Enzymatic Reduction of 2,4,6-Trinitrotoluene and Related Nitroarenes: Kinetics Linked to One-Electron Redox Potentials

ARTICLE *in* ENVIRONMENTAL SCIENCE AND TECHNOLOGY · AUGUST 2000

Impact Factor: 5.33 · DOI: 10.1021/es991422f

2 AUTHORS:

R. Guy Riefler
Ohio University
17 PUBLICATIONS **290** CITATIONS
SEE PROFILE

Barth F Smets
Technical University of Denmark
181 PUBLICATIONS **3,761** CITATIONS
SEE PROFILE

Environ. Sci. Technol. **2000**, *34,* 3900−3906

Enzymatic Reduction of 2,4,6-Trinitrotoluene and Related Nitroarenes: Kinetics Linked to One-Electron Redox Potentials

R. GUY RIEFLER† AND BARTH F. SMETS*,†,‡

Environmental Engineering Program, Department of Civil and Environmental Engineering, and Microbiology Program, Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06269-2037

At military bases and munitions factories, 2,4,6-trinitrotoluene (TNT) is a common soil and groundwater contaminant. Although the reduction of nitro groups in TNT and related nitroarenes has been extensively investigated, few researchers have studied the link between reduction rates and the electrochemical properties of these compounds. In this work, the standard one-electron redox potentials at pH 7 ($E_1^{\circ\prime}$) for six important nitroarenes have been measured by pulse radiolysis. The internally consistent values were −0.253 V for TNT, −0.417 V for 2-amino-4,6-dinitrotoluene, −0.449 V for 4-amino-2,6-dinitrotoluene, −0.397 V for 2,4-dinitrotoluene, −0.402 V for 2,6-dinitrotoluene, and −0.502 V for 2,4-diamino-6-nitrotoluene. The reduction kinetics of these nitroarenes was investigated using a bacterial nitroreductase, NAD(P)H:FMN oxidoreductase that uses NADH·H^+ as a cosubstrate. A log-linear relationship was observed between the $E_1^{\circ\prime}$ values and the enzymatic reduction rates for five nitroarenes, suggesting that transfer of the first electron is the rate-limiting step in nitroreduction.

Introduction

2,4,6-Trinitrotoluene (TNT), a widely used explosive, is a common contaminant at military bases and munitions production and handling facilities. In addition to its explosion hazard, TNT causes red blood cell abnormalities, liver dysfunction, and cancer in mammals (*1, 2*). TNT is found as a pollutant in soil and groundwater often in combination with 2,4-dinitrotoluene (24DNT) and 2,6-dinitrotoluene (26DNT), both precursors in the TNT manufacturing process (*3*). Although some bacterial cultures are capable of partial mineralization of TNT (*4*−*6*), TNT is most commonly biotransformed by reduction of the nitro groups (*7, 8*). By the sequential addition of two electrons, a nitro group is reduced to a nitroso, a hydroxylamino, and finally an amino group (*9*). Through successive reductions of the three nitro groups, many bacterial species sequentially transform TNT to aminodinitrotoluene, diaminonitrotoluene, and finally triaminotoluene (TAT) (*9*). However, in some bacterial cultures hydroxylamino-substituted compounds persist without production of amino-substituted compounds (*10*). Two monoamino isomers, 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT), and only one diamino isomer, 2,4-diamino-6-nitrotoluene (DANT), form in significant amounts from TNT reduction (*11*). As the electron-withdrawing nitro groups are sequentially replaced with electron-donating amino groups, the remaining nitro groups become less susceptible to reduction. Thus, TNT is reduced faster than ADNTs which, in turn, are reduced faster than DANT (*12*). Complete anaerobic transformation of TNT to TAT appears limited by the DANT to TAT transformation.

Many enzymes in both prokaryotic and eukaryotic cells are capable of mediating TNT, ADNT, DANT, and DNT reduction (*13*). These have been divided in type I nitroreductases that reduce in two-electron increments and type II nitroreductases that reduce in one-electron increments. These enzymes have other physiological roles but also exhibit activity with nitro groups (*13*). Type II nitroreductases are oxygen sensitive because the nitro anion radical formed after one-electron reduction can react with oxygen, resulting in electron transfer to oxygen yielding a superoxide radical and the original nitro group (*9*). Most nitroreductases purified from bacteria are type I soluble flavoproteins that use nicotinamide adenine dinucleotide (NADH·H^+) or nicotinamide adenine dinucleotide phosphate (NADPH·H^+) as electron donors (*13*). In redox reactions, these compounds accommodate the transfer of two protons and two electrons to yield NAD^+ and $NADP^+$, respectively. Many purified enzymes and cell-free extracts exhibit activity against TNT using either NADH·H^+ or NADPH·H^+ as electron donors including enzymes from *Pseudomonas pseudoalcaligenes* (*14*), *P. aeruginosa* (*15*), *Bacillus* sp. (*15*), *Staphylococcus* sp. (*15*), *Ralstonia eutropha* (*16*), *Enterobacter cloacae* (*17, 18*), and *Clostridium thermoaceticum* (*19*).

In several abiotic reduction studies, the rate of reduction of nitroarenes and quinones was linked to the one-electron redox potentials of the compounds (*17, 20*−*26*). Log-linear relationships were observed between the reduction rates and the one-electron redox potentials of the nitroarenes or quinones. None of these studies, however, investigated the compounds of interest here, namely, TNT, 24DNT, 26DNT, 4ADNT, 2ADNT, and DANT, because the one-electron reduction potentials for these compounds had not been determined. One-electron redox potentials have since been estimated for 24DNT from a linear free energy relationship (LFER) established with 10 nitroarenes with known one-electron redox potentials (nitrobenzene, methylnitrobenzenes, chloronitrobenzenes, and acetylnitrobenzenes) (*22*) and for TNT, 2ADNT, 4ADNT, and DANT from a LFER established with five nitroarenes (4-methylnitrobenzene, 4-chloronitrotoluene, 4-acetylnitrotoluene, 1,3-dinitrobenzene, and 1,4-dinitrobenzene) (*26*).

