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Oxidation of Thiamine on Reaction with Nitrogen Dioxide Generated by Ferric Myoglobin and Hemoglobin in the Presence of Nitrite and Hydrogen Peroxide

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Abstract—It is shown that nitrogen dioxide oxidizes thiamine to thiamine disulfide, thiochrome, and oxodihydrothiochrome (ODTch). The latter is formed during oxidation of thiochrome by nitrogen dioxide. Nitrogen dioxide was produced by incubation of nitrite with horse ferric myoglobin and human hemoglobin in the presence of hydrogen peroxide. After addition of tyrosine or phenol to aqueous solutions containing oxoferryl forms of the hemoproteins, thiamine, and nitrite, the yield of thiochrome greatly increased, whereas the yield of ODTch decreased. In the presence of high concentrations of tyrosine or phenol compounds ODTch was not formed at all. The neutral form of thiamine with the closed thiazole cycle and minor tricyclic form of thiamine do not enter the heme pocket of the protein and do not interact with the oxoferryl heme complex Fe(IV=O) or porphyrin radical. The tricyclic form of thiamine is oxidized to thiochrome by tyrosyl radicals located on the surface of the hemoprotein. The thiol form of thiamine is oxidized to thiamine disulfide by both hemoprotein tyrosyl radicals and oxoferryl heme complexes. Nitrite and also tyrosine, tyramine, and phenol readily penetrate into the heme pocket of the protein and reduce the oxyferryl complex to ferric cation. These reactions yield nitrogen dioxide as well as tyrosyl and phenoxyl radicals of tyrosine molecules and phenol compounds, respectively. Tyrosyl and phenoxyl radicals of low molecular weight compounds oxidize thiamine only to thiochrome and thiamine disulfide. The effect of oxoferryl forms of myoglobin and hemoglobin, nitrogen dioxide, and phenol on thiamine oxidative transformation as well as antioxidant properties of the hydrophobic thiamine metabolites thiochrome and ODTch are discussed.

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Key words: nitrite, oxoferryl forms of hemoproteins, hydrogen peroxide, thiamine, thiochrome, thiamine disulfide, oxodihydrothiochrome

Nitric oxide (NO) is involved in various physiological functions including smooth muscle relaxation, inhibition of thrombocyte aggregation, neurotransmission, and immune response [1, 2].

Nitrite is known to be one of the products of NO metabolism: in some cases its local concentration corresponds to NO concentration in tissues. For example, it was shown that the increased NO production in pathophysiological inflammatory processes is accompanied by increased nitrite concentration [3]. Very high nitrite concentration was found in synovial fluid of patients with rheumatic arthritis [4]. Nitrite concentration in blood plasma of viral-infected patients with interstitial pneumonia increased to 36 μM (more than 70-fold) [5]. In the human body nitrite is oxidized to the stable nitrate and also participates in other conversions, e.g. it nitrates tyrosyl and tryptophanyl residues of proteins. Nitration of aromatic amino acid residues by nitrite proceeds in acidic medium at pH ≤ 6.0 [6] and is catalyzed by peroxidases and hemoproteins in the presence of nitrite and hydrogen peroxide [7].

Recently it has been shown that endogenous nitrite is a signal molecule sensitive to oxygen gradient in tissues

Abbreviations: ODTch, oxodihydrothiochrome; T, neutral form of thiamine with closed thiazole cycle; Tch, thiochrome; TDP, thiamine diphosphate; TDS, thiamine disulfide; Tp, tricyclic form of thiamine; TSH, thiol form of thiamine.

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and is reduced to NO at decreased oxygen concentration in tissues [8-12]. As noted earlier, deoxyhemoglobin and deoxymyoglobin reduce nitrite to NO with formation of ferric and nitroso-forms of the hemoproteins [9, 13, 14]. In the presence of NO methemoglobin catalyzes nitrite transformation to gaseous N_2O_3, and this compound is readily soluble in hydrophobic sites of cell membranes. The N_2O_3 causes nitrosylation of sulfhydryl groups of low molecular weight thiols and sulfhydryl groups of proteins [15].

Under extreme conditions (acute hypoxia) pH value in tissues of the ischemic heart decreases to 5.5 and even lower [8], and nonenzymatic reduction of nitrite to NO can be observed. It is supposed that during acute ischemia additional mechanisms of NO generation with participation of hemoglobin and myoglobin [8, 9], xanthine oxidase [16], cytochrome *c* oxidase [17], and endothelial NO-synthase [18] are switched on.

Nitrite at low concentrations can fulfill vasodilatory functions *in vivo* and protect tissues from damage during ischemia–reperfusion [19-21]. Toxic effects of nitrite drastically increase at high H_2O_2 concentrations and high level of ferric hemoproteins [22].

The tissues damage mechanism caused by reperfusion of ischemic tissues is not well studied. It is supposed that on ischemia–reperfusion the formation of the active forms of oxygen and nitrogen such as superoxide anions, hydroxyl radicals, hydrogen peroxide, nitrogen dioxide, and peroxynitrite drastically increase [23-25].

The action of hydroxyl radicals, peroxynitrite, and NO_2 on proteins causes formation of long-lived free radicals localized on tyrosine and tryptophan residues and also formation of thiyl radicals [26]. The interaction between free radicals results in interprotein cross-linking and formation of dityrosine; intermolecular disulfide bonds damaging the native structure of proteins are also formed.

Highly oxidized forms of hemoglobin [27, 28], myoglobin [29], cytochrome *c*, and other hemoproteins [30, 31] play a very important role in the destructive processes developing in the course of oxidative stress. Using EPR it was shown that in the case of the oxoferryl form of human hemoglobin (compound I), free radicals localized on the S and/or O atoms [32, 33] and also long-lived tyrosyl radicals [34] are formed on the protein. Long-lived free radicals localized on Tyr103 and Tyr151 are formed on interaction of horse heart metmyoglobin with H_2O_2 [35]. These tyrosyl residues of myoglobin participate in formation of interprotein cross-links, e.g. between myoglobin and lactoperoxidase macromolecules in the presence of H_2O_2 [36].

The steady-state H_2O_2 concentration in blood under normal conditions does not exceed 0.2 nM due to its destruction by catalase and glutathione peroxidase [37]. However, even at such a low H_2O_2 concentration methemoglobin (or hemoglobin) is oxidized with formation of the oxoferryl forms of hemoglobin with a radical localized on the protein. Both oxoferryl forms of hemoglobin, compound I [38] and compound II [39], were detected in blood under physiological conditions. The H_2O_2 concentration in blood and tissues drastically increases in pathological states, e.g. on ischemia–reperfusion, and it may reach 10 μM and even higher in ischemic myocardium [40].

In the presence of nitrite the oxoferryl forms of myoglobin and hemoglobin generate NO_2, which causes nitration of tyrosine or tyrosyl residues of proteins and formation of dityrosine [29, 31, 41]. Inclusion of NO_2 into the tyrosine molecule decreases pK_a of the hydroxyl group from 10.2 to 7.2. This increases the total negative charge localized on nitrated tyrosyl residues, which is accompanied by structural change in the macromolecule and initiates protein unfolding or denaturation and enzyme inactivation [3].

Thiamine (or vitamin B_1) is known to be an important indispensable nutritional factor and is used in the organism as a structural component of the thiamine diphosphate (TDP) molecule. TDP is a cofactor of such important enzymes of energetic metabolism as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. TDP is also a cofactor of transketolase, a key enzyme of the pentosophosphate cycle [42].

Recently, along with elucidation of mechanisms of the main reactions catalyzed by TDP [42], data on high biological activity of thiamine metabolites have accumulated [43]. It was shown that the thiol form of thiamine releases NO from S-nitrosoglutathione, and NO causes vascular relaxation [44]. Although the antioxidant capacity of thiamine is still not completely characterized, certain experimental data indicate that thiamine can act as an antioxidant under oxidative or nitrosyl stress [43].

The lack of thiamine during prolonged oxidative stress accompanied by generation of active forms of oxygen causes selective neuronal damage [45]. Enhanced NO synthesis against the background of lack of thiamine is accompanied by the nitration of tyrosine with formation of 3-nitrotyrosine and by nerve tissue damage [46]. Thiamine inactivates tyrosyl radicals and inhibits formation of dityrosine and interprotein cross-linking in reactions catalyzed by the oxoferryl forms of hemoglobin [47, 48].

Earlier it was shown that thiamine and especially its hydrophobic derivatives (thiochrome and oxodihydrothiochrome (ODTch)) are traps for peroxynitrite [49] and destroy the toxic oxoferryl forms of hemoproteins [50].

