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Biotransformation Routes of Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine by Municipal Anaerobic Sludge

JALAL HAWARI,*,† ANNAMARIA HALASZ,† SYLVIE BEAUDET,† LOUISE PAQUET,† GUY AMPLEMAN,‡ AND SONIA THIBOUTOT‡

Biotechnology Research Institute, National Research Council, 6100 Royalmount Avenue, Montreal, Quebec, H4P 2R2 Canada, and Defence Research Establishment Valcartier, National Defence, 2459 Boulevard Pie XI Nord, Val Bélair, Quebec, G3J 1X5, Canada

Recently we demonstrated that hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a trimer of methylene nitramine (CH_2═N—NO_2) undergoes spontaneous decomposition following an initial microbial attack using a mixed microbial culture at pH 7 in the presence of glucose as carbon source. The present study describes whether the second cyclic nitramine octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), a more strained tetramer of CH_2═N—NO_2, degrades similarly using sludge of the same source. Part of HMX biotransformed to give products that are tentatively identified as the nitroso derivatives octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine (mNs-HMX) and octahydro-1,3-dinitroso-5,7-dinitro-1,3,5,7-tetrazocine and its isomer octahydro-1,5-dinitroso-3,7-dinitro-1,3,5,7-tetrazocine (dNs-HMX). Another fraction of HMX biotransformed, apparently via ring cleavage, to produce products that are tentatively identified as methylenedinitramine ($O_2NNHCH_2NHNO_2$) and bis(hydroxymethyl)nitramine ($(HOCH_2)_2NNO_2$). None of the above intermediates accumulated indefinitely; they disappeared to predominantly form nitrous oxide (N_2O) and formaldehyde (HCHO). Formaldehyde biotransformed further to eventually produce carbon dioxide ($^{14}CO_2$). Nitrous oxide persisted in HMX microcosms containing glucose but denitrified rapidly to nitrogen in the absence of glucose. The presence of nitrous oxide was accompanied by the presence of appreciable amounts of hydrogen sulfide, a known inhibitor of denitrification.

Introduction

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 highly energetic chemicals that are widely used in various commercial and military activities (*1*, *2*). Both cyclic nitramines are toxic (*3*, *4*) and have adverse effects on the central nervous system of mammals (*5*). The widespread contamination by these explosives necessitates that contaminated soil and groundwater be remediated. Physicochemical techniques such as incineration (*2*) and alkaline hydrolysis (*6*, *7*) are neither cost-effective nor environmentally safe. Several studies on the biodegradation of RDX and HMX have been reported (*8*−*15*), but in most cases very little information is available on the type of products and the degradation pathways of these chemicals.

Earlier McCormick et al. (*8*) proposed a degradation pathway for RDX based on the sequential reduction of the energetic chemical to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine, and hexahydro-1,3,5-trinitroso-1,3,5-triazine. The nitroso metabolites are suggested to undergo further reduction prior to ring cleavage to yield HCHO, CH_3OH, NH_2NH_2, and $(H_3C)_2NNH_2$. Most recently Hawari et al. (*16*) reported the dominant formation of CO_2 and N_2O as end products from the biodegradation of RDX with a domestic anaerobic sludge and identified at least two pathways for its degradation. One route involved reduction of the nitro groups in RDX to the nitroso derivatives, and another novel route involved a direct ring cleavage to produce methylenedinitramine ($O_2NNHCH_2NHNO_2$) and bis(hydroxymethyl)nitramine ($(HOCH_2)_2NNO_2$) (*16*). The nitramines undergo spontaneous chemical decomposition in water to nitrous oxide and formaldehyde.

It is our objective in the present study to apply a combination of analytical techniques (LC/MS, SPME/GC−MS, capillary electrophoresis/UV, and other GC methods) to identify most possible intermediate products involved in the (bio)transformation of HMX with anaerobic sludge taken from the same source used to treat RDX (*16*). A comprehensive knowledge of the degradation products of this family of cyclic nitramine explosives would help to understand their degradation pathways and, consequently, would improve our understanding of the metabolic routes to their mineralization.

