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# DT-diaphorase catalyzes N-denitration and redox cycling of tetryl

Žilvinas Anusevičius[a], Jonas Šarlauskas[a], Henrikas Nivinskas[a], Juan Segura-Aguilar[b], Narimantas Čėnas[a,*]

[a]*Institute of Biochemistry, Mokslininkų 12, Vilnius 2600, Lithuania*
[b]*Division of Biochemistry, Department of Pharmaceutical Biosciences, BMC, Uppsala University, S-751 23 Uppsala, Sweden*

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**Abstract Rat liver DT-diaphorase (EC 1.6.99.2) catalyzed reductive N-denitration of tetryl (2,4,6-tri-nitrophenyl-*N*-methylnitramine) and 2,4-dinitrophenyl-*N*-methylnitramine, oxidizing the excess of NADPH. The reactions were accompanied by oxygen consumption and superoxide dismutase-sensitive reduction of added cytochrome *c* and reductive release of $Fe^{2+}$ from ferritin. Quantitatively, the reactions of DT-diaphorase proceeded like single-electron reductive N-denitration of tetryl by ferredoxin:$NADP^+$ reductase (EC 1.18.1.2) (Shah, M.M. and Spain, J.C. (1996) Biochem. Biophys. Res. Commun. 220, 563–568), which was additionally checked up in this work. Thus, although reductive N-denitration of nitrophenyl-*N*-nitramines is a net two-electron (hydride) transfer process, DT-diaphorase catalyzed the reaction in a single-electron way. These data point out the possibility of single-electron transfer steps during obligatory two-electron (hydride) reduction of quinones and nitroaromatics by DT-diaphorase.**



*Key words:* DT-diaphorase; Tetryl; *N*-nitramine; Explosive; Redox cycling; Electron transfer

## 1. Introduction

Mammalian DT-diaphorase (NAD(P)H:quinone oxidoreductase, EC 1.6.99.2) is a cytosolic dimeric flavoprotein containing one molecule of FAD per subunit [1]. It catalyzes two-electron reduction of quinones and aromatic nitro compounds using NADH or NADPH as an electron donor. Among biomedically important substrates of DT-diaphorase, one should mention anticancer drugs EO9, diaziquone [2] and 5-(aziridinyl-1-yl)-2,4-dinitrobenzamide (CB-1954) [3], environment pollutant dinitropyrenes [4]. In contrast with single-electron reactions of flavoenzyme electron transferases, e.g. NADPH:cytochrome P-450 reductase (EC 1.6.2.4) or ferredoxin:$NADP^+$ reductase (EC 1.18.1.2) [5–7], two-electron reactions of DT-diaphorase in most cases do not initiate redox cycling of quinones and nitro compounds [1–3], and their bioreductive activation by DT-diaphorase is attributed to the formation of alkylating products [2,3].

DT-diaphorase reduces quinones according to a 'ping-pong' mechanism with high turnover rates approaching 2000 $s^{-1}$ [8,9]. On the contrary, enzyme nitroreductase activity does not exceed 5 $s^{-1}$ in most cases [3,10,11]. According to a 0.21-nm resolution crystal structure of DT-diaphorase [12], quinones occupy the nicotinamide binding pocket in the vicinity of the isoalloxazine ring of FAD, and may accept hydride from the N5 position of isoalloxazine. The interaction of DT-diaphorase with nitroaromatics has been studied less thoroughly, although there exist data that quinones and nitroaromatics bind on different enzyme sites [10,11]. In general, the mechanism of two-electron reduction of quinones and nitroaromatics by DT-diaphorase has been studied insufficiently. It is of particular interest to check up whether two-electron (hydride) transfer proceeds in a single step, or follows a multistep, e.g. electron-proton-electron mechanism.

The explosive tetryl (2,4,6-trinitrophenyl-*N*-methylnitramine) (Fig. 1) is an important environment pollution factor [13], possessing genotoxic properties [14,15]. The enzymatic reductive activation of tetryl is almost uninvestigated, except for the recently described single-electron reductive N-denitration by ferredoxin:$NADP^+$ reductase [16].