The goal of the present work was to study the oxidation of thiamine by NO_2 generated by ferric hemoglobin and myoglobin in the presence of H_2O_2 and nitrate. We have shown that NO_2 oxidizes the thiol form of thiamine (TSH) to thiamine disulfide (TDS) and the tricyclic form of thiamine to thiochrome (Tch). Thiochrome is oxidized to ODTch by the action of nitrogen dioxide.

MATERIALS AND METHODS

In this work we used thiamine, thiamine monophosphate, thiamine diphosphate, thiamine disulfide, and thiochrome from Sigma (USA); horse skeletal muscle myoglobin, L- and D-tyrosine, tyramine, glutathione, ferric cyanide, and hemin from Fluka (USA); Sephadex G-25 from Pharmacia (Sweden). All other reagents were of extra pure grade and produced in Belarus or Russia.

Oxyhemoglobin was obtained from fresh donor blood according to a known procedure [51]. Oxyhemoglobin concentration in solution was determined using molar extinction coefficients $\varepsilon_{412} = 125{,}000\ M^{-1}\cdot cm^{-1}$ and $\varepsilon_{542} = 14{,}250\ M^{-1}\cdot cm^{-1}$. Methemoglobin concentration was determined using $\varepsilon_{630} = 3700\ M^{-1}\cdot cm^{-1}$, pH 7.2 [52]. Methemoglobin was obtained by addition of 10-20-fold excess of ferric cyanide to oxyhemoglobin. The protein was separated from low molecular weight compounds by gel filtration on Sephadex G-25.

The oxoferryl forms of hemoglobin were obtained by addition of 100-1000-fold molar excess of H_2O_2 to methemoglobin. The hemoglobin Soret band with maximum at 407 nm and $\varepsilon_{407} = 190{,}000\ M^{-1}\cdot cm^{-1}$ was shifted to 418 nm after addition of H_2O_2. This indicates that the oxoferryl form of hemoglobin ($\varepsilon_{418} = 110{,}000\ M^{-1}\cdot cm^{-1}$, pH 7.5) is formed. Concentrations of methemoglobin and oxoferryl forms of hemoglobin in solution were determined by decomposition of the absorption spectrum into individual spectra using the least-squares method. The absorption spectra of methemoglobin and the oxoferryl form of hemoglobin were used as the standards. Metmyoglobin concentration in neutral and weakly acidic media was determined via optical absorption at 408 and 630 nm ($\varepsilon = 188{,}000$ and $3900\ M^{-1}\cdot cm^{-1}$, respectively), and $\varepsilon_{411} = 119{,}000\ M^{-1}\cdot cm^{-1}$ was used at pH 9.5 for the Soret band [53]. Concentration of the oxoferryl forms of myoglobin was determined via optical absorption at 421 nm ($\varepsilon_{421} = 111{,}000\ M^{-1}\cdot cm^{-1}$) [54]. The molar extinction coefficients given above correspond to concentration of the hemoproteins recalculated per heme.

The oxoferryl form of Mb(IV=O) (compound II) was obtained by addition of 10-100-fold molar excess of H_2O_2 in 0.05 M phosphate buffer, pH 7.0, to 1-10 μM metmyoglobin. After mixing the reagents and 5-10 min incubation, the yield of oxoferryl form was monitored spectrophotometrically via the position of the Soret band. Excess H_2O_2 was decomposed by addition of catalase to the reaction mixture.

H_2O_2 concentration was measured spectrophotometrically ($\varepsilon_{240} = 39.4\ M^{-1}\cdot cm^{-1}$ [55]) or by addition of excess of KI and subsequent measurement of concentration of thus formed molecular I_2 ($\varepsilon_{350} = 26{,}000\ M^{-1}\cdot cm^{-1}$). Formation of dityrosine was monitored spectrophotometrically ($\varepsilon_{315} = 5000\ M^{-1}\cdot cm^{-1}$, pH 7.5 [56]) or fluorimetrically. Fluorescence was excited at 315 nm, and fluorescence intensity was measured at 410 nm [57]. Tyrosine concentration was measured spectrophotometrically ($\varepsilon_{277} = 1500\ M^{-1}\cdot cm^{-1}$ [58]) or fluorimetrically ($\lambda_{max} = 303$ nm, $\lambda_{ex} = 280$ nm [59]).

Fluorescent products (Tch and ODTch) obtained after oxidation of thiamine by H_2O_2 in the presence of methemoglobin or metmyoglobin were identified by ascending paper chromatography (*n*-butanol–ethanol– water, 2 : 1 : 1) [60]. At pH 7.5 Tch and ODTch have absorption maxima at 365 and 342 nm, respectively, and also fluorescence maxima at 450 and 440 nm, respectively [47, 49].

Tch and ODTch concentrations were measured using fluorescence spectroscopy. Since the emission spectra of Tch and ODTch overlap significantly, the fluorescence spectrum of a mixture of these two compounds was separated to Tch and ODTch individual spectra using the least-squares method. Commercial Tch preparation from Sigma and ODTch obtained as described earlier [60] were used as the standards. TDS concentration was determined after incubation with excess glutathione and subsequent oxidation of the formed thiamine [44]. ODTch was isolated by gel filtration on a column with Sephadex G-25 from the reaction mixture containing metmyoglobin, H_2O_2, thiochrome, and nitrite.

To determine quantum yield of Tch and ODTch fluorescence, the Williams comparative method was used [61]. Solution of coumarin I in ethanol with quantum yield $F = 0.73$ was used as a standard [62]. Absorption spectra of solutions were obtained using a SPECORD-200 PC double-beam spectrometer (Analytik Jena, Germany). Fluorescence spectra were recorded using a CM2203 spectrofluorimeter from Solar (Belarus). Kinetics of fluorescence decay was measured using a pulse fluorimeter described earlier [63]. Data analysis of fluorescence decay curves and determination of kinetic parameters were carried out using a developed software package on the base of the Marquardt method.

RESULTS

Formation of Tch, ODTch, and TDS after reaction of NO_2 with thiamine. After addition of H_2O_2 to an aqueous solution of metmyoglobin, formation of the oxoferryl forms of myoglobin $^{+\bullet}$Mb(IV=O) (compound I) and Mb(IV=O) (compound II) was observed. This was accompanied by a shift of the Soret band to the longer wavelength from 410 to 422 nm [41].

$$Mb(III) + H_2O_2 \rightarrow {}^{+\bullet}Mb(IV{=}O) + H_2O \qquad (1)$$

On interaction of horse heart metmyoglobin with H_2O_2, free radicals localized on heme porphyrin are first formed. Then long-lived free radicals localized on tyrosine residues on the protein are formed due to intramolecular electron transfer [34, 35]. The oxoferryl form of myoglobin $^{+\bullet}$Mb(IV=O) formed in reaction (1) is het-

erogenous in composition. It contains at least two reaction centers: free radical localized on the protein and the oxoferryl complex Fe(IV=O).

Interaction of H_2O_2 with a free radical localized on protein outside the heme pocket results in formation of Mb(IV=O):

$$^{+\bullet}Mb(IV=O) + H_2O_2 \rightarrow Mb(IV=O) + {}^{\bullet}HO_2. \quad (2)$$

To reduce the oxoferryl form of Mb(IV=O) to the ferric state, an electron donor is required. Phenol compounds including tyrosine residues [41] and also functional groups of some other amino acid residues of the protein polypeptide chain [34, 35] are efficient donors.

The oxoferryl form of Mb(IV=O) (compound II) with a certain probability captures an electron from neighboring favorably oriented functional groups with formation of metmyoglobin with a radical localized on the protein $^{+\bullet}$Mb(III) [34, 38]:

$$Mb(IV=O) \rightarrow {}^{+\bullet}Mb(III). \quad (3)$$

This free-radical form of metmyoglobin $^{+\bullet}$Mb(III) is transformed to metmyoglobin on reaction with H_2O_2:

$$^{+\bullet}Mb(III) + H_2O_2 \rightarrow Mb(III) + {}^{\bullet}HO_2, \quad (4)$$

$$2{}^{\bullet}HO_2 \rightarrow H_2O_2 + O_2. \quad (5)$$

Interprotein electron transfer in solution also results in reduction of the oxoferryl form of myoglobin (compound II) to metmyoglobin.

Thus formed metmyoglobin interacts again with H_2O_2 (reaction (1)). That is why both oxoferryl and ferric forms of the protein (reactions (1)-(4)) are present in solution. However, the steady-state concentration of the oxoferryl forms is higher as can be seen from the absorption spectra. This is probably caused by the lower rate of reaction (3) compared to the rates of reactions (1) and (2).

Reactions of H_2O_2 with metmyoglobin and oxoferryl forms of myoglobin cause rapid H_2O_2 decomposition in solution (reactions (1), (2), and (4)). For example, after incubation of 10 μM metmyoglobin with 1 mM H_2O_2 for 30 min H_2O_2 concentration decreased about 2-fold. Decomposition of H_2O_2 by metmyoglobin was accelerated in the presence of nitrite, phenol, and tyrosine.

