The two cyclic nitramine explosives hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are powerful energetic compounds that are commonly used in conventional munitions and various military applications. Activities associated with manufacturing, training, waste disposal, and closures of bases have resulted in severe soil and groundwater contamination with the two explosives (9, 14, 21). RDX and HMX are both toxic (25, 29), and the degradation of RDX has received considerable attention and thus to provide insight about the RDX degradation pathway.

Previous studies have focused on the degradation of RDX by microorganisms under anaerobic conditions (1, 17, 18, 20, 24, 30). McCormick et al. (20) reported the formation of the three nitroso derivatives, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MXN), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX), which after further reduction to the corresponding hydroxylamines undergo ring cleavage to produce hydrazine, dimethyl hydrazine, and methanol, respectively. No microorganisms or enzymes were identified in the study by McCormick et al. (20). Recently, Kitts et al. (19) reported the involvement of a type I nitroreductase in the degradation of RDX, but no products were identified. Using anaerobic sludge, Hawari et al. (11) reported that in addition to the occurrence of a ring cleavage via the nitroso route, other ring cleavage pathways, such as direct ring cleavage and/or denitrification followed by ring cleavage, might be possible. In the latter study, several key intermediate ring cleavage products, including bis(hydroxymethyl)nitramine, methylenedinitramine (MEDINA), nitrous oxide, and formaldehyde, accumulated, but hydrazines were not detected (11, 12).

Several groups described RDX biodegradation under aerobic conditions, but little information was provided on the degradation pathway (5, 6, 16, 27). Products from biodegradation of cyclic nitramine explosives under aerobic conditions are poorly understood, particularly ring cleavage products (5, 10). Jones et al. (16) isolated a Rhodococcus sp. strain A from explosives-contaminated soil and demonstrated its potential for the degradation of RDX but did not report any products. Coleman et al. (6) reported the isolation and characterization of another Rhodococcus sp. strain DN22, which efficiently degrades RDX with the production of NO2-. No other products have been identified during RDX biodegradation with DN22. The aim of the present study was to identify key intermediates produced during aerobic degradation of RDX by Rhodococcus sp. strain DN22. In addition, uniformly labeled [U-14C]-RDX and ring-labeled [15N]-RDX were used in incubation mixtures with strain DN22 to determine the stoichiometry of the reaction and thus to provide insight about the RDX degradation pathway.

Rhodococcus sp. strain DN22 can convert hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) to nitrite, but information on degradation products or the fate of carbon is not known. The present study describes aerobic biodegradation of RDX (175 µM) when used as an N source for strain DN22. RDX was converted to nitrite (NO2-) (30%), nitrous oxide (N2O) (3.2%), ammonia (10%), and formaldehyde (HCHO) (27%), which later converted to carbon dioxide. In experiments with ring-labeled [15N]-RDX, gas chromatographic/mass spectrophotometric (GC/MS) analysis revealed N2O with two molecular mass ions: one at 44 Da, corresponding to 14N14NO, and the second at 45 Da, corresponding to 15N14NO. The nonlabeled N2O could be formed only from NO2-, whereas the 15N-labeled one was presumed to originate from a nitramine group (15N-14NO2) in RDX. Liquid chromatographic (LC)-MS electrospray analyses indicated the formation of a dead end product with a deprotonated molecular mass ion [M-H] at 118 Da. High-resolution MS indicated a molecular formula of C6H12N3O5. When the experiment was repeated with ring-labeled [15N]-RDX, the [M-H] appeared at 120 Da, indicating that two of the three N atoms in the metabolite originated from the ring in RDX. When [U-14C]-RDX was used in the experiment, 64% of the original radioactivity in RDX incorporated into the metabolite with a molecular weight (MW) of 119 (high-pressure LC/radioactivity) and 30% in 14CO2 (mineralization) after 4 days of incubation, suggesting that one of the carbon atoms in RDX was converted to CO2 and the other two were incorporated in the ring cleavage product with an MW of 119. Based on the above stoichiometry, we propose a degradation pathway for RDX based on initial denitration followed by ring cleavage to formaldehyde and the dead end product with an MW of 119.
**Title:** Determination of Key Metabolites during Biodegradation of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine with Rhodococcus sp. Strain DN22

