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Analysis of 2,4,6-trinitrotoluene and its transformation products in soils and plant tissues by high-performance liquid chromatography

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ABSTRACT

Previous studies have failed to provide an acceptable mass balance for 2,4,6-trinitrotoluene (TNT) in soils and plants due to deficiencies in analytical methodology. A high-performance liquid chromatographic (HPLC) method for soil analysis is reported which allowed for a mass balance in excess of 88% during a 2-month study. A method for plant analysis was developed which involved fractionation of organic extracts on Florisil adsorbent, to remove interfering pigments, followed by HPLC analysis of TNT and the primary metabolites, 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. Chromatographic recovery of TNT from spiked tissues was 85 ± 6%. The methodology was utilized to investigate TNT uptake and metabolism in plants grown in TNT hydroponic solutions.

INTRODUCTION

Wastewaters contaminated with 2,4,6-trinitrotoluene (TNT) are produced by munitions manufacturing and packaging facilities, which use water to rinse TNT residues from equipment, rejected shells, and the interior surfaces of the facilities. The waste effluents contain up to 100 mg/l TNT [1,2]. The fact that approximately $2 \cdot 10^6$ liters of such wastewater are produced daily from a single facility emphasizes the magnitude of the potential pollution problem [3]. In the past, wastewater has been directed to lagoons for settling of solid material prior to release to rivers and streams [4,5]. Over time, explosive residues and their transformation products have accumulated in large areas of soil formerly occupied by the settling lagoons. Concerns about the environmental fate of these residues are now intensified because recent revegetation of these contaminated plots allows for the possible introduction of TNT, TNT transformation products, and plant-produced TNT metabolites into the food chain.

There is a basis for concern about the toxicity of TNT. Cases of liver damage and anemia among chronically exposed munition workers are well documented [6–8]. Exposure to TNT has been shown to be toxic to a large variety of biota including green algae, oyster larvae [9], fungi, Gram-positive bacteria [5], fish [10,11], rats and mice [12] as well as plants [1]. In addition, TNT has been found to be mutagenic in the Ames bacterial assay [13].

A number of investigations have examined the metabolism of TNT in bacterial, animal, and plant systems. In a study conducted by McCormick *et al.* [6], bacteria

were shown to reduce TNT to 2-amino-4,6-dinitrotoluene (2-AMDNT) and 4-amino-2,6-dinitrotoluene (4-AMDNT) through the successive formation of nitroso and hydroxylamino intermediates. Interestingly, reduction was found to occur most extensively in the para position [6]. Several studies examining the uptake and metabolism of TNT by plants have identified the AMDNT isomers as TNT metabolites [1,14,15]. The metabolic pathway in plants also favors the formation of 4-AMDNT over reduction to 2-AMDNT. Metabolic studies involving animals have identified 4-AMDNT, 2,4-diamino-6-nitrotoluene, 2,2′,6,6′-tetranitro-4,4′-azoxytoluene, or the corresponding gulcuronide conjugates in urine of experimental animals that had been fed TNT [6]. There has been no evidence for the biological cleavage of the aromatic ring of TNT [6]. This universal metabolism of TNT by bacteria, animals, and plants to the AMDNT isomers is significant, as both the toxicity and mutagenicity of the AMDNT isomers have been demonstrated [13].

In order to assess the impact of TNT on food-chain transfer, the transformations of TNT in soils, as well as the uptake and metabolism of parent compound and associated transformation products by plants, need to be delineated. Although a variety of analytical methods have been described for recovery and analysis of TNT and tranformation products in soils, demonstration of an acceptable mass balance has not been realized. Analysis for TNT, TNT transformation products, and TNT-related metabolic products in plant tissues presents a new dimension in difficulty due to the highly complex nature of biological matrices. It is not surprising that previous attempts to delineate these complex phenomena have been plagued by analytical interferences, poor reproducibility, and low material balance [1,14,15]. The analytical approach to these problems mandate the use of both high-resolution analytical techniques and radiolabeled analyte. For instance, a study by Palazzo and Leggett [1] utilized tissue from plants grown in hydroponic solutions containing cold TNT. The plant tissues were subjected to acid hydrolysis, benzene extraction, and analysis by gas chromatography (GC) with electron-capture detection. These authors were able to identify and quantitate both the AMDNT isomers and TNT; however, since radiolabeled TNT was not used, it was impossible to determine what percentage of plant-sequestered TNT was ultimately present in these three chemical forms. Radiolabeled studies have the additional advantage of allowing for unambiguous identification of metabolic transformation products.

Accurate assessment of environmental and health risks demand analytical methods that provide for an acceptable mass balance. The primary goal of the study was therefore to develop an analytical methodology for the examination of TNT and TNT transformation and/or metabolic products in soils and plant tissues that would satisfy the mass balance criterion. Due to the limited thermal stability of explosives residues, a method utilizing high-performance liquid chromatography (HPLC) was developed.

