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BIODEGRADATION OF NITROAROMATIC COMPOUNDS

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CONTENTS

ABSTRACT

Nitroaromatic compounds are released into the biosphere almost exclusively from anthropogenic sources. Some compounds are produced by incomplete combustion of fossil fuels; others are used as synthetic intermediates, dyes, pesticides, and explosives. Recent research revealed a number of microbial systems capable of transforming or biodegrading nitroaromatic compounds. Anaerobic bacteria can reduce the nitro group via nitroso and hydroxylamino intermediates to the corresponding amines. Isolates of *Desulfovibrio* spp. can use nitroaromatic compounds as their source of nitrogen. They can also reduce

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2,4,6-trinitrotoluene to 2,4,6-triaminotoluene. Several strains of *Clostridium* can catalyze a similar reduction and also seem to be able to degrade the molecule to small aliphatic acids. Anaerobic systems have been demonstrated to destroy munitions and pesticides in soil. Fungi can extensively degrade or mineralize a variety of nitroaromatic compounds. For example, *Phanerochaete chrysosporium* mineralizes 2,4-dinitrotoluene and 2,4,6-trinitrotoluene and shows promise as the basis for bioremediation strategies.

The anaerobic bacteria and the fungi mentioned above mostly transform nitroaromatic compounds via fortuitous reactions. In contrast, a number of nitroaromatic compounds can serve as growth substrates for aerobic bacteria. Removal or productive metabolism of nitro groups can be accomplished by four different strategies. (*a*) Some bacteria can reduce the aromatic ring of dinitro and trinitro compounds by the addition of a hydride ion to form a hydride-Meisenheimer complex, which subsequently rearomatizes with the elimination of nitrite. (*b*) Monooxygenase enzymes can add a single oxygen atom and eliminate the nitro group from nitrophenols. (*c*) Dioxygenase enzymes can insert two hydroxyl groups into the aromatic ring and precipitate the spontaneous elimination of the nitro group from a variety of nitroaromatic compounds. (*d*) Reduction of the nitro group to the corresponding hydroxylamine is the initial reaction in the productive metabolism of nitrobenzene, 4-nitrotoluene, and 4-nitrobenzoate. The hydroxylamines undergo enzyme-catalyzed rearrangements to hydroxylated compounds that are substrates for ring-fission reactions. Potential applications of the above reactions include not only the biodegradation of environmental contaminants, but also biocatalysis and synthesis of valuable organic molecules.

INTRODUCTION

Natural organic compounds are readily biodegradable and can serve as sources of carbon and energy for microorganisms that have evolved over geological time to exploit them. In contrast, xenobiotic compounds synthesized and released into the biosphere only recently by humans can present daunting challenges to heterotrophic microorganisms. The inclusion of unusual chemical bonds or substitution with halogens or other functional groups can render a molecule resistant to microbial degradation. A surprisingly large number of halogenated organic compounds are produced in nature (45, 85). As a result, there are correspondingly large numbers of microorganisms able to degrade halogen-substituted compounds that bear some similarity to natural substances. The mechanisms of enzymatic attack on halogenated compounds have been studied extensively and are relatively well understood (36, 63). In contrast, only a few natural nitro-substituted compounds have been reported (125), and they are mostly antibiotics. The vast majority of the nitroaromatic compounds

detected in the environment are anthropogenic and released because of their extensive use in the synthesis of dyes, plasticizers, pesticides, and explosives. They are also produced by incomplete combustion of fossil fuels (93, 123).

The toxicity of nitroarenes and their metabolites has been studied in a variety of systems (9, 38, 59, 130, 132). Both the nitro group and the amino group are relatively stable in biological systems. Interconversion between the two, however, involves the intermediate production of the corresponding nitroso and hydroxylamino derivatives—which are very reactive and, in many instances, more toxic than the parent molecules. Polycyclic nitroaromatic compounds are not very toxic or carcinogenic, but can be activated not only by reduction of the nitro group by intestinal microflora, but also by mammalian cytochrome P-450-mediated oxidation of the aromatic ring (9, 38).

Currently, the most visible environmental problem caused by contamination with nitroaromatic compounds is the widespread contamination of soil by explosives. Sites of former manufacturing, loading, and storage facilities in the United States and Europe are heavily contaminated with munitions from World War II. In addition, the more water-soluble nitroaromatic solvents and pesticides may be appropriate targets for bioremediation. Several barriers must be overcome before biodegradation can provide an effective treatment strategy for nitroaromatic compounds: (*a*) the toxicity of nitroaromatic compounds to microorganisms, (*b*) low bioavailability due to insolubility or sorption of the contaminant, (*c*) complications caused by mixtures of nitroaromatic contaminants, and (*d*) lack of catabolic systems able to degrade the nitroaromatic compound in the microbial community. The discovery and development of appropriate catabolic systems is the subject of this review.