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**Performing Organization Report Number:**

**Sponsoring/Monitoring Agency Name(s) and Address(es):**

**Distribution/Availability Statement:** Approved for public release; distribution unlimited

**Supplementary Notes:**

**Abstract:**

**Subject Terms:**

**Security Classification of:**

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**Limitation of Abstract:** Same as Report (SAR)

**Number of Pages:** 8

**Name of Responsible Person:**

MATERIALS AND METHODS

Chemicals. Commercial-grade RDX (purity of >99%) and ring-labeled [15N]-RDX (purity of >98%) (2) were provided by Defense Research Establishment Valcartier, Quebec, Canada. [U-14C]-RDX with chemical and radiochemical purities reaching 98 and 97% with a specific activity of 28.7 μCi/mmol (3) was also provided by Defense Research Establishment Valcartier (3). We determined the chemical purity of [U-14C]-RDX by high-performance liquid chromatography (HPLC)/UV analysis and radiochemical purity by collecting the HPLC fractions corresponding to RDX for subsequent radioactivity measurements. D2O (99% purity), 18O2 (97%), and [15N]CH3OH (53-mCi/mm specific activity as provided by the supplier) were from Aldrich, Oakville, Ontario, Canada. All other chemicals used were reagent grade.

Organisms and growth conditions. Rhodococcus sp. strain DN22, previously isolated from a soil contaminated with RDX, 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and heavy metals, was kindly provided by Nicholas V. Coleman. The strain was grown in a mineral salt medium previously described by Coleman et al. (6). Unless specified otherwise, succinate (2.4 mM) was used as the carbon source and RDX (175 μM) (from a concentrated acetone stock solution) was added as the sole nitrogen source. Acetone was removed by evaporation prior to the addition of the aqueous medium. Growth was monitored spectrophotometrically at 530 nm (A530) (Thermo Spectronic, Rochester, N.Y.). Cultures were protected from light and agitated at 250 rpm at 25°C.

Mineralization of RDX by growing cultures of Rhodococcus sp. strain DN22 was determined as previously described (11). Briefly, the assays were performed with 120-ml serum bottles containing 10 ml of mineral salt medium and RDX (175 μM) containing [U-14C]-RDX (0.038 μCi). A 5-ml tube containing 1 ml of KOH (0.5 M) was placed in the serum bottle to act as a CO2 trap. The serum bottles were crimp sealed under a blanket of air with Telfon-coated rubber septa and incubated at room temperature under agitation (150 rpm). The KOH traps were regularly sampled for radioactivity determination (14CO2) using a Tri-Carb 4530 liquid scintillation counter (LSC) (model 2100 TR; Packard Instrument Company, Meriden, Conn.). After each sampling of the KOH trap, a fresh volume (1 ml) of the original KOH solution was added to the trap to compensate for the withdrawn volume. When the stationary phase was reached, the microcosms were measured to determine the remaining radioactivity in the culture supernatant and in the biomass.

Resting cells assays were performed using mid-log phase culture (A600 = 0.4 to 0.5). Cells were washed and resuspended in the mineral salt medium described above to an absorption equal to 1.2 without the addition of any carbon or nitrogen source apart from RDX. In some cases, (NH4)2SO4 (1 mM) was added above to an absorption equal to 1.2 without the addition of any carbon or nitrogen source. In other cases, (NH4)2SO4 (1 mM) was added to DN22 resting cells to prevent the uptake of NO2− produced during RDX degradation (6, 8). For some cell suspensions, we added ring-labeled [15N]-RDX to DN22 cultures to determine which nitrogen atoms were incorporated into metabolites.

