Liquid Chromatographic Characterization of 2,4,6-Trinitrotoluene Bound Residues Extracted from *Eisenia fetida*

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Abstract

The nitroaromatic compound TNT is a common component in military munitions because of its chemical stability and comparatively safe method of manufacture. In living systems, TNT is rapidly metabolized into four identifiable and extractable compounds (2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, 2,4-diamino-6-nitrotoluene, and 2,6-diamino-4-nitrotoluene). Exposing model organisms to TNT forms unextractable biotransformed products, commonly called "bound residues." This project evaluates methods for removal of bound residues from tissue. Liquid chromatography used in combination with radiotracer analysis will provide preliminary insight into the chemical structure(s) of these extractable bound residues.

Introduction

Nitroaromatic Compounds

The toxicological properties of nitroaromatic compounds have been studied for over 50 years. As early as the 1950s and 1960s, 4-nitroquinoline-1-oxide was determined to be carcinogenic (Purohit et al., 2000). It was one of the first nitroaromatic compounds in which nitro-reduction was thought to be responsible for the compound's carcinogenic activity. Since then, the same mechanism has been attributed to the initiation of carcinogenicity in most, if not all, nitroaromatics.

Nitroaromatics are present in a large range of substances, from cigarette smoke and diesel exhaust to grilled foods, pesticides, and even pharmaceutical drugs. The military synthesized and utilized a series of nitroaromatic munitions. This series includes hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine (HMX), N-methyl-N-2,4,6-tetranitroaniline (tetryl), 2,4,6-trinitrotoluene (TNT), and their "by products and degradation products" (S.S. Talmage et al., 1999). Out of these nitroaromatics, TNT has the highest overall

concentrations (μg/L) in soil, surface, and ground water at former and current military installations. The presence of TNT in military institutions directly reflects TNT's popularity in explosives due to its chemical stability, low sensitivity to impact, and comparatively safe methods for manufacture (Urbanski, 1984).

Trinitrotoluene's History

TNT is an odorless, yellow, synthetic solid first made by German chemist Joseph Wildbrand in 1863. In the early 1900s the German military began investigating the munitions potential of TNT. Through World War II, TNT was the most popular high power explosive in use (Reddy et al., 2000). Today, the strength of an explosive is still measured against that of TNT.

Unfortunately, TNT can contaminate water sources through various pathways. The production and purification of the explosive under military-controlled conditions results in the discharge of TNT in wastewater (Reddy et al., 2000; Larson et al., 1999). Because of the recent introduction of Base Realignment and Closure procedures, which return many military institutions to the private sector, there has been increased concern about contamination via TNT uptake by organisms (Krämer et al., 2005). At military installations currently producing TNT near open-burning, open-detonation sites, soil and water contamination poses a very real problem with unknown consequences.

Chemical Structure of TNT

The molecular symmetry of TNT makes it more persistent than mononitrotoluene or dinitrotoluene. TNT contains three nitro groups ($-NO_2$) that consist of two electronegative elements, nitrogen and oxygen. The symmetrical arrangement of TNT's three nitro groups around the aromatic ring often hinders the attack of biological enzymes that normally function in the metabolism of aromatic compounds (Esteve-Núñez et al., 2001). The electronegativity of these nitro groups attracts the *pi*-electrons from the aromatic ring. At the same time, the nitro group itself is polarized. Oxygen is more electronegative than nitrogen, so nitrogen's electrons attract the two oxygen molecules with which it shares a bond. This redistribution of electrons imparts a partial positive charge to nitrogen and a partial negative charge to oxygen. The electronegativity and partial positive charge of nitrogen makes reduction of the group a common reaction in living organisms (Lotufo et al., under

review; Esteve-Núñez et al., 2001).

In Figure 1, reduction of the first nitro group occurs at a much faster rate than that of the other two nitro groups. This is due to the fact that replacing a nitro group with an amino group on the ring increases the *pi*-electrons available around the nitroaromatic ring itself. Available electrons around the nitroaromatic ring help counteract the partial positive charges on the nitrogen of a nitro-group and consequently lower the redox potential required for reduction of the ring's remaining nitro groups. Additionally, the nitro group in the *para* position (on the carbon opposite of the methyl group) in the TNT molecule is much easier to reduce than the two groups in the *ortho* positions (to the immediate left and right of the methyl group), due to both electronic and steric factors (Esteve-Núñez et al., 2001). These structural factors help to explain the relative concentrations of biotransformation products seen in the metabolism of TNT.

Metabolism of TNT

Typically, four main reduced compounds are found and easily identified within the total extracted metabolites of TNT from an organism: 2-Amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2,4-diamino-6-nitrotoluene (2,4-DANT), and 2,6-diamino-4-nitrotoluene (2,6-DANT) along with parent TNT. The reduction may also result in the dimerization of the hydroxy molecules seen in Figure 1 in azoxy compounds (2, 2'-azoxy or 4, 4'-azoxy), but these dimers have not yet been looked for in oligochaetes (Honeycutt et al., 1996). Figure 2 provides an example of this dimerization. TNT and its degradation products have been identified in certain aquatic plants, and they eventually enter terrestrial organisms through the food chain (Lotufo et al., under review; Larson et al., 1999; Esteve-Núñez et al., 2001; Reddy et al. 2000).

Dangers of TNT and its Bioaccumulation Products

Currently, the U.S. Environmental Protection Agency (EPA) has set a Lifetime Health Advisory value for TNT in drinking water at ~2µg/L and classified TNT as a C-substance, a possible human carcinogen (Krämer et al., 2005). TNT can cause hemolytic anemia in both humans and rats due to "histologic changes in the spleen and liver" (Reddy et al., 2000, p.174). In humans, chronic TNT exposure is toxic to the liver, eye, and the entire hematopoietic system, which is necessary for

the production of blood and is composed of the bone marrow, spleen, tonsils, and lymph nodes (Lotufo et al., under review).