To study kinetics of reduction of the oxoferryl forms excess H_2O_2 should be removed from solution using catalase. After this the oxoferryl forms of myoglobin stable for several hours were detected spectrophotometrically (Table 1). High stability of the oxoferryl forms of myoglobin is due to the fact that reactions (2) and (4) yielding the ferric myoglobin are blocked in the absence of free H_2O_2. Earlier it was shown that after removal of excess H_2O_2 with catalase mainly Mb(IV=O) was formed in metmyoglobin/H_2O_2 solution [29, 41].

In the absence of free H_2O_2 in solution, high (≥1 mM) thiamine concentrations caused transformation of the oxoferryl form of hemoprotein Mb(IV=O) (compound II) to the ferric form in several minutes. Thus, the half-time of conversion was ~14 min in the presence of thiamine (Table 1).

This slow reduction of the oxoferryl form of myoglobin to metmyoglobin was accompanied by oxidation of

Table 1. Half-times of conversion of the oxoferryl form of myoglobin to the ferric form in the absence of free H_2O_2 in solution

Solution composition	Half-time of conversion of the oxoferryl form of myoglobin to metmyoglobin, min	k*
MetMb + H_2O_2 + catalase	180	$6.3 \cdot 10^{-5}$ sec^{-1}
MetMb + H_2O_2 + catalase + 1 mM thiamine	14	1.2 $M^{-1} \cdot sec^{-1}$
MetMb + H_2O_2 + catalase + 1 mM L-tyrosine	3.2	5.2 $M^{-1} \cdot sec^{-1}$
MetMb + H_2O_2 + catalase + 0.1 mM nitrite	3.5	48 $M^{-1} \cdot sec^{-1}$
MetMb + H_2O_2 + catalase + 0.5 mM nitrite	0.67	50 $M^{-1} \cdot sec^{-1}$
MetMb + H_2O_2 + catalase + 1 mM GSH**	–	–

Note: The oxoferryl form of myoglobin was obtained by addition of H_2O_2 to metmyoglobin solution. Excess H_2O_2 was removed by addition of catalase. Concentrations: metmyoglobin, 10 μM; H_2O_2 (initial), 1 mM; catalase, 0.1 mg/ml (1 mg of catalase decomposed 12.5 mM H_2O_2 per min). Thiamine, L-tyrosine, nitrite and glutathione were added to the reaction mixture after preincubation of metmyoglobin and H_2O_2 with catalase for 10 min, pH 6.0 ± 0.5.

* k, the first- and second-order reaction rate constants of metmyoglobin formation.

** Metmyoglobin formation was not detected during 60 min incubation of the given mixture. Autooxidation of glutathione yielding oxidized glutathione and H_2O_2 proceeded in aqueous solutions of glutathione in air. We suppose that H_2O_2 thus generated stabilizes the oxoferryl form of myoglobin.

Table 2. Formation of Tch and TDS on the reaction of thiamine with the oxoferryl form of myoglobin in the absence and in the presence of tyrosine

Solution composition	[TDS], μM	[Tch], μM	[Tch]/[TDS]	[Dityrosine], μM
(A) + 1 mM thiamine	4.8	0.12	1 : 40	–
(A) + 1 mM thiamine + 1 mM D-tyrosine	3.30	0.4	1 : 8.2	1.31
(A) + 1 mM D-tyrosine	–	–		5.0
(A) + 1 mM D-tyrosine + 0.1 mM GSH				1.0

Note: Concentrations: metmyoglobin, 10 μM; H_2O_2, thiamine and tyrosine, 1 mM; catalase, 0.03 mg/ml. (A) is MetMb + H_2O_2 + catalase solution.

thiamine with formation of Tch and TDS. After complete reduction of the oxoferryl form of myoglobin the yield of Tch was very low: the concentration of Tch did not exceed 2.5% of the initial concentration of the oxoferryl form of myoglobin (Table 2). This means that TDS is the main product of thiamine oxidation, and the oxoferryl form of myoglobin was in fact reduced to the ferric form due to interaction with the thiol form of thiamine.

Using the rate constant of thiamine interaction with the oxoferryl form of myoglobin Mb(IV=O) (Table 1), one can easily determine the half-time of conversion of the oxoferryl form of myoglobin to the ferric form and the yield of thiamine oxidation products for any thiamine concentration.

Concentration of the thiol form of thiamine increases on pH increase. At pH 9.2 this concentration is 50% total thiamine concentration. At pH ≥ 8 in solution containing thiamine, metmyoglobin and H_2O_2 TDS is the main product of thiamine oxidation, whereas Tch is almost not formed. That is why we suppose that unlike the tricyclic form of thiamine (Tp), the thiol form of thiamine (TSH) penetrates into heme pocket and reduces the oxoferryl complex to ferric cation, which results in formation of metmyoglobin and TDS. As compared to thiamine, tyrosine, tyramine and other phenol compounds and especially nitrite cause more rapid transformation of the oxoferryl form of myoglobin to the ferric form. Half-time of conversion of the oxoferryl form in the presence of tyrosine and nitrite was only several minutes (Table 1). After addition of tyrosine the rate of thiamine oxidation to Tch increases, whereas the yield of dityrosine decreases (Table 2).

At high phenol concentrations the yield of Tch for solution containing thiamine, metmyoglobin, H_2O_2 increased more than 120 times compared with that for phenol-free solutions. Other monophenol compounds also enhanced catabolism of thiamine 1-2 orders of magnitude, but reaction of thiamine oxidation in the presence of phenol compounds always stopped at the stage of Tch and TDS formation (Table 3). Earlier it was shown that tyrosyl and phenoxyl radicals oxidized the tricyclic form of thiamine (Tp) to Tch and TSH to TDS [47]:

$$2^{\cdot}\mathrm{Tyr} + \mathrm{Tp} \rightarrow \mathrm{Tch} + 2\mathrm{Tyr}, \quad (6)$$

$$2^{\cdot}\mathrm{Tyr} + 2\mathrm{TSH} \rightarrow \mathrm{TDS} + 2\mathrm{Tyr}. \quad (7)$$

The increased yield of Tch and decreased yield of TDS in the presence of tyrosine (Table 2) suggests that tyrosyl radicals more efficiently oxidize Tp to Tch (reaction (6)) than TSH to TDS (reaction (7)).

The same tendencies in kinetics of formation and reduction of the oxoferryl forms were observed in the case of hemoglobin. The Soret band maximum of methemoglobin in the presence of excess H_2O_2 shifts to 417 nm; this indicates that the oxoferryl form of hemoglobin is formed. Addition of thiamine (0.01-1 mM) almost does not effect the Soret band position. In the presence of excess H_2O_2 thiamine is oxidized with the constant rate during prolonged time period up to complete consumption of H_2O_2 in solution.

The data indicate that hemoglobin is almost completely in the oxoferryl form in solutions containing high H_2O_2 concentrations. Tch yield in the absence of tyrosine or other phenol compounds was very low even after prolonged incubation of solutions containing thiamine, H_2O_2 and metmyoglobin (or methemoglobin). In fact, absorption increase (Fig. 1) in the wavelength range 360-370 nm, where Tch absorption maximum is located, was rather low (Fig. 1), and Tch concentration was measured by fluorescence spectroscopy (Table 3).

TDS was the main product of thiamine oxidation. We have shown that thiamine is oxidized to Tch in peroxidase reactions catalyzed by metmyoglobin or methemoglobin at concentrations beginning from 1 μM. In the presence of phenol compounds we observed oxidation of thiamine to Tch beginning from thiamine concentration 0.1 μM. However, in experiments we usually used higher thiamine concentrations (≥1 mM). This is due to the fact that some products of thiamine oxidation do not fluoresce at room

Table 3. Yield of Tch and ODTch after incubation of thiamine (T) in aqueous solutions containing metmyoglobin, methemoglobin and H_2O_2 in the presence and in the absence of phenol compounds, glutathione and nitrite

Solution composition	[Tch], μM	[ODTch], μM	[Tch] + [ODTch], μM
T + H_2O_2	0.01	–	0.01
10 μM Mb(III) + T + H_2O_2	0.45	0.01	0.46
10 μM Mb(III) + T + NO_2^- + 5 mM H_2O_2	2.8	8.5	11.3
10 μM Mb(III) + T + L-tyrosine + H_2O_2	25.1	–	25.1
10 μM Mb(III) + T + phenol + H_2O_2	62.2	–	62.2
1 μM Mb(III) + T + tyramine + H_2O_2	3.8	–	3.8
1 μM Mb(III) + T + phenol + H_2O_2	12.6	–	12.6
1 μM Hb(III) + T + H_2O_2	0.21	–	0.21
1 μM Hb(III) + T + 0.02 mM GSH + H_2O_2	0.10	–	0.10
1 μM Hb(III) + T + 0.10 mM GSH + H_2O_2	0.002	–	0.002
1 μM Hb(III) + T + NO_2^- + H_2O_2	3.5	0.4	3.9
1 μM Hb(III) + 5 mM T + 2 mM NO_2^- + 10 mM H_2O_2	32.0	28.0	60.0
1 μM Hb(III) + T + L-tyrosine + H_2O_2	3.0	–	3.0
10 μM Hb(III)+ T + 5 mM H_2O_2	0.42	0.01	0.43
10 μM Hb(III) + T + L-tyrosine + 5 mM H_2O_2	5.1	–	5.1

Note: Concentrations of thiamine, nitrite, H_2O_2, and phenol compounds were 1 mM unless otherwise specified in the table. Incubation time 30 min.

temperatures (e.g. TDS) or have low-intensive fluorescence (e.g. ODTch). That is why the yield of these products was rather high and after identification their concentrations were measured by absorption spectroscopy.

