Environ. Sci. Technol. **1999**, *33,* 446−452

Confirmation of Conjugation Processes during TNT Metabolism by Axenic Plant Roots

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This paper examines processes in plants for the formation of fate products of TNT beyond its aminated reduction products, 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. TNT metabolites were isolated and characterized in combination with temporal analyses of product profiles and ^{14}C distribution, in microbe-free, axenic root cultures of *Catharanthus roseus*. Four unique TNT-derived compounds were isolated. Using evidence from ^{1}H NMR, mass spectroscopy, HPLC, acid hydrolysis, and enzymatic hydrolysis with β-glucuronidase and β-glucosidase, they were established as conjugates formed by reactions of the amine groups of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. From the mass spectral evidence, at least a six-carbon unit from the plant intracellular millieu was involved in conjugate formation. Mass balance analysis indicated that, by 75 h after TNT amendment of the initial TNT radiolabel, extractable conjugates comprised 22%, bound residues comprised another 29%, 2-amino-4,6-dinitrotoluene was 4%, and the rest remained unidentified. Isolates from TNT-amended roots versus monoamino-dinitrotoluene-amended roots were not identical, suggesting numerous possible outcomes for the plant-based conjugation of 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene. This study is the first direct evidence for the involvement of the primary reduction products of TNT—2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene—in conjugation processes in plant detoxification of TNT.

Introduction

Explosives contamination of soil and groundwater at manufacturing, loading, and packaging sites poses serious environmental risk. Of the explosives contaminants, 2,4,6-trinitrotoluene (TNT) is the most widespread and persistent. The imperative for cleanup is strong, and in situ, cost-effective risk abatement strategies are being sought. Phytoremediation appears to satisfy many of these criteria, though much remains to be determined of the ability of plants to transform TNT as well as other explosives to environmentally acceptable end points (*1*). In particular, there is interest in determining transformation pathways, products, and toxicological profiles during the metabolism of TNT by plants. Recent efforts directed at bridging the knowledge gaps of TNT phytotransformation have included investigations with a variety of plant systems: yellow nutsedge (*2*), bush beans (*3*), aquatic and wetland species (*4*−*6*), axenic root cultures (*4*), garden vegetables (*7*), hybrid poplar (*8*), and other terrestrial species (*9*). In most studies, TNT transformation by plants has been accompanied by the appearance of its monoamino derivatives: 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. Little is known about the product profile of TNT biocatalysis by plants beyond these early products. 2,4-Diamino-6-nitrotoluene, one of the reduction isomers of either 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene, has been reported recently in TNT-treated hybrid poplars but only accounting for a maximum of 0.4% of initial TNT moles (*8*).

In previous studies with whole plants of *Myriophyllum aquaticum* and axenic root cultures of *Catharanthus roseus*, using [U-^{14}C]TNT, we reported the ability of plant metabolism to sequester or integrate TNT metabolites into biomass (*4, 5*). Harvey et al. (*3*) obtained an analogous outcome on treatment of bush bean plants with TNT and suggested the formation of acid-hydrolyzable conjugates. Plants are able to attach glucose (by glucosyltransferases) or malonate (by malonyltransferases) to amino groups, thus forming "conjugates" (*10*). The formation of conjugates of organic xenobiotics is an important protective phase in plant detoxification metabolism that results in the formation of non-phytotoxic or less toxic species. Some of these conjugates are compartmentalized into plant organelles such as the vacuoles and also eventually may be incorporated into "bound" (i.e., nonextractable) residues (*10*). However, the disconnect in our understanding of plant metabolism of TNT, between the early products of TNT phytocatalysis—such as 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene—and bound residues still remains. Polar metabolites have been observed but remain unidentified (*3, 8*). The objective of studies presented herein was to determine processes by which these formed during TNT metabolism by plants. To accomplish this, TNT metabolites were isolated and characterized in combination with temporal analyses of product profiles and ^{14}C distribution, in axenic (microbe-free) root cultures of *C. roseus*. The analysis presented here provides evidence for the conjugation of TNT's monoamino derivatives as a significant pathway during plant metabolism of TNT.

Materials and Methods

Chemicals, Materials, and Enzymes. 2,4,6 Trinitrotoluene (TNT), 4-amino-2,6-dinitrotoluene, and 2-amino-4, 6-dinitrotoluene (Chemservice, Westchester, PA) were used for culture amendment. The following chemicals were employed as reference standards in analytical quantification and/or product identification: TNT, 2-amino-4,6-dinitrotoluene, and 4-amino-2,6-dinitrotoluene from Chemservice (Westchester, PA); 2,4-diamino-6-nitrotoluene, 2,6-diamino-4-nitrotoluene, 4-hydroxylamino-2,6-dinitrotoluene, 2-hydroxylamino-4,6-dinitrotoluene, 2,2′,6,6′-tetranitro-4,4′-azoxytoluene (4,4′-azoxy), and 4,4′,6,6′-tetranitro-2,2′-azoxytoluene (2,2′-azoxy) gifted by Dr. R. J. Spanggord (SRI International, Menlo Park, CA); 2,4-dihydroxylamino-6-nitrotoluene by catalytic biosynthesis according to Hughes et al. (*11*); and, 2,4,6-triaminotoluene from Sigma-Aldrich (St. Louis, MO). [ring-U-^{14}C]-2,4,6-trinitrotoluene (Chemsyn, KS), with specific activity of 21.58 mCi/mmol and purity of 98%, was used for fate and product analyses. Reference standards of CD_3OD (99.8 atom %) and CD_3CN (99.8 atom %) obtained from Aldrich

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10.1021/es980635m CCC: $18.00
Published on Web 12/17/1998