Experimental Section

Materials and Methods. Commercial grade HMX (with a purity >99%) was provided by Defense Research Establishment Valcartier, Quebec, Canada. Uniformly labeled [UL-^{14}C]HMX was synthesized according to the procedure described in ref *17*. The chemical and radiochemical purity as measured in our laboratory reached 94% and 91%, respectively. The specific activity of the radioactive compound was 93.4 μCi/mmol. All other chemicals were reagent grade. The municipal sludge, which in the past proved to be an excellent source of microorganisms, particularly methanogens (*18*), was obtained from a food factory (Cornwall, ON, Canada) and was used as the exogenous source of microorganisms. The sludge was always obtained fresh and stored at 4 °C when not in use. The viability of the sludge was measured using a glucose activity test (*19*). On average, the biomass concentration of the sludge was 8 g of VSS/L (volatile suspended solid) with a 0-mV reduction potential (E_h) before incubation that dropped down to a range of −250 to −300 mV during the biodegradation of HMX. The drop in E_h is possibly related to several fermentative processes such as those leading to the production of hydrogen from other cosubstrates. The sludge was also found to contain several heavy metals (mg/kg dry weight) including iron (8300), copper (44), nickel (36), and manganese (11). A BBL dry anaerobic indicator (VWR, Canlab, ON, Canada) was placed inside the microcosm to detect air leaks to ensure anaerobic conditions.

Microcosms Description for the Degradation of HMX. In a typical setup, a serum bottle (100 mL) was charged with anaerobic sludge (5 mL) and a mineral salt medium (10 mL) composed of 0.15 g/L NaH_2PO_4, 0.45 g/L K_2HPO_4, 0.02 g/L $MgSO_4$, and 0.24 g/L Na_2SO_4. Glucose (2.1 g/L) was used to serve as a carbon source and HMX (100 mg/L) as the only

* Corresponding author telephone: (514)496-6267; fax: (514)496-6265; e-mail: jalal.hawari@nrc.ca.
† National Research Council.
‡ National Defence.

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extraneous N-source for the degrading microorganisms using a final volume of 50 mL. However, we expect the sludge to contain other organic nitrogenous compounds that could also serve as a nitrogen source to the degrading microorganisms. We used high concentrations of HMX in an attempt to generate sufficient amounts of metabolites for detection. To account for an insoluble or suspended portion of HMX [water solublity is ca. 5 mg/L (*4*)], all the content of the microcosm was extracted in acetonitrile. Some microcosms were supplemented with [UL-^{14}C]HMX (100 000 dpm) and then fitted with a small test tube containing 1.0 mL of 0.5 M KOH to trap liberated carbon dioxide ($^{14}CO_2$). The headspace in each microcosm was flushed with nitrogen-free argon gas to maintain anaerobic conditions and then sealed with butyl rubber septa and aluminum crimp seals to prevent the loss of CO_2 and other volatile metabolites. For the analysis of N_2 and N_2O, sampling of the gaseous products from the headspace was performed using a gas-tight syringe. Two control microcosms were prepared: one contained the sludge without HMX, and the second contained HMX and an autoclaved sludge. Each microcosm was wrapped with aluminum foil to protect the mixture against photolysis. Microcosms with [UL-^{14}C]HMX were routinely sampled (on hourly and/or daily basis) for the determination of $^{14}CO_2$ in the KOH trap using a Packard, Tri-Carb 4530 liquid scintillation counter (model 2100 TR, Packard Instrument Company, Meriden, CT).

Analysis of HMX and Its Intermediate Products. The analytical part was worked out as described in ref *16*. Briefly, acetonitrile (50 mL) was added to the HMX-treated culture medium and mixed for few minutes at room temperature. Aliquots (1 mL) from the above treated culture medium were filtered through a 0.45-μm pore-size Millex-HV filter. The filtered culture medium (50 μL) was analyzed for the remaining HMX using a Waters HPLC system (Waters Chromatography Division) composed of a model 600 pump, an auto-injector (model 717 plus), and a Model 996 photodiode array detector. Detection and quantification was accomplished at λ 254 nm (*16*).

