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Bioremediation of Explosives

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Introduction

Several explosives have been used extensively in the United States by activities conducted in private industry, in the Department of Defense and in the Department of Energy. This extensive manufacture, packing, and use of explosives has often resulted in significant contamination of soils and ground waters near these activities. Congressional mandate has now required that such sites be remediated. The manufacture and packing of explosives also generates aqueous effluent containing the explosive. Efficient, complete and cost-effective means of dealing with all of these problems must be developed. While no single technology can solve all problems, an especially promising technology for this explosives problem is biotechnology. When applicable, biotechnology is cheap and provides complete conversion of hazardous compounds to harmless biomass or carbon dioxide. The focus of this paper will be on our present understanding of the microbial metabolism of the explosives, TNT and RDX, which have been used most extensively in the United States. The engineering phase of this problem has been discussed in detail in several reports by the US Army.

Nitroaromatics are xenobiotics, or manmade compounds, which play a very large and important part in the chemical industry. Although many drugs and dyes are nitroaromatics, the largest applications of these compounds are as pesticides, insecticides, and as explosives (Rickert, 1985). The mutagenic and carcinogenic potential of this class of compounds or their derivatives (Dilley *et al.*, 1979; Ellis *et al.*, 1978; Kaplan and Kaplan, 1982a) has recently brought an interest in the risks and hazards that these compounds present to the environment and human populations (Spanggord *et al.*, 1985; Lee *et al.*, 1975; Nay *et al.*, 1974; Osimon and Klausmeier, 1972; Sax 1963; Won *et al.*, 1976). Of course, the risks presented also depend on the persistence of these chemicals in the environment they contaminate. According to Spanggord and coworkers (1985), there are four environmental processes affecting the loss of organic chemicals in an aquatic environment. These include volatilization, chemical transformation, sediment sorption and biological transformation or biodegradation. However, we are concerned with the last of these, biological transformation. We define degradation to mean complete degradation, i.e., mineralization, of a compound in which the compound is metabolized

stoichiometricly to biomass and/or carbon dioxide. We define transformation to mean only a chemical modification of a compound, thus transformation can occur without degradation.

Microbial degradation of many diverse organic compounds has been extensively studied. Some of these microbes isolated from soil and water, have been successfully applied to large scale decontamination efforts. This bioreclamation approach is often much less expensive than existing conventional cleanup technology. However, no completely developed bioreclamation technology exists for explosives. In order to complete the development of treatment systems for large scale remediation of explosives contaminated soils and waters, we must acquire a better understanding of the microbial breakdown of explosive compounds such as TNT (2,4,6-trinitrotoluene), HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) (Figure 1). Because the metabolism of TNT has been the most extensively studied, we will begin with the biodegradation of TNT

TNT has been used extensively as an explosive by the US military for several decades. Contamination of soils and groundwater has occurred from munitions manufacturing, loading, assembling, and packing operations (Kaplan and Kaplan, 1982b) and from disposal activities. TNT is an environmental hazard because it has toxicological effects on a number of organisms in addition to its explosive character. Toxic effects that have been reported include liver damage and anemia in workers engaged in large scale manufacturing. TNT is also toxic to freshwater fish (McCormick *et. al.*, 1976). Inhalation of the dusts or vapors of TNT can be fatal; skin contact with absorption of explosives can result in simple discoloration of the skin, dermatitis, headaches, and poisoning (Bongiovanni *et. al.*, 1984). TNT reduction products also pose an environmental danger because they have been reported to be toxic and mutagenic as well (Dilley *et. al.*, 1979; Ellis *et. al.*, 1978; Kaplan and Kaplan, 1982a). Because reduction products of this explosive are dangerous, we are examining oxidative degradation by microbial communities growing aerobically.

