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THE ANAEROBIC BIOTRANSFORMATION OF RDX, HMX, AND THEIR ACETYLATED DERIVATIVES

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AND
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This report describes the isolation and identification of the products formed from the biotransformation of RDX and HMX and their acetylated derivatives. The reactions occurred only under anaerobic conditions. Products were identified as the mono- and dinitroso derivatives, as well as formaldehyde and methanol. Traces of hydrazine and 1,1-dimethylhydrazine were detected.

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PREFACE

This report describes studies undertaken to ascertain the fate of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), its homolog, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and their N-acetylated derivatives when subjected to biodegradation by mixed cultures under batch conditions. The nature of the biotransformation products and the mutagenic properties of the products and starting materials have been determined.

Microbiological treatment has been proposed for the elimination of pollutants present in effluents from the manufacture of RDX and its cogeners. The data obtained in this study are needed for the development of the proposed process and to make sure that the waste waters receive an adequate treatment to meet environmental standards. [illegible]

This work was supported by the US Army Toxic and Hazardous Materials Agency under project number 1L162720D048, Task W-20.

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THE ANAEROBIC BIOTRANSFORMATION OF RDX, HMX, AND THEIR ACETYLATED DERIVATIVES

INTRODUCTION

During the manufacture of RDX* [121-82-4] and its homolog, HMX [2691-41-0], low concentrations of these compounds as well as similar levels of the N-acetylated derivatives of these compounds, AcRDX [14168-42-4] and AcHMX [13980-00-2], may be discharged into the environment at concentrations that may be toxic. Microbiological treatment of effluents from the manufacture of RDX has been proposed as a means of eliminating these and other components which might otherwise constitute a threat to the environment. This investigation has been undertaken to determine whether RDX and its cogeners can be biodegraded, the conditions for efficient transformation, and the nature of any biotransformation products and their mutagenic properties, if any. Previous studies on the biotransformation of RDX have been reported[1-4].

Little information is available in the literature on the biotransformation of HMX and the two acetylated derivatives. Spanggord et al.[5] reported the formation of mono-, di-, tri-, and tetranitroso-HMX during the anaerobic biotransformation of HMX. The evidence was based on mass spectral data from metabolites isolated from culture media by HPLC. In this study we report similar findings with HMX, AcRDX, and AcHMX.

MATERIALS AND METHODS

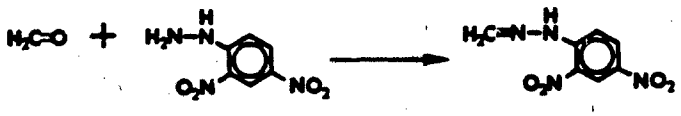
Cultures and Media: Biodegradation studies were carried out in nutrient broth (8 g/L) (Difco Laboratories, Detroit, MI). For the anaerobic studies the vessels were filled to approximately 95% of their capacity, inoculated with anaerobic sewage sludge (obtained from the Nut Island Sewage Treatment Plant, Boston, MA), and incubated as stationary cultures at 37°C. The inocula were prepared by diluting the sludge with two volumes of distilled water and filtering through glass wool. A 2% (v/v) inoculum was used.

Due to the relative insolubilities of the compounds used as substrates, weighed amounts were added to empty flasks and dissolved in a small amount of acetone. The acetone was evaporated by a stream of N_2, leaving a thin film of material on the inside surface of the flask. The culture medium was deoxygenated by boiling, poured into the flask containing the deposit of material, and stirred vigorously until solution was attained. The medium was allowed to cool to 35°C to 40°C before inoculation. Samples were removed from the culture vessels after various periods of incubation, centrifuged, and the supernatant solutions filtered through 0.22 u membrane filters for HPLC analysis.

*Abbreviations used: hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX); hexahydro-1-N-acetyl-3,5-dinitro-1,3,5-triazine (AcRDX, TAX); octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX); octahydro-1-N-acetyl-3,5,7-trinitro-1,3,5,7-tetrazocine (AcHMX, SEX); 3,7-dinitro-1,3,5,7-tetraza-bicyclo-[3.3.1]-nonane (DPT); 1-acetoxy-methyloctahydro-3,5,7-trinitro-1,3,5,7-tetrazocine (acetoxy-MeHMX); ocatahydro-1,3,5-trinitro-7-nitroso-1,3,5,7-tetrazocine (mononitroso-HMX); 1-acetyloctahydro-3,7-dinitro-5-nitroso-1,3,5,7-tetrazocine (mononitroso-AcHMX).

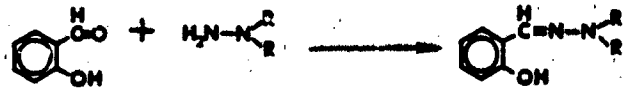
Liquid Chromatography: For routine analysis of reaction products, samples of clarified culture medium filtrate (CCMF) were injected without further treatment into a Model 6000A liquid chromatograph (HPLC), equipped with a 3.9 mm x 30 cm uBondapak C_{18} column and a Model 450 variable wavelength detector (Waters Associates, Inc., Milford, MA). For analytical separations the solvent was 10% methanol/90% water. In some cases 20% acetonitrile/80% water was used for separations of HMX reaction mixtures. The materials were detected at 230 nm. For isolation and purification purposes CCMF was continuously extracted for 24 to 48 hr with either chloroform or methylene chloride, the solvent extracts evaporated to dryness, the residue taken up in acetone, methanol or acetonitrile, and multiple injections made onto a "semiprep" 7.8 mm x 30 cm C_{18} column. For the collection of individual peaks from AcRDX and AcHMX CCMF the solvent system was 20% acetonitrile/80% water, and for HMX CCMF, the solvent system was 30% acetonitrile/70% water. Single peaks were collected separately, concentrated to small volumes and the retention times reaffirmed on the smaller analytical column. These samples were used for further analytical tests.

Determination of Formaldehyde: Determinations of formaldehyde during the early part of the study used the chromotropic acid method described by Grant[6]. An HPLC method was developed based on a method described by Beasley et al.[7] The method involved the addition of 1.0 mL of a filtered 0.1% solution of 2,4-dinitrophenylhydrazine in 2 N HCl, to 1.0 mL of CCMF contained in a small test tube. The contents were mixed and allowed to react for 10 min, and 10 to 20 uL were injected directly into the HPLC and chromatographed at a flow rate of 2.5 mL/min using 60% methanol in water. The formyl derivative was detected at 340 nm (see Fig. 1). The reaction is shown by equation 1.



(1)

Determination of Hydrazines: Earlier estimations of hydrazines were qualitative determinations by thin layer chromatography (TLC). An HPLC method was developed for the determination of hydrazine and 1,1-dimethylhydrazine, based on the method described by Abdou et al.[8] To a small test tube were added: 1.0 mL of hydrazine sample in ethanol, 0.1 mL of glacial acetic acid, and 0.1 mL of 0.2 M salicylaldehyde in ethanol. The contents were mixed well, the tube was heated in a 60°C water bath for 20 min., 2.8 mL of ethanol were added to bring the total volume to 4.0 mL, and 10 uL samples were injected directly into the HPLC for analysis. The solvent system for hydrazine was 70% methanol in water and for 1,1-dimethylhydrazine the solvent was 50% methanol in water. Both were detected at 293 nm. The reaction is shown by equation 2, where R = H or R = CH_3.



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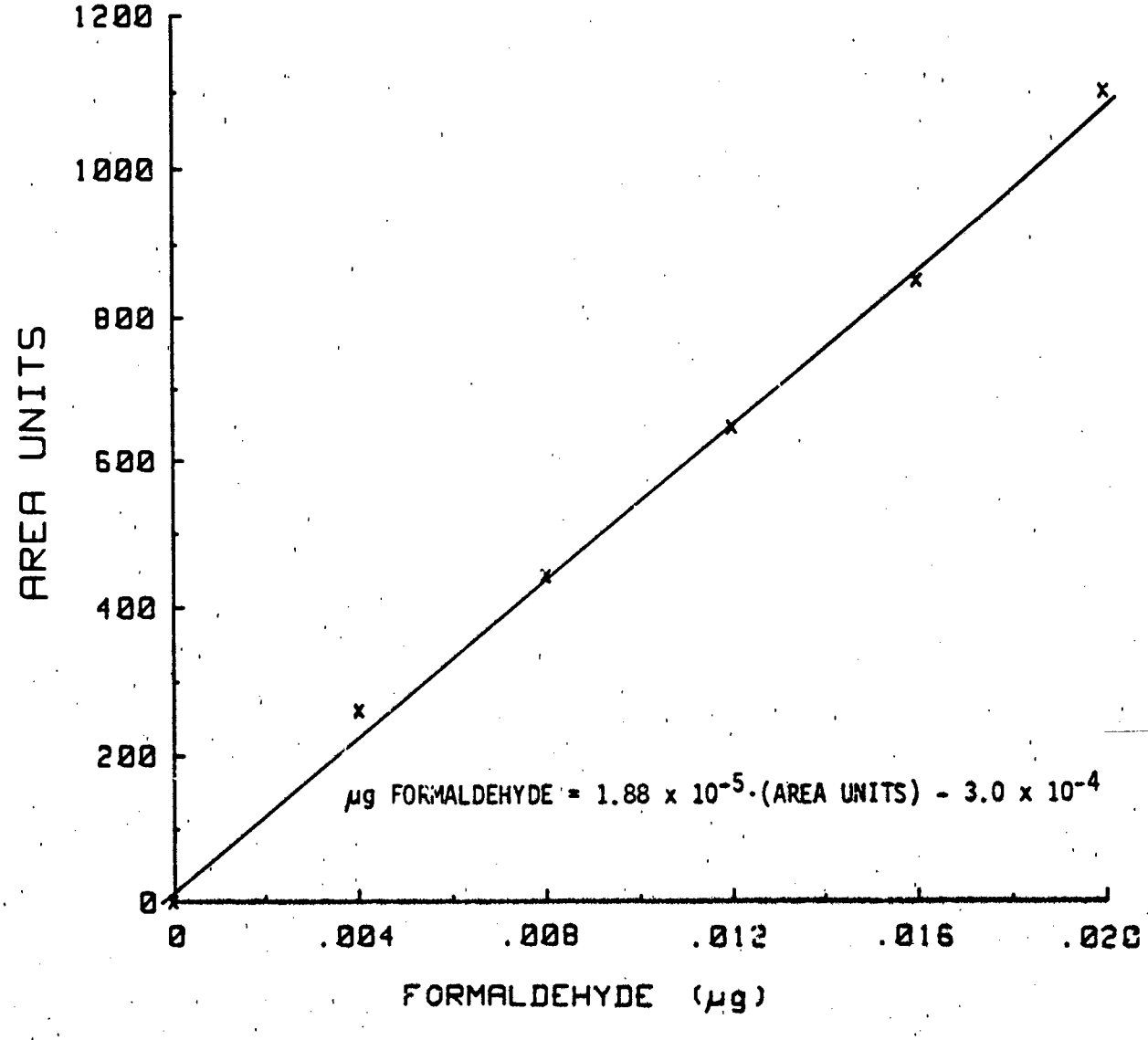


Figure 1. Standard curve for the determination of formaldehyde.