After addition of nitrite to solution containing methemoglobin (or metmyoglobin), thiamine and H_2O_2 we observed that the yield of products of thiamine oxidation absorbing in the wavelength range >300 nm drastically increased (Fig. 2). Unlike this, the equilibrium ratio of concentrations of the oxoferryl forms of hemoproteins and their ferric forms in the presence of high nitrite concentrations and excess H_2O_2 shifts to the ferric forms, and the oxoferryl forms are present in negligible amounts. For example, this is evidenced by position of the Soret band maximum in absorption spectra of solutions of methemoglobin and H_2O_2 in the presence of nitrite (Fig. 2). The Soret band maximum in this case is at 407 nm; this indicates that the equilibrium concentration of the ferric hemoglobin is high.

Nitrite is known to interact with the oxoferryl forms of myoglobin or hemoglobin with NO_2 formation [41]. For example, interaction of nitrite with the oxoferryl form of myoglobin $^{+\bullet}$Mb(IV=O) (compound I) results in formation of NO_2 and the oxoferryl form Mb(IV=O) (compound II):

$$^{+\bullet}\text{Mb(IV=O)} + NO_2^- \rightarrow \text{Mb(IV=O)} + {}^{\bullet}NO_2, \quad k_8 = 10^3\ M^{-1}\cdot sec^{-1}. \qquad (8)$$

In this case nitrite interact with free radicals localized on heme porphyrin [41].

Nitrite also interacts with the oxoferryl form Mb(IV=O) (compound II) and reduces it to metmyoglobin [29, 41]. In this case nitrite interacts with the oxoferryl complex Fe(IV=O):

$$\text{Mb(IV=O)} + 2H^+ + NO_2^- \rightarrow \text{Mb(III)}OH_2 + {}^{\bullet}NO_2, \quad k_9 = 50\ M^{-1}\cdot sec^{-1}. \qquad (9)$$

The rate constant of nitrite interaction with $^{+\bullet}$Mb(IV=O) (reaction (8)) is higher than that of nitrite interaction with Mb(IV=O) (reaction (9)) [29, 41].

NO_2 forming in reactions (8) and (9) oxidizes thiamine. The yield and content of thiamine oxidation

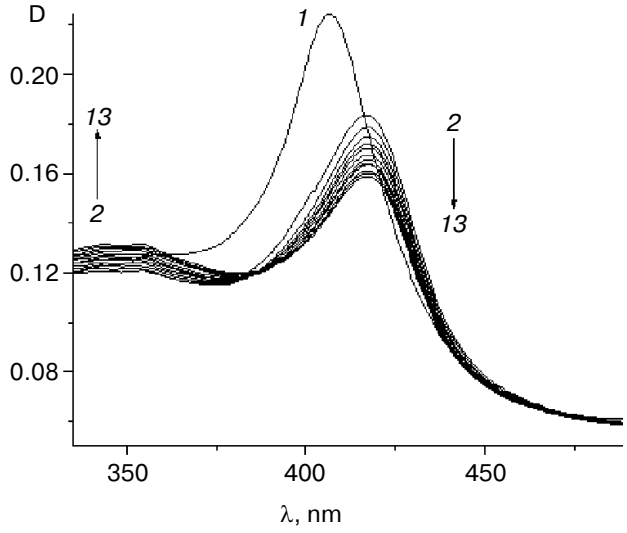


Fig. 1. Absorption spectrum of solution containing methemoglobin and thiamine in the absence of H_2O_2 (*1*) and changes in absorption spectrum of methemoglobin and thiamine in the presence of H_2O_2 dependent on incubation time (*2-13*). Records of absorption spectra were repeated after 2 min. Concentrations: methemoglobin, 1 μM; thiamine, 1 mM; H_2O_2, 1 mM; potassium phosphate buffer, 0.05 M, pH 7.5.

products depend on the thiamine/nitrite ratio in solution. In fact, position of the total absorption maximum of thiamine oxidation products being a superposition of absorption of individual products changed from 370 to 342 nm and depended on the ratio of incubation mixture components.

If thiamine concentration exceeded that of nitrite, absorption maximum of oxidation products was shifted to the long-wavelength range (370 nm), and fluorescence with the maximum at 450 nm typical for thiochrome was observed. It was shown chromatographically that under these conditions thiochrome is the main fluorescent product of thiamine oxidation (Table 3). At lower thiamine (or higher nitrite) concentrations ODTch was formed along with thiamine disulfide and thiochrome on reaction with NO_2 (Table 3). If solution contained ODTch along with Tch, absorption spectrum of the mixture of thiamine oxidation products was shifted to the short-wavelength range (Fig. 2).

This is caused by the fact that ODTch absorbs in the shorter range with the maximum at 342 nm compared with Tch which absorbs at 365 nm.

Nitrogen dioxide oxidizes Tch to ODTch. Thiochrome is stable in acidic and basic media in the presence of methemoglobin or metmyoglobin. We did not observe any changes in Tch absorption spectrum as well as intensity changes in its fluorescence spectrum after incubation with hemoproteins under these conditions for a day or more. After addition of excess H_2O_2 to aqueous solution containing methemoglobin or metmyoglobin and Tch we observed that fluorescence intensity of the latter slowly decreased; this was caused by hemin destruction and release of Fe ions. In the presence of H_2O_2, Fe(II) ions generate hydroxyl radicals (Fenton reaction), which oxidize Tch with formation of the products of deeper destruction. For example, in solution containing 0.03 mM Fe(II), 0.02 M Tch, and 1 mM H_2O_2, Tch was 72% oxidized during 5 min to non-fluorescent products absorbing in the range <300 nm.

In the presence of 1 mM EDTA concentration of the oxidized Tch did not exceed 0.5% the initial value. Consequently, in the presence of excess EDTA we were able to inhibit and almost completely block Tch oxidation in the Fenton reaction due to complexation of Fe ions with EDTA. After addition of NO_2^- to the solution containing methemoglobin, H_2O_2, and Tch we observed significant acceleration of Tch oxidation (Fig. 3).

The long-wavelength absorption band of Tch (maximum 365 nm) was shifted to 342 nm, and fluorescence intensity decreased significantly. An isobestic point at 355 nm was observed in the absorption spectrum of the reaction mixture (Fig. 3). For this wavelength the molar extinction coefficients of Tch and ODTch are equal. This means that Tch is oxidized only to ODTch on reaction with NO_2. The same reaction was observed in solution containing metmyoglobin. ODTch was isolated from the reaction mixture by gel filtration on a column with Sephadex G-25. Excess EDTA at concentration higher than that of metmyoglobin was added to the reaction mixture. After elution with phosphate buffer we observed the release of peaks I-III ascribed to metmyoglobin, ODTch, and Tch, respectively (Fig. 4a). Absorption spectra of metmyoglobin and low molecular weight compounds that were eluted after the release of the proteins

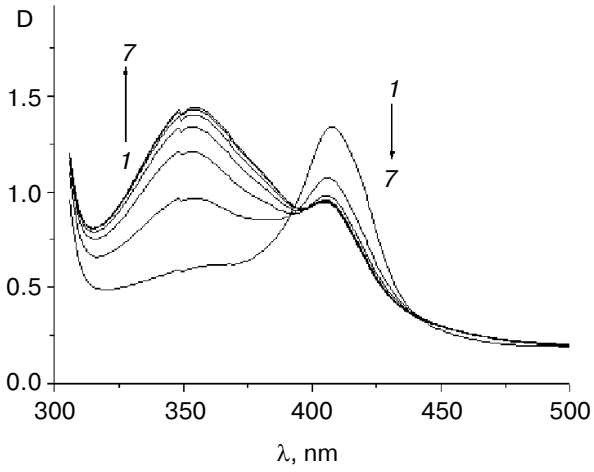


Fig. 2. Kinetics of formation of the products of thiamine oxidation by methemoglobin in the presence of nitrite and H_2O_2. Absorption spectra (*1-7*) were recorded with time interval 3 min. Concentrations: methemoglobin, 10 μM; thiamine, 5 mM; nitrite, 2 mM; H_2O_2, 10 mM.

