]).

The concentration ratio of GS-NO (1 mM) and reduced glutathione (1 mM), which disappeared from the solution virtually completely, correlated with the stoichiometry of the reaction of GS-NO synthesis from protonated nitrite (Reaction 1).

Fig. 3 (panels b and c) displays the elution band 2 characteristic of oxidized glutathione present in reduced glutathione preparations as an admixture. The intensity of the extrinsic band did not change in the course of GS-NO synthesis. Ferrosulfate added to the solution did not interfere with the course of GS-NO synthesis, but initiated the formation of DNIC with an increase in pH to neutral values (Step 2 in the DNIC synthesis protocol).

Fig. 3 (right) shows the time-dependent changes in the HPLC chromatograms of the test solution (panel c) 20, 80 and 180 min after the increase of pH to 7.2, inducing DNIC synthesis (panels d – f, respectively). A comparison of the chromatograms depicted in panels c and d revealed significant depression of the elution bands of reduced glutathione and GS-NO (panel d, bands 1 and 4, respectively) and a drastic increase of the elution band of oxidized glutathione (panel d, band 2) during the very first 20 min of the reaction.

80 min after pH increase, the changes in the chromatogram progressed further in the same direction (panel e) and after 3 h the elution band of oxidized glutathione (band 2) was predominant against the background of a complete disappearance of the elution band of reduced glutathione and only a very slight appearance of the elution band of GS-NO (band 4). After the 20 h of pH increasing and DNIC synthesis finishing (stage 2) the band of GS-NO decreased completely (data not shown). At the same time a weak band of nitrite (band 3) which correspondent to its concentration less than 0.035 mM, was appeared on the chromatogram (panel f). There was the same nitrite concentration as it was in the DNIC with glutathione solution to the end of the synthesis (according to the HPLC data). There will be proposed below (see Discussion) that the appearance of weak elution band of nitrite (band 3) can be attributed to the minor decomposition of DNIC formed in the process of HPLC chromatography. As regards the weak band 5 (panels d–f), its appearance could be initiated by mixing of mobile phase (5 mM phosphate buffer, pH 5.8) with the test solution on the chromatographic column with an increase in pH, e.g., to 8.0. This effect was less significant during the interaction of the acidic solution used for GS-NO synthesis with the column (Fig. 3, left).

The chromatograms shown in Fig. 3 (right) did not contain the elution band of newly synthesized B-DNIC. Neither was this band detected in our previous studies [20] designed to investigate HPLC chromatograms of ready-to-use B-DNIC with glutathione. In the aforecited studies, we failed to detect anything other than the elution bands of reduced and oxidized glutathione, which led us to conclude that the lack of an elution band of B-DNIC with glutathione might be due to their conversion into DNIC with cetyltriammonium chloride (the ligand retained on the chromatographic column). As a result of such conversion, DNIC remained bound to the column, while its constituent component glutathione was released from the complex into the surrounding solution [20].

The optical analysis of the solution whose chromatogram is displayed in panel f established that B-DNIC with glutathione were indeed formed at the concentration of 0.5 mM (as calculated per one iron atom). Since in this complex the ratio between the constituent components (iron, glutathione, nitrosyl ligands) was equal to 1:1:2, respectively, their concentrations were determined as 0.5, 0.5 and 1 mM, respectively. Therefore, only 50% of reduced glutathione and

Fe^{2+} initially present in the solution (1 mM) were incorporated into B-DNIC, while nitrosyl ligands from GS-NO controlling DNIC synthesis were incorporated virtually completely. The remaining 50% of glutathione (0.5 mM) was in the free state, while 50% of iron (0.5 mM) precipitated from the solution in the form of water-insoluble hydroxide iron complexes.

Since the interaction of B-DNIC with the column could be accompanied by their conversion into DNIC with cetyltrimethylammonium chloride (CTAC) [20] with a simultaneous release of glutathione from the test solution, there appeared an additional amount (0.5 mM) of free glutathione, as a result of which its total concentration including that of glutathione initially present in DNIC reached 1 mM. The oxidation of free glutathione could be responsible for the appearance in the test solution of 0.5 mM oxidized glutathione. This compound could additionally be formed in the same amount during interaction of GS-NO with iron (Scheme 1) as a result of which the total concentration of oxidized glutathione would reach 1 mM, which correlated with its concentration determined by the chromatographic method (Fig. 3, panel f).