TNT is a nitroaromatic compound. Several different microbes have been reported to metabolize selected nitroaromatic compounds (structures are shown in Figure 2) using a number of different reactions. Nitrobenzoic acids undergo complete microbial degradation by strains of *Norcardia* (Cartwright and Cain, 1959). The degradation of nitroanilines can be accomplished by a single *Pseudomonad* (Zever and Kearney, 1983). In 1953 Simpson and Evans isolated

two organisms which oxidatively and completely degraded ortho-nitrophenol and para-nitrophenol. The degradation of the herbicide, dinitro-o-cresol and several structurally related compounds, including para-nitrophenol, 2,4-dinitrophenol, and 2,4,6-trinitrophenol (picric acid) was carried out by *Arthrobacter simplex* (Gunderson and Jensen, 1956); these bacterial cultures clearly used dinitro-o-cresol as a sole source of carbon and nitrogen. Further studies on the degradation of dinitro-o-cresol by two mixed soil bacterial cultures were carried out several years later (Jensen and Lautrup-Larsen 1967); these mixed bacterial cultures appeared to be utilizing dinitro-o-cresol via a pathway unlike that used by *Arthrobacter simplex*. Complete degradation of dinitro-o-cresol was also accomplished by a pure culture of a Pseudomonad; this degradation of dinitro-o-cresol was enhanced by the presence of hydrogen donors (Tewfik and Evans, 1966). The Pseudomonad degraded 3,5-dinitro-o-cresol as follows:

3,5-Dinitro-o-cresol -->3-amino-5-nitro-o-cresol-->3-methyl-5-nitrocatechol-->
3-methyl-5-aminocatechol-->2,3,5-trihydroxytoluene--> ring cleavage.

The *Arthrobacter simplex* that degraded 3,5-dinitro-o-cresol employed a much simpler pathway in which the initial steps are unlike the first steps used by the Pseudomonad; this pathway is as follows:

3,5-Dinitro-o-cresol -->3-methyl-5-nitrocatechol-->2,3,5-trihydroxytoluene-->
ring cleavage.

Raymond and Alexander (1971) demonstrated the capability of unspecified soil bacteria to use para-nitrophenol as a carbon and energy source as well as the capability of the same bacteria to cometabolize meta-nitrophenol. Cometabolism has been defined as a reaction sequence in which the microorganism enzymatically transforms a compound that it cannot utilize as a sole source of energy. Para-nitrophenol was also transformable via oxidation by a *Moraxella* species, which converted it to hydroquinone and nitrite (Spain *et al.*, 1979). The enzyme catalyzing this reaction was a monooxygenase, as indicated by the requirement of an electron donor and the incorporation of 1 atom of molecular oxygen into the substrate (Spain *et al.*, 1979).

The insecticide, parathion (o,o-diethyl-o,p-nitrophenyl phosphorothioate) is an excellent example of a toxic compound that can be degraded only by a microbial community. This organophosphate has been used extensively as a general insecticide, and with such wide use came the problem of detoxifying the wastes resulting from surplus pesticide, pesticide containers, aircraft spray tanks, resulting waste waters, and contaminated soils (Munnecke and Hsieh, 1975).

Biological oxidation by a mixed culture is one way to effectively detoxify parathion. Munnecke and Hsieh (1975) determined the crucial parameters for the complete and rapid degradation of parathion by this mixed culture. Mixed bacterial cultures provide several advantages when compared with monocultures for degradation of some xenobiotics. Combinations of different bacteria can provide unique arrays of biodegradative enzymes to accomplish degradations not carried out by any of the enzyme systems in the individual bacteria. The rate of biodegradation can often be increased by the acclimation of mixed cultures and sometimes a simpler pathway of degradation can be accomplished when compared with metabolism by a pure culture (Munnecke and Hsieh, 1976).

In contrast to the mineralization of parathion by a mixed culture, toluene is degraded by a pure culture. Toluene is the substrate that is nitrated to produce TNT. The biodegradation of toluene has been studied extensively and thus, the pathway of biodegradation is known. *Pseudomonas putida* F1 utilizes toluene as a sole carbon source for growth. Toluene is catabolized by the dihydrodiol pathway; the initial reaction of toluene catabolism is carried out by a multi-component enzyme system designated as toluene dioxygenase. Toluene dioxygenase transfers electrons from NADH to the terminal dioxygenase, 3-methylcatechol-2,3-dioxygenase (Zylstra and Gibson, 1989). The products of the initial steps of degradation are 2-hydroxypenta-2,4-dienoate and acetate (Zylstra *et. al.*, 1988), demonstrating that the aromatic ring is cleaved by a pure culture of *Pseudomonas putida* F1.

