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Bamberger Rearrangement during TNT Metabolism by *Clostridium acetobutylicum*

J. B. HUGHES,*,† C. WANG,† K. YESLAND,‡ A. RICHARDSON,† R. BHADRA,† G. BENNETT,‡ AND F. RUDOLPH‡

Department of Environmental Science and Engineering and Department of Biochemistry and Cell Biology, Rice University, 6100 Main, Houston, Texas 77005-1892

Studies were conducted to isolate and identify polar and oxygen-sensitive intermediates of 2,4,6-trinitrotoluene (TNT) transformation by *Clostridium acetobutylicum*. Studies conducted in anaerobic cell extracts demonstrated that a polar product formed from the transformation of 2,4-dihydroxylamino-6-nitrotoluene by a mechanism known as the Bamberger rearrangement. The product was stabilized by derivatization with acetic anhydride, and the structure was confirmed by mass spectroscopy, ^{1}H NMR, and IR spectroscopy techniques. The reaction occurred in the presence of cell extract and H_2 but did not occur in cell extract-free controls. From spectroscopic data, the product of 2,4-dihydroxylamino-6-nitrotoluene rearrangement was identified as either 2-amino-4-hydroxylamino-5-hydroxyl-6-nitrotoluene (4-amino-6-hydroxylamino-3-methyl-2-nitrophenol) or 2-hydroxylamino-4-amino-5-hydroxyl-6-nitrotoluene (6-amino-4-hydroxylamino-3-methyl-2-nitrophenol). Acid-catalyzed rearrangement of 2,4-dihydroxylamino-6-nitrotoluene resulted in a single product, which after derivatization, was identical to a derivatized product from cell extracts. Acid-catalyzed Bamberger rearrangement occurs with the hydroxyl addition para to the participating hydroxylamine, indicating that the 2-amino-4-hydroxylamino-5-hydroxyl-6-nitrotoluene (4-amino-6-hydroxylamino-3-methyl-2-nitrophenol) was the product isolated form cell extracts. This product was also confirmed in whole cell systems that had been fed TNT. Following derivatization of the culture broth, a product was isolated that was identical to those isolated from crude cell extracts and acid catalysis experiments.

Introduction

The development of biological processes for treatment of 2,4,6-trinitrotoluene (TNT) contaminated soils or groundwaters has been an area of research and testing for several decades (*1*). These studies have demonstrated that TNT can be biotransformed under a range of conditions and that the final products of metabolism are often difficult to determine (*1, 2*). Generally, the initial stages of TNT metabolism involve the reduction of one or more aryl nitro groups via nitroso and hydroxylamino intermediates forming aryl amines (*3*). Commonly cited intermediates of TNT metabolism are aminodinitrotoluenes and diaminonitrotoluenes (*3*−*5*). Under strong reducing conditions, 2,4,6-triaminotoluene (TAT) can be formed and undergo further metabolism (*6*−*8*). Reaction mechanisms other than reduction to the amine that may influence the fate of TNT in anaerobic bioremediation systems, include condensation forming azoxy dimers (*1, 6*), substitution of aminated intermediates (*9*), and ring reduction (*10*). Despite the numerous known products of TNT transformation, determining the fate of TNT in bioremediation systems remains challenging (*3, 8, 11*). TNT fate is generally evaluated by the final distribution of ^{14}C-labeled products (*3, 12*). Mineralization of TNT has been observed, but is often only a small percent (*8, 13*). Many products "bind" to soil in a poorly reversible manner (*1, 14*), and undefined "polar metabolites" may reside in the aqueous phase (*12, 15, 16*).

Recent studies have demonstrated the temporal accumulation of hydroxylamino intermediates during the reduction of TNT (*12, 16, 17*). Because hydroxylamines are mutagenic and exhibit chemical properties quite different than the amine group (*18*), their formation and further metabolism is of interest in TNT bioremediation systems. The bioorganic chemistry of arylhydroxylamines has been reviewed by Corbett and Corbett (*18*). Possible reactions include reduction to the amine, nucleophilic, and/or electrophilic substitutions or rearrangement (e.g., Bamberger rearrangement) to phenolic amines (*4, 18, 19*). The formation of phenolic amines through the Bamberger rearrangement is particularly interesting since many phenols can undergo anaerobic ring cleavage (*2*). The formation of phenolic metabolites from TNT has been proposed (*20*) through the conversion of TAT to 2,4,6-trihydroxytoluene (methylphloroglucinol) and further metabolism to 4-hydroxytoluene (*p*-cresol). However, this processes does not involve hydroxylamino intermediates, and unlike the Bamberger rearrangement this reaction eliminates N-functionalities from the ring.

