

Two Mechanisms for Toxic Effects of Hydroxylamines in Human Erythrocytes: Involvement of Free Radicals and Risk of Potentiation

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ABSTRACT: The toxic potency of three industrially used hydroxylamines was studied in human blood cells *in vitro*. The parent compound hydroxylamine and the O-ethyl derivative gave very similar results. Both compounds induced a high degree of methemoglobin formation and glutathione depletion. Cytotoxicity was visible as Heinz body formation and hemolysis. High levels of lipid peroxidation occurred, in this respect O-ethyl hydroxylamine was more active than hydroxylamine. In contrast H₂O₂ induced lipid peroxidation was lowered after O-ethyl hydroxylamine or hydroxylamine treatment, this is explained by the ferrohemoglobin dependence of H₂O₂ induced radical species formation. Glutathione S-transferase (GST) and NADPH methemoglobin reductase (NADPH-HbR) activities were also impaired, probably as a result of the radical stress occurring. The riboflavin availability was decreased. Other enzyme activities glutathione reductase (GR), glucose 6-phosphate dehydrogenase (G6PDH), glucose phosphate isomerase and NADH methemoglobin reductase, were not or only slightly impaired by hydroxylamine or O-ethyl hydroxylamine treatment.

A different scheme of reactivity was found for N,O-dimethyl hydroxylamine. This compound gave much less methemoglobin formation and no hemolysis or Heinz body formation at concentrations up to and including 7 mM. Lipid peroxidation induction was not detectable, but could be induced by subsequent H₂O₂ treatment. GST and NADPH-HbR activities and riboflavin availability were not decreased. On the other hand GR and G6PDH activities were inhibited. These results combined with literature data indicate the existence of two different routes of hematotoxicity induced by hydroxylamines. Hydroxylamine as well as O-alkylated derivatives primarily induce methemoglobin, a process involving radical formation. The radical stress occurring is probably responsible for most other effects. N-alkylated species like N,O-dimethyl hydroxylamine primarily lead to inhibition of the protective enzymes G6PDH and GR. Since these enzymes play a key role in the protection of erythrocytes against oxidative stress a risk of potentiation during mixed exposure does exist.

Keywords: hydroxylamines, human erythrocytes, oxidizing effects, potency ranking, structure activity relations

INTRODUCTION

Hydroxylamines are derivatives of the parent compound hydroxylamine (chemical formula H₂NOH). Hydroxylamine itself is used in the synthesis of caprolactam, which is used for the

production of Nylon 6TM. It is also used as a reducing agent in photography, as an antioxidant for soaps and fatty acids, as a tanning agent and as an intermediate in the production of pesticides and pharmaceuticals (1,2). Derivatives of hydroxylamine often serve as intermediates in

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chemical synthesis. The main toxic effect of hydroxylamine is of hematological nature (1,2). Methemoglobin formation was found *in vitro* (3-5). In *in vivo* animal studies anemia with increased levels and sometimes with sulfhemoglobinemia was consistently found (6,7). In some studies this was found to be accompanied by remarkable splenomegaly (8-10). In a subchronic (ninety days) study with rats, performed according to guidelines of the Organization for Economic Co-Operation and Development (OECD), exposure to 250 ppm hydroxylamine-sulfate lead to hematological effects and increased spleen weight and some other effects (11). In the 50 ppm treated groups small increases in reticulocyte numbers and some other marginal effects were found. The "no effect level" in this study was between 10 and 50 ppm for both sexes.

In his review Gross (2) quotes a single incidence of human hydroxylamine poisoning described in the Russian literature. A woman who had drunk about "two swallows" of hydroxylamine solution in water developed rapidly progressing hemolytic anemia which required five weeks for recovery.

Martin et al. (12) described the development of serious anemia in five laboratory workers working with methylated hydroxylamines (O-methyl, N,O-dimethyl and trimethyl hydroxylamine). In the most acute patient lowered hemoglobin and increased serum iron levels were found, indicating hemolytic anemia. All patients had shown jaundice symptoms and had increased numbers of bone marrow erythroblasts. Reticulocyte numbers were increased in two patients. Heinz bodies were not found and could not be detected spectrophotometrically. These findings prompted the authors to further *in vitro* studies. Interestingly trimethyl hydroxylamine in these studies gave by far the highest hemolytic activity, while it did not lead to increased methemoglobin levels. In contrast the far less hemolytic O-methyl hydroxylamine and the N,O-dimethyl compound did strongly induce methemoglobin formation. The higher hemolytic

potency of trimethyl, and to a much lesser extent N,O-dimethyl hydroxylamine, was found to be accompanied with strong glucose 6-phosphate dehydrogenase (G6PDH) inhibition and loss of reduced glutathione (GSH).

We recently described the *in vitro* hematotoxic effects of three methylated hydroxylamines: O-methyl hydroxylamine, N-methyl hydroxylamine and N,N-dimethyl hydroxylamine (13). This study also suggested that different mechanisms for the hematotoxicity of hydroxylamines may exist. In one route methemoglobin formation is a major effect, while G6PDH inhibition is predominant in the other. Both routes seem to be able to give rise to hemolytic anemia. The purpose of the present study was to further elucidate these two mechanisms. Three industrially used hydroxylamines were tested: hydroxylamine, O-ethyl hydroxylamine and N,O-dimethyl hydroxylamine. Next to the effects described above we were especially interested in the fact that the methemoglobin formation by hydroxylamine does involve a radical mechanism (14,15), and therefore might give rise to free radical induced lipid peroxidation.

