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# Transformation and mineralization of 2,4,6-trinitrotoluene (TNT) by manganese peroxidase from the white-rot basidiomycete *Phlebia radiata* *

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**Abstract**

The degradation of the nitroaromatic pollutant 2,4,6-trinitrotoluene (TNT) by the manganese-dependent peroxidase (MnP) of the white-rot fungus *Phlebia radiata* and the main reduction products formed were investigated. In the presence of small amounts of reduced glutathione (10 mM), a concentrated cell-free preparation of MnP from *P. radiata* exhibiting an activity of 36 nkat/ml (36 nmol Mn(II) oxidized per sec and per ml) transformed 10 mg/l of TNT within three days. The same preparation was capable of completely transforming the reduced derivatives of TNT. When present at 10 mg/l, the aminodinitrotoluenes were transformed in less than two days and the diaminonitrotoluenes in less than three hours. Experiments with $^{14}$C-U-ring labeled TNT and 2-amino-4,6-dinitrotoluene showed that these compounds were mineralized by 22% and 76%, respectively, within 5 days. Higher concentrations of reduced glutathione (50 mM) led to a severe inhibition of the degradation process. It is concluded that *Phlebia radiata* is a good candidate for the biodegradation of TNT as well as its reduction metabolites.

*Abbreviations:* ADNT – aminodinitrotoluene; 2-A-4,6-DNT – 2-amino-4,6-dinitrotoluene; 4-A-2,6-DNT – 4-amino-2,6-dinitrotoluene; DANT – diaminonitrotoluene; 2,4-DA-6-NT – 2,4-diamino-6-nitrotoluene; 2,6-DA-4-NT – 2,6-diamino-4-nitrotoluene; GSH – reduced glutathione; LiP – lignin peroxidase; MnP – manganese peroxidase; OHADNT – hydroxylaminodinitrotoluene; TNT – 2,4,6-trinitrotoluene

## Introduction

The ligninolytic white-rot fungi – among which *Phanerochaete chrysosporium* is the best studied representative – are known to degrade and occasionally to mineralize a great variety of organic pollutants (Bumpus & Aust 1987; Paszczynski & Crawford 1995). The ability of these organisms to degrade many persistent xenobiotics correlates with their ability to extensively metabolize lignin (Barr & Aust 1994). The mechanistic basis for both processes appears to be related to the synthesis and secretion of nonspecific oxidative enzymes, including lignin peroxidases (LiP, Kuwahara et al. 1984) and manganese-dependent peroxidases (MnP, Tien & Kirk 1983). The production of these ligninolytic enzymes is triggered in response to nitrogen or carbon starvation (Lamar 1992; Field et al. 1993).

*P. chrysosporium* has also been studied for its ability to degrade the nitroaromatic explosive 2,4,6-trinitrotoluene (TNT) (Bumpus et al. 1985; Fernando et al. 1990; Spiker et al. 1992). Production of munitions and decommissioning of many former military

* Dedicated to Prof. Annele I. Hatakka on the occasion of her 50th birthday.

bases have led to widespread pollution of water and soils by nitroaromatics at numerous sites of the world (Walsh 1990). TNT is a toxic and mutagenic compound for both microorganisms and higher organisms (Won et al. 1976; Styles & Cross 1983). The TNT molecule includes three electron-withdrawing groups reducing the electron density of the aromatic ring. TNT is thus resistant to oxidative attack by oxygenases and tends to persist in the environment (Rieger & Knackmuss 1995).

*Phlebia radiata*, another ligninolytic white-rot fungus (Hatakka & Uusi-Rauva 1983), was shown to secrete both a LiP and a MnP, which are biochemically very close to those of *P. chrysosporium* (Kantelinen et al. 1988; Kantelinen et al. 1989; Hatakka 1994), plus a laccase (benzenediol:oxygene oxidoreductase; Niku-Paavola et al. 1988; Lundell et al. 1990). In a previous paper, we showed that *P. radiata* is able to transform TNT and its main reduction products (Van Aken et al. 1997).