MATERIALS AND METHODS

Uniformly ring-labeled [^{14}C]TNT (specific activity of 5.3 mCi/mmol) was obtained from E.I. du Pont de Nemours (Boston, MA, U.S.A.). Radiopurity, based on HPLC radiochromatography, was 99.86%. 2-Amino-4,6-dinitrotoluene was a Standard Analytical Reference Material (SARM) obtained from the U.S. Army Toxic and Hazardous Materials Agency (Aberdeen Proving Ground, MD, U.S.A.).

Soil characterization and sampling

Palouse soil, representing a typical Washington State agricultural soil, was used for all studies. Palouse is a silt-loam, mixed mesic Pachic Ultic Haploxeroll. The sample was collected at Pullman, WA, U.S.A., and consisted of the Ap horizon [23]. This soil is 77% silt, and 21% clay, contains 2% organic matter, and has a cation-exchange capacity of 23.8 mequiv./100 g and a pH of 5.6. For soil experiments, a solution containing appropriate proportions of labeled and vacuum-desiccated unlabeled TNT (Chem Service, West Chester, PA, U.S.A.) was prepared in 2.0 ml of methanol and amended with 400 g of air-dried soil to give a final concentration of 60 ppm TNT containing 10 μCi of labeled TNT. Amended soils were immediately brought to and maintained at 0.66 of field capacity with water. After initial sampling, which occurred no later than 2 h after amendment, the soils were maintained in a growth chamber environment that simulated the luminous intensity and spectral dispersion of sunlight during the 16-h daily light cycle. Sampling occurred at 0, 10, and 60 days and consisted of placing approximately 10 g of soil into a pre-weighed Soxhlet extraction thimble.

Plant cultivation and sampling

The chemical fate of TNT in plants was evaluated using bush beans (*Phaseolus vulgaris*, tendergreen) grown from seed. Plants were grown for 21 to 26 days on hydroponic nutrient solutions as described previously [16], at which time solutions were amended with a total of 10 ppm TNT, containing 5 μCi of radiolabel per 500 ml. These solutions were filter sterilized and placed in autoclaved 500-ml beakers to minimize bacterial contamination, which could promote transformation of TNT. Bush bean plants were placed in these solutions and maintained in a growth chamber until harvested at 1 and 7 days. The beakers were jacketed in an opaque sheath to protect the roots from light, as well as to minimize the photolysis of TNT. Solutions were analyzed by HPLC and liquid scintillation spectrometry at 0, 1 and 7 days. At harvest, plants were removed from the hydroponic solutions and the roots rinsed with 0.10 *M* calcium chloride followed by a rinse in methanol–water (80:20). Plants were then separated into roots, stems, and leaves; the tissues were minced, thoroughly mixed, and stored at -80°C until analysis.

Soil extraction

Soils were subjected to exhaustive Soxhlet extraction with 200 ml of methanol (J. T. Baker HPLC Grade) for 48 h. The soil extracts were filtered through a 0.22-μm nylon 66 filter (Alltech, Deerfield, IL, U.S.A.) before reducing the volume to approximately 20 ml by rotary evaporation. The concentrated extract was again filtered through a 0.22-μm filter, and the final volume was adjusted to a total of 25.0 ml. Extracted soils were dried at 105°C overnight, cooled in a desiccator, and weighed to obtain an accurate oven-dry weight. Portions of the extracted soils were further analyzed by total combustion in a Packard Model 306 oxidizer (Packard, Downers Grove, IL, U.S.A.) to determine the amount of irreversibly bound TNT residues that were not removed by Soxhlet extraction. The extraction efficiency of the procedure was obtained by comparing the amount of radiolabel contained in the final methanol extract to the amount of label originally added to the soil (both values were determined by liquid scintillation spectrometry). The methanol extract was subsequently analyzed for TNT and transformation products by HPLC as described below.

Tissue extraction and fractionation

The extraction and fractionation scheme for plant tissues is outlined in Fig. 1. A 1.0-g sample of fresh weight tissue was homogenized for 2.5 min in a Sorvall Omni-Mixer (Newtown, CT, U.S.A.) with 10.0 ml of 1 *M* hydrochloric acid and approximately 0.5 g of dry ice. After transfer to a 25-ml Corex centrifuge tube, the tissue was subjected to acid hydrolysis by immersing the tube into a boiling water bath for 1 h. After cooling to room temperature, the hydrolyzed material was extracted with 10.0 ml of diethyl ether. The phases were separated by centrifugation for 10 min at 3000 *g*. The volume of each layer was recorded and 100-μl aliquots were removed for liquid scintillation spectrometry. The radioactivity remaining in the pellet was determined by oxidation followed by liquid scintillation spectrometry. To

