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Role of Thiamine Thiol Form in Nitric Oxide Metabolism

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Abstract—In alkaline media the thiamine cyclic form is converted into a thiol form (pK_a 9.2) with an opened thiazole ring. The thiamine thiol form releases nitric oxide from S-nitrosoglutathione (GSNO). Thiamine disulfide, mixed thiamine disulfide with glutathione, and nitric oxide are produced in the reaction. Free glutathione was recorded in small amounts. The concentration of formed nitric oxide agreed well with the concentration of degraded GSNO. The concentration of released nitric oxide was determined under anaerobic conditions spectrophotometrically by production of nitrosohemoglobin. In air, the release of nitric oxide was recorded by the production of nitrite or the oxidation of oxyhemoglobin to methemoglobin. The concentration of the thiol form in the body under physiological pH values (7.2-7.4) did not exceed 1.5-2.0%. We believe that due to the exchange reactions between the thiamine thiol form and S-nitrosocysteine protein residues, nitric oxide can be released and mixed thiamine–protein disulfides are formed. The mixed thiamine disulfides (including thiamine ester disulfides) as well as the thiamine disulfide form are quite easily reduced by low molecular weight thiols to form the thiamine cyclic form with a closed thiazole ring. A possible role of the thiamine thiol form in releasing deposited nitric oxide from low-molecular-weight S-nitrosothiols and protein S-nitrosothiols and in regulation of blood flow in the vascular bed is discussed.

Key words: thiamine thiol form, nitric oxide, S-nitrosoglutathione, thiamine disulfide, thiochrome, nitrosohemoglobin, nitrite

Nitric oxide functions as one of the universal regulators of cellular and tissue metabolism. Furthermore, it also functions as one of the basic cytotoxic effectors of the system of cellular immunity [1].

Nitric oxide and its metabolites readily react with different molecules, such as ferro and ferri forms of iron, both within a heme and in a free state [2], as well as with superoxide, molecular oxygen [3, 4], glutathione, cysteine, and cysteine residues in proteins [5].

The high reactivity of nitric oxide determines its short lifetime (several seconds) in the organism [6].

Nitric oxide molecules affect metabolic processes both in host cells and those located in the neighborhood. This means that, despite their high activity, NO molecules can diffuse in the bound state or be transferred to distances that are at least several times greater than cellular sizes. Therefore, it is suggested that NO can be reversibly included into compounds capable of transferring NO from donor cells to target cells. At present, nitrosothiols are supposed to be candidates for the role of such carriers [7, 8]. Under physiological conditions, S-nitrosothiols are much more stable compounds compared to nitric oxide. The half-life time of, for example, S-nitrosoglutathione (GSNO) is about 10 h, whereas that of S-nitrosocysteine is over 90 min [7].

Blood plasma contains micromolar concentrations of GSNO and S-nitrosoalbumin [9]. In the presence of glutathione (GSH) and cysteine, NO release from S-nitrosothiols is enhanced [10, 11]. The interactions between S-nitrosothiols and low-molecular-weight thiols and sulfhydryl group-containing proteins are suggested to play an important role in control of NO functions in the organism [11]. Thiamine and its derivatives are known to easily produce their thiol forms in alkaline media [12-14]. In this work, we studied the interaction of the thiol form of thiamine with GSNO under aerobic and anaerobic conditions and showed that the thiol form released NO from GSNO and S-nitrosoproteins.

MATERIALS AND METHODS

The concentration of aqueous thiamine solutions in neutral medium was calculated according to the molar

Abbreviations: TSST (TS-ST)) thiamine disulfide; GSST (GS-ST)) mixed thiamine disulfide with glutathione; TSH) thiamine thiol form; Hb) hemoglobin; oxyHb) oxyhemoglobin; nitrosoHb) nitrosohemoglobin; TTP) thiamine triphosphate; TMP) thiamine monophosphate; TS˙) thiyl radical; GSNO) S-nitrosoglutathione; GSH) glutathione.

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extinction coefficient at 233 nm, ε = 12,000 $M^{-1}\cdot cm^{-1}$, pH 7.0 [12]. The concentration of the thiol form of thiamine in alkaline media was determined spectrophotometrically according to the increase in absorption at 250 nm upon transition from neutral to alkaline medium. The content of the disulfide form of thiamine was measured after its incubation with excess of cysteine or glutathione in neutral medium. Thiamine disulfide due to the thiol disulfide exchange reaction is converted to a form with closed thiazole ring [13, 14]. The concentration of thiamine with closed thiazole ring was measured using a fluorescence method after the oxidation of thiamine to thiochrome by alkaline ferricyanide [14]. The maximum of the fluorescence excitation spectrum of thiochrome in neutral medium is at 365 nm, while the maximum of fluorescence is at 450 nm [15].

The amount of NO released during degradation of GSNO by the thiol form of thiamine in the absence of oxygen was monitored spectrophotometrically by formation of nitrosohemoglobin (nitrosoHb) [16]. Amount of sulfhydryl groups was determined by the Ellman reagent [17]. In air, nitric oxide releasing from GSNO by the thiamine thiol form was recorded by the nitrite formation. Nitrite concentration was measured using the Griess reagent, recording absorption at 530 nm. Nitrate concentration was also measured by the Griess reagent, but after the preliminary incubation of nitrates with metallic cadmium plates (Fluka, Switzerland) [11, 18].

Nitrosoglutathione was obtained by incubation of glutathione and nitrite in equimolar concentrations in acidic medium, and then pH of the medium was adjusted to neutral value. The concentration of nitrosoglutathione was determined by absorption at 340 nm using molar extinction coefficient of 800 $M^{-1}\cdot cm^{-1}$. S-Nitrosoalbumin was obtained after incubation of human serum albumin with an excess of nitrite in acidic medium [11]. The excess of nitrite was removed from the obtained S-nitrosoalbumin by gel filtration on Sephadex G-50 (Pharmacia, Sweden) in 0.05 M sodium-phosphate buffer, pH 7.0.

Nitrosylation of the hemoglobin sulfhydryl groups was carried out by prolonged incubation of oxyhemoglobin aqueous solutions with 20-fold molar excess of nitrite in acidic medium at pH 3.5. During several minutes, oxyhemoglobin was oxidized to methemoglobin. The obtained aqueous solutions of methemoglobin were incubated with nitrite during 3 h. Then the solution was neutralized and separated from the nitrite excess on Sephadex G-50 in phosphate buffer. The absorption spectra were recorded with a Specord M-500 spectrophotometer (Carl Zeiss Jena, Germany), and fluorescence measurements were carried out on SFL 1211a spectrofluorimeter (Solar, Belarus). A Jasco J-20 spectropolarimeter (Jasco, Japan) was used for CD-spectral measurements.

Glutathione, L-cysteine, serum albumin, and thiamine were purchased from Sigma (USA); other chemicals (of analytical grade) were produced in Russia.