The mineralization of TNT by white-rot fungi involves two distinct steps. As with all microorganisms, it begins with reduction (Won et al. 1976; Fernando et al. 1990; Bumpus & Tatarko 1994) to hydroxylaminodinitrotoluenes (OHADNT) and aminodinitrotoluenes (ADNT). The latter can be further reduced into diaminonitrotoluenes (DANT) (Stahl & Aust 1993; Bumpus & Tatarko 1994; Michels & Gottschalk 1994). These first reduction steps require the mycelial material and probably involve membrane-bound nitroreductases (Rieble et al. 1994). In further, oxidative steps, reduction products from TNT are oxidized by LiP and/or MnP (Stahl & Aust 1993; Michels & Gottschalk 1994; Van Aken et al. 1997). OHADNT are substrates for LiP (Bumpus & Tatarko 1994; Michels & Gottschalk 1994), whereas ADNT are not (Bumpus & Tatarko 1994). However, it was postulated that ADNT could be substrates for MnP (Stahl & Aust 1993). Recently, Scheibner & Hofrichter (1998) showed that cell-free preparations of MnP from the white-rot basidiomycete *Nematoloma frowardii* and from the litter decaying basidiomycete *Stropharia rugosoannulata* convert TNT and its main reduction products. Moreover, this *in vitro* system is able to mineralize a mixture of reduction products from $^{14}$C-TNT (Scheibner & Hofrichter 1998) as well as $^{14}$C-2-amino-4,6-dinitrotoluene (Scheibner et al. 1997). The authors reported that the addition of reduced thiols like glutathione (GSH) strongly enhances the degradation rates (Scheibner et al. 1997; Hofrichter et al. 1998). The main role of MnP is to catalyze the oxidation of Mn(II) to Mn(III) which is likely the ultimate oxidizing agent acting on the target molecules. If GSH is present in the system, it could be oxidized in its turn by Mn(III) to give the highly reactive thiyl radical ($GS^{\bullet}$). The latter should be a much more powerful degrading agent than Mn(III) itself (Scheibner et al. 1997; Hofrichter et al. 1998).

However, *N. frowardii* and *S. rugosoannulata* (order Agaricales, family Strophariaceae) are basidiomycetes with gills while *P. radiata* (order Meruliales, family Meruliaceae) – as well as *P. chrysosporium* (order Meruliales, family Phanerochaetaceae) – are corticioid basidiomycetes (Corticiaceae; Ginns & Lefebvre 1993; Hawksworth et al. 1995). The aim of this work was to show that MnP from *P. radiata*, a well-known, "mainstream" ligninolytic white-rot fungus (Hatakka 1994), is able to transform TNT and its main reduction products 2-amino-4,6-dinitrotoluene (2-A-4,6-DNT), 4-amino-2,6-dinitrotoluene (4-A-2,6-DNT), 2,4-diamino-6-nitrotoluene (2,4-DA-6-NT) and 2,6-diamino-4-nitrotoluene (2,6-DA-4-NT) in a cell-free system.

## Materials and Methods

*Fungus and Culture Conditions. Phlebia radiata* Fr. strain 79 (ATCC 64658) was grown on the following nitrogen-limited liquid medium: 10 g/l glucose, 78 mg/l L-asparagine, 40 mg/l $NH_4NO_3$ (2.2 mM total N), 7.5 g/l sodium tartrate (50 mM; pH 4.5) as buffer, 0.2 g/l $KH_2PO_4$, 0.5 g/l $mgSO_4.7H_2O$, 0.1 g/l $CaCl_2 \cdot 2H_2O$, 42 mg/l $MnSO_4 \cdot H_2O$ as an inducer for MnP, 1 ml/l mineral solution and 0.5 ml/l vitamin solution (Hatakka & Uusi-Rauva 1983). All cultures were carried out in 1-liter Roux bottles each containing 150 ml culture medium inoculated with 10 ml of a mycelium suspension which were then incubated under stationary conditions at 28 °C. After two weeks, the extracellular liquid was separated from the mycelial material by filtration on glass fiber. This ligninolytic fluid exhibited a MnP activity of 24 ± 3 nkat/ml (1 nkat corresponds to 1 nmol substrate transformed in 1 sec). The inoculum was prepared as follows: *P. radiata* was maintained on Petri dishes containing 2.5% yeast malt extract agar (ISP Medium 2; Difco Laboratories, Detroit, MI, USA). Ten pieces of agar (approx. 0.5 $cm^2$) were cut and grown on the surface of the liquid medium under the conditions described above. After one week, the mycelial

mat was collected, resuspended in sterile water, and homogenized.