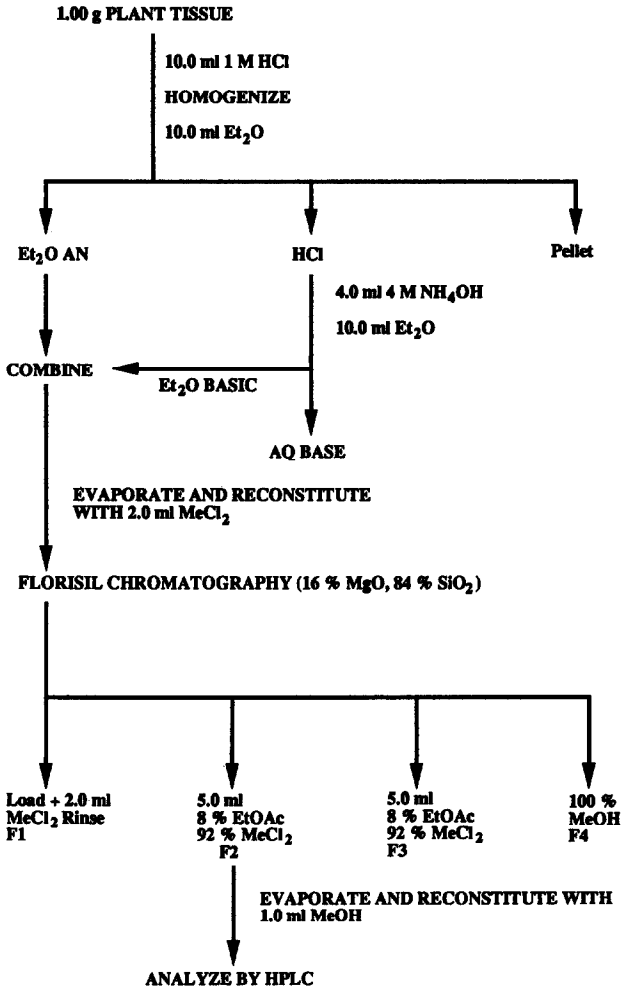

Fig. 1. Flow chart outlining the extraction and fractionation of plant tissues. AN = Acid–neutral; Et_2O = diethyl ether; $MeCl_2$ = methylene chloride; EtOAc = ethyl acetate; MeOH = methanol; AQ = aqueous.

obtain an extract of the basic organics, the hydrochloric acid layer was made basic by the addition of 4.0 ml of 4 *M* NH_4OH and extracted with a second 10.0-ml portion of diethyl ether. The layers were separated by centrifugation, the volumes recorded, and subsamples taken for liquid scintillation spectrometry. The two ether extracts were combined, evaporated to dryness with a stream of dry nitrogen, and reconstituted with 2.0 ml of methylene chloride.

The methylene chloride sample was applied to a Sep-Pak Florisil column (Waters, Milford, MA, U.S.A.) which had previously been equilibrated with methylene chloride. An additional 2.0 ml of methylene chloride was used to rinse the remaining residue from the sample vial to the Florisil column. The column eluate collected during the application of the sample comprised fraction 1. Fractions 2 and 3 were eluted from the column with successive 5.0-ml portions of methylene chloride–ethyl acetate (92:8). The final fraction (fraction 4) was eluted from the Florisil column by 5.0 ml of methanol. This strong solvent was chosen to strip the column of the maximal amount of adsorbed material. Volumes of the Florisil fractions were recorded and 100-μl aliquots were taken for liquid scintillation spectrometry. Prior to HPLC analysis, fraction 2 was evaporated to dryness and the residue was dissolved in 1.0 ml of methanol.

Residue analysis

The HPLC system consisted of a Waters Model 600E pump and system controller. The methanol extract (20 μl) of soil or plant tissue extracts was injected by a Waters WISP 710 automatic injector onto a Beckman Ultrasphere 5-μm octadecyl silica column and the components separated by a linear solvent program at a flow-rate of 1.0 ml/min. The solvent system was water–acetonitrile, with a 20-min gradient from 40% to 100% acetonitrile. Components were detected by UV absorption at 254 nm (Waters Model 490E detector), with a detector sensitivity of 0.008 a.u.f.s. Peak areas obtained from a Hewlett-Packard 3390 integrator were used for quantitative measurements.

Radiochromatographic detection was extensively used for unambiguous identification of transformation products arising from TNT. During selected chromatographic runs, the column eluate was collected in 0.5-ml increments for a total of 30 min. Each fraction was assayed for radioactivity by liquid scintillation spectrometry. Radiochromatograms were generated by plotting the disintegrations per min (dpm) in each successive aliquot as a function of retention time. Transformation products and/or metabolites identified in this manner were collected by repetitive HPLC runs to accumulate enough material for sebsequent mass-spectral studies.