Because TNT is a more complex molecule than toluene, individual bacterial cultures may not be capable of degrading TNT or the degradation may not be as rapid as it could be if it were catalyzed by a mixed culture. Biotransformation of TNT has been demonstrated by a microbial community in laboratory culture (Won *et. al.*, 1974; McCormick *et. al.*, 1976), in activated sludge (Carpenter *et. al.*, 1978), in compost (Doyle *et. al.*, 1986; Isbister *et. al.*, 1982; Kaplan and Kaplan, 1982b), and in aqueous aerobic culture (Spanggord *et. al.*, 1985). However, except for the activities investigated by Spanggord and coworkers (1985) and Won and coworkers (1974), most of these transformations appeared to occur through the reductive pathway of TNT in which the nitro group is first reduced to an hydroxamate and further reduced to an amino group. The nitroamine is then converted to the hydroxyl compounds which are presumably activated for the subsequent ring cleavage (McCormick *et. al.*, 1976) (Figure 3). These resulting intermediates are often highly reactive with one another and form

azoxy compounds (Kaplan and Kaplan, 1982b). The scheme for transformation of TNT through this reductive pathway as developed by Kaplan and Kaplan is illustrated in Figure 2. These TNT-like polymers are apparently essentially intractable to further microbial degradation and could be toxic themselves (Carpenter et al., 1978; Rogers and Kaplan, 1971).

Traxler and coworkers (1975) reported that certain gram-negative bacteria isolated by enrichment culturing were capable of using TNT as a sole source of carbon. This activity was enhanced by the addition of yeast extract, which implies that some sort of cometabolism was occurring. Some of these cultures were also capable of using TNT as their sole source of nitrogen. These cultures may have been capable of cleaving the ring, although this activity was not shown to be a major reaction in these cultures. Attempts to demonstrate stoichiometric conversion of [^{14}C]TNT to carbon dioxide were unsuccessful; only a small fraction of the radiolabel was recovered as carbon dioxide. Significant radioactivity was associated with the biomass. These studies demonstrated a rapid removal of TNT from the solution by the culture; this removal of TNT could have been accomplished by association of the TNT with the bacterial cell walls; this explanation is also consistent with the detection of most of the radioactivity in the cell mass.

Additional evidence in support of cometabolism of TNT was obtained by Won and coworkers (1974) and by Won and Heckley (1974). These studies examined the capability of pseudomonas-like organisms to metabolically oxidize TNT. Cometabolism (or transformation) of TNT was also observed by Osmon and Klausmeier (1973). The production of carbon dioxide from TNT carbon was not demonstrated. These cultures were also examined for their capability to degrade RDX; degradation of RDX was not carried out by these bacteria.

Examination of compost containing TNT indicate transformation but not degradation of the TNT (Doyle *et. al.*, 1986; Isbister *et. al.*, 1982; Kaplan and Kaplan, 1982b). These composts degraded RDX, which is only known to be accomplished in anaerobic conditions, suggesting that these composts were not truly aerobic.

Although biotransformation by reduction of the nitro groups of TNT, may lower or even abolish the initial toxicity of the compound, and will certainly abolish its explosive character, this simple modification of TNT it is not a good solution to the problem. Failure to cleave the aromatic ring will probably result in the persistence of other toxic aromatic compounds. In order for the reclamation of

contaminated sites to be accomplished, the complete removal of the nitro groups and cleavage of the aromatic ring are necessary. Some bacteria use an oxidative pathway for the removal of nitro groups, such as in the metabolism of dinitrotoluene as reported by Spanggord (1985). This pathway may be useful in the degradation of TNT.

Fungi have significant degradative capabilities, some of which are unlike those found in bacteria, thus, fungi may be capable of transforming or degrading explosives. Fungi have been obtained that transform TNT (Parrish, 1977). No evidence of ring cleavage was obtained using [¹⁴C]TNT; this study detected only reduction of the 4-nitro group. Studies using the white rot fungus (*Phanerochaete chrysosporium*), a lignin-degrading fungus that is capable of degrading an array of organic compounds, are being conducted at Utah State University; we await their complete report.

In 1944, Channon *et al.* found in rabbits what was probably an oxidation mechanism that transformed TNT to trinitrobenzyl alcohol. This oxidation of the methyl group is catalyzed by a cytochrome P450 dependent reaction. Bacteria do not contain such cytochrome P450 dependent enzymes and thus can not catalyze this same oxidation.

RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) is also a major explosive used by the US military. Soil and water contamination has resulted from manufacturing, packing, loading, and assembly operations. RDX has some toxicity towards mammals (Schneider *et al.*, 1977; Hathaway and Buck, 1977; Ross 1976); these studies suggest that the metabolic products derived from RDX may be the actual toxins. The biodegradation of RDX is much less well studied than is the biodegradation of TNT. Most of the studies attempted anaerobic degradation and in those that attempted aerobic degradation, no evidence was obtained for aerobic biodegradation. As is frequently the case with TNT, the disappearance of RDX was monitored rather than the actual biodegradation or a stoichiometric conversion to carbon dioxide or biomass. In other words, the mass balance has not often been determined for microbial transformations or degradations of either of these explosives.

Anaerobic microbial transformation of RDX has been reported. Osmon and Klausmeier (1973) reported disappearance of RDX during soil enrichment studies, but presented no evidence for the degradation of RDX. Soli (1973) also reported the disappearance of RDX in anaerobic cultures containing the purple photosynthetic bacteria of the genera *Chromatium*, *Rhodospirillum*, and

Rhodopseudomonas and possibly others. These photosynthetically active cultures, which do not release oxygen, were supplemented with sodium acetate and ammonium chloride. Soli postulated that the RDX was being reduced in this reductive environment.

McCormick and coworkers (1981) examined anaerobic metabolism of RDX by anaerobic sewage sludge. RDX (50-100 µg/ml) was rapidly removed from the cultures. A number of intermediates and products of this metabolism were identified; these were hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine, hexahydro-1,3,5-trinitroso-1,3,5-triazine, hydrazine, 1,1-dimethylhydrazine, 1,2-dimethylhydrazine, formaldehyde, and methanol. [¹⁴C]RDX was used to monitor the fate of the RDX carbons; approximately 62% of the radioactivity was recovered by ether extraction of the culture and 42% remained in the aqueous phase. No [¹⁴C]-labeled volatiles evolved from the culture during incubation. The intermediates were produced in the culture in the following order: first, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine; second, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine; and third, hexahydro-1,3,5-trinitroso-1,3,5-triazine. A pathway for this degradative metabolism was proposed that begins with successive reduction of the nitro groups until the compound is destabilized, at which time the ring is cleaved (Figure 4). It is worthy of note that this metabolism of RDX includes the generation of a reactive radical which is presumably capable of reacting with other compounds in the mixture. It is the reaction of a radical that causes the polymerization of TNT intermediates in compost. The fate of such radicals should be considered if anaerobic digestion of RDX is developed for practical implementation.

Spanggard *et al.*, (1980) observed significant RDX transformation only when yeast extract was added to his anaerobic culture. Sikka and coworkers (1978) also observed RDX disappearance only in cultures from activated sludge were supplemented with yeast extract. These observations imply that transformation of RDX required a cometabolite in those systems.

Aerobic transformations or degradation of RDX have not been observed in the several laboratory studies that examined this possible route of RDX metabolism.

RDX and HMX are of the basic chemical structural class designated as triazines. Triazine degradation has been reviewed by Cook and Hutter (1981), Esser *et al.*, (1975), and Kaufman and Kearney (1970). Cook and Hutter (1981) present a positive assessment of biodegradation in general pointing out that "only

a small part of the reservoir of biodegradative enzymes available in nature has yet been tapped," although they do not claim that all compounds are biodegradable. The most rapid biodegradation of triazines has been observed for cyanuric acid and ammeline whereas N-alkylated hydrazines were degraded slowly (Zeyer, 1979). Cook and Hutter (1981) have obtained bacteria that use triazines quantitatively as nitrogen sources. These studies provide us with an example that more creative approaches may be effectively utilized to achieve efficient biodegradation of compounds that we now consider to be either recalcitrant to biodegradation or those that are biodegraded slowly.

Recent Results

At Los Alamos have been examining the ability of several soil bacteria to grow in liquid culture with TNT as their sole source of carbon. Several bacteria that proliferate in these TNT-dependent cultures have been isolated and identified. Our cultures were often grown initially with succinate and TNT as carbon sources; in the later stages of the culture growth, TNT serves as a carbon source to sustain the cultures after the succinate is exhausted (Figure 5); succinate is exhausted approximately 40 hours after inoculation. The mixed cultures without TNT (controls) begin to decline after 40 hours (Figures 5). Cultures growing with succinate and TNT as carbon sources continue to grow for at least 400 hours (Figure 5). Each of these cultures grows to approximately the same cell density during the first 40 hours.

