Methemoglobin Formation in Human Erythrocytes by Nitroaromatic Explosives

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We have examined the structure-activity relationships in methemoglobin (MetHb) formation by high explosives 2,4,6-trinitrotoluene (TNT), 2,4,6-trinitrophenyl-*N*-nitramine (tetryl) and 2,4,6-trinitrophenyl-*N*-nitraminoethylnitrate (pentryl), and a number of model nitrobenzenes. In lysed human erythrocytes the rate constants of oxyhemoglobin (OxyHb) oxidation increased with an increase in single-electron reduction potential ($E^1{}_7$) or with a decrease of the enthalpies of single-electron reduction of nitroaromatics. Tetryl and pentryl oxidized OxyHb almost 3 times faster than TNT. Although the initial rates of MetHb formation in intact erythrocytes by tetryl, pentryl, and TNT matched their order of reactivity in the oxidation of OxyHb in lysed erythrocytes, TNT was a more efficient MetHb forming agent than tetryl and pentryl during a 24-h incubation. The decreased efficiency of tetryl and pentryl was attributed to their reaction with intraerythrocyte reduced glutathione (GSH) producing 2,4,6-trinitrophenyl-Sglutathione, which acted as a less efficient OxyHb oxidizing agent.

Introduction

Nitroaromatic compounds have been used as antimicrobial agents, raw materials in industry, pesticides and explosives. As a result, they are widely distributed in the environment. Most of these compounds are toxic, mutagenic or carcinogenic (Purohit and Basu, 2000). Apart from the redox cycling of free radicals or the formation of alkylating nitroso- and hydroxylamine species caused by one- or two-electron enzymatic reduction, respectively (Guissani *et al.,*1990; Wardman *et al.,* 1995; Purohit and Basu, 2000), the formation of methemoglobin (MetHb) and the subsequent erythrocyte hemolysis and anemia is another important mechanism of toxicity of nitroaromatic compounds. MetHb is produced either under the action of nitrosobenzenes and hydroxylamines formed during the reductive metabolism of nitroaromatics by the intestine microflora, or by direct oxidation of oxyhemoglobin (OxyHb) by nitrocompounds (Facchini and Griffiths, 1981; Cossum and Rickert, 1985; Vasquez *et al.,* 1995; Chandra *et al.,* 1995).

2,4,6-Trinitrotoluene (TNT) and other polynitroaromatic explosives such as 2,4,6-trinitrophenyl-*N*-nitramine (tetryl) and 2,4,6-trinitrophenyl-*N*-nitraminoethylnitrate (pentryl) (Fig. 1) comprise an important group of potentially cytotoxic and mutagenic environmental pollutants (Whong *et al.,* 1980; Tan *et al.,* 1992; Lachance *et al.,* 1999). The mechanisms of TNT toxicity involve redox cycling with the formation of reactive oxygen species (Kong *et al.,* 1989), covalent binding to proteins (Leung *et al.,* 1995), and the induction of methemoglobinemia (Levine *et al.,* 1984; Djerassi, 1998). The mechanisms of toxicity of tetryl and pentryl are studied insufficiently.

The aim of this work was to examine methemoglobin formation in isolated human erythrocytes

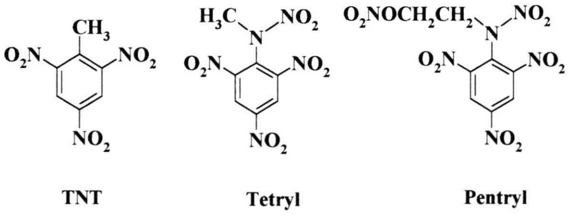

Fig. 1. Structural formulae of explosives studied in this paper.

Abbreviations: TNT, 2,4,6-trinitrotoluene; MetHb, methemoglobin; OxyHb, oxyhemoglobin; $E^1{}_7$, single-electron reduction potential; $\Delta Hf(ArNO_2^{-\cdot})$, enthalpy of single-electron reduction of nitroaromatic compound; GSH, reduced glutathione; GSSG, oxidized glutathione; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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under the action of TNT, tetryl and pentryl, which represents a potential mechanism of their toxicity. Since the structure-activity relationships in the MetHb-forming ability of nitroaromatic compounds are poorly understood, emphasis was made on the comparison of TNT, tetryl, and pentryl with a series of nitrobenzene derivatives with variable electron-accepting potency.