RESULTS

Acceleration of transformation of thiamine thiol form into disulfide form by GSNO. Aqueous solution of thiamine has characteristic absorption in the ultraviolet region. In neutral medium, the absorption spectrum consists of two bands with maxima at 233 and 267 nm and molar extinction coefficients of 12,000 and 9,600 $M^{-1}\cdot cm^{-1}$, respectively [12]. Upon acidification, an asymmetric absorption band with a maximum at 247 nm is observed.

These spectral changes are related to protonation of the N-1 nitrogen atom in the pyrimidine ring, pK_a 4.9 [13, 14]. The isolated pyrimidine component of thiamine (2-methyl-4-amino-5-aminomethyl-pyrimidine) shows similar changes in the absorption spectrum in acidic medium. The thiamine absorption spectrum agrees quite well with the sum of absorptions of equimolar concentrations of 2-methyl-4-amino-pyrimidine and N-methylthiazole [12]. This is probably related to the fact that pyrimidine and thiazole components are interconnected by a methylene bridge in thiamine and the conjugated systems of rings are isolated from each other. In addition, according to X-ray data, the angle between the rings in the crystal is close to 90° [19] and the effect of conjugation of their electronic systems is low in the crystal state and also, possibly, in solutions. In neutral and acidic aqueous media, thiamine is stable, but in alkaline solution, it produces a thiol form with an opened thiazole ring [13, 14]. During the transition to thiol form absorption at 250 nm grows and the minimum in the absorption spectrum of the initial thiamine solution is smoothed out. At the initial periods of time the reaction is reversible, and after acidification of the medium the thiazole ring is closed to produce again the cyclic thiamine form, which can be easily monitored spectrophotometrically. Pseudobase (II) serves as an intermediate form between the quaternary ammonium base of thiamine (I) and the open thiol form (III) (Fig. 1). A pseudobase can also produce the tricyclic form of thiamine (IV) in small amounts. At pH 10.0-10.5 (at higher pH values the sulfhydryl group is ionized), practically only the thiamine thiol form is present in solution. At the pH values involved, the thiamine form with closed thiazole ring is converted into the thiol form within several minutes. In highly alkaline medium (pH 11.5 and above), the yellow thiamine thiol form is also produced concurrently [13, 14]. This reaction proceeds cooperatively as a result of the practically simultaneous release of two protons [14]. The yellow thiol form is unstable (it is not shown in the scheme (Fig. 1)) and is easily transformed to give thiol or an intermediate tricyclic form. The thiol form is hydrolyzed and oxidized by air oxygen to yield thiamine disulfide (V). The tricyclic form is oxidized to thiochrome (VI), which has absorption at 365 nm and a fluorescence maximum at 450 nm [15]. Thiamine oxidation into thiochrome by treatment

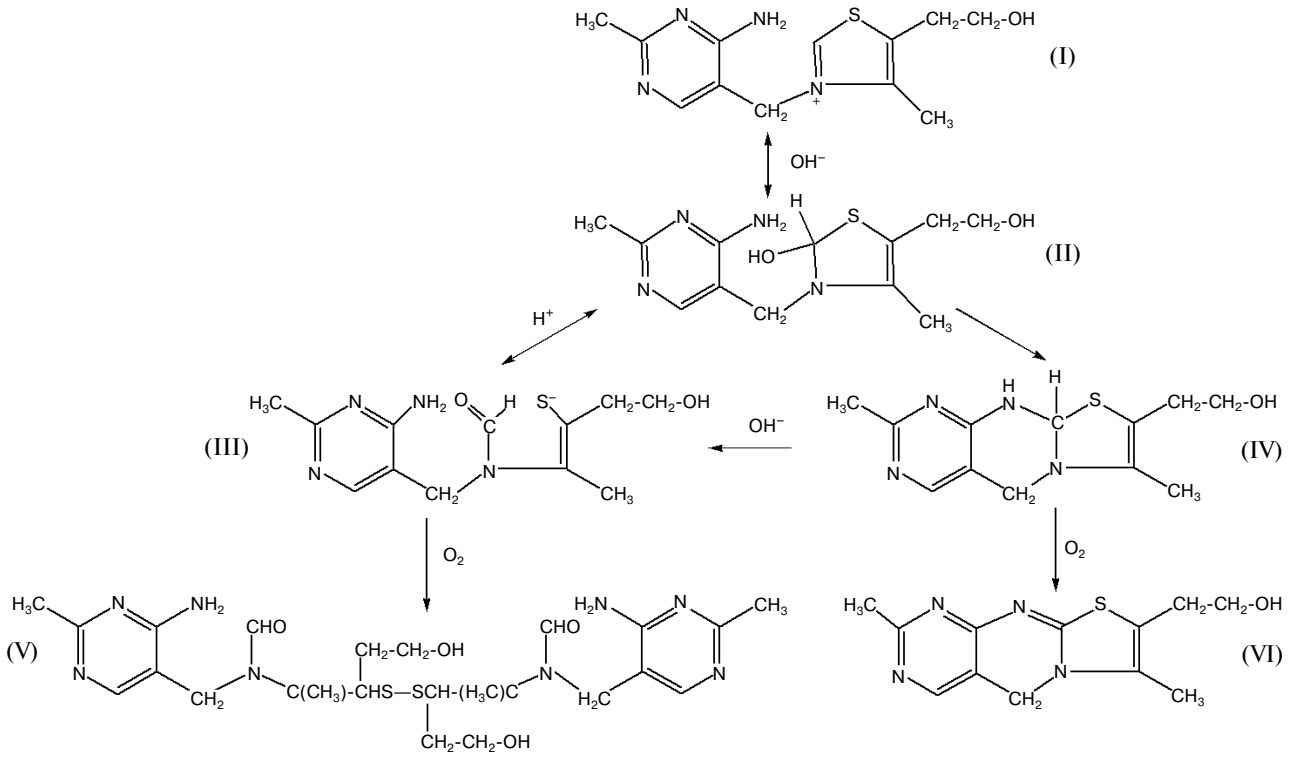

Fig. 1. Scheme of formation of thiamine thiol and tricyclic forms in alkaline medium of gradually increasing pH value and their oxidations to thiamine disulfide and thiochrome, correspondingly.

with alkaline ferricyanide is widely used for photometric thiamine assay [13, 14]. The amount of thiol form (III) at given pH and reversibility of back transformation of thiol form into cyclic form of thiamine (I) in neutral medium were monitored using the fluorescence method by the thiochrome formation.