MATERIALS AND METHODS

Chemicals

The following chemicals were used: N,O-dimethyl hydroxylamine (methoxymethylamine, CAS-nr 1117-97-1, purity 99.5%) and O-ethyl hydroxylamine (ethoxyamine, CAS-nr 624-86-2, 55% (w/v) solution in water), were a gift from DSM Special Products (Geleen, NL). Brilliant cresyl blue was from BDH (Poole, UK), malonaldehyde bis(diethylacetal) (used as precursor for malondialdehyde reference) was from Janssen Chimica (Beerse, Belgium), 2-thiobarbituric-acid (TBA) was from Merck (Darmstadt, FRG), glucose 6-phosphate dehydrogenase (grade XI from *Torula* yeast) (G6PDH), glutathione reductase (type III) (GR), hydroxylamine HCl (Hydroxylamine, CAS-nr: 5470-11-1 (free base CAS-nr: 7803-49-8) (ACS

reagent, purity 99.1%) and all other biochemicals were from Sigma (St. Louis, Mo., USA). All inorganic chemicals were of analytical quality. Only microfiltrated deionized water was used.

Blood Samples

Human blood samples were collected in vacuum tubes containing K_3EDTA as an anticoagulant. For the determinations of Heinz body formation, lipid peroxidation, peroxidation resistance, glutathione S-transferase and glutathione reductase activities fresh samples from individual persons were used. For all other experiments pooled blood samples from three to five persons with blood group O-positive were used. The latter samples were checked for viral infections and stored at 4C until the next day.

Incubations

To 1 ml blood 100 μ l hydroxylamine solution in 100 mM KH_2PO_4/Na_2HPO_4 of pH 7.4 were added, and the samples were incubated in a shaking waterbath (80 rpm) for 1 hour at 37C. All experiments were performed three times on separate days and with different blood samples, each day all incubations were performed in duplicate. Except for the determinations of Heinz bodies, hemolysis and lipid peroxidation, erythrocytes were washed three times with five volumes of cold phosphate buffered saline (15 mM KH_2PO_4/Na_2HPO_4 + 130 mM NaCl; pH=7.4). Where applicable the erythrocytes were lysed by addition of three to five volumes of cold water, after 15 minutes storage on ice cellular debris was removed by centrifugation at 13000 g for 10 min.

Analyses

Heinz body formation

Heinz bodies were stained by incubation of 1 ml blood with 0.5 ml 1% (w/v) brilliant cresyl blue in saline. More severe cellular damage leads

to detectable numbers of Heinz bodies after shorter incubation times and at lower temperatures. For this reason, one of two identical samples was incubated at room temperature and the other at 37C and samples were dried and evaluated by light microscopy (1000x) under oil immersion (16) after 20, 60 and 120 minutes of coloring.

Hemolysis

After incubation, the hemoglobin (Hb) content of the plasma was used to determine the degree of hemolysis (17).

Methemoglobin

The percentage present in hemolysates was calculated from the absorbance change after addition of KCN compared to the same change in a sample fully converted to the methemoglobin form by the addition of $K_3Fe(CN)_6$ (18).

Total glutathione (GSH+GSSG)

Total glutathione was determined after precipitation of protein (200 μ l packed cells plus 200 μ l 8% (w/v) trichloroacetic acid) using the cyclic oxidation-reduction method essentially as described by Anderson (19). Especially for the determination of GT, it was of interest whether the Torula yeast GR enzyme used was itself inhibited by any of the hydroxylamines studied. This was tested separately with hydroxylamine concentrations of 0, 2.5 and 7 mM, no significant inhibition of Torula yeast GR was found under the assay conditions used.

Reduced glutathione (GSH)

GSH was determined as non-protein sulfhydryl after trichloroacetic acid precipitation of erythrocytes by measurement of the reactivity towards 5,5'-dithio-bis(2-nitrobenzoic acid) as previously described (20).

Release of thiobarbituric-acid reactive substances (TBARS)

The accumulation of lipid peroxidation products in the extracellular medium was assessed by determination of the amount of TBARS released and was expressed as malondialdehyde equivalents (21). After incubation of 1.5 ml fresh blood with hydroxylamines, the plasma was collected after centrifugation (1500 g for 10 min). The protein was precipitated by addition of an equal volume of 10% (w/v) trichloroacetic acid and subsequent centrifugation. 1.5 ml of the supernatant was added to 1 ml TBA solution (1% (w/v) in 50 mM NaOH) and heated in a boiling waterbath for 15 min. After forced cooling, 1.25 ml butanol was added, and the two phases were thoroughly mixed. The absorbance of the butanol phase at 535 nm was determined and corrected for the background absorbance at 590 nm.

Resistance to H₂O₂ forced lipid peroxidation

The remaining resistance to H₂O₂ induced lipid peroxidation of erythrocytes pre-treated with hydroxylamines was assessed. After incubation of fresh blood samples with hydroxylamines the plasma was removed by centrifugation. The erythrocytes were washed three times to remove remaining hydroxylamines and were diluted in phosphate buffered saline to a cell concentration of 5% (v/v). To 2.5 ml erythrocyte suspension an equal volume of 20 mM H₂O₂ plus 1 mM NaN₃ was added. TBARS release was determined after reincubation at 37°C for 0, 15, 30, 45 and 60 min (22).

Free hemoglobin sulfhydryl (HbSH)

Free hemoglobin sulfhydryl groups were determined in hemolysates from the reactivity towards 4,4'-dithiodipyridine as previously described (20).

NADH methemoglobin reductase (NADH-HbR)

NADH-HbR activity in hemolysates was determined using DEAE-cellulose purified hemoglobin converted to the methemoglobin-ferrocyanide complex as substrate with the method described by Hegesh et al. (23) and some small modifications according to Bauer (24).

NADPH methemoglobin reductase (NADPH-HbR)

NADPH-HbR activity in hemolysates was determined from the reduction of methylene blue by NADPH (25). Disappearance of NADPH was followed spectrophotometrically at 340 nm. Corrections were made for spontaneous methylene blue reduction.

Glutathione S-transferase (GST)

GST (EC 2.5.1.18) activity with 1-chloro-2,4-dinitrobenzene as substrate was determined in hemolysates prepared from incubates of fresh blood samples by the addition of three volumes 1.4 mM neutralized dithiothreitol, using the method of Habig and Jakoby (26) with previously described modifications (27).