The nitroso derivatives and ring cleavage products of HMX were analyzed using a Micromass Plattform benchtop single quadrupole mass detector fronted by a Hewlett-Packard 1100 series HPLC system connected to a Supelcosil LC−CN column (25 cm × 4.6 mm; 5 μm particle size). The solvent system consisted of a methanol/water gradient at a flow rate of 1 mL/min. Analyte ionization was done in a negative electrospray ES(−) ionization mode producing mainly $[M - H]^-$. Further details on the method can be found in ref *16*.

Formaldehyde was detected as its oxime derivative using an SPME fiber coated with poly(dimethylsiloxane)/divinylbenzene (Supelco) and the derivatizing agent *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine as described by Martos and Pawliszyn (*20*) and Hawari et al. (*16*). Formic acid, HCOOH, was analyzed by capillary electrophoresis (CE) and UV detection using a Hewlett-Packard ^{3D}HPCE system consisting of a photodiode array detector following the procedure that was described by Chen et al. (*21*) and developed latter for the analysis of RDX metabolites (*16*). Formic acid was initially detected and quantified at 340 nm (210 nm reference) with a limit of detection of 200 μg/L. The identity of HCOOH as a degradation product of HMX was confirmed using [UL-^{14}C]HMX and by collecting the product by HPLC fractionation for subsequent radioactivity measurement.

Measurement of HMX Gaseous End Products N_2O, N_2, and $^{14}CH_4$. A SRI 8610 GC (INSUS Systems Inc.) connected to a Supelco Porapack Q column (2 m) and coupled with either a electron capture detector (ECD) (330 °C) for the detection of N_2O or a radioactivity detector (RAM) for the detection of $^{14}CH_4$ were used. The gaseous products from the headspace of the culture medium were sampled using a gas-tight syringe for subsequent injection inside the GC using helium as a carrier gas (21 mL/min) at 60 °C. Gas identification was confirmed by comparison with reference materials. The detection limit for RAM and ECD was 150 dpm and 12 ppm, respectively. Gaseous nitrogen was analyzed with an HP GC connected to Supelco Chomosorb 102 column coupled with a thermal conductivity detector (TCD).

Attempted Analysis of Hydrazine, Dimethyl Hydrazine, and Other Nitrogen Species Such as NH_4^+ and NO_2^-. Ion chromatography (Dionex model DX-500 ion chromatograph system) consisting of a GP40 gradient pump and coupled with electrochemical detector (pulsed detection mode) was used to analyze hydrazines as described by Larson and Strong (*22*). Samples (25 μL) from the culture medium were injected into a Hamilton PRP-X200 (250 mm × 4.1 mm × 10 μm) analytical cation-exchange column using 30% methanol in 4 mM nitric acid at a flow rate of 1 mL/min. Standards of hydrazine and dimethylhydrazine were employed for confirmation. A more sensitive technique (SPME/GC−MS) with a picogram detection limit was also employed to confirm the absence of hydrazines as HMX metabolites. Ammonium cation was analyzed for in the aqueous phase of the culture medium using an SP 8100 HPLC system equipped with a Waters 431 conductivity detector and a Hamilton PRP-X200 (250 mm × 4.1 mm × 10 μm) analytical cation-exchange column using 30% methanol in 4 mM nitric acid at a flow rate of 0.75 mL/min. The NO_2^- was analyzed using the CE as described by Okemgbo et al. (*23*) using sodium borate (25 mM) and hexamethonium bromide (25 mM) as an electrolyte at pH 9.2.

Results and Discussion

HMX Nitroso Metabolites. The LC/MS ES(−) of HMX after treatment with domestic anaerobic sludge over a period of 6 days (Figure 1) showed several peaks with deprotonated molecular mass ions $[M - H]$ appearing at m/z 279, 263, and 263 Da, matching molecular mass formulas of $C_4H_8N_8O_7$, $C_4H_8N_8O_6$, and $C_4H_8N_8O_6$, respectively. Using the above obtained deprotonated molecular mass ions $[M - H]$, the peaks are tentatively identified as mono nitroso, octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine [279 Da] (mNs-HMX), and two isomers of the dinitroso derivative, (octahydro-1,3-dinitroso-5,7-dinitro-1,3,5,7-tetrazocine) [263 Da] and (octahydro-1,5-dinitroso-3,7-dinitro-1,3,5,7-tetrazocine) [263 Da] (dNs-HMX). We did not detect any of the trinitroso derivative, although this compound was formed as a major product during biodegradation of HMX in soil slurry (30% w/v) using the same type of sludge (*24*).