As follows from the shown scheme (Fig. 1), only thiamine with the closed thiazole ring (I) (due to further formation of tricyclic form) is easily oxidized by alkaline ferricyanide into thiochrome (VI), and thiol form of thiamine (III) does not form thiochrome under the action of ferricyanide. Therefore, if thiol form is present in solution of thiamine then decrease in fluorescence intensity, observed after thiamine oxidation in alkaline media, is proportional to the content of thiol form. Hence, to determine the amount of thiol form alkaline solution of thiamine must be oxidized to thiochrome. Decrease in fluorescence intensity of thiochrome corresponds to thiol form concentration. Fluorescence intensity of thiochrome, obtained after thiamine oxidation at by alkaline ferricyanide pH 9.0-9.4, is ~2 times lower than of thiochrome obtained by thiamine oxidation in neutral medium. Changes in fluorescence intensity of thiochrome, produced by thiamine oxidation at different pH values, have good correlation with the closed ring form–thiol form transition (pK_a 9.2). In strongly alkaline media (pH > 10.0) when thiamine in solution exists practically completely in thiol form, fluorescence intensity of thiochrome produced after reaction with ferricyanide is negligible. At pH ⩾ 10.0 reaction with ferricyanide results in formation of non-fluorescent thiamine disulfide (V). On acidification of the medium, thiol form is converted into cyclic form. When the pH of alkaline thiamine solution was adjusted to 7.0 or lower with subsequent oxidation of thiamine to thiochrome, then fluorescence intensity was practically the same as the fluorescence of thiochrome produced by oxidation of an equal concentration of thiamine in neutral media. This indicates that complete reversibility of transition between thiamine forms takes place when incubation times of thiamine solution are relatively short, especially for solutions with pH ⩽ 10 (Table 1, upper row).

After prolonged incubation of thiamine in alkaline solution in air, gradual oxidation of the thiol form by air oxygen takes place to form thiamine disulfide (Fig. 1). However, thiamine disulfide formation due to thiamine thiol form oxidation by dissolved oxygen is low. After 2-h incubation of thiamine at pH 10.0, production of ~5-7% of thiamine disulfide was observed. Even after 24-h incubation at pH 10.0, the content of thiamine disulfide in aqueous solution did not exceed 25%. Notable decrease in thiochrome output was observed only after long incu-

Table 1. Content (mM) of thiamine (I), thiamine thiol form (III), and thiamine disulfide (V) after incubation of thiamine aqueous solutions in air for 3 h in the presence of GSNO at pH 10.0 and 11.0 (the initial thiamine concentration was $3.5 \cdot 10^{-4}$ M)

GSNO, mM	I		III		V	
	pH 11	pH 10	pH 11	pH 10	pH 11	pH 10
0	0.01	0.06	0.30	0.25	0.04	0.04
0.36	—	0.03	0.11	0.12	0.24	0.20
0.72	—	0.01	0.04	0.04	0.31	0.31
1.1	—	—	0.01	0.01	0.34	0.34

bation of thiamine in aqueous solution at pH $\geqslant$ 8.0. GSNO addition led to acceleration of the oxidation of thiamine thiol form to disulfide and after incubation thiamine concentration in solution was reduced to zero (Table 1). Increase in GSNO concentration was followed by proportional growth of thiamine disulfide (TSST) (or mixed thiamine disulfide with glutathione, GSST) formation in alkaline solution of thiamine (Fig. 2).

Formation of TSST in the presence of GSNO decreased when medium pH was lowered and had good correlation with initial content of thiol form in solution. At pH 7.0 and lower incubation of thiamine solution with GSNO did not result in significant TSST formation. Therefore, it can be concluded that only the thiol form of thiamine can be oxidized by GSNO to form TSST. TSST

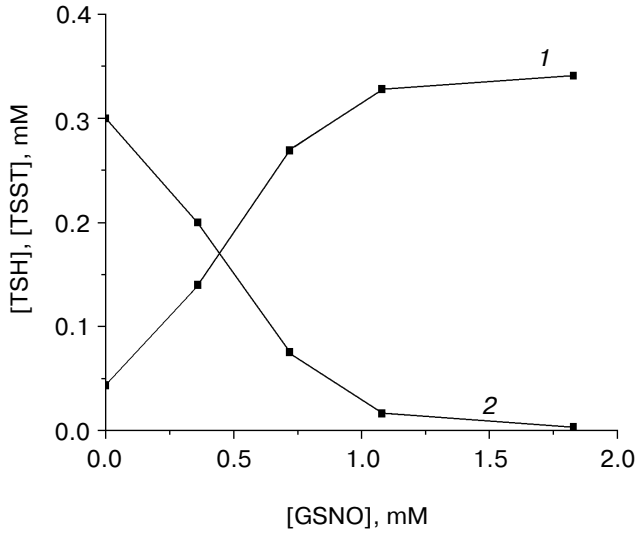

Fig. 2. Thiamine disulfide formation (*1*) on incubation of thiamine thiol (*2*) in alkaline environment in the presence of various GSNO concentrations in air. Time of the incubation, 2 h (pH 11.0). Concentration of thiamine, $3.5 \cdot 10^{-4}$ M. Thiamine in solution was almost completely in the thiol form.

can be easily reduced by glutathione or cysteine to produce thiol form, which in neutral medium is rapidly transformed to thiamine form with closed thiazole ring. Therefore, TSST amount can be monitored by measuring concentration of thiamine after incubation with thiols. TSST reduction probably takes place in two stages. In the first stage, free thiamine is effectively formed after reaction with small amounts of glutathione on TSST, and then the efficiency of thiamine reduction is lowered. We showed that the first equivalent of thiamine was released from TSST in the presence of 3-4-fold molar excess of glutathione. The thiol disulfide exchange reaction occurs between TS-ST and glutathione (GSH):

$$GSH + TS\text{-}ST \rightarrow TSH + GS\text{-}ST, \quad (1)$$

where GS-ST is the mixed disulfide of thiamine and glutathione. Thiamine is produced in thiol form (TSH), which in neutral medium is rapidly converted into cyclic form of thiamine with closed thiazole ring (T):

$$TSH + 2H^+ \rightarrow T. \quad (2)$$

To release the second equivalent of thiamine from GS-ST, higher concentrations of glutathione are needed:

$$GS\text{-}ST + GSH \rightarrow GSSG + TSH. \quad (3)$$

After complete reduction of TSST two equivalents of thiamine must be formed (see Scheme in Fig. 1). Excess addition of cysteine and glutathione results in practically complete TS-ST reduction to thiamine. Indeed, the fluorescence intensity of thiochrome, produced after oxidation of the reduced disulfide form by ferricyanide, was practically equal to thiochrome fluorescence of equimolar thiamine concentrations. In this case, thiamine also was oxidized to thiochrome by ferricyanide.