Researchers at Merck Labs demonstrated that ~2% of an administered dose of TNT binds to globin, plasma, hepatic, and renal proteins (Leung et al., 1995). This binding of TNT and, as more often occurs, its biotransformation products to key cellular proteins, is critical for determining the detrimental effects of TNT.

TNT is an environmentally persistent explosive with proven toxic effects in many organisms (Larson et al., 1999; Reddy et al., 2000). In mammals, 2-ADNT, 4-ADNT, and 2, 4-DANT are the most common biotransformation products, though 2, 6-DANT and 4-hydroxylamino-2, 6-dinitrotoluene (4-HA) are also metabolized and expelled. Although 2, 4, 6-triaminotoluene (TAT) has not yet been isolated as a metabolic product in the aerobic breakdown of TNT, its production is also possible (Leung et al., 1995; Honeycutt et al., 1996). These biotransformation products have been found in laboratory animals and in the urine of munitions workers, suggesting that the metabolic pathway for TNT transformation is similar in many higher species (Leung et al., 1995).

Significant, rapid biotransformation of parent TNT occurs in worms. So far, research concerning TNT in earthworms has mainly concentrated on the identification and effects of the known reduced metabolites (2-ADNT, 4-ADNT, 2,4-DANT, and 2,6-DANT) and the original TNT compound (Ownby et al., 2005; Renoux et al., 2000). In these previous studies, research has mainly focused on the toxicological effects of aqueous exposure or a combination of aqueous and dietary exposure. When both pathways are present, dietary intake has a negligible effect on TNT accumulation in worms, but there is still the potential for significant dietary uptake of TNT and dermal exposure (Belden et al., 2005.2; Lotufo et al., under review).

In general, the sum metabolites obtained are reported along with quantity of identified metabolites extracted (Belden et al., 2005.2; Dodard et al., 2004; Ownby et al., 2005; Renoux et al., 2000). The identified metabolites extracted do not account for the total number of metabolites collected, indicating that there are other unidentified metabolites extracted from the worms (Belden et al., 2005.2; Dodard et al., 2004). Research on the subject has paid little attention to the structure of the currently unidentified extractable metabolites or to the subsequent identification of any unextractable biotransformation products. A nearly identical metabolic breakdown of TNT has been established in tested

mammalian species, but a similar breakdown of TNT in earthworms has not yet been proven (Leung et al., 2005). Furthermore, relative concentrations of TNT's identified biotransformation products are not identical between mammals and earthworms, despite the rapid biotransformation of the explosive demonstrated in fish, mammals, and earthworms (Ownby et al., 2005). For example, in earthworms, the biotransformation products are formed within five minutes of initial TNT uptake (Renoux et al., 2000). Additionally, while the sum metabolites removed from a worm are reported, there is a mass-balanced quantity of TNT that is still missing in previous work.

These currently unidentified extractable compounds may have detrimental effects when they reenter the environment as waste products and when they enter the food chain via intake of worms by predators. Bioaccumulation in the food chain is a real threat to higher organisms. Previous studies have hypothesized that bound residues exist and account for the loss of radioactivity and TNT, but recently a cojoint study confirmed their existence by demonstrating their removal. Systems composed of acid and phosphate buffer demonstrated cleavage of bound residues. Residues may be inert in lower organisms but become malignant as they accumulate in higher organisms. The identified biotransformation products of TNT accumulate to a greater degree than parent TNT in aquatic invertebrates, including the two benthic invertebrates *Chironomus tentans* and *Lumbriculus variegatus*, as well as other, more complex, organisms (Belden et al., 2005.2).

Identified extractable biotransformation products, especially 4-ADNT, accumulate to a greater degree than parent TNT, and have also been found to be equally or more toxic than parent TNT in organisms (Lachance et al., 1999; Krämer et al., 2005). Catfish expel these toxic biotransformation products (like 2-ADNT and 4-ADNT) at a greater concentration than unaltered parent TNT (Belden et al., 2005.2; Ownby et al., 2005). Consumption of earthworms exposed to TNT may expel toxic biotransformation products, contaminating the soil and water. To enable future research on the uptake and biotransformation of these products, the products must first be identified.

Glutathione's Potential Role in TNT Metabolism

Glutathione (Figure 3) is a tripeptide consisting of the amino acids glutamine, cystine, and glycine. The thiol (sulfur) group in glutathione participates in conjugation reactions with electrophilic compounds,

including nitroaromatic compounds (Field & Thurman, 1996; Leung et al., 1995; Esteve-Núñez et al., 2001). These conjugation reactions serve as a defensive detoxification method in mammals, fish, insects, higher plants, and microorganisms to prevent oxidative stress (Field & Thurman, 1996).

Nitromusks have a similar structure to TNT and have been shown to form adducts with hemoglobin in rainbow trout (Mottaleb et al., 2004; Mottaleb et al., 2005). However, adducts of this type have not been isolated or characterized for TNT. While this adduct possibility should be investigated, it is also possible that TNT forms adducts with carbohydrates like glucuronic acid or proteins like glutathione or DNA. These adducts are especially relevant in earthworms because they do not have blood but another heme-system. However, these organisms do contain DNA, glutathione, and glucaronide.

Previous studies in weed science, biochemistry, toxicology, microbiology, and molecular biology support the role of glutathione in the metabolism of TNT in earthworms, which are found in aquatic and terrestrial environments (Field & Thurman, 1996). TNT and its metabolites may very likely react with glutathione and then become integrated into protein (Belden et al., 2005.2). They may also be excreted from the body as waste.