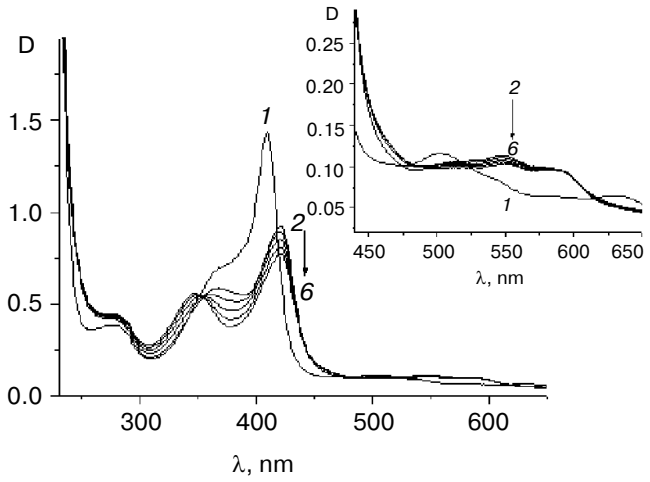


Fig. 3. Absorption spectra of solutions containing methemoglobin, Tch, and NO_2^- in the absence (*1*) and in the presence (*2-6*) of H_2O_2 dependent on incubation time. Absorption spectra (*2-6*) were recorded with time interval 6 min. Concentrations: methemoglobin, 10 μM; thiochrome, 10 μM; NO_2^-, 1 mM; H_2O_2, 5 mM.

(peaks II and III) are presented in Fig. 4b. Unlike Tch, ODTch fluorescence intensity in aqueous solutions is low. Along with fluorescence spectral methods, we used ascending paper chromatography to identify Tch and ODTch with chemically synthesized Tch and ODTch as the standards (under the given conditions R_f for Tch and ODTch were 0.58 and 0.78, respectively). Nitrite anion has low molar extinction coefficient ($\varepsilon_{275} = 21\ M^{-1}\cdot cm^{-1}$) [58], so at concentration <1 mM its absorption is almost absent in the absorption spectra. EDTA absorbs in at shorter wavelength, and its absorption does not overlap with the long-wavelength absorption maxima of Tch and ODTch.

Like thiamine, thiamine disulfide absorbs at wavelengths <300 nm. That is why the absorption spectrum of the products formed on oxidation of thiamine by NO_2 is a superposition of Tch and ODTch spectra in the wavelength region 300-450 nm (Fig. 2).

Oxidation of Tch to ODTch in the presence of nitrite is caused by reaction with NO_2:

$$Tch + {}^{\bullet}NO_2 \rightarrow {}^{\bullet}Tch + NO_2^-. \quad (10)$$

Thiochrome radical ˙Tch is oxidized to ODTch by a second NO_2 molecule:

$${}^{\bullet}Tch + {}^{\bullet}NO_2 \rightarrow ODTch + NO_2^-. \quad (11)$$

The ˙Tch radicals probably yield Tch and ODTch also in the disproportion reaction:

$$2{}^{\bullet}Tch \rightarrow Tch + ODTch. \quad (12)$$

The presence of ODTch in solution may indicate that Tch (or thiamine) interacts with NO_2 (Table 3).

At pH ≥ 10.5 TDS was the main product of thiamine oxidation by NO_2. Figure 5 presents a scheme of oxidative transformations on reaction with NO_2 of the thiol and tricyclic forms of thiamine, which are in equilibrium with

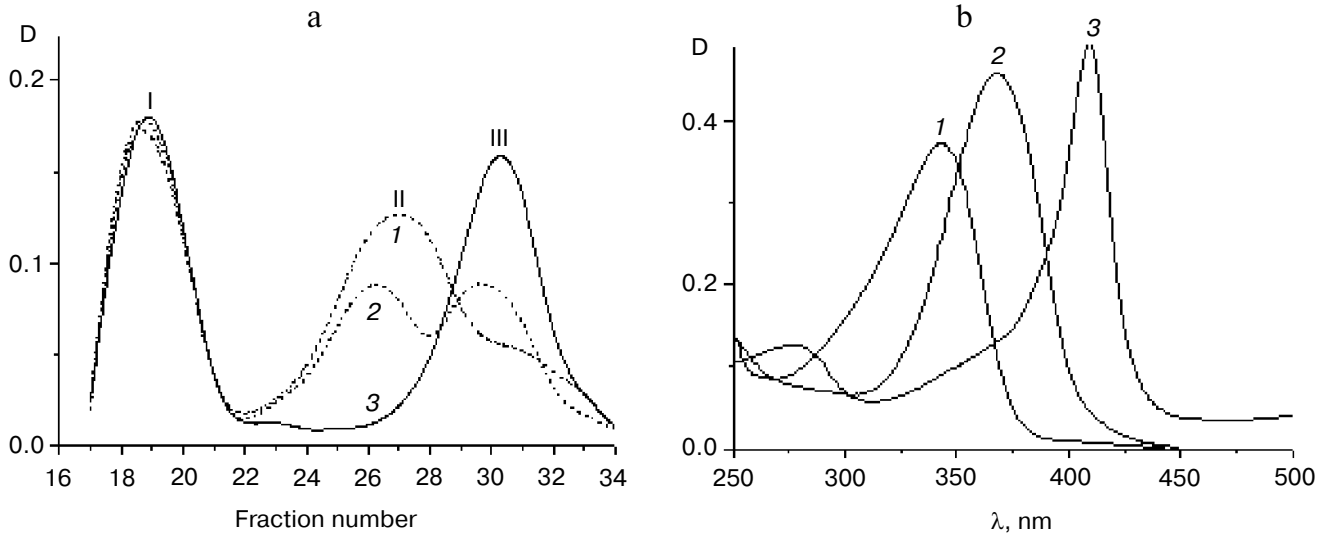


Fig. 4. a) Chromatographic separation of mixtures containing metmyoglobin, Tch, nitrite, H_2O_2, and EDTA for various incubation times (*1*, *2*) and a mixture containing metmyoglobin, Tch, H_2O_2, and EDTA (*3*) on Sephadex G-25. Concentrations: metmyoglobin, 10 μM; thiochrome, 15 μM; nitrite, H_2O_2, and EDTA, 1 mM. Incubation time (min): *1*) 20; *2*) 30; *3*) 20. Then each solution (1 ml) was applied on a column with Sephadex G-25. Elution was performed with 0.05 M sodium-phosphate buffer. Fraction volume 3 ml, absorption of fractions was recorded at 355 nm. Peaks I, II, and III were ascribed to metmyoglobin, ODTch, and Tch, respectively. b) Absorption spectra of ODTch, Tch, and metmyoglobin (*1-3*, respectively) in 0.05 M phosphate buffer, pH 7.0. Concentrations: ODTch and Tch, 20 μM; metmyoglobin, 2.5 μM.