For experiments with 18O2 labels, the culture medium was first flushed with nitrogen to remove air and then 18O2 (97% pure) was added to the headspace with a gas-light syringe followed by the addition of DN22 cells. The final oxygen concentration was varied from 0 to 20% (vol/vol) (O2/N2) as measured by a gas chromatograph (GC) connected to a thermal conductivity detector (TCD).

In experiments with deuterated water, RDX grown cells were harvested by centrifugation and then resuspended in pure D2O (5 ml) in the presence of RDX. Maximum cell density (A530, 1.14) was attained after 14 h of incubation, during which RDX disappeared completely. In the uninoculated controls, no RDX removal was observed.

The removal of RDX and the formation NO2− was accompanied by the formation of N2O, which continued to accumulate long after the disappearance of NO2− (Fig. 1 top). After 4 days of incubation, the amount of N2O attained a plateau of 4.5 μmol (not shown). We did not observe N2O in controls containing RDX in the absence of Rhodococcus. The presence of N2O was confirmed by comparison with a reference standard material and by measuring its molecular mass ion at 44 Da by GC-MS. Incubation of RDX grown cells with NaN2O3 in the presence of succinate also lead to the accumulation of N2O also in trace amounts. When the above biodegradation experiment was repeated using ring-labeled [15N]-RDX as the nitrogen source for DN22, we detected a molecular mass ion at 45 Da, corresponding to 14N15ONO, and another at 44 Da, corresponding to 14N14NO almost with the same intensity. The results indicate that the major products of N2O were produced from RDX or from RDX metabolites, such as NO2−.

We did not detect any of the RDX nitroso derivatives, such as MNX, DXN, and TXN, although such products have been frequently observed during biodegradation of RDX with aerobic sludge (11, 20) and with P. chrysosporium (27).

(ii) Carbon-containing products. Incubation of RDX with cultures led to the disappearance of RDX and the formation of formaldehyde (HCHO) (Fig. 1 bottom). Formaldehyde accumulated during the time of rapid RDX removal and was subsequently mineralized as determined by the accumulation of 14CO2 from [U-14C]-RDX (Fig. 1 bottom). In a separate experiment, incubation of H14CHO with RDX grown cultures...
under the same conditions led to the degradation of the aldehyde and the accumulation of $^{14}$CO$_2$ in very high yield (88%).

(iii) Accumulation of a dead end product. During the growth of strain DN22 on RDX (Fig. 2), there was a concurrent formation and accumulation of another soluble metabolite as detected by HPLC/UV analysis and LC-MS(ES$^-$/H11002). The early chromatographic elution of the metabolite (retention time of 3.6 min) implies that the compound is more polar than RDX. The compound was not degraded by strain DN22. The metabolite yielded a deprotonated molecular mass ion [M-H] at 118 Da (MW, 119 Da) (Fig. 3A). The fragmentation pattern of the metabolite shows two other relevant mass ions at 61 and 46 Da representing fragments -NHNO$_2$ and -NO$_2$ (Fig. 3A). When ring-labeled $^{15}$N-RDX was used, the [M-H] ion of the metabolite shifted to 120 Da (Fig. 3B), indicating that the metabolite contained two of the three ring-labeled $^{15}$N atoms in RDX. Also, the mass ion fragment -NHNO$_2$ shifted from 61 to 62 Da, confirming that the fragment still contains one atom of $^{15}$N. High-resolution MS of the metabolite yielded a [M-H] at 118.0331, matching a deprotonated molecular formula of C$_2$H$_4$N$_3$O$_3$. The calculated mass of the deprotonated mass ion was 118.0253, suggesting that the metabolite had a molecular formula of C$_2$H$_4$N$_3$O$_3$ (MW, 119 Da) containing three N atoms (half of the total N content of RDX), two C atoms (two third of the total C content of RDX), and five H atoms.