Glutathione may function in a similar way within the earthworm. The conjugates and sulfonated metabolites formed can be potentially damaging to the environment. In sulfonated metabolites, the sulfonic acid functional group is often ionized at the pH of most environmental systems due to its low pK_a. This charge on the molecule increases the metabolite's solubility in water, making it more mobile in an aqueous environment than the parent compound, in this case TNT (Field & Thurman, 1996; Larson et al., 1999). It can be somewhat difficult to detect and identify sulfonates because of their low volatilities, which can make identification through traditional analytical chemistry techniques of gas chromatography and mass spectrometry difficult but not impossible. This difficulty can result in sulfur-incorporating reactions being overlooked (Field & Thurman, 1996).

Adding a compound containing a free sulfhydryl group like cysteine, glutathione (GSH) or 3, 4-dichlororbenzenethiol decreases the level of covalent adducts available in TNT, indicating these compounds may be involved in the formation of TNT adducts. In the study by K.H. Leung, et al., adding GSH decreased covalent binding by 34.7% in [^{14}C]

TNT, again suggesting the involvement of protein sulfhydryl groups in TNT adduct formation. In this same study, a dual product was detected after the incubation of [^{14}C] 4-hydroxylamino-2, 6-dinitrotoluene (4HA), and [^{3}H] GSH, but the amount of product created was not enough for isolation and identification. This is possibly because the created product is too unstable and readily rearranges to form sulfonamide, which is then hydrolyzed into sulfinic acid and an amine. This process can be seen in Figure 5.

Glucuronic acid (Figure 4), though a sugar and not a protein like glutathione, also acts as an agent of detoxification in biological systems. Biological contaminants interact with the carboxylic acid functional group of the acid. Once bound in a glucuronide adduct, the complex is very water-soluble and can easily pass through an organism. It is not known, however, if glucuronide-TNT biotransformation product adducts form in organisms, or if this is even a detoxification pathway used with the explosive.

High-performance liquid chromatography (HPLC) has been successful at separating and identifying many of the biotransformation products of TNT (Belden et al., 2005.2; Ownby et al., 2005). The extent of transformation that explosive compounds can undergo *in vivo* can create biotransformation products that have very different molecular weights and polarities than the parent explosive. This makes putting together a method to detect all potential metabolites with adequate separation in HPLC more of an art than science at times (Larson et al., 1999). Thus alternative analytical techniques must be used for the final identification of TNT biotransformation products, especially those that have not yet been identified. A mass spectrometer (MS) paired with a HPLC can be used to obtain spectra for each of these metabolites and then compared to spectra of identified molecules to determine the structure of the previously unidentified metabolites.

Objectives of This Study

In this particular study, the removal and isolation of bound TNT biotransformation products was attempted with the use of two hydrolysis techniques: (1) hydrochloric acid with a pH of approximately two and (2) a phosphate buffer with a pH of approximately six. Concurrent studies by Bolner and coworkers have established that these techniques would effectively remove the previously unextractable TNT derivatives from tissue samples. After confirming removal of these bound residues from

the tissue using radioactive tracers, separation and identification of the extracted biotransformation products took place. Radioactive tracers and HPLC were used for this separation and identification.

Experimental Methods

Experimental Set-Up

Nine worms were dermally exposed to 30 µL ^{14}C-TNT (63,000 dpm/µL) for 72 hours. A single worm was placed in a sampling jar for 72 hours as well, but not exposed to any ^{14}C-TNT. During this time each worm was fed 25 mg of ground cereal grass every 24 hours.

After 72 hours, 3 X 15 mL acetonitrile extractions were performed on all 10 worms. The extract was then blown down to ~5 mL, parafilmed, and saved for further analysis. All tissue samples were then individually dried under a nitrogen gas stream. Samples were then combined to make 3 triplicate lots of tissue (**1**-[1-3], **2**-[4-6], **3**-[7-9]), while the tissue from the worm not exposed to TNT was kept separate as a control. Tissue samples from each lot were then taken to make (a) a 10 mg pellet for LSC analysis, (b) a 100 mg pellet for hydrolysis with phosphate buffer, and (c) a 100 mg pellet for hydrolysis with 0.01 M HCl.

From each triplicate lot, 10 mg of tissue was taken, put in a Liquid/Solid Chromatography (LSC) vial, covered with tissue solubilizer, and allowed to digest for 24 hours. Then 10 mL of scintillation cocktail was added to the vial, and the sample was analyzed with LSC. From each lot, 140 mg of tissue was placed in a centrifuge tube with 8 mL of phosphate buffer and sonicated, then allowed to sit for 24 hours. The same procedure was carried out with the second 140 mg sample from each lot, only 8 mL of 0.01 M HCl (pH ~2) was used in place of the phosphate buffer.

After 24 hours, the samples were all centrifuged, and the liquid was pulled off and filtered. Each sample was then concentrated under a nitrogen gas stream to 4 mL and 1 mL was taken for HPLC analysis. Each sample was run on a 100 µL full loop with fractions collected. This process is summarized in Figure 7.

High Performance Liquid Chromatography (HPLC) Methodology

Reverse-phase chromatography was run on a Varian ChromSep HPLC column SS (150 X 4.6 mm, Lake Forest, CA). The liquid phase was an acetonitrile/nanopure mixture in a gradient elution. The acetonitrile

was ramped in order to better separate the two ADNTs and DANTs respectively and to better allow for the separation of any bound residues removed after digestion that elute later from the column.

Separation of TNT and metabolites that may have been present was performed on an HPLC (Varian Prostar 210, Australia) with the UV-VIS detector set to 261nm. Gradient elution began at 10% ACN, increased to 50% ACN over the first 8 minutes and held until 12 minutes, increased to 70% at 17 minutes, increased to 100% at 18 minutes and then held until 22 minutes, then decreased to 10% ACN at 24 minutes and held for the remainder of the run, giving enough time for the column to return to initial conditions for the next run. Using this method, 2, 4-DANT eluted at 8.2 minutes, 2, 6-DANT eluted at 9.1 minutes, ADNTs between 13.7 and 14.1 minutes, and TNT at 17.5 minutes. All elution times are an average determined from an average of multiple chromatography runs, an example of which can be seen in Figure 6 with peak times for all metabolites labeled.