A typical standard curve of hydrazine is shown in Fig. 2. The curve is linear up to about 0.5 ug after which there is some leveling off. The assay for 1,1-dimethylhydrazine is fairly linear up to about 1.0 ug (Fig. 3). The sensitivity of the method is approx. 50 ng.

Preparation of DPT[1] [949-56-4]: This intermediate was prepared from hexamethylenetetramine (HMT) by the method of Bachmann et al.[9] (see Fig. 4). The crude product sintered at 181°C; mp 207-208°C (dec.); Bachmann et al.[10] obtained mp of 198°C (dec.) for this product but a mp as high as 216°C has been reported for recrystallized samples. The product gave a single spot on thin layer chromatography (TLC) developed with nitromethane/toluene (1:1). Eastman Chromagram silica gel sheets with fluorescent indicator were used for this and subsequent preparations. Visualization of the chromatograms was by fluorescence quenching. The infrared (IR) and ^{13}C nuclear magnetic resonance (NMR) spectra were consistent with the structure. This intermediate was used without purification for the following syntheses.

Preparation of Acetoxymethyl-HMX [5755-28-2]: The foregoing intermediate was prepared by treatment of DPT with acetic anhydride in the presence of a catalytic amount of nitric acid according to the method of Bachmann and Jenner[10]. The crude product begins to sublime at 137°C; mp 157-160°C. Bachmann and Jenner[10] report mp 152°C for the crude product. The IR spectrum was consistent with the structure.

Preparation of Mononitroso-HMX [2755-28-2]: Mononitroso-HMX was prepared by the treatment of acetoxymethyl-HMX with nitrosyl chloride and acetic anhydride according to the method of Bachmann and Deno[11]. The recrystallized product had mp 236-238°C (lit. 236-237°C). The gas chromatography/mass spectrometry (GC/MS), IR and ^{13}C NMR spectra were consistent with the structure.

The preparation of mononitroso-HMX was also carried out in a single step starting with DPT. Into a solution of 3 mL of NOCl and 10 mL of acetic anhydride was suspended 0.5 g (2.29 mM) of DPT with stirring. Nitric acid (0.1 mL of 99%) was added and the mixture was stirred at room temperature for one hour. An additional 0.2 mL of nitric acid was then added, and the reaction mixture stirred for an additional hour. At the end of this time 30 mL of water were added with continued stirring. A white precipitate of NO-HMX formed which was isolated by filtration and allowed to dry at room temperature (0.213 g, 31.5% yield). The crude product was homogeneous by HPLC and TLC analysis; its chromatographic behavior was identical to the sample prepared by the method of Bachmann and Deno[11]. The GC/MS and IR spectra of the mononitroso-HMX prepared by this procedure were identical with those of the authentic sample. The recrystallized product had mp 236-238°C.

Preparation of Mononitroso-AcHMX [13980-02-4]: The preparation of mononitroso-AcHMX by the procedure of Bachmann and Deno[11] (see Fig. 4) did not give the expected product. The starting DPT did not dissolve in the reaction mixture as reported and was recovered unchanged. We surmised that our reaction did not proceed because of the absence of catalytic impurities in the commercial nitrosyl chloride (Matheson) which we used. Bachmann and Deno[11]

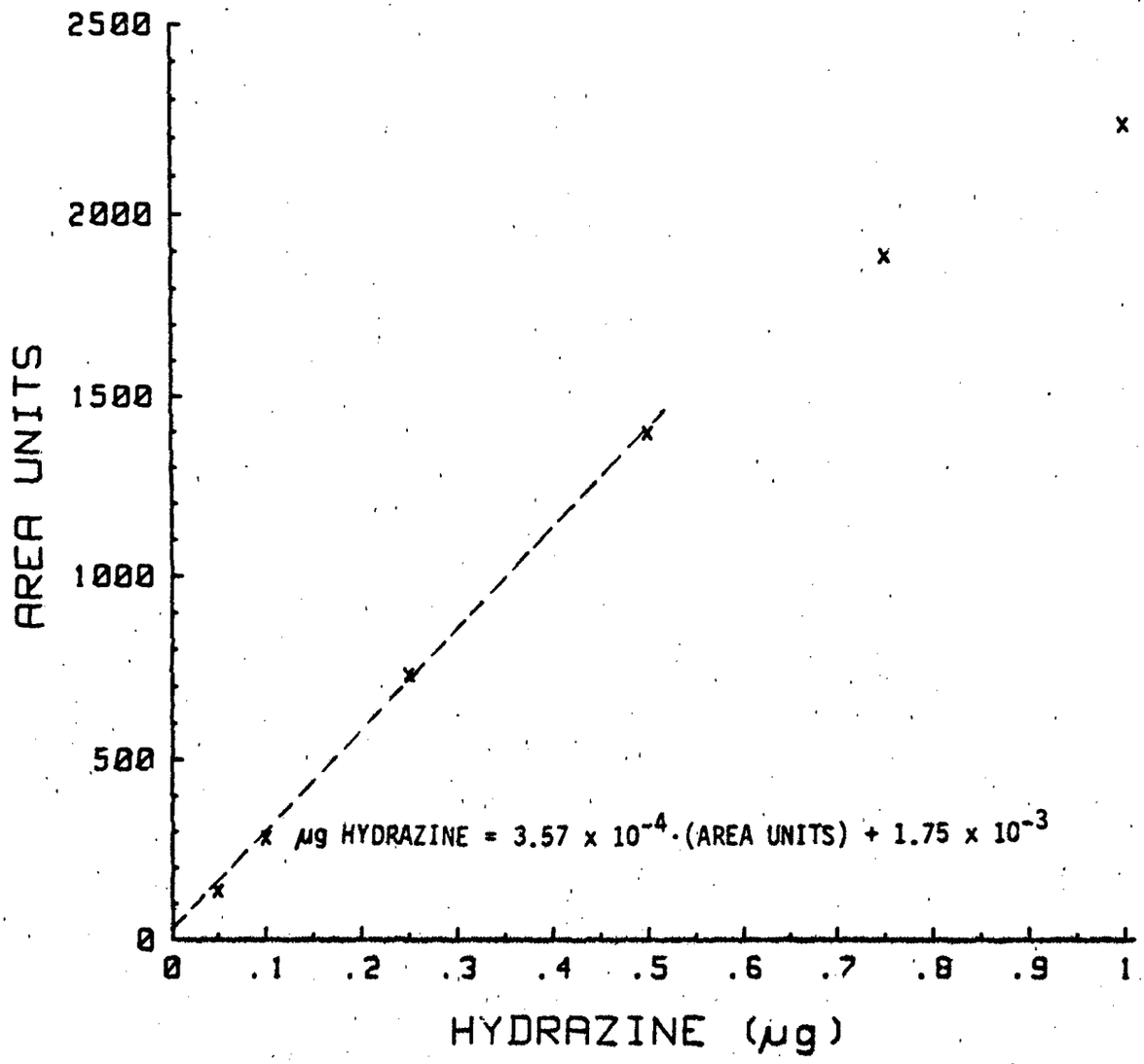


Figure 2. Standard curve for the determination of hydrazine.

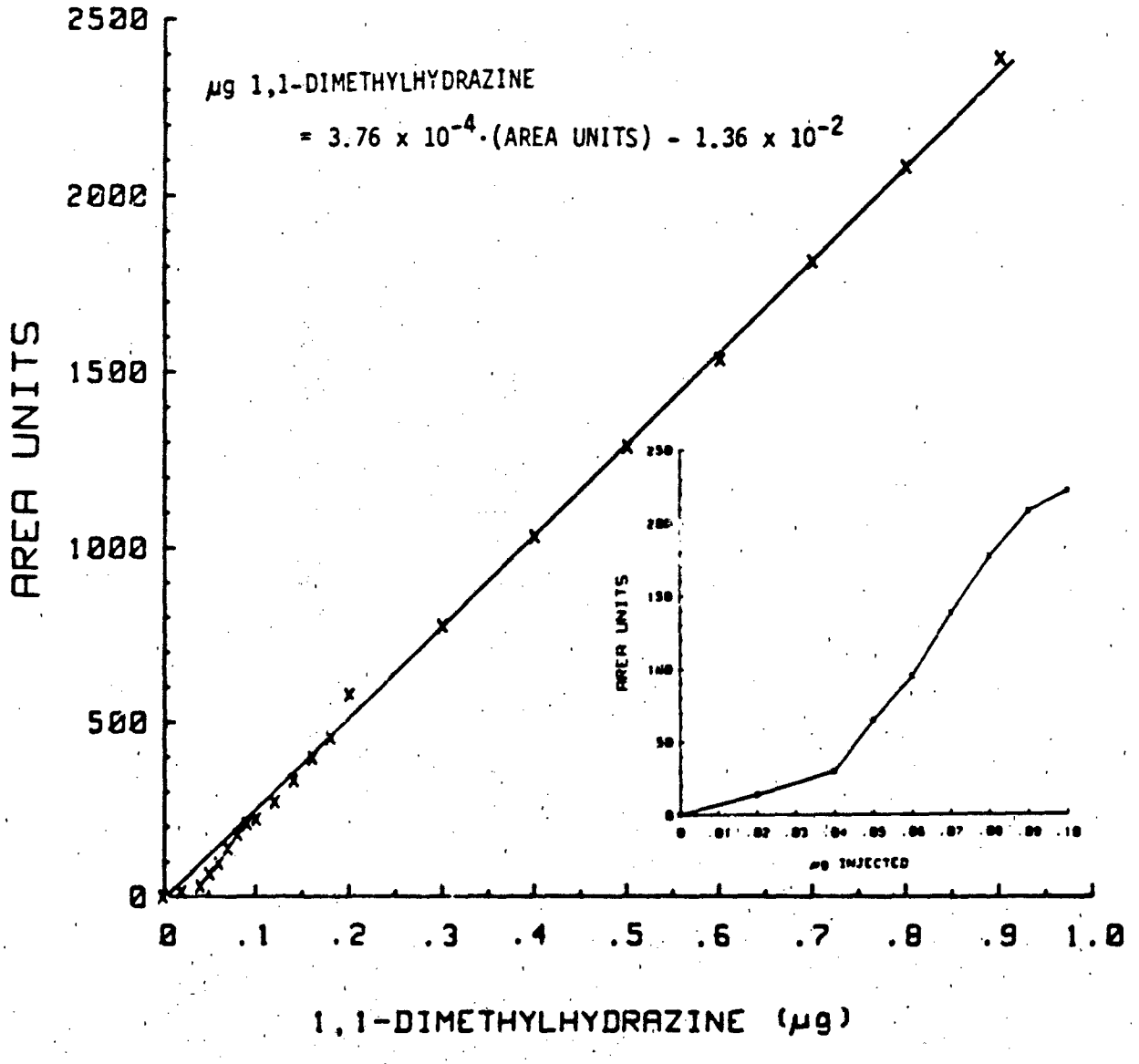


Figure 3. Standard curve for the determination of 1,1-dimethylhydrazine.