Separate experiments done in this laboratory have demonstrated that several individual bacteria grow in solid culture with TNT as their sole source of carbon. We also see variability among these bacteria in their capability of degrading TNT. Perhaps one bacterium's slow step will be complemented by another bacterium, to provide a quicker degradation if the bacteria are combined as a mixed culture.

We have bacteria that grow on TNT as a sole source of carbon and understand some of their growth characteristics, thus our future studies will include the detection and identification of the intermediates in the degradation. These studies will be carried out using high pressure liquid chromatography (HPLC) because it provides many advantages over other techniques sometimes used for detection of explosives (Krull & Camp, 1980). It is also more useful in assaying for the presence of any volatile by products of TNT. These studies will

ensure that TNT is being safely oxidized to useful products so that large scale clean up of munitions waste from the production of TNT can be carried out.

To assure that an efficient process is developed for TNT biodegradation, are conducting appropriate lab scale tests with TNT contaminated soil. First, we are testing their efficiency in soil / water slurries; we are also testing their efficiency in a column system designed to simulate composting conditions. A pilot scale test of this bacterial degradation will be conducted as soon as weather permits.

Discussion and Summary

We have shown that several soil bacteria grow aerobically with TNT as their sole source of carbon. The LANL mixed culture were capable of degrading 2,4- and 2,6 DNT, methyl resourcinol, and 2,4,6-trihydroxytoluene. Our isolated strains were obtained from soil that had been contaminated with TNT for approximately forty years. It must be noted that the TNT concentration in these cultures is fairly high and as such, is above the solubility of TNT in water. Furthermore, biodegradation of most nitroaromatics is done with concentrations of about 100 ppb or at least 500 fold less than the TNT concentrations we have used. In order to be useful for remediation of TNT-contaminated soils and waters we must develop biodegradation of these high concentrations of TNT.

The metabolism of TNT and RDX by microorganisms have been much more well studied than has the metabolism of other explosives. Several general characteristics of these metabolic process appear to be emerging as general properties of these systems. The first of these is the beneficial effect of the presence of a cometabolite. The most commonly used cometabolite has been yeast extract, which is generally considered to be a good cometabolite for many other microbial transformations or degradations of xenobiotics. Cometabolites are useful when the compound being degraded does not provide sufficient energy or is limiting in a particular element.

TNT has been degraded by both anaerobic and aerobic metabolism. The relative utility of these two distinctly different metabolic routes in different systems - compost, aqueous slurries, or aqueous digestion of waste streams - have not been determined. The aerobic degradation provides the distinct advantage of avoiding the polymerization reactions by the intermediates observed in anaerobic digestion of TNT. These polymerization reactions lead to intractable compounds.

Unfortunately, mass balance has not been demonstrated for anaerobic digestion of TNT in compost and studies have shown toxic or mutagenic compounds resulting from this digestion. On the other hand, composting of TNT is clearly an attractive system, that has had the benefit of considerable engineering development. With regards the soil / water system, efficient reactors for aerobic digestions of soil / water slurries are now being marketed and are being further developed. Therefore aerobic digestions of not only TNT but other compounds as well, will be more attractive than they have been in the past.

In contrast with TNT, RDX has only been broken down by anaerobic metabolism. This may not accurately reflect the biodegradative capabilities of the total bacterial community, but rather it may only reflect those that have been surveyed in the several studies of RDX metabolism. It may also be a function of the general chronology of the discovery of biodegradation. Anaerobic degradation of xenobiotics was discovered long before aerobic degradation was identified. Consequently, many researchers pursued the development and discovery of new anaerobic biodegradation reactions and pathways.

With the national focus on remediating the environment and the subsequent allocation of funds for understanding biodegradation, we will undoubtedly solve the problems of many presently recalcitrant compounds. This will be done by employing our standard approach along with new and novel approaches. The metabolic diversity in microorganisms is enormous and is at present a little utilized resource in biotransformations or biodegradations.

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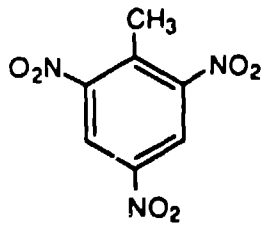
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Zeyer, J., and P. C. Kearney. 1984. Degradation of *o*-nitrophenol and *m*-nitrophenol by a *Pseudomonas putida*. *J. Agric. Food Chem.* 32:238-242.