A 100 µL loop was then utilized on the HPLC using the method described above and fractions obtained. Fourteen fractions were collected:

Fraction	Time (minutes)	Material
1	0-2	
2	2-4	
3	4-6	
4	6-8	
5	8-8.8	2,4-DANT
6	8.8-10	2,6-DANT
7	10-13	
8	13-14	ADNT's
9	14-14.8	
10	14.8-16	TNT
11	16-18	
12	18-20	
13	20-22	
14	22-27	

After collection, 10mL of ScintiSafe Plus™ 50% was added to each vial, then Liquid Scintillation Counting analyzed fractions for ^{14}C activity.

Results

Each worm was initially exposed to 1.89 x 10^6 dpm of radioactivity via ^{14}C-TNT (30 µL, 63,000 dpm/µL). Previous work has demonstrated a 26% uptake of TNT (and thereby radioactivity) through dermal exposure (Bolner, unpublished). It was also established that three acetonitrile extractions maximized the amount of TNT removed from the organism with only an additional 5% increase in material for an additional extraction with acetonitrile. As Table 1 shows, the triplicate acetonitrile extractions recovered on average only 57.7 ± 6.2 % of TNT and biotransformation products with 42.2 ± 5.9% of the TNT remaining in the tissue sample. This indicated that any radioactivity pulled out later by a hydrolysis technique was indeed from a previously termed unextractable residue.

The acid hydrolyzed an average of 36.1 ± 14.7% of the previously unextractable residues. Based on the counts of radioactivity per milligram in tissue of each lot, the percentages of radioactivity hydrolyzed by the acid for each sample were determined as follows, with additional information in Table 4.

Summary of Acid Hydrolysis Effectiveness

	Radioactivity in each tissue sample (dpm)	Radioactivity in extract (dpm)	Percent extracted by acid hydrolysis
A1	109,531.3	34,187.2	31.2%
A2	66,856.98	37,621.8	56.3%
A3	145,431.9	44,327.5	30.5%
AVERAGE	107,272.4	38,712.2	36.1 ± 14.7%

The acid hydrolysis for each lot's extract was also analyzed via HPLC fraction collection with LSC data collected for all samples and summarized in Table 5. Figure 8, constructed from this data, demonstrates radioactivity as a function of fraction (and thereby elution time). The graph shows a definite peak at fraction 8 (13 to 14 minutes) in all lots with an average of 44.8 ± 3.5% of the radioactivity collected off the column eluting in that fraction. A smaller but still noticeable peak with an average percentage of 22.0 ± 0.7% of the total radioactivity is also present for fraction 2, corresponding to an elution time of two to four minutes.

Based on the counts of radioactivity per milligram of tissue

for each lot seen in Table 3, an average of 75.8 ± 37.3% of the initial radioactivity in the tissue was extracted. This data is summarized below for each lot, with additional information in Table 6.

Summary of Phosphate Buffer Hydrolysis Effectiveness

	Radioactivity in each tissue sample (dpm)	Radioactivity in extract (dpm)	Percent extracted by acid hydrolysis
B1	109641.5	79325.0	72.3%
B2	63249.1	78601.8	124.3%
B3	112550.4	58435.2	51.9%
AVERAGE	95146.9	72120.7	75.8 ± 37.3%

The buffer hydrolysis for each lot's extract was also analyzed via HPLC fraction collection with LSC data collected for all samples and summarized in Table 7. From this data, Figure 9, showing radioactivity as a function of fraction (and thereby elution time), was constructed. Figure 9 shows a definite peak at fraction 8 (13 to 14 minutes) in all lots with an average of 21.0 ± 4.1% of the total radioactivity collected off the column eluting in that fraction. Another peak with an average percentage of 19.9 ± 3.0% of the total radioactivity was present for fraction 5, corresponding to an elution time of 8.0-8.8 minutes. The third largest percentage of radioactivity was found in fraction 2, corresponding to two to four minutes, with an average value of 17.6 ± 5.4 %. In the buffer hydrolysis, an average of 66 percent of the radioactivity that eluted off the column was found between two and 8.8 minutes (fractions 2-5).

Discussion

Analysis of Data

The purpose of this study was to first extract TNT biotransformation products from worm tissue samples that remained in the tissue after acetonitrile extractions. This extraction of the bound residues was done with a hydrochloric acid solution of pH 1.85 and phosphate buffer of pH 5.92. HPLC was then used to separate and examine the potential identities of these biotransformation products. Comparisons among residues cleaved by each hydrolysis technique were carried out using radiotracers.

The acid's pH is comparable to that of a mammalian digestive system. The buffer's pH is closer to that of a mammalian blood stream. While these hydrolysis models are crude at best, they can be used to predict which and to what degree bound residues may be released into mammalian systems. This model is especially relevant when dealing with indirect exposure through the food chain rather than direct contact with the chemical.

Acid hydrolysis removed an average 36.1% of the radioactivity found in tissue that had already undergone three acetonitrile extractions. Though there is a significant deviation in the individual percentages, this result is still substantially higher than the 5% of radioactivity removed with an additional acetonitrile extraction, indicating that the acid hydrolyzes additional biotransformation products from the tissue. The elution times of radioactivity, and the consequent TNT biotransformation products, correspond to the previously identified ADNTs and the elution of cold ADNTs that spiked the extract. However, there is an appreciable amount of radioactivity that elutes off the column within the first eight minutes of the HPLC run, before any of the known metabolites would elute.

Phosphate buffer hydrolysis removed an average of 75.8% of the radioactivity previously bound into worm tissue. Again, despite the large variation in the results for the individual samples (from 51.9% to 124.3%), the data maintains that the buffer is indeed removing additional TNT biotransformation products from the tissue, which were not extractable with acetonitrile. In HPLC analysis, the majority of the radioactivity for each buffer extract eluted in the fractions between 8 and 8.8 minutes and 13 and 14 minutes. These fractions correspond to the elution times of the cold DANTs and ADNTs respectively. Also, radioactivity elutes off the column in fractions two and four, which together correspond to a four minute period from two to six minutes.