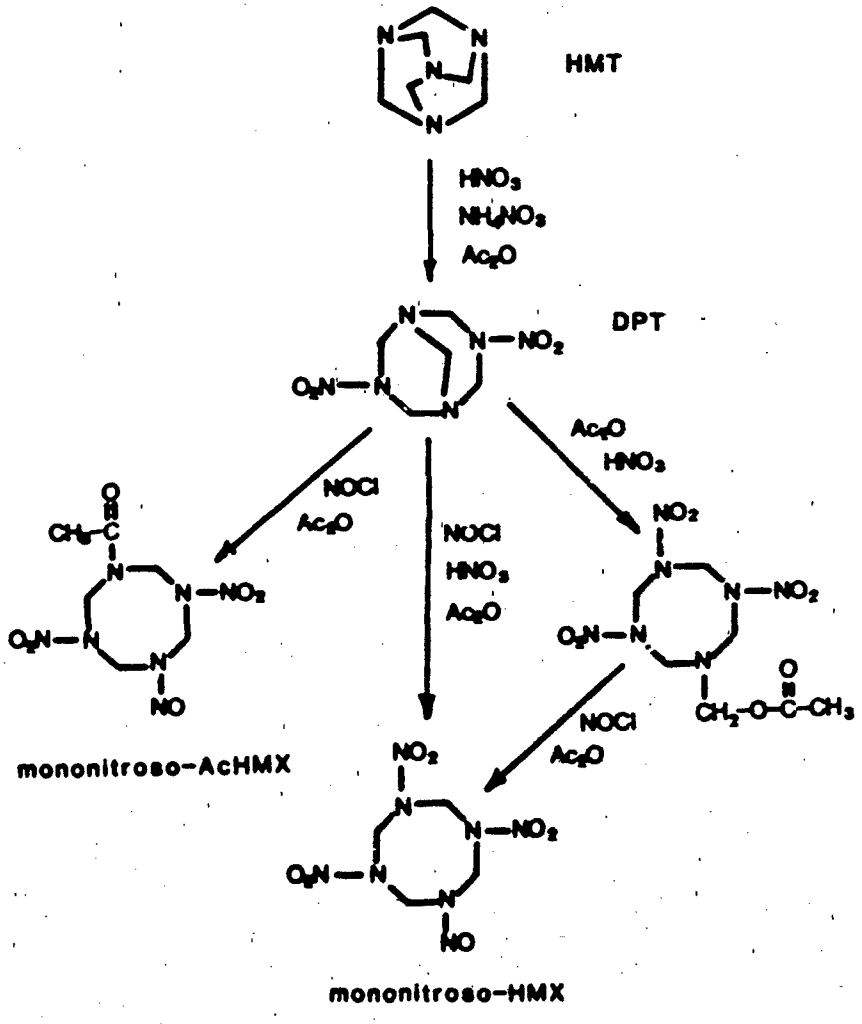


Figure 4. Preparation of mononitroso-HMX and mononitroso-AcHMX.

had prepared their nitrosyl chloride by the reaction of nitrosyl sulfuric acid with sodium chloride[12]. This product probably contained acidic impurities which catalyzed the formation of the product. We attempted to catalyze this reaction by the addition of acids, such as nitric, sulfuric and acetic in various concentrations. The following is a modification of the Bachmann and Deno procedure, which yields both mononitroso-HMX and mononitroso-AcHMX.

A suspension of 2.5 g of DPT, in a solution of 3 mL of nitrosyl chloride (collected by condensation from the gas phase from the Matheson product) in 10 mL of acetic anhydride, was prepared. To the stirred suspension was added one drop of 10% (vol/vol) of concentrated sulfuric acid in acetic anhydride. Stirring was continued at room temperature until the DPT went into solution and then for 2 hours longer. The reaction mixture was poured on 30 mL of water with stirring. A white precipitate formed after about 30 min. The reaction mixture was allowed to stand 36 hrs at 6°C. The precipitate was collected by filtration. A portion was dissolved in acetone and examined with ethyl acetate as developer. Three spots were observed by UV quenching: a major spot (Fraction B) near the origin and a major spot (Fraction A) with a smaller one (Fraction C) in front of the solvent front.

The precipitate was taken up in ethyl acetate and the solution was filtered to remove a small amount of solid. The components were then separated by column chromatography on silica gel (Davison Grade 62, 60 to 200 mesh). The column was developed with ethyl acetate/acetone (1:1). Arbitrary fractions were taken which were monitored by TLC; those containing only one of the major components were combined and recrystallized from boiling methanol/ethanol. Fraction A was mononitroso-HMX (0.13 g): mp 238-241°C (lit. mp 236-237°C). The IR spectrum and MS were identical with those of the authentic sample. It was homogeneous on LC and TLC and behaved in an identical manner as the authentic sample. Fraction B was mononitroso-AcHMX (0.21 g); mp 180°C (dec.), lit mp 180° and MS spectra were consistent with the structure. The minor Fraction C was not examined.

Mutagenicity Testing: The Ames screening test for mutagenicity was conducted on RDX, trinitroso-RDX, HMX, AcRDX, and AcHMX according to standard procedures[13]. Five strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537, TA1538) were used to test these compounds over a range of concentrations both in the presence and absence of metabolic activation. The tests were run in triplicate and a two- to threefold increase in back mutations was considered as the criterion for a positive test for mutagenicity. Dimethylsulfoxide was used to achieve solution at the higher concentrations of test compound.

Nuclear Magnetic Resonance (NMR): NMR measurements were carried out with a Model XL200 NMR Spectrometer (Varian Associates, Palo Alto, CA); pulse width, 8 usec; delay time, 5-20 sec; number of transients, 10; 50 MHz. Samples were dissolved in deuterated acetone or deuterated dimethylsulfoxide.

Mass Spectral Analysis (MS): Gas chromatography/mass spectroscopy was conducted with a Finnegan GC/MS. Chemical ionization (CI) was used in all analyses except for dinitroso-AcRDX, in which case electron impact (EI) was used. The carrier gas was methane except for mononitroso-AcRDX, in which case ammonia was used.

RESULTS

RDX. As reported previously[3] RDX disappeared from nutrient broth cultures inoculated with anaerobic sewage sludge and incubated anaerobically (Fig. 5). The disappearance of RDX was accompanied by the appearance of several products which were identified as the mono-, di- and trinitroso derivatives of RDX formed by sequential reductions of the nitro groups to nitroso groups (Fig. 6). In addition, the formation of formaldehyde and methanol was reported[3]. After extraction and concentration, the presence of hydrazine, 1,1-dimethylhydrazine and 1,2-dimethylhydrazine was confirmed by GC/MS analysis.

HMX. Inoculation of anaerobic nutrient broth cultures containing 50 mg/L of HMX with anaerobic sewage sludge, and subsequent analysis by HPLC, showed a gradual reduction of HMX concentration. The reduction was accompanied by the appearance of three peaks in the HPLC tracing (Fig. 7). The initial rate of disappearance of HMX was less than that found for RDX and leveled off so that complete elimination of HMX did not occur even after several weeks of incubation. Intermediates A and B appeared to be stable over a several week period.

Biotransformation of ^{14}C-labeled HMX and RDX resulted in the distribution of radioactivity illustrated in Fig. 8. With RDX all of the radioactivity was detected in the supernatant, with none being found in the cell pellet. With HMX the radioactivity was initially associated with the pellet and then became solubilized. The increase up to the maximum value at day 2 to 3 is due to the increase in cell numbers during growth to the stationary phase. It thus appears either that HMX undergoes biotransformation only after absorption to cellular material or that there is a permeability barrier to be overcome that evidently doesn't exist in the case of RDX.

The assumption was made, upon finding several intermediate peaks by HPLC analysis of culture filtrates, that these were likely to be analogous to the nitroso derivatives observed with RDX. Consequently, the working hypothesis was that the compounds were mono- and dinitroso derivatives of HMX (Fig. 9) and that they arose by sequential reductions of the nitro groups. As seen in Fig. 10 there is the possibility for the formation of two isomeric forms of dinitroso-HMX.