In previously reported studies of TNT transformation by *Clostridium acetobutylicum*, we have observed the rapid reduction of TNT to 2,4-dihydroxylamino-6-nitrotoluene and the subsequent the formation of a polar product (*15, 16, 21*) without detection of intermediate aminated forms. Attempts to isolate this product(s) were unsuccessful due to the complexities of purification from an organic-rich nutrient broth and the compound's oxygen sensitivity. This paper presents results of studies using *C. acetobutylicum* cell extracts coupled with chemical derivatization of intermediates to identify the mechanism of 2,4-dihydroxylamino-6-nitrotoluene metabolism by this organism. Studies are also presented confirming product formation in cell cultures.

Materials and Methods

Chemicals. Nitroaromatics used were 2,4,6-TNT (99%; purity; Chem Service, West Chester, PA), [U-ring-^{14}C]TNT (Chemsyn Science, Lenexa, KS) purified (98.6%) as described previously (*16*), and 4-hydroxylamino-2,6-dinitrotoluene and 2,4-dihydroxylamino-6-nitrotoluene [synthesized and purified as described previously (*16*)]. Other chemicals included acetic anhydride (Reagent ACS 97%, TITR, ACROS), hydrochloric acid (37.5%, Fisher scientific), and sodium acetate (anhydrous, Fisher Scientific). HPLC grade solvents included ethyl acetate, hexane, and methylene chloride (99.9%, Fisher scientific). NMR was conducted in chloroform-*d* (99.8 atom %, Aldrich).

* To whom correspondence should be addressed. Telephone: (713)285-5903; fax: (713)285-5203; e-mail address: hughes@owlnet.rice.edu.

† Department of Environmental Science and Engineering.

‡ Department of Biochemistry and Cell Biology.

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Preparation of *Clostridium acetobutylicum* Crude Extract. Cells were grown as described previously (*15*) to mid log phase (o.d. 595 = 1.2) and collected by centrifugation (12000*g*) for 10 min. Cells were washed in 5 mM Tris (pH = 7.8) and again collected by centrifugation (12000*g* for 10 min). The cells were resuspended in 5 mM Tris (pH 7.8) using a volume equal to twice that of the cell pellet, and lysozyme (Sigma) was added (10 mg/g of cells). After mixing, the suspension was incubated at 37 °C for 30 min and then sonicated for 12 min. The lysate was centrifuged (15000*g*) for 15 min to remove cellular debris. The supernatant (i.e., crude extract) was frozen with liquid nitrogen and stored at −80 °C. All steps were carried out under anaerobic conditions. Transfers and the sonication of cells were conducted in an anaerobic glovebox (90%/10% (v/v) N_2 and H_2, respectively). Centrifuge tubes were sealed in the glovebox prior to removal for centrifugation. The protein concentration of crude extract aliquots was determined by the Lowery assay before use.

Analytical Methods. An HPLC equipped with a diode-array UV/vis detector (Waters, Milford MA) was used to quantify and fractionate TNT and its transformation products. Monitoring of analyte concentration during transformation studies was conducted at room temperature with a Waters Nova-Pak-C_8 column (3.9 × 150 mm) and an isocratic mobile phase mixture of 82% water and 18% 2-propanol at 1 mL/min. Spectra were acquired continuously between 200 and 400 nm, and chromatograms were extracted at 230 nm for quantitation. Purification of derivatized products (derivatization procedure is described in the next section) was conducted at room temperature with a preparative-scale Waters Nova-Pak-C_8 column (7.8 × 300 mm) using an isocratic mobile phase of 75% water and 25% 2-propanol at 2 mL/min.

The measurement of ^{14}C in HPLC fractions and stock solutions was performed with a Beckman LS3801 scintillation counter after addition of samples to ReadyGel scintillation cocktail (Beckman). ^1H NMR spectra were obtained on a Bruker AC-250 spectrometer. Chemical shifts are reported in ppm relative to $CDCl_3$ (7.26 ppm). Chemical ionization (CI) mass spectroscopy and high-resolution mass spectroscopy were conducted with a MAT95 mass spectrometer. Infrared (IR) spectra were obtained with a Nicolet 205 FTIR spectrophotometer.

Derivatization Procedure. A derivatization procedure capable of acetylating hydroxylamino derivatives of TNT was developed and tested initially using 4-hydroxylamino-2,6-dinitrotoluene. A solution containing 4-hydroxylamino-2,6-dinitrotoluene (10 mg) dissolved in 1 mL of methanol and 4 mL of distilled water was chilled in an ice bath and acidified to pH = 1 by slowly adding 3 mL of HCl. Acetic anhydride (3 mL) was then added dropwise while maintaining the solution temperature at 0 °C . As the reaction proceeded (approximately 10 min), the solution changed from yellow to nearly colorless. The reaction was stopped by the addition of sodium acetate (3.5 mg) dissolved in 5 mL of water. After repeated extraction with ethyl acetate, the organic phases were washed with water and dried over $MgSO_4$. The solvent was evaporated and the product isolated by TLC plate separation (silica) with 1:1:1 (v/v/v) ethyl acetate/methylene chloride/hexane eluant. Following purification by the preparative-scale HPLC method, the compound was identified using ^1H NMR, CI mass spectroscopy, and infrared spectroscopy.