At first glance it would seem that the enzymes involved in the inorganic nitrogen cycle could catalyze transformations of aromatic nitro substituents. Closer examination reveals that the chemistry of the molecules and their reactions are very different and that the enzymes involved in their transformation are unrelated (6). Fortuitous transformation of nitroaromatic compounds has been studied extensively. Many, and perhaps most, living organisms contain enzymes that can catalyze the transformation of the aromatic nitro group. The most common examples are the wide variety of redox enzymes that can serve as nitroreductases (17). Less common are enzymes and complete catabolic pathways that allow bacteria to use nitroaromatic compounds as their source of nitrogen or carbon for growth. A number of such systems were discovered recently, however, and are the focus of intensive research to discover not only their mechanisms and evolutionary origins, but also their potential for biotechnology applications.

The microbial degradation of explosives and nitroaromatic compounds has been reviewed by several authors (43a, 58, 67, 68, 78a, 109, 129); therefore, most of the historical work is not discussed here. In the past four years, the

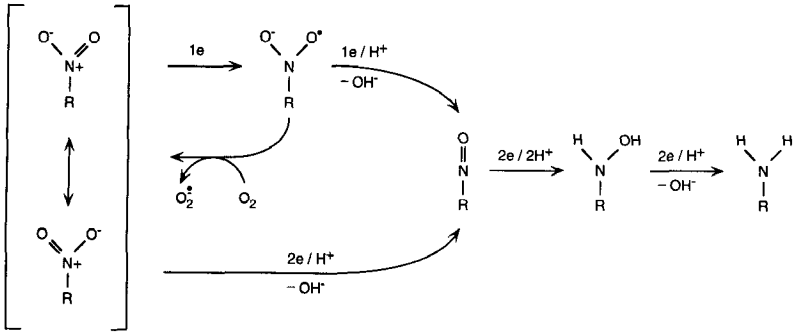

Figure 1 Reduction of nitro groups by one-electron or two-electron mechanisms.

intensive research in this area has led to dramatic progress in understanding the microbial strategies and associated mechanisms for the degradation of nitroaromatic compounds. These recent developments and the questions they have raised are the focus of this review.

CHEMISTRY OF THE AROMATIC NITRO GROUP

The nitro group exists as a resonance hybrid (Figure 1). Because the oxygen atoms are more electronegative than the nitrogen atom, the polarization of the nitrogen-oxygen bond causes the nitrogen atom to carry a partial positive charge and to serve as an electrophile. Therefore, the most common reaction of the nitro group in biological systems is reduction, which can proceed either by one-electron or two-electron mechanisms (Figure 1). In addition, iron(II), other metals and reduced sulfur compounds (31, 44, 56, 94) can serve as reductants for the nonenzymatic reduction of nitroaromatic compounds. Both the nitro group and the amino group are relatively stable. The sequence of reactions involved in reduction of the nitro group to the amine produces highly reactive intermediates, however. The nitroso and hydroxylamino groups are electrophiles that can interact with biomolecules to cause toxic, carcinogenic, and mutagenic effects (9, 59).

The one-electron reduction of the nitro group produces a nitro radical anion, which can be oxidized to the starting material by molecular oxygen with the concomitant production of superoxide. This futile cycle leads to the designation of enzymes that catalyze one-electron reduction of the nitro group as "oxygen sensitive" (17). Enzymes from a variety of sources—including strictly anaerobic bacteria such as *Clostridium* spp. (4), facultative bacteria such as *Es-*

cherichia coli (92) and *Enterobacter* spp. (17), as well as plants and animals (17)—catalyze one-electron reduction of the nitro group.

Reduction of the nitro group by the sequential addition of pairs of electrons is "oxygen insensitive" because no radicals are produced (17). Nitroreductases of this type convert nitro groups to either hydroxylamines or amines by the addition of electron pairs donated by reduced pyridine nucleotides. The reaction pathway includes the nitroso derivatives, but they are difficult to detect because they are so reactive and unstable. The high reactivity of the nitroso and hydroxylamino intermediates is responsible for much of the toxicity and carcinogenicity attributed to nitroaromatic compounds (130). Both structures can react readily with a variety of biological materials and they can also undergo condensation reactions. For example, partial reduction of the nitro group in the presence of oxygen leads to the nonenzymatic production of azoxy compounds as condensation products (79).

The ease of reduction of the aromatic nitro group depends on the nature of the other substituents on the ring and on the reducing potential of the environment. Electron-withdrawing groups activate the molecule for reduction of the nitro group, whereas electron-donating groups make the ring more susceptible to electrophilic attack. In the case of the nitrotoluenes, the probability of reduction increases and the probability of electrophilic attack decreases as the number of nitro groups increases. Therefore, the reduction of one nitro group of TNT is very rapid under a variety of conditions, including those prevalent in growing cultures of aerobic bacteria. In contrast, reduction of 2-amino-4,6-dinitrotoluene (ADNT) requires a lower redox potential, and reduction of 2,4-diamino-6-nitrotoluene (DANT) requires a redox potential below –200 mV (40), because the electron-donating properties of the amino groups lower the electron deficiency of the molecule.