AcHMX. The kinetics of disappearance of AcHMX is illustrated in Fig. 11. The disappearance of AcHMX occurs much more slowly than any of the other compounds studied. If more or less equivalent extinction coefficients are presumed for AcHMX and the several intermediate peaks (curves A, B and C) observed on HPLC analysis then it is obvious that the sum total of

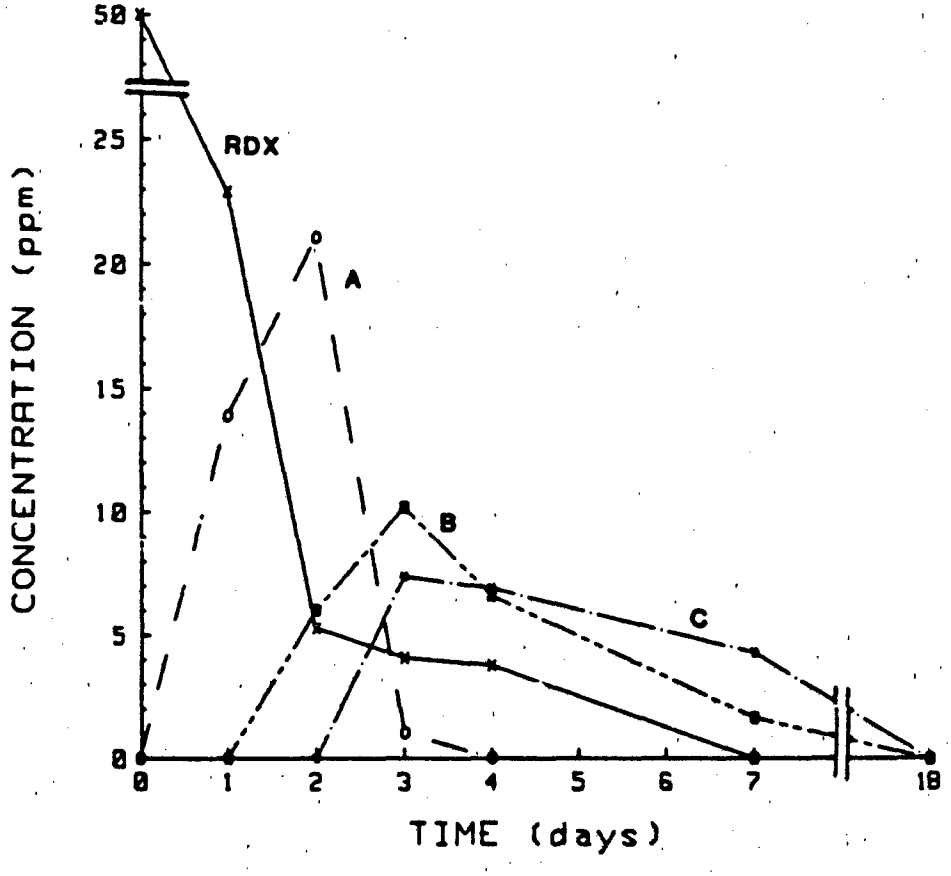


Figure 5. Kinetics of disappearance of RDX and production of intermediates during anaerobic incubation. A = mononitroso-RDX; B = dinitroso-RDX: C = trinitroso-RDX.

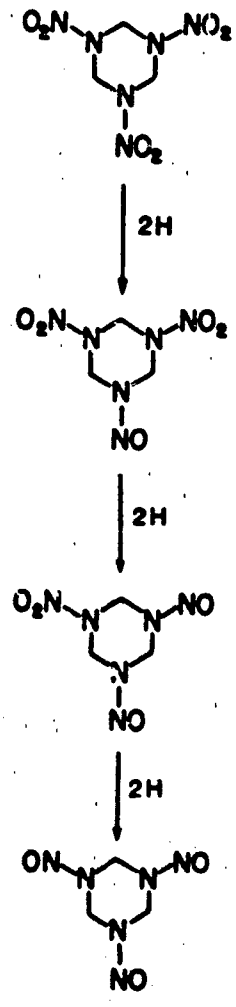


Figure 6. Stepwise reduction of nitro groups on RDX.

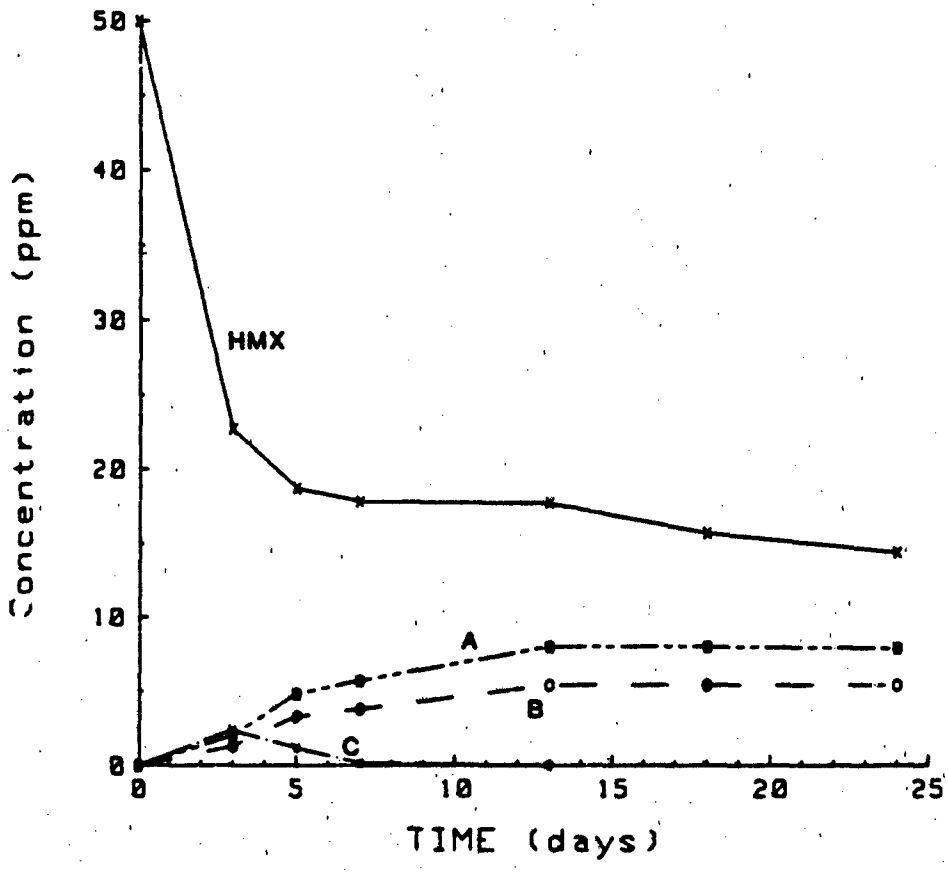


Figure 7. Kinetics of disappearance of HMX and production of intermediates during anaerobic incubation.

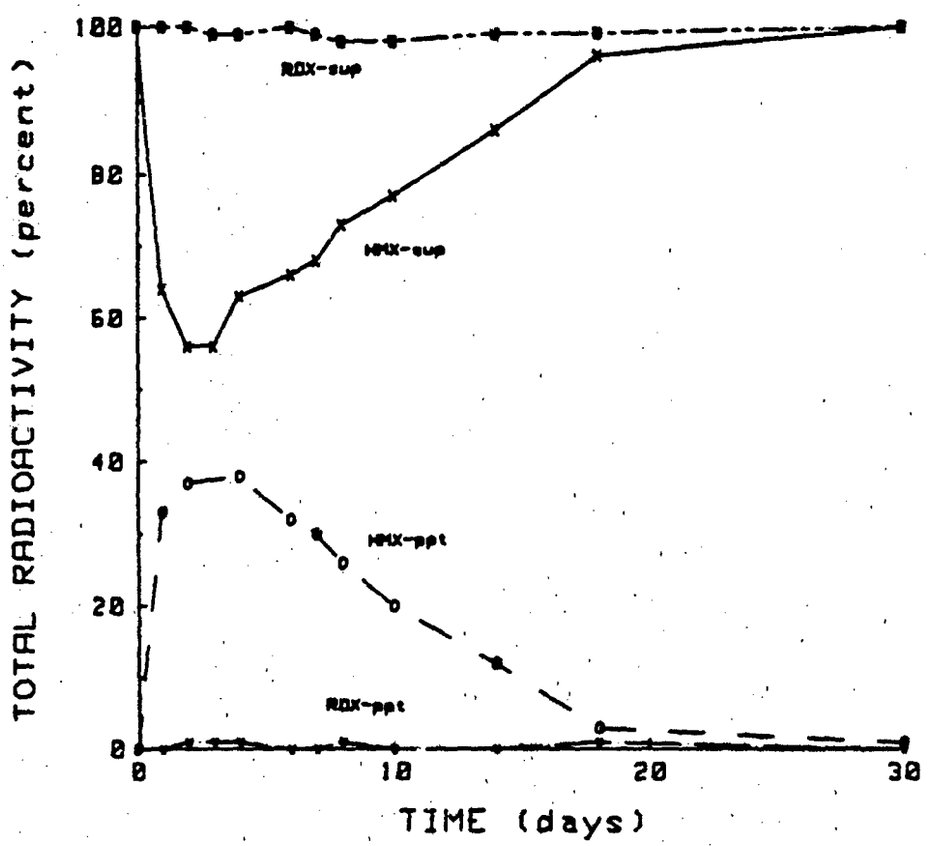


Figure 8. Distribution of ^{14}C between soluble and particulates in the biotransformation of ^{14}C-labeled RDX and of ^{14}C-labeled HMX.

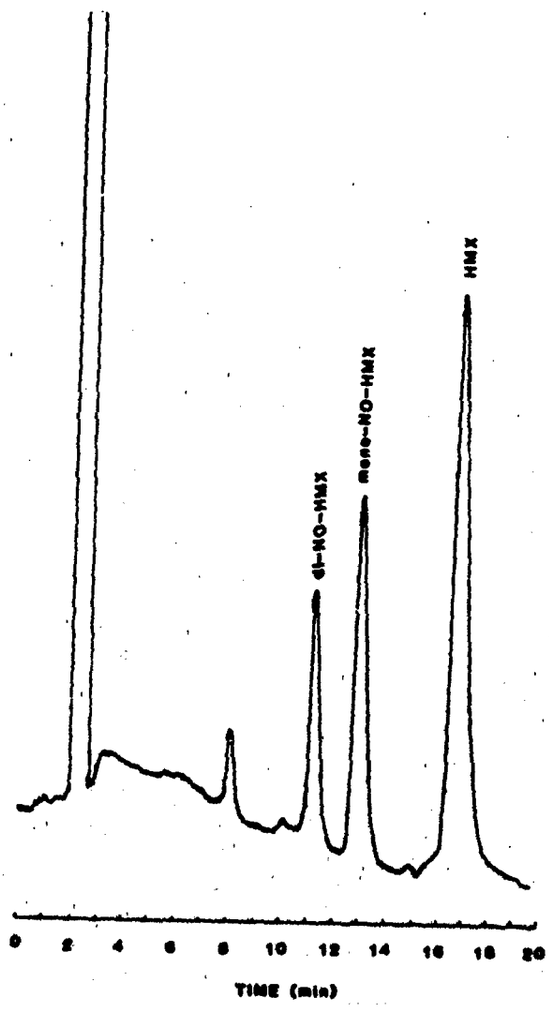


Figure 9. Separation of nitroso derivatives of HMX by HPLC.