*Enzymatic Analysis.* MnP activity was measured photometrically by the direct oxidation of Mn(II)-malonate to Mn(III)-malonate complexes at 270 nm (Glenn et al. 1986; Wariishi et al. 1992). LiP activity was measured by the oxidation of veratryl alcohol to veratryl aldehyde at 310 nm (Tien & Kirk 1983). Because the addition of $H_2O_2$ to initiate the oxidation of veratryl alcohol to veratryl aldehyde could lead to the consumption of Mn(III)-malonate complex and a decrease of the absorbance at 310 nm – thus masking a potential LiP activity – we were careful to monitor in parallel the lack of formation of veratryl aldehyde by HPLC (310 nm). Laccase activity was measured by the oxidation of 2,2′-azinodi-3-ethyl-benzothiazoline-6-sulphuric acid (ABTS) to a colored cationic radical at 436 nm (Niku-Paavola et al. 1988). 0.1 mg/ml catalase was added in order to prevent the possible oxidation of ABTS by peroxidases (Lundell & Hatakka 1994). All activities are expressed in nkat/ml.

*Preparation of MnP.* The culture fluid was concentrated 10-fold by ultrafiltration on a 10-kDa cutoff membrane (Pall Filtron Corp., Northborough, MA, USA). In order to remove low molecular weight compounds and especially Mn ions, the ultrafiltration was repeated twice more after redilution of the concentrate fluid in 10 volumes of acetate buffer (25 mM, pH 5.0; Scheibner et al. 1997). The final MnP preparation exhibited a MnP activity of 219 $\pm$ 11 nkat/ml and only negligible LiP (0.0 nkat/ml) or laccase (0.2 nkat/ml) activities. For degradation experiments with unlabeled compounds, the MnP preparation was diluted four times with acetate buffer (25 mM, pH 5.0) to a final MnP activity of 48 $\pm$ 4 nkat/ml. Mineralization tests with labeled compounds were performed using the initial MnP preparation without any dilution.

*Degradation Experiments with Unlabeled Compounds.* Experiments were conducted in 10-ml serum vials containing a 2.5 ml reaction mixture of the following composition: 1.6 g/l malonate buffer (30 mM; pH 4.5), 0.2 g/l $MnSO_4 \cdot H_2O$ (1 mM), 0 to 15.4 g/l reduced glutathione (GSH; 0 to 50 mM), 10 mg/l nitroaromatic substrate, 43 U/l glucose oxidase (1 U corresponds to 1 $\mu$mol substrate transformed in 1 min) and 2.7 g/l glucose as $H_2O_2$-generating system, and 75% (v/v) MnP preparation (Scheibner et al. 1997). This mixture exhibited a final MnP activity of 36 $\pm$ 5 nkat/ml and was filter-sterilized (pore size 0.22 $\mu$m; Millipore Corp., Bedford, MA, USA). The reaction flasks were incubated in the dark at 35 °C on a gyratory shaker (200 rpm). Control experiments contained 10 mM GSH and were carried out with autoclaved MnP.

In order to investigate the inhibitory effect of high concentrations of GSH on the degradation process, we performed degradation experiments on 2-A-4,6-DNT, using different dilutions (1/1, 1/2, 1/4, 1/8) of the initial MnP preparation (MnP activity 219 $\pm$ 11 nkat/ml) and different concentrations of GSH (0 to 30.7 g/l; 0 to 100 mM). The final reaction mixtures exhibited MnP activities reduced according to their dilution factors. The 2-A-4,6-DNT concentrations remaining after 24 h were plotted as a function of the GSH concentration and fitted based on logistic curves (SigmaPlot, Jandel Scientific Software, San Francisco, CA, USA). All degradation experiments were performed in triplicate.