(Milwaukee, WI), were used for NMR analysis. HPLC-grade 2-propanol, acetonitrile, methanol, hexane, and ethyl acetate supplied by EM Science (Gibbstown, NJ) were used for HPLC mobile phase formulations. Supelclean LC-8 (Supelco, Bellefonte, PA) and silica were employed for solid-phase extraction. For enzymatic hydrolysis of isolated conjugates, β-glucuronidase from *Helix pomata* (EC 3.2.1.31), and β-glucosidase from almonds (EC 3.2.1.21) (Sigma Chemicals, St. Louis, MO) were employed. Stock solutions for culture amendment were prepared in HPLC-grade methanol and filter sterilized: TNT (15.6 mg/mL; ^{14}C activity, 1263 dpm/μL), 2-amino-4,6-dinitrotoluene (10 mg/mL), and 4-amino-2,6-dinitrotoluene (10 mg/mL).

Tissue Cultures. Hairy root cultures of *C. roseus* were propagated and maintained in a culture volume of 50 mL in 250-mL Erlenmeyer flasks following the method of Bhadra and Shanks (*12*), where their growth characteristics are also detailed.

Analytical Methods. TNT and derivatives including 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, 4-hydroxylamino-2,6-dinitrotoluene, 2-hydroxylamino-4, 6-dinitrotoluene, 2,4-diamino-6-nitrotoluene, 2,6-diamino-4-nitrotoluene, and 2,4-dihydroxylamino-6-nitrotoluene were analyzed by C_8 reverse-phase HPLC with PDA detection as described in Hughes et al. (*4*). The azoxy compounds, 4,4′-azoxy and 2,2′-azoxy, were analyzed by C_{18} reverse-phase HPLC with UV/Vis detection using a Bondclone C_{18} column (300 × 3.9 mm, 10 μm; Phenomenex, Torrance, CA) and an isocratic mobile phase of 60/40 (% v/v), CH_3CN/H_2O, at 1.2 mL/min. Spectra of azoxy analytes were acquired from 200 to 500 nm, and the chromatograms at 230 nm were employed for quantification. Peak identification in all cases was based on comparisons of retention times and UV/Vis spectra with authentic standards. The detection limit using this method was found to be 0.2 ppm. An independent HPLC method was also developed to confirm the purity of conjugates isolated from plant biomass or media: stationary phase, Luna C_{18} (250 × 4.0 mm, Phenomenex, Torrance, CA); mobile phase, 86/14% (v/v) 2-propanol/H_2O at 1 mL/min; and detection, UV/Vis between 200 and 400 nm with chromatogram extraction at 230 nm. Isolated conjugates were spiked with each other and with standards to verify identity.

^1H NMR spectra were acquired on a Bruker AC-250 MHz spectrometer relative to either CD_3OD (^1H δ: 3.31 and 4.78 ppm) or CD_3CN (^1H δ: 1.94 ppm). Chemical shifts are reported in ppm, and coupling constants are given in hertz (Hz). Mass spectroscopy was performed with two systems: (a) by HPLC−MS on a HP1100 system using either electrospray (ES) or chemical ionization (CI) at atmospheric pressure and (b) by direct probe chemical ionization mass spectroscopy on a Finnigan MAT 95 spectrometer with methane as the ionizing gas.

Separation of Transformation Products. Products of plant transformation of TNT or monoaminodinitrotoluenes were separated and purified from methanol extracts of root tissues by using a series of extraction steps: solid-phase extraction (SPE) with silica, followed by SPE with C_8, and where necessary, final purification by preparative scale C_{18} HPLC. Visual observation of eluant bands and HPLC analysis of eluant fractions were employed to monitor eluant fractions. First, the methanol extract of nitroaromatic-amended plant tissue was concentrated to ≤5 mL. It was then loaded on a hand-packed silica column, and the components of the mixture eluted with a series of mobile phase. The first eluant was hexane/ethyl acetate, 60/40% (v/v), which removed the nitraromatics (TNT and reduction products only) selectively. This was followed by elution with methanol to collect the desired TNT metabolite. Emerging from the Si−SPE, the fractionated extract contained the nitroaromatic metabolite(s) as well as plant-specific compounds (alkaloids, etc.). The subsequent separation was C_8−SPE on a hand-packed column with combinations of water and 2-propanol. The initial composition was 100/0% (v/v) water/2-propanol, with a gradient to 75/25% (v/v) water/2-propanol. The desired nitroaromatic metabolites in eluant fractions were collected, extracted with ethyl acetate, evaporated to dryness, and redissolved in methanol for further analysis of purity. If adequately pure, ~95% or more, chemical analysis followed. Otherwise for further purification, preparative HPLC was employed: stationary phase, C_{18} (5 μm, 19 × 150 mm, Bondapak, Waters, Milford, MA); mobile phase, water/methanol, 75/25% (v/v) at 10 mL/min; and, detection, UV/Vis. Following final purification, the fractions containing the nitroaromatic metabolite were collected, extracted with ethyl acetate, evaporated to dryness, and dissolved in methanol for chemical analysis.

Mass Balance Analysis. Mass balance on the root systems was performed by determining ^{14}C in each of the following compartments—extracellular ^{14}C, intracellular extractable ^{14}C, and intracellular bound ^{14}C. Radioactivity was detected and quantified using a Beckman LS 6500 scintillation counter as described in Hughes et al. (*4*). Extracellular ^{14}C was determined from a measured aliquot of the aqueous medium. To determine the level of unbound ^{14}C in root biomass (intracellular extractable ^{14}C) as well as extractable nitroaromatics, duplicate 80-mg aliquots of freeze-dried root samples were extracted three times, each time for 4−6 h, by ultrasonication in 5-mL aliquots of methanol. The extract was separated from the solid residue by centrifuging (17000*g*). Following three extractions of each sample replicate, the extracts were pooled, evaporated, redissolved in a known volume of methanol, and analyzed. Unextractable TNT-derived ^{14}C that was bound to root biomass—intracellular bound ^{14}C—was determined by combusting weighed aliquots of previously extracted samples in an OX-600 biooxidizer (R.J. Harvey, NJ), and measuring the ^{14}C activity of the resultant $^{14}CO_2$ as described in Hughes et al. (*4*). Standards of mannitol and dried tissue materials spiked with predetermined ^{14}C-labeled TNT levels indicated an average recovery efficiency of 95%.