In the present study, we show that reductive N-denitration of tetryl and its 2,4-dinitro analog by DT-diaphorase is accompanied by redox cycling and other events, characteristic of a single-electron transfer. Apart from possible toxicological significance, these data point to the possibility of single-electron transfer steps in the reduction of quinones and nitroaromatics by DT-diaphorase.

## 2. Materials and methods

### 2.1. Enzymatic assays

Rat liver DT-diaphorase (EC 1.6.99.2) was prepared as described [17]. Ferredoxin:$NADP^+$ reductase from *Anabaena* (FNR), prepared as described [18], was a generous gift from Dr. M. Martinez-Julvez and Professor C. Gomez-Moreno (Zaragoza University, Spain). The enzyme concentrations were determined using $\varepsilon_{460} = 11$ $mM^{-1}$ $cm^{-1}$ (DT-diaphorase), and $\varepsilon_{459} = 9.4$ $mM^{-1}$ $cm^{-1}$ (FNR). All the kinetic measurements, unless otherwise specified, were carried out in 0.1 M K-phosphate buffer solution (pH 7.0) containing 1 mM EDTA, 0.01% Tween 20, and 0.25 mg/ml bovine serum albumin, at 25°C. The activity of DT-diaphorase was monitored according to the increase in absorbance of reduced cytochrome *c* at 550 nm as described [17], in the presence of 10 μM menadione, 50 μM cytochrome *c*, and 200 μM NADPH. The enzyme activity was equal to 2000 $s^{-1}$. The activity of ferredoxin:$NADP^+$ reductase, determined according to the reduction of 1 mM ferricyanide by 200 μM NADPH [18], was equal to 200 $s^{-1}$.

The rates of nitroreductase reactions of DT-diaphorase and FNR were monitored following NADPH oxidation ($\Delta\varepsilon_{340} = 6.2$ $mM^{-1}$ $cm^{-1}$). The corrections were introduced for absorbance rise due to *N*-methylpicramide and 2,4-dinitro-*N*-methylaniline formation (see Section 3). The catalytic constant ($k_{cat}$) and the bimolecular rate constant ($k_{cat}/K_m$) of nitro compound reduction, correspond to the reciprocal intercepts and slopes of plots [E]/v, 1/[S], where [E] is enzyme concentration, and [S] is concentration of nitro compound. $k_{cat}$ expresses the number of NADPH oxidized by the active center of enzyme per 1 s.

The reductive mobilization of $Fe^{2+}$ from ferritin (1.5–1.6 mM $Fe^{3+}$) was monitored according to the increase in absorbance of the $Fe^{2+}$-

*Corresponding author. Fax: (370) (2) 72 91 96.
E-mail: ncenas@bchi.lt

*Abbreviations:* FNR, ferredoxin:$NADP^+$ reductase; $k_{cat}$, catalytic constant; $k_{cat}/K_m$, bimolecular rate constant; SOD, superoxide dismutase; TLC, thin layer chromatography


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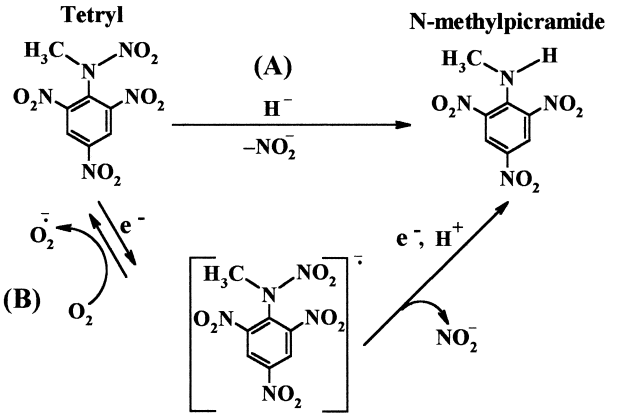


Fig. 1. Scheme of the single step (A) and the multistep (B) hydride transfer during the reductive N-denitration of tetryl.

α,α′-bipyridyl complex ($\Delta\varepsilon_{522} = 8.43\ mM^{-1}\ cm^{-1}$) [19] in 0.1 M Tris-HCl, pH 7.0, containing 2 mM of bipyridyl, as described [20].

