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Anaerobic Biotransformation of 2,4-Dinitrotoluene and 2,6-Dinitrotoluene by *Clostridium acetobutylicum:* A Pathway through Dihydroxylamino Intermediates

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Experiments were conducted to isolate and identify the intermediates and products of 2,4-dinitrotoluene and 2,6-dinitrotoluene metabolism by *Clostridium acetobutylicum*. Transformation of both dinitrotoluenes initially resulted in the formation of hydroxylaminonitrotoluenes. Subsequent transformation favored the formation of dihydroxylaminotoluenes, with a limited reduction to aminonitrotoluene isomers. In cell cultures, metabolism beyond the level of dihyroxylaminotoluene was not observed. In cell extracts, where activity could be maintained for periods in excess of those in cell cultures, further transformation yielded aminohydroxylaminotoluenes and eventually diaminotoluenes. These findings further demonstrate the potential for hydroxylamines to be significant intermediates of nitroaromatic transformation under anaerobic fermentative conditions. Interestingly, the rearrangement of dihydroxylaminotoluenes was not observed, as was the case in previous studies of 2,4-dihydroxylamino-6-nitrotoluene metabolism (e.g., the dihydroxylamino metabolite of 2,4,6-trinitrotoluene transformation by *C. acetobutylicum*). Dihydroxylaminotoluenes were found to be quite unstable, decomposing rapidly upon exposure to oxygen, complicating the assessment of their fate in remediation processes.

Introduction

The development of biological processes for treatment of TNT-contaminated soil and groundwater has been an area of active research and testing for several decades (*1*). As principal impurities of TNT production, 2,4-dinitrotoluene and 2,6-dinitrotoluene are often found together with TNT in explosives-contaminated media. Each is classified as a priority pollutant, and their fate must also be considered in TNT bioremediation systems.

Recently, the role of *Clostridia* in anaerobic TNT-bioremediation systems has received considerable attention due to their ability to rapidly reduce aryl nitro groups (*2*−*6*). However, the ability of *Clostridia* to transform 2,4-dinitrotoluene and 2,6-dinitrotoluene has not been rigorously examined. The formation of aminonitrotoluenes under anaerobic conditions has been observed from both 2,4-dinitrotoluene and 2,6-dinitrotoluene (*7*−*14*). Interestingly, the primary products of TNT transformation by *Clostridia* are not aminated derivatives. Instead, transformation results in the formation of aryl hydroxylamines (*3*−*6*). Hydroxylamino intermediates of dinitrotoluene metabolism have been proposed during anaerobic metabolism of dinitrotoluenes, but have not been isolated (*14, 15*).

The transient formation of hydroxylamino compounds is of interest in bioremediation systems due to the complex fate imparted by this functional group (*16*). Hydroxylamines can be reduced to amines, or may undergo more complex fate processes, including: binding with the organic fraction of soils (*17, 18*); condensation with nitroso groups forming substituted azoxytoluenes (*19*); or rearrangement forming aminophenols (*16*). Furthermore, hydroxylamino intermediates are often more mutagenic than their parent nitro compounds, and their accumulation in remediation systems may represent a concern, depending on their eventual fate (*16*).

The purpose of studies presented herein was to isolate and identify transformation products of 2,4-dinitrotoluene and 2,6-dinitrotoluene metabolism by the saccharolytic fermentor *Clostridium acetobutylicum*. *C. acetobutylicum* has previously been studied for its ability to transform nitroaromatics, most notably TNT (*4*−*6*). The focus of the present study was to evaluate the potential for hydroxylamine formation during dinitrotoluene transformation, and to assess the potential for further metabolism either through hydroxylamine reduction or through a rearrangement process. Results demonstrate that dihydroxylaminotoluenes are the primary intermediates of transformation for both dinitrotoluene isomers. Further metabolism of dihydroxylaminotoluenes, when it occurred, resulted in the formation of arylamines through hydroxylamine reduction, not via rearrangement.

Materials and Methods

Chemicals. 2,4-Dinitrotoluene and 2,6-dinitrotoluene were obtained from Aldrich (purity 97%). [U-ring-^{14}C]-2,4-Dinitrotoluene and [U-ring-^{14}C]-2,6-dinitrotoluene were generously provided by Dr. J. C. Spain (Tyndall Air Force Base, FL) and further purified by HPLC to a purity of 96% and 97%, respectively. 2-Amino-6-nitrotoluene, 4-amino-2-nitrotoluene, 2-amino-4-nitrotoluene, 2,4-diaminotoluene, and 2,6-diaminotoluene with a purity of >97% were obtained from Aldrich and used without further purification. Acetic anhydride (Reagent ACS 97%, ACROS), HCl (37.5%, Fisher), and sodium acetate (Fused, anhydrous, Fisher) were used as received. The solvents used were HPLC grade: ethyl acetate, hexane, and methylene chloride (99.9%, Fisher). Chloroform-*d* (99.8 atom %) was purchased from Aldrich.

