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Reduction of Nitroaromatic Explosives by Oxygen-Insensitive NAD(P)H:Nitroreductases: Implications for their Cytotoxicity and Biodegradation*)

Jonas ŠARLAUSKAS, Lina MISEVIČIENĖ,
Henrikas NIVINSKAS, Žilvinas ANUSEVIČIUS,
Narimantas ČĖNAS*

Institute of Biochemistry, Mokslininkų 12, LT-08662 Vilnius, Lithuania
**E-mail: ncenas@bchi.lt*

Aušra NEMEIKAITĖ-ČĖNIENĖ

Institute of Immunology of Vilnius University,
Molėtų Pl. 29, LT-08409, Vilnius, Lithuania

Evaldas MALDUTIS

The General J. Žemaitis Military Academy of Lithuania,
Šilo 5a, LT-10322 Vilnius, Lithuania

Richard J. HARRIS, Nigel S. SCRUTTON

Faculty of Life Sciences, The University of Manchester,
Manchester M13 9PT, UK

Abstract: Nitroaromatic explosives are toxic and mutagenic to humans and other mammalian species. The first step(s) in the biodegradation/bioremediation of the explosive residues in soil or groundwater is their reduction by bacterial oxygen-insensitive nitroreductases to the relatively stable metabolites. Here we analyze the quantitative structure-activity relationships in the reduction of nitroaromatic explosives and model nitroaromatic compounds by mammalian DT-diaphorase (NQO1) and *Enterobacter cloacae* NAD(P)H:nitroreductase (NR), which performs

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the four-electron reduction of nitrogroups to corresponding hydroxylamines, and by *Enterobacter cloacae* PB2 NADPH: pentaerythritol tetranitrate reductase (PETNR), which performs nitroreduction and reduction of benzene ring with the formation of hydride-Meisenheimer adducts. Our data show that in all the cases the reduction rate of nitroaromatics mainly depends on the energetics of the charge transfer.

Keywords: nitroaromatic explosives, enzymatic reduction of, biodegradation of

Introduction

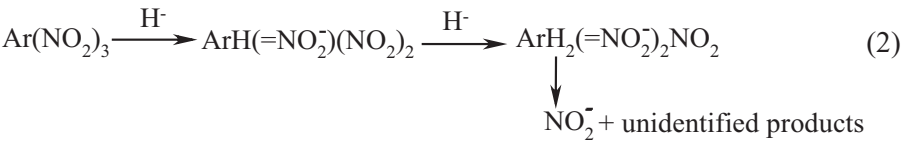
Nitroaromatic explosives are toxic and mutagenic to humans and other mammalian species. Their toxicity is partly attributed to the single-electron enzymatic reduction to radicals accompanied by the formation of the reactive oxygen species (oxidative stress type of cytotoxicity) [1, 2]. Single-electron reduction of nitroaromatics is performed by flavoenzymes dehydrogenases-electrontransferases, e.g., NADPH:cytochrome P-450 reductase (P-450R, EC 1.6.2.4) or ferredoxin:$NADP^+$ reductase (FNR, EC 1.18.1.2). In this enzyme group, the flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) cofactors are characterized by the high stability of their neutral (blue) radicals at the equilibrium [3]. The reduction of nitroaromatic compounds by P-450R and FNR follows an outer-sphere electron-transfer model, i.e., the reactivity increases with an increase in their single-electron reduction potential (E^1_7), and is relatively insensitive to their structural pecularities [2, 3].

Another important mechanism of cytotoxicity of nitroaromatic compounds is their two-(four)-electron reduction to nitroso and hydroxylamino metabolites, which covalently bind to proteins and DNA [4]:

$$ArNO_2 \xrightarrow{+2e^- + 2H^+} ArNO \xrightarrow{+2e^- + 2H^+} ArNHOH \qquad (1)$$
$$\searrow H_2O$$

In mammalian organisms, this type of reaction is catalyzed by NAD(P)H: quinone oxidoreductase (DT-diaphorase, NQO1, EC 1.6.99.2), which is localized mainly in the liver. Another source of the formation of toxic aromatic hydroxylamines in mammals is the gastrointestinal tract, where the nitroaromatic compounds are efficiently reduced by the nitroreductases of *Escherichia* and *Enterobacter* sp. Because of the relative stability of the nitroreduction products under aerobic conditions, these enzymes are named the ‘oxygen-insensitive‘ nitroreductases.