Glutathione reductase (GR)

GR (EC 1.6.4.2) activity in hemolysates was determined by a modification of the method described by Carlberg and Mannervik (28). 100 µl Hemolysate were added to 2 ml 100 mM potassium phosphate buffer, 50 µl 80 mM EDTA, 100 µl 2 mM NADPH and 100 µl 0.3 mM flavin adenine dinucleotide (FAD). After 2 min pre-incubation (37°C), the reaction was started by addition of 100 µl 7.5 mM oxidized glutathione. After 15 sec the reaction was followed spectrophotometrically at 340 nm for 2 min. The FAD was added in order to convert all enzyme to its holo form.

Glutathione reductase riboflavin activity coefficient (GR_{coeff})

The coefficient of erythrocyte GR activity with and without addition of FAD to the assay was used as an indicator of the riboflavin content. The determinations were carried out exactly as described for GR above except that FAD was left out from one of the 2 samples (18).

Glucose 6-phosphate dehydrogenase (G6PDH)

G6PDH (EC 1.1.1.49) activity in hemolysates was determined spectrophotometrically; the reduction of $NADP^+$ was followed at 340 nm (18).

Glucose-phosphate isomerase

Glucose-phosphate isomerase (EC 5.3.1.9) activity in hemolysates was determined using fructose 6-phosphate as substrate in the presence of $NADP^+$, G6PDH and 6-phosphogluconic dehydrogenase. Under these circumstances each molecule of fructose 6-phosphate converted will give rise to reduction of two molecules $NADP^+$, which was followed spectrophotometrically at 340 nm (24).

Hemoglobin (Hb)

The Hb concentrations were determined with the hemoglobin cyanide procedure (17).

Statistical Evaluation

Significance of concentration dependent changes was evaluated using linear regression analysis. In each experiment incubations were performed with 0, 1, 2.5, 5 and 7 mM hydroxylamine concentrations. All experiments were performed three times. Duplicate values obtained during a single experiment were averaged before statistical analysis since these values are not independent. This leads to a total of 3 (experiments) x 5 (concentrations) = 15 data

points for each regression analysis. The regression model used corrects for interexperimental variations in control values. For this reason the standard errors shown in the figures are given as the standard error of the mean difference between sample value and its control (i.e. the corresponding value at concentration 0).

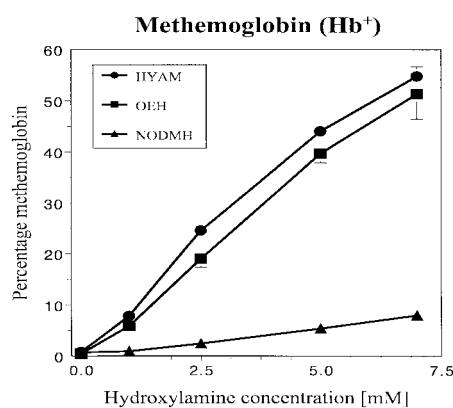


Figure 1. Formation of methemoglobin in human erythrocytes after incubation with hydroxylamines (1 hour, 37C). The error bars represent the standard error of the mean difference between the sample value and its control. Regression analyses showed significant ($P < 0.0001$) increases in methemoglobin formation with increasing concentration for all three hydroxylamines.

RESULTS

Oxidative Effects

A concentration dependent methemoglobin formation was found for all three hydroxylamines (Figure 1). The effects of hydroxylamine and O-ethyl hydroxylamine (about 8% methemoglobin formation for each mM addition) were much stronger than the effect caused by N,O-dimethyl hydroxylamine. GT decreased for all three hydroxylamines (Figure 2A). GT depletion caused by O-ethyl hydroxylamine (0.34 (SE 0.04) μ moles GT/g Hb decrease for each mM O-ethyl hydroxylamine) was much more pronounced than the effect caused by the other two hydroxylamines (0.17 (SE 0.03) and 0.12 (SE 0.02) μ moles GT/g Hb decrease for each mM for hydroxylamine and N,O-dimethyl hydroxylamine respectively). The decreases in reduced GSH also

were strongest for O-ethyl hydroxylamine (Figure 2B). The regression coefficient of this GSH decrease amounted to 0.26 (SE 0.03) $\mu\text{moles/g Hb}$ for each mM O-ethyl hydroxylamine. For hydroxylamine and N,O-dimethyl hydroxylamine these depletions were only about 0.11 (SE 0.03) and 0.07 (SE 0.03) $\mu\text{moles/g Hb}$ for each mM of the respective hydroxylamines added. Plasma GT was found to increase for all three

hydroxylamines (Figure 2C). Contrary to what might be expected from erythrocyte depletions, the increase was strongest for N,O-dimethyl hydroxylamine (about 5.4 μM increase/mM N,O-dimethyl hydroxylamine). For O-ethyl hydroxylamine and hydroxylamine increases were not more than half that amount. Loss of hemoglobin sulfhydryl group availability was not found for any of the chemicals studied.