*Mineralization Experiments with $^{14}$C-U-Ring Labeled Compounds.* Experiments were conducted in anaerobic 10-ml test tubes tightly closed with rubber septa and containing 1 ml of the reaction mixture described above except that it showed a final MnP activity of 162 $\pm$ 14 nkat/ml. $^{14}$C-U-ring labeled TNT and 2-A-4,6-DNT were added to final activities of approx. 55 000 and 10 500 dpm, respectively. Both were mixed with their corresponding unlabeled compound in order to reach a total concentration of 10 mg/l. The mixture was sterilized and incubated as described above. Control tubes contained 10 mM GSH and autoclaved MnP. The reaction tubes were flushed periodically with pure oxygen for 30 min at a flow rate of 50 ml/min. Any gas released was bubbled into two sequential flasks. The first contained Opti-Fluor (Packard Instrument B.V., Groningen, The Netherlands) and served as a trap for volatile organic compounds. The second contained an alkaline scintillation cocktail with Opti-Fluor and Carbosorb (Packard) mixed in a 2:1 ratio and was used as a trap for $^{14}CO_2$ (Scheibner et al. 1997).

*Analysis of TNT and its Reduction Products.* Quantitative determination of TNT and its reduction products was performed by reverse phase HPLC (model HP 1090, Hewlett-Packard, Waldbronn, Germany), on a $C^{18}$ ODS Hypersil column (5 mm; 4.6 $\times$ 100 mm; Chrompack International, Middelburg, The Netherlands). The mobile phase was an isocratic solvent system consisting of phosphate buffer (10 mM, pH 3.2): acetonitrile 60:40 at a flow rate of 0.75 ml/min. Compounds were monitored via UV $A_{230}$ on a photodiode array detector. Intermediates and products were identified by comparison of both retention times and UV spectra with authentic standards.

*Chemicals.* All chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany), Sigma (St Louis, MO, USA) or Aldrich (Milwaukee, WI, USA). Glucose oxidase (Sigma) was low in catalase (< 0.032% of the glucose oxidase activity). 2,4,6-Trinitrotoluene (TNT) was obtained from Nobel Explosives (Chatelet, Belgium). 2-Amino-4,6-dinitrotoluene (2-A-4,6-DNT) and 4-amino-2,6-dinitrotoluene (4-A-2,6-DNT) were synthesized from their corresponding dinitrotoluidic acids (Sigma) through the Schmidt reaction (Zbarskii et al. 1971). TNT, 2-A-4,6-DNT and 4-A-2,6-DNT were purified by recrystallization in pure ethanol. 2,4-Diamino-6-nitrotoluene (2,4-DA-6-NT) was graciously donated by Dr. D. Bruns-Nagel (University of Marburg, Germany). 2,6-Diamino-4-nitrotoluene (2,6-DA-4-NT) was purchased from Sigma. $^{14}$C-U-ring labeled TNT (21.58 mCi/mmol) was obtained from Chemsyn Science Laboratories (Lenexa, KS, USA). $^{14}$C-U-ring labeled 2-A-4,6-DNT (2.2 mCi/mmol) was provided by Prof. W. Fels (Department of Organic Chemistry, University of Paderborn, Germany). All these compounds exhibited a purity higher than 98% (as determined by HPLC).

## Results

The transformation and mineralization experiments were carried out with a cell-free enzymatic system which included a MnP preparation from *P. radiata*, Mn(II), reduced GSH and a $H_2O_2$-generating system (glucose plus glucose oxidase). This *in vitro* system was able to transform TNT and its main reduction products 2-A-4,6-DNT, 4-A-2,6-DNT, 2,4-DA-6-NT and 2,6-DA-4-NT (Figures 1A–E). The dose-response of the GSH effect was tested over a range of concentrations from 0 to 50 mM. With 10 mM reduced GSH, the disappearance of DANT occurred very rapidly (100% in less than 3 h; Figures 1A,B). ADNT were converted more slowly (100% in approx. 24 h; Figures 1C,D), while TNT underwent only partial transformation over the time of the experiment (43% in 3d; Figure 1E). Without GSH, the degradation rates of all the compounds under study were at least 5 times lower (Figures 1A–E). However, the degradation was greatly reduced for GSH concentrations above 20 mM (Figures 1A–E). No degradation was observed in control flasks (Figures 1A–E). HPLC analysis performed during the degradation processes also revealed the presence of some small peaks likely corresponding to unknown intermediates.