It is important to note that the reaction (1) being catalyzed by the 'oxygen-insensitive' nitroreductases of numerous bacteria, microorganisms, and fungi, also make a first step in the biodegradation of nitroaromatic explosives in the environment [5]. In parallel, certain nitroreductases reduce the aromatic ring with the formation of Meisenheimer-type hydride adducts, which further undergo denitration [5]:



(2)

Typically, all the 'oxygen-insensitive' nitroreductases including NQO1 possess the flavin cofactor with the destabilized anionic (red) semiquinone state at the equilibrium. One may note that in contrast to the reactions of single-electron reduction of nitroaromatics by flavoenzymes, the mechanisms and the substrate specificity of their two-(four)-electron reduction still remain insufficiently understood.

In this paper, we summarize the data of our previous [2, 6-11] and ongoing studies on the mechanisms of two-electron reduction of nitroaromatic explosives (Figure 1) and model compounds by 'oxygen-insensitive' nitroreductases: rat NQO1 and *Enterobacter cloacae* NAD(P)H:nitroreductase (NR) which reduce the nitrogroup(s) into hydroxylamine(s) (reaction (1)), and *Enterobacter cloacae* PB2 NADPH:pentaerythritol tetranitrate (PETN) reductase (PETNR), which in parallel performs the aromatic ring reduction with the formation of Meisenheimer-type adducts (reaction (2)). The methods of synthesis of nitroaromatic explosives and model nitroaromatic compounds, purification of NQO1 and NR, enzyme kinetics and cytotoxicity studies, and quantum mechanical calculations were reported in our previous papers [2, 7-11]. Because the directly determined E^1_7 values are unavailable for a large number of nitroaromatic explosives, we also used their E^1_7 values calculated from the data of their single-electron enzymatic reduction ($E^1_{7(calc.)}$) (www.bchi.lt/potential.pdf).