^{14}C-Radiochromatographs and ^{14}C-Based LC Response Factors. The C_8-HPLC profile of ^{14}C in a sample—aqueous medium or root extract—was determined by collecting eluant aliquots corresponding to peaks at 230 nm and measuring by scintillation counting as described earlier.

^{14}C-based response factors (extinction coefficients at 230 nm) of TNT metabolites identified in this study were determined by collecting eluant aliquots corresponding to their C_8-HPLC retention and scintillation counting. Purity of collected fractions was ensured by employing peak-purity analysis with the PDA detector. Response factors were used to estimate the concentrations of TNT metabolites.

Hydrolysis of TNT Metabolites. To evaluate the formation of hydrolyzable metabolites of TNT, enzyme and acid treatments were employed. Enzymes used include β-glucuronidase and β-glucosidase. Extracts of 2-amino-4,6-dinitrotoluene- or 4-amino-2,6-dinitrotoluene-amended roots were dried and redissolved in acetate buffer (pH 5). Before enzyme addition, the aqueous extracts were analyzed for levels of various analytes—starting materials such as TNT, 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene, and TNT metabolites. Following this, 20 mg of each enzyme was added to 2-mL aliquots of extracts and incubated at 37 °C. For the acid treatment, no additions were made, although the same reaction volume was incubated. The profiles of analytes were monitored by HPLC analysis.

Transformation Studies. The 4−5-week-old, stationary phase root cultures were employed to study nitroaromatic transformation: 50-mL cultures for transient studies of TNT transformation and 1.5-L cultures for the isolation and analysis of nitroaromatic transformation products. Prior to

TABLE 1. Balance on ^{14}C in Hairy Root Cultures of *C. Roseus* over an 8-day Period

time	extracellular ^{14}C (fraction initial)	intracellular extractable ^{14}C (fraction initial)	intracellular bound ^{14}C (fraction initial)	total ^{14}C (fraction initial)
0 h	1.00	0	0	1.00
7 h	0.57 ± 0.02	0.38 ± 0.03	0.08 ± 0.02	1.03 ± 0.05
24 h	0.36 ± 0.08	0.49 ± 0.09	0.14 ± 0.02	0.99 ± 0.04
31 h	0.29 ± 0.11	0.52 ± 0.08	0.14 ± 0.07	0.97 ± 0.05
47 h	0.20 ± 0.03	0.60 ± 0.02	0.25 ± 0.01	1.05 ± 0.02
75 h	0.16 ± 0.03	0.57 ± 0.03	0.29 ± 0.01	1.02 ± 0.01
8 day	0.10 ± 0.06	0.63 ± 0.06	0.29 ± 0.13	1.01 ± 0.03

the start of the transient studies, autoclaved Milli-Q water (Millipore Inc., MA) was added to the small-scale cultures to adjust the volume of the medium to 50 mL. To amend cultures with a nitroaromatic, the appropriate filter-sterilized stock solution was added to axenic roots: transient studies of TNT transformation, 80 μL was added to 50 mL of culture for an initial concentration of 25 mg/L; product analysis of TNT metabolites, 3 mL/1.5 L of culture for an initial concentration of 31 mg/L; product analysis of metabolites of 4-amino-2,6-dinitrotoluene or 2-amino-4,6-dinitrotoluene, 4 mL/1.5 L of culture for an initial concentration of 27 mg/L. Nitroaromatic-amended cultures were maintained in darkness at the culture conditions as before.

In transient studies of TNT transformation, the levels of extracellular nitroaromatics and ^{14}C at various times were analyzed by sampling 250 μL of the medium of at least three cultures. For mass balance, the root biomass was analyzed at 7, 24, 31, 47, and 75 h from amendment by sacrificing triplicate cultures at each time point. Sacrificed roots were rinsed with water, freeze-dried, and extracted as described previously. Root extracts were analyzed for levels of nitroaromatics and TNT-derived ^{14}C, and residues of extracted tissue were analyzed for bound ^{14}C by biooxidation.

Results

Temporal Analysis of TNT and Aminated Derivatives. The temporal analysis of TNT-treated roots for transformation products points toward the formation of fate products not found in studies with microbial (both bacterial and fungal) cultures. As reported previously (*4*), TNT applied to root cultures decreased to below detection limit in the medium. 2-Amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene were the only known transformation products that appeared, but neither accumulated in stoichiometric proportion to the disappearance of TNT from the medium (Figure 1A). The present study shows in addition that the buildup of TNT in the plant root matrix was minimal: the transient maximum was 5% of initial at 7 h, and it decreased to below the detection limit within 75 h from addition (Figure 1B). In related studies with initial TNT levels of 80 mg/L (unpublished data), maximum intracellular buildup of TNT was about 12% of initial at 6 h from addition. In the present study, both monoamino derivatives of TNT, 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene, were detected as well in the root biomass, but there too their levels were low (Figure 1B). The maximum combined level of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene was 12% of initial TNT at 24 h from TNT amendment. Other reported products of TNT reduction or transformation were not detected in TNT-treated roots, including 2,4-diamino-6-nitrotoluene and 2,6-diamino-4-nitrotoluene; 4,4′-azoxy; 2,2′-azoxy; 2-hydroxylamino-4,6-dinitrotoluene and 4-hydroxylamino-2,6-dinitrotoluene; and 2,4-dihydroxylamino-6-nitrotoluene.