Materials and Methods

TNT, tetryl, and pentryl were synthesized according to the established methods (Urbanski, 1964). The purity of nitroaromatic compounds was determined using melting points, TLC, NMR, IR, and elemental analysis. All the other compounds were obtained from Sigma or Aldrich and used as received.

Freshly prepared suspensions of erythrocytes from healthy patients obtained from Vilnius Blood Transfusion Center were washed twice by centrifugation, resuspended in 0.01 M K-phosphate (pH 7.0) containing 0.137 M NaCl, 0.0027 M KCl, 10 mM glucose and 1 mM EDTA, and stored at 4 °C for not more than 7–10 days. For the kinetics studies, the erythrocytes were lysed in a buffer solution containing 40 μg/ml digitonin. The oxyhemoglobin (OxyHb) concentration was adjusted to 10–30 μM (ε_{577} = 15 $mM^{-1}cm^{-1}$ (Winterbourn, 1985)). The kinetics of methemoglobin (MetHb) formation were monitored according to the absorbance rise at 630 nm and the absorbance decrease at 577 nm ($\Delta\varepsilon_{630}$ = 3.46 $mM^{-1}cm^{-1}$, $\Delta\varepsilon_{577}$ = 10.55 $mM^{-1}cm^{-1}$ (Winterbourn, 1985)) after the addition of excess oxidant (molar ratio 1:10–100) using a Hitachi-557 spectrophotometer at 37 °C.

Intact erythrocytes were incubated with various concentrations of nitroaromatics for 24 h. The aliquots of the reaction mixture were lysed in a digitonin solution, the OxyHb and MetHb concentrations (μM) were calculated according to the absorbance at 577 nm and 630 nm: [OxyHb] = 66 A_{577} – 80 A_{630}, and [MetHb] = 279 A_{630} – 3.0 A_{577} (Winterbourn, 1985). The amount of lysed erythrocytes was determined by recording the absorbance spectra of the supernatant after centrifugation of erythrocyte suspension. The content of reduced glutathione (GSH) in erythrocytes was determined according to a modified procedure of thiol determination, assuming that GSH represents more than 95% of the nonprotein thiols in erythrocytes (Beutler and Dale, 1988). Erythrocytes at 15% hematocrite (15% v/v in buffer solution) were incubated with 300 μM of nitroaromatic compounds for 24 h at 37 °C, then cooled to 4 °C and mixed with equal volume of cold 5% sulfosalicylic acid. After the centrifugation, the supernatant (0.1 ml) was added to the 1.9 ml 1.0 mM solution of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The GSH concentration was determined spectrophotometrically, using $\Delta\varepsilon_{412}$ = 13.6 mM^{-1} cm^{-1}. The control level of GSH (2.1±0.1 μmol/ml erythrocytes) was in the established reference range of 2.0–2.5 μmol/ml (Beutler and Dale, 1988).

The products of the reaction of tetryl and pentryl with GSH were identified as follows. Tetryl or pentryl (0.5 mM) were allowed to react with 10 mM GSH in 0.01 M K-phosphate (pH 7.0) containing 1 mM EDTA and 10% v/v acetonitrile for 1 h. The reaction mixture (10 μl) was injected into Hewlett Packard 1100 series HPLC-MSD system equiped with a single-quadrupole mass spectrometer, and analyzed using Lichrosphere RP-8 column (Merck, 125 × 4 mm, 5 μm diameter particles). Solvents A (0.1% trifluoroacetic acid (TFA) in water) and B (TFA/water/ acetonitrile = 0.1:9.9:90 (v/v/v)) were used for a gradient elution. The column was initially equilibrated with solvent A at a 1 ml/min flow rate. After the injection, the column was eluted with a 2 min linear gradient to 20% B, followed by a 22 min linear gradient to 30% B, and 20 min linear gradient to 50% B at a 1 ml/min flow rate. The compounds were detected by their absorbance at 214 nm. In the mass spectrometry analysis, the mobile phase was 6% acetic acid in isopropanol (flow rate, 0.5 ml/min). A positive electron spray ionization, the process which produces mainly protonated molecular mass ion $[M+H]^+$ (recorded in the mass range of 50–1500 Da), was achieved using the capillary voltage of 4.5 kV, and the skimmer voltage of 120 V.