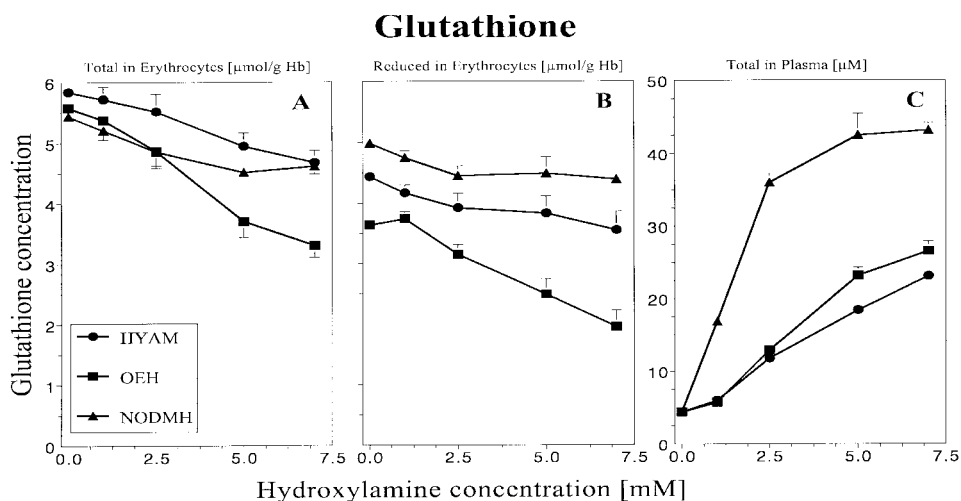


Figure 2. Availability of A: total glutathione (reduced plus oxidized) in human erythrocytes; B: reduced glutathione in human erythrocytes and C: total glutathione in human plasma, after incubation with hydroxylamines (1 hour, 37°C). The error bars represent the standard error of the mean difference between the sample value and its control. Separate experiments were performed to obtain the data for panel A, B and C. Regression analyses showed that all concentration dependent changes were significant ($P < 0.0002$ in all cases, except the decrease in reduced glutathione with increasing hydroxylamine and N,O-dimethyl hydroxylamine (panel B) where the P values were 0.007 and 0.015 respectively).

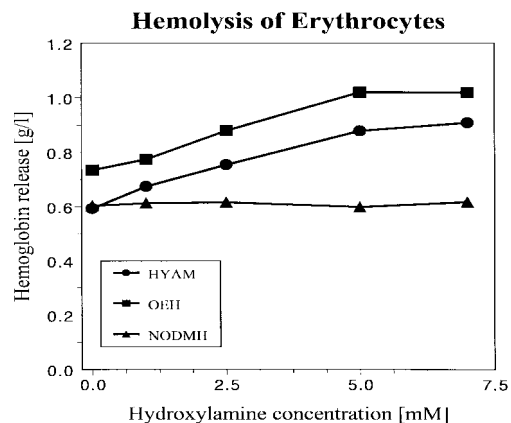


Figure 3. Occurrence of hemolysis of human erythrocytes after incubation with hydroxylamines (1 hour, 37°C). The errors bars represent the standard error of the mean difference between sample value and its control. Regression analyses showed significant ($P < 0.0001$) increases in hemolysis with increasing hydroxylamine and O-ethyl hydroxylamine concentrations.

Cell Damage

Increased hemolysis occurred for hydroxylamine and O-ethyl hydroxylamine (Figure 3). Both compounds liberated about 45 mg Hb/l from the erythrocytes for each mM added. For N,O-dimethyl hydroxylamine no increased hemolysis was detectable. Heinz bodies were visible in all blood samples treated with 2.5 mM hydroxylamine or O-ethyl hydroxylamine (Table 1). In the 2.5 mM hydroxylamine treated samples blue spots, normally indicating Heinz bodies, were also visible extracellularly. The same indications for cell breakdown were also present in the 2.5 mM O-ethyl hydroxylamine treated samples, but only when they were incubated at 37C. Minor Heinz body formation was present in the 1 mM hydroxylamine treated samples incubated at 37C or incubated for 60 min or more at room temperature as well as in the 1 mM O-ethyl hydroxylamine treated samples incubated at 37C for 60 min or more. The occurrence of Heinz bodies at a lower temperature and after shorter incubation times for hydroxylamine compared to O-ethyl hydroxylamine, indicates that the effect is strongest for hydroxylamine. In N,O-dimethyl hydroxylamine treated blood samples no Heinz body formation could be detected even at concentrations up to 10 mM.

Lipid Peroxidation

In Figure 4 the mean release of lipid peroxidation products from erythrocytes from three individuals is shown. Although there were clear interindividual differences in both susceptibility and background, the relative effect of the three chemicals was consistent in all three individuals. O-ethyl hydroxylamine clearly gave the largest release of lipid peroxidation products. The regression coefficient was 117 (SE 13) nM/mM O-ethyl hydroxylamine. The effect of hydroxylamine was about two times smaller, but still highly significant. N,O-dimethyl hydroxylamine gave only minor increases with borderline significance (P = 0.093).

The remaining resistance of erythrocytes towards H₂O₂ induced lipid peroxidation after hydroxylamine and O-ethyl hydroxylamine pre-treatment is shown in Figure 5. Fresh blood samples from three individuals were used. Although there were some interindividual differences in sensitivity the qualitative phenomena were comparable for all three persons and averaged data are shown. For both hydroxylamines pre-treatment lead to a hydroxylamine-concentration dependent decrease in the H₂O₂ induced lipid peroxidation

Table 1: Presence of Heinz bodies in human erythrocytes after incubation with hydroxylamines (1 hour, 37C). After addition of brilliant cresyl blue the erythrocytes were incubated at room temperature or 37C for a specified amount of time. Visibility of Heinz bodies is indicated as positive (+), negative (-) or visible but minor (±).

Sample	Room Temperature			37C		
	20 min	60 min	120 min	20 min	60 min	120 min
Blank	-	-	-	-	-	-
N,O-dimethyl hydroxylamine 10 mM	-	-	-	-	-	-
hydroxylamine 1 mM	-	±	±	±	±	±
hydroxylamine 2.5 mM	± ¹	± ¹	± ¹	± ¹	± ¹	± ¹
O-ethyl hydroxylamine 1 mM	-	-	-	-	±	±
O-ethyl hydroxylamine 2.5 mM	+	+	+	+ ¹	+ ¹	+ ¹

¹Blue spots were also visible extracellularly.

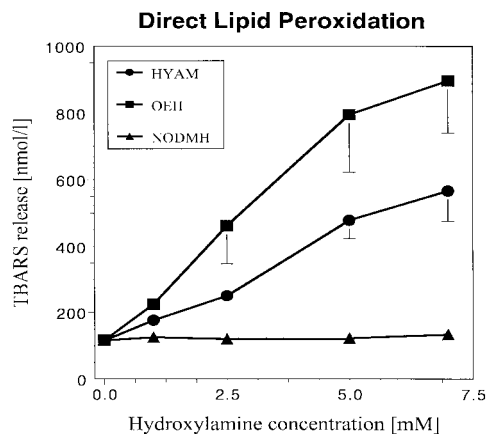


Figure 4. Lipid peroxidation, expressed as malondialdehyde equivalents in reactivity towards TBA, in blood incubated with hydroxylamines (1 hour, 37C). Regression analyses on the combined data showed significant increases in TBARS release with increasing O-ethyl hydroxylamine and hydroxylamine concentrations ($P < 0.0001$).