Temporal Mass Balances on ^{14}C. The temporal mass balance on ^{14}C in TNT-amended roots further emphasizes the formation of TNT metabolites that are not within the currently known range of transformation products. Presented

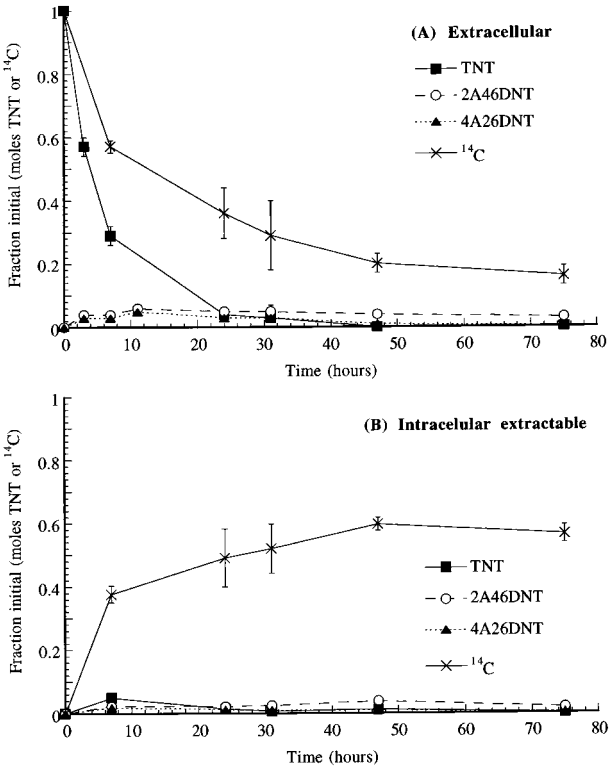


FIGURE 1. Temporal profiles of TNT, 2-amino-4,6-dinitrotoluene (2A46DNT), 4-amino-2,6-dinitrotoluene (4A26DNT), and TNT-derived ^{14}C in (A) extracellular, aqueous medium and (B) intracellular phase or biomass of TNT-amended hairy root cultures of *C. roseus*. Data points are the average of triplicate cultures, and error bars represent standard deviation.

in Table 1 is a breakdown of fractions comprising the transient ^{14}C balances using triplicate cultures at each time point: extracellular ^{14}C, intracellular extractable ^{14}C, and intracellular bound ^{14}C. Extracellular ^{14}C decreased in concert with TNT in the aqueous medium (Table 1 and Figure 1A). However, whereas extracellular TNT decreased to below detection limit within 47 h, extracellular ^{14}C decreased to ~20% of its initial level (Table 1). During the same period, the intracellular profile of extractable ^{14}C mirrored this trend, increasing to ~60% of the initial ^{14}C level (Table 1). The increasing fraction of unknown compounds formed over time from TNT is underscored by this disparity between total levels, intracellular and extracellular, of known analytes as compared to that of ^{14}C (Figure 1). Significant binding processes were observed within the initial 75-h period, with the bound fraction of TNT-derived ^{14}C increasing to 29% of initial (Table 1).

Metabolites of TNT: Detection by ^{14}C and UV/Vis Analyses. In TNT-amended roots, TNT-derived ^{14}C was associated with UV-absorbing analytes other than the parent compound, 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. As shown in Figure 2, significant levels of ^{14}C

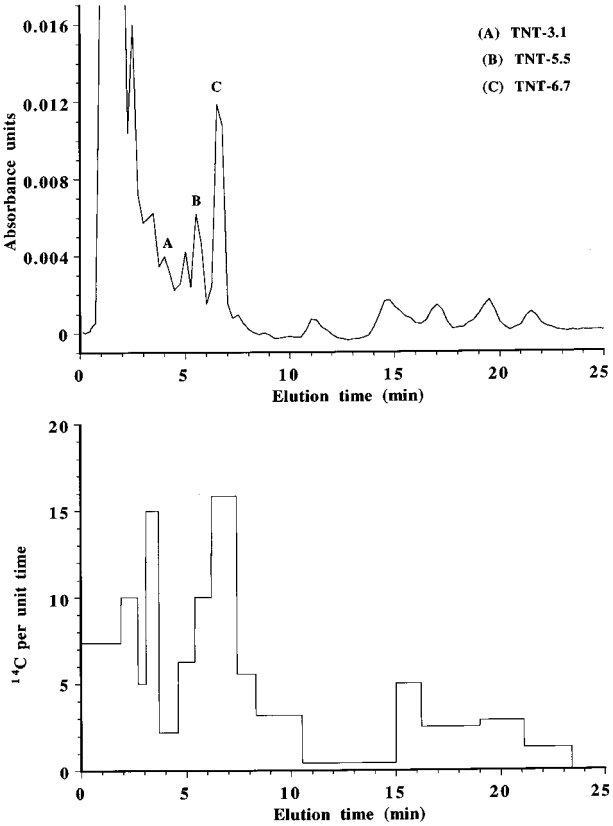


FIGURE 2. C_8-HPLC profiles of an extract of TNT-treated *C. roseus* hairy roots, 31 h from amendment with TNT: (A) chromatogram at 230 nm and (B) TNT-derived ^{14}C-radiolabel plotted as percent of ^{14}C per unit of HPLC retention time. To determine total percent of ^{14}C associated with a retention time window, multiply the duration of retention window by the ordinate value for that interval.