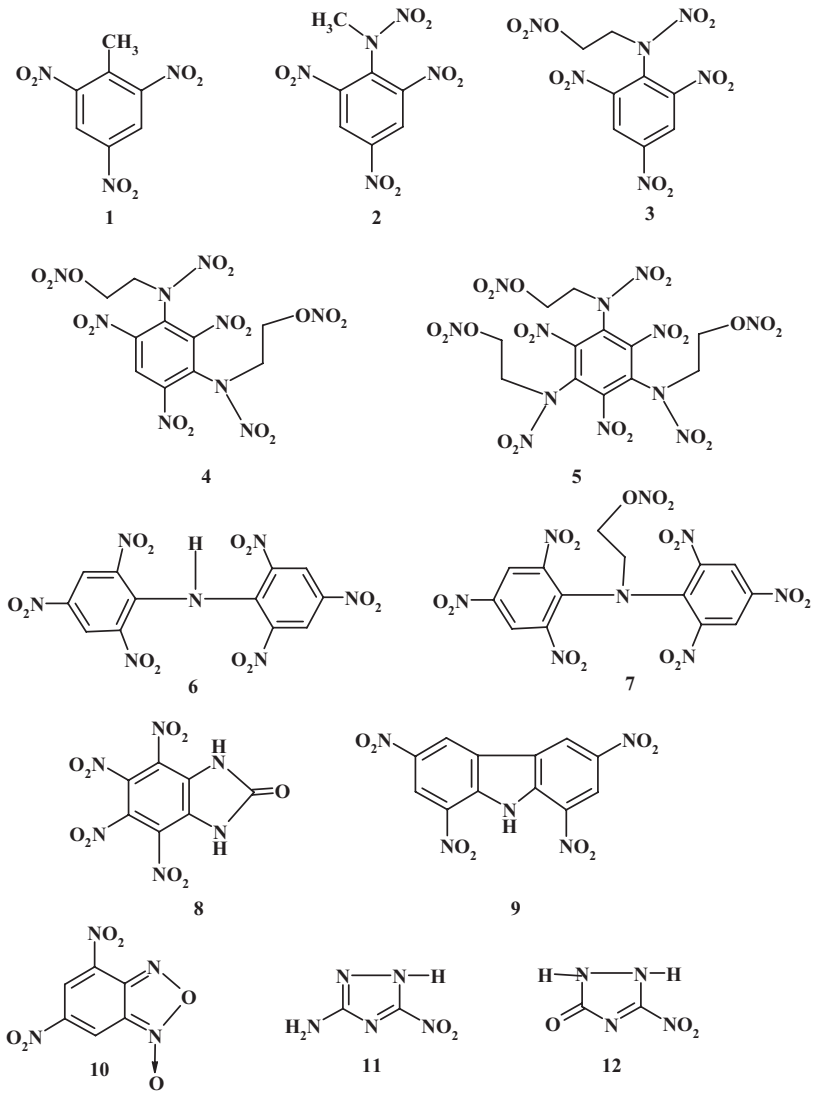


Figure 1. Structural formulae of examined nitroaromatic explosives: 2,4,6-trinitrotoluene (TNT) (1), 2,4,6-trinitrophenyl-*N*-methylnitramine (tetryl) (2), 2,4,6-trinitrophenyl-*N*-nitroxyelthylnitramine (pentryl) (3), dipentryl (4), tripentryl (5), 2,2',4,4',6,6'-hexanitrodiphenylamine (hexyl) (6), hexyl-*N*-ethylnitrate (7), 4,5,6,7-tetranitrobenzimidazolone (8), 1,3,6,8-tetranitrocarbazole (9), 4,6-dinitrobenzofuroxane (10), 3-amino-5-nitro-1,2,4-triazole (11), and 5-nitro-1,2,4-triazol-3-one (12).

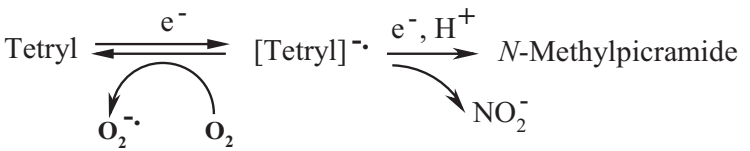
Nitroreductase reactions of rat liver NQO1

Mammalian NQO1 is a dimeric (2 x 31 kD) enzyme containing one molecule of FAD per subunit. The midpoint potential of FAD at pH 7.0 is -0.159 V, whereas the potentials of FAD/$FAD^{\cdot-}$ and $FAD^{\cdot-}$/$FADH^{-}$ couples are -0.200 and -0.118 V, respectively [12]. This provides ca. 7% $FAD^{\cdot-}$ stabilization at the equilibrium. Quinone reduction by NQO1 follows a 'ping-pong' scheme and often proceeds at a high maximal rate, > 1000 s^{-1}. The reactivity of quinones increases with an increase in their single-electron reduction potential and decreases with an increase in their Van der Waals volume (VdWvol) above 200 $Å^3$, due to the sterical hindrances [13]. Recent X-ray and computer modeling studies provided evidence that mono- and bicyclic quinones, an inhibitor of NQO1, dicumarol, and dihydronicotinamide ring of NADPH bind at the same domain in the active center [14]. These compounds interact with tyrosine-126', histidine-161, and phenylalanine-106, and form π-π complex with the isoalloxazine ring of FAD at a distance of 3.5 Å by displacement of tyrosine-128'.

Nitroaromatic compounds, although being less reactive than quinones, comprise another important group of substrates for NQO1. NQO1 plays a considerable role in mammalian cell cytotoxicity and genetoxicity of nitroaromatic pollutants – the components of exhaust gases or the explosive residues in the environment. However, the mechanism and substrate specificity of nitroreductase reactions of NQO1 are still poorly understood. There is no X-ray data on the complexes of nitroaromatic compounds in the active center of NQO1, except several computer modeling studies [15, 16].