The rate of oxygen consumption during enzymatic reactions was monitored using a Clark electrode, at 25°C. The concentrations of nitrite were determined spectrophotometrically at 540 nm, monitoring the formation of azo dye in the presence of sulfanilamide, naphthylethylene diamine dihydrochloride, and 4-fold diluted reaction mixture, as described [21]. The 10–200-μM $NaNO_2$ solutions were used for the calibration curve.

*2.2. Thin-layer chromatography*

During the thin-layer chromatography (TLC) analysis of tetryl and 2,4-dinitrophenyl-*N*-nitramine enzymatic reduction products, compounds (200 μM) were incubated in 0.1 M K-phosphate, pH 7.0, with 5 μM NADPH, 5 mM glucose-6-phosphate, 30 μg/ml glucose-6-phosphate dehydrogenase, 20 nM DT-diaphorase or FNR for 1–2 h. At the end of the reaction, 2 ml of reaction mixture was extracted by 0.2 ml chloroform. Organic fraction was applied on Kieselgel 60 $F_{254}$ plates (Merck), and eluted by chloroform and toluene mixture (1:1).

*2.3. Chemicals*

Tetryl and *N*-methylpicramide were synthesized according to the described methods [22]. 2,4-Dinitrophenyl-*N*-methylnitramine and 2,4-dinitro-*N*-methylaniline were prepared as described [23]. 4,5,6-Trinitrobenzimidazolone was prepared as described in our previous paper [11].

Glucose-6-phosphate dehydrogenase and catalase were obtained from Fermentas (Vilnius), while other reagents and enzymes were from Sigma.

## 3. Results

The reductive N-denitration of tetryl with the formation of *N*-methylpicramide (Fig. 1) may proceed either as a substitution of *N*-nitro group by hydride ion [24] (Fig. 1, route A), or involve the initial electron-transfer step (Fig. 1, route B), as in FNR-catalyzed reaction [16]. In the latter case, the first electron transfer yields tetryl anion-radical which is reoxidized by oxygen with the formation of $O_2^{-\cdot}$ [16]; the redox cycling competes with the subsequent reactions of anion radical, i.e. the elimination of nitrite ion, and the acceptance of a second redox equivalent.

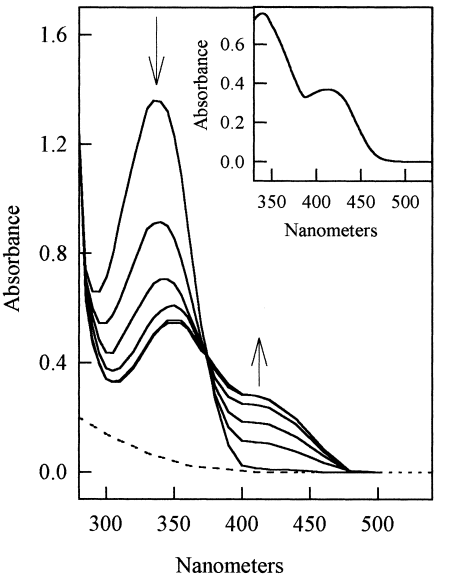


Fig. 2. Spectral changes during the oxidation of 200 μM NADPH by 50 μM tetryl in the presence of 10 nM ferredoxin:$NADP^+$ reductase. The spectra were recorded every 20 min. The dashed line shows the initial absorbance of the reaction mixture before the addition of NADPH. The inset shows the absorbance spectrum of 50 μM *N*-methylpicramide.

The single-step hydride transfer (Fig. 1, route A) should not be accompanied by redox cycling, since the reaction product *N*-methylpicramide or the mixtures of tetryl and *N*-methylpicramide (50:50 μM) neither autooxidized in the solution, nor reduced the added cytochrome *c* or ferritin.