Mineralization experiments with $^{14}$C-U-ring labeled compounds showed a release of $^{14}CO_2$, faster from $^{14}$C-2-A-4,6-DNT than from $^{14}$C-TNT (Table 1). Without GSH, the mineralization extent after 5 d reached only 16% of the initial activity with $^{14}$C-2-A-4,6-DNT and 3% with $^{14}$C-TNT (Table 1). In the presence of GSH, at levels up to 20 mM, the radioactivity released was considerably higher and resulted in maximal percentages of 76 and 22 from $^{14}$C-2-A-4,6-DNT and $^{14}$C-TNT respectively (Table 1). For a GSH concentration of 50 mM, the extent of mineralization was lower: 23% for $^{14}$C-4-A-2,6-DNT and 4% for $^{14}$C-TNT (Table 1). HPLC analysis of the reaction mixture after 5 d incubation showed that the remaining radioactivity was shared between nondegraded $^{14}$C-TNT or $^{14}$C-2-A-4,6-DNT and unidentified $^{14}$C-metabolites (Table 1). It can be seen from Table 1 that only insignificant amounts of $^{14}CO_2$ were released from control flasks or found in the volatile organics trap. Mass balances resulted in 94 to 102% recoveries of the initial radioactivity (Table 1).

The inhibitory effect of GSH was observed when GSH was present at 10–20 mM in the reaction mixture with lower MnP activity ($36 \pm 5$ nkat/ml; Figures 1A–E) and between 20 and 50 mM in that with higher MnP activity ($162 \pm 14$ nkat/ml; Table 1). We determined the GSH concentration above which inhibition of the degradation occurred at different MnP activities. The results showed that the concentration of GSH corresponding to this inhibitory threshold – defined as the inflection point of the logistic curves (Figure 2) – increases with the MnP activity (Figure 3).

Our MnP preparation was obtained by concentration of a crude extracellular fluid. Even though it could contain other enzymes, degradation experiments performed in controls successively without $MnSO_4$, without $H_2O_2$-generating system or without MnP did not show any transformation (data not shown).

## Discussion

Bumpus & Tatarko (1994) and Michels & Gottschalk (1994) have previously shown that 4-OHA-2,6-DNT is a substrate for LiP. It was also suggested that ADNT do not serve as substrates for LiP (Bumpus & Tatarko 1994), but possibly for MnP (Stahl & Aust 1993). On the other hand, Valli et al. (1992) observed that MnP is the only peroxidase able to oxidize aminonitro-