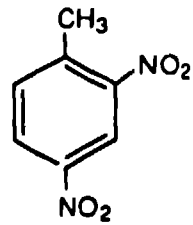
Zylstra, G. J., W. R. McCombie, D. T. Gibson, and B. A. Finette. 1988. Toluene degradation by *Pseudomonas putida* F1: genetic organization of the *tod* operon. *Appl. Environ. Microbiol.* 54(4):1498-1503.

Zylstra, G. J., and D. T. Gibson. 1989. Toluene degradation by *Pseudomonas putida* F1. *J. Biol. Chem.* 264(25):1494-14946.

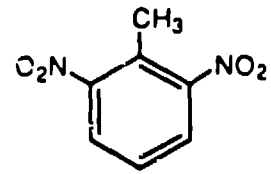
High Explosives



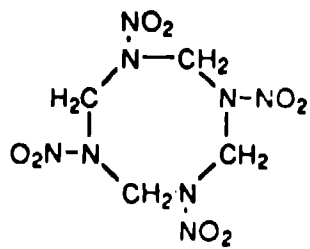
Trinitrotoluene



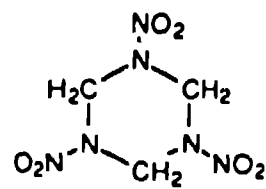
2,4-Dinitrotoluene



2,6-Dinitrotoluene



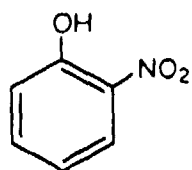
HMX



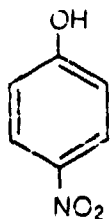
RDX

Figure 1) Chemical structures of the high explosives discussed in this report.