In summary, the reduction of TNT and related nitroarenes has been extensively investigated; however, few researchers have studied the link between reduction rates and the electrochemical properties of these compounds. In this work, it was hypothesized that the reduction rates of TNT, 2ADNT, 4ADNT, DANT, 24DNT, and 26DNT are correlated to their one-electron redox potentials. A single enzyme, NAD(P)H:FMN oxidoreductase isolated from *Photobacterium fischeri*, was employed in this research. This enzyme's physiological role is the coupled oxidation of NAD(P)H·H^+ and reduction of flavin mononucleotide (FMN), but it has been shown to reduce TNT and other nitroarenes (*27*). The objectives of this research were (i) to measure the one-electron redox potentials for TNT, 2ADNT, 4ADNT, DANT, 24DNT, and 26DNT and (ii) to verify the hypothesis that the enzymatic

* Corresponding author e-mail: barth.smets@uconn.edu; phone: (860)486-2270; fax: (860)486-2298.
† Department of Civil and Environmental Engineering.
‡ Department of Molecular and Cell Biology.

10.1021/es991422f CCC: $19.00
Published on Web 08/04/2000

reduction rates of these compounds are related to their one-electron redox potentials.

Materials and Methods

Chemicals. TNT was purchased from Chemservice (Westchester, PA). 2ADNT, 4ADNT, and DANT were purchased from Accustandard Inc. (New Haven, CT); 24DNT, 26DNT, duraquinone (DQ), anthraquinone-2-sulfonate (AQS), and methyl viologen (MV) were purchased from Aldrich (Milwaukee, WI). $NADH{\cdot}H^+$ and NAD^+ were purchased from Sigma (St. Louis, MO), and *Photobacterium fischeri* NAD(P)H:FMN oxidoreductase was purchased from Roche (Indianapolis, IN). Neat 2-hydroxylamino-4,6-dinitrotoluene, 4-hydroxylamino-2,6-dinitrotolune, and 2,4-dihydroxylamino-6-nitrotoluene were generously provided by Dr. Joseph Hughes (Rice University, Houston, TX) for identification of TNT reduction products. TNT, 2ADNT, 4ADNT, DANT, and $NADH{\cdot}H^+$ were 99% pure, and NAD^+ was 98% pure. Nitroarenes were dissolved in deionized water (DI) and autoclaved at 121 °C to create stock solutions. Spectrophotometric and HPLC analysis confirmed that no transformation occurred with this treatment. $NADH{\cdot}H^+$ and NAD^+ stock solutions were prepared in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (TRIZMA) buffer at pH 7 and stored at 4 °C. $NADH{\cdot}H^+$ concentration was confirmed before each experiment by spectrophotometric measurement. NAD(P)H:FMN oxidoreductase was dissolved in 40% (by volume) glycerol containing 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol, and 50 mM potassium phosphate buffer at pH 7, per manufacturer instructions. The enzyme's reported activity was 100 U/mg of protein at 25 °C and pH 7 with FMN and $NADH{\cdot}H^+$ as substrates.

Nitroarene concentrations were quantified using reversed-phase HPLC with photodiode array detection (PU980 and FP920 Jasco Inc., Easton, MD). Compounds were separated on a Betasil Cyano column (Keystone Scientific Inc., Bellefonte, PA) using a methanol/DI mobile phase (1 mL/min) buffered with 20 mM phosphate at pH 4, with a linear gradient from 30% methanol to 45% methanol in 15 min. Absorbance spectra from 200 to 400 nm ($\Delta = 1$ nm) were measured at 0.8-s intervals to ensure accurate identification of compounds, while quantification was performed at 254 nm. $NADH{\cdot}H^+$ concentrations were determined by UV/VIS spectrophotometry (Varian Cary Bio50, Sugar Land, TX) at 340 nm ($\epsilon_{NADH{\cdot}H^+} = 6{,}220\ M^{-1}\,cm^{-1}$). $NADH{\cdot}H^+$ strongly absorbs at this wavelength, while NAD^+ does not. The nitroarenes absorb weakly at 340 nm, so concentrations of $NADH{\cdot}H^+$ were kept 5 times higher than the nitroarene concentrations. For experiments with TNT, 24DNT, 26DNT, 4ADNT, and 2ADNT paired with $NADH{\cdot}H^+$, 2, 1, 1, 6, and 6%, respectively, of the absorbance at 340 nm was due to interference from the nitroarenes initially. Inspection of absorbance spectra of nitroarene transformation products separated by HPLC revealed insignificant change in the nitroarene-contributed absorbance at 340 nm during the course of the experiments.

Pulse Radiolysis. Pulse radiolysis was performed according to established procedures (*28, 29*). All experiments were conducted at the U.S. Department of Energy Radiation Laboratory at Notre Dame University (South Bend, IN). Briefly, a nitroarene (S) and a quinone or methyl viologen (Q) were dissolved in a 2-methyl-2-propanol ($(CH_3)_3COH$) solution (0.2 M) buffered at pH 7 with 5 mM phosphate. TNT was paired with DQ; 2ADNT, 4ADNT, 24DNT, and 26DNT were paired with AQS; and DANT was paired with MV. Solutions were irradiated by 2.5-ns pulses of 2.8 MeV electrons using the Titan Beta model TS-8/16-1 S electron linear accelerator (*30*). Solution absorbance was recorded at the wavelength of maximum absorbance for the quinone radical (445 nm for DQ, 505 nm for AQS, and 395 nm for MV) (*31*).