GC–mass spectrometry (MS) studies utilized a Hewlett-Packard 5970 mass-selective detector interfaced to a Hewlett-Packard 5990A gas chromatograph. Transformation products were purified by HPLC as described above, evaporated to dryness with dry nitrogen, and dissolved in a small aliquot of hexane. Analysis consisted of a 1-μl splitless injection onto a 30 m × 250 μm I.D. DB-5 column containing a 1.0-μm film of stationary phase (J & W Scientific, Folsom, CA, U.S.A.). The separation was accomplished with helium carrier gas and a temperature program from 40 to 280°C at 6°C/min. Nominal resolution mass spectra were obtained by scanning the quadrupole mass spectrometer from 40 to 600 a.m.u. at a rate of 200 a.m.u./s.

Determination of $^{14}CO_2$ and volatile products

Evolution of volatile organics and $^{14}CO_2$ from plants and soils were examined by a previously described technique [17]. Soils were placed in a sealed canister and plants in a specially designed chamber that isolated the aerial portions from the roots. Air was drawn by vacuum sequentially through the chamber, two XAD columns (to trap volatile organics), and four bubbler traps (to trap $^{14}CO_2$) at a rate of 500 ml/min. Each bubbler initially contained 10.0 ml of 3 *M* sodium hydroxide. After each 24-h collection period, volumes remaining in the sodium hydroxide traps were recorded and material adsorbed on the XAD columns (100 × 5.0 cm) eluted with methanol. Radioactivity contained in methanol and sodium hydroxide samples were determined by liquid scintillation spectrometry.

Palouse soil, amended with 10-ppm TNT containing 20 μCi ^{14}C/400 g, was allowed 21 days to equilibrate prior to examining the evolution of volatiles and $^{14}CO_2$. The soil experiment was conducted for 6 consecutive days. Volatiles emanating from a mature hydroponically-grown bush bean plant were collected for three consecutive days. The plant was maintained on a 10-ppm TNT nutrient solution containing 5 μCi/500 ml during this experiment.

RESULTS

Purity of the radiolabeled TNT was determined to be 99.86% by radiochromatography. This purity was judged to be sufficient for subsequent metabolic studies and was used without further purification.

Soil fate of TNT

Chromatograms of methanol extracts from Palouse soil, a soil containing an intermediate organic content, produced blanks which were devoid of any interfering peaks. Chromatographic profiles of Palouse soil containing 60 ppm TNT are shown in Fig. 2. The top chromatogram is from an extraction initiated immediately after amendment of the soil with TNT. The retention time of TNT under the chromatographic conditions utilized in this study was 13.46 min. The chromatogram shown in the bottom of Fig. 2 is from a methanol extract of soil aged for 10 days with TNT. This chromatogram shows the presence of TNT as well as a peak that elutes immediately prior to TNT. The presence of radioactivity was verified in the first eluting peak, indicating that TNT had been transformed in the soil during the 10-day period. Co-injection experiments showed that 2-amino-4,6-dinitrotoluene and the unknown peak co-eluted. Further evidence for the identity of the unknown peak was provided by GC–MS studies. GC–MS analysis of this HPLC peak gave a chromatogram containing two peaks having identical mass spectra. One peak (retention time 29.24 min) was identified as 2-amino-4,6-dinitrotoluene as evidenced by a match of retention time and mass spectra with the SARM authentic standard. The mass spectrum of the second GC–MS peak (retention time of 28.41 min) indicated a structural isomer of 2-amino-4,6-dinitrotoluene, most likely the 4-amino-2,6-dinitrotoluene isomer. The 4-amino-2,6-dinitrotoluene isomer was found to be present at nearly twice (1.8 times) the abundance of the 2-amino-4,6-dinitrotoluene isomer.

The mass balance for TNT aged in Palouse soil for a 3-month period is presented in Table I. Average values from the triplicate analyses and the associated standard

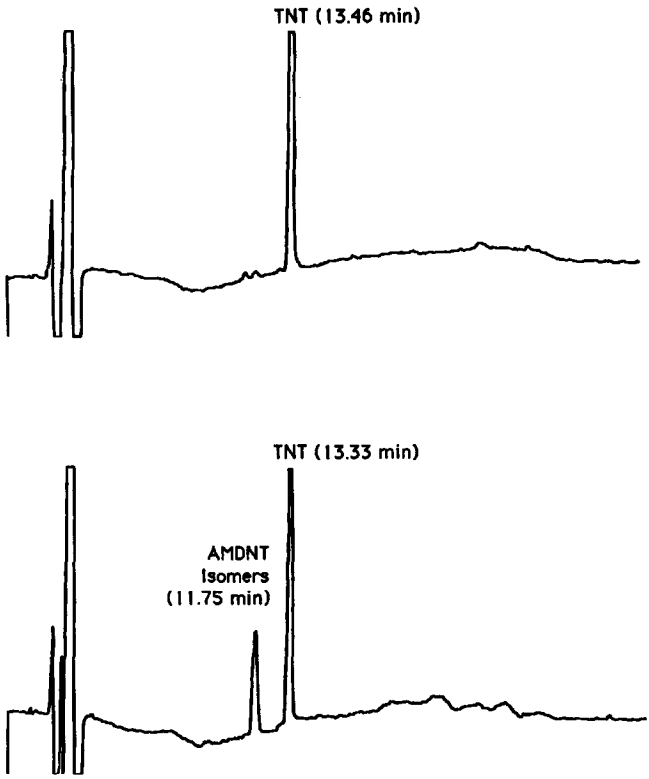

Fig. 2. Chromatographic profiles of methanol extracts of TNT in Palouse soil. The upper chromatogram is from an extract performed immediately after amendment; the bottom chromatogram is from an extract of soil aged with TNT for 10 days.