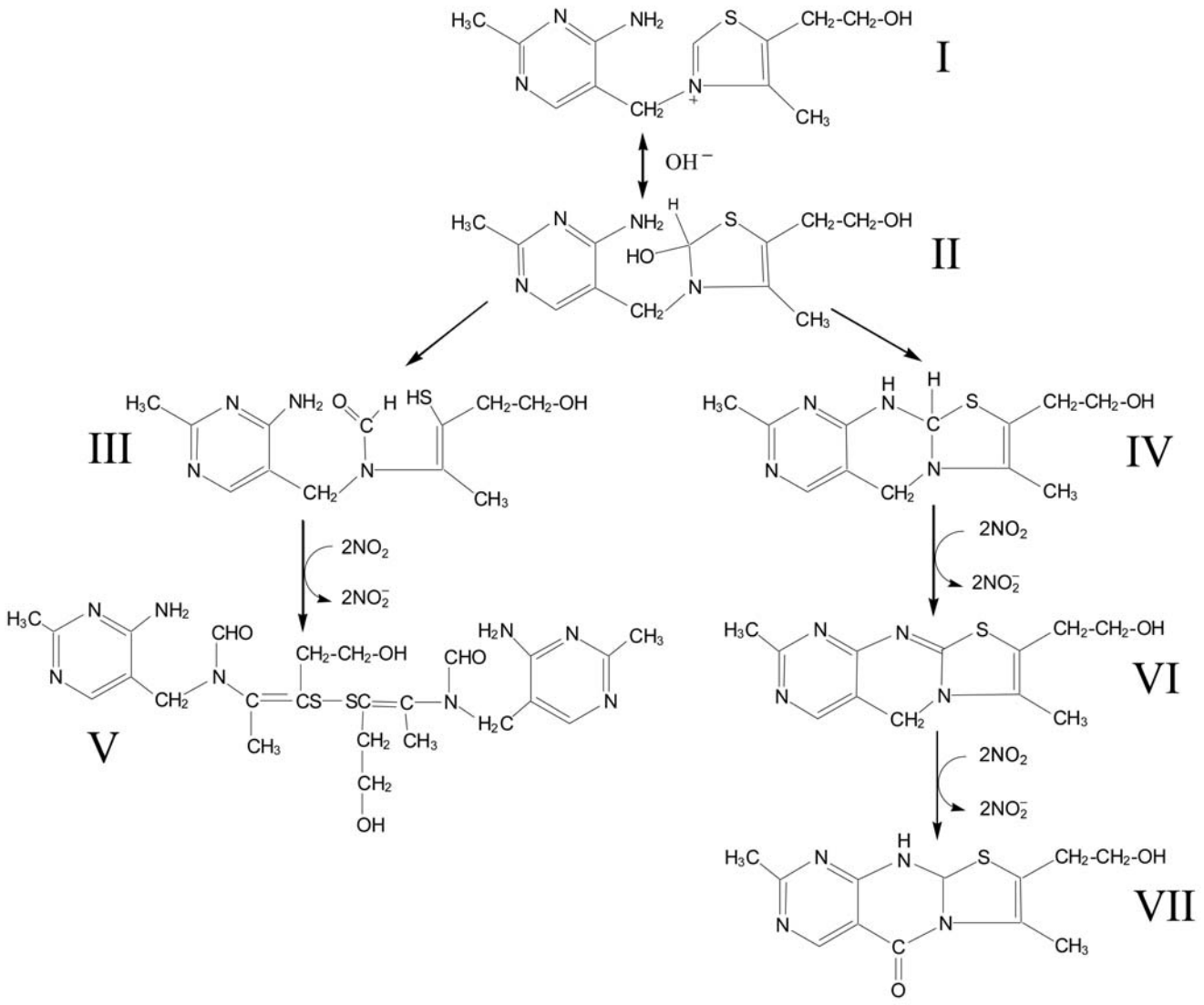


Fig. 5. Scheme of oxidative transformations of thiamine in neutral and basic media on reaction with NO_2. I) neutral form of thiamine with the closed thiazole cycle; II) thiamine pseudo-base; III) TSH; IV) Tp; V) TDS; VI) Tch; VII) ODTch.

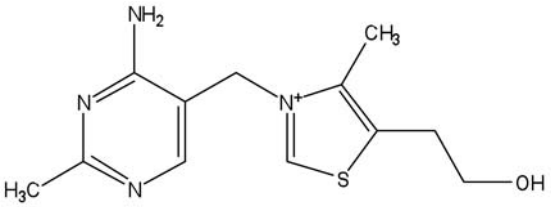
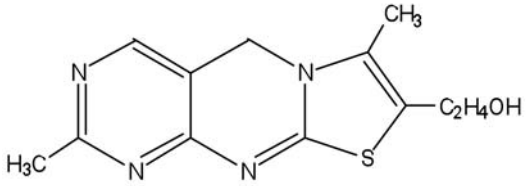
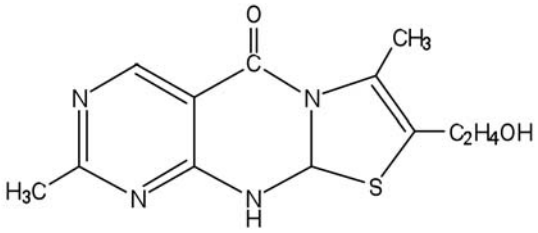
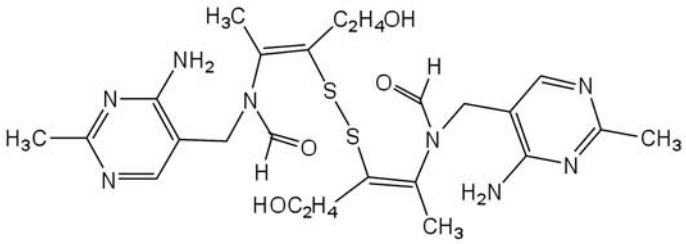
the thiamine form with the closed thiazole cycle in neutral and basic media.

The data indicate that Tch is not a final product of Tp oxidation on reaction with NO_2 but is further transformed to ODTch. TSH is oxidized to TDS by NO_2. The yield of TDS increased with increasing pH proportionally to TSH concentration in solution.

Fluorescence spectral properties of Tch and ODTch were studied in several polar solvents and polyvinyl alcohol films (structural formulas of the compounds are given in Table 4). It was found that fluorescence kinetics of Tch neutral form could be satisfactorily described by monoexponential decay (Fig. 6).

Fluorescence decay lifetimes τ for aerobic solutions of Tch in water and ethanol are presented in Table 5. The maximal τ values were observed for Tch ethanol solutions, whereas in aqueous solutions at neutral pH the τ values decrease 2.1-2.2-fold. Fluorescence quantum yields of ODTch in butanol, ethanol, and water at pH 7.4 were 0.26, 0.30, and 0.03, respectively. For ODTch, the kinetics of fluorescence decay in solutions was also monoexponential, and the lifetime was 1.9 nsec (polyvinyl alcohol), ~1.8 nsec (ethanol, butanol), and <0.3 nsec (water, pH 7.4). Analysis of fluorescence spectral characteristics of Tch and ODTch indicates that in water at physiological pH the absorption spectra of Tch and ODTch overlap significantly, and the fluorescence maxima of Tch and ODTch are practically equal (444 and 445 nm, respectively). Fluorescence quantum yield of ODTch in water is an order of magnitude lower than that of Tch. That is why it is rather difficult to detect ODTch in the presence of Tch, especially at low concentrations.

Table 4. Structural formulas of thiamine and products of its oxidative transformation

Structural formulas	Compound name
	thiamine
	thiochrome
	oxodihydrothiochrome
	thiamine disulfide

DISCUSSION

Oxidation of thiamine by long-lived tyrosyl radicals localized on the protein of the oxoferryl forms of myoglobin and hemoglobin. Free radicals are known to be intermediates in some enzymatic reactions; they are localized on amino acid residues of the protein [64]. For example, it was shown that tyrosyl and tryptophanyl radicals are formed during catalysis by prostaglandin-H-synthase and cytochrome *c* peroxidase, respectively [65]. In these cases the free radicals interact with substrate molecules and play an important role in the enzymatic reactions. However, it is well known that free radicals including free radicals on proteins are responsible for development of pathological processes [66-68].

During ischemia–reperfusion and inflammation the concentrations of active forms of nitrogen and oxygen increase greatly. In these cases free protein radicals are formed due to the attack of the active forms of nitrogen and oxygen on the functional groups of amino acid residues of the polypeptide chains. Free protein radicals cause formation of intermolecular crosslinks with free-radical forms of other macromolecules or biomolecules, initiate formation of protein peroxides and reactions of

Table 5. Fluorescence spectral properties of Tch and ODTch in aqueous solution at neutral pH and in ethanol

Compound	In water				In ethanol			
	λ_{abs}, nm	λ_{fl}, nm	*F*	τ, nsec	λ_{abs}, nm	λ_{fl}, nm	*F*	τ, nsec
Tch	374	445	0.28	1.7	369	430	0.75	3.8
ODTch	349	444	0.03	–	351	415	0.3	1.8

Note: *F*, fluorescence quantum yield; τ, fluorescence decay lifetime.

nitration and hydroxylation of amino acid residues, and also cleavage of peptide bonds [66].

NO interacts with hemoproteins, oxygen, and especially efficiently with superoxide and free protein radicals. Interaction with hemoproteins and with free protein radicals modulates the local NO concentration. These reactions are accompanied by formation of the active forms of nitrogen peroxynitrite and NO_2, which are more powerful oxidants than NO. NO_2 is formed during autooxidation of NO, on decomposition of peroxynitrite [69], on oxidation of nitrite by peroxidases, and also by the oxoferryl forms of myoglobin and hemoglobin [41].