TNT Transformation and Polar Product Characterization. A methanol stock solution was prepared containing 50 mg/mL unlabeled TNT and 5.0×10^5 dpm/mL [U-ring-^{14}C]-TNT. In a glovebox (90%/10% N_2 and H_2, respectively), methanol stock solution (2 mL) was added to 500 mL of anaerobic Tris buffer (5 mM, pH = 7.8) containing cell extract at a concentration of 0.05 mg of protein/mL. The reaction mixture was continuously mixed by bubbling gas (90%/10% N_2 and H_2, respectively) through the solution using an aquarium pump and a glass frit.

The transformation process was monitored by HPLC analysis of samples taken from the solution. Samples were placed in HPLC autosampler vials, sealed within the glovebox, cooled in an ice bath, and analyzed immediately. After 72 h, the remaining cell extract system was derivatized in the glovebox with deoxygenated reagents.

The crude cell extract suspension containing the polar product was cooled in an ice bath to 0 °C, and hydrochloric acid was added slowly to reach pH = 1. With the solution still cold, acetic anhydride (12 mL) was added drop by drop. After 15 min, sodium acetate was added to adjust the pH to 7, and the solution was removed from the glovebox. After extraction with ethyl acetate (three times, total 500 mL), the combined organic phases were dried over anhydrous $MgSO_4$, filtered, and evaporated to yield a brown residue. Two ^{14}C-labeled compounds were isolated by silica column chromatography eluted with 3:1:2 (v/v/v) ethyl acetate/methylene chloride/hexane. These fractions were then purified using the preparative-scale HPLC method and characterized using ^1H NMR, CI mass spectroscopy, high-resolution mass spectroscopy, and IR spectroscopy.

Cell Cultures. A cell culture (300 mL) of *C. acetobutylicum* was grown as described previously (*15*). During the log-growth phase, the culture was spiked with TNT and ^{14}C-TNT predissolved in methanol (20 mg of TNT and 3 130 000 dpm/mL) resulting in an initial TNT concentration of 100 mg/L (1.56×10^3 dpm/mL). Temporal samples (2 mL) were removed aseptically and centrifuged (13000*g* for 4 min), and the supernatant was analyzed by HPLC for TNT and its reduced products. After 100 min of incubation, neither TNT or its hydroxylamino derivatives were detected. The culture was transferred to an anaerobic chamber (100% N_2), cooled in an ice/water bath (0 °C), and acidified to pH 1 with HCl; 20 mL of acetic anhydride was added. After 30 min, the pH was adjusted (pH = 7) with sodium acetate, and the suspension was removed from the glovebox and centrifuged at 25000*g* for 10 min in sealed, nitrogen-purged polypropylene centrifuge tubes. The supernatant was recovered, gravity-filtered through a 1-μm glass fiber filter (Gelman Sciences, Ann Arbor, MI), and extracted three times with ethyl acetate (each volume of ethyl acetate equal to supernatant volume). The extracts were combined and evaporated to dryness.

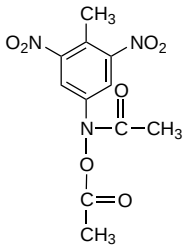
Two methods were used for comparison of products from cell extracts and cell cultures. First, the residue from cell cultures was analyzed by HPLC using the preparative-scale method described previously comparing retention time and UV spectra of eluting peaks. Secondly, ^1H NMR was conducted on the ^{14}C-labeled fraction separated by silica column chromatography (mobile phase was 3:1:2 ethyl acetate/methylene chloride/hexane). Desired fractions were evaporated to dryness, dissolved in $CDCl_3$, and ^1H NMR conducted.

Results and Discussion

Derivatization of 4-Hydroxylamino-2,6-dinitrotoluene. A single product was isolated from the acetylation of 4-hydroxylamino-2,6-dinitrotoluene and identified as 2,6-dinitro-4-(*N*-acetoxyacetamido)toluene. The structure of this compound and relevant spectroscopic data are presented in Table 1. Purification yielded 12.2 mg of product (87.5% of initial).