Presence of 2-methyl-2-propanol ensured that after each pulse, radicals produced by water radiolysis ($H^{\bullet}$, $OH^{\bullet}$) were converted to $(CH_3)_3COH^{\bullet}$. The remaining solvated electrons (e_{aq}^-) and $(CH_3)_3COH^{\bullet}$ reacted with either Q or S to produce $Q^{\bullet-}$ or $S^{\bullet-}$. These two redox couples quickly attain equilibrium with an equilibrium constant calculated by

$$K = \frac{\{Q^{\bullet-}\}\{S\}}{\{S^{\bullet-}\}\{Q\}} = f\frac{[Q^{\bullet-}][S]}{[S^{\bullet-}][Q]} \tag{1}$$

where {A} is the activity of *A*, [A] is the molar concentration of *A*, and *f* is the composite activity coefficient for the four dissolved compounds.

The quinone radical concentration is proportional to the absorbance measured at the quinone radical peak wavelength corrected for interference by nitroarene absorbance. Because the electron pulse is highly reproducible, the same total number of radicals is produced in each run. The concentration of nitroarene radicals is proportional to the decrease in the absorbance at the quinone radical peak wavelength in the solution when nitroarenes were present. In other words, the difference in absorbance at the quinone radical peak of a quinone-only experiment and a quinone-plus-nitroarene experiment is proportional to the electrons transferred to the nitroarene (*28*). Thus

$$\frac{[Q^{\bullet-}]}{[S^{\bullet-}]} = \frac{A - A_s}{A_q - A} \tag{2}$$

where A, A_s, and A_q are the average absorbance at the quinone radical peak wavelength of the test solution (quinone plus nitroarene), the nitroarene-only solution, and the quinone-only solution, respectively. Substituting eq 2 into eq 1 gives

$$K = f\frac{[S](A - A_s)}{[Q](A_q - A)} \tag{3}$$

The one-electron redox potential in reference to the hydrogen electrode (E_1), at standard conditions (E_1°), at pH 7 ($E_1^{\circ\prime}$) for the nitroarenes can then be determined by

$$E_1^{\circ\prime}(S) = E_1^{\circ\prime}(Q) - 0.059\log(K) \tag{4}$$

Activity coefficients are computed for the four dissolved compounds using the Debye−Hückel expression with unity coefficients assumed for neutral species (*32*). At 5 mM phosphate at pH 7, $I = 5.40 \times 10^{-3}$ M. For nitroarenes and DQ, activity coefficients are $\gamma_S = \gamma_Q = 1$ and $\gamma_{S^{\bullet-}} = \gamma_{Q^{\bullet-}} = 0.917$. For AQS, which has a −1 charge, $\gamma_Q = 0.917$ and $\gamma_{Q^{\bullet-}} = 0.708$. For MV, which has a +2 charge, $\gamma_Q = 0.708$ and $\gamma_{Q^{\bullet-}} = 0.917$. Thus, for solutions with DQ, $f = 1$; with AQS, $f = 0.842$; and with MV, $f = 1.41$.

Mixtures of a single nitroarene at concentrations ranging from 0.01 to 0.2 mM and a quinone or methyl viologen at concentrations ranging from 0.0001 to 1.0 mM were prepared to yield concentration ratios, [S]/[Q], from 0.1 to 200. After preparation, the mixture was prepurged and continually purged with N_2 as the solution was discharged to the radiolysis flow cell. Absorbance versus time profiles were averaged over five pulses. At least three individual profiles were used at each concentration ratio to determine average equilibrium concentrations of quinone or methyl viologen radicals. Four to six different concentration ratios were used to compute a mean value and standard deviation of the equilibrium coefficient. Mean and standard deviations for $E_1^{\circ\prime}$ were computed from equilibrium coefficient estimates using eq 4 and appropriate error propagation formulas (*33*).

Because a fraction of the original compound was transformed into radicals, [Q] and [S] were not equivalent to the

starting concentrations. Using a known standard, potassium thiocyanate, it was determined that 3.47×10^{-6} M solvated electrons were formed in each pulse experiment. Given that $[Q^{\bullet -}]/[S^{\bullet -}]$ was computed with eq 2, and $[Q^{\bullet -}] + [S^{\bullet -}] = 3.47 \times 10^{-6}$ M, values for $[Q^{\bullet -}]$ and $[S^{\bullet -}]$ were determined, and [Q] and [S] were likewise computed. $E_1^{\circ\prime}$ calculations for the six nitroarenes used the reported values $E_1^{\circ\prime}(\mathrm{DQ}) = -0.260$ V, $E_1^{\circ\prime}(\mathrm{AQS}) = -0.390$ V, and $E_1^{\circ\prime}(\mathrm{MV}) = -0.448$ V (*34*).

Enzymatic Transformation Experiments. Enzymatic transformation of the nitroarenes was performed in 3-mL sealed quartz spectrosil cuvettes (Aldrich, Milwaukee, WI) with solution volumes of 1.5 mL. A TRIZMA buffered solution of $NADH{\cdot}H^+$, nitroarene, and enzyme was mixed in a cuvette kept at 25 °C in a water-jacketed cuvette holder. To exclude oxygen, the solution was initially sparged with water vapor saturated N_2 for 5 min, while N_2 flush of the headspace continued throughout the experiment. Cap septa were replaced on a daily basis to maintain airtight seals. Initially, control experiments were conducted to verify the absence of transformations in solutions containing mixtures of enzyme/$NADH{\cdot}H^+$, enzyme/TNT, and TNT/$NADH{\cdot}H^+$. $NADH{\cdot}H^+$ and TNT were at 500 and 100 μM, respectively, and the enzyme was at 0.533 mg of protein/L. $NADH{\cdot}H^+$ and TNT concentrations were measured by absorbance at 340 and 254 nm, respectively.

An additional control experiment was conducted to confirm that $NADH{\cdot}H^+$ depletion was an accurate measure for nitroarene depletion. Enzymatic 2ADNT transformation was used as a worst-case scenario, because 2ADNT has the highest interference with $NADH{\cdot}H^+$ absorbance at 340 nm. The experiment was initiated by adding the enzyme at 7.5 mg of protein/L to a solution of 2ADNT and $NADH{\cdot}H^+$ at 100 and 500 μM, respectively. $NADH{\cdot}H^+$ consumption was monitored spectrophotometrically, and periodic samples were removed for HPLC analysis.