Acceleration of GSNO degradation by the thiamine thiol form. Now let us consider how thiamine affects GSNO in aqueous solution. S-Nitrosothiols are known to

possess characteristic absorption in the region of 340 and 540 nm [11]. Optical activity is induced in the absorption bands of S-nitrosothiols. The sign of the Cotton effect in the CD spectra of S-nitrosoglutathione and S-nitrosocysteine is negative for the band with maximum at 340 nm and positive for the band with maximum at 540 nm. The band at 340 nm in the absorption spectrum of GSNO ($\varepsilon = 800\ M^{-1}\cdot cm^{-1}$) overlaps the long-wavelength absorption band of the thiol form of thiamine possessing nearly the same intensity of absorption in this spectrum region, which hampers registration of GSNO in the mixture with thiamine. However, the thiamine thiol form does not have optical activity. Therefore, it is convenient to monitor GSNO decay in the presence of the thiamine thiol form using CD spectra. GSNO is known to be stable in neutral and alkaline media. For example, we did not observe noticeable GSNO destruction at pH 7.0 in phosphate buffer at 5°C during 1-2 days. On addition of thiamine to the GSNO aqueous solution at neutral pH, no changes were seen in the stability of the nitroso compound. However, when pH was increased GSNO degradation was enhanced simultaneously with an increase in the content of thiamine thiol form (Fig. 3). The results convincingly suggest that the thiol form of thiamine accelerates GSNO degradation (Fig. 3). In turn, GSNO accelerates the oxidation of the thiamine thiol form into the thiamine disulfide form (Fig. 2). This suggests that on the reaction of the thiamine thiol form GSNO is reduced to form glutathione and nitric oxide. The reduced glutathione most probably forms mixed disulfide as a result of interaction with the disulfide form of thiamine (reaction (1)).

After the incubation of thiamine with GSNO in alkaline medium in air, the nitrite content in the solution rose symbatically with GSNO destruction. Nitrates were not recorded in the solution. Therefore, it is possible to assume that in the presence of oxygen the released nitric oxide was mainly transformed into nitrite (see results below).

Nitrite formation on incubation of S-nitrosoalbumin and S-nitrosohemoglobin with thiamine in alkaline medium in air. Human serum albumin (HSA) contains one cysteine residue, Cys34 [20]. In serum albumin isolated from blood plasma using Ellman's reagent we determined 0.33 mol of free sulfhydryl group per mol of protein. The absorption maximum of serum albumin at 280 nm is due to the absorption of aromatic amino acids residues, primarily tryptophan and tyrosine residues. Moreover, the protein absorption can be approximated very well by the sum of independent absorptions of aromatic residues and disulfide bonds of cystines [21]. The band in the absorption spectrum of the initial HSA corresponding to aromatic amino acids has a maximum at 280 nm. In the absorption spectrum of nitrosylated albumin, this band shifts to shorter wavelength by 11 nm. This may indicate modification of the aromatic residues of the protein. Also, a weak long-wavelength band with a maximum at 340 nm was seen in the absorption spectrum of S-nitrosylated HSA. Addition of excess of thiamine in thiol form to aqueous solution of S-nitrosylated albumin (pH 8.5) resulted in formation of nitrite, which was recorded by the Griess reagent. The yield of nitrite after the reaction of the thiamine thiol form with HSA-SNO reached 0.28 mol of nitrite per mol of protein. Analogically, addition of 5- to 6-fold molar excess of thiamine to S-nitrosylated hemoglobin also led to increase in nitrite concentration in solution (pH 8.5). In our experiments, the amount of nitrite released from S-nitrosomethemoglobin was 1.4 mol per mol of the protein. Therefore, incubation of S-nitrosoproteins with the thiamine thiol form results in increase in nitrite concentration in the solution. This indicates that nitric oxide released from S-nitrosoproteins is oxidized by air oxygen to nitrite. Since the amount of degraded S-nitrosoprotein, as recorded by decreased absorption, is greater than the amount of the formed nitrite, one can suggest that some part of the nitric oxide is released from the solution into air (see results below).

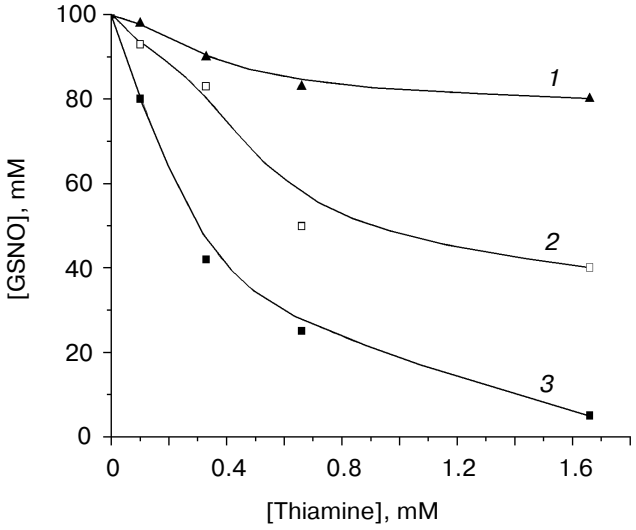

Fig. 3. Kinetics of GSNO degradation in alkaline environment in the presence of various thiamine concentrations: *1*) pH 8.0; *2*) pH 9.0; *3*) pH 10.0. Concentration of GSNO, 0.35 mM. Time of incubation of GSNO mixture with thiamine, 3 h.

Nitric oxide release from GSNO under anaerobic conditions in the presence of thiamine thiol form. Spectrophotometric determination of nitric oxide using nitrosohemoglobin formation. A modified Thunberg cell was used to monitor NO under anaerobic conditions (Fig. 4) [16]. HbO_2 was placed into a rectangular cell. A Warburg vessel (*3*) with a side branch was attached to the cell (*1*). GSNO aqueous solution was placed into the central Warburg vessel, and thiamine solution was poured into the side branch. Then by the turning of plug (*2*), vessel and pump were connected (*4*), and a vacuum pump was used to remove air from the vessel volume. OxyHb

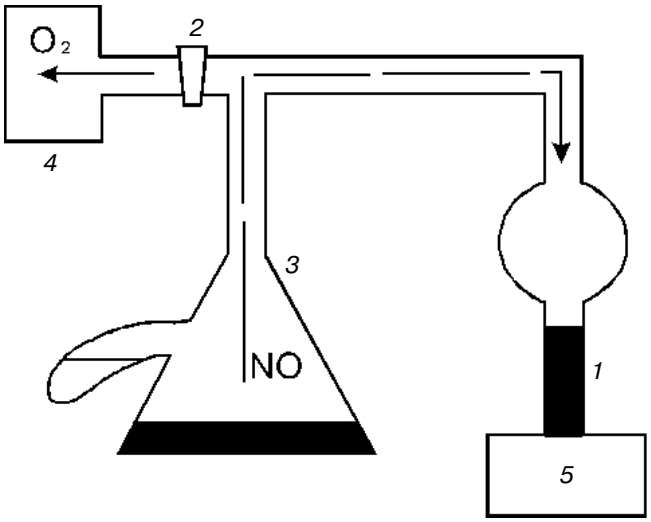

Fig. 4. Scheme of device for spectrophotometric determination of NO in vacuum: *1*) glass cell (*l* = 1 cm); *2*) valve which is used to connect the desaturation device with a vacuum pump or air; *3*) Warburg vessel; *4*) vacuum pump; *5*) magnetic mixer. The desaturation device after air removal was placed into the cuvette compartment of a spectrophotometer with magnetic mixer, which mixed a Hb solution in the glass cuvette.

was converted into deoxyHb, which was accompanied by characteristic changes in the absorption spectrum. Then the thiamine solution was poured from the side branch into the central Warburg vessel containing aqueous GSNO solution. After mixing the GSNO and thiamine, we observed release of gas bubbles of NO, which diffused into the rectangular cell (*1*) containing deoxyHb (Fig. 4).