Interestingly, this procedure was capable of completely acetylating the hydroxylamine at both reactive centers (N- and O-). Without excess acetic anhydride, addition at the N- is generally favored, although O-derivatization is sometimes observed (*18*). No information could be located in the

TABLE 1. Structure of Product [2,6-Dinitro-(4-(*N*)-acetoxyacetamido)toluene] Isolated from the Derivatization of 4-Hydroxylamino-2,6-dinitrotoluene and Pertinent Spectroscopic Data



CI Mass Spectroscopy	mass
molecular ion (M + 1)[b]	298.0
fragment ions (F + 1)[b]	256.0, 238.0, 196.0

^{1}H NMR	ppm (relative intensity) splitting
Ar-CH_3	2.38 (3) singlet
Ar-H	8.16 (2) singlet
R-$NCOCH_3$ [a]	2.23 (3) singlet
R-$NOCOCH_3$ [a]	2.55 (3) singlet

FTIR Spectroscopy	wavelength (intensity)
NO_2 asymmetric stretch	1543.6 (very strong)
NO_2 symmetric stretch	1370.0 (strong)

[a] Groups from derivatized aryl hydroxylamine. [b] Molecular and primary fragment ions reported from CI/MS include the mass of the ionizing proton.

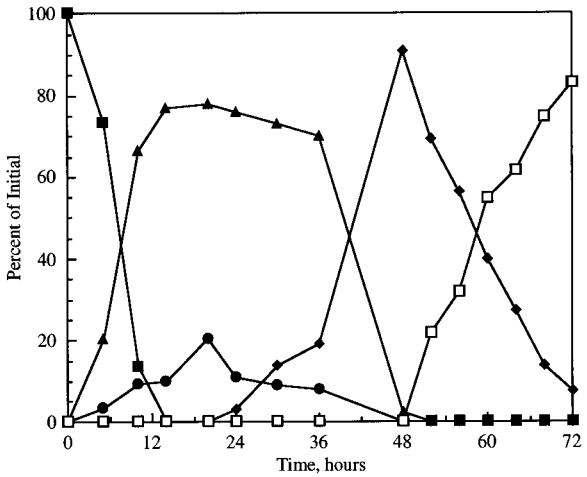


FIGURE 1. Temporal transformation of TNT (■) to 4-hydroxylamino-2,6-dinitrotoluene (▲), 2-hydroxylamino-4,6-dinitrotoluene (●), 2,4-dihydroxylamino-6-nitrotoluene (♦), and polar product (□) in crude cell extracts.

literature to determine the ^{1}H NMR chemical shift corresponding to an aryl *N*-acetoxyacetamido group. On the basis of substituent effects for aryl nitro ($\Delta_{ortho} = 0.94$, $\Delta_{para} = 0.39$) and methyl groups ($\Delta_{meta} = -0.09$) (*22*), the calculated Δ_{ortho} for this group was −0.35.

TABLE 2. Results from CI Mass Spectrometry, ^{1}H NMR Spectroscopy, and FTIR Analysis of ^{14}C-Labeled Derivatization Products Obtained from Crude Cell Extracts

CI mass spectroscopy	I mass[a]	II mass[a]
molecular ion (M + 1)[b]	368.3	326.2
fragment ions (F + 1)[b]	326.2, 308.2, 266.2	282.4, 268.2, 226.2

^{1}H NMR	I ppm (relative intensity) splitting	II ppm (relative intensity) splitting
Ar-CH_3	2.20 (3) singlet	2.12 (3) singlet
Ar-H	7.86 (1) singlet	7.55 (1) singlet
Ar-$OCOCH_3$	2.39 (3) singlet	2.38 (3) singlet
R-$NCOCH_3$ [c]	2.24 (3) singlet	2.25 (3) singlet
R-NH[c]	8.07 (1) broad singlet	7.52 (1) broad singlet
R-$NOCOCH_3$ [d]	2.49 (3) singlet	2.25 (3) singlet
R-$NCOCH_3$ [d]	2.24 (3) singlet	not applicable
R-NOH [d]	not applicable	8.93 (1) broad singlet

FTIR Spectroscopy	I wavelength (intensity)	II wavelength (intensity)
NO_2 asymmetric stretch	1538.6 (very strong)	1536.4 (very strong)
NO_2 symmetric stretch	1370.6 (strong)	1369.8 (strong)

[a] **I** empirical formula $C_{15}H_{17}N_3O_8$; **II** empirical formula $C_{13}H_{15}N_3O_7$. [b] Molecular and primary fragment ions reported from CI/MS include the mass of the ionizing proton. [c] Groups from derivatized arylamine. [d] Groups from derivatized aryl hydroxylamine.

TNT Transformation in Cell Extracts and Polar Product Identification. The results from sampling and HPLC analysis of crude cell extracts spiked with TNT are shown in Figure 1. TNT was rapidly transformed to 4-hydroxylamino-2,6-dinitrotoluene and 2-hydroxylamino-4,6-dinitrotoluene, with a greater extent of initial reduction at the 4-position. After approximately 24 h, the 4-hydroxylamino-2,6-dinitrotoluene and 2-hydroxylamino-4,6-dinitrotoluene were converted to the 2,4-dihydroxylamino-6-nitrotoluene, which then decreased in concentration with a commensurate accumulation of the polar product. After 72 h, 83% of the ^{14}C was present in the polar fraction. The UV spectra obtained for the polar product during HPLC analysis is presented in Figure 2.