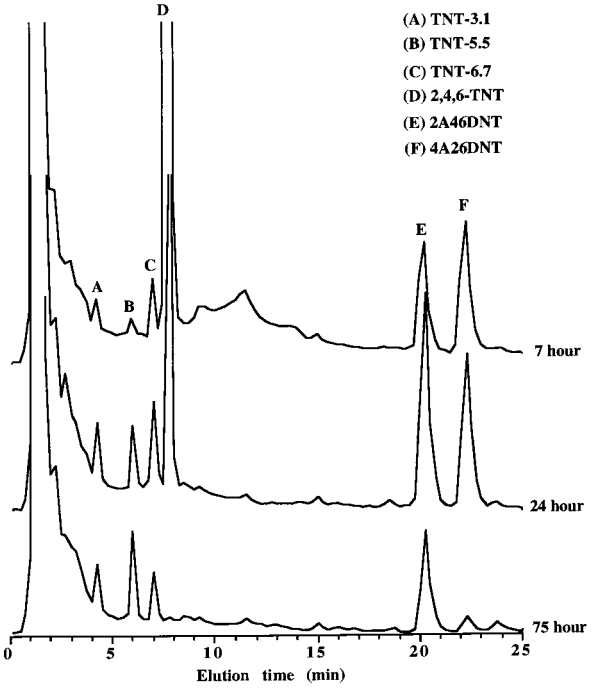


FIGURE 3. Stacked chromatograms (at 230 nm) of C_8-HPLC analysis of the extracellular, aqueous medium of TNT-treated root systems of *C. roseus* at 7, 24, and 75 h from amendment with TNT, showing the appearance of unique UV-absorbing analytes. Abbreviations: 2A46DNT, 2-amino-4,6-dinitrotoluene; 4A26DNT, 4-amino-2,6-dinitrotoluene.

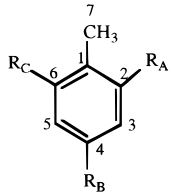
coeluted with three prominently detected analytes at C_8-HPLC retention times of 3.1, 5.5, and 6.7 min. The levels of TNT, 2-amino-4,6-dinitrotoluene, and 4-amino-2,6-dinitrotoluene in this extract of a 31-h TNT-treated roots were extremely low—0.8%, 2.1%, and 1.3%, respectively. Other known TNT transformation products were not detected. Of interest is the close similarity of the UV/Vis absorbance spectrum of the analytes at 3.1, 5.5, and 6.7 min to that of either 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene. The spectra of analytes eluting at 3.1 and 6.7 min were strikingly similar to that of 4-amino-2,6-dinitrotoluene: coincident maxima at 229 nm and slightly different spectral absorbances up to 360 nm. Similarly, the spectrum of the analyte eluting at 5.5 min was similar to that of 2-amino-4,6-dinitrotoluene: coincident maxima at 224 and 368 nm and slightly different spectral absorbance between 245 and 300 nm. These compounds are labeled as TNT-3.1, TNT-5.5, and TNT-6.7, respectively. These three TNT metabolites appeared both in the extracellular medium (Figure 3) and in the matrix of TNT-treated roots. All were detected in the extracellular medium at 7 h after the addition of TNT, as were 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. Over time, TNT, 2-amino-4,6-dinitrotoluene, and 4-amino-2,6-dinitrotoluene concentrations in the medium decreased as TNT-3.1, TNT-5.5, and TNT-6.7 increased (Figure 3).

Transformation products with UV/Vis spectra similar to the respective monoaminodintrotoluene also appeared in root cultures amended with either 4-amino-2,6-dinitrotoluene or 2-amino-4,6-dinitrotoluene only. 4-Amino-2,6-dinitrotoluene-amended roots accumulated a prominent, spectrally similar analyte (coincident maximum at 229 nm and spectral features as described previously) that eluted in C_8-HPLC at a retention time of 6.6 min. 2-Amino-4,6-dinitrotoluene-treated roots accumulated a prominent spectrally similar analyte (coincident maxima at 224 nm and spectral features as described previously) that eluted in C_8-HPLC at a retention time of 5.6 min. These transformation products are labeled as 4A-6.6 and 2A-5.6 min, respectively.

NMR Analysis of Nitroaromatic Metabolites. The ^{1}H NMR chemical shifts of the purified isolates, TNT-5.5, TNT-6.7, 2A-5.6, and 4A-6.6, obtained from the amendment of axenic roots with either TNT, 2-amino-4,6-dinitrotoluene, or 4-amino-2,6-dinitrotoluene as the starting material are summarized in Table 2. The ^{1}H chemical shifts of TNT, 2-amino-4,6-dinitrotoluene, and 4-amino-2,6-dinitrotoluene are also presented to facilitate comparison. All four compounds TNT-6.7, TNT-5.5, 4A-6.6, and 2A-5.6 contain two aromatic protons and an intact methyl group based upon their ^{1}H NMR spectra. Therefore, the aromatic ring structure is intact, and the similarity of the substituted methyl ^{1}H resonances in the isolated compounds to those of 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene confirms that the methyl group in these isolates has not been modified.