None of the above nitroso derivatives accumulated in the system indefinitely. They biotransformed with no clear indication of their actual fate. We did not observe any hydroxylamino derivatives (HOHN−HMX) described earlier during biodegradation of RDX with anaerobic sludge by McCormick et al. (*8*). Other studies reported the formation of nitroso derivatives during biodegradation of RDX and/or HMX with either soil isolates (*Providencia rettgeri*, *Citrobacter freundii*, *Morganella morganii*) of the *Enterobacteriaceae* family (*11*) or consortia of a horse manure under O_2-depleting conditions (*12, 13*).

Ring Cleavage Intermediates. The LC/MS chromatogram of the sludge-treated HMX also showed the initial presence of several other peaks and presumed to be ring cleavage products of the original HMX molecule (Figure 1). For instance, one LC/MS peak showed a deprotonated molecular mass ion $[M - H]$ at 135 Da, matching a molecular mass formula of $CH_4N_4O_4$ (MW 136 Da). Another characteristic mass ion was detected at m/z 61 Da, representing the fragment mass ion -$HNNO_2$. Whereas another LC/MS peak

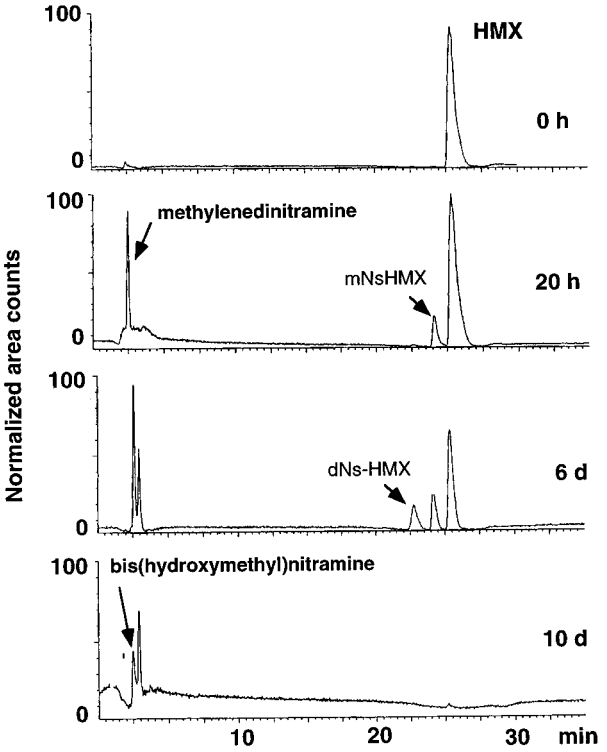

FIGURE 1. LC/MS (ES−) time course of biotransformation of HMX (100 mg/L) with a domestic anaerobic sludge in the presence of glucose as a carbon source at pH 7.0. The *y*-axis represents normalized area counts.

showed a deprotonated molecular mass ion [M − H] at 121 Da matching a molecular mass formula of $C_2H_6N_2O_4$ (MW 122 Da). Other relevant mass ions included one at *m*/*z* 139 Da representing ([M − H] + H_2O). We work out the identities of both peaks by comparing their chromatographic and mass data (retention times and mass ions) with those obtained earlier from the degradation of RDX with sludge taken from the same source (*16*). Both RDX and HMX gave the two products at the same retention time (3.09 and 3.88 min, respectively). In the case of RDX, the two peaks were tentatively identified using ring-labeled [^{15}N]RDX as the ring cleavage products methylenedinitramine ($O_2NNHCH_2NHNO_2$) and bis(hydroxymethyl)nitramine [$(HOCH_2)_2NNO_2$], respectively. Likewise, we tentatively identified the two LC/MS peaks observed during treatment of HMX with the sludge as the ring cleavage products methylenedinitramine and bis(hydroxymethyl)nitramine, respectively. This is not surprising in view of the fact that the two cyclic nitramines RDX and HMX are structurally similar. Both compounds are cyclic oligomers (trimer and tetramer, respectively) of the same chemical unit methylenedinitramine, $CH_2{=}N{-}NO_2$ [$(CH_2NNO_2)_3$ for RDX and $(CH_2NNO_2)_4$ for HMX].