After derivatization, two ^{14}C-labeled products were isolated by silica column chromatography. These compounds were purified using the preparative scale HPLC technique—with a combined yield of nearly 80% of initial—and subjected to spectroscopic analysis. The ^{1}H NMR of each compound indicated that only one aryl proton remained on the molecule and IR spectra for each yielded characteristic absorption of the nitro group. These results indicate that the metabolism of 2,4-dihydroxylamino-6-nitrotoluene involved a substitution at either the 3- or 5-position and not a continued reduction of the remaining nitro group to trihydroxylaminotoluene.

CI mass spectroscopy yielded molecular ion peaks (M + 1) of 368.3 and 326.2, respectively. The compound with a molecular ion of 368.3 was obtained in a greater yield

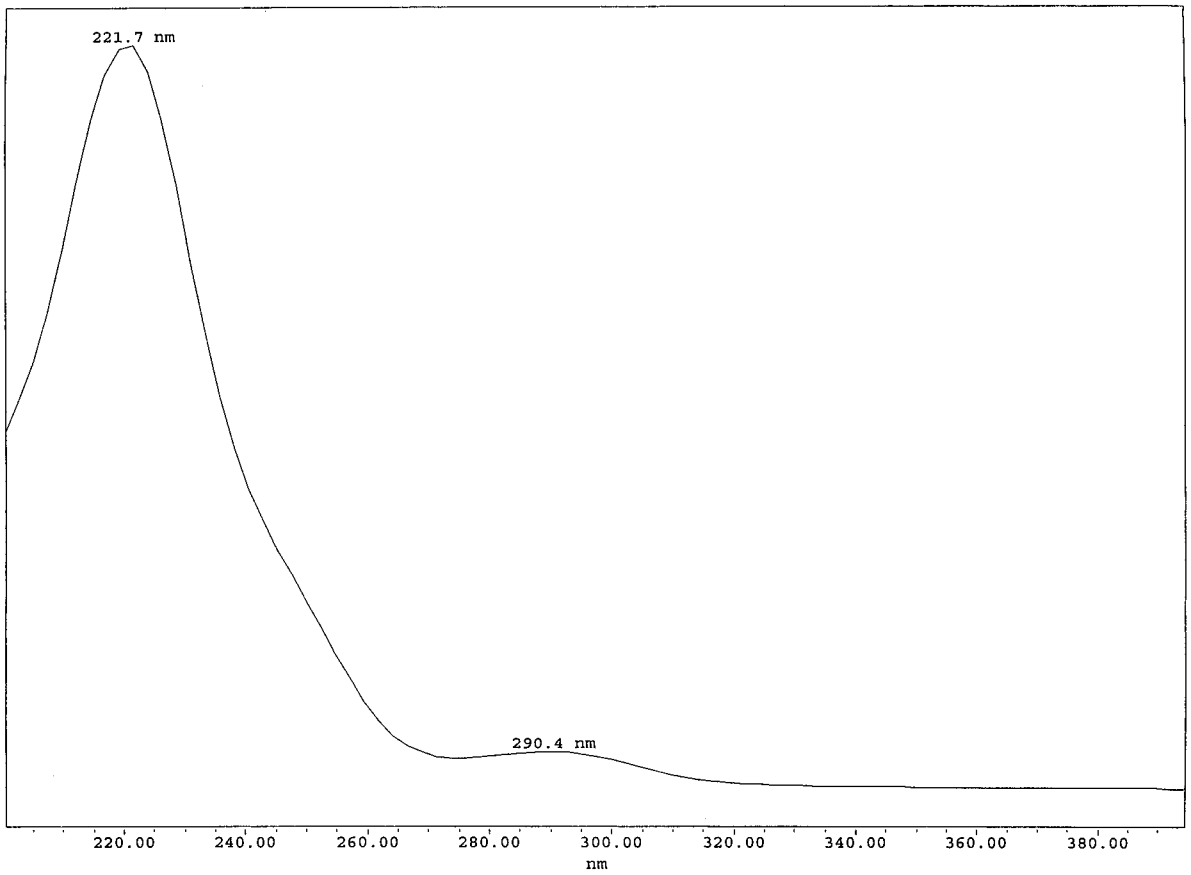


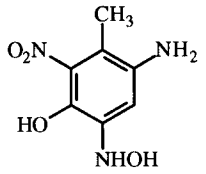
FIGURE 2. UV/vis spectra of polar product obtained during HPLC analysis of TNT transformation in cell extracts.