ANAEROBIC BIODEGRADATION

The reactions of nitroaromatic compounds in anaerobic systems almost exclusively involve the reduction of the nitro group. McCormick et al (79) clearly demonstrated that *Viellonella alkalescens* could reduce TNT and described some of the enzymes involved. Subsequently, a variety of other bacteria have been shown to reduce aromatic nitro compounds under anaerobic (4, 79, 90, 97) and even under aerobic (105) conditions (Table 1). The most interesting recent advances are the discoveries that pure cultures of *Desulfovibrio* and *Clostridium* species can extensively degrade TNT. Although a variety of bacteria, soils, and sediments can catalyze the reduction of TNT, the studies with *Desulfovibrio* and *Clostridium* species provide novel insight into the enzymes responsible and the mechanism. Therefore, they are discussed in some detail here.

Table 1 Representative bacteria reported to reduce nitroaromatic compounds[a]

Organism	Nitroaromatic compounds transformed	Reference
Clostridium acetobutylicum	Chloramphenicol, 2-/3-nitrophenol, 2-/3-/4-nitrobenzoate, 2-nitrobenzaldehyde	89
Clostridium pasteurianum, E. coli; *Veillonella alkalescens*	TNT; 40 nitro compounds, including nitrophenols, nitrobenzoates, nitrotoluenes	79
Clostridium kluyveri, Clostridium sp. La1, *C. pasteurianum*	4-Nitrobenzoate, 2-nitroethanol	3
C. propionicum, C. butyricum, C. barkeri, Proteus mirabilis, Micrococcus lactilyticus, Peptostreptococcus anaerobius	4-Nitrobenzoate, 2-nitroethanol	2
Bacteroides fragilis	1-Nitropyrene	72
Clostridium leptum, C. paraputrificum, C. clostridiiforme, Eubacterium sp.	1-Nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene	97
Haloanaerobium praevalens, Sporohalobacter marismortui	Nitrobenzene, 2-/3-/4-nitrophenol, 2-/3-/4-nitroaniline, 2,4-dinitrophenol, 2,4-dinitroaniline	90
Desulfovibrio sp. (B. strain)	TNT, 2,4-/2,6-dinitrotoluene, 2,4-dinitrophenol	11, 12
Desulfovibrio sp.	TNT, 2,6-/3,4-dinitrotoluene, 2-/4-nitrotoluene, nitrobenzene	94
Methanobacterium formicicum, Methanobacterium thermoautotrophicum, Methanospirillum hungatei, Methanosarcina sp. KS2002, *Methanosarcina barkeri, Methanosarcina frisia, Methanogenium tationis, Desulfovibrio desulfuricans*, D. gigas, *Desulfovibrio* sp. AS, *Desulfovibrio* sp. HB, *Desulfotomaculum* sp. GROL	3-/4-Nitrophenol, 2,4-dinitrophenol, 4-nitrobenzoate, 4-nitroaniline	44
Desulfotomaculum orientis, Desulfococcus multivorans	4-Nitrophenol	44
Methanococcus	TNT	13
Pseudomonas	2-/3-/4-Chloronitrobenzene, 2-/3-/4-nitrobenzoate, 2-/3-/4-nitrophenol, 1-chloro-2,4-dinitrobenzene, TNT	105

[a] Modified from Reference 95.

Sulfate-Reducing Bacteria

Two strains of *Desulfovibrio* have been studied extensively because of their ability to transform nitroaromatic compounds. Boopathy & Kulpa studied a strain designated *Desulfovibrio* sp. strain B that uses TNT (11) and a variety of other nitroaromatic compounds (12) as the source of nitrogen for growth and also as the terminal electron acceptor. The authors provided strong evidence that the nitro compounds are reduced to the corresponding amines and proposed that the amino groups are removed from the aromatic ring by a reductive deamination mechanism analogous to that described by Schnell & Schink (106). The evidence (11, 14) that *Desulfovibrio* sp. strain B converts 2,4,6-triaminotoluene (TAT) to toluene is preliminary, and no experimental evidence was provided for the operation of the deamination mechanism, but the proposed reductive deamination would be an exciting discovery if confirmed. Tentative evidence for reductive deamination of aminotoluenes was also provided by studies of bacteria from rumen fluid (25).

Preuss et al (94) isolated a strain of *Desulfovibrio* by selective enrichment with pyruvate as the carbon source, sulfate as the terminal electron acceptor, and TNT as the source of nitrogen. The strain fixes atmospheric nitrogen and can also use ammonia as its nitrogen source. Under growth conditions the sulfide in the medium chemically reduces the TNT to DANT. The DANT is then reduced by the *Desulfovibrio* sp. to TAT, which subsequently disappears from the culture fluid.