Preparation of Crude Cell Extract of *C. acetobutylicum*. Cells of *Clostridium acetobutylicum* (ATCC 824) were grown and maintained anaerobically as described previously (*6*). Crude cell extracts were prepared by the addition of lysozyme (Sigma) according to the procedure described previously (*5*), except that buffer was added to a concentration of 1 g of cells (wet basis) per 10 mL. This lysate was centrifuged at 15000*g* for 15 min to remove cellular debris, which gave an extract concentration of 10 mg of protein/mL as determined by the absorbance at 280 nm. The clear extract was frozen with liquid nitrogen and stored at −80 °C.

Analytical Methods. HPLC (Waters, Milford, MA) equipped with a diode-array UV−visible detector (Model 996), a fluid

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pump (Model 600E), and an autosampler (Model 717 plus) was employed to quantify and fractionate dinitrotoluenes and their transformation products. Spectra were acquired continuously between 200 and 600 nm and chromatograms extracted at 230 nm for quantitation. Analytes were separated on a reverse-phase Waters Nova-Pak-C_8 column (3.9 × 150 mm) at room temperature with an isocratic mobile phase of 82/18 (v/v) or 90/10 (v/v) water/2-propanol at 1 mL/min. The ^{14}C measurements were performed on a Beckman LS6500 scintillation counter (Beckman Instruments Inc., Fullerton, CA).

The mass spectra were obtained using either (a) a HP 1100 Series LC/MSD (Hewlett-Packard Co., Palo Alto, CA) interfaced to an atmospheric pressure ionization with either positive or negative scan modes, (b) a Finnigan MAT 95 spectrometer (Finnigan Co., San Jose, CA) with either direct probe chemical ionization (CI) or desorption chemical ionization (DCI) using methane as the ionizing gas, or (c) a Waters Integrity System HPLC equipped with a LC/Therma-Beam Mass Detector (TMD). ^1H NMR spectra were obtained on a Bruker AC-250 spectrometer relative to $CDCl_3$ (7.26 ppm) or CD_3OD (3.31 and 4.78 ppm). Chemical shifts are reported in ppm and coupling constants in hertz (Hz).

Cell Cultures. Cell cultures (50 mL) of *Clostridium acetobutylicum* were grown as described previously (*6*). During the log-growth phase, the cultures were spiked with either 2,4-dinitrotoluene (5 mg dissolved in 0.5 mL of methanol containing 7.5×10^5 dpm of [U-ring-^{14}C]-2,4-dinitrotoluene) or 2,6-dinitrotoluene (5 mg dissolved in 0.5 mL of methanol containing 1.2×10^6 dpm of [U-ring-^{14}C]-2,6-dinitrotoluene). Samples (1 mL) were removed aseptically and centrifuged (13000*g* for 6 min), and the supernatants were analyzed by HPLC for 2,4-dinitrotoluene, 2,6-dinitrotoluene, and metabolic products. Fractions were collected based on UV absorbance, and concentrations were quantified by liquid scintillation analysis.

Isolation of Transformation Intermediates. Experiments were conducted in cell extract systems as previously described (*5*) to avoid the analytical complexities associated with the isolation of metabolites from a rich fermentation broth. A series of cell extract systems containing individual dinitrotoluenes were maintained for varying durations, allowing for the isolation and identification of primary intermediate compounds formed during the transformation of each dinitrotoluene. No reactions were noted for controls without hydrogen or without cell extract.

The initial intermediates from the anaerobic transformation of 2,4-dinitrotoluene and 2,6-dinitrotoluene were obtained in cell extract systems [10 mL of extract and 300 mL of Tris (10 mM, pH 7.8)] spiked with 100 mg of individual dinitrotoluenes and 4.1×10^5 dpm of ^{14}C (both predissolved in 10 mL of methanol). The reaction mixture was bubbled with H_2 and transformation monitored by HPLC (C_8 reverse-phase isocratic mode with a mobile phase mixture of 82/18 H_2O/2-propanol). Nearly all of the 2,4-dinitrotoluene was transformed in 2 h, forming two products (molar ratio of 4/1) at a combined yield of 74%. A longer reaction time was required (3.5 h) for a similar extent of 2,6-dinitrotoluene transformation, which resulted in a single product with a yield of 75%. In each case, transformation was stopped by exposing the reaction mixture to air. Solutions were then extracted with ethyl acetate 3 times (total 600 mL) and the combined organic fractions dried with anhydrous $MgSO_4$. After filtering, the ethyl acetate was evaporated, leaving yellow residues. Intermediates were purified by silica column chromatography (ethyl acetate/hexane = 1/2) and subjected to ^1H NMR and MS analysis.