H₂O₂ Forced Lipid Peroxidation after Hydroxylamine Pre-Treatment

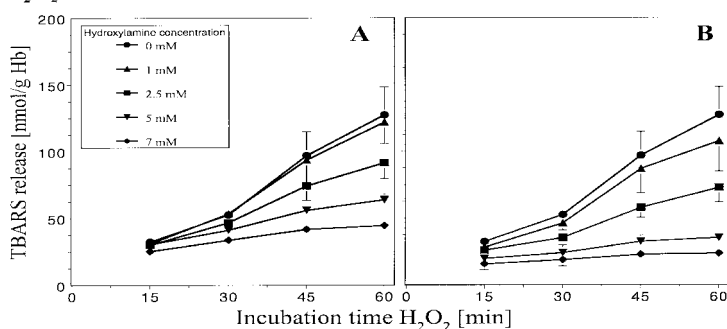


Figure 5. Hydrogen peroxide (10 mM) forced lipid peroxidation, expressed as malondialdehyde equivalents in reactivity towards TBA after pre-incubation of blood with various concentrations of A: hydroxylamine and B: O-ethyl hydroxylamine (1 hour, 37C).

H₂O₂ Forced Lipid Peroxidation after NODMH Pre-Treatment

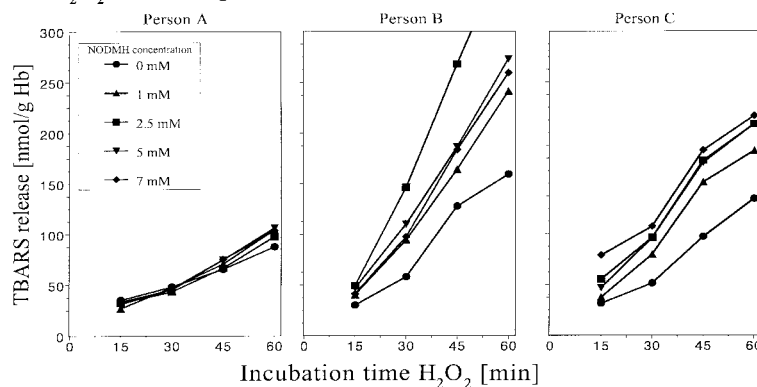


Figure 6. Hydrogen peroxide (10 mM) forced lipid peroxidation, expressed as malondialdehyde equivalents in reactivity towards TBA, in erythrocytes of three individuals after pre-incubation of blood with various concentrations of N,O-dimethyl hydroxylamine (1 hour, 37C).

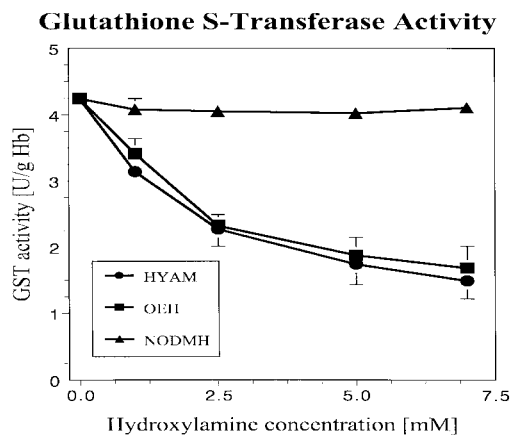


Figure 7. Activity of glutathione S-transferase in human erythrocytes after incubation with hydroxylamines (1 hour, 37C). The error bars represent the standard error of the mean difference between sample value and its control. Regression analyses showed significant decreases in GST activity with increasing hydroxylamine and O-ethyl hydroxylamine concentrations ($P < 0.0001$).

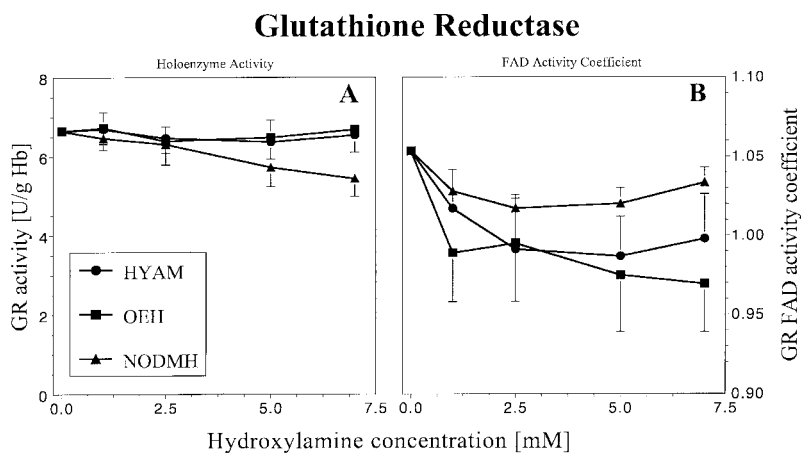


Figure 8. A: Activity of glutathione reductase (holoenzyme) and B: FAD activity coefficient of glutathione reductase in human erythrocytes after incubation with hydroxylamines (1 hour, 37C). The coefficients were calculated by division of the activity in the presence of FAD (11.75 μ M) by the activities determined in a parallel assay without FAD addition. The error bars represent the standard error of the mean difference between sample value and its control. Regression analyses showed a significant decrease in GR activity with increasing N,O-dimethyl hydroxylamine concentration ($P = 0.0002$) as well as significant decreases in FAD activity coefficients with increasing hydroxylamine ($P = 0.025$) and O-ethyl hydroxylamine ($P = 0.010$) concentrations.