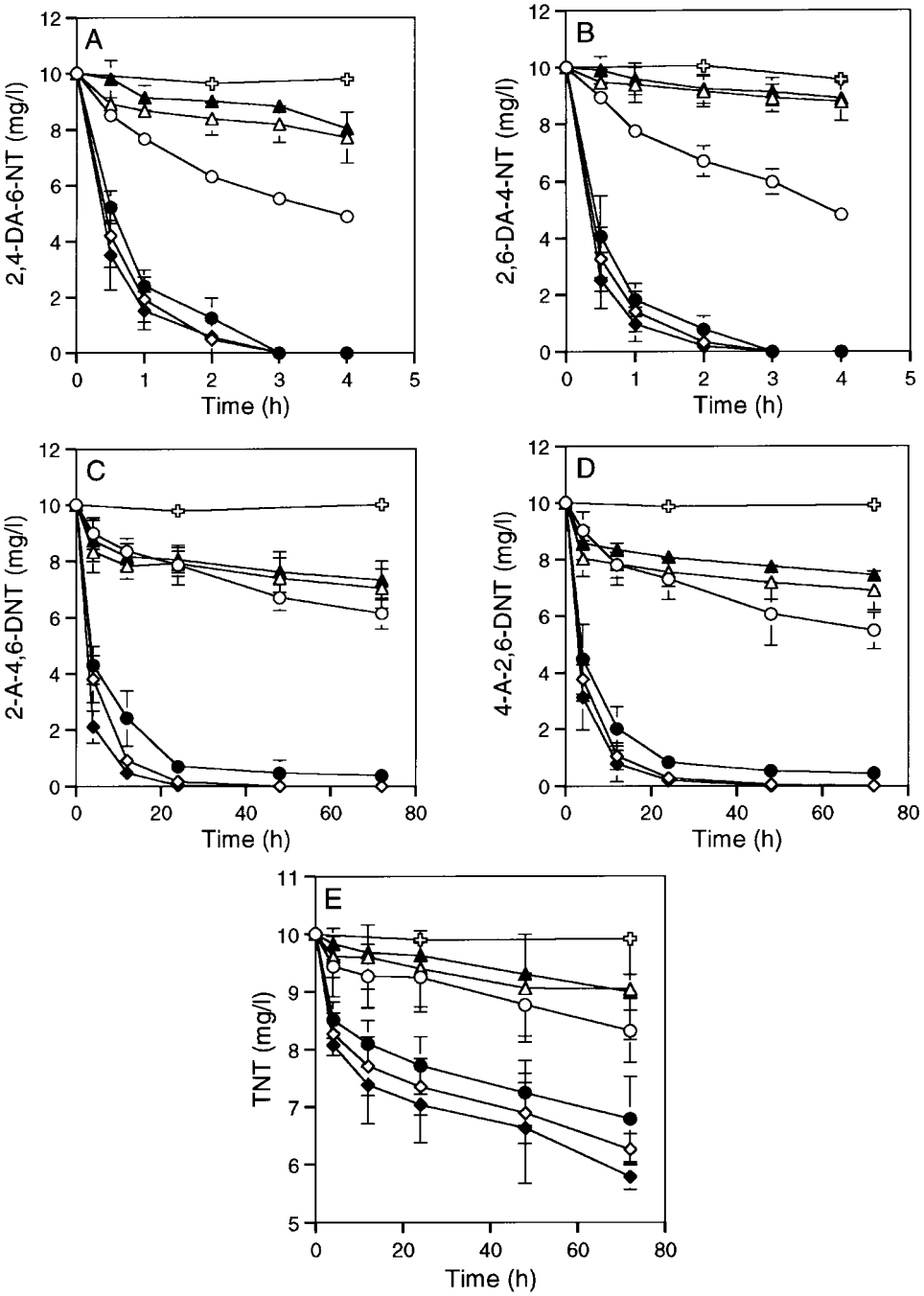


*Figure 1.* Conversion of (A) 2,4-DA-6-NT, (B) 2,6-DA-4-NT, (C) 2-A-4,6-DNT, (D) 4-A-2,6-DNT and (E) TNT at a concentration of 10 mg/l in the MnP enzymatic system (MnP/Mn(II)/GSH in malonate buffer 30 mM, pH 4.5, final MnP activity 35.5 ± 4.6 nkat/ml). Samples were incubated at 35 °C, under stirring at 200 rpm. Residual metabolite concentrations are shown for different levels of GSH: 0 mM (open circle), 1 mM (closed circle), 5 mM (open diamond), 10 mM (closed diamond), 20 mM (open triangle), 50 mM (closed triangle) and control (open cross).

*Table 1.* Mass balances for mineralization of $^{14}$C-2-A-4,6-DNT and $^{14}$C-TNT in the MnP enzymatic system after 5 days of incubation

| | Activity recovered from $^{14}$C-TNT (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| GSH (mM) | 0 | 1 | 5 | 10 | 20 | 50 | Contr. |
| $^{14}CO_2$ trap | 3 | 6 | 10 | 18 | 22 | 4 | 1 |
| Volatile organics trap | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Reaction mixture | | | | | | | |
| Nontransformed $^{14}$C-TNT | 76 | 60 | 50 | 47 | 46 | 91 | 96 |
| Unidentified $^{14}$C-Metabolites | 17 | 35 | 35 | 32 | 30 | 5 | 3 |
| Total | 96 | 101 | 95 | 97 | 98 | 101 | 100 |
| | Activity Recovered from $^{14}$C-2-A-4,6-DNT (%) | | | | | | |
| GSH (mM) | 0 | 1 | 5 | 10 | 20 | 50 | Contr. |
| $^{14}CO_2$ trap | 16 | 36 | 45 | 57 | 76 | 23 | 1 |
| Volatile organics trap | 1 | 1 | 2 | 1 | 1 | 1 | 0 |
| Nontransformed $^{14}$C-2-A-4,6-DNT | 52 | 7 | 0 | 0 | 0 | 72 | 98 |
| Unidentified $^{14}$C-metabolites | 31 | 54 | 49 | 42 | 17 | 6 | 0 |
| Total | 100 | 98 | 96 | 100 | 94 | 102 | 99 |