Suspensions of cells containing little or no sulfide catalyze the reduction of TNT to TAT when pyruvate is supplied as the electron donor. The rate of reduction of each successive nitro group decreases dramatically because amino groups deactivate the molecule for further reduction. The authors suggested that the facile reduction of TNT to DANT is mediated by nonspecific enzymes that reduce low-potential electron carriers such as ferredoxin. Therefore, the authors focused their subsequent investigations on the mechanism of conversion of DANT to TAT, because it seems to be the rate-determining step in the overall process.

Hydrogen, pyruvate, or carbon monoxide can serve as electron donors for the reduction of DANT by cell suspensions of the *Desulfovibrio* sp. Therefore, the corresponding ferredoxin-reducing enzymes hydrogenase, pyruvate-ferredoxin-oxidoreductase, and carbon monoxide dehydrogenase are probably responsible for the reduction of DANT. When hydrogen or pyruvate serves as the electron donor, DANT is reduced to TAT. In contrast, DANT is only partially reduced, to 2,4-diamino-6-hydroxylaminotoluene (DAHAT), when carbon monoxide serves as the electron donor. Furthermore, with pyruvate as the electron donor, the addition of carbon monoxide inhibits the production of TAT and causes the accumulation of DAHAT. Similarly, inhibition results

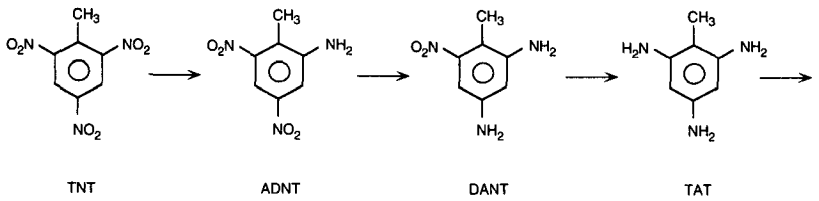

Figure 2 Reduction of TNT by anaerobic bacteria.

when hydroxylamine is included in cell suspensions using hydrogen as the electron donor (94). The above observations led the authors to suggest that sulfite reductase might be responsible for the reduction of DAHAT to TAT, because sulfite reductase is known to be inhibited by carbon monoxide. Subsequent experiments revealed that the reduction of sulfite by cells of the *Desulfovibrio* sp. was inhibited by carbon monoxide, DAHAT, DANT, and hydroxylamine. These results provide strong circumstantial evidence that sulfite reductase is responsible for the reduction of DAHAT to TAT in the *Desulfovibrio* sp. (94). In contrast to the sequence postulated by Boopathy & Kulpa (11), TAT was not further metabolized by the *Desulfovibrio* sp. The overall scheme of TNT reduction by the *Desulfovibrio* sp. is outlined in Figure 2.

Clostridium

Several strains of clostridia have been studied because of their ability to reduce nitroaromatic compounds (4, 79, 94, 97). Angermaier & Simon (4) provided evidence that hydrogenase and ferredoxin in *Clostridium kluyveri* are responsible for a one-electron reduction of nitroaromatic compounds. Rafii et al (97) characterized oxygen-sensitive enzymes from several strains of *Clostridium* isolated from human fecal material. The enzymes reduced 4-nitrobenzoate and several nitropyrenes to the corresponding amines. It is not clear whether the reaction catalyzed by the enzyme is oxygen sensitive because it is a one-electron transfer or because the reaction involves transfer of two electrons catalyzed by an enzyme that is oxygen labile.

Hydrogenase from *Clostridium pasteurianum* and carbon monoxide dehydrogenase from *Clostridium thermoaceticum* reduce DANT to DAHAT when ferredoxin is included in the reaction mixture (94). The reduction also takes place with reduced ferredoxin or methyl viologen in the absence of enzymes, which suggests that the enzymes are not specific for the nitroaromatic compound but only serve to reduce the ferredoxin. Thus the wide distribution of nonspecific, oxygen-sensitive nitroreductases among biological systems seems

to reflect the wide distribution of redox enzymes. It follows that much of the reduction under such conditions can be attributed to the ability of many nitroaromatic compounds to be reduced nonenzymatically by a variety of reductants including iron(II), sulfhydryl compounds, and small electron carriers such as ferredoxin. In contrast, the conversion of DAHAT to TAT is catalyzed more slowly by the clostridia and seems to require the action of specific enzymes such as the ones in *Desulfovibrio* spp. (94).