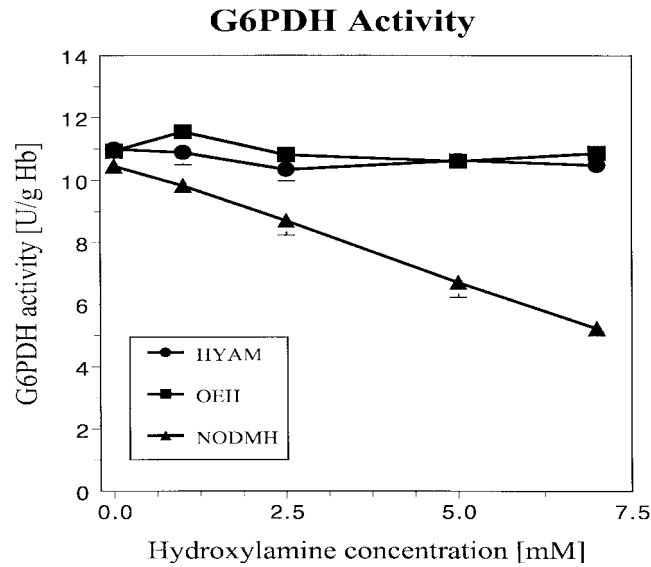


Figure 9. Activity of glucose 6-phosphate dehydrogenase in human erythrocytes after incubation with hydroxylamines (1 hour, 37C). The error bars represent the standard error of the mean difference between sample value and its control. Regression analyses showed significant decreases in G6PDH activity with increasing N,O-dimethyl hydroxylamine concentrations ($P < 0.0001$).

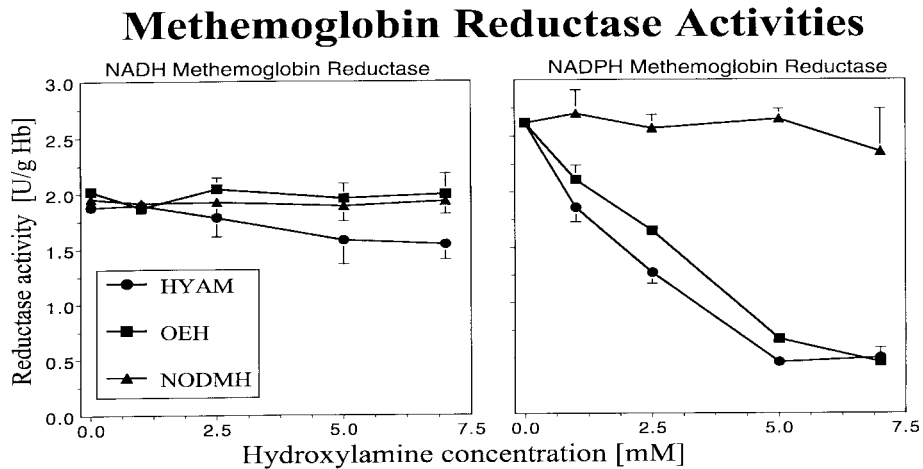


Figure 10. Activity of NADH methemoglobin reductase and NADPH methemoglobin reductase in human erythrocytes after incubation with hydroxylamines (1 hour, 37C). The error bars represent the standard error of the mean difference between sample value and its control. Regression analyses showed a significant decrease in NADH methemoglobin reductase with increasing hydroxylamine concentrations ($P = 0.002$) and significant decreases in NADPH methemoglobin concentrations with increasing hydroxylamine and O-ethyl hydroxylamine concentrations ($P < 0.0001$).

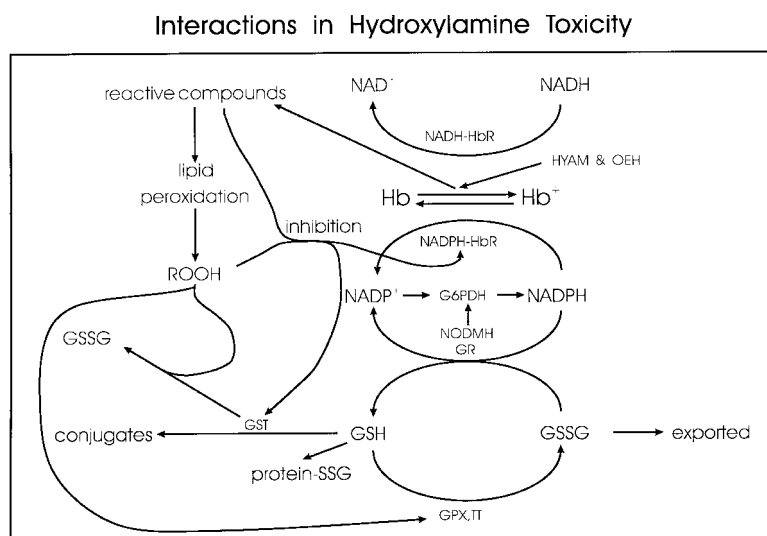


Figure 11. Schematic representation of the proposed mechanisms for hydroxylamine erythrotoxicity. hydroxylamine and the O-ethyl derivative (O-ethyl hydroxylamine) induce formation of methemoglobin. During this process free radicals are formed that induce lipid peroxidation. The free radicals plus the lipid peroxides (ROOH) formed lead to inhibition of glutathione S-transferase (GST) and NADPH methemoglobin reductase (NADPH-HbR). The oxidative stress occurring leads to depletion of glutathione (GSH) by various routes. N-substituted hydroxylamines like N,O-dimethyl hydroxylamine inhibit glucose 6-phosphate dehydrogenase (G6PDH), and to a lesser extent glutathione reductase (GR). The resulting inability to reduce NADP^+ and glutathione renders the erythrocyte more vulnerable to oxidative stress, including that caused by hydroxylamine and O-ethyl hydroxylamine. Other abbreviations used: GPX = glutathione peroxidase, Protein-S-SG = protein-glutathione mixed disulfides, TT = thiol transferase.