The deoxyHb was gradually converted to nitrosoHb, which was accompanied by characteristic changes in the absorption spectrum. The initial deoxyHb Soret band at 429 nm shifted to 418 nm and a broad asymmetric band with maximum at 555-560 nm was replaced by two bands with maxima at 545 and 574 nm belonging to nitrosoHb (Fig. 5). Hence, the thiamine thiol form promoted release of nitric oxide from GSNO, and it lead to the formation of nitrosoHb:

$$\text{Hb} + \text{NO} \rightarrow \text{HbNO}. \qquad (4)$$

In the absorption spectrum of the deoxyhemoglobin and nitrosohemoglobin mixture, we observed the presence of isobestic points at 546 and 590 nm, where the extinction coefficients were equal to 12,340 and 6770 $M^{-1} \cdot cm^{-1}$, respectively (Fig. 5). The positions of the isobestic points, characteristic for the equilibrium mixture of deoxy- and nitrosoHb, were used for the quantitative calculations of the concentrations of nitrosohemoglobin [16].

In the presence of GSNO, significant acceleration of the oxidation of the thiamine thiol form to thiamine disulfide was observed (Fig. 2). Summarizing the results, the interaction of the thiamine thiol form (TSH) with GSNO can be assumed to proceed as follows:

$$\text{GSNO} + \text{TSH} \rightarrow \text{GSH} + \text{TS}^{\cdot} + \text{NO}, \qquad (5)$$

$$\text{TS}^{\cdot} + \text{TS}^{\cdot} \rightarrow \text{TS-ST}, \qquad (6)$$

where TS-ST is thiamine disulfide. Then GSH reacts with TS-ST in a thiol–disulfide exchange reaction (reaction (1)). Furthermore, as a result of the interaction of GSH with the thiamine thiyl radical ($TS^{\cdot}$) an anion-radical of mixed disulfide GS(H)ST can be formed. In air, the latter is easily transformed into the mixed disulfide. High concentrations of GSNO practically completely oxidized the thiamine thiol form to thiamine disulfide (Fig. 2). The concentration of the glutathione formed was insignificant and comprised only several percent of the initial GSNO concentration. This is probably connected with the mixed disulfide formation (reaction (1)).

Interaction of the thiamine thiol form with nitrosoglutathione in aqueous solution in air. Nitric oxide release into air. Nitric oxide output resulting from the interaction of the thiamine thiol form with nitrosoglutathione in the presence of oxygen was determined in a device, which is schematically represented in Fig. 6. The measurements were conducted under normal atmospheric conditions.

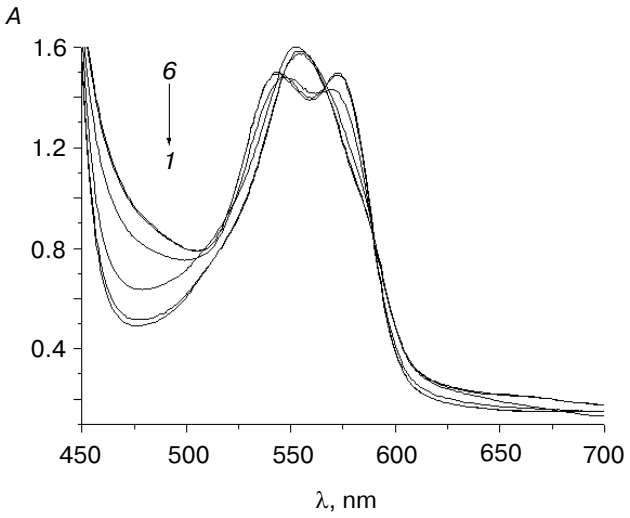

Fig. 5. Nitrosohemoglobin formation kinetics measured using the desaturation device shown in Fig. 4. The initial deoxyHb absorption spectrum (*1*) and spectra of deoxyHb after 10- (*2*), 20- (*3*), 30- (*4*), 45- (*5*), and 60-min (*6*) incubation under anaerobic conditions following the mixing of thiamine and GSNO at 37°C in the Warburg vessel. The thiamine concentration was $2 \cdot 10^{-3}$ M; GSNO concentration was 10^{-3} M, pH 8.7. The reaction was started after pouring the thiamine from the side branch into the center of the GSNO-containing Warburg vessel.

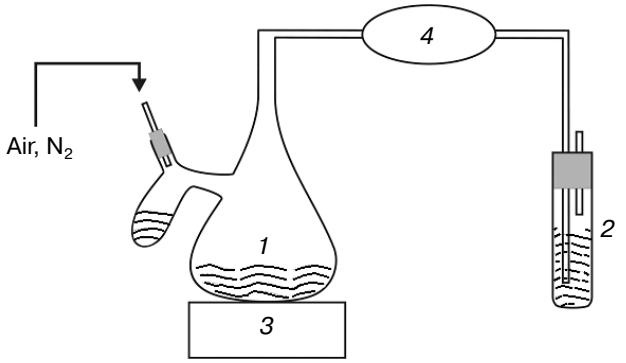

Fig. 6. Device for spectrophotometric measurements of nitric oxide formed after the interaction of the thiamine thiol form with S-nitrosoglutathione or other nitrosothiols in air: *1*) Warburg vessel containing in a side branch a water solution of thiamine (in the central vessel, a water solution of nitrosoglutathione); *2*) vessel containing water or Griess reagent; *3*) magnetic mixer; *4*) pump.

In the central part of the Warburg vessel GSNO aqueous solution was placed, and aqueous solution of thiamine thiol form was placed into the side branch. The Warburg vessel was connected with atmospheric air by a glass tube of the same diameter as the vessel (*2*). Then the evacuating pump (*4*) was switched on and thiamine was mixed with GSNO by inclining the Warburg vessel (*1*) (Fig. 6).

The released nitrogen compounds were continuously pumped out from the Warburg vessel (*1*) with the aid of pump (*4*), and then bubbled through vessel (*2*), which contained water. The pump rate was 50 ml of air per sec and the volume of the vessel was 50 ml. This means that during approximately 1 sec the entire volume of air in the vessel (*1*) was substituted by new air. In vessel (*2*), nitrite was formed (Fig. 7) proportionally to the time of incubation of the solution containing GSNO and thiamine in the Warburg vessel (*1*). Nitrite concentration in the vessel (*2*) (Fig. 7) increased with increasing thiamine concentration in the Warburg vessel (*1*) (Fig. 6).