Kaake et al used an anaerobic mixed culture in the development of a strategy for the biodegradation of dinoseb (2-*sec*-butyl-4,6-dinitrophenol) (65, 66) under methanogenic conditions with starch as the electron donor. Similar enrichment cultures degraded RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) and TNT to nondetectable levels in contaminated soil (39, 40). Pure cultures of *Clostridium bifermentans* isolated from the consortium (100) and similar strains isolated from other enrichments (26) degrade both RDX and TNT. The pathway for reduction of TNT to TAT by the consortium and the isolated *Clostridium* strains seems to be the same as that proposed by other workers (94). However, the TAT is subsequently degraded extensively not only by the consortium but also by the isolates. The authors provide evidence that TAT is first converted to 2,4,6-trihydroxytoluene by hydrolysis of the amino groups. They propose (26) that reductive dehydroxylation reactions convert 2,4,6-trihydroxytoluene to 4-hydroxytoluene (*p*-cresol), which is then converted to simple organic acids readily assimilated by the culture. The evidence for the proposed pathway is preliminary, and exploration of the mechanism involved will provide an exciting area for future research.

The studies described above indicate that reduction of the nitro group is the major reaction controlling the behavior and fate of nitroaromatic compounds in ecosystems that contain bacteria. The reactions that convert TNT to TAT can be catalyzed by a variety of bacteria, but the subsequent metabolism of TAT is controversial at present.

BIODEGRADATION BY FUNGI

The white rot fungus, *Phanerochaete chrysosporium,* produces a complex system of extracellular peroxidases, small organic molecules, and hydrogen peroxide for the degradation of lignin. The ligninolytic system is nonspecific and can biodegrade a wide range of synthetic chemicals including nitroaromatic compounds. Barr & Aust (8) have provided an excellent review of the mechanisms used by the fungus for transformation of synthetic chemicals. Several groups (18, 35, 80, 114–116, 124) have reported the degradation and even mineralization of nitroaromatic compounds by *P. chrysosporium.*

The initial steps in the fungal degradation of both 2,4-dinitrotoluene and TNT involve reduction of the nitro groups. Valli et al (124) suggested that

2,4-dinitrotoluene is reduced predominantly to 2-amino-4-nitrotoluene by intracellular reductase enzymes that are not part of the ligninolytic system. Evidence for the pathway was provided by transformation experiments with mycelia and purified enzymes. Manganese peroxidase catalyzes the conversion of 2-amino-4-nitrotoluene to 4-nitro-1,2-benzoquinone. The authors suggest that the quinone is reduced to 4-nitrocatechol, but provide no strong evidence for the reaction. 4-Nitrocatechol serves as a substrate for oxidative removal of the nitro group in a reaction catalyzed by manganese peroxidase. Alternatively, methylation reactions and subsequent removal of the nitro group can be catalyzed by lignin peroxidase. The methylated and dihydroxy intermediates were readily degraded by the mycelium, and subsequent reactions in the pathway were demonstrated in cell extracts or with purified enzymes.

Most fungi can catalyze the reduction of at least one nitro group of TNT (91). Mycelia of *P. chrysosporium* reduce TNT to a mixture of 2-amino-4, 6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, and 2,4-diamino-6-nitrotoluene (115). Under ligninolytic conditions the amino compounds disappear, and mineralization can be fairly extensive. Stahl & Aust (116) provided evidence that the reduction of TNT requires live, intact mycelia and that extracellular enzymes and enzymes in cell extracts cannot catalyze the reaction even when supplemented with reduced pyridine nucleotides. They proposed that the reduction reaction is closely coupled to the proton export system used by the fungus to maintain an external pH of 4.5. Generation of protons would produce electrons that could be used in the reduction reaction. In contrast, Valli et al (124) suggested that 2,4-dinitrotoluene is reduced by intracellular enzymes in *P. chrysosporium*. Similarly, Michels & Gottschalk (80) indicated that TNT is reduced by an NADH-dependent nitroreductase in extracts prepared from *P. chrysosporium*. Neither of these latter groups, however, provided data on the reduction reactions. All of the above studies support the conclusion that the ligninolytic system is not involved in the initial reduction of nitroaromatic compounds.

Ligninolytic cultures of *P. chrysosporium* mineralize TNT, whereas nonligninolytic cultures produce primarily the amino derivatives and azoxy condensation products. Nonligninolytic cultures can also mineralize TNT to a lesser extent (80, 94, 114, 115). The mechanism by which the fungi mineralize TNT is not known, but several recent investigations provided preliminary information about the process. The amino derivatives of TNT are not substrates for the ligninolytic system (18), but reduction of the molecule is required prior to mineralization. Michels & Gottschalk (81) provided strong evidence that under nonligninolytic conditions TNT is reduced to 4-amino-2,6-dinitrotoluene via the corresponding hydroxylamino intermediate (Figure 3). The 4-amino-2,6-dinitrotoluene is coverted to 4-formamido-2,6-dinitrotoluene, which is subsequently reduced to 2-amino-4-formamido-6-nitrotoluene and finally con-