Results and Discussion

The kinetic analysis of OxyHb oxidation by nitroaromatic compounds is complex, since the reaction product MetHb slows down the reaction (Bates and Winterbourn, 1982; Čėnas and Öllinger, 1994). Therefore, the reaction rate con-

Table I. Rate constants of oxyhemoglobin oxidation by nitroaromatic compounds (k), their single-electron reduction potentials (E^1_7) and enthalpies of single-electron reduction ($\Delta Hf(ArNO_2^{\cdot -})$), amounts of methemoglobin formed in erythrocytes at 40% hematocrite, and the relative efficiencies of methemoglobin formation by nitroaromatic compounds at 1.5–1.7% hematocrite after a 24-h incubation ([MetHb]/[$ArNO_2$]).

No. Compound	k [$M^{-1}s^{-1}$]	E^1_7 [mV][a]	$\Delta Hf(ArNO_2^{\cdot -})$ [kJ/mol] AM1	PM3	Amount of MetHb formed in erythrocytes at 40% hematocrite (%)[b]	[MetHb]/[$ArNO_2$]
1. Pentryl	9.43 ± 0.51	–	−394.8	−376.6	4.9 ± 0.5	3.2 ± 0.5
2. Tetryl	8.9 ± 0.50	–	−362.5	−367.9	4.7 ± 0.5	3.0 ± 0.5
3. TNT	3.30 ± 0.25	–	−310.8	−316.5	9.0 ± 1.0	8.0 ± 1.0
4. *o*-Dinitrobenzene	3.33 ± 0.20	−287	−257.4	−261.1	26 ± 2.0	7.2 ± 0.9
5. *p*-Dinitrobenzene	2.89 ± 0.15	−257	−273.4	−281.1	9.0 ± 1.0	4.5 ± 0.7
6. *m*-Dinitrobenzene	1.78 ± 0.07	−345	−254.7	−262.2	7.5 ± 1.0	1.35 ± 0.15
7. 3,5-Dinitrobenzamide	0.28 ± 0.02	−355	−284.2	−289.2	2.5 ± 0.3	0.24 ± 0.03
8. 4-Nitrobenzaldehyde	0.48 ± 0.03	−325	−222.2	−223.2	≥1.0	0.21 ± 0.20
9. 2,4-Dinitrochlorobenzene	0.39 ± 0.02	–	−274.2	−277.1	2.2 ± 0.2	1.30 ± 0.15
10. 4-Nitroacetophenone	0.25 ± 0.02	−355	−217.1	−218.1	2.3 ± 0.2	0.32 ± 0.04
11. 4-Nitrobenzoic acid	0.30 ± 0.02	−425	−228.2	−236.6	–[c]	0.05 ± 0.01
12. Nitrobenzene	0.10 ± 0.02	−485	−167.7	−172.1	–	0.024 ± 0.005

[a] From Wardman (1989);
[b] 24 h incubation in the presence of 300 μM of each nitro compound;
[c] Close to the MetHb level in the absence of nitro compounds, 0.3–0.4%.

stants (k, Table I) of OxyHb oxidation in lysed erythrocytes were calculated according to the initial reaction rates. In general, the reactivity of nitroaromatics increased with an increase in their single-electron reduction potential (E^1_7) (Fig. 2A), although the linear correlation between log k and E^1_7 was poor (r^2 = 0.7158). Since the E^1_7 values for tetryl, pentryl and TNT are currently not available, we used the enthalpies of anion-radical formation ($\Delta Hf(ArNO_2^{\cdot -})$) obtained by means of quantum mechanical calculation in our previous studies (Nivinskas *et al.,* 2001) (Table I). It is known that these parameters exhibit a correlation with single-electron transfer redox potentials (Lien *et al.,* 1999). The rough linear correlations between log k and $\Delta Hf(ArNO_2^{\cdot -})$ were characterized by r^2 = 0.7030 (PM3, Fig. 2B), and by r^2 = 0.6899 (AM1, data not shown). Thus, the high rate of oxidation of OxyHb by TNT, tetryl and pentryl is at least partially determined by their strong electron-accepting properties.