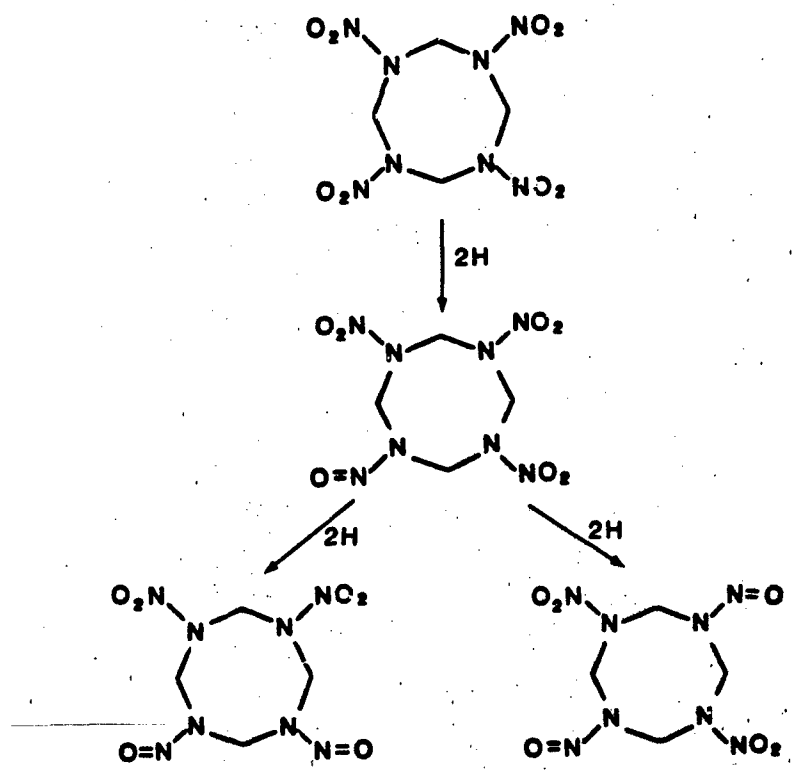


Figure 10. Stepwise reduction of nitro groups on HMX.

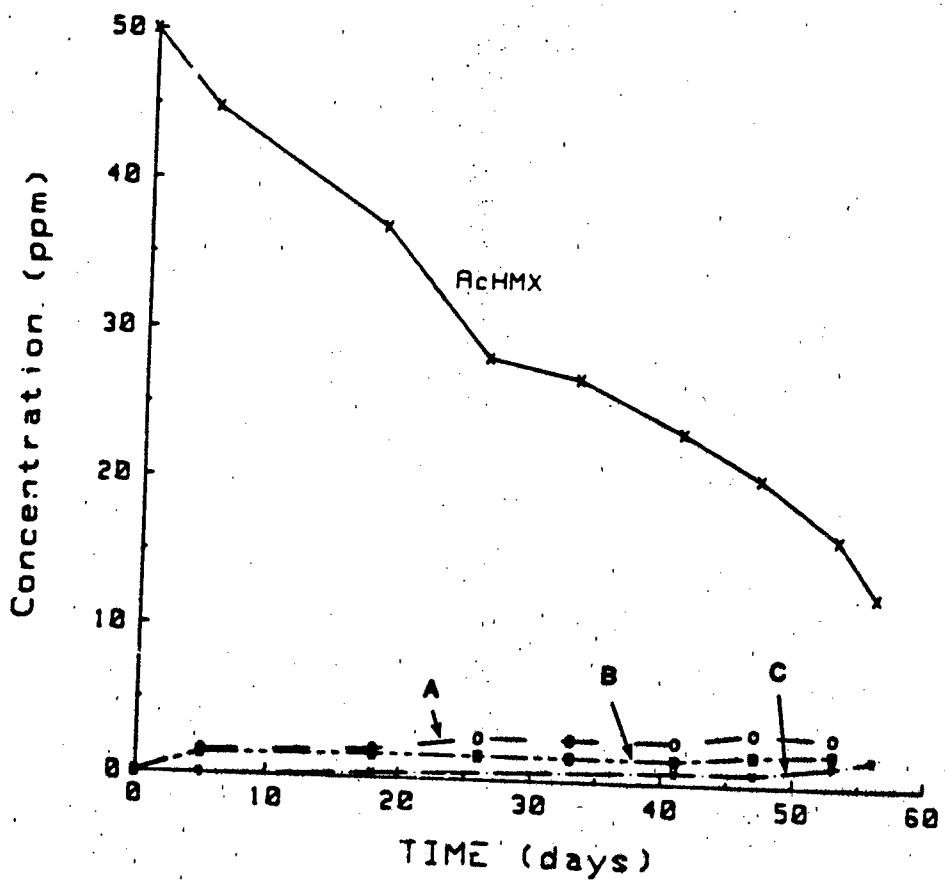


Figure 11. Kinetics of disappearance of AcHMX and production of intermediates during anaerobic incubation.

intermediates falls short of the amount of AcHMX that has disappeared. Nevertheless, sufficient quantities of the two major intermediates (Fig. 12) were collected to conduct a mass spectral analysis for confirmation. Fig. 13 shows the pathways of reduction for AcHMX, and the formation of two different isomers of dinitroso-AcHMX.

AcRDX. Inoculation of anaerobic nutrient broth cultures containing 50 mg/L of AcRDX caused the disappearance of AcRDX. In some experiments the concentration of AcRDX was reduced to below detectable limits, and in others a residual amount remained after several weeks (Fig. 14). Two intermediates, A and B, were observed on HPLC analysis and were presumed to be mono- and dinitroso-AcRDX (Fig. 15), arising from the stepwise reduction of the nitro groups to nitroso groups (Fig. 16) in a manner analogous to that reported for RDX[3].

The residue resulting from evaporation of solvent from pooled fractions containing the mononitroso-AcRDX HPLC peak, was dissolved in hot isopropanol and crystallized. The material appeared homogeneous on HPLC analysis and had a mp 115-116°C. An infrared (IR) spectrum is presented in Fig. 17, exhibiting a strong amide band at 1668 cm^{-1}, and strong bands in the $N-NO_2$, N-NO region between 1280 cm^{-1} and 1520 cm^{-1}.

The proton NMR spectrum (PMR) of AcRDX at 25°C is presented in Fig. 18. The three equivalent methyl group proton resonances are seen at 3.04 δ, four methylene proton resonances from those adjacent to the acetyl group are seen at 5.76 δ, and two methylene resonances from those opposite the acetyl group are seen at 6.22 δ. The PMR spectrum of mononitroso-AcRDX (Fig. 19) exhibited two methyl resonances (three protons) and a complicated six proton methylene group pattern spread over five resonances.

Nuclear Magnetic Resonance. The ^{13}C NMR spectra of DPT, HMX, mononitroso-HMX, AcHMX and mononitroso-AcHMX are presented in Table 1. The spectra for DPT and HMX are entirely consistent with the structures for these compounds (Fig. 20). However in the case of mononitroso-HMX the resonances C_D and C_C exhibit displacements that suggest a lack of free rotation around the N-N bond of the nitrosamino group, which allows it to assume a nonsymmetrical configuration in relation to the adjacent methylene groups[14]. The slight opposite displacements of the C_A and C_B resonances are probably due to some restricted rotation of the acetamido group around the C-N bond.

The spectrum of mononitroso-AcHMX is consistent with the presence of an acetamido group. Apparently in this compound the resonances for C_B and C_D fortuitously coincide, producing a single peak at 64.8 ppm. The small displacements observed for C_A and C_B can be ascribed to the absence of free rotation in the acetamido and nitrosamino groups as in AcHMX and mononitroso-HMX.

Mass Spectral Studies. Table 2 shows the various ions detected from analysis of the compounds in purified fractions from HMX, AcRDX and AcHMX biotransformation reactions. In most cases the ion can be accounted for by loss of the specific fragments listed in Table 3.

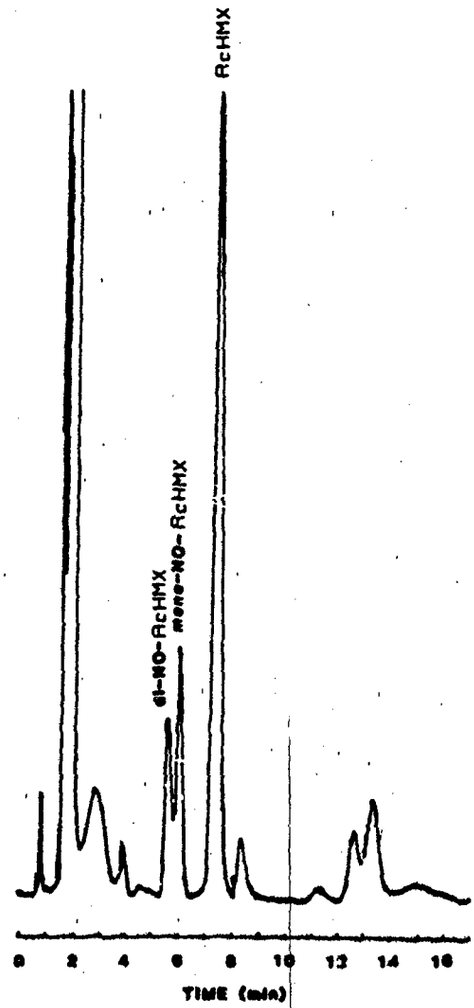


Figure 12. Separation of nitroso derivatives of AcHMX by HPLC.