NO in air is known to be transformed to NO_2, which is then converted to N_2O_3 and/or N_2O_4. N_2O_3 formed in water is converted only into nitrite, and N_2O_4 with H_2O forms, in equal proportion, both nitrite and nitrate [4]. The oxidation of NO to NO_2 is very slow and includes several stages [22, 23]:

$$NO + O_2 \rightleftarrows OONO \quad (k_1 = 10^6\ M^{-1}\cdot sec^{-1};\ k_{-1} = 2\cdot10^5\ M^{-1}\cdot sec^{-1}), \tag{7}$$

$$NO + OONO \rightarrow ONOONO \quad (k_2 = 5.8\cdot10^5\ M^{-1}\cdot sec^{-1}), \tag{8}$$

$$ONOONO \rightarrow 2NO_2 \quad (k_3 = 5\cdot10^3\ sec^{-1}). \tag{9}$$

A rapid reaction occurs between NO_2 and NO:

$$NO_2 + NO \rightleftarrows N_2O_3 \quad (k_4 = 1.1\cdot10^9\ M^{-1}\cdot sec^{-1};\ k_{-4} = 8\cdot10^4\ M^{-1}\cdot sec^{-1}), \tag{10}$$

$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (k_5 = 5.3\cdot10^2\ sec^{-1}). \tag{11}$$

Summing these equations, the process of nitrite formation in water, as a result of the oxidation of nitric oxide by oxygen, can be represented by the following equation:

$$4NO + O_2 + 2H_2O \rightarrow 4H^+ + 4NO_2^-. \tag{12}$$

The molecules of nitrogen dioxide interact among themselves to form N_2O_4, which forms nitrite and nitrate in water:

$$2NO_2 \rightarrow N_2O_4, \tag{13}$$

$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^-. \tag{14}$$

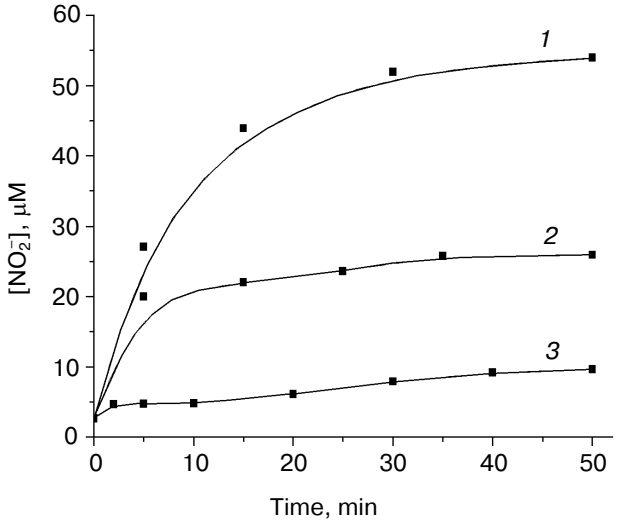

Fig. 7. Release of nitric oxide (nitrite) from GSNO aqueous solution during the reaction of the thiamine thiol form as a function of thiamine concentration (measurements were carried out in air). The amount of nitric oxide released was measured as the amount of nitrite formed in the vessel (*2*) with water after the gas mixture from the reaction system was bubbled through it (see Fig. 6); pH of the mixture was 9.8; thiamine concentration was 10^{-3} (*1*), $5\cdot10^{-3}$ (*2*), and $3\cdot10^{-4}$ M (*3*). The GSNO concentration was 10^{-3} M.

Therefore, as follows from the equations given above, the quantity of nitrate and nitrite must correspond to the quantity of nitric oxide released from the vessel in which the thiamine thiol form and GSNO were located. Certainly, this is true only when all of the nitric oxide after interaction with atmospheric oxygen is completely transformed into N_2O_3 and N_2O_4. After bubbling of air (Fig. 6) from the vessel (*1*) in which thiamine and GSNO reacted through the vessel with water (*2*) practically only nitrite formation in vessel (*2*) was observed. Nitrate was not recorded. Consequently, nitric oxide is released from vessel (*1*), which in air is transformed (reactions (7)-(11) or overall reaction (12)) into nitrous anhydride to form nitrite in water (Fig. 7).

It should be noted that not all of the nitric oxide was released into the atmosphere from GSNO aqueous solution under the reaction of the thiamine thiol form. The amount of degraded GSNO was greater than the amount of nitric oxide released into air (Table 2). The solubility of oxygen in water is 0.25 mM at 20°C, while the solubility of nitric oxide is approximately three times higher. Therefore, for low concentrations of GSNO degraded in the reaction of the thiamine thiol form mainly transformation of nitric oxide into nitrite was observed.

Further GSNO increase led to an increase in released nitric oxide into the atmosphere (Table 2). Lowering of nitric oxide release into the atmosphere at high concentrations of thiol form can be connected with formation of NO_x compounds.

Acceleration of oxyhemoglobin oxidation to methemoglobin on reaction of GSNO in the presence of the thiamine thiol form. S-Nitrosoglutathione accelerated the transformation of oxyhemoglobin to methemoglobin (Fig. 8). GSNO is known to be slowly degraded to release nitric oxide [7, 11, 24]. It is possible to assume that acceleration of oxyHb transformation to metHb in the presence of GSNO is connected with the reaction between NO and oxyHb. OxyMb and oxyHb are known to be oxidized by NO into metMb and metHb, respectively, at a high rate constant with formation of nitrate [25]:

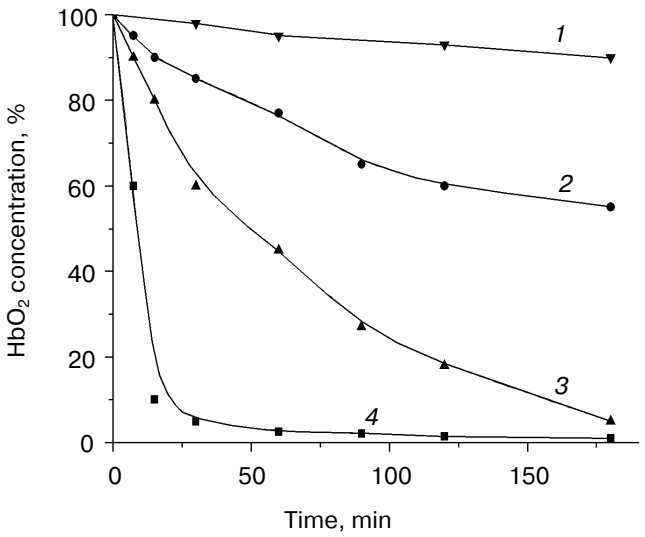

Fig. 8. Kinetics of (*1*) oxyhemoglobin autooxidation to methemoglobin, (*2-4*) oxyhemoglobin oxidation to methemoglobin: in the presence of 1 mM GSNO (*2*), 1 mM GSNO and 0.1 mM thiamine (*3*), 1 mM GSNO and 1 mM thiamine (*4*). The oxyhemoglobin concentration was 0.1 mM, pH 8.0.

$$HbO_2 + NO \rightarrow Hb(III) + NO_3^-. \qquad (15)$$

The thiamine thiol form accelerated GSNO degradation (Fig. 3) and release of nitric oxide (Fig. 7). This led to acceleration of oxyHb oxidation to metHb (Fig. 8). Especially rapid oxyHb oxidation in the presence of GSNO was observed when equimolar or excess thiamine concentrations were used (Fig. 8, curve *4*). In air, nitric oxide is transformed into nitrite (reactions (7)-(11)). OxyHb is also known to be oxidized by nitrite to metHb in acidic and neutral media [26]. The reaction kinetics is