The TNT-derived isolate (TNT-6.7) and the 4-amino-2,6-dinitrotoluene-derived isolate (4A-6.6) each have a singlet resonance for two protons in the aromatic region, similar to 4-amino-2,6-dinitrotoluene. This demonstrates a symmetry of the two aromatic protons in TNT-6.7 and 4A-6.6, as in 4-amino-2,6-dinitrotoluene. On the other hand, the TNT-derived isolate (TNT-5.5) and the 2-amino-4,6-dinitrotoluene-derived isolate (2A-5.6) each bear two unique resonances for two aromatic protons that indicate their nonsymmetry similar to the aromatic protons of 2-amino-4,6-dinitrotoluene. As in 2-amino-4,6-dinitrotoluene, both aromatic proton resonances are split as doublets, with coupling constants of 2—2.5 Hz arising from long-range coupling of these protons. Therefore ^{1}H NMR provides strong evidence for the relationship of the isolates to either 2-amino-4,6-dinitrotoluene or

TABLE 2. Summary of NMR Resonances of Isolated Analytes and Standards



analyte	H-3[d]	H-5[d]	H-7[d]
2,4,6-trinitrotoluene[a] ($R_A = R_B = R_C = NO_2$)	8.95(s)	*c*	2.64 (s)
4-amino-4,6-dinitrotoluene[a] ($R_A = R_C = NO_2$; $R_B = NH_2$)	7.21 (s)	*c*	2.21 (s)
2-amino-4,6-dinitrotoluene[a] ($R_B = R_C = NO_2$; $R_A = NH_2$)	7.7 (d) $J = 2.25$ Hz	7.8 (d) $J = 2.35$ Hz	2.22 (s)
TNT-6.7[b] (product from TNT-amended roots)[b]	7.71 (s)	*c*	2.29 (s)
4A-6.6[b] (product from 4-amino-2,6-initrotoluene-amended roots)	7.31 (s)	*c*	2.21 (s)
TNT-5.5[b] (product from TNT-amended roots)	8.5 (d) $J = 2.5$ Hz	8.1 (d) $J = 2.5$ Hz	2.21 (?)
2A-5.6[b] (product from 2-amino-4,6-dinitrotoluene-amended roots)	7.76 (d) $J = 2.0$ Hz	7.86 (d) $J = 2.0$ Hz	2.24 (s)[b]

[a] Chemical shifts relative to CD_3OD (^1H: δ, 3.31, and 4.87). [b] Chemical shifts relative to CD_3OD (^1H: δ, 3.31, and 4.87) and CD_3CN (^1H: δ, 31.94). [c] Chemical shift equivalent to H-3 due to aromatic symmetry. [d] s, singlet; d, doublet.

4-amino-2,6-dinitrotoluene. But, numerous additional multiplet resonances in the 3−4 ppm region of their ^1H spectra demonstrate their difference from either TNT reduction product. From the aromatic resonances of each set of isolates (i.e., 4-amino-2,6-dinitrotoluene-related or 2-amino-4,6-dinitrotoluene-related), it is also evident that the isolate from TNT-amended roots is not identical to that from the monoaminodinitrotoluene-amended roots (e.g., analyte TNT-6.7 versus analyte 4A-6.6), indicating the possibility for the formation of a range of derivatives of monoaminodinitrotoluenes in the metabolism of TNT by plants.

Recovery of Aminodinitrotoluenes from TNT Metabolites by Hydrolysis. To investigate the relationship of the identified nitroaromatic metabolites to monoaminodinitrotoluenes, TNT-6.7, extracts of 2-amino-4,6-dinitrotoluene-amended roots containing 2A-5.6, and extracts of 4-amino-2,6-dinitrotoluene-amended roots containing 4A-6.6 were subjected to several hydrolysis treatments. Three treatments were employed for all isolates in this phase of the investigation: enzymatic hydrolysis with β-glucuronidase (from *Helix pomatia*); enzymatic hydrolysis with β-glucosidase (from almonds); and acid hydrolysis. All three treatments resulted in a decrease of the TNT or the monoaminodinitrotoluene-derived metabolite over an 80-h treatment period and an increase of the corresponding monoaminodinitrotoluene concentration. Therefore, in the case of TNT-6.7, 4-amino-2,6-dinitrotoluene appeared, and its level increased over the hydrolysis duration. In the case of extracts containing either 2A-5.6 or 4A-6.6, 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene increased, respectively. The limited availability of TNT-5.5 did not allow a similar treatment of this 2-amino-4,6-dinitrotoluene-related compound. Enzymatic hydrolysis appeared more effective than simply acid hydrolysis in regenerating either 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene from the nitroaromatic metabolites. The outcome of these hydrolysis treatments provides further evidence that the isolated compounds are hydrolyzable conjugates of the initial reduction products of TNT.

MS Analysis of TNT Metabolites. Mass spectroscopic analysis of selected purified isolates (TNT-6.7, 2A-5.6, and 4A-6.6) indicates the formation of TNT metabolites in plants with higher molecular mass than TNT. Several ions with molecular mass greater than 197 (monoamino derivatives of TNT) or 227 (TNT) were detected by either chemical ionization mass spectroscopy or electrospray mass spectroscopy. Both ionization modes were employed in conjunction with HPLC at atmospheric pressure (i.e., APCI and API-ES), while the former was also employed independently with direct probe and methane as the ionizing gas. Electrospray ionization in the negative ion mode resulted in the detection of ions of higher molecular mass than TNT and its amines: for 4A-6.6, 472 and 238; for 2A-5.6, 472 and 238; and for TNT-6.7, 488, 472, and 374. When the ionizing voltage was nearly doubled in this mode of analysis of TNT-6.7, there was an increase in the abundance of the ions with masses of 197, identical to the monoaminodinitrotoluenes. With chemical ionization in the positive ion mode, the most abundant ions (M + 1) detected were for 4A-6.6, 360 and 323; for 2A-5.6, 360 and 323; and for TNT-6.7, 360 and 395. Chemical ionization is a stronger ionizing method than electrospray. Consequently, smaller ions were observed but were nevertheless larger than TNT and its amines. The ion with M + 1 of 360 could be that of a monoaminodinitrotoluene-hexose conjugate ($M_w = 359$), while the ion with M + 1 of 395 is likely the condensation ion of 4-amino-2,6-dinitrotoluene (*11*).