Initially, we examined the MetHb formation in erythrocyte suspension at 40% hematocrite (40% v/v), which was close to their content in human

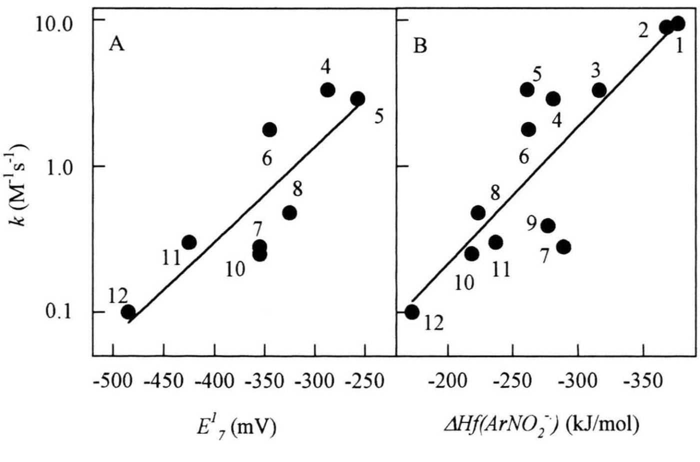

Fig. 2. The dependence of the rate constant (k) of the oxyhemoglobin oxidation in lysed erythrocytes on the single-electron reduction potential (E^1_7) of nitroaromatic oxidants (A), and on their enthalpies of single-electron reduction ($\Delta Hf(ArNO_2^{\cdot -})$, PM3) (B). The numbers of compounds are taken from Table I.

blood. The content of MetHb after the 24 h incubation of erythrocytes with 300 μM of each nitrobenzene derivative, the concentration adjusted close to the limit of solubility of tetryl and pentryl is given in Table I. We failed to detect a measurable amount of choleglobin, the denatured derivative of hemoglobin absorbing at 675 nm. The extent of erythrocyte lysis accounted for 5–7% of MetHb level. Interestingly, tetryl and pentryl were less efficient inducers of MetHb in erythrocytes than TNT or dinitrobenzenes, although the former were more efficient oxidants of OxyHb (Table I).

For a more quantitative insight into MetHb formation in erythrocytes, we used their concentration adjusted to 300 μM OxyHb (1.5–1.7% hematocrite). The initial rates of MetHb formation determined for several most active oxidants matched their reactivity in the OxyHb oxidation in lysed erythrocytes (Table I), i.e., tetryl, pentryl > TNT, *o*- and *p*-dinitrobenzenes > *m*-dinitrobenzene (Fig. 3A). However, the initial rates were 30–50 times lower than in lysed erythrocytes, evidently, due to the action of MetHb-reducing enzymes such NADH:cytochrome b_5 reductase and cytochrome b_5 (Shirabe *et al.,* 1994), NADPH: methemoglobin reductase (Xu *et al.,* 1992), and other erythrocyte antioxidant systems. One must note that after 24 h incubation, the intermediate concentrations of tetryl (10–50 μM) formed lower amounts of MetHb, as compared to TNT and dinitrobenzenes (Fig. 3B). The relative efficiencies of MetHb formation during 24 h, expressed as mole of MetHb formed per mole of nitrocompound ([MetHb]/[$ArNO_2$]), and calculated from the data linearization in double-reciprocal coordinates 1/[MetHb], 1/[$ArNO_2$] are given in Table I. It is evident that [MetHb]/[$ArNO_2$] vary almost in accordance with the rate constants of OxyHb oxidation in lysed erythrocytes, except that the efficiency of tetryl and pentryl was markedly lower than expected. Looking for possible causes of this discrepancy, we examined the depletion of erythrocyte reduced glutathione (GSH) by tetryl and other nitroaromatic compounds.