Subsequent experiments were carried out to isolate other metabolites of dinitrotoluene transformation using longer incubations and a higher cell extract concentration. Cell extract systems [10 mL of extract and 100 mL of Tris (10 mM, pH 7.8)] were spiked with 50 mg of individual dinitrotoluenes and 4.1×10^5 dpm of ^{14}C (both predissolved in 5 mL of methanol). Transformation was monitored by HPLC (C_8 reverse-phase isocratic mode with a mobile phase mixture of 82/18 H_2O/2-propanol). After 6 h, a polar and oxygen-sensitive compound was obtained in high yield (65%) from the 2,4-dinitrotoluene system. After 10 h, a polar and oxygen sensitive intermediate was obtained from the 2,6-dinitrotoluene system at a yield of 70% as measured by ^{14}C recovery.

Further direct purification was not possible due to the instability of these metabolites. Samples were subjected to LC-MS analysis for direct characterization and were also derivatized with acetic anhydride, under anaerobic conditions, using a procedure similar to that reported previously (*18*). HCl (5 mL of 37.5%) was added slowly to the aqueous solutions containing the oxygen-sensitive compounds, followed by dropwise addition of acetic anhydride (5 mL). The products (a light brown residue) were isolated by column chromatography with silica gel packings (70−230 mesh) and 4/1 (v/v) ethyl acetate/hexane as the mobile phase, and further purified by HPLC with a C_8 reverse-phase column. Derivatization products were subjected to ^1H NMR and CI mass spectroscopy.

Further metabolism was evaluated under conditions identical to those used in experiments that yielded oxygen-sensitive products. These experiments were conducted for 20 h with 2,4-dinitrotoluene and for 24 h with 2,6-dinitrotoluene. In each case, a single oxygen-stable product was obtained at high yields (89% for 2,4-dinitrotoluene and 94% for 2,6-dinitrotoluene as measured by ^{14}C recovery). These compounds were extracted with ethyl acetate 3 times (total 600 mL) and the combined organic fractions then dried with anhydrous $MgSO_4$. After filtering, the ethyl acetate was evaporated. The resulting residues were then subjected to ^1H NMR analysis for structural confirmation.

Temporal Pathway Analysis in Cell Extracts. Cell extract (10 mL for 2,4-dinitrotoluene systems and 14 mL for 2,6-dinitrotoluene systems) was added to 50 mL of Tris buffer (10 mM, pH 7.8) and subsequently spiked with individual dinitrotoluenes (5 mg with 1.4×10^6 dpm of ^{14}C dissolved in 0.5 mL of methanol). Bottles were supplied with a continuous addition of H_2. HPLC analysis was conducted with complete fractionation of the elution profile and subsequent quantification by liquid scintillation analysis. Also, samples were analyzed by HPLC/MS.

Results

Transformation of Dinitrotoluenes in Cell Cultures. The results from temporal sampling and HPLC analysis of cell culture spiked with 2,4-dinitrotoluene and 2,6-dinitrotoluene are shown in Figure 1. Both dinitrotoluenes were rapidly transformed in cell cultures with a limited production of aminated nitrotoluenes. In the 2,4-dinitrotoluene-fed system, 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene were observed, with the highest concentrations (% of initial ^{14}C) observed at the first sampling (t = 0.9 h)—5.6% and 5.4%, respectively. Similarly, the highest concentration of 2-amino-6-nitrotoluene (13%) was observed in the first sampling (t = 0.9 h). At the end of both incubations, only 2-amino-6-nitrotoluene was present in detectable concentrations (2.6%).

Other ^{14}C-labeled intermediates were observed in both systems. In systems fed 2,4-dinitrotoluene, two intermediates (labeled A_{24DNT} and B_{24DNT}) were initially observed at high concentrations with a combined yield of 82% at 0.9 h. Subsequently, a single unidentified product (labeled C_{24DNT}) was formed with a yield of 92%. Additionally, a nonidentifiable, non-UV-absorbing metabolite (labeled D_{24DNT}) was observed in cell systems at low levels. In the 2,6-dinitrotoluene system, one primary intermediate (labeled A_{26DNT}) appeared