The elution of radioactivity off the column so early indicates the presence of the original Carbon-14 from TNT in molecules that are water soluble to a greater degree than any of the identified TNT biotransformation products. In reverse-phase HPLC, glucuronide conjugates are expected to elute before the known metabolites because sugars are more polar than the other metabolites. Similar retention times would be expected for glutathione derivatives as well on a reverse phase column. If the TNT had not formed water-soluble molecules and had been completely broken down into hydrocarbons by the hydrolysis techniques, radioactive products would have been collected after the

general elution time for the parent TNT molecule. It is still possible that there was a complete breakdown after the incorporation of TNT's carbon into cellular materials. These currently unknown compounds (unknown both in existence and structure) could also elute before any of the known metabolites, and it is likely that they also contribute to the radioactivity eluting off the column in the first six minutes.

Sources of Error

With a dermal exposure, it is difficult to determine the exact amount of ^{14}C-TNT each worm is exposed. The amount of radioactivity varies based on the homogeneity of the TNT/acetonitrile mixture and, more so, on the reproducibility of the 30 μL syringing of the solution. This is why quantitative preliminary exposure and uptake data were obtained in previous experiments. In this experiment, the focus was not as quantitative however and more concerned with the qualitative properties (structures) of the TNT's metabolism. Consequently, errors associated with quantitative variations and mass balancing of the exposure, while potentially large, are not very influential on the final experimental data.

Replicates in this experiment show too much of a deviation and are too few to make any claims about the quantities of each hydrolyzed TNT biotransformation product formed in each type of hydrolysis. However, the data exhibits a definite trend in the elution times of the majority of radioactivity for both hydrolysis techniques.

Final Conclusions and Future Work

While radioactive products were recovered before the elution times for any of the standard TNT metabolites, the amount of radioactivity measured after the TNT was negligible. However, radioactivity eluted at times corresponding to the aminated biotransformation products of TNT. The hydrolysis methods may form 4-ADNT (or 2-ADNT) from a glutathione-TNT conjugate in the worm tissue. It is also possible that the early eluting radioactivity indicates that the TNT is completely broken down by the worm tissue and then incorporated into other cellular materials within the organism. While this theory cannot be confirmed through the current experimental setup, it is logical that the bound residues only become extractable through a change in their molecular structure or formula, which could include cleavage of the sulfonamide

(Figure 5) back into a TNT metabolite and glutathione derivative. LC-MS analysis of the extract could also help to further confirm the removal of ADNTs from the tissue by each hydrolysis and, to some extent, identify the structure of the compounds containing the Carbon-14 from the initial TNT that elutes before the DANTs on the chromatogram.

Nuclear magnetic resonance (NMR) is another method that can be used to identify the structures of TNT metabolites and help to characterize any products that may form between TNT and other molecules within an organism. If a trinitrotoluene-glutathione semimercaptal can be proven to exist through ^{1}H-NMR or other conclusive methods, it proves the occurrence of this particular reaction within organisms as well. This means that the amount of glutathione in an organism affects its metabolism of TNT. This could be why certain organisms appear to rid themselves of the chemical faster than others. It would also give researchers NMR and GC data on a molecule to look for the in metabolism of TNT, which may lead to the identification of the unknown metabolites of TNT.

Again, data from this experiment does not confirm an extractable TNT-glutathione or glucuronide biotransformation product. The experiment does indicate that bound residues stemming from the parent TNT compound can be extracted from worm tissue using both acid hydrolysis and phosphate buffer hydrolysis.

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APPENDIX OF TABLES AND FIGURES

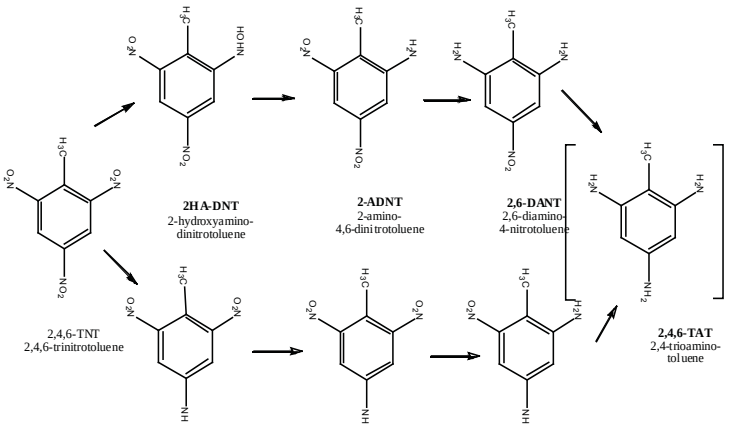

Figure 1. Potential degradation products for TNT and their respective paths. 2,4-triaminotoluene is seen only in anaerobic conditions for metabolism of TNT and has yet to be identified in mammalian excretions. Adapted from Lachance et al. 2004.