Contribution of Conjugate-Metabolites to Mole Balance. While the ^{14}C balance described previously accounts for the TNT-derived ^{14}C in the various compartments (extracellular, intracellular extractable, and intracellular bound), it does not differentiate between molecular species. Therefore, an additional analysis (summarized in Table 3) was performed to quantitate the 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene-related conjugate metabolites identified in this study. They were estimated by employing ^{14}C-based response factors. Since the NMR analysis established the aromaticity of the TNT metabolites, with one aromatic moiety per conjugate molecule, the molar ^{14}C activity per mole of conjugate was assumed the same as that of the ^{14}C-labeled TNT. From a comparison of the mole fractions of conjugate metabolites over a 75-h period to those of the known nitroaromatics (TNT, 2-amino-4,6-dinitrotoluene, and 4-amino-2,6-dinitrotoluene), it is evident that the identification of the conjugate metabolites in this study is a significant step toward analyzing the fate of TNT in plants. The total level of conjugate metabolites was comparable to or greater than that of TNT, 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene, beyond 7 h from TNT amendment. The conjugate metabolites accounted for 15−26% of initial TNT added during the course of the study.

Discussion

The objective of this study was to further investigate the formation of fate products of plant metabolism of TNT beyond its aminated reduction products, 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. The results

TABLE 3. Mole Balance on TNT, 2-Amino-4,6-dinitrotoluene, 4-Amino-2,6-dinitrotoluene, Conjugate Metabolites, and Bound Fractions Expressed as Fraction Initial TNT[a]

time (h)	total[a]				
	nitroaromatics[c]	conjugates	bound	known[d]	recovered[e]
0	1.00	0	0	1.00	1.00
7	0.45 ± 0.02	0.15 ± 0.04	0.08 ± 0.02	0.68 ± 0.05	1.03 ± 0.05
24	0.18 ± 0.01	0.20 ± 0.06	0.14 ± 0.02	0.52 ± 0.07	0.99 ± 0.04
31	0.15 ± 0.02	0.22 ± 0.06	0.14 ± 0.07	0.51± 0.12	0.97 ± 0.05
47	0.12 ± 0.02	0.26 ± 0.01	0.25 ± 0.01	0.63 ± 0.01	1.05 ± 0.02
75	0.05 ± 0.01	0.22 ± 0.01	0.29 ± 0.01	0.56 ± 0.02	1.02 ± 0.01

[a] The fraction of TNT fate that remains unknown is represented by the difference between total recovered and total known. [b] Represents sum of extracellular medium and intracellular biomass phase. [c] Represents sum of TNT, 2-amino-4,6-dinitrotoluene, and 4-amino-2,6-dinitrotoluene. [d] Represents sum of nitroaromatics (TNT, 2-amino-4,6-dinitrotoluene, and 4-amino-2,6-dinitrotoluene), conjugates, and bound metabolites. [e] Represents mass balance on TNT by ^{14}C analysis.

presented here, using microbe-free or axenic root tissues of the terrestrial plant *Catharanthus roseus,* confirm conjugation as a metabolic process for TNT disposition in plants. The central role of the TNT reduction products 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene is also an interesting outcome of this study.

In previous studies of TNT metabolism in a variety of plants (*3, 8*) and tissue culture (*4*), temporal levels of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene have been measured in 1−2 week studies, but they accounted for a small percentage of initial TNT transformed: 15% or less. The implication is that the formation of these first set of TNT reduction products is a relatively rapid kinetic process in plants or possibly a minor pathway. Since the diaminonitrotoluene derivatives of TNT have been rarely reported (*8*) and triaminotoluene was never detected in whole plant systems, the first possibility would require the formation of additional metabolites. Acid-hydrolyzable conjugates, bound moieties, and polar metabolites have been alluded to but not isolated or identified (*3*−*5, 8*).

In this study, four unique plant-based conjugates of TNT-derived compounds were isolated, and their relationship to the initial TNT reduction products was established. These conjugates, are formed by reactions of the amine groups of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. A combination of evidence supports this conclusion: ^1H NMR; higher molecular mass than TNT, 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene; similarity of UV/Vis spectra; and finally, chemical or enzymatic hydrolysis to return the respective monoaminodinitrotoluene building block. From the mass spectral evidence, it is likely that at least a six-carbon unit from the plant intracellular millieu was involved in conjugate formation. The conjugates are significant since mole balance enumerates their free or unbound contribution at 15−26% of initial TNT moles over a 75-h period (Table 3). Furthermore, these conjugates are likely "gateways" to bound residues (*13*) and, therefore, could also account for the fraction bound into plant matter. From the perspectives of ecotoxicology and the detoxification potential of plant metabolism, the bioavailability of bound residues of TNT and its transformants is of great interest; published studies in the herbicide and pesticide areas indicate lowered bioavailability of resultant bound residues (*14*).

The four products isolated in this study serve to underscore the potential for conjugation of 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene in TNT metabolism by plants, but it is not exhaustive. The conjugates isolated in this study simply serve to illustrate the numerous possible outcomes for the plant-based conjugation of 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene due to the variety of molecules (sugars, amino acids, and organic acids) available for substitution at reactive sites, in this case $-NH_2$. When the roots were treated with TNT, the 4-amino-2,6-dinitrotoluene-derived TNT-6.7 and the 2-amino-4,6-dinitrotoluene-derived TNT-5.5 were formed; however, when they were treated with 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene, 2A-5.6 and 4A-6.6, respectively, were formed. This result may be expected since plants contain more than one type of enzyme for conjugation reactions to amino groups. For example, in *Dorotheanthus bellidiformis,* depending on the xenobiotic concentration, the product spectrum differed due to the competition of two glucosyltransferases (with different kinetic properties) for substrate (*15*). In the TNT-amended root study versus the monoaminodinitrotoluene-amended root studies, differences in the fluxes of the monoaminodinitrotoluenes likely occurred and thus may have possibly contributed to the differences observed in the product spectrum. Furthermore, the possibility of other reactive sites besides the amino group also exists, such as obtained by the modification of the methyl group. The imperative to elucidate conjugation processes may lie not in determining specific molecules capable of conjugation with reactive sites on initial TNT metabolites but in investigating the reactive centers that are susceptible to conjugation and their long-term stability.