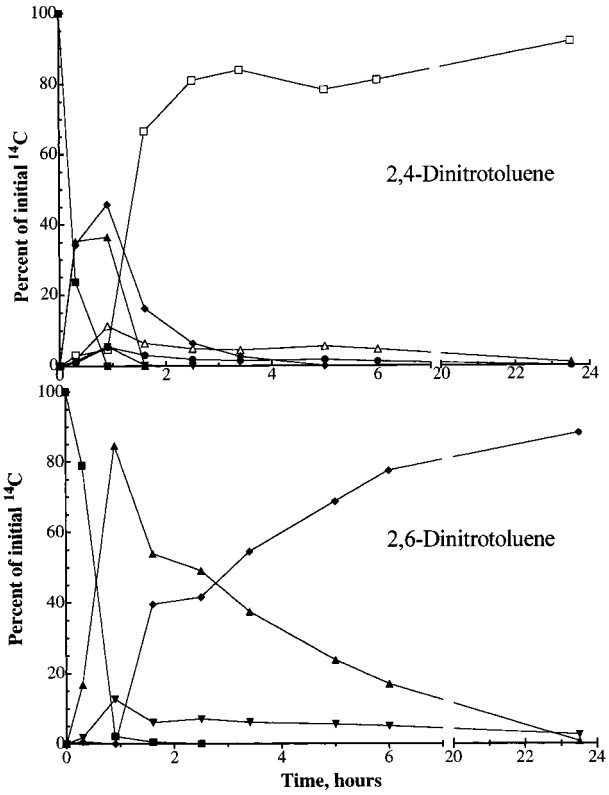

FIGURE 1. Temporal concentrations of metabolites during the transformation of 2,4-dinitrotoluene (top) and 2,6-dinitrotoluene (bottom) in cell cultures of *C. acetobutylicum*. Symbols in the top figure denote percent of ^{14}C present as 2,4-dinitrotoluene (■), 2-amino-4-nitrotoluene (▼), 4-amino-2-nitrotoluene (●), metabolite A_{24DNT} (▲), metabolite B_{24DNT} (◆), metabolite C_{24DNT} (□), and metabolite D_{24DNT} (△). Symbols in the bottom figure denote percent of ^{14}C present as 2,6-dinitrotoluene (■), 2-amino-6-nitrotoluene (▼), metabolite A_{26DNT} (▲), and metabolite B_{26DNT} (◆).

TABLE 1. Mass Spectra of Metabolites from 2,4-Dinitrotoluene, 2,6-Dinitrotoluene, and Their Derivatives

metabolites or derivatives	observed molecular ion	method[a]
4-hydroxylamino-2-nitrotoluene	169 (M+1)	1
2-hydroxylamino-4-nitrotoluene	169 (M+1)	1
2,4-dihydroxylaminotoluene	154 (M)	2
2-hydroxylamino-4-aminotoluene	138 (M)	2
2,4-di(*N*-acetoxyacetamido)toluene	323 (M+1)	1
2-hydroxylamino-6-nitrotoluene	169 (M+1)	1
2,6-dihydroxylaminotoluene	155 (M+1)	3
2-hydroxylamino-6-aminotoluene	139 (M+1)	3
2,6-di(*N*-acetoxyacetamido)toluene	323 (M+1)	1

[a] Methods of ion formation and sample introduction: (1) desorption chemical ionization (DCI), (2) LC-MS (EI), (3) LC-MS (CI).

rapidly [yield of 85% (t = 0.9 h)], which was further transformed to a single major product (labeled B_{26DNT}) with a yield of 88%.

Determination of Intermediates and Products of Dinitrotoluene Transformation. The results of mass spectroscopy and ^1H NMR for compounds isolated from cell extract systems (with or without derivatization) are presented in Tables 1 and 2, respectively. Table 2 also contains ^1H chemical shifts for parent compounds and corresponding aminated compounds available as standard materials.

HPLC analysis of initial transformation products obtained from cell extract systems demonstrated identical retention times and UV spectra to those observed in whole cell systems. Isolation and spectroscopic analysis of initial reduction intermediates of 2,4-dinitrotoluene (i.e., A_{24DNT} and B_{24DNT} in whole cell studies) revealed that each had molecular weights corresponding to a hydroxylaminonitrotoluene. Confirmation was obtained through ^1H NMR analysis where the aromatic protons were intact and shifted upfield as compared to the parent compound. The assignment of structures between the two possible hydroxylaminonitrotoluene isomers was based upon further examination of ^1H NMR spectra, HPLC elution characteristics, and UV spectra.

From the ^1H NMR results, a clear difference in the chemical shift of the benzylic protons of the methyl group results from the reduction at either the 2-position or the 4-position. A greater upfield shift is expected from the reduction at the 2-position (as occurs with 2-amino-4-nitrotoluene standards), which was observed for the two isolated compounds (∂ = 2.20 ppm versus ∂ =2.44 ppm). This assignment was further supported by the HPLC characteristics and UV spectra. A characteristic of this HPLC method demonstrated by the aminonitrotoluene standards is the propensity of the 2-amino-4-nitrotoluene to elute more rapidly than the 4-amino-2-nitrotoluene isomer. Also, 2-amino-4-nitrotoluene shows a strong absorbance in the 300 nm region not apparent with 4-amino-2-nitrotoluene. The hydroxylamino isomer identified by ^1H NMR (∂ = 2.20) as the 2-hydroxylamino-4-nitrotoluene (assigned as A_{24DNT} in cell culture studies) eluted more rapidly than the 4-hydroxylamino-2-nitrotoluene (assigned as B_{24DNT} in cell culture studies), and both displayed similar UV spectra as compared to their corresponding aminonitrotoluenes.