Next, we checked up the reaction of tetryl with ferredoxin:$NADP^+$ reductase, as an example of a single-electron transfer (Fig. 1, route B) [16]. Tetryl (50 μM) oxidized the excess of NADPH (200 μM) in the presence of FNR (Fig. 2), yielding the characteristic spectrum of *N*-methylpicramide (Fig. 2, inset) at the end of the reaction. The analogous spectrum of *N*-methylpicramide was obtained in the presence of tetryl, FNR, 5 μM NADPH, and NADPH regeneration system (3 mM glucose-6-phosphate and 30 μg/ml glucose-6-phos-

Table 1
Kinetic characteristics of tetryl and 2,4-dinitrophenyl-*N*-methylnitramine reactions with ferredoxin:$NADP^+$ reductase (FNR) and DT-diaphorase, pH 7.0, 25°C

| Parameters | Tetryl | | 2,4-Dinitrophenyl-*N*-methylnitramine | |
|---|---|---|---|---|
| | FNR[a] | DT-diaphorase[b] | FNR[a] | DT-diaphorase |
| $k_{cat}$ ($s^{-1}$) | 56 ± 8 | 73 ± 10 | 47 ± 3 | 4.4 ± 0.5 |
| $k_{cat}/K_m$ ($M^{-1}\ s^{-1}$) | (1.1 ± 0.2) × $10^5$ | (2.6 ± 0.3) × $10^5$ | (2.5 ± 0.4) × $10^5$ | (5.2 ± 0.7) × $10^4$ |
| Rate of cytochrome *c* reduction (% of NADPH oxidation rate)[c] | 180 ± 5 | 132 ± 10 | 182 ± 3 | 157 ± 12 |
| Rate of ferritin reduction (% of NADPH oxidation rate) | 2.5 ± 0.5 | 2.6 ± 0.3 | 3.3 ± 0.7 | 4.2 ± 0.6 |

[a]Concentration of NADPH, 200 μM.
[b]Concentration of NADPH, 200 μM. The rate constants were determined assuming that spectral changes at 340 nm were the same as for the formation of the single reaction product, *N*-methylpicramide.
[c]Concentration of cytochrome *c*, 50 μM.

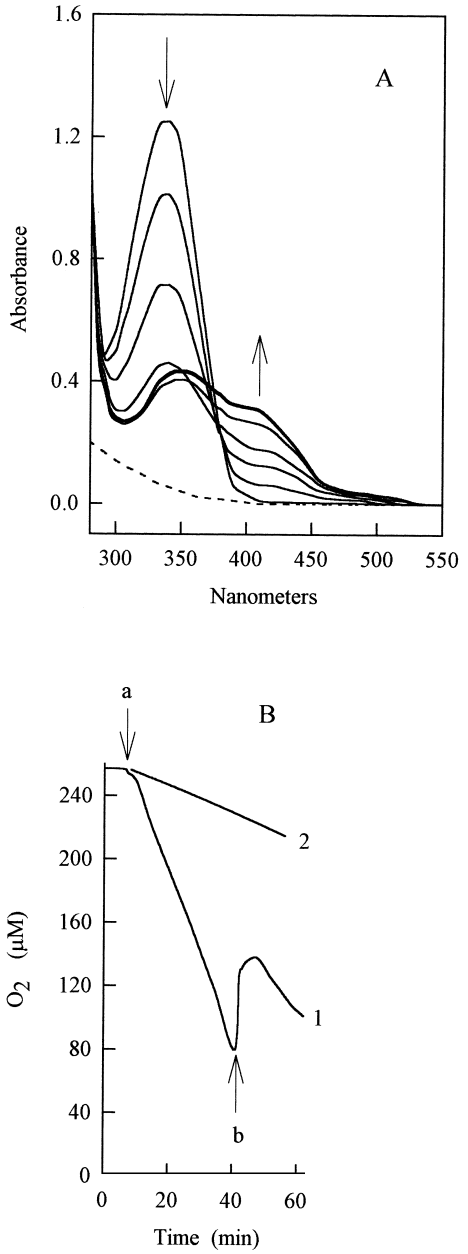


Fig. 3. A: Spectral changes during oxidation of 200 μM NADPH by 50 μM tetryl in the presence of 10 nM DT-diaphorase. The spectra were recorded every 20 min. The dashed line shows the initial absorbance of the reaction mixture before the addition of NADPH. B: The oxygen consumption during the oxidation of 300 μM NADPH by 50 μM tetryl and 10 nM DT-diaphorase (1), the same in the presence of 2 μM dicoumarol (2). The arrow a indicates the moment of DT-diaphorase addition, arrow b indicates the moment of 30 μg/ml catalase addition.