The formation of conjugates of xenobiotic compounds is an important component of their metabolism in plants, leading to their lowered toxicity to the plant (*10*). The model often proposed is that of the 'green liver' (*16*), and it originates from studies of herbicide disposition by plants (*17*). In this model, toxic compounds uptaken by plants are metabolized in three stages: transformation, conjugation, and sequestration. The evidence for the first and last stages of the green liver model is available for TNT disposition by plants (*2*−*5, 8*): the formation of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene; and the formation of bound residues, respectively. To the best of our knowledge, our present study is the first direct evidence for the involvement of the primary reduction products of TNT (2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene) in conjugation processes in plant detoxification of TNT.

From the standpoint of environmental impact assessment of TNT and remediation processes based on plant metabolism (phytoremediation), understanding plant conjugation, which is situated between inital transformation processes, and the formation of bound moieties is critical for several reasons. It could facilitate a knowledge-based approach to assessing 'true' toxicity, mutagenicity, and toxic mechanisms beyond the stage of TNT 'disappearance'. In the same vein, it may speed the development of analytical tools for the realistic assessment of nitroaromatic fate in the environment. From the standpoint of remediation design, conjugate analysis may be the route to assessing intrinsic kinetics of TNT disposition and the bioavailability of metabolic products.

Acknowledgments

We would like to express our appreciation to Dr. Terry Marriot (Director, Mass Spectrometry Center at Rice University) for his assistance with direct probe mass spectrometric analysis of samples. We would also like to thank Dr. Ernest Lykissa and Matthew Neely (Department of Pathology, Baylor College of Medicine) for their assistance with LC−MS. We thank Dr. Ron J. Spanggord (SRI International) for the gift of several reference standards. We thank John Lauritzen for the (unpublished) preliminary studies with 80 mg/L TNT. This material is based in part upon work supported by the Texas Advanced Technology Program under Grant 003604-045, by NSF Young Investigator Award BCS-9257938 to J.V.S., and by DSWA Project 01-97-1-0020.

Literature Cited

(1) Schnoor, J. L.; Licht, L. A.; McCutcheon, S. C.; Wolfe, N. L.; Carreira, L. H. *Environ. Sci. Technol.* **1995**, *29*, 318.
(2) Palazzo, A. J.; Leggett, D. C. *J. Environ. Qual.* **1986**, *15*, 49.
(3) Harvey, S.; Fellows, R. J.; Cataldo, D. A.; Bean, R. M. *J. Chromatogr.* **1990**, *518*, 361.
(4) Hughes, J. B.; Shanks, J. V.; Vanderford, M.; Lauritzen, J.; Bhadra, R. *Environ. Sci. Technol.* **1997**, *31*, 266.
(5) Vanderford, M.; Shanks, J. V.; Hughes, J. B. *Biotechnol. Lett.* **1997**, *19*, 277.
(6) Best, E. P. H.; Zappi, M. E.; Fredrickson, H. L.; Sprecher, S. L.; Larson, S. L.; Ochman, M. In *Bioremediation of Surface and Subsurface Contamination*; Bajpai, R. K., Zappi, M. E., Eds.; Annals of the New York Academy of Science: New York, 1997; Vol. 829, p 179.
(7) Larson, S. L. In *Bioremediation of Surface and Subsurface Contamination*; Bajpai, R. K., Zappi, M. E., Eds.; Annals of the New York Academy of Science: New York, 1997; Vol. 829, p 195.
(8) Thompson, P. L.; Ramer, L.; Schnoor, J. L. *Environ. Sci. Technol.* **1998**, *32*, 975.
(9) Scheidemann, P.; Klunk, A.; Sens, C.; Werner, D. *J. Plant Physiol.* **1998**, *152*, 242.
(10) Coleman, J. O. D.; Blake-Kalff, M. M. A.; Davies, T. G. E. *Trends Plant Sci.* **1997**, *2*, 1144.
(11) Hughes, J. B.; Wang, C. Y.; Bhadra, R.; Richardson, A. *Environ. Toxicol. Chem.* **1998**, *17*, 343.
(12) Bhadra, R.; Shanks, J. V. *Biotechnol. Tech.* **1995**, *9*, 681.
(13) Lamoureaux, G. L.; Rusness, D. G. In *Xenobiotic Conjugation Chemistry*; Paulson, G. D., Ed.; American Chemical Society: Washington, DC, 1986; p 62.
(14) Khan, S. U. In *Environmental Chemistry of Herbicides*; Grover, R., Cessna, A. J., Eds.; CRC Press: Boca Raton, 1988; Vol. 2, p 265.
(15) Heuer, S.; Vogt, T.; Bohm, H.; Strack, D. *Planta* **1996**, *199*, 244.
(16) Sandermann, J. H. *Pharmacogenetics* **1994**, *4*, 225.
(17) Hatzios, K. K. In *Environmental Chemistry of Herbicides*; Grover, R., Cessna, A. J., Eds.; CRC Press: Boca Raton, 1991; Vol. 2, p 141.

Received for review June 22, 1998. Revised manuscript received October 16, 1998. Accepted October 27, 1998.

ES980635M