Biodegradation of RDX by resting cells resuspended in pure D$_2$O yielded two products with deprotonated molecular mass ions [M-H] at 118 and 119 Da. The percentage of 119 to 118 Da was 18% (data not shown). We did not account for the water residue that were retained with biomass after centrifugation. The D-labeled experiment indicated the incorporation of only one deuterium in the metabolite based on the assumption that H rather than D was lost during ionization in the mass spectrometer. The suggested molecular formula of the above metabolite would be C$_2$H$_4$DN$_3$O$_3$.

Figure 3C shows that alkaline hydrolysis of RDX with NaOH at pH 12 produced a product which had the same chromatographic (retention time of 3.6 min) and mass ([M-H] at 118 Da) data as observed earlier for the MW 119 RDX metabolite. Alkaline hydrolysis of RDX (pH 12) in the presence of D$_2$O also resulted in the formation of a product with [M-H] at 119 Da, indicating the inclusion of one D atom in the product.

When RDX was incubated with cells in the presence of $^{18}$O$_2$, we did not observe any mass change in the MW 119 Da metabolite. It was very difficult to search for $^{18}$O in HCHO, but no $^{18}$O was detected in carbon dioxide when the latter was trapped in an alkaline medium and analyzed on LC-MS.
Stoichiometry. We used resting cells assays to calculate the distribution of nitrogen in RDX metabolites produced during incubation with DN22 in the presence of ammonium sulfate (1 mM). The latter was added to prevent the uptake of NO$_2^-$ produced as an RDX metabolite (6, 8). In experiments supplemented with ammonium sulfate, the concentrations of ammonia as an RDX product was calculated as the difference between initial and final concentrations of NH$_4^+$. Table 1 summarizes the percentage of N-containing products produced following RDX removal. We found an N-mass balance of 91.2% distributed as follows: NO$_2^-$ (30%), N$_2$O (3.2%), NH$_3$ measured as NH$_4^+$ (10%), and a metabolite with an MW of 119 (48%). Table 1 also summarizes the carbon stoichiometry in a 4-day culture. After 4 days of incubation, 97.6% of the original radioactivity in RDX was found as follows: 30% was CO$_2$ and 67.6% was in the liquid phase. However, Table 1 shows a 94% carbon mass balance distributed as follows: CO$_2$ (30%) and a metabolite with an MW of 119 (64%), leaving 3% of measured radioactivity unidentifiable.

The stoichiometry shown in Table 1 indicates that following the removal of RDX (C$_3$H$_6$N$_6$O$_6$), 30% of its C content (one atom) was used to produce $^{14}$CO$_2$ and 64% (two C atoms) were incorporated in the dead end product with an MW of 119 (C$_2$H$_5$N$_3$O$_3$). Controls containing RDX without strain DN22 retained close to 99.5% of the original radioactivity in the form of unreacted RDX.

**DISCUSSION**

The formation of nitrite concurrent with RDX disappearance during incubation with growing DN22 cells (Fig. 1 top) clearly indicated the occurrence of an important early enzymatic denitration step in the degradation process (Fig. 4). The absence of any of the familiar nitroso products MNX, DNX, and TNX also supported the hypothesis that denitration was the most critical initial microbial (enzymatic) step in RDX degradation.

Nitrite did not accumulate in the system and its disappearance was accompanied by microbial growth and the formation of N$_2$O (Fig. 1), suggesting that nitrite was assimilated by the bacteria. Nitrogen assimilation via ammonia can be accompanied by the production of N$_2$O (31).