Furthermore, we detected several other LC/MS peaks including one that showed a [M − H] at *m*/*z* 121 Da, matching a molecular mass formula of $CH_6N_4O_3$ (MW 122). Other relevant mass ions in this peak included one at *m*/*z* 139 Da representing the solvent adduct ([M − H] + H_2O) and another at *m*/*z* 46 Da, representing the fragment mass ion -NO_2 (*m*/*z* 46 Da). The peak was tentatively identified as *N*-nitramino-*N*′-hydroxylaminomethylamine ($O_2NHNCH_2NHNHOH$). We are not sure at the present time whether the product is a reduced form of the previously formed methylenedinitramine ($O_2NHNCH_2NHNO_2$) or is a ring cleavage product of the hydroxylamine derivative of HMX (HOHN−HMX). McCormick et al. (*8*) postulated the presence of HOHN−RDX during biotreatment of RDX with anaerobic sludge but did not observe them.

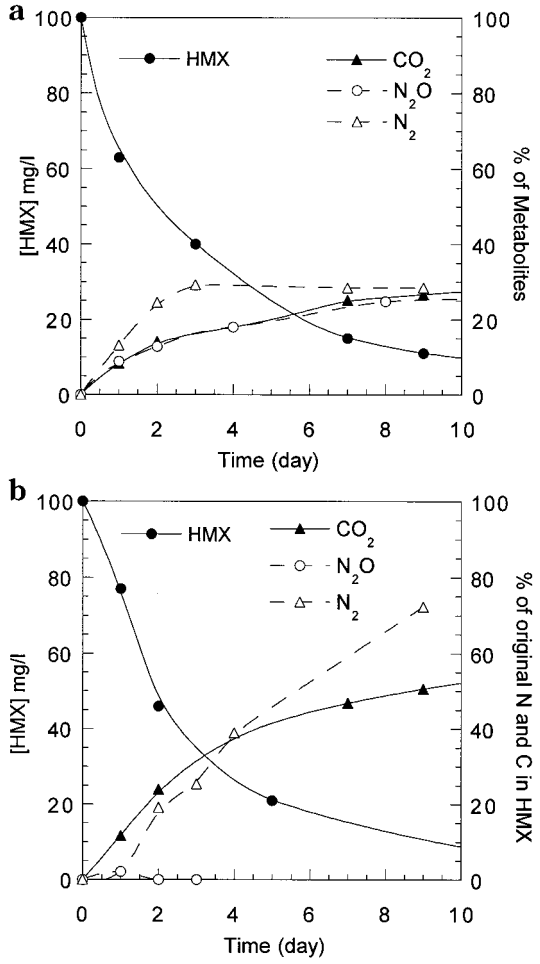

FIGURE 2. Time course study for the biotransformation of HMX with the domestic anaerobic sludge (a) in the presence of glucose and (b) in the absence of glucose.

None of the above HMX ring cleavage products accumulated indefinitely. They all transformed and produced predominantly formaldehyde (HCHO) and nitrous oxide (N_2O). The presence of formaldehyde as HMX degradation product was confirmed by the detection of H^{14}CHO using [UL-^{14}C]HMX. We were also able to detect formic acid and confirmed its presence by the detection of H^{14}COOH. Eventually HCHO (bio)transformed to carbon dioxide (detected as $^{14}CO_2$).

Neither N_2O nor $^{14}CO_2$ was observed in controls containing either the buffer and HMX or HMX and dead biomass. Roughly 40% of total HMX initial concentration (100 mg/L) was attributed to mineralization ($^{14}CO_2$) after 40 d of incubation. Furthermore, methane ($^{14}CH_4$) was detected and confirmed by the use of uniformly labeled [UL-^{14}C]HMX. Neither hydrazine nor dimethylhydrazine [$(CH_3)_2NNH_2$] reported earlier by McCormick et al. (*8*) was observed in either the present study or the previously described RDX study (*16*).