deviations are shown in Table I. The first column lists the percentage of total radioactivity added to the soil that was extracted by methanol during the Soxhlet extraction procedure. The second column presents the percentage of TNT that was recoverable in an unaltered form as determined by HPLC studies. The third column is the percentage of total TNT that was left in the soil after Soxhlet extraction as determined by oxidation of the extracted soil and quantitation of the resulting $^{14}CO_2$ by

TABLE I

MASS BALANCE OF PALOUSE SOIL CONTAINING TNT

Time (days)	Radiolabel in methanol extract (%)	Unaltered TNT (%)	Radiolabel in soil after extraction (%)	Mass balance deficit
0	94±1	88±1	0.7±0.1	5%
10	71±2	57±4	17±3	12%
61	60±2	36±3	30±1	10%

liquid scintillation spectrometry. The last column shows the percentage of TNT that could not be accounted for in either the methanol extract or the extracted soil [100 − (column 1 + column 3)].

A small amount of $^{14}CO_2$ was observed to be evolved from Palouse soil during the 6-day collection of volatiles. Assuming a constant CO_2 generation rate during the 2-month study, a quantity equal to 4% of the TNT was oxidized to CO_2. Emission of volatile organics from Palouse soil was not observed.

Analysis of hydroponic solutions

Aerated control solutions of TNT that were shielded from the growth chamber lights showed only minimal losses of TNT during the 7-day study. Control solutions that were exposed to the growth chamber lights (both non-aerated and aerated) displayed a stable amount of radiolabel (average of 4.7 μCi, $n = 6$); however, the concentration of TNT was found to decline to an average of 3.0 ppm ($n = 5$) after 7 days. The disparity between radiolabel and TNT concentration was coindicent with the appearance of a pink photodecomposition product. The pink product was not analyzed but was believed to be the same photodecomposition product observed and characterized by Spanggord *et al.* [18]. The aminodinitrotoluene isomers were not observed in control solutions even after 7 days.

Analysis of solutions used to support bush beans showed both plant uptake and root catalytic transformations. Radiolabel decreased from the initial 5.0 μCi/beaker to 3.5 and 0.6 μCi/beaker at 1 and 7 days, respectively. The amount of TNT as determined by HPLC decreased from 10 to 2 ppm within 24 h and was below the detection limit of 0.1 ppm by 7 days. Transformation products observed in the chromatographic profiles included the aminodinitrotoluene isomers and a smaller amount of an unknown compound eluting with a retention time of 17.38 min. Incorporation of radiolabel in this unknown compound was verified by radiochromatography.

Fate of TNT in plant tissue

Initial plant studies examined the extent of conjugation of the TNT-derived radiolabel present in plant tissues. Leaf tissue from a bush bean grown in hydroponic culture for 7 days was homogenized with water and subsequently extracted with diethyl ether. Only 4% of the total label was found to partition into the diethyl ether layer, while 57% of the label remained in the water layer. This indicated that the TNT was metabolized to very polar and perhaps conjugated forms. In an attempt to free possible conjugates, an acid hydrolysis was implemented immediately after homogenization. A subsample of the above leaf tissue was hydrolyzed with 1 *M* hydrochloric acid for 1 h prior to extraction with diethyl ether. It was found that 23% of the radiolabel was now ether extractable; 41% remained in the hydrochloric acid layer. By either extraction with acid hydrolysis or water extraction, slightly over 60% of the total radiolabel was found to be solubilized in the aqueous or ether layers. In all subsequent experiments the plant tissue was homogenized, hydrolyzed with acid, and then extracted with diethyl ether. The fractions resulting from the partitioning between the 1 *M* hydrochloric acid and the diethyl ether layers are referred to as the hydrochloric acid and the diethyl ether acid–neutral fractions, respectively.

Initial methanol and chloroform–methanol extraction of plant tissue indicated the need for further fractionation before HPLC analysis due to the the co-extraction

of interfering pigments. Radiolabeled TNT was spiked (2.93 ppm fresh weight) onto bush bean leaves, the tissue homogenized, and the mixture extracted with methylene chloride. The organic layer was then subjected to fractionation on Florisil adsorbent with the collection of four fractions (F1–F4). Radiolabeled TNT eluted in fraction F2; the plant pigments were found to elute in fractions F3 and F4. In order to demonstrate the recovery and reproducibility of this separation, triplicate samples were fractionated and fraction F2 was further examined by HPLC and liquid scintillation spectrometry. The recovery of TNT in fraction F2 as based on chromatographic peak areas was 85 ± 6%, whereas the recovery of radiolabel was 83 ± 4%. It was subsequently found that the aminodinitrotoluene isomers co-elute from the Florisil column in fraction F2 along with the TNT.