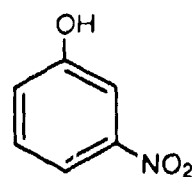
Nitrophenols



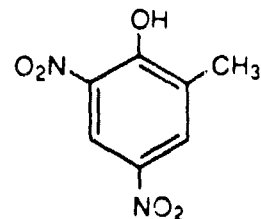
o-Nitrophenol



p-Nitrophenol

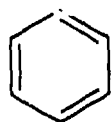


m-Nitrophenol

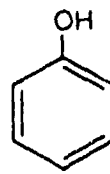


2,6-Dinitro-*o*-cresol

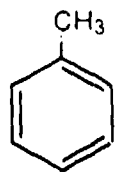
Aromatic Substrates



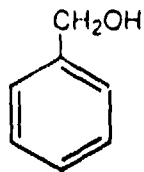
Benzene



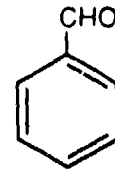
Phenol



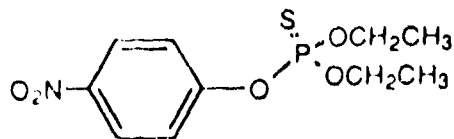
Toluene



Benzylalcohol

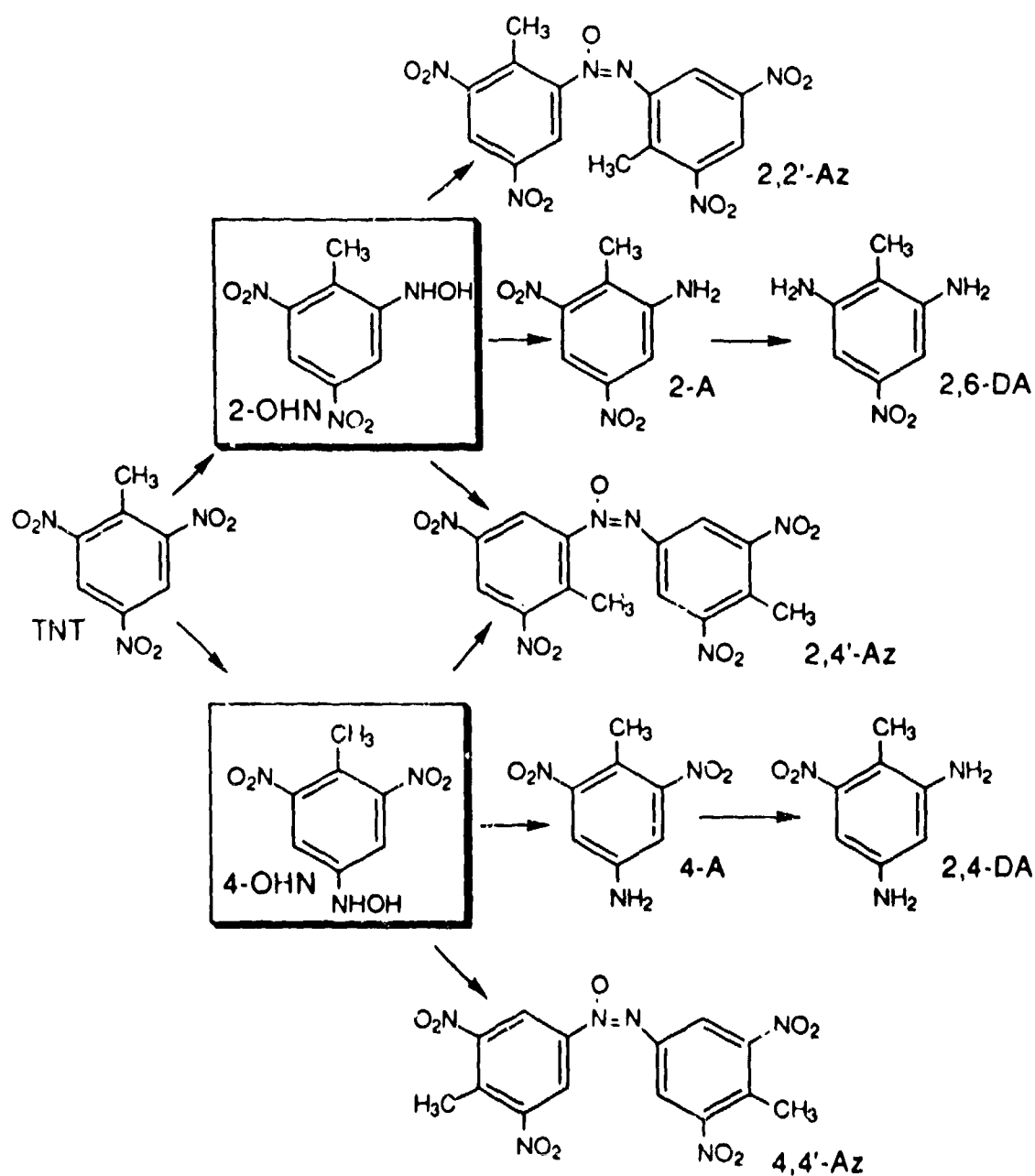


Benzaldehyde



Parathion

Figure 2) Chemical structures of aromatic compounds discussed in this report.