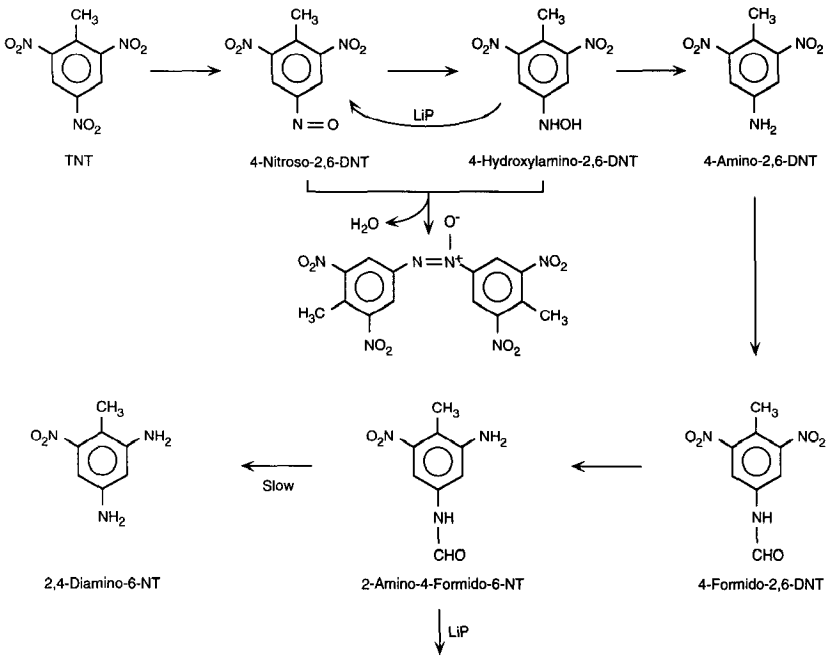

Figure 3 Transformation of TNT by *Phanerochaete chrysosporium*. 4-Hydroxylamino-2,6-DNT and 2-amino-4-formamido-6-nitrotoluene are substrates for lignin peroxidase (LiP) (80, 115).

verted to DANT. They found no evidence for the direct reduction of ADNT to DANT. Both 4-hydroxylamino-2,6-dinitrotoluene and 2-amino-4-formamido-6-nitrotoluene are substrates for oxidation by the ligninolytic system and purified lignin peroxidase. This preliminary work suggests a possible mechanism that would explain previous, seemingly conflicting, observations by several groups. There remain, however, a number of questions concerning the mineralization pathway and the participation of the other isomers of the intermediates produced during reduction of TNT. Even under optimum conditions for mineralization, the majority of the carbon from TNT is not converted to carbon dioxide, and the final products of biodegradation other than carbon dioxide have not been characterized.

The major impediment to the practical application of white rot fungi for biodegradation of TNT has been its toxicity to the fungus. Investigations into the mechanism of toxicity of TNT have revealed that the parent compound and its amino derivatives are not toxic to *P. chrysosporium* (18, 80). In contrast, 4-hydroxylamino-2,6-dinitrotoluene and 2-hydroxylamino-4,6-dinitrotoluene dramatically inhibited veratryl alcohol oxidation by lignin peroxidase. The

conversion of veratryl alcohol to veratryl aldehyde is essential for the production of the organic radicals involved in the oxidation of chemicals that are not primary substrates for lignin peroxidase (55). The hydroxylamino compounds are good substrates for the enzyme and competitively inhibit the oxidation of veratryl alcohol. In some studies (80), oxidation of hydroxylaminodinitrotoluenes led to the accumulation of azoxy condensation products; other workers (18) suggest that TNT is the final product of the oxidation. It is clear that development of an approach that avoids the accumulation of hydroxylamino intermediates is the key to practical application of the fungus for biodegradation of TNT.

AEROBIC BIODEGRADATION BY BACTERIA

In contrast to the nonspecific metabolism by fungi and anaerobes, some aerobic bacteria can use nitroaromatic compounds as growth substrates. They can often derive carbon, nitrogen, and energy from degradation of nitroaromatic substrates. Therefore, the isolation and study of such bacteria is much easier than are comparable studies of anaerobes and fungi able to transform nitro compounds. It is remarkable that the capabilities of such aerobic bacteria have only recently come to be appreciated.

Bacteria able to degrade nitrophenols and nitrobenzoates were reported many years ago, but the mechanisms they use for metabolism of the nitro group were not well understood. Recently, bacteria able to degrade a wide range of polar and nonpolar nitroaromatic compounds were reported. Such bacteria use a variety of strategies for the removal or transformation of the nitro group. These include (*a*) monooxygenase-catalyzed elimination of the nitro group as nitrite; (*b*) dioxygenase-catalyzed insertion of two hydroxyl groups with subsequent elimination of the nitro group as nitrite; (*c*) partial reduction of the nitro group to a hydroxylamine, which is the substrate for rearrangement or hydrolytic reactions and elimination of ammonia; and (*d*) partial reduction of the aromatic ring to form a Meisenheimer complex and subsequent elimination of the nitro group as nitrite. Each of these strategies is described in detail below.