Bush bean tissue samples were acid hydrolyzed, extracted into diethyl ether, and subjected to Florisil fractionation as described above. The amount of radiolabel contained in the tissues and the percentage of total radiolabel in each chemical fraction for bush bean leaves, stems, and roots are shown in Table II. Interestingly, it was found that the aminodinitrotoluene isomers were extracted from 1 *M* hydrochloric acid into diethyl ether. The combined electron-withdrawing property of the nitro substituents is responsible for the neutral behavior of these compounds. This property permitted the co-extraction and quantitation of the aminodinitrotoluene isomers along with TNT. Representative HPLC chromatograms of the F2 fractions from bush bean leaf, stem, and root tissues are shown in Figure 3; Table III gives the concentrations based on the tissue fresh weight for TNT and the aminodinitrotoluene isomers.

TABLE II

TOTAL RADIOACTIVITY (AS BASED ON OXIDATION), PERCENTAGES OF TOTAL RADIOACTIVITY IN CHEMICAL FRACTIONS AND MATERIAL BALANCE FOR THE ANALYSIS OF BUSH BEAN TISSUES

Values are the averages from analysis of 3 plants, with the exception of stem values, which are based on 1 plant.

	Day 1			Day 7		
	Leaves	Stem	Roots	Leaves	Stem	Roots
Activity (dpm/g)	$(5\pm2)\cdot10^4$	$6\cdot10^4$	$(4\pm1)\cdot10^5$	$(6\pm2)\cdot10^4$	$4\cdot10^4$	$(6\pm3)\cdot10^5$
Fraction (% total activity)						
Hydrochloric acid	38±6	23	23±1	41±1	31	17±2
Aqueous base	25±1	18	17±0	27±3	22	11±1
Diethyl ether acid–neutral	28±3	32	69±8	19±4	32	38±6
Diethyl ether base	7±4	6	3±1	6±3	8	4±1
F1	0	0	0	0	0	0
F2	6±1	11	23±4	5±1	10	14±2
F3	3±2	2	6±2	1±1	3	3±1
F4	12±5	12	26±3	6±5	13	14±3
Pellet	18±2	37	26±2	24±5	45	40±5
Material balance (Hydrochloric acid + diethyl ether acid–neutral + pellet)	84	92	118	84	108	95

TABLE III

CONCENTRATIONS OF TNT AND THE AMDNT ISOMERS IN BUSH BEAN TISSUE AFTER 1 AND 7 DAYS OF GROWTH IN HYDROPONIC CULTURE

	Concentrations (ppm fresh weight)	
	TNT	AMDNT isomers
Day 1		
Leaves	0.32±0.11	0.23±0.07
Stem	0.63	0.35
Root	6.07±2.19	4.06±1.13
Day 7		
Leaves	0.18±0.16	0.15±0.08
Stem	0.88	0.12
Root	7.44±3.14	1.49±0.62

Results obtained during the oxidative metabolism study indicated that bush bean plants did not transpire volatile organics containing TNT-derived radiolabel or $^{14}CO_2$.

DISCUSSION

Soil fate of TNT

The results presented in Table I reveal that the extraction efficiencies for soils were quite high at time 0, and nearly all the radiolabel was recovered as TNT. As TNT aged in soil, the amount of extractable radiolabel decreased with an increase in the amount of non-extractable radiolabel that was irreversibly bound to the soil. It is unclear whether sorption of TNT and/or its transformation products are responsible for the decreased extractability of radiolabel over the 60-day period. The decrease in extractable radiolabel as a function of time in soil may result from sorption of organic residues to soil minerals and/or soil organic matter. This phenomenon has been described for chemically related compounds by several authors [19–21]. Discrepancies between the percent of extractable radiolabel and the amount of unaltered TNT represent the extent of transformation of parent TNT to the aminodinitrotoluene isomers. Transformation to these isomers continued throughout the 2-month study period. Radiochromatographic studies did not detect the presence of other extractable transformation products in this soil.

Previous studies have failed to provide a reasonable mass balance for TNT and TNT transformation products in soils. For example, Pennington [15] was able to account for only 50% of the added TNT radiolabel after TNT had aged in soils for 65 days. This low mass balance was attributed to the formation of volatile transformation products. In support of this view, our data show a trend for an increasing mass balance deficit with time (Table I). This may represent the formation of volatile transformation products and/or mineralization of the TNT in the soil to CO_2. Small amounts of CO_2 were evolved from TNT-amended Palouse soil which accounted for approximately 4% of the radiolabel during the 2-month study period. The methodology presented in this study provides for an acceptable mass balance over the study

period (Table I). An additional important feature of the present methodology is the high degree of reproducibility, as indicated by the small standard deviations of the triplicate analyses (Table I).