In the case of the initial 2,6-dinitrotoluene metabolite (assigned as A_{26DNT} in whole cell studies), the molecular weight obtained by DCI-MS analysis, and the upfield shift of the aromatic protons obtained by ^1H NMR, supports the identification of the first intermediate as a hydroxylaminonitrotoluene. Due to the symmetry of the molecule, the only possible structure of this intermediate is 2-hydroxylamino-6-nitrotoluene. HPLC analysis also demonstrated a more rapid elution of the hydroxylamino intermediate relative to the 2-amino-6-nitrotoluene standard.

The polar, highly oxygen-sensitive compounds obtained from the reduction of hydroxylaminonitrotoluenes in cell extracts had identical HPLC retention times and UV spectra as compared to the primary end-products observed in whole cell experiments (i.e., C_{24DNT} and B_{26DNT}). Through LC-MS, these were tentatively identified as 2,4-dihydroxylaminotoluene and 2,6-dihydroxylaminotoluene—from 2,4-dinitrotoluene and 2,6-dinitrotoluene, respectively—based upon molecular ions obtained. These results could not be used as confirmation of structure since standards of neither 2,4-dihydroxylaminotoluene nor 2,6-dihydroxylaminotoluene were available and similar molecular ions would be expected if a dihydroxylaminotoluene had undergone a Bamberger rearrangement (resulting in the formation of possible aminohydroxlaminomethylphenol isomers). Therefore, these intermediates were stabilized through acetylation, and further characterization was conducted.

The mass spectroscopy data for derivatized products listed in Table 1 confirm that the acetylation of intermediates was complete, regardless if the starting molecule was a dihydroxylaminotoluene or a corresponding rearrangement product. However, results from ^1H NMR analysis of derivatized products do allow for this differentiation. Three aromatic protons are intact for both derivatization products, dismissing any possibility of a rearrangement and confirming that the analytes prior to derivatization were in fact 2,4-dihydroxylaminotoluene and 2,6-dihydroxylaminotoluene.

Extended reaction periods of 2,4-dinitrotoluene (20 h) and 2,6-dinitrotoluene (24 h) in cell extracts resulted in the formation of products not observed in whole cell studies.

TABLE 2. Comparison of ^{1}H NMR Chemical Shifts of Isolated Compounds and Standards[a]

standard/product*	methyl group	aromatic protons			R-NH_2
2-amino-4-nitrotoluene	2.23 (s)	7.56 (dd, J = 2.5, 7.5 Hz)	7.50 (d, J = 2.5 Hz)	7.17 (d, J = 7.5 Hz)	3.90 (s, b)
2-hydroxylamino-4-nitrotoluene*	2.20 (s)	7.71 (dd, J = 2.5, 7.5 Hz)	7.62 (d, J = 2.5 Hz)	7.52 (d, J = 7.5 Hz)	**
4-amino-2-nitrotoluene	2.46 (s)	7.29 (dd, J = 2.5, 7.5 Hz)	7.10 (d, J = 7.5 Hz)	6.83 (dd, J = 2.5, 7.5 Hz)	3.82 (s, b)
4-hydroxylamino-2-nitrotoluene*	2.44 (s)	7.24 (d, J = 7.5 Hz)	7.21 (d, J = 2.5 Hz)	7.09 (dd, J = 2.5, 7.5 Hz)	**
2-hydroxylamino-6-nitrotoluene*	2.25 (s)	7.56 (d, J = 7.5 Hz)	7.45 (d, J = 7.5 Hz)	7.34 (d, J = 7.5 Hz)	**
2-amino-6-nitrotoluene	2.24 (s)	7.18 (dd, J = 1.0, 7.5 Hz)	7.10 (d, J = 7.5 Hz)	6.87 (dd, J = 1.0, 7.5 Hz)	3.90 (s, b)
2,4-diaminotoluene*	2.05 (s)	6.82 (d, J = 7.5 Hz)	6.10 (dd, J = 2.5, 7.5 Hz)	6.06 (d, J = 2.5 Hz)	3.39 (s, b)
2,6-diaminotoluene*	1.96 (s)	6.83 (d, J = 7.5 Hz)	6.21 (2H, dd, J = 2.5, 7.5 Hz)		3.55 (s, b)

derivatized compounds	methyl group	aromatic protons		methyl group for *N*-ester or *N*-amide		
2,4-di(*N*-acetoxyacetamido)toluene*	2.13 (s)	7.54 (2H, s)	7.36 (s)	2.38 (s)	2.18 (2H, s)	2.04 (s)
2,6-di(*N*-acetoxyacetamido)toluene*	2.18 (s)	7.57 (2H, s)	7.33 (s)	2.18 (2H, s)	2.37 (s)	2.08 (s)

[a] NMR spectra were taken in $CDCl_3$ and CD_3OD; s, singlet; d, doublet; b, broad; **, chemical shift varies.

The compounds obtained have identical HPLC retention times and UV spectra to authentic standards of 2,4-diaminotoluene and 2,6-diaminotoluene, respectively. ^{1}H NMR analysis confirmed this, as identical shifts from isolated products and standard materials were obtained. The transformation potential of 2,4-diaminotoluene and 2,6-diaminotoluene was further tested in cell extract systems (data not shown). No transformation was observed over a 24 h period.