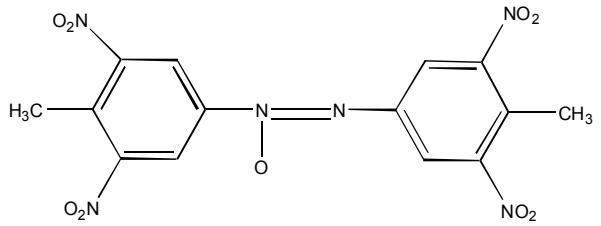

Figure 2: 2,2',6,6'-Tetranitro-4,4'-azoxytoluene

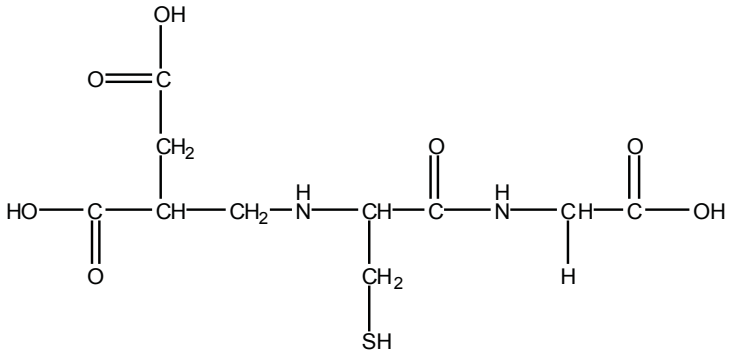

Figure 3: Gluathione, [N-(N-L-glutamyl-L-cysteinyl)glycine]

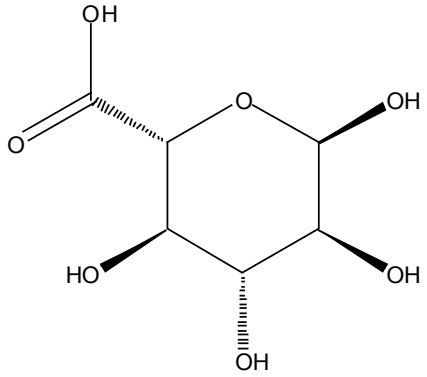

Figure 4: Glucuronic Acid

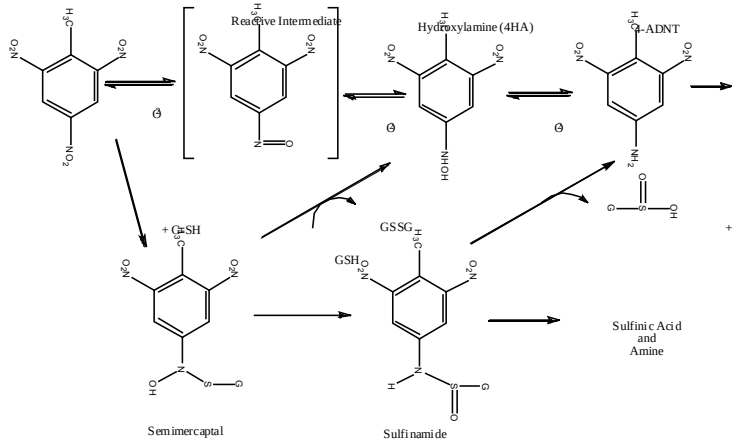

Figure 5. Proposed pathway for TNT's reactions with the cellular protein glutathione. Reaction demonstrated with the reactions occurring at the 4-position (para) of the molecule but could also occur with the nitro groups located ortho to the methyl. Figure adapted from mechanism proposed by K.H. Leung et al.