For reaction rate measurements, experiments were initiated by adding the enzyme to solutions that contained $NADH{\cdot}H^+$ and a single nitroarene at 500 and 100 μM, respectively. $NADH{\cdot}H^+$ consumption was monitored by spectrophotometry. An enzyme aliquot was injected into the capped cuvette through the septum, and the solution was rapidly mixed before continuing spectrophotometric observations. Initial and final aliquots were collected for HPLC analysis to determine final concentrations of nitroarenes and to identify transformation products. $NADH{\cdot}H^+$ consumption profiles were translated into nitroarene reduction profiles using the stoichiometry calculated from measured initial and final substrate concentrations. Experiments were conducted with TNT, 2ADNT, 4ADNT, DANT, 24DNT, and 26DNT. Initial reaction rates were determined by estimating the slope of the initial profiles of nitroarene consumption (≤2 min) using standard linear regression. Rates were normalized with respect to the enzyme and initial substrate concentrations. If an initial linear region was not observed, the experiment was repeated with a different enzyme concentration until a clear initial linear region was observed. Experiments were conducted at several different initial enzyme concentrations.

Transition state theory was used to relate reaction rates with $E_1^{\circ\prime}$. For reactions that have a common rate-limiting step, a correlation between the free energy of activation for that rate-limiting step, $\Delta G^{\ddagger}$, and the reaction rate, k, is often observed (*35*):

$$\Delta G^{\ddagger} = -2.3RT \log k + c \qquad (5)$$

where R is the universal gas constant, T is the absolute temperature, and c is a constant. Additionally, the free energy of activation is often proportional to the free energy change of the first electron transfer, $\Delta G_1^{\circ\prime}$:

$$\Delta G^{\ddagger} = \alpha \Delta G_1^{\circ\prime} + \beta \qquad (6)$$

where α and β are constants (*36*). Finally, the free energy of one-electron transfer can be related to $E_1^{\circ\prime}$ using the Faraday constant, F:

$$\Delta G_1^{\circ\prime} = F(E_1^{\circ\prime}(\mathrm{oxidant}) - E_1^{\circ\prime}(\mathrm{reductant})) \qquad (7)$$

Combining these three relationships, using $T = 25$ °C, and using a constant value for $E_1^{\circ\prime}$(reductant) results in the linear free energy relationship

$$\log k = A\frac{E_1^{\circ\prime}(\mathrm{oxidant})}{0.059} + B \qquad (8)$$

where A and B are constants (*36*). Values for A and B were determined by least-squares regression and minimizing absolute deviations (MAD) (*37*).

The Marcus theory of outer-sphere electron transfer was used to interpret A values obtained from eq 8. Marcus theory assumes that a reversible electron-transfer reaction takes the form

$$A + D \underset{k_{-d}}{\overset{k_d}{\leftrightarrow}} (AD) \underset{-k_{el}}{\overset{k_{el}}{\leftrightarrow}} (A^{\bullet -}D^{\bullet +}) \Rightarrow \text{products} \qquad (9)$$

where k_d is the rate constant for complex formation, k_{-d} is the rate constant for complex disassociation, k_{el} is the rate constant for electron transfer, and k_{-el} is the rate constant for back electron transfer (*35*). The Marcus equation relates the observed overall reaction rate, k_{obs}, and the pH-corrected standard Gibbs free energy of the electron-transfer step, $\Delta G_1^{\circ\prime}$. Three significant scenarios are observed: (i) a diffusion-limited case where k_{obs} is controlled by k_d; (ii) an activation-limited case where k_{obs} is controlled by k_{el}; and (iii) an equilibrium-controlled case where k_{-el} is significant (*35*). When the reaction is equilibrium controlled (case iii), the slope of log k versus $\Delta G_1^{\circ\prime}$ is $-1/2.3RT$, the slope of log k versus $E_1^{\circ\prime}$ is $nF/2.3RT$, and the slope of log k versus $E_1^{\circ\prime}$/0.059 V is 1. When the slope of log k versus $E_1^{\circ\prime}$/0.059 V is near 0, the overall reaction rate is controlled by the rate of precursor formation (case i). Slope values between 1 and 0 may indicate electron-transfer control (case ii).

TABLE 1. Measurement of $E_1^{\circ\prime}$ (V) by Pulse Radiolysis and Other Reported Estimates (Average ± SD)

nitroarene	redox-coupled compd	*n*	*K*	$E_1^{\circ\prime}$ (V)	reported $E_1^{\circ\prime}$ (V)
TNT	DQ	5	0.760 ± 0.040	−0.253 ± 0.022	−0.300[a]
2ADNT	AQS	5	2.91 ± 0.65	−0.417 ± 0.096	−0.390[a]
4ADNT	AQS	4	9.98 ± 1.38	−0.449 ± 0.060	−0.430[a]
DANT	MV	6	8.36 ± 5.30	−0.502 ± 0.275	−0.515[a]
24DNT	AQS	5	1.31 ± 0.17	−0.397 ± 0.057	−0.360[b]
26DNT	AQS	4	1.62 ± 0.37	−0.402 ± 0.098	na[c]

[a] From ref *26*. [b] From ref *22*. [c] na, no estimate available.