The continued formation of N$_2$O (total yield, 3.6%) long after the complete removal of RDX and nitrite indicated that in addition to nitrite assimilation by DN22, the presence of other RDX intermediate(s) might have been directly responsible for its formation. Also, the detection of both $^{15}$N$^\text{NO}$ (m/z, 44 Da) and $^{15}$N$^{14}$NO (m/z, 45 Da) during biodegradation of ring-labeled [$^{15}$N]-RDX indicated that in addition to -NO$_2^-$ assimilation there must be another route for its production. The formation of $^{15}$N$^{14}$NO requires the direct participation of

![FIG. 3. LC/MS (ES−) spectra of the metabolite with an MW of 119 produced during RDX degradation using resting DN22 cells (A), DN22 cells and ring-labeled [$^{15}$N]-RDX (B), and sodium hydroxide (pH 12) (C).](image)

**TABLE 1. Stoichiometry of nitrogen and carbon during RDX degradation calculated based on percentage of reacted RDX using total theoretical numbers on N and C atoms**

<table>
<thead>
<tr>
<th>Element (total no. of atoms)</th>
<th>NO$_2^-$</th>
<th>N$_2$O</th>
<th>NH$_3^a$</th>
<th>CO$_2$</th>
<th>MW 119</th>
<th>% Recovered</th>
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<tr>
<td>Carbon (3)</td>
<td>30.0 (1.7)$^e$</td>
<td>1.5 (8.5)$^e$</td>
<td>10.0</td>
<td>30.0$^b$ (0.8)$^f$</td>
<td>64.0$^b$</td>
<td>94.0$^b$</td>
</tr>
<tr>
<td>Nitrogen (6)</td>
<td></td>
<td></td>
<td></td>
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$^a$ No ammonium salt was added to the resting-cell suspension.
$^b$ Based on $^{14}$CO$_2$ measured using [U-$^{14}$C]-RDX.
$^c$ Based on HPLC/radioactivity measurement using [U-$^{14}$C]-RDX.
$^d$ Total of the measured values was 97.6%, distributed as follows: 30% was CO$_2$ and 67.6% was in the liquid phase, leaving 3% unidentified.
$^e$ Value in parentheses represents relative standard deviation of a triplicate.
$^f$ Calculated based on the empirical formula C$_2$H$_5$N$_3$O$_3$ found by LC/MS (ES-) and high-resolution MS.
an RDX nitramine group (\(^{15}\text{N},^{14}\text{NO}_2\)). Earlier, we reported the formation of N\(_2\)O from the spontaneous decomposition of nitramide, NH\(_2\)NO\(_2\), considered as a ring cleavage product of RDX biodegradation with anaerobic sludge (11). Regardless of which mechanism leads to the production of N\(_2\)O, its formation as a secondary product in a small yield (3.6%) does not provide insight about early steps in RDX metabolism.

The formation of 2 mol of NO\(_2^-\)/H\(_2\)O per molecule of RDX that disappeared is consistent with the stoichiometry reported earlier by Coleman et al. (6). A plausible hypothesis would be that the first loss of NO\(_2^-\)/H\(_2\)O produced the cyclohexenyl product (I) whereas the second denitration produced the cyclohexadienyl intermediate (II) (Fig. 4).

The transient accumulation of HCHO and the subsequent formation of CO\(_2\) clearly indicate the cleavage of the RDX ring following its denitration. The concurrent formation of carbon dioxide with the disappearance of HCHO (Fig. 1 bottom) might indicate the direct involvement of the aldehyde in the formation of CO\(_2\). The above conclusion is supported by the rapid and efficient degradation of H\(^{14}\)CHO to \(^{14}\)CO\(_2\) (88%) under the same conditions. The total yield of CO\(_2\) indicated that roughly one carbon atom in each RDX molecule mineralized.