Fate of TNT in plant tissues

Initial plant studies examining the effects of acid hydrolysis on the amount of ether-extractable TNT metabolites clearly indicated that 19% of the TNT metabolites in leaf tissue were present as hydrochloric acid hydrolyzable conjugates. Interestingly, acid hydrolysis did not increase the amount of radiolabel that was solubilized. In both the acid hydrolysis and water experimental conditions, approximately 60% of the radiolabel was solubilized, while about 40% remained in the pellet. It can only be assumed that the fraction of radiolabel remaining in the pellet following extraction was tightly bound, and probably conjugated.

The total activity per gram of fresh weight tissue is summarized in Table II. It is clear from the data that the majority of radiolabel was localized in the root tissue. Roots from both the 1- and 7-day bush bean plants contained approximately ten times the amount of radiolabel as an equal weight of the leaf tissue. Stem tissue contained an amount of radiolabel per gram of tissue similar to that of the leaf tissue. Localization of radiolabel primarily in the root tissue, with minimal translocation to the shoot, has been noted in studies of dinitroaniline herbicides [22]. The chemical similarity of the aminodinitrotoluene isomers, which were observed in hydroponic solutions containing bush beans, and the dinitroaniline herbicides may account for the similarity in radiolabel distribution.

Since the aminodinitrotoluene isomers were observed in all plant-containing hydroponic solutions and were absent from control solutions, it can be concluded that the presence of the root prompted formation of these compounds. It is not clear whether this transformation was due to metabolism of TNT by the root or to microorganisms associated with the root. Additionally, it is not clear whether plant uptake involves both TNT and the aminodinitrotoluene isomers or uptake of TNT followed by metabolic alteration to the aminodinitrotoluene isomers. These issues are difficult to experimentally address since, short of utilizing aseptic plants, root sterilization would damage the tissue resulting in impaired plant uptake. However, deconvolution of the above processes may not be imperative. If root microflora are responsible for transformation of TNT in hydroponic solutions, microorganisms normally associated with the rhizosphere would be expected to promote similar transformations in the environment.

The material balance summarized in Table II compares the percentage of total activity that can be accounted for in the hydrochloric acid, diethyl ether and pellet fractions. The average of these values is 97%, which indicates that practically all of the radiolabel can be accounted for at this stage of the fractionation. It should be noted that the sum of the diethyl ether acid–neutral and diethyl ether base fractions should equal the sum of the Florisil fractions. The actual material recovered in the Florisil fractions only accounts for an average of 65% of the material applied to the columns. This discrepancy is due either to radiolabel that was not solubilized when the ether-extractable residue was taken up in methylene chloride (before Florisil chromatography), adsorptive losses on the Florisil adsorbent, or to mechanical losses.

Fractionation on Florisil adsorbent prior to HPLC analysis of fraction F2

produced blanks with very little interference from indigenous plant components. Advantages of Florisil fractionation prior to HPLC analysis include prolonged analytical column life and the ability to determine TNT and the aminodinitrotoluene isomers without analytical interferences. The method was shown, by analysis of TNT-spiked leaf tissue, to give both high recoveries (84%) and good reproducibility.

The distribution of radiolabel among the chemical fractions (Table II) emphasizes the previously unknown polar nature of TNT plant metabolites. After acid hydrolysis, an average of 12% of the radiolabel over all analyses was found in fraction F2. Large quantities of more polar metabolites, found primarily in fraction F4 (average of 15% of the radiolabel) and the non-ether-extractable aqueous base (average of 29% of the radiolabel) fraction, accounted for an average of 44% of the radiolabel. The amount of radiolabel sequestered in non-extractable forms in the pellets averaged 29%. There is a clear indication from the data that the percentage of radiolabel found in fraction F2 was higher in the root tissue than in the leaves. The leaf tissue contained higher proportions of water-soluble metabolites present in the aqueous base fraction. These data suggest that TNT and its primary metabolites (the aminodinitrotoluene isomers) were transported from the roots to the aerial portions of the plant, where they underwent further modification to more polar metabolites. It should be emphasized that once plant uptake occurs, radiolabel remains within the plant tissues. Studies specifically designed to examine the release of $^{14}CO_2$ and ^{14}C-containing volatile organics failed to detect emission of such compounds by bean plants grown in ^{14}C-TNT containing solutions.