(approximately 3:1) and subjected to high-resolution mass spectroscopy confirming its elemental composition ($C_{15}H_{17}N_3O_8$, MW = 367.32, error = 1.2 ppm). The difference in mass between the two products ($\Delta_{mass} = 42$) corresponds to the absence of an acetyl group ($C_{13}H_{15}N_3O_7$; MW = 325.28), which would occur if derivatization was not complete. The ^{1}H NMR of this smaller compound contained a broad downfield proton (8.93 ppm) that is consistent with an $(R)_2$-N-O-H proton remaining from only the N-acetylation of a hydroxylamine group. Spectroscopic information for both derivatized products (labeled **I** and **II**) are presented in Table 2. These results are consistent with a toluene ring substituted with the following groups: nitro-, acetoxy- (formed from the acetylation of a hydroxyl group), acetamide- (from the acetylation of an amino group), and either a N-acetoxyacetamide or N-acetamidohydroxylamine (depending on the degree of acetylation of a hydroxylamine group).

On the basis of the results of spectroscopic analysis, four possible structures for the parent compound(s) prior to derivatization are shown in Figure 3. All are consistent with a Bamberger rearrangement of the 2,4-dihydroxylamino-6-nitrotoluene. Structure A would result from the addition of a hydroxyl group at the 5-position with participation from—and subsequent reduction of—the *p*-hydroxylamine. Structure B would result from the addition at the 5-position with participation of the *o*-hydroxylamine. Structures C and D correspond to addition at the 3-position with participation of either *o*-hydroxylamine. Calculation of ^{1}H NMR chemical shift for the aryl proton using literature values (*22*) and the shift obtained of the completely acetylated product are consistent with only two of the possible four isomers. After complete acetylation, the predicted ∂ for a proton remaining in the 3-position (structures A and B) is 7.83 ppm, while a proton remaining in the 5-position would be either 8.22 (structure C) or 7.97 ppm (structure D). The observed value ($\partial = 7.86$) indicates that the hydroxyl group addition is at the 5-position and the parent compound is either structure A or B. Similar rearrangement mechanisms have been reported in studies of the microbial metabolism of nitrobenzene by *Pseudomonas pseudoalcaligenes* (*19*) and *Ralstonia eutropha* (*23*) but not for TNT. In *P. pseudoalcaligenes*, the addition of the hydroxyl group occurred exclusively ortho to the intermediate hydroxylaminobenzene forming 2-aminophenol. Rearrangement catalyzed by *R. eutropha* resulted in the formation of 2-aminophenol (33%) and 4-aminophenol (63%).

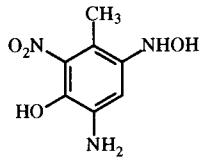
From the spectroscopic results alone, the assignment of groups at the 2- or 4-positions (e.g., 2-amino-4-hydroxylamino or 2-hydroxylamino-4-amino) is difficult; however, experimental results indicate that structure A is more likely. When derivitizing 2,4-dihydroxylamino-6-nitrotoluene using the method employed for the acetylation of 4-hydroxylamino-2,6-dinitrotoluene, a rapid transformation was observed in the cold acid solution (pH = 1) (characterized by a rapid dissipation of the yellow color of the 2,4-dihydroxylamino-6-nitrotoluene solution) prior to the addition of acetic anhydride. After derivatization, the product had an HPLC retention time (14.3 min using the preparative-scale technique), UV spectra, and ^{1}H NMR [peaks obtained with relative areas in parentheses were 2.24 (6), 2.39 (3), 2.49 (3), 7.86 (1), and 8.07 (1)] identical to **I** from crude cell extract studies. This result is consistent with the acid-catalyzed Bamberger rearrangement in which the para rearrangement is strongly favored (*24*). Only the 2-hydroxylamine group is capable of para rearrangement with this molecule since the aryl methyl group is para to the 4-hydroxylamine group. The ortho rearrangement of the 4-hydroxylamine group is unlikely without enzymatic catalysis, and under identical conditions, rearrangement of the 4-hydroxylamino-2,6-dinitrotoluene was not observed.

A)



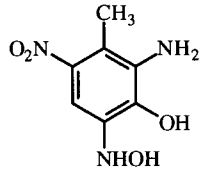
2-amino-4-hydroxylamino-5-hydroxyl-6-nitrotoluene
(4-amino-6-hydroxylamino-3-methyl-2-nitrophenol)

B)



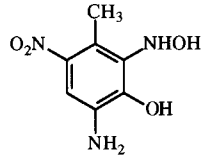
2-hydroxylamino-4-amino-5-hydroxyl-6-nitrotoluene
(6-amino-4-hydroxylamino-3-methyl-2-nitrophenol)

C)



2-amino-3-hydroxyl-4-hydroxylamino-6-nitrotoluene
(6-amino-2-hydroxylamino-5-methyl-4-nitrophenol)

D)



2-hydroxylamino-3-hydroxyl-4-amino-6-nitrotoluene
(2-amino-6-hydroxylamino-5-methyl-4-nitrophenol)

FIGURE 3. Possible structures resulting from the Bamberger rearrangement of 2,4-dihydroxylamino-6-nitrotoluene.