We examined NQO1-catalyzed reduction of 39 nitroaromatic compounds, including 16 explosive compounds (2,4,6-trinitrotoluene (TNT) and its metabolites, tetryl, pentryl, dipentryl, hexyl, tetranitrocarbazole, nitrobenzimidazolones, nitrobenzofuroxanes, and nitrotriazoles) [11]. The obtained multiparameter regressions show that the reactivity of compounds increases with an increase in their $E^1_{7(calc.)}$ with the coefficient Δlog rate constant /$\Delta E^1_{7(calc.)}$ ~ 10 V^{-1}, and the highest torsion angle between the nitrogroup(s) and the aromatic ring. Another although less certain factor increasing the reactivity of nitroaromatic compounds, is their affinity to the oxidized form of NQO1. However, the use of the above parameters resulted in the relatively uncertain (r^2 ~ 0.800) correlations. Another important but poorly understood factor influencing the reactivity of nitroaromatics is their ability to bind at the different domains in the active center of NQO1. Our data [11] partly contradict the computer modeling data [15, 16] which suggest that nitroaromatic compounds should bind at the dicumarol/quinone binding domain, because: i) the entropies of activation

($\Delta S^{\neq}$) of nitroreductase reactions of NQO1 are much less negative than $\Delta S^{\neq}$ of quinone reduction, thus demonstrating the less efficient electronic coupling of reactants, ii) increase in VdWvol of nitroaromatics up to 250-300 $Å^3$ does not decrease their reactivity, but increases it, and iii) dicumarol acts as incomplete inhibitor towards several nitroaromatic substrates, including tetryl. It shows that a number of nitroaromatic compounds may bind outside the dicumarol/quinone binding domain. However, dicumarol acts as a complete (linear) inhibitor with respect to 4,5,6,7-tetranitrobenzimidazolone, whose reactivity is much higher than expected. This is probably caused by its binding at the dicumarol/quinone binding domain. Another important aspect of catalysis of NQO1 is that it catalyzes reductive *N*-denitration of tetryl in a single-electron way, with the formation of *N*-methylpicramide, nitrite, superoxide, and oxidation of excess NADPH [2]:



(3)

Reactions of *E. cloacae* nitroreductase

Enterobacter cloacae NAD(P)H:nitroreductase (NR, EC 1.6.99.7) is a dimeric (2 x 24.5 kD) protein, containing a molecule of FMN per subunit. The midpoint potential of FMN at pH 7.0 is -0.190 V, with less than 1% $FMN^{-\cdot}$ stabilization at the equilibrium [17]. *E. cloacae* NR shares a significant sequence homology with nitroreductases from *Escherichia coli, Salmonella typhimurium,* and FMN reductase from *Vibrio fischeri*. *E. cloacae* NR reduces nitroaromatic compounds according to a 'ping-pong' scheme with strong competitive to NAD(P)H inhibition by the nitroaromatic substrates. It shows that NAD(P)H and nitroaromatic compounds bind at the same domain. NR reduces nitroaromatic compounds at high maximal rates, exceeding 100 s^{-1} or even 1000 s^{-1} for tetryl and pentryl [7]. The reduction of polynitroaromatic compounds (tetryl, TNT, dinitrobenzenes) occurs biphasically, with a fast consumption of 2 moles NAD(P)H per mole of nitrocompound, and subsequent slower oxidation of further excess NAD(P)H. The second phase of reaction is accompanied by O_2 uptake. It shows that polynitroaromatic compounds are first reduced to monohydroxylamines. According to the studies of *E. coli* NR,

the reduction of nitroso compounds into hydroxylamines is 10^4 times faster than the reduction of nitrogroup into nitroso [18]. We found that monohydroxylamine metabolites of TNT are further reduced by *E. cloacae* NR, but at slower rates than TNT [8]. The reduction of TNT is not accompanied by nitrite formation. *E. cloacae* NR and NQO1 reduce tetryl in different ways: NR reduces tetryl to unidentified product(s) other than *N*-methylpicramide, nitrite is formed not simultaneously with the NAD(P)H oxidation, but after a lag-period.