It is known that 2,4-dinitrochlorobenzene rapidly depletes erythrocyte GSH in a glutathione S-transferase-catalyzed reaction with the formation of 2,4-dinitrophenyl-SG (Awasthi *et al.,* 1981). The slow formation of nitrophenyl-SG by *o*- and *p*-dinitrobenzenes was also reported (Cossum and Rickert, 1987). The GSH depletion by nitroaromatics creates more prooxidant conditions (Awasthi *et al.,* 1981), but the nitrophenyl-SG conjugates are transported from erythrocytes (Bartosz *et al.,* 1993). We have found that during 24-h incubation 300 μM 2,4-dinitrochlorobenzene depleted a stoichiometric amount of GSH, whereas 300 μM *o*-dinitrobenzene depleted 50 ± 10 μM GSH, and TNT, *m*- and *p*-dinitrobenzenes depleted 20–30 μM GSH. In contrast, 300 μM tetryl or pentryl depeleted 220 ± 20 μM GSH (n = 3). Although it is not known whether tetryl and pentryl may act as the substrates for glutathione S-transferase, we have previously shown that tetryl may directly react with GSH (k = 0.6 $M^{-1}s^{-1}$, pH 7.0), giving an unidentified product absorbing at 340–440 nm with

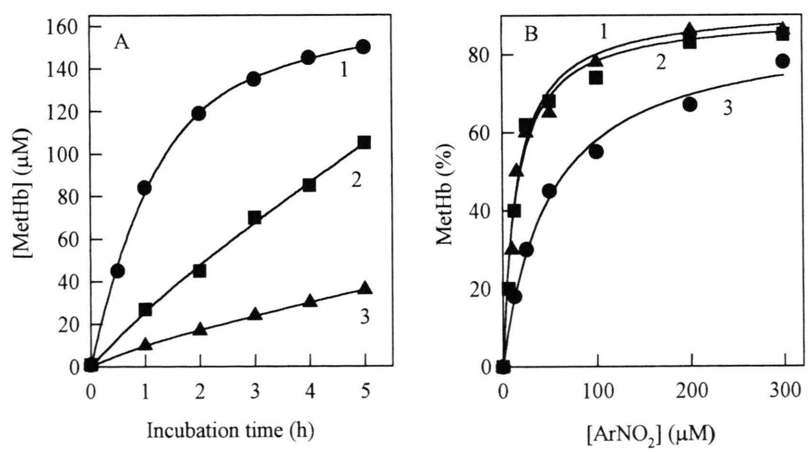

Fig. 3. Methemoglobin (MetHb) formation in intact erythrocytes at 1.5–1.7% hematocrite (initial oxyhemoglobin concentration, 300 μM) under the action of nitroaromatic compounds. A) Kinetics of MetHb formation after the addition of 300 μM tetryl (1), 300 μM TNT (2), and 300 μM *m*-dinitrobenzene (3). The reaction course in the presence of 300 μM pentryl coinci-ded with curve (1), whereas in the presence of 300 μM *o*- and *p*-dinitrobenzenes it coincided with curve (2). B) The extent of MetHb formation after 24 h erythrocyte incubation with different concentrations of *o*-dinitrobenzene (1), TNT (2), and tetryl (3).

ε_{340} = 8.5 mM^{-1} cm^{-1} (Miškinienė *et al.*, 1998). In this study, we found that pentryl also reacted with GSH in a second order reaction (k = 1.0 $M^{-1}s^{-1}$) giving the product with analogous absorbance spectra. Further, we detected the 340–440 nm absorbing species in the erythrocyte supernatant after their 24-h incubation with tetryl or pentryl, and subsequent protein precipitation by sulfosalicylic acid (Fig. 4A).