Monooxygenase-Catalyzed Initial Reactions

Some of the earliest reports on the biodegradation of nitroaromatic compounds involved studies of bacteria able to grow on nitrophenol. Simpson & Evans (108) provided preliminary evidence in 1953 that a strain of *Pseudomonas* could convert 4-nitrophenol to hydroquinone with concomitant release of nitrite. The details of the pathway (Figure 4) only became clear almost 40 years later (110). Studies with a partially purified enzyme (111) revealed that a strain of *Moraxella* degrades 4-nitrophenol by an initial oxygenase attack that results

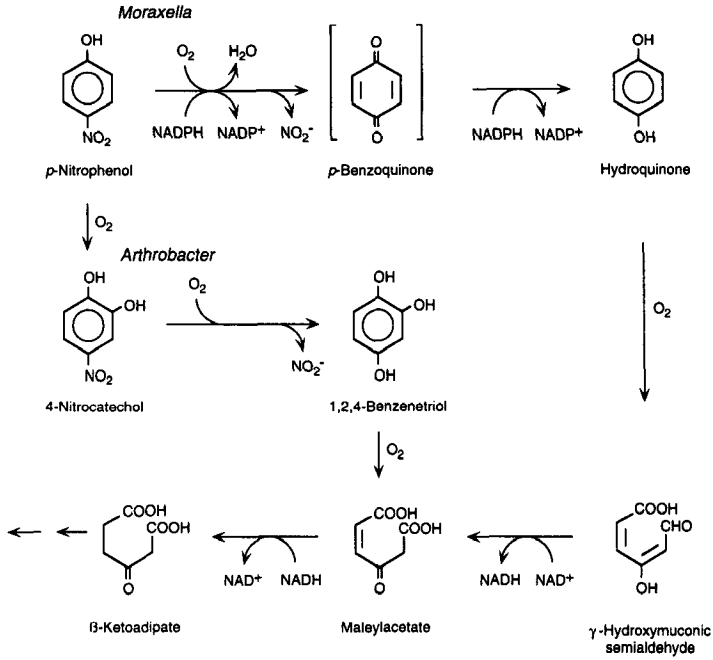

Figure 4 Biodegradation of 4-nitrophenol by *Moraxella* sp. and *Arthrobacter* sp. (62, 110).

in the release of nitrite and the accumulation of hydroquinone. Two moles of NADPH are required to oxidize each mole of 4-nitrophenol. Experiments with $^{18}O_2$ provided rigorous evidence that the mechanism of the reaction is a monohydroxylation (111), and preliminary evidence suggests that the enzyme is a flavoprotein monooxygenase. The stoichiometry of the reaction and the accumulation of hydroquinone as the first detectable intermediate suggest that the initial product of the reaction is 1,4-benzoquinone. An inducible quinone reductase present in cells grown on 4-nitrophenol is not easily separated from the membrane-bound oxygenase that catalyzes the initial reaction. Therefore the quinone could not be detected during the reaction. A similar reaction mechanism has been proposed for the elimination of anionic leaving groups catalyzed by other flavoprotein monooxygenases, including *p*-hydroxybenzoate hydroxylase (60) and pentachlorophenol monooxygenase (134).

The hydroquinone produced by the initial reactions serves as the substrate for a ring-fission reaction catalyzed by a ferrous iron–dependent dioxygenase. The ring-fission product is oxidized to maleylacetic acid, which is then reduced to β-ketoadipate. Only catalytic amounts of NAD^+ are required for the con-

version of the ring-fission product to β-ketoadipate in cell extracts because the two reactions recycle the cofactor. Cells of the *Moraxella* sp. grown on 4-nitrophenol also contain an enzyme that catalyzes the oxidation of 1,2,4-benzenetriol. The presence of this enzyme suggests that the nitrophenol could be degraded by an alternate pathway via 4-nitrocatechol, but there is no evidence for the production of 4-nitrocatechol from 4-nitrophenol by this strain. Furthermore, the monooxygenase that initiates the conversion of 4-nitrophenol to hydroquinone is present at levels sufficient to account for the growth of the cells. It is possible that small amounts of 4-nitrocatechol are produced by nonspecific hydroxylases. In support of this hypothesis, 4-nitrocatechol is oxidized slowly by 4-nitrophenol-grown cells with concomitant release of nitrite. It is also possible that a nonspecific hydroxylase converts hydroquinone to 1,2,4-benzenetriol during the operation of the primary pathway. In either case the primary pathway in the *Moraxella* sp. (110) and in an *Arthrobacter* and a *Nocardia* sp. studied by Hanne et al (53) involves the conversion of 4-nitrophenol to hydroquinone. The presence of 1,2,4-benzenetriol dioxygenase in the *Moraxella* remains to be explained.