phate dehydrogenase) (data not shown). At the end of the reaction, 30 ± 3 μM nitrite ($n = 3$) and a close amount of *N*-methylpicramide (determined using $\Delta\varepsilon_{420} = 6.3$ $mM^{-1}$ $cm^{-1}$) was formed. In TLC analysis of the reaction mixture, a single yellow non-fluorescent spot of *N*-methylpicramide ($R_f = 0.91$), in addition to traces of tetryl (UV-fluorescent spot with $R_f = 0.97$), was observed. The kinetic constants of tetryl reduction by FNR (Table 1), were determined according to a NADPH oxidation rate monitored at 340 nm and corrected for the increase in the absorbance due to *N*-methylpicramide formation ($\Delta\varepsilon_{340} = 12.8$ $mM^{-1}$ $cm^{-1}$). During the reaction, oxygen was consumed at rates close to the NADPH oxidation rate; the addition of catalase during the reaction caused the return of oxygen, indicating that $H_2O_2$ was formed as the final reaction product (data not shown). In separate assays, the reduction of the added cytochrome *c* and the reductive release of $Fe^{2+}$ from ferritin was observed (Table 1). Superoxide dismutase (SOD, 120 μg/ml), inhibited these reactions by 20–35%, pointing to the equilibrium between $O_2/O_2^{\bullet-}$ and tetryl/tetryl anion radical (Fig. 1, route B).

The DT-diaphorase-catalyzed oxidation of NADPH by tetryl proceeded similarly to FNR-catalyzed reaction, since tetryl oxidized the excess of NADPH (Fig. 3A). The reaction was accompanied by oxygen consumption, which was inhibited by the specific inhibitor of DT-diaphorase, dicoumarol (Fig. 3B). In separate assays, the reduction of cytochrome *c* and the reductive mobilization of $Fe^{2+}$ from ferritin (Table 1) was observed, which was inhibited by SOD (20–25%), and by dicoumarol. The spectral changes during DT-diaphorase-catalyzed reaction (Fig. 3A) were similar to FNR (Fig. 2); however, the absence of the isosbestic point at 375 nm at the end of the reaction pointed to the formation of several products. Indeed, in addition to *N*-methylpicramide, TLC data showed a minor dark red spot of a second reaction product with $R_f = 0.79$. For this reason, only approximate kinetic parameters of the reaction were determined (Table 1), assuming that the spectral changes at 340 and 420 nm were the same as in case of the formation of the single reaction product *N*-methylpicramide. Kinetic parameters of the reaction (Table 1) did not depend on the NADPH concentration, 10–200 μM. The oxidation of 200 μM NADPH by 50 μM tetryl and DT-diaphorase was accompanied by the formation of 17 ± 2 μM nitrite ($n = 3$).

The identification of the additional product(s) of tetryl re-

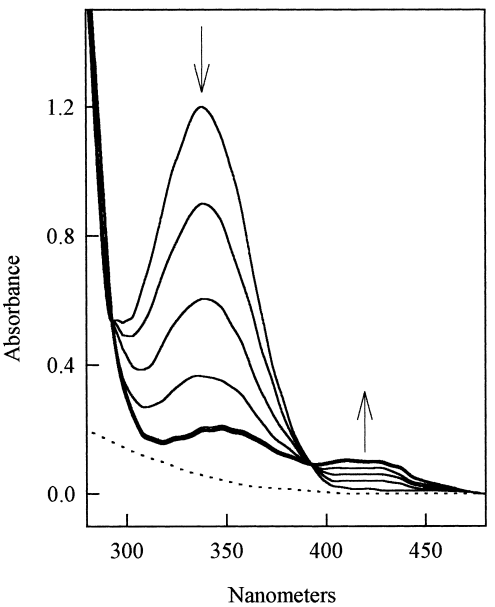


Fig. 4. Spectral changes during oxidation of 200 μM NADPH by 50 μM 2,4-dinitrophenyl-*N*-methylnitramine in the presence of 10 nM DT-diaphorase. The spectra were recorded every 30 min. The dashed line shows the initial absorbance of the reaction mixture before the addition of NADPH.