We tentatively identified the molecular structure of the detected C\(_3\)H\(_7\)N\(_3\)O\(_3\) metabolite as the aldehyde (IVA) or the amine (IVB) (Fig. 5). We based our conclusion on the assumption that the denitrated intermediate II would first react with water to produce the hypothetical hydroxylated structure III. The subsequent spontaneous decomposition of the resulting \(\alpha\)-hydroxylated product (III) would then produce both IV A and IVB (Fig. 5). The autodecomposition of the unstable \(\alpha\)-hydroxylated product (III) would also produce NH\(_3\), HCHO in addition to the dead end product IV (C\(_3\)H\(_7\)N\(_3\)O\(_3\)) (Fig. 4). No nitrite assimilation occurred during resting-cell assays. Therefore, the formation of ammonia in these assays was presumed to be generated directly through the decomposition of the hydroxylated product III or via one of its ring cleavage intermediates possibly formamide (NH\(_2\)CHO). Pseudomonas \textit{putida} biodegrades NH\(_2\)CHO to NH\(_3\) and CO\(_2\) (4). \(\alpha\)-Hydroxylation of cyclic and acyclic dialkyl nitrosamines catalyzed by a mixed function oxidase can lead to the production of unstable carbinol products which decompose to nitrogen and cationic alkyl groups, R\(^+\) (26, 22).

Our hypothesis of the occurrence of a rapid hydrolytic ring cleavage (II to III to IV) following RDX initial denitrification (RDX to I to II) was supported by our observation of deuterium in the MW 119 metabolite during RDX biodegradation in the presence of D\(_2\)O. The evidence does not support a simple exchange with the solvent because when we added an HPLC-purified sample of C\(_3\)H\(_7\)N\(_3\)O\(_3\) to D\(_2\)O, we did not observe any
change in the MW. Furthermore, we observed the MW 119 product during alkaline hydrolysis of RDX at pH 12. The absence of $^{18}$O in the metabolite produced during RDX transformation in the presence of $^{18}$O$_2$ clearly supports the formation of MW 119 products via a hydrolytic step. Interestingly, we did not observe any $^{18}$O incorporation in CO$_2$. However, further spectroscopic evidence on the identity of the 119-Da metabolites is needed to provide insight about the mechanism of its formation and also its subsequent reactions.

Previously, we reported that biodegradation of RDX with another isolated Rhodococcus strain produced the cyclohexenyl intermediate (I) which autodecomposed to produce the dead end product MW 119 (IV) that was tentatively identified as the aldehyde IV A (10). In fact, both mono denitration followed by ring cleavage (13) and successive elimination of two HNO$_2$ molecules followed by ring cleavage (15) have been reported for the destruction of RDX under alkaline conditions.

Most of the products (NO$_2^-$, NH$_3$, N$_2$, HCHO, and CO$_2$) detected during RDX biodegradation with DN22 have also been detected during the alkaline hydrolysis of RDX, suggesting a resemblance in the degradation mechanisms of both reactions. RDX hydrolyzes in an alkaline solution (pH 12) via a bimolecular elimination of HNO$_2$ to initially produce a 1,3,5-triaza-1,3,5-trinitrocyclohexane (I) (7). The intermediate (I) decomposes at a rate $10^5$ times faster than the initial rate of RDX degradation by E$_2$ (13). As Fig. 3C shows, we detected a product with an MW of 119 with the same retention time (3.6 min) as observed during incubation with Rhodococcus. It is possible that intermediate I was also formed by strain DN22, but its rapid decomposition would have prevented detection.

The nitrogen and carbon stoichiometry shown in Table 1 is consistent with the scheme in Fig. 4 in which we assumed that both RDX denitration steps occurred prior to ring cleavage. Figure 4 thus represents the best explanation for the detected RDX degradation products and their time course of production and stoichiometry. Future work should focus on the actual enzymes involved in the initial attack on RDX and the role of enzymes versus abiotic mechanisms in the subsequent complex reactions that take place following ring cleavage.

Acknowledgments

We thank Louise Paquet, Carl Groom, Alain Corriveau, and Chantal Beaulieu for their technical assistance and Sylvie Beaudet for mass analysis. We also thank Nick Coleman and Sandra Trotter for providing the strain and for reviewing the manuscript. We are grateful to Sonia Thiboutot and Guy Ampleman from the Defense Research Establishment Valcartier, Quebec, Canada for providing us with the energetic chemicals.

Funding was provided by the U.S. DoD/DoE/EPA Strategic Environmental Research and Development Program (SERDP # 1213).

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