These HPLC data provide additional evidence in favour of our hypothetical mechanism of B-DNIC synthesis performed in accordance with the novel protocol and the reactions involved therein including the addition of bivalent iron, reduced glutathione and nitrite to the initial solution in distilled water.

Discussion

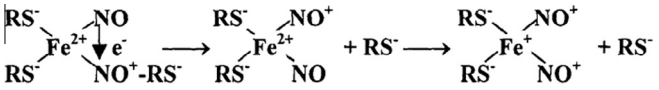
The main advantage of the novel protocol for the synthesis of B-DNIC with glutathione described in the foregoing chapters is the ease of performance and that the synthetic procedure is complete through a mere addition of reagents (glutathione, a Fe^{2+} salt and nitrite) to a specified volume of distilled water. However, the order of their addition is of crucial importance, viz., glutathione has to be added first, then iron, and, finally, nitrite. The reason is that glutathione or cysteine added to distilled water cause acidification of the medium, while further introduction of a Fe^{2+} salt does not induce its precipitation in the form of hydroxide complexes, but, rather, causes an additional decrease of pH. As a result, nitrite addition to an acidified (pH 3.8 ± 0.1) solution triggers the synthesis of RS-NO.

It should be noted that the GS-NO formed thereupon could undergo decomposition under the catalytic effect of contaminant copper ions present in the same acidic medium. The mechanism of this decomposition is shown in Scheme 3 [18]:

As a result, NO released from GS-NO, might leave the solution in the gaseous form, which might inevitably cause the reduction of the whole bulk of DNIC formed in the solution with a further rise of pH to neutral values. However, we did not observe any release of NO vesicles from our test solutions. Most probably, their absence was due to the presence of Fe^{2+} ions, which competed with the copper admixture for GS-NO. The binding of the latter to Fe^{2+} ions was accompanied by the formation, in acidic solutions, of mononitrosyl iron complexes (MNIC) with non-thiol ligands (H_2O molecules or amino- and carboxy groups of glutathione or cysteine). These MNIC had a spin $S = 3/2$ and were detected by a characteristic EPR signal with a component at $g = 4.0$ [11]. As a result, NO incorporated into MNIC remained in the solution and could be

$$\begin{aligned} &GS\text{-}NO + Cu^{+} \longrightarrow GS^{\cdot} + NO + Cu^{2+} \\ &Cu^{2+} + GS^{-} \longrightarrow GS^{\cdot} + Cu^{+} \\ &GS\text{-}NO \xrightarrow[Cu+/2+]{} NO + \tfrac{1}{2}(GS\text{-}SG) \end{aligned}$$

Scheme 3. GS-NO decomposition under the catalytic effect of contaminant copper.



Scheme 4. Mechanism of M-DNIC formation from MNIC with glutathione (RS^-) and GS-NO (NO^+-RS^-).

incorporated into M-DNIC with a subsequent increase in pH to neutral values (Scheme 4). .

The reaction mechanism of M-DNIC formation in this case was similar to that depicted in Scheme 1:

We hypothesized that with an increase of pH, NO molecule released from MNIC with non-thiol ligands and one GS-NO molecule as well as two glutathione molecules (RS^-) were included into the coordination sphere of Fe^{2+} that resulted in M-DNIC formation. The molecular orbitals formed thereupon, which included d-orbitals of iron and π-orbitals of NO and GS-NO, provided the electron transfer from NO to GS-NO. The latter favoured the appearance, in the complex thus formed, of nitrosonium ions (NO^+) and an unstable $NO\text{-}GS^-$ ($NO\text{-}RS^-$) molecule. This molecule underwent decomposition with a release of GS^- (RS^-) and NO molecules. Binding of the latter to iron promoted the formation of paramagnetic M-DNIC with a d^7 (Fe^+) electronic configuration of the iron atom; its dimerization yielded B-DNIC.