Comparisons between 1 and 7 day plants (Table II) show a trend consistent with metabolic immobilization of radiolabel in both leaf and root tissues. Leaf and root tissues from 1-day plants contained a higher percentage of radiolabel in the diethyl ether acid–neutral fraction than plants exposed for 7 days. After fractionation of the 1-day plant extracts on Florisil, the larger percentage of extractable radiolabel was reflected in fraction F4. Conversely, plants exposed for 7 days showed a higher percentage of immobilized radiolabel in the leaf and root pellets. These results suggest that a fundamental process associated with TNT metabolism in plants is the detoxification of TNT metabolic products by sequestration in non-extractable forms.

The chromatograms of the F2 fraction of bush bean leaf tissue exposed hydroponically in TNT solutions for 7 days show both TNT and the aminodinitrotoluene isomers at concentrations slightly above the detection limit of the analytical scheme (Fig. 3 top). The chromatogram in the bottom of Fig. 3 is from similarly exposed bush bean root tissue. Large quantities of these compounds are evident. Bush bean stem tissue contains concentrations of TNT and the aminodinitrotoluene isomers that are intermediate to those found in the leaves and roots (Fig. 3 center and Table III). The concentrations of TNT and the aminodinitrotoluene isomers given in Table III are consistent with the distribution of radiolabel as summarized in Table II. Note that only 5–6% of the radiolabel contained in leaves was speciated as TNT or the aminodinitrotoluene isomers.

Radiochromatograms of the F2 fraction from bush bean root tissue indicated the presence of an unknown TNT metabolite eluting with a retention time of 21.95 min. The peak corresponding to this metabolite is marked with an asterisk in Fig. 3. Incorporation of radiolabel into discrete metabolites, such as the unknown component identified above and the aminodinitrotoluene isomers, argues towards a defined

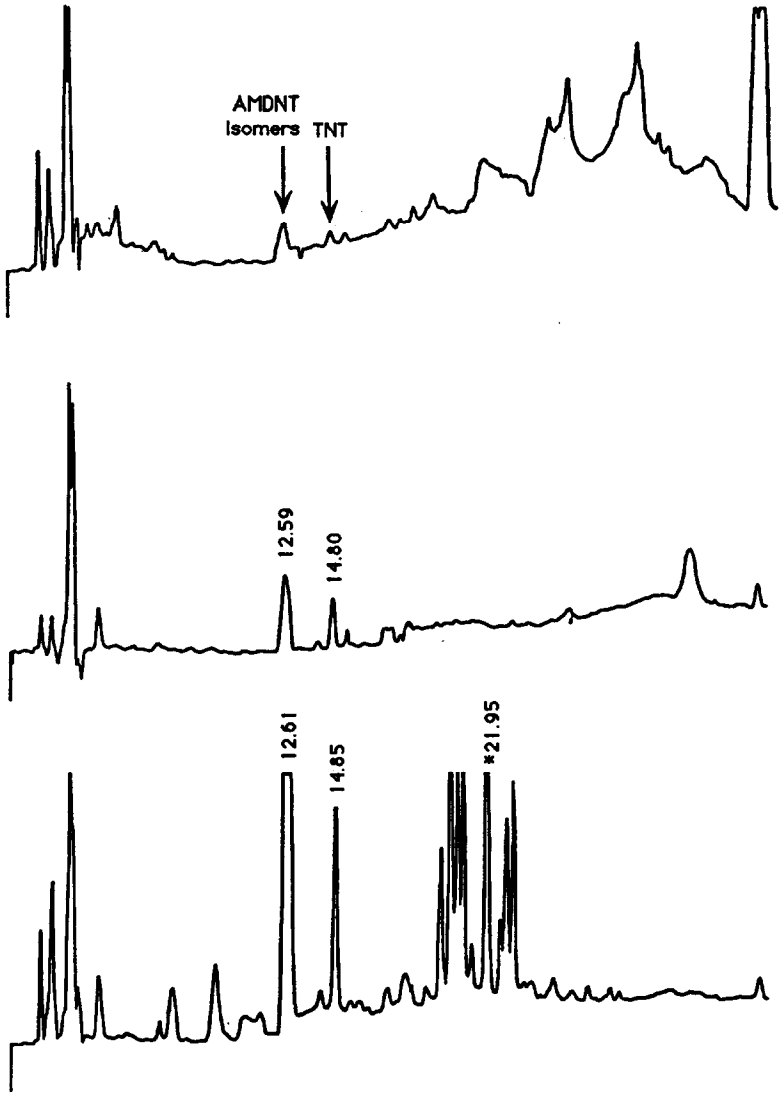

Fig. 3. Chromatograms of the F2 fraction of bush bean plants grown in hydroponic solutions containing 10 ppm TNT. The top chromatogram is from leaves, the center from stem, and the bottom from root tissue. The peak marked with an asterisk contains radiolabel and represents an unknown TNT metabolite. Numbers at peaks indicate retention times in min.

metabolism of TNT rather than nondescript incorporation of the radiolabel into a variety of metabolic paths. Structural elucidation of this metabolite, as well as the polar metabolites contained in the F4 and aqueous base fractions, should be the focus of future investigations.

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