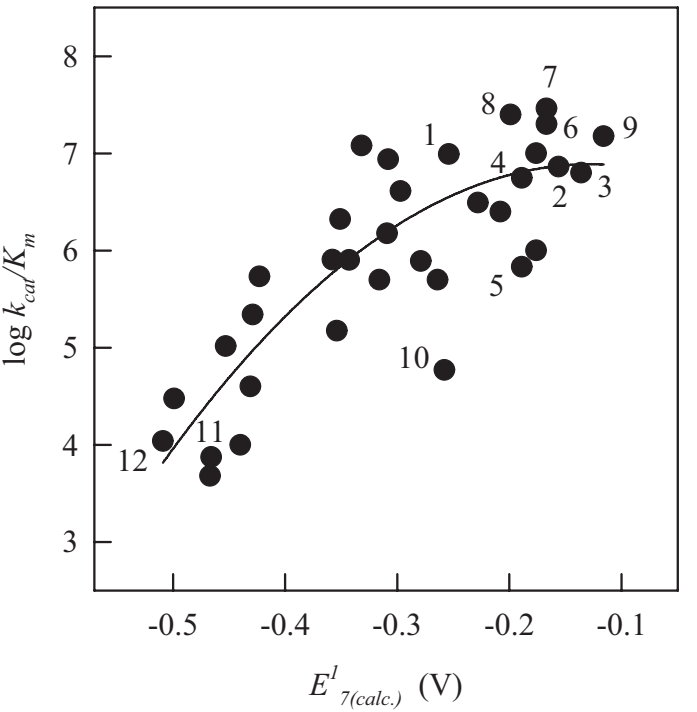


Figure 2. The dependence of bimolecular rate constants (k_{cat}/K_m) of reduction of nitroaromatic compounds by *E. cloacae* nitroreductase on their single-electron reduction potentials ($E^1_{7(calc.)}$) at pH 7.0 and 25 °C. The numbers correspond to the numbers of explosive compounds (Figure 1), the rest are the model nitroaromatic compounds.

In general, the reactivity of nitroaromatic compounds towards *E. cloacae* NR increases with an increase in their electron-accepting potency, which may be expressed either as $\Delta E^1_{7(calc.)}$, or as the enthalpies of free radical ($\Delta Hf(ArNO_2^{\cdot-})$) or hydride adduct (anionic *N,N*-dihydroxylamine) formation ($\Delta Hf(ArN(OH)O^-)$) [7]. The observed parabolic log rate constant vs. potential dependence is relatively

uncertain (Figure 2). However, at present we are unable to characterize the structural peculiarities of nitroaromatic compounds prerequisite for their efficient reduction by *E. cloacae* NR. The X-ray data of *E. cloacae* and *E. coli* NR show that pyrimidine ring of FMN is close to the surface of protein globule [19]. The X-ray data of the complex of oxidized *E. coli* NR with nitroaromatic compound nitrofurazone shows that the compound binds at the isoalloxazine ring of FMN displacing phenylalanine-70 and phenylalanine-124', but with an unproductive orientation [18]. These factors may impose flexibility and multiple orientations in the binding of nitroaromatic substrates. It is also possible that hexyl and hexyl-*N*-ethylnitrate may bind at several domains of NR, e.g., at the catalytic and intersubunit domain, because their strong substrate inhibition ($K_i \leq 1$ μM at 200 μM NADH) is characterized by the nonlinear (parabolic) Dixon plots. On the other hand, the reactivity of 4,6-dinitrobenzofuroxane is much lower than one may expect from its $\Delta E^1_{7(calc.)}$ value (Figure 2). A possible reason for its low reactivity is a loss of aromaticity of benzene ring, because this compound exists in a form of Meisenheimer-type hydroxy adduct.