Results

Pulse Radiolysis. Average and standard deviations of experimental K and $E_1^{\circ\prime}$ values for TNT, 2ADNT, 4ADNT, 24DNT, 26DNT, and DANT are reported in Table 1. Calculated values of K varied little over an order of magnitude range of concentration ratios for all chemicals except DANT, which had a coefficient of variation (CV) of 63.4%. $E_1^{\circ\prime}$ values varied from −0.253 for TNT to −0.502 for DANT. As with K, much greater uncertainty was observed in the $E_1^{\circ\prime}$ estimate of DANT,

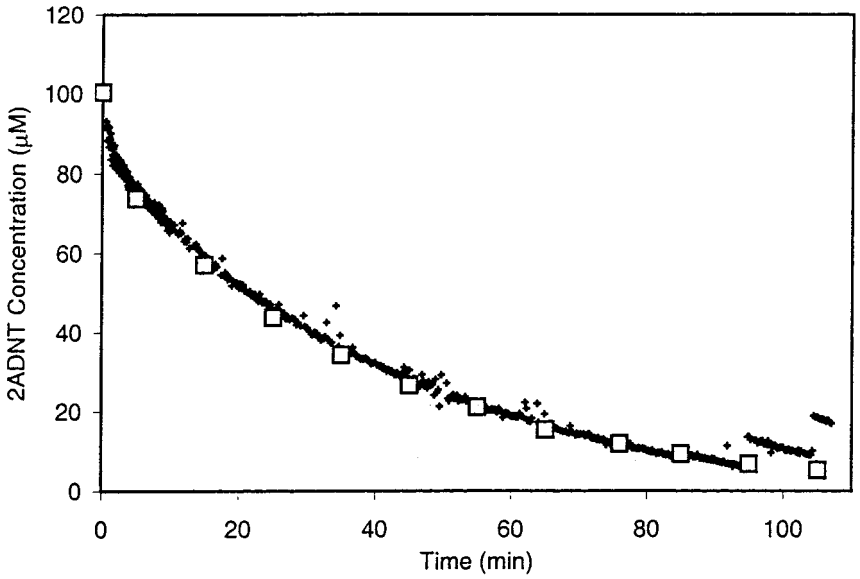

FIGURE 1. 2ADNT depletion profile during an enzyme assay calculated from NADH·H$^+$ depletion (+) and measured directly via HPLC (□).

CV of 54.8%, than of the remaining compounds, CVs of 8.7−24%. Maximum precision was achieved with TNT, CV of 8.7%.

Enzymatic Experiments. Initially, control experiments were performed to confirm that NADH·H$^+$, TNT, and enzyme were all necessary components for reaction. When only two of the three components were mixed, no activity was observed during the time scale of the experiment, indicating that no unexpected oxidation/reduction reactions occurred in this system.

An experiment was conducted to validate the use of an NADH·H$^+$ consumption profile for calculating a nitroarene consumption profile. NADH·H$^+$ and 2ADNT were concurrently measured during an enzymatic reaction by UV/VIS spectrometry and HPLC, respectively. After 85 min, 91.1 μM 2ADNT and 178 μM NADH·H$^+$ were transformed, resulting in a stoichiometric ratio of 1.95 mol of NADH·H$^+$/mol of 2ADNT. Three moles of NADH·H$^+$ are required for reduction of a nitro group to an amino group, while a 2:1 ratio is indicative of reduction to a hydroxylamino group. HPLC chromatograms revealed two peaks that did not match the elution time or spectra for DANT. These peaks presumably were 2-amino-4-hydroxylamino-6-nitrotoluene and 2-amino-6-hydroxylamino-4-nitrotoluene, although confirmatory standards could not be obtained. Assays with TNT, for which appropriate standards were available, resulted in the formation of 2-hydroxylamino-4,6-dinitrotoluene and 4-hydroxylamino-2,6-dinitrotoluene without formation of 2ADNT or 4ADNT. These results indicate that this enzyme transforms nitro groups to the hydroxylamino level, not the amino level, as has been observed in other bacterial systems (*10*).

The 2ADNT consumption curve was calculated from NADH·H$^+$ consumption data using the calculated 1.95 stoichiometric ratio. The HPLC measured 2ADNT values match the calculated values well, indicating a consistent stoichiometry between the two reactants (Figure 1). The large number of high-quality data points obtained by absorbance measurements, particularly at the beginning of the experiment, illustrates the advantage of measuring NADH·H$^+$ instead of the nitroarene and facilitates measuring initial reaction rates. Furthermore, over the considered concentration ranges, standard deviations of the HPLC (3.27 μM 2ADNT) and spectrophotometric measurements (0.0283 ABU corresponding to 2.60 μM 2ADNT) were very close.

Experiments were performed to determine the transformation rates of TNT, 2ADNT, 4ADNT, 24DNT, 26DNT, and DANT (Table 2). Average reaction rates were determined over a range of initial conditions by varying the initial enzyme concentration. Normalized reaction rates were not impacted by the initial enzyme concentrations (data not shown). TNT was reduced most rapidly, followed by 24DNT, 26DNT, 2ADNT, and 4ADNT. No transformation of DANT was observed with as much as 26.7 mg of protein/L of enzyme. This trend of decreasing activity coincides with the trend of decreasing one-electron redox potentials (Table 1). Log reaction rate was plotted versus $E_1^{\circ\prime}/0.059$ for the five nitroarenes tested (Figure 2). Kinetics of TNT, 26DNT, 2ADNT, and 4ADNT reduction obeyed a linear free energy relationship (eq 8) quite well (Figure 2). 24DNT however deviated from this relationship, i.e., 24DNT was reduced faster than predicted by its $E_1^{\circ\prime}$. The best-fit linear regression equation for the five pairs is

$$\log k = \frac{0.509E_1^{\circ\prime}}{0.059} + 5.01 \qquad (10)$$

with an $R^2 = 0.906$ and standard deviations for A and B of 0.095 and 0.11, respectively. This fit is disproportionately influenced by the TNT result because the $E_1^{\circ\prime}/0.059$ for that data point is much higher than the remaining data points. Methods to reduce the influence from this point include removing the outlier or using a fitting method that puts less relative weight on outliers (*37*). Removing the TNT data point greatly changes the resulting linear fit because of greater influence from the 24DNT data point ($A = 0.829$, $B = 7.28$). Because the TNT data point has the greatest reliability (i.e., smallest standard deviation), it should be retained in the fit. The MAD, which puts less relative weight on outliers, was employed, and very similar regression results were obtained ($A = 0.524$, $B = 5.02$).