For N,O-dimethyl hydroxylamine the effect that occurred was more complex. The amounts of lipid peroxidation products released from erythrocytes from person A were not markedly influenced by N,O-dimethyl hydroxylamine pre-treatment. Blood samples from this individual did generally have the lowest induced lipid peroxidation both after direct treatment with hydroxylamines and after hydroxylamine and O-ethyl hydroxylamine pre-treatment followed by H_2O_2 treatment. The erythrocytes from the other two individuals showed a hydroxylamine-concentration dependent increase in H_2O_2 induced lipid peroxidation after 1 and 2.5 mM pre-treatment. In samples from person B however, the response to H_2O_2 was lower in the 5 and 7 mM pre-treated samples than the (very high) response in the 2.5 mM pre-treated samples.

Enzyme Inhibitions

GST activity was strongly decreased in both hydroxylamine and O-ethyl hydroxylamine treated samples (Figure 7). For hydroxylamine and O-ethyl hydroxylamine the GST activity decreased with about $0.35 \text{ U} \cdot \text{g Hb}^{-1} / \text{mM}$ addition. As a consequence about two-thirds of the initial GST activity was lost at the highest ($=7 \text{ mM}$) hydroxylamine and O-ethyl hydroxylamine concentrations used. For N,O-dimethyl hydroxylamine no significant changes in the GST activity were found. In contrast the GR activity was only decreased for N,O-dimethyl hydroxylamine, for this substance GR activities in 7 mM incubations were about 20% lower than the $6.7 \text{ U} \cdot \text{g Hb}^{-1}$ found in control incubations (Figure 8A). The GR_{coeff} was slightly decreased in both hydroxylamine ($P = 0.025$) and O-ethyl

hydroxylamine ($P = 0.01$) treated samples (Figure 8B). This indicates a decrease in riboflavin availability in these samples (18).

The G6PDH activity was strongly impaired by N,O-dimethyl hydroxylamine (0.76 (SE 0.04) U·g Hb⁻¹ lost/mM N,O-dimethyl hydroxylamine), leading to about 50% reduction at the highest (=7 mM) N,O-dimethyl hydroxylamine concentration tested (Figure 9). Hydroxylamine and O-ethyl hydroxylamine did not influence the G6PDH activity. The activity of another pentose-phosphate shunt enzyme tested, glucose-phosphate isomerase, was not significantly influenced by any of the three hydroxylamines. NADH-HbR activity was only impaired by hydroxylamine. The control activity of 1.9 U·g Hb⁻¹ fell to about 1.5 U·g Hb⁻¹ in 7 mM hydroxylamine incubations (Figure 10A). NADPH-HbR activity was strongly decreased in incubations with both hydroxylamine and O-ethyl hydroxylamine. For both substances the decrease amounted to about 0.3 U·g Hb⁻¹ for each mM addition, and as a result less than 20% of the control activity was found in the 7 mM incubations (Figure 10B).

DISCUSSION

On the whole the effects caused by hydroxylamine and O-ethyl hydroxylamine were very much like the effects that we recently described for O-methyl hydroxylamine (13), while the effects of N,O-dimethyl hydroxylamine were comparable to those of N-methyl hydroxylamine. A schematic representation of the mechanisms involved is given in figure 11. For hydroxylamine and O-ethyl hydroxylamine massive methemoglobin formation was found, for N,O-dimethyl hydroxylamine this was much lower. Hydroxylamine forms complexes with hemoproteins, for instance leading to inactivation of catalase (29-31). Methemoglobin formation *in vitro* was previously described for hydroxylamine (4,5,14,15,32) and N,O-dimethyl hydroxylamine (4). The mechanism of methemoglobin formation by hydroxylamine is known to differ from that

induced by nitrite (5). Reaction of hydroxylamine with hemoglobin involves Hb/Hb⁺ cycle reactions (3,30), and leads to decomposition of hydroxylamine. The methemoglobin forming reaction, which produces NH₃ from hydroxylamine, was found to be very fast, explaining the high methemoglobin concentrations found. During the other step of the cycle N₂ is formed. Stolze et al. (14,15) proved with electron spin resonance spectroscopy techniques that free hydronitroxide radicals (H₂NO•) are formed as intermediates in the methemoglobin formation induced by hydroxylamine. Stoichiometric considerations lead them to the expectation that H₂O₂ and active oxygen species might also be formed. Moreover, they proved the existence of a compound I type ferryl species, probably formed by H₂O₂ (33). This radical formation can be one of the causes for lipid peroxidation.

The reduced availability of GSH will also facilitate lipid peroxidation. GSH consumption can result from direct oxidation and from use in protective reactions catalyzed by GST and glutathione peroxidase (34). In this way lipid peroxidation may not only be facilitated by GSH depletion (35) but may also provoke it (34). This agrees with our finding that O-ethyl hydroxylamine as the substance with the highest lipid peroxidation activity also gives the highest GT depletion. Loss of 4,4'-dithiodipyridine reactive HbSH groups was not found. After treatment with electrophilic agents such a loss can be clearly demonstrated (20). However, oxidative loss of HbSH groups for instance by formation of mixed disulfides with GSH, will probably not be picked up by this assay, as dithiodipyridines are able to break GSH sulfhydryl bindings (36). We recently provided evidence that such a formation of mixed disulfides does indeed occur after hydroxylamine treatment (37) and this explains why the increases in extracellular GT (mainly present as oxidized glutathione) did not make up for the loss of GT from the erythrocytes.

Methemoglobin formation, GT depletion and

membrane damage, as indicated by lipid peroxidation, are all strongly associated with cellular damages like Heinz body formation and hemolysis. Induction of this kind of damage by the two substances with high potencies for methemoglobin formation, GT depletion and lipid peroxidation is therefore in accordance with expectations. For hydroxylamine Heinz body formation in mice (38) and in pigs (6) were previously reported.