Table 2. Ratio between a quantity of formed nitrite in the solution and the nitric oxide released into the atmosphere after the addition of the thiol form of thiamine to S-nitrosoglutathione aqueous solution (concentration of thiamine, $5 \cdot 10^{-3}$ M, pH 8.67)

Content of GSNO in solution	GSNO*	NO_2^-**	NO_2^-** / GSNO*, (%)***	NO****	NO**** / GSNO*, (%)*****
$1.92 \cdot 10^{-4}$ M	141.92 ± 11.0	101.2 ± 5.0	71.6 ± 2.54	25.4 ± 2.4	18.0 ± 1.2
$7.46 \cdot 10^{-4}$ M	682.18 ± 50.0	321.63 ± 20.0	47.1 ± 1.0	300 ± 15.0	43.0 ± 2.0

* Decrease in GSNO concentration in the solution after addition of TSH (μM).
** Growth of nitrite concentration in solution after addition of TSH (μM).
*** Fraction of nitrite formed from the concentration of GSNO* destroyed.
**** NO release into the atmosphere (μM).
***** Fraction of nitric oxide released into the atmosphere of the concentration of destroyed GSNO*.

complex and includes a lag-period and an autocatalytic stage. During the reaction oxygen free radicals, hydrogen peroxide, and hemoglobin ferryl form are produced. However, to initiate oxyHb oxidation the complex between deoxyHb and NO_2 must be formed. But this is possible only after protonation of the distal histidine residue. We showed that in alkaline medium under the conditions of our experiments (pH 8.0), nitrite did not oxidize oxyHb into metHb and only the process of oxyHb autooxidation was observed. Consequently, oxyHb oxidation to metHb in the presence of GSNO and the thiamine thiol form (Fig. 8) occurs only on the reaction with nitric oxide, which is transformed into nitrate (reaction (15)).

DISCUSSION

Thiamine is a biologically active compound necessary for the vital activity of man and animals. In the organism, the coenzyme form of thiamine participates in a number of enzymatic reactions.

Thiamine pyrophosphate catalyzes oxidative and non-oxidative decarboxylation of α-keto acids and the transketolase reaction [14, 27].

Breslou showed by NMR that the proton of the second carbon of the thiazole component was easily exchanged with deuterium. The carbanion, resulting from ionization of C-2, interacted with substrate carbonyl group and substrate decarboxylation occurred [28, 29]. The role of the amino group in the pyrimidine component is to stabilize the bipolar thiazolyl ion [30]. Within the enzyme favorable orientation of pyrimidine and thiazole rings in thiamine pyrophosphate occurs. The iminopyrimidine tautomer formation is peculiar to this coenzyme conformation [30, 31]. The aminopyrimidine ring in combination with a nucleophilic group of an enzyme glutamine residue composes the proton transfer system from the C-2 carbon in the thiazole ring, which controls the degree of C-2 proton dissociation [30].

However, not only the coenzyme form of thiamine are deposited or contained in tissues and liquid medium of organisms, but also free thiamine as well as thiamine monophosphate and thiamine triphosphate [32]. This is probably caused by the fact that thiamine and its phosphoric ethers play important roles, for example, in the functioning of nerve cells [14]. It is assumed that thiamine diphosphate and also thiamine triphosphate participate in the sodium ion transport system through the neuronal membrane [33]. Free thiamine also possesses regulator functions. It was shown that thiochrome and also carbonyl-containing products of its further oxidation possessed high antioxidant activity, they effectively interacted with oxygen free radicals [34]. The possible participation of the thiamine thiol form in reactions of enzymatic catalysis is now excluded. The experimental data presented in this work indicate that the thiamine thiol form participates in nitric oxide release from S-nitrosoglutathione and S-nitrosoproteins.

The thiamine thiol form is known to be oxidized into thiamine disulfide on reaction with ferricyanide and hydrogen peroxide. Alkaline aqueous thiamine solution can be also oxidized to disulfide by dissolved oxygen (Fig. 1) [13]. However, the reaction of the thiamine disulfide formation on reaction with oxygen proceeds very slowly. In alkaline medium thiamine practically completely exists in the thiol form (Table 1). Addition of GSNO accelerates the oxidation of the thiamine thiol form to the disulfide. High concentrations of GSNO practically completely oxidized the thiamine thiol form to give thiamine disulfide (Fig. 2). Thiamine disulfide formation on reaction with GSNO was accelerated only in alkaline medium.

The addition of excess cysteine or glutathione to thiamine disulfide reduced it to thiamine. In neutral medium GSNO did not influence significantly the stability of the thiamine cyclic form. On the other hand, thiamine accelerated GSNO degradation only in alkaline medium (Fig. 3). S-nitrosoalbumin and S-nitrosomethemoglobin degradation rate increased in the presence of the thiamine thiol form. The destruction of GSNO on the reaction of thiamine thiol was accompanied by nitric oxide release, which was recorded under anaerobic conditions by nitrosoHb formation (Figs. 4 and 5). In air, nitric oxide was transformed to nitrite (Figs. 6 and 7), or it was oxidized in a reaction with oxyHb to nitrate. The thiamine thiol form accelerated the oxidation of oxyHb into metHb in the presence of GSNO (Fig. 8). Nitric oxide released from GSNO during the reaction of the thiamine thiol form oxidized oxyHb to metHb with the formation of nitrate. The yield of nitric oxide (nitrites) from the aqueous GSNO solution during the reaction of the thiamine thiol form increased with increasing thiamine concentration (Fig. 7).

Is it possible for the thiamine thiol form to participate in nitric oxide release from the S-nitrosothiols in the human body? Thiamine and phosphoric ethers of thiamine are deposited in tissues, probably with the participation of proteins. The highest level in tissue is of thiamine pyrophosphate, the coenzyme form of thiamine that is incorporated into, for example, the pyruvate dehydrogenase multienzyme complex. The maximum depot of thiamine in such tissues as heart and liver reaches 700-800 μg per 100 g tissue. As a rule, the content of free thiamine does not exceed 15% of the total concentration of tissue thiamine forms [32]. However, in some tissues the content of free thiamine can be higher. For example, 90% of thiamine in skin is present as non-phosphorylated derivatives. Content of thiamine phosphates of non-coenzyme nature—thiamine monophosphate (TMP) and thiamine triphosphate (TTP)—is also approximately 10 times lower than that of thiamine pyrophosphate [32].