On the other hand, we detected nitrogen when HMX was treated with the sludge under a blanket of argon. Earlier we confirmed that RDX biodegradation with the same sludge gave N_2O and N_2 as end products. The formation of the two gases was confirmed by using ring-labeled [^{15}N]RDX and the detection of $^{15}N^{14}NO$ (*m*/*z* 45 Da) and $^{15}N^{14}N$ (*m*/*z* 29 Da) (*16*, *25*). No ^{15}N-labeled HMX was available at the time of the present experiment, but the presence of N_2O and N_2 was confirmed by comparing their GC retention times with reference standards and also by their absence in the controls (described above).

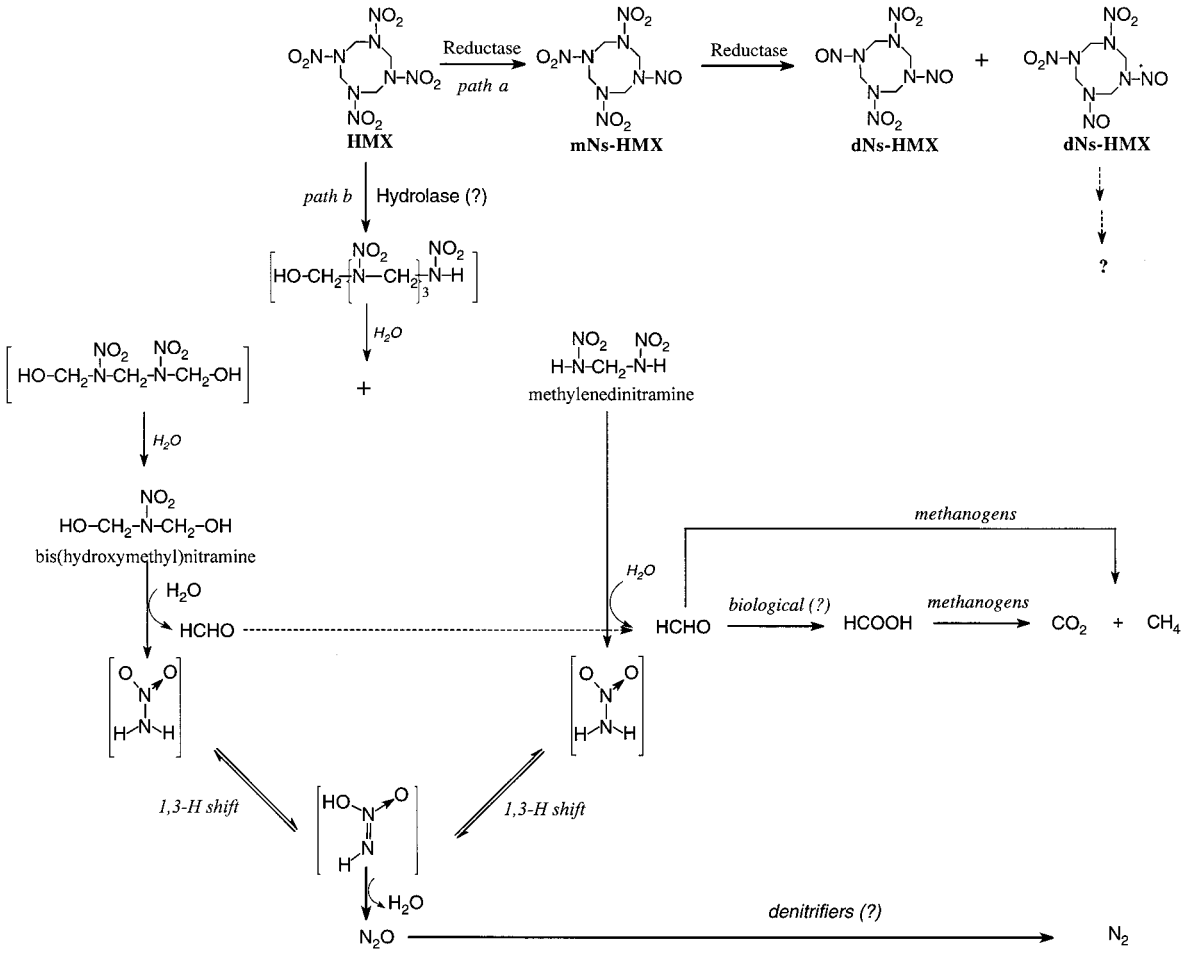

FIGURE 3. Potential biodegradation routes of HMX during treatment with anaerobic sludge. Path a: Reduction via nitroso route who were later removed from the system without identifying their products (?). Path b: Ring cleavage followed by competing chemical and biochemical transformations. A square bracket indicates unidentified product whereas a question mark indicates potential presence that requires further experimental verification. In the case of RDX, McCormick et al. (*8*) reported the formation of hydrazines via this route.

Time Course Study and the Degradation Pathway. Figure 2, panels a and b, represents time course studies for the disappearance of HMX and the appearance of N_2O, N_2, and CO_2 in the presence and absence of glucose, respectively. Roughly 50% of the total nitrogen content of reacted HMX (90%) was detected as gaseous nitrogen and nitrous oxide. Using solubility data, another 4% of N_2O could be accounted for as a dissolved fraction in the aqueous phase (*26*). Formaldehyde did not accumulate, and its disappearance was accompanied by the formation of formic acid, HCOOH, methane (CH_4), and carbon dioxide (CO_2). The concentrations of HCHO and HCOOH were not high enough and thus are excluded from Figure 2. This is in contrast to RDX where we successfully measured HCHO and the ring cleavage product methylendinitramine with time (*16*). This is possibly caused by a lower biological reactivity of HMX as compared to RDX. It is known that HMX is less water soluble (5 mg/L) than RDX (40 mg/L) (*4*) and is chemically more stable (*27*).