Transformation of Dinitrotoluenes in Cell Extracts. The transformation of 2,4-dinitrotoluene and 2,6-dinitrotoluene in cell extract systems allowed for the quantitation of intermediates through HPLC fractionation ^{14}C analysis. Tentative identification of minor intermediates through additional HPLC/MS analysis was also conducted (these tentative assignments are represented in italics throughout this section). Results of ^{14}C analysis from HPLC fractions, with assigned structures from MS analysis, in systems fed 2,4-dinitrotoluene and 2,6-dinitrotoluene are shown in Figure 2.

In the case of 2,4-dinitrotoluene, a transformation pathway from 2,4-dinitrotoluene to the final product 2,4-diaminotoluene, through various hydroxylamino intermediates, was observed. 2,4-Dinitrotoluene disappeared rapidly with a concomitant accumulation of hydroxylaminonitrotoluene intermediates (i.e., 4-hydroxylamino-2-nitrotoluene and 2-hydroxylamino-4-nitrotoluene). Further reduction of these hydroxylamino intermediates resulted in the formation of 2,4-dihydroxylaminotoluene. Subsequent transformation of 2,4-dihydroxylaminotoluene to other metabolites was relatively slow. From 3 h and 10 h, an increase in *2-hydroxylamino-4-aminotoluene* and *4-hydroxylamino-2-aminotoluene* was observed with the decrease of 2,4-dihydroxylaminotoluene. Fourteen hours later, the 2,4-diaminotoluene level reached its maximum. No further degradation products were found after the formation of 2,4-diaminotoluene. Besides the major pathway through 2,4-dihydroxylaminotoluene, 4-amino-2-nitrotoluene was observed as a minor product accumulating to a maximum concentration of 25% at 0.5 h, but had completely disappeared at hour 7. Additionally a fraction without UV absorbance had a maximum concentration of 17% at hour 5 and then decreased to background levels. The combined recovery of ^{14}C in the form of 2,4-dihydroxylaminotoluene, 2,4-diaminotoluene, and the tentatively identified *hydroxylamino-aminotoluene* intermediates was 99% at the final sampling.

Similar to 2,4-dinitrotoluene, the transformation of 2,6-dinitrotoluene was noted to proceed with the transient formation of 2-hydroxylamino-6-nitrotoluene and the subsequent formation of 2,6-dihydroxylaminotoluene. Through LC-MS, *2-hydroxylamino-6-aminotoluene* was tentatively identified as a precursor to the end-product of 2,6-diaminotoluene. Also, 2-amino-6-nitrotoluene was observed as a minor metabolic product with a maximum concentration

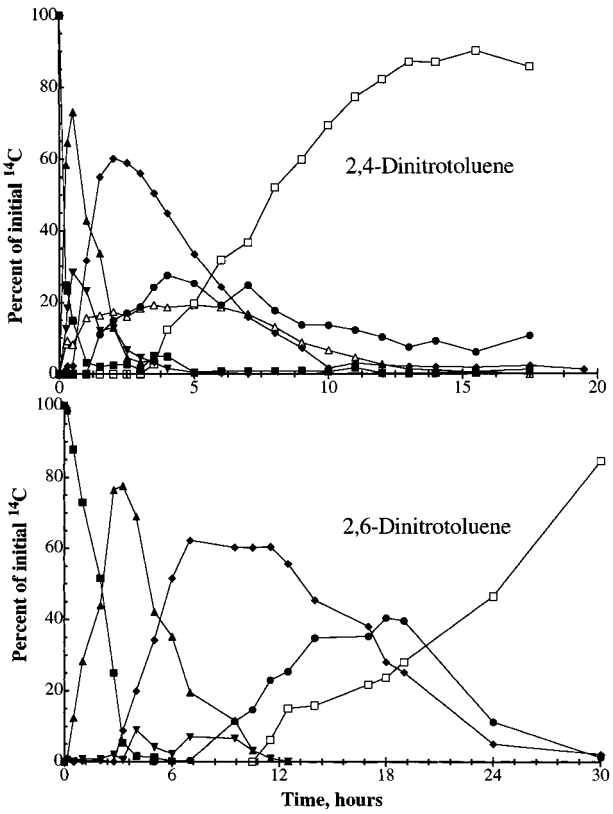

FIGURE 2. Temporal concentrations of identifiable compounds during the transformation of 2,4-dinitrotoluene (top) and 2,6-dinitrotoluene (bottom) in cell extracts of *C. acetobutylicum*. Symbols in the top figure denote percent of ^{14}C present as 2,4-dinitrotoluene (■), hydroxylaminonitrotoluenes (▲), 4-amino-2-nitrotoluene (▼), 2,4-dihydroxylaminotoluene (◆), aminohydroxylaminotoluene (●), 2,4-diaminotoluene (□), and an unknown (△). Symbols in the bottom figure denote percent of ^{14}C present as 2,6-dinitrotoluene (■), 2-hydroxylamino-6-nitrotoluene (▲), 2-amino-6-nitrotoluene (▼), 2,6-dihydroxylaminotoluene (◆), 2-amino-6-hydroxylaminotoluene (●), and 2,6-diaminotoluene (□).