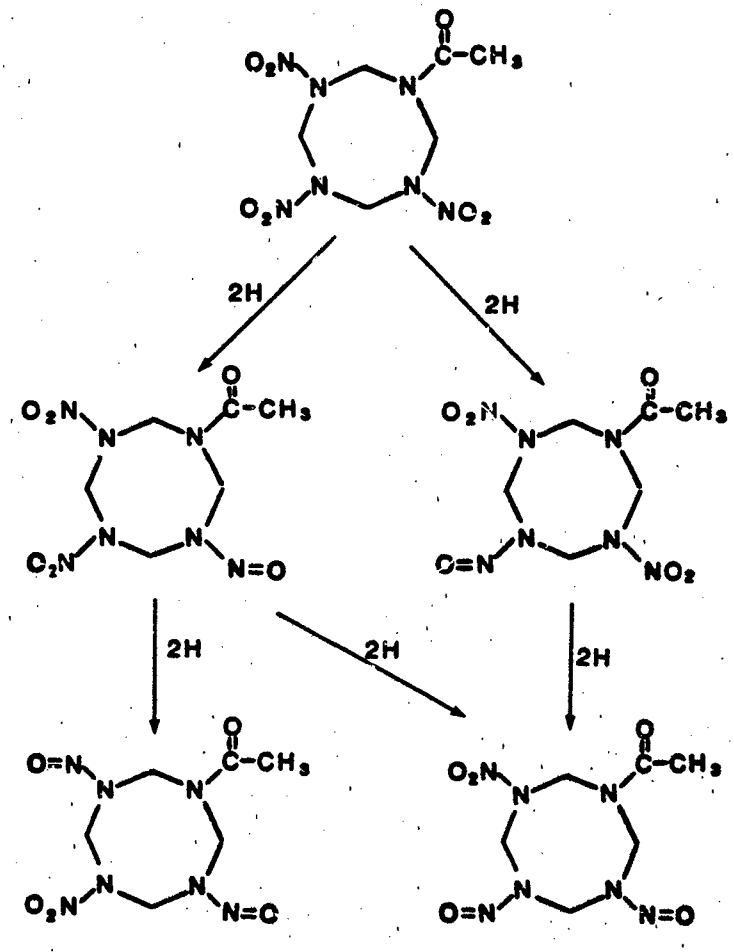


Figure 13. Stepwise reduction of nitro groups on AcHMX.

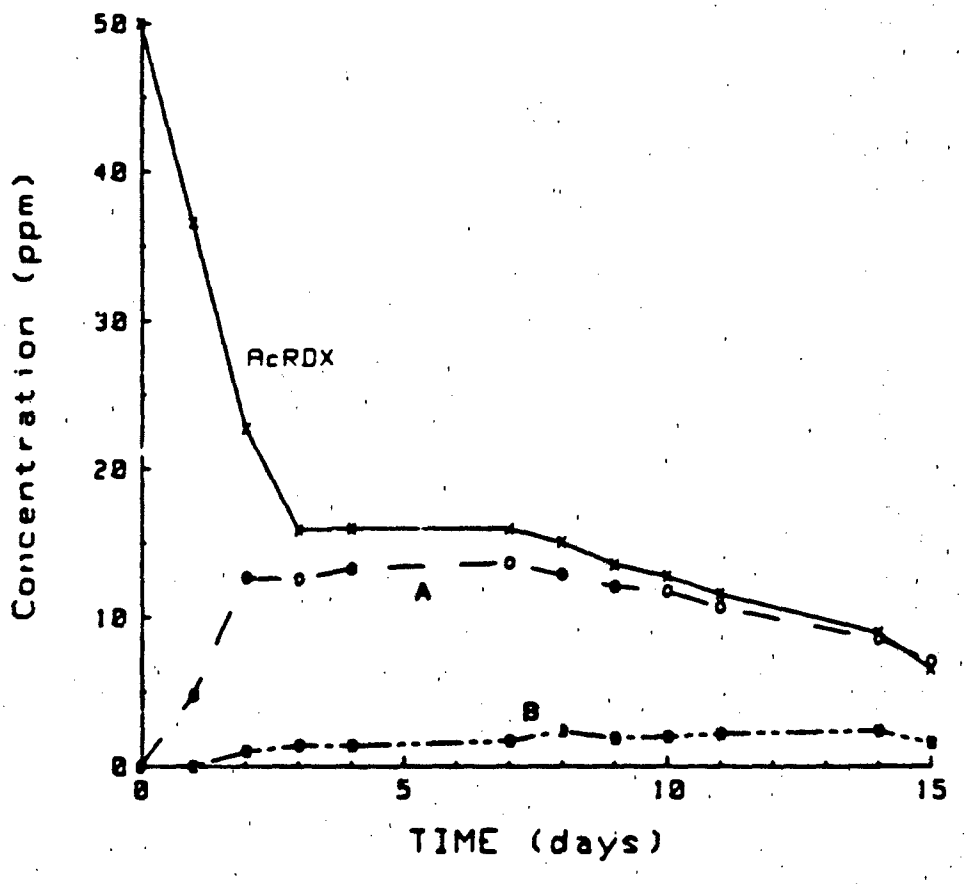


Figure 14. Kinetics of disappearance of AcRDX and production of intermediates during anaerobic incubation.

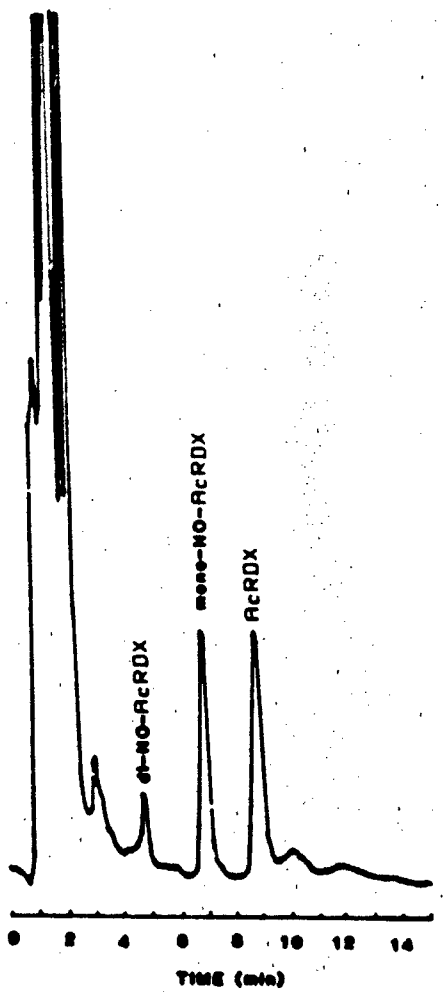


Figure 15. Separation of nitroso derivatives of AcRDX by HPLC.

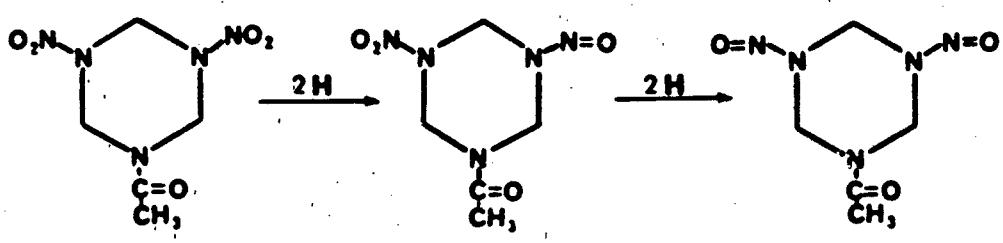


Figure 16. Stepwise reduction of nitro groups on AcRDX.

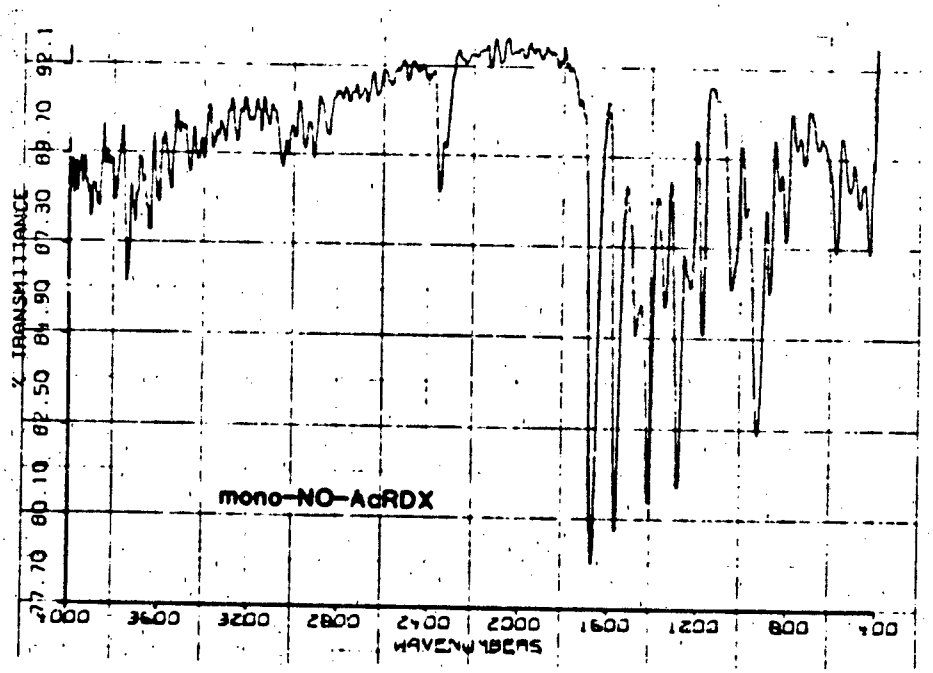


Figure 17. FTIR spectrum of mononitroso-AcRDX.