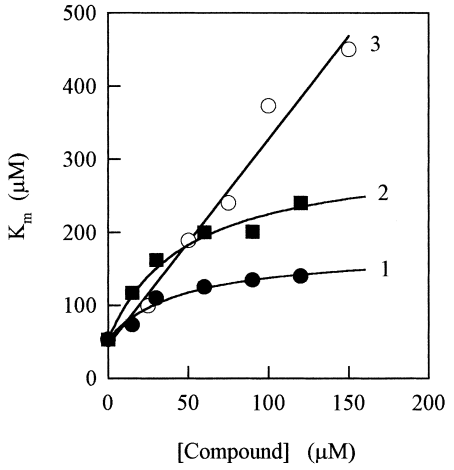


Fig. 5. The competitive to NADPH inhibition of NADPH:menadione reductase reaction of DT-diaphorase by tetryl (1), 2,4-dinitrophenyl-*N*-methylnitramine (2) and 4,5,6-trinitrobenzimidazolone (3). $K_m$ of NADPH were calculated varying the concentration of NADPH (40–400 μM) in the presence or in the absence of inhibitors. The concentration of menadione was 10 μM, the concentration of cytochrome *c* was 50 μM.

action with DT-diaphorase was beyond the scope of the present work. We have found that *N*-methylpicramide was not reduced by DT-diaphorase at a measurable rate. However, it is known that during tetryl transformation by soil bacteria, the reduction of nitro group(s) in the tetryl benzene ring may occur, in addition to the formation of the main reaction product *N*-methylpicramide [13]. One may suppose that the easiness of nitroreduction will increase upon the increase in electron affinity of the compound, i.e. the increase in the number of nitro groups. In an attempt to avoid parallel reactions, we have studied enzymatic reactions of tetryl dinitro analog, 2,4-dinitrophenyl-*N*-methylnitramine (Table 1). Indeed, the reaction of this compound with DT-diaphorase yielded a single reaction product, 2,4-dinitro-*N*-methylaniline ($R_f = 0.76$). The spectral changes during the reaction of 2,4-dinitrophenyl-*N*-methylnitramine with DT-diaphorase (Fig. 4) were analogous to the reaction with FNR (data not shown), and closely resembled the spectral data of tetryl reaction with FNR (Fig. 2). The reaction was also accompanied by the reduction of cytochrome *c* and the reductive mobilization of $Fe^{2+}$ from ferritin (Table 1), which were inhibited by SOD by 22–24%. The yield of nitrite in this case made up 10% of the amount of NADPH oxidized.

In the reactions of tetryl and 2,4-dinitrophenyl-*N*-methylnitramine with FNR or DT-diaphorase, the rate of *N*-methylpicramide or 2,4-dinitro-*N*-methylaniline formation and the rate of reduction of the added cytochrome *c*, used to be always directly proportional to the enzyme concentration, 5–50 nM. Typically, the rate of *N*-methylpicramide or 2,4-dinitro-*N*-methylaniline formation was equal to 15–20% of the NADPH oxidation rate.

Since nitroaromatic compounds are competitive to NADPH inhibitors in the menadione reductase reaction of DT-diaphorase [3,11], the inhibition properties of tetryl and its 2,4-dinitro analog were tested. These compounds were competitive to NADPH inhibitors, increasing $K_m$ of NADPH (Fig. 5). However, the non-linear Cleland plots were observed (Fig. 5), pointing to the incomplete character of the inhibition. In contrast, other nitro compounds, e.g. the previously described inhibitor of DT-diaphorase 4,5,6-trinitrobenzimidazolone [11], showed linear Cleland plots (Fig. 5). The plotting of the data in the coordinates 1/inhibition degree, 1/inhibitor concentration [25], yielded the values of maximal inhibition degree, 73% (tetryl), and 83% (2,4-dinitrophenyl-*N*-methylnitramine), and 50% inhibiting concentrations of 40 μM and 14 μM, respectively. In the control experiments, we have found that incomplete inhibition was not related to tetryl-dependent reduction of cytochrome *c* by DT-diaphorase.