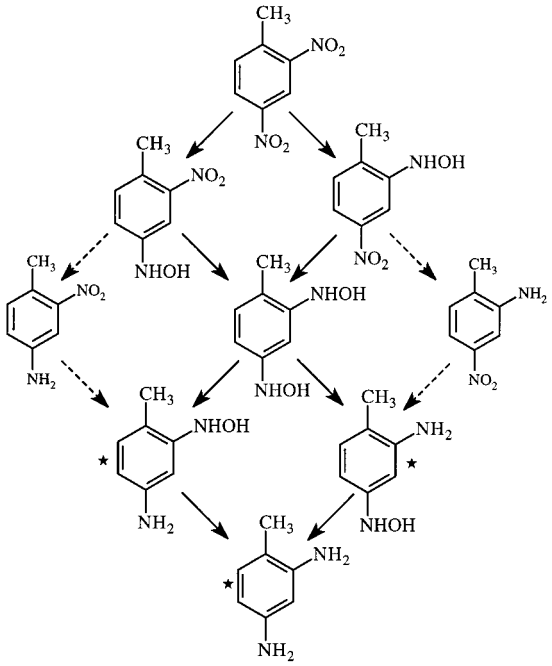

FIGURE 3. Observed transformation pathway for the transformation of 2,4-dinitrotoluene in cell culture and cell extracts. Dashed arrows represent minor pathways. The symbol ★ is placed by compounds observed only in extract systems.

of 10% at 5 h and then completely disappeared by hour 11. As compared to 2,4-dinitrotoluene, no ^{14}C-labeled fraction without UV absorbance was detected in 2,6-dinitrotoluene systems. The combined yield of identified and tentatively identified products at the last sampling was 88%.

Discussion

The transformation of both dinitrotoluenes in cell cultures and cell extracts resulted in rapid reduction of aryl nitro groups to hydroxylamino derivatives. Nitroso intermediates were not observed in any systems. After the formation of hydroxylaminonitrotoluenes, further reduction of all isomers favored the formation of dihydroxylaminotoluenes, although aminonitrotoluene formation was observed. In cell cultures, the primary products of 2,4-dinitrotoluene and 2,6-dinitrotoluene transformation were 2,4-dihydroxylaminotoluene and 2,6-dihydroxylaminotoluene, respectively. In cell extracts, further and complete reduction to diaminotoluenes was observed. The combined results of transformation from cell cultures and cell extracts are depicted in Figure 3 for 2,4-dinitrotoluene and in Figure 4 for 2,6-dinitrotoluene.

Perhaps the most interesting difference between the cell cultures and the cell extract systems was the varying extent of transformation observed. In cell cultures, reduction beyond the level of dihydroxylaminotoluenes was not detected. In cell extract, dihydroxylaminotoluenes did accumulate, but were subsequently reduced to diaminotoluenes. In previous studies (*6*), we observed the cessation of TNT reduction as cell cultures shifted into solventogenic growth, which generally occurred within 6 h under the conditions reported in these studies. This may explain the incomplete reduction observed in cell cultures. In cell extracts, nitro-reduction activity can be sustained for longer periods through the continuous addition of hydrogen to the system, apparently allowing for the eventual formation of arylamines from both dinitrotoluenes. The hypothesis regarding the cessation of further reduction of dihydroxylaminotoluenes in cell systems as a result of the shift into solventogenic growth phase could not be tested due to the oxygen sensitivity of both dihydroxylaminotoluenes. Attempts to isolate either dihydroxylaminotoluene (without derivatization) and add them to acetogenic cell cultures were unsuccessful, as decomposition occurred rapidly during isolation.

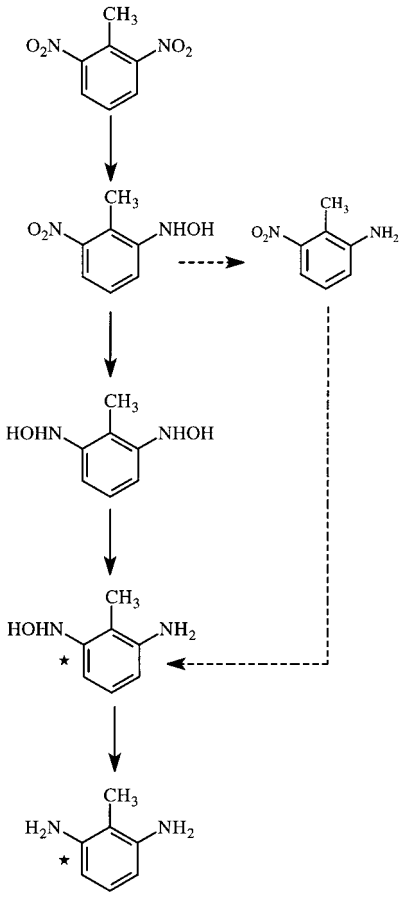

FIGURE 4. Observed transformation pathway for the transformation of 2,6-dinitrotoluene in cell culture and cell extracts. Dashed arrows represent minor pathways. The symbol ★ is placed by compounds observed only in extract systems.