Reactions of *E. Cloacae* PB2 PETN reductase

E. cloacae PB2 NADPH:PETN reductase (PETNR) is a monomeric 40 kD protein, containing a molecule of FMN per subunit. The midpoint potential of FMN at pH 7.0 is -0.267 V, with less than 1% $FMN^{\cdot-}$ stabilization at the equilibrium [20]. PETNR belongs to a structurally separate group of flavoenzymes, the Old Yellow Enzyme (OYE, *Saccharomyces carlsbergensis* NADPH:oxidase) family, which also includes *Pseudomonas putida* M10 morphinone reductase (MR), *Pseudomonas fluorescens* xenobiotic reductase B (XenRB), and *E. coli* *N*-ethylmaleimide reductase (NEMR) [21]. All the enzymes of the OYE family perform two independent reactions, the reduction of aromatic nitrogroup(s) into hydroxylamine(s) (reaction (1)), and the aromatic ring reduction with the formation of Meisenheimer-type hydride adducts, which further undergo denitration (reaction (2)). The moles of nitrite formed per mole of TNT consumed vary from 0.25 (NEMR, XenRB) and 0.18 (PETNR), to 0.03 (OYE, MR) [21]. The pathways of nitrite formation during the reductive degradation of TNT require more thorough studies. It has been proposed that NO_2^- may be liberated during the nonenzymatic reaction between 4-hydroxylamino-DNT and Meisenheimer-type TNT-hydride diadduct [22]. However, according to the latter studies, the denitration occurs in the absence of 4-hydroxylamino-DNT, and its rate depends on enzyme concentration [21]. A specific feature of the OYE family members

is that apart from the reduction of nitroaromatics, they perform the reductive denitration of PETN and glycerol trinitrate, although with different substrate preference. The transgenic tobacco plants expressing PETNR perform the biodegradation of nitroaromatic and nitroaliphatic explosives [23].

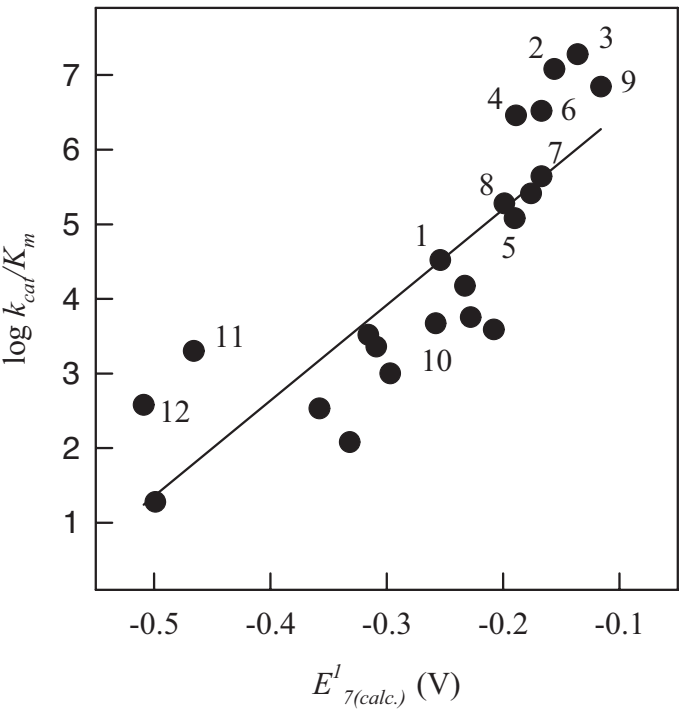


Figure 3. The dependence of bimolecular rate constants (k_{cat}/K_m) of reduction of nitroaromatic compounds by *E. cloacae* PETNR on their single-electron reduction potentials ($E^1_{7(calc.)}$) at pH 7.0 and 25 °C. The numbers correspond to the numbers of explosive compounds (Figure 1), the rest are the model nitroaromatic compounds.

We examined the reduction of 28 nitroaromatic compounds by PETNR, including 20 explosive compounds. For 23 compounds with available $\Delta E^1_{7(calc.)}$ values, there exists an uncertain linear log rate constant vs. $\Delta E^1_{7(calc.)}$ relationship ($r^2 = 0.6535$) with the coefficient Δlog rate constant/$\Delta E^1_{7(calc.)} \sim 12\ V^{-1}$ (Figure 3). It points to the important role of charge transfer energetics in the reactivity of compounds. The maximal reduction rates of the most reactive oxidants, tetryl, pentryl and hexyl, are close to 35 s^{-1}, and their bimolecular reduction rate constants are above $10^7\ M^{-1}s^{-1}$. In contrast to *E. coli* NR, the reactivity