The ability of 2,4-dihydroxylamino-6-nitrotoluene to undergo rearrangement during the derivatization procedure implies that some of the products isolated may have resulted from the small amount of 2,4-dihydroxylamino-6-nitrotoluene remaining in the crude cell extract (7.5 mol %). The yield after purification approached 80%, thus the product isolated can not be a result of this potential artifact.

Attempts to further confirm structures with ^{13}C spectroscopy were not successful (data not shown). The similarity of chemical shifts resulting from derivatized amino or derivatized hydroxylamino groups and the low response of the ^{13}C NMR without isotope enrichment did not allow for positive identification. In theory, compound A is a more likely product of the rearrangement since the intermediate structures for rearrangement of B would favor hydroxyl addition at the 3-position and could result in mixed products—neither of which were observed.

The initial stages of TNT transformation in *C. acetobutylicum* crude cell extracts are presented in Figure 4. Reduction of the aryl nitro group yields aryl hydroxylamines and eventually an aryl amine via the Bamberger rearrangement. Since the Bamberger rearrangement can be a strict chemical reaction or can be catalyzed by enzymes, an experiment was conducted to evaluate which may be occurring in crude cell extract systems. Two small-scale (20 mL) test systems (in triplicate) were spiked with 2,4-dihydroxylamino-6-nitrotoluene to a concentration of 0.5 mM. One contained deoxygenated 5 mM Tris (pH = 7.8); the other contained deoxygenated 5 mM Tris (pH = 7.8) and 0.05 mg of protein/mL. After 24 h of incubation in an anaerobic glovebox (90% N_2/10% H_2), no less than 95% of the original 2,4-dihydroxylamino-6-nitrotoluene was observed in extract-free systems (i.e., loss in any one system never exceed 5%). In the systems containing cell extract, transformation always exceeded 82%. These results clearly demonstrated that the rapid rearrangement observed in these systems is catalyzed by biological components of the crude cell extract, similar to studies of hydroxylaminobenzene rearrangement to aminophenols.

This result is particularly interesting, since *C. acetobutylicum* was not grown in the presence of any nitroaromatic compound, yet the enzyme that catalyzes the rearrangement is present without a nitroaromatic inducer. In studies mentioned previously (*19*, *23*) where the Bamberger rearrangement of hydroxylaminobenzene was observed, cell extracts were prepared from cultures actively growing on nitroaromatics as their carbon and energy source. Conditions might favor expression of nitroaromatic catabolic gene. Such conditions would not be expected in *C. acetobutylicum* growth on glucose.

Any discussion of the enzymes directly involved in the TNT transformation process by *C. acetobutylicum* is speculative; however, activity without the presence of a nitroaromatic-inducing substrate may imply that the enzyme(s) responsible are constitutive. Others studying the transformation of TNT by *Clostridia* in whole cell systems have also observed that inducing substrates are not required for TNT transformation to aminated derivitives (*5*). Previously, we reported that the rate of TNT transformation in cell cultures appears to be related to acidogenic hydrogenase activity (*15*).

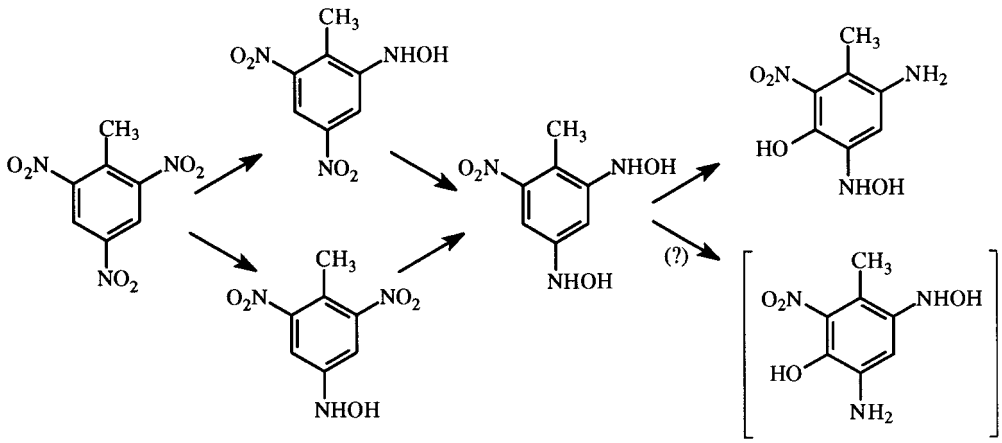


FIGURE 4. Proposed pathway of transformation observed in *C. acetobutylicum* crude cell extracts. Brackets around the 6-amino-4-hydroxylamino-3-methyl-2-nitrophenol Bamberger rearrangement product denote that this structure is possible but less likely than the 4-amino-6-hydroxylamino-3-methyl-2-nitrophenol product.