TABLE 2. Average Initial Reaction Rates for the Reduction of Five Nitroarenes (Average ± SD)

compd	*n*	range of enzyme concn (mg of protein/L)	normalized reaction rate (mL (mg of protein)$^{-1}$ min^{-1})
TNT	8	0.133−0.533	594 ± 104
24DNT	2	4.00, 5.33	91.1 ± 24.5
26DNT	3	5.33−8.00	26.1 ± 14.6
2ADNT	2	6.67	21.0 ± 1.4
4ADNT	3	6.67−13.3	10.8 ± 4.9

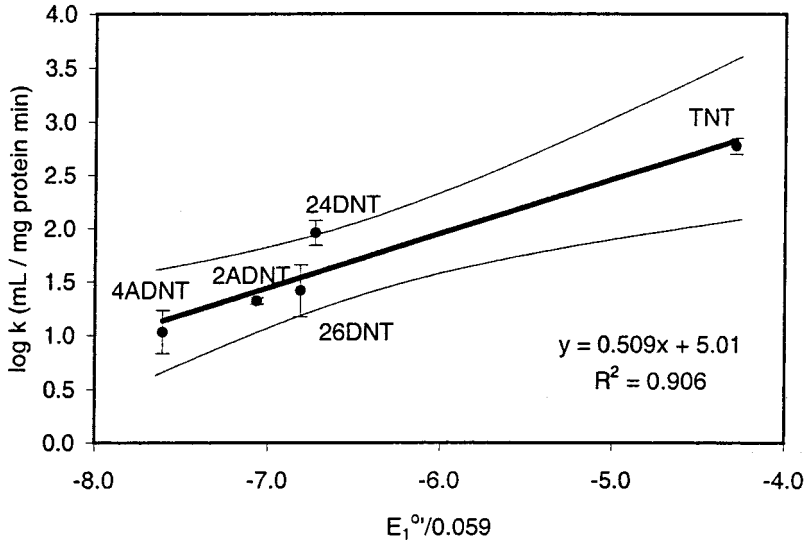

FIGURE 2. Average-normalized initial reaction rate as a function of $E_1^{\circ\prime}$ for five nitroarenes and best-fit linear regression. The 95% confidence interval for the linear regression is shown, and error bars indicate 1 SD.

TABLE 3. Linear Free Energy Relationships between Nitro Reduction Rates and $E_1^{\circ\prime}$

rate	catalyst	A[a]	ref
first-order reaction rate	magnetite/Fe^{2+}	0.22−0.42	*25*
initial reaction rate	cytochrome P-450 reductase	0.360	*23*
first-order reaction rate	hydrogen sulfide	0.45−1.0	*22*
initial reaction rate	*Enterobacter cloacae* nitroreductase	0.475	*17*
initial reaction rate	NAD(P)H:FMN oxidoreductase	0.509	this research
V_{max}/K_m	flavocytochrome b_2	0.581	*24*
V_{max}/K_m	adrenodoxin	0.599	*24*
first-order reaction rate	iron(II) porphyrin	0.60	*25*
first-order reaction rate	iron(II) porphin	0.60−0.93	*21*
V_{max}/K_m	cytochrome P-450 reductase	0.677	*24*
V_{max}/K_m	ferredoxin oxidoreductase	0.820	*20*
V_{max}/K_m	cytochrome P-450 reductase	0.885	*20*
first-order reaction rate	*Streptomyces* exudates	0.985	*41*
first-order reaction rate	2-hydroxy-1,4-naphthoquinone	0.99	*21*
first-order reaction rate	8-hydroxy-1,4-naphthoquinone	1.00	*21*
first-order reaction rate	8-hydroxy-1,4-naphthoquinone	1.0	*22*
first-order reaction rate	8-hydroxy-1,4-naphthoquinone	1.25	*26*

[a] Parameter from the linear regression equation $\log k = AE_1^{\circ\prime}/0.059 + B$.

Discussion

Qualitative relationships between aromatic nucleus substituents and one-electron redox potentials of the nitroarenes are apparent (*38*) (Table 1). Comparing TNT with 4ADNT and 2ADNT, as electron-withdrawing nitro groups are exchanged with electron-donating amino groups, the electron densities of remaining nitro groups increase. Thus, it is more difficult to reduce the remaining nitro groups, and the $E_1^{\circ\prime}$ decreases. A similar trend is observed comparing 4ADNT and 2ADNT with DANT and comparing TNT with 24DNT and 26DNT—electron-withdrawing nitro groups are exchanged with electron-donating hydrogen groups. Also, 2ADNT and 4ADNT have lower $E_1^{\circ\prime}$ than 24DNT and 26DNT because the amino group is a stronger electron donor than the hydrogen group. Thus, all six results are consistent with trends expected from the respective group contributions.

Comparing the aminodinitrotoluene isomers, 4ADNT has a more negative $E_1^{\circ\prime}$ value than 2ADNT. This may be explained by both steric and solvent effects. The nitro groups ortho to the methyl group likely experience steric hindrance forcing the resonance structures out of the benzene ring plane (*38, 39*). The result is lower stability of the one-electron reduced 4ADNT species than the one-electron reduced 2ADNT species (*38*). Alternatively, reduction may be enhanced by the ability of the dissolved compound to support localized charge on a nitro group (*39*). Nitro groups ortho to the methyl group are shielded from stabilizing solvent effects by the hydrophobic methyl group, thus resisting polarization (*39*). Again, the result is lower stability of the one-electron reduced 4ADNT species than the one-electron reduced 2ADNT species. Consistent with these explanations, increased reduction rates for 2ADNT over 4ADNT have been observed (*40*) (Table 2). The same trends of lower $E_1^{\circ\prime}$ values for compounds with only ortho nitro groups are observed for the DNT isomers. Again, the results presented here are consistent with trends expected from the respective group contributions; however, the difference in $E_1^{\circ\prime}$ values for the pairs 2ADNT/4ADNT and 24DNT/26DNT are not statistically significantly.