It is well known that lipid peroxidation products can give rise to inhibition of several enzymes and to protein damage in general (39). Purified rat GST-P (7-7), which is equivalent to human GST π , the form of GST predominating in human erythrocytes (40) is very vulnerable to oxidative stress (41). The human enzyme itself is also inhibited by H_2O_2 (42). This was confirmed in control experiments where erythrocytes were treated directly with H_2O_2 (data not shown). Previously we showed that GST activity in human erythrocytes is impaired by occupational exposure to coal tar products (43) and to the pesticide dichloropropene (27), in miners with early forms of pneumoconiosis (44) and after long-distance running (45). At least in the latter two cases oxidative stress is the most likely cause for GST activity loss. Therefore, activity loss of GST under conditions causing lipid peroxidation was expected beforehand. In fact these experiments were included in order to examine the possible use of GST activity loss as a biomarker for hydroxylamine exposure. As expected, GST inactivation was only found for the strong lipid peroxidation causing substances hydroxylamine and O-ethyl hydroxylamine and not for N,O-dimethyl hydroxylamine. NADPH-HbR was also strongly inhibited by hydroxylamine and O-ethyl hydroxylamine and not by N,O-dimethyl hydroxylamine. Inhibition of NADPH-HbR by hydroxylamine was previously shown by Layne and Smith (46), and does explain the lowered effectiveness of methylene blue treatment in case of hydroxylamine poisoning in mice (7). The absence of inhibition by N,O-dimethyl

hydroxylamine indicates a possible relation between radical stress and inhibition of this reductase. Impairment of NADPH-HbR did indeed occur in control experiments where erythrocytes were treated directly with H_2O_2 (data not shown). The loss of riboflavin, as indicated by the decreases in GR_{coeff} after hydroxylamine and O-ethyl hydroxylamine treatment, might also be caused by consumption during radical scavenging.

N,O-dimethyl hydroxylamine showed far less activity in most aspects discussed so far. Methemoglobin formation was low, lipid peroxidation, hemolysis and Heinz body formation were not found, GST and NADPH-HbR inhibition were absent, and riboflavin loss was not found. On the other hand N,O-dimethyl hydroxylamine treatment of erythrocytes resulted in a decrease of G6PDH activity, consistent with the earlier findings by Martin et al. (12). GR activity was also decreased by N,O-dimethyl hydroxylamine, but since more than 80% of GR activity was still present at N,O-dimethyl hydroxylamine concentrations of 7 mM this may be of less importance. Interestingly GT concentrations in erythrocytes were decreased by N,O-dimethyl hydroxylamine while GT in plasma was even more increased than for the two other hydroxylamines. GT depletion by N,O-dimethyl hydroxylamine can be the result of oxidation in glutathione peroxidase and thiol transferase reactions, combined with lower availability of NADPH reduction equivalents for GR activity, due to G6PDH inhibition. Even the G6PDH inactivation alone will render the erythrocyte more vulnerable to oxidative stress from subsequent exposure to other oxidative compounds. This is confirmed by the fact that N,O-dimethyl hydroxylamine treated erythrocytes did show a higher vulnerability towards subsequent H_2O_2 induced lipid peroxidation. Contrary to what might have been expected hydroxylamine and O-ethyl hydroxylamine pre-treatment did not lead to an increased vulnerability to H_2O_2 . This is however easily explained. For H_2O_2 induced lipid peroxidation in

erythrocytes ferrous-hemoglobin is a prerequisite (47). After hydroxylamine and O-ethyl hydroxylamine treatment a large fraction of hemoglobin is converted to the ferric form, necessarily leading to a decrease in lipid peroxidation. It should be noted that changes in H₂O₂ resistance cannot be the result of catalase inhibition by the hydroxylamines, since NaN₃ was added to the assays in order to obtain a full inhibition of catalase. Hydroxylamine is known to inhibit catalase both *in vitro* (29,30) and *in vivo* in mouse liver (31), and this inhibition will in itself increase the vulnerability of erythrocytes to oxidative stress.

The occurrence of lipid peroxidation in erythrocytes exposed to hydroxylamine and O-ethyl hydroxylamine indicates the availability of free radicals, possibly including active oxygen species. The presence of active oxygen species and of the resulting peroxides *in vivo* is associated with increased cancer risks and accelerated aging (48-50). It should be noted however that radical production in this case seems to be strongly linked to the presence of hemoglobin. In experiments with hydroxylamine we did not see any lipid peroxidation in washed erythrocyte membranes or in isolated rat hepatocytes (51). Since erythrocytes do not contain a nucleus, direct DNA damage is not possible. On the other hand reactive products formed in erythrocytes might be able to reach other cells and provoke damage there, and similar radical mechanisms might be induced by hydroxylamines at hemoproteins elsewhere. Considering the above, the most serious threat resulting from possible exposure of humans to hydroxylamines seems to come from the direct erythrotoxic effects. Methemoglobin formation, lipid peroxidation, GT depletion and inhibition of protective enzymes can lead to faster senescence of erythrocytes and increased sequestration in the spleen. Next to clinical parameters for anemia and increases in reticulocytes, determination of GST and NADPH-HbR and assessment of lipid peroxidation *in vivo* (52) may be useful biomarkers to monitor human exposure to

hydroxylamines with direct oxidative activity, like hydroxylamine and O-ethyl hydroxylamine. Two special aspects should be noted: 1) due to the inhibition of NADPH-HbR by hydroxylamines like hydroxylamine and O-ethyl hydroxylamine and due to the inhibition of G6PDH by compounds like N,O-dimethyl hydroxylamine and trimethyl hydroxylamine, treatment of accidental methemoglobinemia with methylene blue may fail, 2) exposure to compounds like N,O-dimethyl hydroxylamine and trimethyl hydroxylamine will inhibit G6PDH and thereby will render the erythrocytes more vulnerable to a subsequent exposure to direct oxidative compounds. Because of the latter, determination of G6PDH activities may also serve as a valuable biomarker when exposure to this group of hydroxylamines can occur.

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