Furthermore, controls containing either HMX in a phosphate buffer (pH 7) in the absence of the sludge or HMX in a killed biomass did not give any of the previously described nitroso or ring cleavage products, indicating the essential role of enzymatic reactions on HMX. We expect the domestic sludge used in the present study to contain a microbial community capable of producing several enzymes including nitroreductases and hydrolases. Recently Kitts et al. (*28*) reported the degradation of RDX by the enteric bacteria *Morganella morganii* via the oxygen-insensitive type I nitroreductase. Reduction of the -NO_2 group in HMX by the same type of nitroreductase would produce the corresponding nitroso derivative. Subsequent reduction of the resulting nitroso group might produce the HOHN−HMX derivative, which was earlier implicated as a prerequisite entity prior to ring cleavage in the RDX pathway described by McCormick et al. (*8*) (Figure 3, path a). Neither the present HMX study nor the earlier study on RDX revealed the presence of these hydroxylamino derivatives (*16*).

On the other hand, we speculate that the presence of a hydrolase enzyme in the sludge might cleave a C−N bond in HMX to give the primary nitramine product $HOCH_2$-[CH_2-NNO_2]$_3$-$NHNO_2$ containing two reactive terminal functional groups, -CH_2OH and -$NHNO_2$ (Figure 3, path b). Such compounds are not stable in water, and their continued spontaneous decomposition (enzymatic or chemical) would eventually produce nitramide (NH_2NO_2) and HCHO (Figure 3, path b). Hydroxyalkylnitramines are known to be unstable in water and exist as equilibrated mixtures with their dissociated products HCHO and NH_2NO_2 (*29*−*31*). The two metabolites methylenedinitramine and bis(hydroxymethyl)-nitramine do not represent the only HMX ring cleavage products in our study. There are possibly other undetected products that were produced in trace amounts. However, the detection of the above two products are particularly important because, as we described above, we were able to identify them using the chromatographic and mass data obtained in our earlier study with RDX (*16*).

Once formed, the nitramide molecule can undergo spontaneous hydrolytic decomposition to produce nitrous

oxide, N_2O (*6*, *29*−*31*) (Figure 3, path b). The chemical decomposition of nitramide to nitrous oxide is well established and is suggested to initially involve a 1,3-H shift from nitrogen to oxygen followed by hydrolysis as shown in Figure 3 (*32*). Using ring-labeled [^{15}N]RDX, we previously found that one nitrogen atom in N_2O originated from the -NO_2 group and the second one originated from the ring (*16*).