To our knowledge, only two studies have previously published values of $E_1^{\circ\prime}$ for TNT, ADNTs, DANT, or DNTs (*22, 26*). In both of these studies, $E_1^{\circ\prime}$ values were inferred from LFERs between the reduction rate of a suite of nitroarenes and 8-hydroxyl-1,4-naphthoquinone (jugalone). The reduction rates of TNT, 2ADNT, 4ADNT, DANT, and 24DNT with jugalone were measured, and $E_1^{\circ\prime}$ values were estimated from the LFER. To develop the LFER, Dunnivant et al. (*22*) used nitrobenzene, 2-nitrotoluene (NT), 3NT, 4NT, 2-chloronitrobenzene (CNB), 3CNB, 4CNB, 2-acetylnitroben-

zene (ANB), 3ANB, and 4ANB. The $E_1^{\circ\prime}$ value for 24DNT we determined via pulse radiolysis and the one computed from the LFER were only 9.3% different (Table 1). Hofstetter et al. used 4NT, 4CNB, 4ANB, 1,3-dinitrobenzene, and 1,4-dinitrobenzene to develop an excellent log-linear LFER fit (*26*). The $E_1^{\circ\prime}$ values for 2ADNT, 4ADNT, and DANT from our pulse radiolysis experiments and the LFER were in close agreement (6.5, 4.2, and 2.6% difference for 2ADNT, 4ADNT, and DANT, respectively; Table 1). The $E_1^{\circ\prime}$ value we measured for TNT deviated by 19% from the value reported by Hofstetter et al. (*26*). These authors supplied electrons for TNT reduction by a H_2S/jugalone system. Because the reduction rate of TNT was 2−4 orders of magnitude faster than for ADNT and DANT, it is possible that the reduction rate was limited by the transport of electrons from H_2S via jugalone to TNT rather than the kinetics of the one-electron transfer, leading to an erroneous low estimate for $E_1^{\circ\prime}$. We believe that our measurement for $E_1^{\circ\prime}$ may be more accurate because the method is more direct. Except for TNT, $E_1^{\circ\prime}$ results presented in this study are very consistent with previously reported values.

Correlations between the $E_1^{\circ\prime}$ of nitroarenes and quinones and reduction rates by a variety of catalysts have been reported with slope values that vary from 0.22 to 1.25 (Table 3). On the basis of the Marcus equation, the slope is largely a function of the solvent reorganization energy, so it is not surprising that slopes vary with different catalysts (*35*). LFERs with the lowest slopes were likely limited by precursor formation as 0.22 was obtained using an aqueous suspension of minerals as electron donors (*25*). Slopes near 1 were obtained only in solutions with dissolved electron donors without enzyme mediation, a situation unlikely to exhibit precursor formation limitation (*21, 22, 26*). Glaus et al. (*41*) also obtained a slope near one (0.985) with *Streptomyces* exudates, although boiling of the solution did not change results, suggesting that low molecular weight dissolved compounds were driving nitro group reduction. LFERs for enzyme-catalyzed reductions exhibit slopes between 0 and 1, indicating possible kinetic limitation from precursor formation (*20, 17, 24*). Similarly, we obtained a slope of 0.509, indicating that precursor formation may limit overall reaction rates. Bryant and DeLuca measured a very similar slope (0.475) with an *Enterobacter cloacae* nitroreductase (*17*). This enzyme has a bound FMN fraction, exhibits FMN activity, and is likely related to the employed NAD(P)H:FMN oxidoreductase (*17*).

All reported catalysts likely mediate one-electron transfers except *E. cloacae* nitroreductase, which mediates two-electron transfers (*17*) (Table 3). It is suspected that NAD(P)H:FMN oxidoreductase also transfers two electrons because oxygen sensitivity was not observed (results not shown), and when reducing FMN, the one-electron reduced flavin was not detected (results not shown). Nevertheless, log-linear relationships between k and $E_1^{\circ\prime}$ were apparent in this research and in Bryant and DeLuca (*17*). This may suggest a correlation between $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$ or that even for enzymes that transfer in two electron increments, the first electron-transfer governs the overall reaction kinetics.

We report $E_1^{\circ\prime}$ values measured by pulse radiolysis for six important soil and groundwater pollutants: TNT, 24DNT, 26DNT, 2ADNT, 4ADNT, and DANT. Previous research has shown that insight into transformation mechanisms and kinetics can be gained from these values (*21*). We have corroborated that the nitroreduction rate of these nitroarenes by NAD(P)H:FMN oxidoreductase is strongly correlated with the compounds' $E_1^{\circ\prime}$ values. Finally, we contend that the availability of these values will drive further mechanistic studies into the biotic and abiotic degradation of TNT and related nitroarenes.

Acknowledgments

We thank the U.S. Department of Energy Radiation Laboratory at the University of Notre Dame, especially Dr. Dan Meisel and Tim Schatz, for operation of the linear electron accelerator and assistance in the $E_1^{\circ\prime}$ measurements.