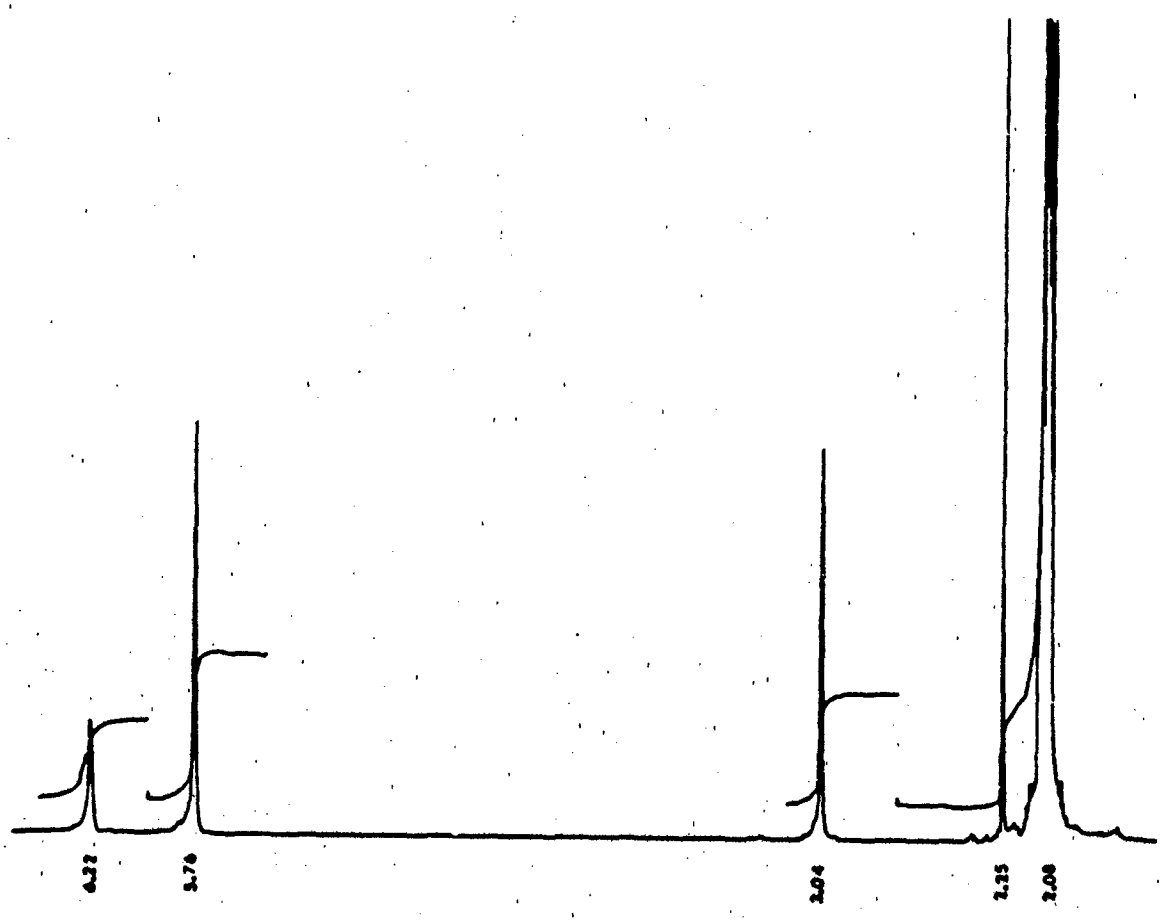


Figure 18. NMR analysis of AcRDX.

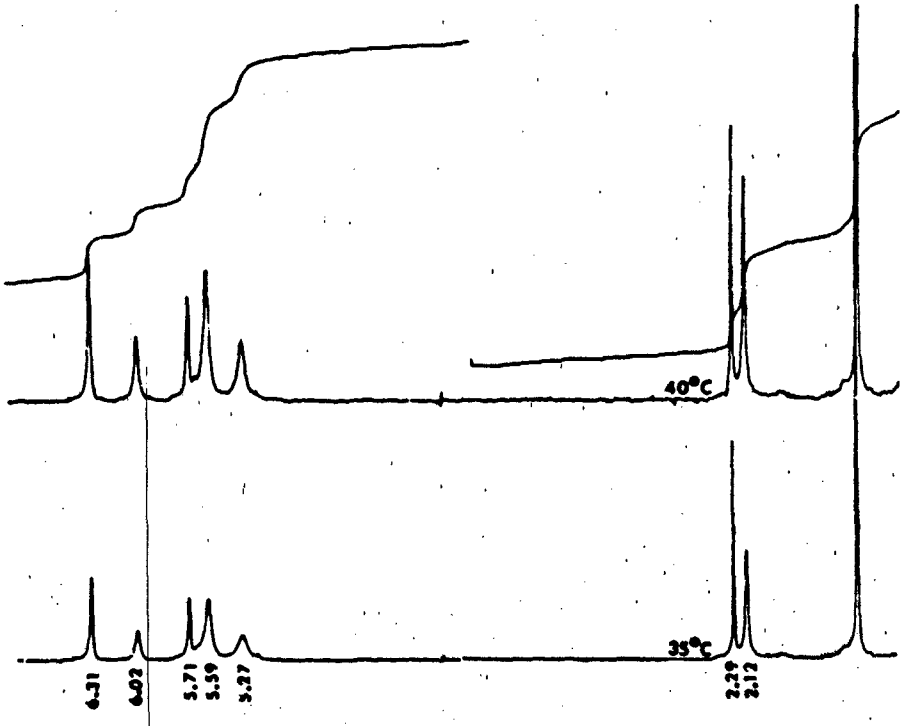


Figure 19. NMR analysis of mononitroso-AcRDX.

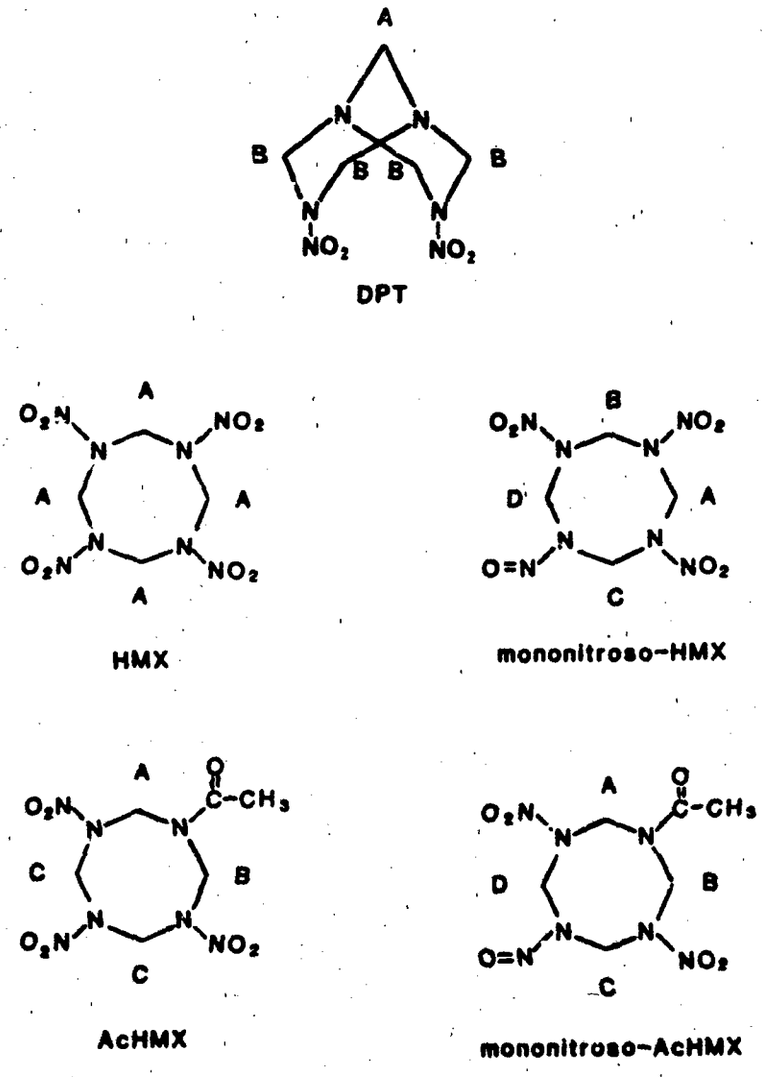


Figure 20. Structures of HMX and related compounds, indicating different methylene hydrogens.

TABLE I

^{13}C Nuclear Magnetic Resonance Data
on HMX and Cogeners

Compound[a]	(ppm)[b]					
	C_A	C_B	C_C	C_D	CH_3	C=O
DPT[c]	66.9	69.4				
HMX[c]	63.8					
mononitroso-HMX[c]	64.1	64.4	56.3	66.2		
AcHMX[d]	60.5	64.4	62.2		21.3	170.7
mononitroso-AcHMX[d]	60.4	64.8	53.4	64.8	20.8	170.2

[a]see Figure 19

[b]internal standard: TMS

[c]solvent: d_6-acetone

[d]solvent: d_6-DMSO

TABLE 2. Mass Spectra of Isolated Intermediates of HMX, AcRDX and AcHMX.

	HMX		AcHMX		AcRDX	
	mono-NO	di-NO	mono-NO	di-NO	mono-NO	di-NO
Molecular weight	280	264	277	261	203	187
Mass spectrum (m/z)	281[a]	235[a]	278[a]	232[a]	221[b]	176[c]
	235	207	262	204	204[a]	145
	207	191	235	183	188	129[d]
	161	164	234	161	161	113
	149	149	232	146	158	101[d]
	133	145	204	142	146	87
	75	133	162	130	130	71
		103	146	100	114	57[d]
			130		100	
			113		87	
			100		72	

[a]Chemical ionization, molecular ion = $\frac{m}{z}$

[b]Ammonia as carrier gas, molecular ion = $\frac{m+18}{z}$

[c]Electron impact, molecular ion = $\frac{m+1}{z}$

[d]Unaccounted for by loss of recognized fragments.

TABLE 3. Typical Fragments Lost During Mass Spectral Analysis.

Fragment lost	Mass (daltons)
$[CH_2-N-NO_2]$	74
$[CH_2-N-NO]$	58
$[NO_2]$	46
$[N-NO]$	44
$[CH_3-C=O]$	30

Biotransformation of Hydrazine and 1,1-dimethylhydrazine. Table 4 shows the results obtained from studies on the disappearance of hydrazine and 1,1-dimethylhydrazine in aerobic and anaerobic batch cultures. In all cases the disappearance of the substrates from sterile control flasks was appreciable, but the disappearance from inoculated flasks was faster. In other experiments in which resting cell suspensions were used in a phosphate buffer (heat-killed cells used for controls) the controls sometimes demonstrated greater activity than the live cell suspensions. These results were difficult to interpret and suggest that unknown factors are involved. No further attempts were made using resting cells.

Formation of Hydrazine and 1,1-dimethylhydrazine. Two-liter quantities of reaction mixtures from AcRDX, HMX and AcHMX were concentrated to 0.1 mL (20,000-fold concentration), and analyzed by HPLC. No trace of hydrazine or 1,1-dimethylhydrazine was detected.

Formation of Formaldehyde and Methanol. The presence of formaldehyde was confirmed in AcRDX reaction mixtures but was not found in those from HMX or AcHMX. Methanol was detected in reaction mixtures of all three compounds.

Mutagenicity Studies. No effects of toxicity or mutagenicity due to RDX, HMX, AcRDX, AcHMX, or trinitroso-RDX were noted in this study, even at the highest levels tested, approximately 200 ug/mL (Table 5).