of 4,6-dinitrobenzofuroxane also follows this relationship. At present, we cannot identify the structural peculiarities of nitroaromatics that are prerequisite for their efficient reduction by PETNR. According to the X-ray crystallographic data, the binding of picric acid and TNT to PETN involves their parallel orientation with the isoalloxazine of FMN, and the interaction of 1-hydroxy or 1-methyl groups with histidines-181,184 [24]. This causes their favourable orientation for hydride transfer with the Meisenheimer-type adduct formation, i.e., the proximity of C-5 carbon to the flavin N-5. However, our data show that the nature of the substituent in the 1-position of TNT, picramide, *N*-methylpicramide, 2,4,6-trinitroanizole, or its absence in 2,4,6-trinitrobenzene does not strongly influence the reactivity of nitroaromatics. In contrast, the maximal reduction rates of tetryl and pentryl are 20 times higher, and their bimolecular reduction rate constants 500 times higher than those of TNT, although the above compounds possess bulky substituents in the 1-position. This may be explained by a higher electron-accepting potency of tetryl and pentryl. The strongest inhibitors of PETNR, hexyl ($K_i < 2$ μM) and tetranitrocarbazole ($K_i = 3.5$ μM) are structurally different. The presence of a bulky *N*-ethylnitrate group in hexyl-*N*-ethylnitrate decreases its affinity to PETNR ($K_i = 70$ μM) and reactivity by 10 times as compared to hexyl. However, the reactivities of hexyl and pentryl are similar, although pentryl is a much weaker inhibitor of PETNR ($K_i = 150$ μM). This shows that nitroaromatic compounds may bind in the active center of PETNR in different ways, and that their efficient interaction with histidines-181,184 is not obligatory. We found that nitrite was formed during the reduction of structurally different compounds, such as TNT, picramide, tetryl, pentryl, tetranitrobenzimidazolone and tetranitrocarbazole. The reductive denitration of tetryl did not follow the single-electron transfer (reaction (3)), and yielded other than *N*-methylpicramide reaction product(s). However, the reduction of *p*-dinitrobenzene was not accompanied by the production of nitrite, yielding *p*-hydroxylaminonitrobenzene. We found that this was not due to the structural peculiarities of *p*-dinitrobenzene, but due to the more favourable energetics of reduction of nitrogroup instead of aromatic ring, which was reverse for the compounds forming nitrite.

Conclusions

The data of this work show that apart from the currently insufficiently understood structural peculiarities, an increase in the electron-accepting potency of nitroaromatic explosives, preferably determined in the aqueous medium, increases their reactivity towards two-electron transferring ‘oxygen-insensitive‘

nitroreductases. In general, the mammalian cell cytotoxicity of nitroaromatic explosives increases with an increase in their E^1_7 or $E^1_{7(calc.)}$ [2], except for the amino- and hydroxylamino- metabolites of TNT, which possess higher than expected cytotoxicity [8, 25, 26]. In this work, we demonstrate the existence of a certain parallelism between the reactivity of nitroaromatic explosives towards the single-electron transferring flavoenzymes and two-electron transferring NQO1. Irrespective of this, the positive relationship between the electron-accepting properties of nitroaromatics and their cytotoxicity should point to the leading role of the oxidative stress-type cytotoxicity, because the inhibitor of NQO1, dicumarol, equivocally affects the cytotoxicity of nitroaromatic compounds [2]. Our data also indicate that the order of reactivity of certain nitroaromatic explosives (nitrotriazoles < TNT < tetryl, pentryl and hexyl) towards two different bacterial nitroreductases, NR and PETNR, is also in line with their relative electron-accepting potency. On the other hand, the enzyme specificity strongly influences the reduction rate of dinitrobenzofuroxane, tetranitrobenzimidazolone and tetranitrocarbazole. Besides, certain nitroaromatic explosives, e.g., hexyl, may act as a strong inhibitors of both nitroreductase types, thus impeding their own biodegradation. Because the nitro- and/or the aromatic ring reduction rates may influence the overall rate of biodegradation of nitroaromatic explosives [5], the data presented may provide some guidelines for the design of specifically adjusted biodegradation systems.

Acknowledgements

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