Certain similarities and significant differences can be identified when comparing the results of dinitrotoluene transformation by *C. acetobutylicum* to identical studies investigating TNT metabolism. Similarities center on the rapid formation of hydroxylamino intermediates and the central role of a dihydroxylaminotoluene (dihydroxylaminonitrotoluene in the case of TNT) in metabolism by this microorganism. As was observed with TNT, initial reduction at the 4-position occurred more rapidly than at the 2-position. In studies with 2,4-dinitrotoluene, 4-hydroxylamino-2-nitrotoluene was formed in higher concentration than 2-hydroxylamino-4-nitrotoluene. The higher propensity for reduction at the 4-position than the 2-position is a probable explanation for the slower transformation rates of 2,6-dinitrotoluene as compared to 2,4-dinitrotoluene.

Unlike studies with TNT, no rearrangement of hydroxylamino intermediates to aminophenols was observed. It is difficult at this time to hypothesize why the Bamberger rearrangement did not occur. The obvious difference between the dihydroxylaminotoluene products of dinitrotoluene transformation and the 2,4-dihydroxylamino-6-nitrotoluene intermediate from TNT transformation is the presence of a strongly electron-withdrawing nitro group in the latter. Certainly, 2,4-dihydroxylamino-6-nitrotoluene is a much more "stable" compound than either 2,4-dihydroxylaminotoluene or 2,6-dihydroxylaminotoluene. For example, isolation, purification, and storage of 2,4-dihydroxylamino-6-nitrotoluene can be conducted in the presence of oxygen

with a negligible loss in yield. How the presence or absence of an electron-withdrawing groups may influence the ability of this organism to catalyze the rearrangement of an arylhydroxylamine group will require further study.

Previous studies using diverse anaerobic inocula have generally cited aminonitrotoluenes as primary end-products from both 2,4-dinitrotoluene and 2,6-dinitrotoluene transformation (*7*−*14*). Complete reduction of 2,4-dinitrotoluene to 2,4-diaminotoluene has been observed in a mixed methanogenic culture with ethanol as the primary substrate (*12, 13*). Under anoxic nitrate-reducing conditions, a limited extent of 2,4-diaminotoluene production has been observed (*9, 10*) along with aminonitrotoluene isomers (primary products), acetamidotoluenes, 6-nitrodazole, 2-nitrotoluene, and 4-nitrotoluene. Other products that have been cited include azoxy dimers resulting from the condensation of 2,4-dinitrotoluene reduction products (*15*) and toluene (*11*).

To the best of our knowledge, hydroxylamino intermediates from the anaerobic transformation of dinitrotoluenes have not previously been identified, although their presence was proposed by Guest et al. (*14*) and McCormick et al. (*15*). Certainly, the formation of arylamines from arylnitro groups requires partial reduction via nitroso and hydroxylamino intermediates (*20*). The identification of these compounds in this study was facilitated by the HPLC method employed, and care was taken to avoid decomposition. Critical to the HPLC approach is the ability to resolve hydroxylamines from corresponding amines since they have very similar spectral properties. Decomposition was avoided by minimizing storage times (most samples were run in "real time") and maintaining anaerobic conditions during sample handling. Had other methods been used, hydroxylamines would have decomposed, leaving only low levels of stable amino forms as the remaining identifiable products.

Hydroxylamino intermediates have been observed from a wide range of mononitroaromatic compounds, including 4-nitrobenzoate (*21, 22*), 4-nitrotoluene (*23*), and nitrobenzene (*24*). The formation of various possible hydroxylamino intermediates during TNT transformation was proposed by Rieger and Knackmuss (*1*), and several have since been isolated (*2, 4*−*6, 25*−*27*). It is also difficult to assess from these studies whether the formation of dihydroxylaminotoluenes in fermentative treatment processes is beneficial or detrimental to meeting remediation goals. Interestingly, the fate of dihydroxylaminotoluene may be similar to that of the previously identified Bamberger rearrangement product of TNT metabolism by *C. acetobutylicum*. All of these compounds are quite unstable in the presence of oxygen and will decompose in anaerobic aqueous environments in a period of days. At this time, the products of decomposition (aerobic or anaerobic) are not known, and their eventual fate in a soil slurry has not been evaluated. Future studies will be directed toward understanding the possible mutagenicity of these compounds and their long-term fate under conditions representative of bioremediation processes.

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