DISCUSSION

As was reported for RDX[2], the biotransformation of HMX, AcRDX and AcHMX occurs only under anaerobic conditions. The first event to occur with all three compounds appears to be a one-step reduction of a nitro group to a nitroso group, followed by the reduction of a second nitro group, as was found with RDX. In the biotransformation of HMX Spanggord et al.[5] have reported the presence of mono-, di-, tri-, and tetranitroso-HMX, from fractions isolated by HPLC. These identifications were based on GC/MS data.

In the present work the reference compounds, mononitroso-HMX and mononitroso-AcHMX, were synthesized by known methods. The GC/MS, IR and NMR spectra of both compounds were consistent with their structures. The retention time and MS of the corresponding unknowns isolated from biological reaction mixtures by HPLC, agreed with those of the reference compounds. The fragmentation patterns were similar to those reported by Bulusu et al.[15]

NMR studies of a number of N-nitramines, including RDX, mononitroso-RDX, HMX and mononitroso-HMX, have been reported[16]. The data presented in Table 1 are in substantial agreement with this report. A compound not previously described, which was purified by recrystallization from biological reaction mixtures containing AcRDX, has been identified as mononitroso-AcRDX. Although the MS of this compound conformed to the proposed structure, its proton NMR (Fig. 19) was considerably more complex than that of AcRDX when both spectra were taken at room temperature (Fig. 18).

TABLE 4. Biotransformation of Hydrazine and 1,1-Dimethylhydrazine.

	Percent Disappearance[1]	
	Hydrazine[2]	1,1-Dimethylhydrazine[2]
Aerobic control	27.1	80.0
Aerobic reaction	97.7	100.0
Anaerobic control	34.0	66.7
Anaerobic reaction	97.5	100.0

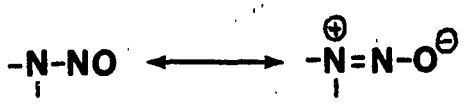
[1]Measured after 3 days of incubation.

[2]Concentration of 10 mg/L.

TABLE 5. Ames Test Results of RDX, HMX, AcRDX, AcHMX, and Trinitroso-RDX.

Compound	Metabolic activation	Micrograms per plate	ppm	TA1535	TA1537	TA1538	TA98	TA100
1. Negative controls	-	0	0	10±2	12±3	10±2	31±8	134±39
	+	0	0	12±3	4±1	14±3	24±9	115±12
2. Positive controls								
Sodium azide	-	1	0.04	200±16				239±25
9-aminoacridine	-	150	6		2047±263			
4-nitrophenylenediamine	-	10	0.4			956±206		
2-nitrofluorene	-	50	2				842±102	
2-anthramine	+	2	0.08	59±4	211±27	1127±95	1206±79	1754±78
3. RDX	-	5000	200	9±1	4±2	9±2	45±12	56±8
	-	500	20	7±5	5±1	10±3	33±3	59±8
	-	50	2	5±0	4±3	7±3	28±3	59±8
	-	5	0.2	4±1	7±4	3±2	22±7	52±10
	+	5000	200	6±2	5±2	6±4	28±4	72±5
	+	500	20	5±4	9±4	8±4	21±5	89±6
	+	50	2	9±0	3±1	8±2	30±8	80±3
	+	5	0.2	7±3	4±2	12±1	38±10	84±8
4. HMX	-	5000	200	7±2	6±3	7±1	20±9	67±14
	-	500	20	9±4	7±3	7±6	21±5	76±11
	-	50	2	17±8	6±1	4±4	20±1	78±8
	-	5	0.2	12±1	7±2	5±1	29±8	73±13
	+	5000	200	14±4	9±2	8±0	35±2	95±8
	+	500	20	10±1	4±2	10±3	30±4	114±19
	+	50	2	9±5	4±2	13±2	25±5	90±12
	+	5	0.2	7±3	4±3	8±1	30±14	69±12
5. SEX	-	5000	200	5±1	8±2	10±2	16±1	91±18
	-	500	20	6±1	8±6	6±1	24±10	96±25
	-	50	2	4±2	11±4	6±2	73±2	94±12
	-	5	0.2	6±2	9±2	5±1	17±4	83±8
	+	5000	200	7±1	3±2	9±2	21±2	127±17
	+	500	20	8±4	4±2	8±1	18±1	106±18
	+	50	2	11±2	6±1	10±6	20±8	102±5
	+	5	0.2	9±2	4±4	6±1	28±18	87±13
6. TAX	-	5000	200	8±7	4±4	5±1	14±3	84±10
	-	500	20	8±1	6±2	4±2	23±5	75±17
	-	50	2	9±4	5±1	8±2	15±6	78±12
	-	5	0.2	5±2	4±4	7±2	20±15	85±18
	+	5000	200	9±6	10±4	8±5	23±4	85±8
	+	500	20	5±5	5±2	8±4	17±6	84±4
	+	50	2	5±4	5±4	6±2	21±2	82±5
	+	5	0.2	6±3	7±2	4±4	14±4	94±20
7. Trinitroso-RDX	-	5000	200	21±10	2±1	7±2	28±5	77±5
	-	500	20	9±4	8±2	6±5	31±2	90±31
	-	50	2	12±3	4±2	9±0	24±2	64±2
	-	5	0.2	11±1	3±2	7±7	28±4	78±6
	+	5000	200	12±1	10±2	13±6	29±7	94±18
	+	500	20	17±6	3±1	14±4	13±11	90±26
	+	50	2	10±2	4±2	12±1	31±5	111±39
	+	5	0.2	18±2	6±3	13±5	27±13	90±6

Cooney[14] has conducted variable temperature proton NMR studies on samples of crystalline mononitroso-AcRDX provided by this laboratory. The spectrum of this compound was observed at 25°C, 0°C, and -80°C in deuteroacetone and remained unchanged. The experiment was repeated at 70°C, 100°C, 120°C and 140°C in deuteronitrobenzene. At 100°C the two methyl group peaks coalesced into a single peak; at 120°C the multiple methylene peaks coalesced into two peaks: and at 140°C the spectrum was very similar to that of AcRDX. This behavior was attributed to lack of free rotation around the nitrogen to nitrogen bond of the nitroso group which is caused by the partial double bond character induced by the resonance structure:



Similar structures can be written for acetyl group in this molecule but they do not appear to contribute significantly to the proton spectrum.

The other nitroso compounds found as metabolites in this work were tentatively identified by the characteristic pattern of the emergence of peaks observed on HPLC of the culture broth at various stages of biotransformation. The same pattern was observed with each of the compounds as was earlier reported for RDX; that is, as the peak area of the starting material decreased, there was the appearance of a second peak with a slightly faster elution time, followed by a third peak with an elution time slightly faster still. In every instance GC/MS analyses confirmed that these were mono-, di-, and in some cases even trinitroso derivatives of the starting compounds.

Although traces of hydrazine, 1,1-dimethylhydrazine and 1,2-dimethylhydrazine were found in broths from RDX, neither hydrazine nor 1,1-dimethylhydrazine were found in 20,000-fold concentrated extracts of reaction mixtures from HMX, AcRDX or AcHMX. The results of the biodegradation experiments with hydrazine and 1,1-dimethylhydrazine show clearly that these hydrazines disappear under anaerobic or aerobic conditions.

RDX and its cogeners probably undergo anaerobic biotransformation via similar routes. The appearance of hydrazine derivatives in the reaction of RDX, but not with the other substrates may be due to the fact that the initial attack on RDX is more rapid than that of the others. In this case accumulation of intermediates that are capable of further biotransformation would occur to a larger extent than if the initial attack took place at a slower rate.

If this interpretation is valid, it is possible that perturbation of one of these systems may allow the formation of significant amounts of hydrazine or hydrazine derivatives. However in this study we have shown that such products would readily be eliminated by further microbial treatment under either aerobic or anaerobic conditions.

Methanol was detected in reaction mixtures of every compound examined to date, and is presumed to have arisen through reduction of the primary product, formaldehyde. Methane was not found in head space gas samples from any of the systems.

We prepared dinitroso-RDX as a reference standard for our studies on the biodegradation of RDX. However no dinitroso or higher nitroso derivative of the other substrates has yet been prepared in sufficient quantities by synthetic means. We attempted the preparation of the nitroso derivatives of AcRDX by reduction of AcRDX with zinc dust and aqueous ammonium chloride. Mixtures of starting material, mononitroso-AcRDX and dinitroso-AcRDX were obtained based on HPLC retention times. These products were separated by semipreparative HPLC, but it was not possible to isolate them in a pure state. On evaporation of the fractions, or even on prolonged standing the individual compounds disproportionated to give mixtures of all three products. The reasons for this behavior in the case of synthetic products, but not the biologically mediated ones, are not clear.

CONCLUSIONS

The biodegradation of RDX, HMX, AcRDX, and AcHMX occurred through reduction of the nitro groups in an anaerobic environment. No activity was observed under aerobic conditions. Products were identified as mono- and di-nitroso derivatives by solvent extraction, HPLC, GC/MS, FTIR and NMR analytical techniques. Under reducing conditions further reductions led to the formation of formaldehyde and methanol. Traces of hydrazine, 1,1-dimethylhydrazine, and 1,2-dimethylhydrazine were detected in the case of RDX upon several thousand fold concentration. Hydrazine and 1,1-dimethylhydrazine were both shown to be biodegradable under either aerobic or anaerobic conditions. No traces of dimethylnitrosamine were detected.

Sufficient quantities of the four substrates were available to conduct an Ames Test on them. Among the identified products only trinitroso-RDX was synthesized in sufficient quantity to permit Ames testing. These were all negative up to the highest concentrations tested (5000 ug/plate).

Studies in progress with anaerobic continuous culture systems show that concentrations of RDX, HMX, AcRDX, AcHMX and nitrate can be reduced significantly (manuscript in preparation). The best results have been obtained with media high in total organic carbon (i.e., nutrient broth, 20% acid-hydrolyzed sewage sludge). Although the products mentioned in the present study have been identified in static, batch culture systems, none has been detected in continuous culture systems. It should be mentioned however, that in the case of a malfunction of a waste stream treatment facility, static conditions may exist which are directly analogous to those extant in a batch culture system. Any treatment facility designed to treat such wastes should have the capability of on-line monitoring of the treated waste stream for intermediates and end-products discussed in this report.

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