## 4. Discussion

The reactions of DT-diaphorase with tetryl and its 2,4-dinitro analog were accompanied by the events characteristic to the single-electron reductive N-denitration by FNR (Fig. 1, route B), i.e. the oxidation of the excess NADPH and redox cycling, the superoxide dismutase-sensitive reduction of cytochrome *c* and ferritin (Table 1, Figs. 2–4). In addition to *N*-methylpicramide, DT-diaphorase forms other, presently unidentified, product(s) of tetryl reduction. However, the use of 2,4-dinitrophenyl-*N*-methylnitramine provided clear evidence of an analogy between FNR- and DT-diaphorase-catalyzed reactions. Quantitatively, the reduction of this compound by DT-diaphorase and FNR gives satisfactorily close ratios between the rates of NADPH oxidation and cytochrome *c* reduction (Table 1). This points to the single-electron reductive N-denitration mechanism of DT-diaphorase (Fig. 1, route B), which is unexpected, since DT-diaphorase is considered to be an obligatory two-electron transfering enzyme [1–3,8,9]. Although DT-diaphorase is rapidly reoxidized by single-electron oxidant ferricyanide [9], the single-electron transfer in tetryl reduction represents a different case, since during N-denitration tetryl may accept two redox equivalents (Fig. 1, route A).

In order to explain the phenomena observed, one may suppose that DT-diaphorase transfers two electrons or hydride from reduced FAD to the oxidant in single-electron steps, e.g. $e^-$-$H^+$-$e^-$, $e^-$-$H^\bullet$, or $e^-$-$e^-$. Although during the reoxidation of reduced DT-diaphorase by ferricyanide the transient FAD semiquinone intermediate was not formed [9], it is possible that the relative rates of the first and second redox equivalent transfer may depend on the intrinsic properties of the electron (hydride) acceptor. In the reactions of tetryl, its slow non-enzymatic conversion to *N*-methylpicramide by hydride donor *N*-benzyl-1,4-dihydronicotinamide, proceeds through the charge-transfer intermediate, and is activated under free-radical initiating conditions [24]. The analogous charge-transfer intermediates are formed in the reductive desulfonation of 2,4,6-trinitrobenzenesulfonate [26] and the reduction of quinones [27,28] by 1,4-dihydronicotinamides. In the latter case, a three-step hydride transfer ($e^-$-$H^+$-$e^-$) takes place [27,28], and the overall reaction rate is partly limited by the first electron and proton transfer rates. Thus, one may assume that during tetryl reduction by DT-diaphorase, the fast first electron transfer is followed by a slower transfer of a second redox equivalent, e.g. $H^+$+$e^-$, or $H^\bullet$. The transfer of a second redox equivalent may be controlled by the rate of covalent bond breaking, i.e. it may occur simultaneously with the release of $NO_2^-$ as shown in Fig. 1 (route B), or only after $NO_2^-$ dissociation from anion-radical. This difference in electron-transfer rates may enable tetryl anion-radical to dissociate

from the enzyme and react with $O_2$ or cytochrome *c*. However, the above reactions of enzyme-bound anion-radical are also not excluded.

The reaction of DT-diaphorase with tetryl proceeded at a high rate (Table 1), which was at least by one order of magnitude higher than for most nitroaromatic compounds [3,11]. Thus, our data point to DT-diaphorase as one of the enzymes possibly responsible for tetryl bioreductive transformation [14,15]. One may suppose that one of the possible reasons of high tetryl reactivity is its specific binding at the presently unidentified site. The incompletely competitive NADPH inhibition by tetryl and its 2,4-dinitro analog (Fig. 5) indicates that these compounds do not share the binding site of the other nitroaromatics, which most probably occupy the adenosine phosphate binding region of the NADPH binding site [11]. It is possible that tetryl and its dinitro analog bind at the immediate vicinity of the NADPH binding site, decreasing its affinity to oxidized DT-diaphorase.

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