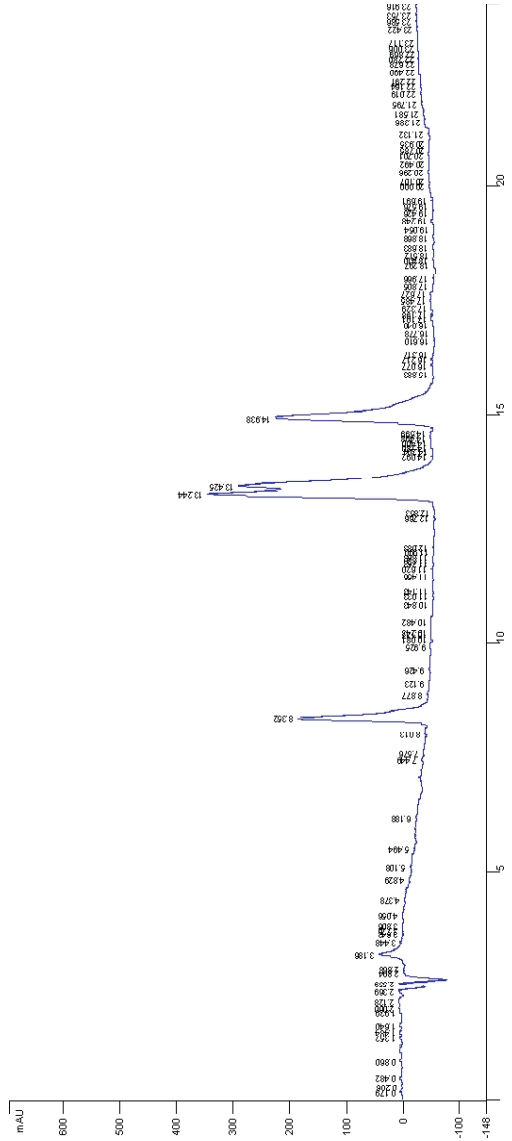

Figure 6. Chromatogram of Cold TNT and Metabolites in Nanopure H2O

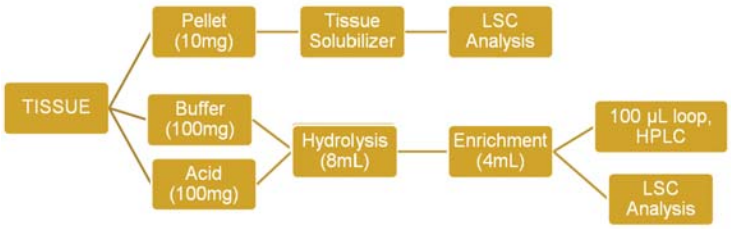

Figure 7. Experimental Setup

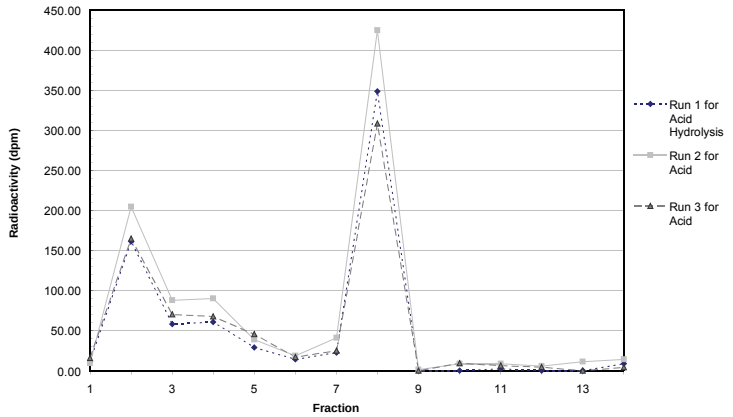

Figure 8. Radioactivity in HPLC Fractions for Acid Hydrolysis

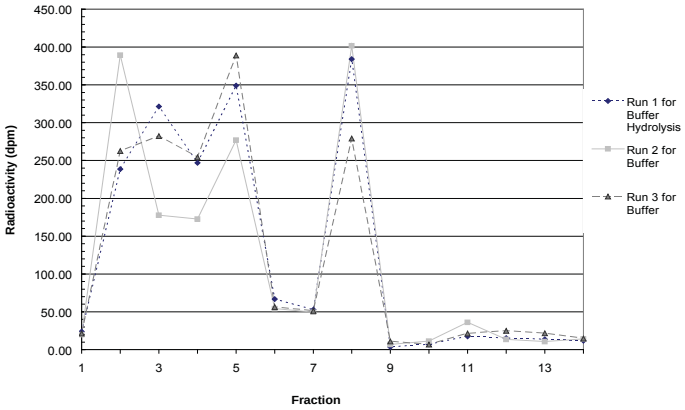

Figure 9. Radioactivity in HPLC Fractions for Buffer Hydrolysis

Table 1. Recovery of Radioactivity after Triplicate Acetonitrile Extractions

Replicate	Tissue Mass (mg)	Sample Size (mg)	Sample Tissue (dpm)	DPM per 25mg (dpm)	TOTAL Tissue (dpm)	Filter (dpm)	1ml Extract (dpm)	TOTAL Extract (dpm)	TOTAL Uptake (dpm)
1	111.90	25.00	3468.92	3468.92	15526.89	544.37	4540.79	22703.95	38775.21
2	73.90	25.00	5985.44	5985.44	17692.96	1408.75	4349.36	21746.80	40848.51
3	129.20	25.00	3834.55	3834.55	19816.95	633.99	6098.78	30493.90	50944.84
4	131.60	25.30	3572.60	3615.47	18583.17	491.92	4297.61	21488.05	40563.14
5	154.60	25.20	4729.70	4767.54	29016.33	329.05	9225.03	46125.15	75470.53
6	111.10	25.80	3484.91	3596.43	15006.72	206.08	6187.45	30937.25	46150.05
7	130.30	25.90	4017.84	4162.48	20213.30	674.70	5532.76	27663.80	48551.80
8	134.70	25.10	5416.87	5438.54	29069.82	1078.96	5655.45	28277.25	58426.03
9	142.40	25.40	2626.87	2668.90	14727.02	363.46	5985.23	29926.15	45016.63
10	98.20	25.00	3194.83	3194.83	12549.29	427.32	3638.37	18191.85	31168.46
11	148.80	25.40	3435.29	3490.25	20124.85	261.93	4446.22	22231.10	42617.88
12	132.90	26.80	1834.78	1966.88	9098.59	199.41	1973.78	9868.90	19166.90
13	201.60	25.40	1800.31	1829.11	14289.07	401.73	4359.06	21795.30	36486.10
14	142.50	25.60	2543.15	2604.19	14156.21	451.84	4428.87	22144.35	36752.40
15	116.20	24.40	3534.48	3449.65	16832.24	485.82	3017.24	15086.20	32404.26
16	92.30	26.20	2791.39	2925.38	9833.79	490.30	1998.18	9990.90	20314.99
17	116.80	24.70	2624.50	2593.01	12410.59	419.87	1962.39	9811.95	22642.41
18	156.40	24.50	1840.15	1803.35	11746.92	107.35	3207.48	16037.40	27891.67
19	124.70	25.10	3146.50	3159.09	15632.21	2616.11	4077.60	20388.00	38636.32
20	138.50	26.10	3116.41	3253.53	16537.27	1003.06	4612.62	23063.10	40603.43
Average				**3390.38**	**16643.21**	**629.80**	**4479.71**	**22398.57**	**38821.90**
Std. Dev.					5279.81	325.91		8607.30	
Percentage of Uptake					**42.9**	**1.6**		**57.7**	
					42.9 ± 5.9 %			57.7 ± 6.2 %	

From study by Michelle Bolner, Baylor University

Table 2. Recovered Radioactivity after Three Acetonitrile Extractions

Worm	Mass Worm (mg)	Radioactivity in 1 mL Extract (dpm)	Total Extracted (dpm)
1	846.3	76907.84	384539.2
2	694.8	49598.96	247994.8
3	1012.3	93633.35	468166.8
4	758.7	89293.57	446467.9
5	596.5	59802.64	299013.2
6	678.5	56682.89	283414.5
7	504.3	33800.56	169002.8
8	823.9	41866.51	209332.6
9	670.5	49150.82	245754.1
AVERAGE	**731.8**	**61193.02**	**305965.1**

Table 3. Radioactivity in Worm Tissue after 3 x 15 mL Acetonitrile Extractions

triplicate lot	dry tissue sample (mg)	radioactivity in sample (dpm)	radioactivity (dpm/mg)
1	10.1	11118.25	1100.8
2	9.7	7142.21	736.3
3	10.0	14485.25	1448.5