Intracellular pH can vary from 4.5 in prostate cells to 8.5 in osteoblasts. However, the normal value of blood pH

does not exceed 7.2-7.4 [35]. Therefore thiamine, and especially its coenzyme form, is in the form with a closed thiazole cycle. The pK_a value of the transition of thiamine into its thiol form is 9.2. In tissues, a small amount of thiamine thiol form is constantly present. As follows from the Hasselbach equation, the thiamine thiol form concentration does not exceed 2% of the total thiamine amount. Intense physical activity and also processes accompanied by hyperventilation are able to raise extracellular pH to 7.65 within a few minutes [35]. In this case, the concentration of the thiol form increases to 3%. Therefore, it is possible to assume that an increase in NO release from both low-molecular-weight S-nitrosothiols and S-nitrosoproteins will be observed.

In blood plasma, GSNO and S-nitrosoalbumin are present at micromolar concentrations [7, 9]. As a rule, the content of free sulfhydryl groups in human serum albumin does not exceed 0.5 per mole of protein. This is caused by the fact that in human blood plasma partial modification of some sulfhydryl groups of albumin molecules occurs, for example, as a result of thiol disulfide exchange with GSSG, cystine, or thiamine disulfides. Formation of serum albumin mixed disulfides decreases the content of free sulfhydryl groups in the albumin molecules per mole of protein. In the absorption spectrum of nitrosylated albumin changes not only in the long-wavelength region at 340 nm, but also in the region of the aromatic amino acid absorption (280 nm) are observed. This makes it possible to assume that not only sulfhydryl groups are modified, but also tryptophan and tyrosine residues. Therefore, we cannot exclude the participation of other amino acid residues of proteins in nitric oxide deposition in addition to the sulfhydryl groups of the cysteine residues.

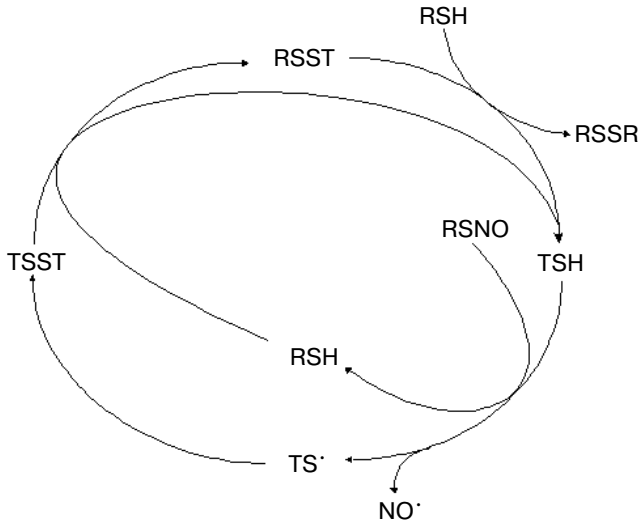

Fig. 9. Scheme of the interaction of thiamine thiol form (TSH) with low-molecular-weight S-nitrosothiols and S-nitrosothiols of proteins (RSNO). TSST, disulfide form of thiamine. RSST, the mixed disulfide.

Hemoglobin contains six sulfhydryl groups. In the β-chain of the hemoglobin molecule, there are two sulfhydryl groups, in the Cys93 and Cys112 residues, and in the α-chain, there is one cysteine, Cys105 residue [36]. The sulfhydryl group of Cys93 is most effectively nitrosylated in the β-chain of the Hb molecule. It is established that nitrosylated hemoglobin is present in small amount in circulating blood under physiological conditions [37].

It should be noted that erythrocytes contain a very high concentration of reduced glutathione [14]. Blood plasma is practically free of low-molecular-weight thiols. The majority of sulfhydryl groups in blood plasma are in serum albumin [14]. We assume that the thiamine thiol form together with intracellular thiols [10, 11], ascorbic acid [38], and also with sulfhydryl groups of cysteic residues in proteins [11] makes a contribution to NO release from the labile physiological depots such as S-nitrosothiols of proteins [9]. The thiamine thiol form (especially thiamine thiol form phosphoric esters) in contrast to glutathione possesses a relatively high unspecific affinity to proteins. The value of the equilibrium association constant of thiamine to human serum albumin is $\sim 10^3$ M^{-1}. The thiamine thiol form rather effectively interacts with disulfide groups of proteins. It is assumed that by mixed disulfide formation up to 30% of thiamine is bound to proteins in heart [32]. We assume that as a result of the exchange reactions between the thiamine thiol form and S-nitrosocysteine residues of proteins, nitric oxide release can occur and mixed thiamine–protein disulfides can be formed (Fig. 9). The efficiency of these reactions especially grows for thiamine phosphates, including non-coenzyme compounds—thiamine monophosphate and thiamine triphosphate. In this case, the interaction of positively charged residues of proteins such as imidazole, arginine, and lysine and negatively charged phosphate groups of thiamine derivatives results in reversible binding of thiamine esters to macromolecules. The high equilibrium constant of thiol thiamine ester binding to proteins can in principle provide effective release of nitric oxide even by low amounts of thiol thiamine esters.

We also suggest participation of thiamine thiol form in redox reactions with heme proteins. Thiamine formation from thiamine disulfide (TSST) was observed during incubation of TSST with mitochondria. The yield of thiamine from TSST increased after inhibition of cytochrome oxidase by cyanide [39]. Nitric oxide binds to cytochrome oxidase and inhibits its activity. The mechanism of cytochrome oxidase inhibition by nitric oxide can probably involve competition between NO and oxygen for binding to the protein heme [40, 41]. Reversible inhibition of the respiratory chain can be also caused by formation of ferricytochrome *c* complexes with NO [42]. The thiamine thiol form participates in reduction of ferricytochrome *c*. This process is accompanied by TSST formation [39]. We showed that the thiamine thiol form effec-

tively reduces not only ferricytochrome *c*, but also the complex of ferricytochrome *c* with nitric oxide.

The inhibition of cytochrome oxidase by nitric oxide and the formation of complexes between NO and ferricytochrome *c* leads to the disturbance of energy metabolism due to ATP level decrease, which in turn reduces contractile function and possibly leads to vessel relaxation. We suggest a possible role of the thiamine thiol form as a regulatory agent in the organism capable of releasing NO from nitrosothiols (mainly proteins) (Fig. 9). Secondarily, the thiamine thiol form is suggested to participate in redox reactions of cytochromes and cytochrome oxidase to decompose NO–heme complexes in proteins of the respiratory chain. In these reactions, the thiamine thiol form produces thiamine disulfide and also mixed disulfides with proteins and low-molecular-weight thiols (Fig. 9). The thiamine disulfide form as well as mixed disulfides of thiamine (including thiamine phosphoric esters disulfides) is sufficiently easily reduced by tissue low-molecular-weight thiols to produce the thiamine cyclic form with the closed thiazole ring.

We suggest that cyclic redox reactions with the participation of the thiamine thiol form ensure stable level of thiamine in the organism in spite of its participation (through the thiamine thiol minor form) in release of deposited nitric oxide from low-molecular-weight S-nitrosothiols and S-nitrosothiols of proteins.

High level of thiamine in heart and liver tissues (together with the coenzyme form of thiamine) probably can be connected with the participation of the thiamine thiol form in blood flow regulation in the tissues of these organs as a result of NO release from S-nitrosothiols.

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