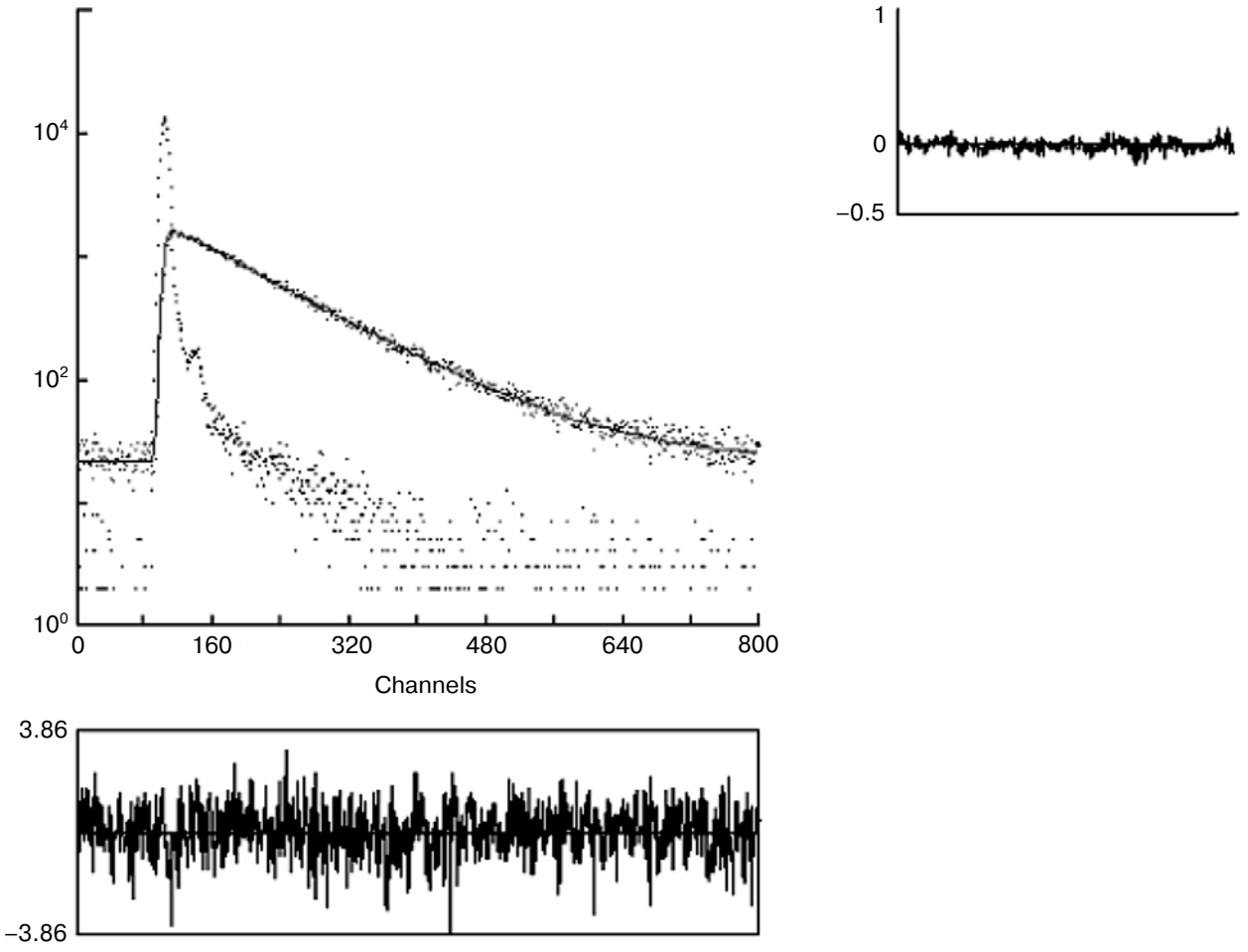


Fig. 6. Kinetics of Tch fluorescence decay in ethanol. The instrument response function and experimental fluorescence decay curve are given by dots. Calculated curve of fluorescence kinetics (line) corresponds to monoexponential decay with τ = 3.8 nsec. Plots of weighted residuals and their autocorrelation function are presented in the lower and right parts of the figure, respectively. Time/channel = 36 psec, λ_{ex} = 405 nm, λ_{fl} = 440 nm.

Mixing of metmyoglobin with H_2O_2 results in formation of the oxoferryl forms of myoglobin, which are easily detected spectrophotometrically [31, 41, 70].

It was shown that there are two oxoferryl forms of myoglobin in solution: an oxoferryl form with a radical localized on porphyrin of the heme or on the protein ($^{+\bullet}$Mb(IV=O), compound I) and also an oxoferryl cation of myoglobin Mb(IV=O) (compound II). Due to intramolecular charge transfer an oxoferryl form with a radical localized on porphyrin of heme, $^{+\bullet}$Mb(IV=O), is transformed to an oxoferryl form containing long-lived tyrosyl radicals, e.g. on Tyr103 residues, which are stable for several hours (Mb(IV=O)–$^{\bullet}$Tyr103) [71, 72]. After removal of excess H_2O_2 by catalase the oxoferryl form of myoglobin Mb(IV=O) exists in solution [29, 41]; it is stable for a rather long time. For example, the half-time of conversion of the oxoferryl form of myoglobin Mb(IV=O) to the ferric form was 180 min under our experimental conditions (Table 1). This slow process suggests that reduction of the oxoferryl complex of the macromolecule Mb(IV=O) to the ferric cation of this macromolecule occurs due to intermolecular charge transfer from a functional group of another macromolecule. High thiamine concentration (≥1 mM) in the absence of free H_2O_2 in solution caused transformation of the oxoferryl form of hemoprotein Mb(IV=O) (compound II) to the ferric form within several minutes (Table 1).

The concentration of the formed Tch did not exceed 3% of the concentration of the reduced oxoferryl form of myoglobin (Table 2). Thus, one can conclude that Tp (as Tch precursor) does not significantly contribute to reduction of the oxoferryl form of myoglobin (2-3%). However, the concentration of the disulfide form of thiamine was almost equal to the concentration of the ferric myoglobin. That is why we suppose that reduction of the oxoferryl form of myoglobin to the ferric form, when only thiamine is present in solution, occurs mainly due to TSH that is oxidized to TDS:

$$Mb(IV{=}O) + TSH \rightarrow TS^{\bullet} + Mb(III)OH_2, \quad (13)$$

$$2TS^{\bullet} \rightarrow TDS. \quad (14)$$

However, the situation is quite different when thiamine interacts with the oxoferryl form of myoglobin or hemoglobin in the presence of tyrosine or phenolic compounds. Tyrosine easily penetrates into the heme pocket and reduces the oxoferryl complex Fe(IV=O) in Mb(IV=O) to the ferric cation, and radicals localized on the porphyrin cycle or tyrosine residues of protein to a neutral porphyrin molecule or to tyrosine residues of the protein, respectively [29, 41]. As a result, the tyrosine molecule is oxidized to tyrosyl radical:

$$^{+\bullet}Mb(IV{=}O) + Tyr \rightarrow Mb(IV{=}O) + {}^{\bullet}Tyr,$$

$$k_{15} = 6{\cdot}10^2\ M^{-1}{\cdot}sec^{-1}, \quad (15)$$

$$Mb(IV{=}O) + H^+ + Tyr \rightarrow Mb(III)OH_2 + {}^{\bullet}Tyr,$$

$$k_{16} = 40\ M^{-1}{\cdot}sec^{-1}. \quad (16)$$

Tyrosyl radicals interact with each other forming a dimer – dityrosine.

After addition of tyrosine to the incubation mixture the rate of thiamine oxidation to Tch increases (Table 2) while the yield of dityrosine in the presence of thiamine decreases. The decreased dityrosine formation and increased Tch yield indicate that Tp along with TSH take part in the reduction of tyrosyl radicals to tyrosine, Tp being oxidized to Tch. Consequently, tyrosyl and phenoxyl radicals oxidize Tp to Tch and TSH to TDS (reactions (6) and (7)) [47, 48].

The contribution of Tp to reduction of tyrosyl radicals thus increases and can exceed the contribution of TSH (Table 2). At high concentrations of tyrosine or phenolic compounds Tch concentration in solution approaches that of the oxoferryl form. This is caused by the fact that tyrosine more efficiently penetrates into the heme pocket and with the higher rate interacts with a radical of the porphyrin cycle (reaction (15)) or the oxoferryl complex Fe(IV=O) in Mb(IV=O) (Table 1, reaction (16)) than TSH (reaction (13)). Reactions (15) and (16) inhibit reaction (13). According to Table 1, nitrite and tyrosine reduce the oxoferryl forms of hemoproteins to ferric forms with the maximal efficiency. Glutathione almost fails to penetrate into the heme pocket and reduce the oxoferryl form to the ferric form (Table 1). However, addition of even low glutathione concentrations (e.g. <0.1 mM) causes significant inhibition of Tch formation (Table 4) and also decreases TDS concentration. Glutathione efficiently interacts with tyrosyl radicals and inhibits formation of dityrosine (Table 2). That is why we suppose that glutathione interacts also with long-lived tyrosyl radicals localized on the protein, reducing them to tyrosine residues; this inhibits formation of Tch as well as TDS. However, glutathione inhibits Tch formation more efficiently than it does TDS formation. In fact, high glutathione concentrations completely inhibit Tch formation but not TDS formation. It is possibly due to TDS formation not only in reaction (7) but also as a result of the interaction of TSH with the oxoferryl complex Fe(IV=O) composing the hemoprotein (reactions (13) and (14)). Thus, the data suggest that the minor thiamine forms, Tp and TSH, are oxidized by long-lived tyrosyl radicals localized on the protein. The oxoferryl complex of compound II of myoglobin or hemoglobin seems to be accessible to TSH but not to Tp.