toluenes. Recently, Scheibner et al. (1997) reported for the first time that cell-free preparations of MnP from the basidiomycetes *N. frowardii* and *S. rugosoannulata* (order of the Agaricales) are able to degrade TNT and its reduction derivatives. Here we demonstrated that MnP from the well-known basidiomycete *P. radiata* (order of the Meruliales), in an *in vitro* system, is also able to degrade and to mineralize these nitroaromatic pollutants (Figures 1A–B, Table 1). Their lack of transformation in the absence of Mn(II), MnP or $H_2O_2$-generating system, as well as the probing with the specific enzymatic assay used, showed that the only enzyme directly involved in the degradation process was MnP.

No metabolites of degradation could be identified either from $^{14}$C-TNT, or from $^{14}$C-2-A-4,6-DNT. However, the radioactivity remaining in the reaction mixture is only partly accounted for by the unchanged initial nitroaromatic compounds. The residual unidentified metabolites could be polar ring fission products like formate, acetate, glyoxalate and/or malonate which could not be observed with the $C^{18}$ column used for the HPLC analysis of nitroaromatic compounds.

The results demonstrate that DANT were transformed much faster than ADNT (almost ten fold) and than TNT itself (twenty-five fold; Figures 1A–E). Similarly, experiments with labeled compounds showed that $^{14}$C-2-A-4,6-DNT was mineralized to a larger extent than $^{14}$C-TNT (three fold; Table 1). When TNT is reduced to ADNT and to DANT, one or two electron-withdrawing nitro groups are replaced by electron-donor amino groups. The electronic density of the aromatic ring increases and the electrophilic oxidative attack by MnP is facilitated (Field et al. 1995).

The enzymatic mineralization of $^{14}$C-TNT and especially of $^{14}$C-2-A-4,6-DNT observed (Table 1) occurred much more rapidly than those previously obtained with whole cultures of *P. chrysosporium* (Fernando et al. 1990; Spiker et al. 1992; Bumpus & Tatarko 1994; Michels & Gottschalk 1994). This could be explained by the fact that the concentrated MnP enzymatic system we used exhibited an activity much higher (162 nkat/ml) than a classical ligninolytic extracellular fluid (24 nkat/ml in our case). However, the major explanation is related to the addition of reduced GSH in the reaction mixture. Small amounts of GSH were shown to strongly enhance transformation of TNT and its reduction derivatives (Figures 1A–B), as well as mineralization of $^{14}$C-TNT and $^{14}$C-2-A-4,6-DNT (Table 1). These results are consistent with previous observations (Scheibner et al. 1997; Scheibner & Hofrichter 1998). Without GSH, the ultimate oxidizing agent in this system could be Mn(III) chelated by malonate (Glenn et al. 1986; Wariishi et al. 1989) or the hydroxyl radical ($HO^{\bullet}$) produced by a Fenton's reaction-like process (Li et al. 1997). In this latter case, the role of the MnP/Mn/$H_2O_2$