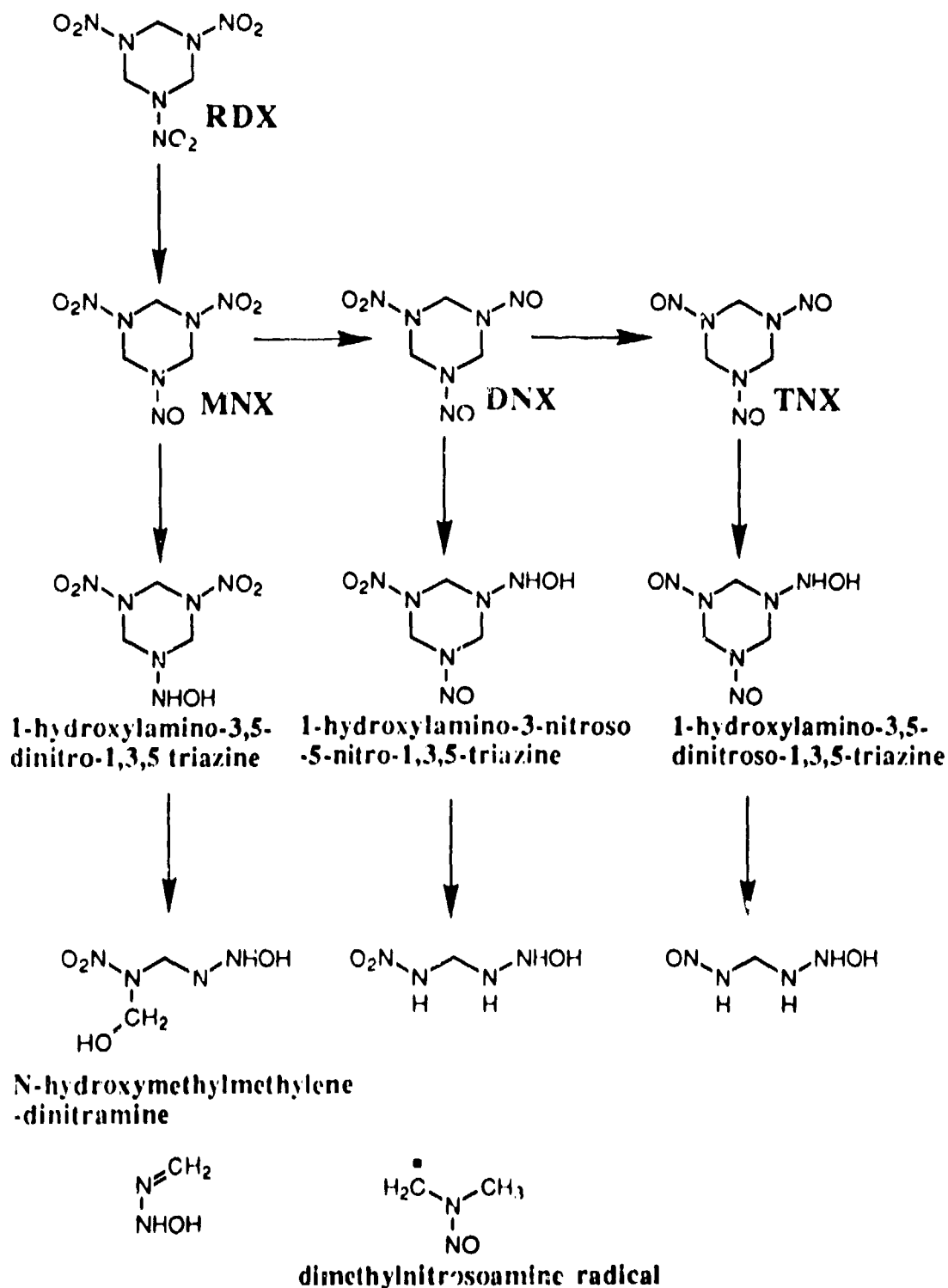


Reductive biotransformation scheme for TNT based on products isolated from compost (Kaplan and Kaplan, 1982). The hydroxylamino intermediates (boxed) were not isolated. Examples of the nomenclature used above are: 4-amino-2,6-dinitrotoluene, 4-A; 2,4-diamino-6-nitrotoluene, 2,4-DA; 2,2',6,6'-tetranitro-4,4'-azoxytoluene, 4,4'-Az; and 4-hydroxylamino-2,6-dinitrotoluene, 4-OHN.

Figure 3

Proposed Pathway for Anaerobic Biodegradation of RDX

McCormick, Cornell, and Kaplan (1981) Appl. Environ. Microbiol. 42, 817-823.



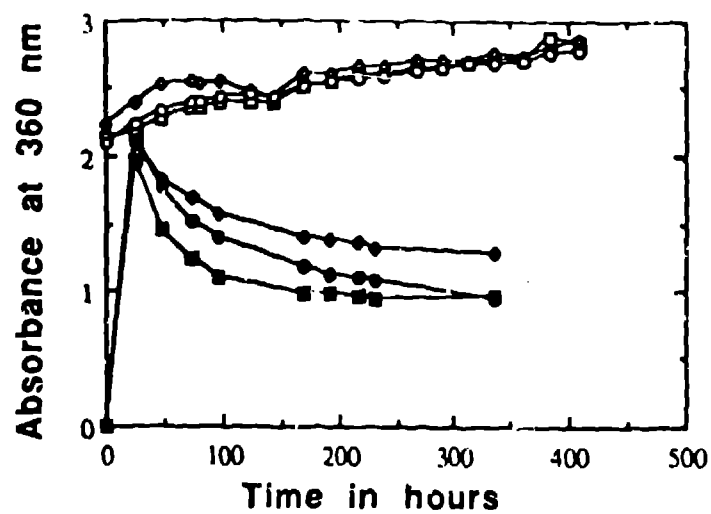


Figure 5. Maintenance of bacterial cultures with succinate as the sole carbon source (closed symbols) and with succinate and TNT as the only carbon sources (open symbols).