In crude cell extracts, TNT transformation to hydroxylamino intermediates and eventually the Bamberger rearrangement product occurred only when H_2 was present as a component of glovebox gas—adding further evidence that hydrogenases active during acidogenic metabolism by this organism are involved, at least indirectly, in TNT transformation.

Cell Cultures. As observed previously (*16*), TNT transformation in cell cultures resulted in the rapid accumulation and subsequent transformation of 2,4-dihydroxylamino-6-nitrotoluene (data not shown). Following the derivatization, samples were analyzed by HPLC comparing elution time and UV spectra of peaks separated from the culture supernatant to purified products obtained from cell extract studies. One large peak was resolved from the culture broth that had an identical retention time (14.3 min) and UV spectra when compared to **I** from crude cell extract studies. This peak was isolated and subjected to ^{1}H NMR. Agreement between the spectra was excellent [peaks obtained with relative areas in parentheses were 2.24 (6), 2.39 (3), 2.49(3), 7.86 (1), and 8.07 (1)], confirming the that a Bamberger rearrangement product of 2,4-dihydroxylamino-6-nitrotoluene was produced in whole cell systems identical to that found in cell extracts.

The ability of bacteria to convert nitroaromatics, including TNT, to aminated phenols may represent an important fate process for these compounds in anaerobic environments. Reduction of aryl nitro groups to the corresponding amine has long been recognized as a reaction that occurs in natural and engineered systems. The formation of aminophenols through rearrangement of hydroxylamines may represent a "competing" reaction that can alter the physical distribution of contaminants, their chemical reactivity, and metabolic pathways required for contaminant destruction. In this case, the presence of a nitroaromatic inducer was not required for activity to be expressed.

To what extent this finding may influence our understanding of TNT bioremediation systems is still unclear. Certainly, anaerobic slurry processes fed sugars are ideal systems for fermentative bacteria, including *Clostridia*. To our knowledge, the analysis of samples for phenolic TNT derivatives has not been conducted in field systems. Both 2,4-dihydroxylamino-6-nitrotoluene and "phenolic hydrolysis products of TAT" were observed in a study of TNT transformation conducted with an anaerobic mixed laboratory enrichment culture (*17*). TAT was also a product in this system, again demonstrating that several TNT transformation processes may result in mixed culture systems.

Interestingly, the fate of Bamberger rearrangement products in anaerobic bioremediation systems may be similar to that of TAT. In our hands, the phenolic product was extremely oxygen sensitive. Even under anaerobic conditions, the compound would decompose to a reddish-brown residue after several days in solution. TAT is also oxygen sensitive and under oxidizing conditions becomes irreversibly "bound" to soils (*1*). The association of TAT with soils is thought to result from ion exchange processes of one or more of its free amino groups. Phenolic products from the Bamberger rearrangement will have a free amine and hydroxyl group that could participate in ion-exchange process as well.

Attempts to recover phenolic product(s) in a variety of nonpolar (e.g., benzene, hexane, etc.) and moderately polar solvents (e.g., ethyl acetate, dichloromethane, etc.) resulted in essentially zero recovery. In comparison, essentially 100% recovery of 2,4-dihydroxylamino-6-nitrotoluene was possible with ethyl acetate extraction of aqueous samples. Estimated pK_a values of phenolic groups are 9.1 and 8.2 (A and B, respectively) and 3.4 and 3.5 (A and B, respectively) for amino groups (*25*, *26*). On the basis of these calculations, neither proton donating/accepting groups of structures A and B (Figure 2) would be extensively ionized in the pH values of experimental systems. Since the product of 2,4-dihydroxylamino-6-nitrotoluene rearrangement was extremely hydrophilic, partitioning to soil organic matter would probably be minimal in remediation systems. At this time, no experiments have been conducted to assess the extent of either ion exchange or partitioning soil systems.

In summary, studies have demonstrated the ability of *C. acetobutylicum* to reduce TNT to 2,4-dihydroxylamino-6-nitrotoluene and then to a phenolic product(s) via the Bamberger rearrangement. The product of transformation is hydrophilic, highly reactive with oxygen, and decomposes in aqueous solution in a matter of days under strict anaerobic conditions. No inducing substances were required for *C. acetobutylicum* to catalyze the transformation reactions, and further evidence for the involvement of acidogenic hydrogenases was obtained. Further study will be required to assess both the propensity of other fermentative organisms to utilize similar pathways for the transformation of nitroaromatics in anaerobic environments and the long-term fate of Bamberger rearrangement products in soil systems.

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