We tried to identify the products of reaction of GSH with tetryl and pentryl. According to the data of HPLC analysis, the single reaction product with sufficiently close retention times (13.77 min, tetryl; 13.52 min, pentryl) was formed (data not shown). The retention times of tetryl and pentryl were 36.26 min and 36.12 min, respectively. The mass spectra of the reaction products were identical in both cases, revealing the major signal of molecular ion with $[M+H^+]^+$ = 519 (Fig. 4B). Their comparison with the mass spectra of GSH and GSSG (data not shown) has enabled us to propose the product fragmentation pattern matching the observed spectra, and to identify the reaction product as 2,4,6-trinitrophenyl-SG (Fig. 4B). The product of the direct reaction of 2,4,6-trinitrochlorobenzene with GSH exhibited analogous absorbance and mass spectra.

Since the reaction of 2,4,6-trinitrochlorobenzene with a stoichiometric amount of GSH is fast ($t_{1/2}$ = 5–6 min at 300 μM of each reagent), we have been able to prepare 2,4,6-trinitrophenyl-SG *in situ* without the use of significant excess GSH. It has been found that 2,4,6-trinitrophenyl-SG was able to oxidize OxyHb in lysed erythrocytes, but only at 20–25% of the tetryl reaction rate.

The concentrations of explosives and other nitroaromatic compounds used in this work might be far above the expected values under physiological conditions. However, our model studies provide some information on the relative MetHb-forming potency of explosives. In comparison with TNT, tetryl and pentryl cause a more rapid initial formation of MetHb in erythrocytes, but they are less efficient during a long incubation time. This may be attributed to their parallel reactions with erythrocyte GSH with the formation of a less reactive MetHb forming agent. Besides, it is possible that 2,4,6-trinitrophenyl-SG is transported from erythrocytes like 2,4-dinitrophenyl-SG as well (Bartosz *et al.*, 1993). Presumably the conjugation of tetryl and pentryl with GSH may have an impact on its other mechanisms of cytotoxicity as well, e.g., flavoenzyme-catalyzed redox cycling and oxidative stress. Preliminarily, we have found that 2,4,6-trinitrophenyl-SG is around 100 times less reactive than tetryl in microsomal NADPH:-cytochrome P-450 reductase-catalyzed redox cycling.

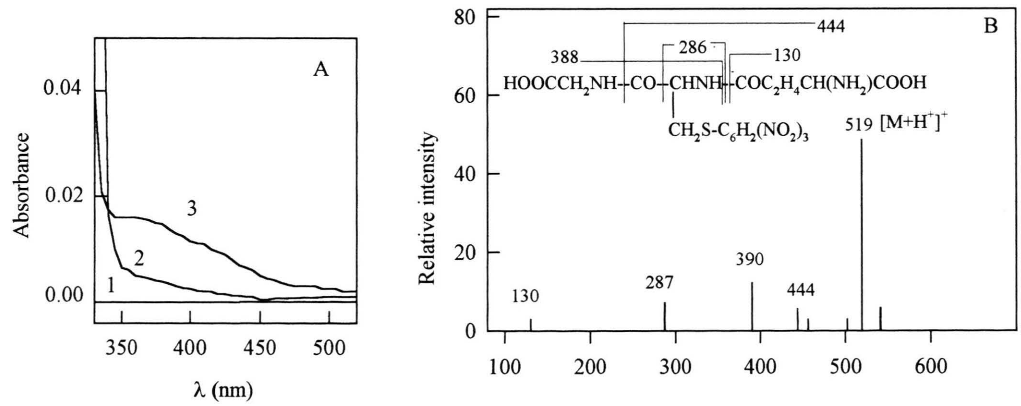

Fig. 4. A) Difference spectra of the erythrocyte supernatant, showing the formation of 340–440 nm absorbing species after the incubation of erythrocytes with tetryl. The sample cell contained 1.8 ml buffer solution and 0.2 ml supernatant, obtained after 24 h incubation of erythrocytes, protein precipitation, and centrifugation. Erythrocytes (5% hematocrite) were incubated in the absence of nitrocompounds (1), in the presence of 300 μM TNT (2), or 300 μM tetryl (3). The reference cell contained identical volume of control supernatant. B) Mass spectra and fragmentation pattern of 2,4,6-trinitrophenyl-SG, the product of the reaction of tetryl and GSH.

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