Table 4. Acid Hydrolysis Results

Sample	Dry Tissue (dpm)	Mass Tissue (mg)	Total Tissue Radioactivity (dpm)	1mL Acid Extract (dpm)	Total Extracted Radioactivity (dpm)	Percent Extracted by Acid	HPLC loop (100uL)	HPLC Radioactivity in 1 mL	Percent Radioactivity off column
1	1100.8	99.5	109531.27	8546.81	34187.2	31.2%	716.40	7164.00	83.8%
2	736.3	90.8	66856.98	9405.45	37621.8	56.3%	964.68	9646.80	102.6%
3	1448.5	100.4	145431.91	11081.88	44327.5	30.5%	739.06	7390.60	66.7%
AVERAGE	**1095.2**	**96.9**	**107273.39**	**9678.05**	**38712.2**	36.1%	**806.71**	8067.13	83.4%
Standard Deviation						*14.7%*			*17.9%*

Table 5. Fraction Collection for Acid Hydrolysis

Sample

	A1			**A2**			**A3**			**AVERAGE**	
Fraction	Radioactivity (dpm)	% Total Radioactivity	Fraction	Radioactivity (dpm)	% Total Radioactivity	Fraction	Radioactivity (dpm)	% Total Radioactivity	% Total Radioactivity	Standard Deviation
1	11.16	1.6%	1	9.23	1.0%	1	15.73	2.1%	1.5%	0.6%
2	161.34	*22.5%*	2	204.51	*21.2%*	2	165.00	*22.3%*	**22.0%**	0.7%
3	58.04	8.1%	3	87.89	9.1%	3	70.12	9.5%	8.9%	0.7%
4	60.47	8.4%	4	90.21	9.4%	4	67.59	9.1%	9.0%	0.5%
5	29.01	4.0%	5	38.73	4.0%	5	45.61	6.2%	4.7%	1.2%
6	14.23	2.0%	6	18.54	1.9%	6	17.12	2.3%	2.1%	0.2%
7	22.60	3.2%	7	40.90	4.2%	7	24.88	3.4%	3.6%	0.6%
8	348.74	*48.7%*	8	425.01	*44.1%*	8	308.57	*41.8%*	**44.8%**	3.5%
9	0.00	0.0%	9	1.37	0.1%	9	0.00	0.0%	0.0%	0.1%
10	Ń	Ń	10	8.58	0.9%	10	9.33	1.3%	1.1%	0.3%
11	2.10	0.3%	11	8.59	0.9%	11	6.55	0.9%	0.7%	0.3%
12	0.00	0.0%	12	5.76	0.6%	12	4.51	0.6%	0.4%	0.3%
13	0.00	0.0%	13	11.34	1.2%	13	0.00	0.0%	0.4%	0.7%
14	8.71	1.2%	14	14.02	1.5%	14	4.05	0.5%	1.1%	0.5%
TOTAL	**716.40**			**964.68**			**739.06**			

With a standard deviation of 137 dpm for the total radioactivity recovered off the column

Table 6. Phosphate Buffer Results

Sample	Dry Tissue (dpm)	Mass Tissue (mg)	Total Tissue Radioactivity (dpm)	1mL Buffer Extract (dpm)	Total Extracted Radioactivity (dpm)	Percent Extracted by Buffer	HPLC loop (100uL)	HPLC Radioactivity in 1 mL	Percent Radioactivity off column
1	1100.82	99.6	109641.36	19831.25	79325.00	72.3%	1753.21	17532.10	88.4%
2	736.31	85.9	63249.06	19650.45	78601.80	124.3%	1632.22	16322.20	83.1%
3	1448.53	77.7	112550.39	19478.39	58435.17	51.9%	1695.98	16959.80	87.1%
AVERAGE	**1095.22**	**87.7**	**95146.93**	**19653.36**	**72120.66**	**75.8%**	**1693.80**	**16938.03**	**86.2%**
Standard Deviation						*37.3%*			*2.8%*

Table 7. Fraction Collection for Phosphate Buffer

Sample

	B1			**B2**			**B3**			**AVERAGE**	
Fraction	Radioactivity (dpm)	% Total Radioactivity	Fraction	Radioactivity (dpm)	% Total Radioactivity	Fraction	Radioactivity (dpm)	% Total Radioactivity	% Total Radioactivity	Standard Deviation
1	23.46	1.3%	1	19.62	1.2%	1	21.24	1.3%	1.3%	0.1%
2	238.73	*13.6%*	2	389.22	*23.8%*	2	262.62	*15.5%*	**17.6%**	5.4%
3	321.53	18.3%	3	177.46	10.9%	3	282.34	16.6%	15.3%	3.9%
4	246.91	14.1%	4	172.46	10.6%	4	254.17	15.0%	13.2%	2.3%
5	349.21	*19.9%*	5	276.74	*17.0%*	5	388.93	*22.9%*	**19.9%**	3.0%
6	66.99	3.8%	6	54.15	3.3%	6	56.23	3.3%	3.5%	0.3%
7	52.98	3.0%	7	49.24	3.0%	7	51.26	3.0%	3.0%	0.0%
8	384.24	*21.9%*	8	401.49	*24.6%*	8	279.13	*16.5%*	**21.0%**	4.1%
9	3.59	0.2%	9	6.70	0.4%	9	10.83	0.6%	0.4%	0.2%
10	6.99	0.4%	10	11.26	0.7%	10	6.67	0.4%	0.5%	0.2%
11	17.57	1.0%	11	35.98	2.2%	11	21.31	1.3%	1.5%	0.6%
12	15.40	0.9%	12	13.21	0.8%	12	24.78	1.5%	1.0%	0.4%
13	13.72	0.8%	13	10.56	0.6%	13	21.70	1.3%	0.9%	0.3%
14	11.89	0.7%	14	14.13	0.9%	14	14.77	0.9%	0.8%	0.1%
TOTAL	**1753.21**			**1632.22**			**1695.98**			

With a standard deviation of 60.5 dpm for the total radioactivity recovered off the column