Whereas the formation of nitrogen during degradation of HMX was considered to be biological because the gas only appeared in microcosms that contained HMX and live sludge (incubated under an atmosphere of argon), possibly unidentified denitrifiers in the sludge caused the transformation of nitrous oxide to nitrogen gas. St. John and Hollocher (*33*) and Garber and Hollocher (*34*) reported the formation of nitrogen as a metabolite from N_2O under anaerobic conditions using *Pseudomonas aeruginosa*. Also it has been reported that facultative and obligate anaerobic bacteria can reduce N_2O to nitrogen (*35*). Also we found that microcosms that contained glucose as a carbon substrate during HMX treatment with the anaerobic sludge showed both nitrous oxide and nitrogen gas as major products (Figure 2a). Unlike microcosms that did not receive glucose, nitrogen concentrations increased drastically and nitrous oxide was beyond the detection limit (Figure 2b). As one of our reviewers suggested, we expected to observe more reduction of N_2O to N_2 by denitrifies in the presence of excess e-donors such as glucose. Interestingly we found relatively high amounts of hydrogen sulfide in microcosms that received glucose. In contrast, only a trace amount of the sulfide was detected in the absence of glucose. It has been reported that sulfides are inhibitory to denitrification processes, and thus their presence might have contributed to the inhibition of denitrification, leading to the accumulation of N_2O (*36*, *37*) (Figure 2a). Furthermore, VSS and the heavy metal cationic species (Fe, Mn, Ni, and Cu) in the sludge, depending on their oxidation states, might have also served as source of electron donors for the denitrification observed in the absence of glucose (Figure 2b).

Formic acid (detected as H^{14}COOH) was detected only in trace amounts that we were unable to measure at accurate concentration with time. We are not sure at the present time how HCOOH is produced in our system, although it has been reported that the acid can be formed as a ring cleavage product following the alkaline hydrolysis of cyclic nitramines. Formaldehyde, another detected HMX product, can also produce HCOOH under alkaline conditions (Canizarro reaction) (*6*, *7*), but the pH during our study stayed near neutral. On the other hand, we speculate that methanogens in the sludge biotransformed formic acid into methane and carbon dioxide (Figure 2). Previously, a sludge of the same origin to the one used here proved to contain several consortia including methanogens (*18*).

Presently we are unable to determine the extent of the nitroso route (Figure 3, path a) relative to the ring cleavage one (Figure 3, path b) for HMX degradation. Also, we did not have any direct evidence of other degradative mechanisms such as reductive denitration. In the case of RDX, nonbiological reductive denitration via a bimolecular elimination (E_2) of HNO_2 yields the unstable intermediate 3,5-dinitro-1,3,5-triazacyclohex-1-ene, which undergoes spontaneous decomposition with a rate constant that is 10^5 times higher than that of RDX itself (*38*). On the other hand, the initial cleavage of an external N−NO_2 or C−H bonds of HMX would destabilize inner C−N chemical bonds (<5 kcal/mol), forcing the molecule to undergo rapid autodecomposition to N_2, N_2O, HCHO, and HCOOH (*32*). The above analysis might help explain why most reported biodegradation studies of cyclic nitramines (*25*) did not describe more than the removal of the explosive and in more specific cases the initial denitration processes as diagnostic tools to describe biodegradation (*39*). Often poor knowledge of intermediate metabolites are described, and without such knowledge the (bio)degradation pathways of RDX and HMX will stay unknown.

In summary, the present study shows that unidentified anaerobic microorganisms from a domestic sludge can convert HMX via at least two independent mechanisms: one involved the familiar reduction of -NO_2 to form the corresponding nitroso derivatives, and the other involved a direct ring cleavage. The present observation confirms the earlier results obtained with RDX using sludge of the same source (*16*). Since both explosives are frequently found together at contaminated sites, the observed similarity in their degradation patterns might help future efforts for their joint successful remediation. Certainly further investigation is needed to learn more about intermediate products of both RDX and HMX and the enzymes that produce them.

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