Literature Cited

(1) Kaplan, C. L.; Kaplan, A. M. *Environ. Sci. Technol.* **1982**, *16*, 566−573.
(2) Hathaway, J. A. In *Chemical Industry Institute of Toxicology Series*; Rickert, D. E., Ed.; Hemisphere Publishing: New York, 1985; pp 262−269.
(3) U.S. Environmental Protection Agency. *Approaches for the remediation of federal facility sites contaminated with explosive or radioactive wastes*; EPA/625/R-93/013; U.S. Government Printing Office: Washington, DC, 1993.
(4) Bradley, P. M.; Chappelle, F. H.; Landmeyer, J. E.; Schumacher, J. G. *Appl. Environ. Microbiol.* **1994**, *60*, 2170−2175.
(5) Boopathy, R.; Manning, J.; Kulpa, C. *Water Environ. Res.* **1998**, *70*, 80−86.
(6) Esteve-Núñez, A.; Ramos, J. L. *Environ. Sci. Technol.* **1998**, *32* (23), 3802−3808.
(7) McCormick, N. G.; Fecherry, F. E.; Levinson, H. S. *Appl. Environ. Microbiol.* **1976**, *31*, 949−958.
(8) Preuss, A.; Fimpel, J.; Diekert, G. *Arch. Microbiol.* **1993**, *159*, 345−353.
(9) Spain, J. C. *Annu. Rev. Microbiol.* **1995**, *49*, 523−555.
(10) Hughes, J. B.; Wang, C. Y.; Bhadra, R.; Richardson, A.; Bennett, G. N.; Rudolph, F. B. *Environ. Toxicol. Chem.* **1998**, *17* (3), 343−348.
(11) Hawari, J.; Halasz, A.; Paquet, L.; Zhou, E.; Spencer, B.; Ampleman, G.; Thiboutot, S. *Appl. Environ. Microbiol.* **1998**, *64* (6), 2200−2206.
(12) Daun, G.; Lenke, H.; Reuss, M.; Knackmuss, H.-J. *Environ. Sci. Technol.* **1998**, *32* (13), 1956−1963.
(13) Cerniglia, C. E.; Somerville, C. C. In *Biodegradation of Nitroaromatic Compounds*; Spain, J. C., Ed.; Plenum Press: New York, 1995; pp 99−115.
(14) Fiorella, P. D.; Spain, J. C. *Appl. Environ. Microbiol.* **1997**, *63* (8), 2007−2015.
(15) Kalafut, T.; Wales, M. E.; Rastogi, V. K.; Naumova, R. P.; Zaripova, S. K.; Wild, J. R. *Curr. Microbiol.* **1998**, *36*, 45−54.
(16) Schenzle, A.; Lenke, H.; Spain, J. C.; Knackmuss, H.-J. *Appl. Environ. Microbiol.* **1999**, *65* (6), 2317−2323.
(17) Bryant, C.; DeLuca, M. *J. Biol. Chem.* **1991**, *266* (7), 4119−4125.
(18) French, C. E.; Nicklin, S.; Bruce, N. C. *Appl. Environ. Microbiol.* **1998**, *64* (8), 2864−2868.
(19) Huang, S.; Lindahl, P. A.; Hughes, J. B. *Appl. Environ. Microbiol.* **2000**, *66* (4), 1474−1478.
(20) Orna, M. V.; Mason, R. P. *J. Biol. Chem.* **1989**, *264* (21), 12379−12384.
(21) Schwarzenbach, R. P.; Stierli, R.; Lanz, K.; Zeyer, J. *Environ. Sci. Technol.* **1990**, *24* (10), 1566−1574.
(22) Dunnivant, F. M.; Schwarzenbach, R. P. *Environ. Sci. Technol.* **1992**, *26* (11), 2133-2141.
(23) Butler, J.; Hoey, B. M. *Biochim. Biophys. Acta* **1993**, *1161*, 73−78.
(24) Cénas, N.; Anusevicius, Z.; Bironaité, D.; Bachmanova, G. I. *Arch. Biochem. Biophys.* **1994**, *315* (2), 400−406.
(25) Klausen, J.; Tröber, S. P.; Haderlein, S. B.; Schwarzenbach, R. P. *Environ. Sci. Technol.* **1995**, *29* (9), 2396−2404.
(26) Hofstetter, T. B.; Heijman, C. G.; Haderlein, S. B.; Holliger, C.; Schwarzenbach, R. P. *Environ. Sci. Technol.* **1999**, *33* (9), 1479−1487.
(27) Zenno, S.; Kobori, T.; Tanokura, M.; Saigo, K. *J. Bacteriol.* **1998**, *180* (2), 422−425.
(28) Meisel, D.; Neta, P. *J. Am. Chem. Soc.* **1975**, *97*, 5198−5203.
(29) Wardman, P.; Clarke, E. D. *J. Chem. Soc., Faraday Trans. 1.* **1976**, *72*, 1377−1390.
(30) Whitham, K.; Lyons, S.; Miller, R.; Nett, D.; Treas, P.; Zante, A.; Fessenden, R. W.; Thomas, M. D.; Wang, Y. Presented at *Particle Accelerator Conference and International Conference on High Energy Accelerators*, Dallas, TX, 1995.
(31) Hug, G. L.; Wang, Y.; Schöneich, C.; Jiang, P.-Y.; Fessenden, R. W. *Radiat. Phys. Chem.* **1999**, *54*, 559−566.
(32) Stumm, W.; Morgan, J. J. *Aquatic Chemistry: Chemical Equilibria and Rates in Natural Water*; John Wiley & Sons: New York, 1996.
(33) Skoog, D.; West, D. M.; Holler, F. J. *Fundamentals of Analytical Chemistry*, 7th ed.; Suanders College Publishing: New York, 1996.

(34) Wardman, P. *J. Phys. Chem. Ref. Data* **1989**, *18* (4), 1637.
(35) Eberson, L. *Electron-Transfer Reactions in Organic Chemistry*; Springer-Verlag: New York, 1987.
(36) Tratnyek, P. G.; Hoigné, J.; Zeyer, J.; Schwarzenbach, R. P. *Sci. Total Environ.* **1991**, *109/110*, 327−341.
(37) Devore, J. L. *Probability and Statistics for Engineering and the Sciences:* 2nd ed.; Brooks/Cole Publishing Company: Monterey, CA, 1987.
(38) Haderlein, S. B.; Schwarzenbach, R. P. In *Biodegradation of Nitroaromatic Compounds*; Spain, J. C., Ed.; Plenum Press: New York, 1995; pp 199−225.
(39) Barrows, S. E.; Cramer, C. J.; Truhlar, D. G.; Elovitz, M. S.; Weber, E. J. *Environ. Sci. Technol.* **1996**, *30* (10), 3028−3038.
(40) Lotrario, J. B.; Woods, S. L. *Biorem. J.* **1997**, *1*, 115−122.
(41) Glaus, M. A.; Heijman, C. G.; Schwarzenbach, R. P.; Zeyer, J. *Appl. Environ. Microbiol.* **1992**, *58* (6), 1945−1951.

Received for review December 19, 1999. Revised manuscript received June 14, 2000. Accepted June 19, 2000.

ES991422F