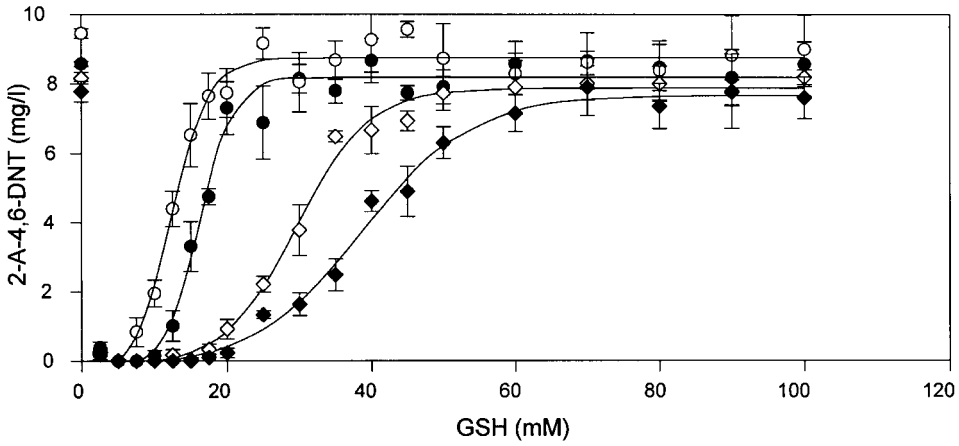


*Figure 2.* Conversion of 2-A-4,6-DNT (10 mg/l) in the MnP enzymatic system (MnP/Mn(II)/GSH in malonate buffer 30 mM, pH 4.5) with different MnP activities: 162 ± 4 nkat/ml (closed diamond), 79 ± 7 nkat/ml (open diamond), 36 ± 6 nkat/ml (closed circle) and 19 ± 4 nkat/ml (open circle). Samples were incubated at 35 °C, under stirring at 200 rpm. Shown are the metabolite concentrations remaining after 24 h of incubation. Curves were fitted according to a logistic function.

system could be simply to reduce Fe(III) to Fe(II) through the oxidative decarboxylation of organic acids (Barr & Aust 1994). Thiol-mediated depolymerizations and oxidations have been previously reported (Forrester et al. 1988; Wariishi et al. 1989; McEldoon & Dordick 1991, D'Annibale et al. 1996) and seem to be related to the one-electron oxidation of thiol by Mn(III) to produce the highly reactive thiyl radical ($GS^{\bullet}$). Besides this effect, we observed that higher concentrations of GSH led to a strong inhibition of the degradation process (Figures 1A–E, Table 1). The inhibitory threshold concentration of GSH was shown to be a function of the MnP activity (Figures 2 and 3). MnP is apparently the initial engine of the overall process. GSH at high concentrations could act adversely at any stage of the process. This phenomenon is currently under investigation.

It is important to note that only the oxidative (or ligninolytic) step of the biodegradation of TNT by white-rot fungi was here under investigation. It must be pointed out that GSH did not act as a reducing agent able to transform TNT, but as a secondary mediator able to generate the highly reactive radical $GS^{\bullet}$. Nitroreductase activity is widespread in nature and nitroaromatic compounds like TNT are rapidly reduced by white-rot fungi – as well as by almost every other organism (Won et al. 1976; Fernando et al. 1990; Bumpus & Tatarko 1994). The rate-limiting step of the TNT biodegradation pathway must be the oxidative conversion of its reduction products (Bumpus & Tatarko 1994).

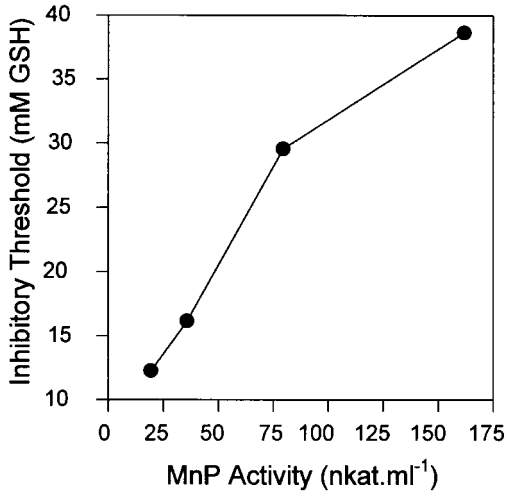


*Figure 3.* Inhibitory threshold concentrations of GSH as a function of the MnP activity in the MnP enzymatic system (MnP/Mn(II)/GSH in malonate buffer 30 mM, pH 4.5). The inhibitory thresholds were computed from the data of Figure 2 (GSH concentrations at the inflection point of the logistic curves).

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