Conjugated oxidation of thiamine and nitrite in reaction catalyzed by metmyoglobin (methemoglobin) and H_2O_2. For a solution containing metmyoglobin and H_2O_2 in the absence of nitrite, the Soret band is at 422 nm, which is typical of the oxoferryl forms of myoglobin. At high nitrite concentrations in solutions containing met-

myoglobin and H_2O_2 or methemoglobin and H_2O_2, the Soret bands are at 408 and 407 nm, respectively (Figs. 1 and 2). This indicates that in the presence of excess nitrite the equilibrium in solution containing hemoprotein and H_2O_2 is shifted towards formation of ferric hemoprotein even at high H_2O_2 concentrations. This means that in this case the rate of reduction of the oxoferryl forms by nitrite is higher (reactions (8) and (9)) than, e.g. the rate of interaction of H_2O_2 with metmyoglobin (reaction (1)).

Nitrite reduces the oxoferryl forms of hemoproteins to the ferric forms (Table 1) more efficiently than tyrosine or TSH and itself is oxidized to NO_2 (reactions (8) and (9)). NO_2 formed on the interaction of nitrite with the oxoferryl forms of hemoproteins (reactions (8) and (9)) causes dismutation to N_2O_4 [41]:

$$2\,^{\cdot}NO_2 \rightarrow N_2O_4,$$

$$k_{17} = 4.5\cdot10^8\ M^{-1}\cdot sec^{-1}. \quad (17)$$

In water N_2O_4 forms nitrite and nitrate anions:

$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^-. \quad (18)$$

Nitrite for the second time interacts with the oxoferryl forms of myoglobin or hemoglobin and finally is gradually oxidized to nitrate.

In neutral aqueous solution thiamine is mainly present in a form with the closed thiazole cycle, and the minor forms Tp and TSH are present in trace amounts. The oxoferryl forms of myoglobin and hemoglobin oxidize the minor forms of thiamine. The yield of the products of thiamine oxidation by the oxoferryl forms of hemoproteins (in the absence of nitrite) was very low (Table 3): it was ≤0.1% of the total thiamine concentration in solution.

In the presence of nitrite the yield of Tch and ODTch in solution containing the ferric hemoprotein (metmyoglobin or methemoglobin), thiamine, and H_2O_2 drastically increased (Fig. 2 and Table 3). In the presence of nitrite and excess H_2O_2, up to 25% of the thiamine in solution was oxidized to Tch or ODTch. ODTch, which is formed via oxidation of Tch by NO_2 (Fig. 3), can be easily isolated from the reaction mixture by gel filtration on Sephadex G-25 (Fig. 4).

The data indicate that NO_2 molecules oxidize the minor Tp form to Tch:

$$Tp + {}^{\cdot}NO_2 \rightarrow {}^{\cdot}Tp + NO_2^-, \quad (19)$$

$$^{\cdot}Tp + {}^{\cdot}NO_2 \rightarrow Tch + NO_2^-, \quad (20)$$

where $^{\cdot}$Tp is a radical of the tricyclic form of thiamine. Tch is oxidized to ODTch by NO_2. Formation of ODTch is also accompanied by regeneration of NO_2 to nitrite (reactions (10) and (11)).

TSH is transformed to TS$^{\cdot}$, the free-radical thiyl form of thiamine:

$$TSH + {}^{\cdot}NO_2 \rightarrow TS^{\cdot} + NO_2^- + H^+, \quad (21)$$

which then forms TDS via a dismutation reaction (14).

Conjugated peroxidase oxidation of thiamine and nitrite occurs in solution containing metmyoglobin (or methemoglobin), H_2O_2, thiamine, and nitrite. In this process NO_2 molecules are reduced to nitrite due to thiamine oxidation to TDS, Tch, and ODTch. Nitrite acts as an intermolecular "shuttle" that provides electron transfer, inactivating a free radical of the heme porphyrin (reaction (8)), reducing the oxoferryl heme complex Fe(IV=O) to ferric cation (reaction (9)), and oxidizing thiamine to Tch and ODTch (Fig. 5).

High nitrite concentrations exceeding thiamine concentrations caused prevailing formation of ODTch. In contrast, if thiamine concentration was significantly higher than nitrite concentration, Tch was the main product of thiamine oxidation.

Thiamine monophosphate and TDP were oxidized by NO_2 to phosphoric esters of TDS, Tch, and ODTch. Unlike Tch (Fig. 6), ODTch in aqueous solutions has low fluorescence intensity, short fluorescence lifetime, and the absorption and fluorescence spectra of ODTch and Tch essentially overlap (Table 5). That is why it is difficult to detect low ODTch concentrations in the presence of Tch. We suppose that the absence of literature data on ODTch formation in the course of oxidative transformation of thiamine is due to these facts.

NO_2 interacts with tyrosine yielding 3-nitrotyrosine and dityrosine and is transformed to N_2O_4 due to a dismutation reaction [41]. At high concentrations of phenolic compounds they completely "intercept" NO_2, and thiamine is oxidized by phenoxyl radicals only to Tch and TDS (Table 3). NO_2 is easily dissolved in hydrophobic components of cell membranes and penetrates into hydrophobic parts of proteins. That is why the presence of a sufficient amount of hydrophobic antioxidants capable of inactivating not only active oxygen but also active nitrogen forms in cell membranes is very important for normal cell functioning. Unfortunately, antioxidants widely used at the present time do not efficiently protect the cardiovascular system from damage during oxidative stress [73].

The data suggest that thiamine and its hydrophobic metabolite Tch can play an important antioxidative role in protection of cell structures from the damaging action of peroxynitrite and NO_2. Structural properties of the thiamine molecule provide the existence of such minor forms as Tp and TSH in equilibrium with the neutral form of thiamine with the closed cycle in neutral medium (Fig. 5). We have shown that the minor thiamine form Tp is easily oxidized to Tch by NO_2, and then NO_2 oxidizes Tch to ODTch.

Consequently, one thiamine molecule in the form of Tp is able to reduce four NO_2 molecules to nitrite (Fig. 5). The solubility of Tch in hydrophobic medium is ~100 times higher than that of thiamine. That is why even low Tch concentrations are able to have protective action on cell membranes. We suppose that thiamine and its non-coenzymatic forms (e.g. thiamine monophosphate) may be "alternate antioxidants". Their antioxidant action manifests itself only on increase in concentrations of NO_2, tyrosyl, and phenoxyl radicals including long-lived tyrosyl protein radicals and on prolonged oxidative stress accompanied by the lack of low molecular weight antioxidants. The equilibrium Tp concentration is very low, and that is why the yield of Tch is also low on interaction with H_2O_2 in aqueous solutions of thiamine in the absence of ferric hemoproteins (Table 3). However, the action of NO_2, tyrosyl or phenoxyl radicals generated by the oxoferryl forms of hemoproteins caused increased Tch yield: it attained 20% of the initial thiamine concentration in solution. So, we observed mutual complimentary action of thiamine and nitrite (or phenolic compounds) on destruction of the toxic oxoferryl forms of myoglobin and hemoglobin and also on reduction of NO_2 to nitrite.

As known, in spite of the action of catalase and glutathione peroxidase, a steady-state H_2O_2 concentration is constantly present in human blood [37]; H_2O_2 oxidizes methemoglobin to the oxoferryl form having free radicals on the protein [38]. The steady-state concentration of free-radical oxoferryl form of hemoglobin in whole human blood is ~1 μM; it depends on the deoxyhemoglobin/oxyhemoglobin ratio [38]. Nitrite concentration in human blood usually does not exceed 1 μM, but it can significantly increase in pathological states and on delivery of nitrite-containing foods and drugs [3].

We have demonstrated *in vitro* that direct interaction of micromolar concentrations of the oxoferryl forms of myoglobin or hemoglobin and also NO_2 and tyrosyl radicals not only with high (≥1 mM) thiamine concentrations but also with its low concentrations beginning with 0.1 μM is observed (Table 3 and Fig. 2).

The data suggest that thiamine and its hydrophobic metabolite Tch together with phenol-containing compounds can be used for maintenance of endothelium function during the development and vascular damage from oxidative and nitrosyl stress.

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