These findings suggest that the synthesis of B-DNIC with glutathione performed according to the novel protocol is controlled by concentration of NO released from GS-NO. As regards the initial concentrations of glutathione and iron, their molar ratios exceeded the amount sufficient for B-DNIC synthesis twofold. This circumstance is of crucial importance for B-DNIC synthesis. We established that the decrease of glutathione concentration to a level characteristic of B-DNIC caused a nearly twofold decrease of DNIC concentration. This finding can be attributed to the fact that the first step of B-DNIC synthesis from GS-NO is the formation of M-DNIC by Scheme 1, which undergoes further dimerization in accordance with Scheme 2. The synthesis of M-DNIC requires two thiol-containing ligands per one $Fe(NO)_2$ molecule and is provided by excess glutathione present in the solution. The twofold excess of glutathione over nitrite in the starting solution is yet another factor responsible for the enhanced synthesis of GS-NO in accordance with the mass action law (Reaction 1). Moreover, the presence of excess iron in the reaction mixture is also prerequisite to the enhanced synthesis of DNIC from GS-NO in accordance with this law (Scheme 1). Besides, iron excess compensates for the partial loss of iron precipitated in the form of hydroxyl iron complexes with an increase in pH to neutral values. The concentration of bivalent iron dissolved in H_2O (5 mM) is sufficient to initiate the synthesis of 2.5 mM B-DNIC containing two iron ions per one B-DNIC.

The question arises whether the new method of the DNIC-glutathione synthesis that we offer can lead to the appearance of a nitrite impurity, as a result of the not whole nitrite utilizing for the synthesis of GS-NO or the result of the partial DNIC disintegration in air. Thereafter the NO molecules released from DNIC may be oxidized by O_2 up to NO_2 and the hydrolysis of the latter may lead to the appearance of nitrite contamination. Nitrite as a NO donating agent possesses the vasodilatation and hypotension activities, on that account, the nitrite contamination could make contribution to the hypotensive action of the complex preparation (Fig. 2).

To solve the problem let us consider the results of chromatographic analysis of the products that appeared in the course of the DNIC-gluthathione synthesis (Fig 3).The consideration of data on b and c panels after 2 h of GSNO synthesis demonstrates that there was no band of elution of nitrite, that was added to initial solution, and there is the evidence of practically full nitrite inclusion in the reaction of GSNO synthesis. Nevertheless in the next

stage of the DNIC synthesis initiated by pH increasing of the solution, containing of GS-NO and the salt of Fe^{2+} (panel e and f), after 3 h after of the start, a weak band of nitrite there was appeared. The intensity of the band was equal to the no more than 7% (0.035 mM) as compared to the initial nitrite concentration (0.5 mM). It appears that this nitrite impurity of DNIC preparation was the result of decay of the part (14%) of this complex (0.035 mM as compared to 0.25 mM DNIC-glutathione) and was a result of its interaction with the component of CTAC column that may lead to transformation of DNIC with glutathione into DNIC with CTAC [20].

It should be noted that in the control experiments, where the DNIC with glutathione was incubated during 3 h in the air in HPLC mobile phase solution (5 mM phosphate buffer, pH 5,8). As optical measurements shown there was no decrease of the DNIC with glutathione content in the solution. Moreover, the content level was stable during at least 10 h. This result allow to suggest that appearance of 14% nitrite impurity in the DNIC solution was due to the influence of the chromatographic methodology used. Thus, proposed simple method for DNIC-glutathione synthesis allows to obtain DNIC preparations with negligibly small contamination of nitrite. That is true for the impurity of GS-NO also.

The complexes synthesized by this method were also stable in the solutions at neutral pH in air during at least 1 week that followed from the intensity of their optical spectra. The process of DNIC degradation bean after two weaks that led to the accumulation of nitrite and nitrate in the solutions. Stabilization of these DNIC with complete preservation of their activity over longer periods of time (e.g., several years) can be achieved through their lyophilization in the presence of polymeric filling reagents using an original procedure developed and patented in our laboratory [21].

In the conclusion we would like to note that as preliminary experiments demonstrate, the proposed protocol of the synthesis of DSNIC with glutathione can be fully applicable to the synthesis of DNIC with cysteine.

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