In contrast to the strains described above, an *Arthrobacter* sp. (62) seems to degrade 4-nitrophenol via 4-nitrocatechol. This pathway (Figure 4) was suggested based on preliminary evidence in an early report (99) on the degradation of 4-nitrophenol by a *Flavobacterium* sp. In cells of the *Arthrobacter* sp., 1,2,4-benzenetriol is produced by the initial reactions and subsequently oxidized to maleylacetic acid. 4-Nitrophenol-grown cells do not contain enzymes capable of oxidizing hydroquinone at detectable rates. The enzyme responsible for the initial attack on 4-nitrophenol has not been detected in cell extracts, so it has not been studied. An enzyme that converts 4-nitrophenol to 4-nitrocatechol has been purified from a *Nocardia* sp. grown on 4-nitrophenol (82), however, and a similar enzyme activity has been demonstrated in another strain of *Nocardia* after growth on phenols (53). The enzyme responsible for the initial attack on 4-nitrophenol in the *Arthrobacter* sp. clearly requires additional study.

The pathway for degradation of 2-nitrophenol, like that of 4-nitrophenol, was suggested based on preliminary evidence in an early report by Simpson & Evans (108). It was confirmed 30 years later by Zeyer & Kearney (135) in a *Pseudomonas putida* isolated from soil. An NADPH-dependent monooxygenase present in extracts of 2-nitrophenol-grown cells catalyzes the conversion of 2-nitrophenol to catechol with the concomitant release of nitrite. Catechol is subsequently oxidized by a 1,2-dioxygenase and degraded via β-ketoadipate. Purified 2-nitrophenol monooxygenase catalyzes the conversion of 2-nitrophenol to catechol with the concomitant oxidation of two moles of NADPH (136). The authors proposed that the enzymatic reaction produces 1,2-benzoquinone by a mechanism analogous to the reaction catalyzed by

4-nitrophenol monooxygenase (111). 2-Nitrophenol monooxygenase is unusual among monooxygenases that catalyze the removal of aromatic nitro groups in that it does not seem to require the participation of a flavin cofactor.

Dioxygenase-Catalyzed Initial Reactions

The catabolism of aromatic hydrocarbons by aerobic bacteria generally requires the activation of the molecule by the addition of two hydroxyl groups to the ring. The reactions are catalyzed by dioxygenase enzymes that introduce two atoms of molecular oxygen on adjacent carbon atoms (42). When the aromatic substrate is a hydrocarbon, the introduction of two hydroxyl groups forms a *cis*-1,2-dihydroxy cyclohexadiene, which is subsequently rearomatized by the action of a dehydrogenase. With substituted aromatic compounds, the introduction of the hydroxyl groups can lead to spontaneous elimination of the substituent and rearomatization of the ring. For example, toluene dioxygenase catalyzes the elimination of hydroxyl groups from phenols (112); naphthalene dioxygenase can remove sulfonyl and hydroxyl groups from substituted naphthalenes (16); 4-sulfobenzoate 3,4-dioxygenase eliminates sulfite from 4-sulfobenzoate (76, 77); and analogous mechanisms have been demonstrated for the removal of chloro (37, 78) and amino (7, 64) substituents.

The removal of aromatic nitro groups by dioxygenase enzymes was first reported by Ecker et al (33) as a result of studies on the transformation of 2,6-dinitrophenol by *Alcaligenes eutrophus*. Additional support for the reaction mechanism came from the observation that the enzyme used by a *Pseudomonas* sp. for the initial oxidation of chlorobenzenes could catalyze the elimination of the nitro group from 2,4,5-trichloronitrobenzene (104). Both of these studies involved fortuitous reactions that resulted in partial transformation of nitroaromatic compounds that could not serve as growth substrates. Subsequently, several groups have isolated aerobic bacteria able to grow on nitroaromatic compounds and remove the nitro group by means of dioxygenase enzymes as the first step in the catabolic pathway.

Nitrobenzene, used extensively as the starting material for synthesis of aniline, is converted to catechol (Figure 5*a*) as the first step in its mineralization by a *Comamonas* sp. isolated from an aerobic waste-treatment plant (88). Experiments with $^{18}O_2$ provided rigorous proof that the initial reaction proceeds by a dioxygenase mechanism. The inducible nitrobenzene dioxygenase is very nonspecific and also catalyzes the oxidation of a variety of nitrophenols, dinitrobenzenes, and nitrotoluenes (SF Nishino & JC Spain, unpublished results). A high activity with naphthalene in intact cells suggests that the dioxygenase might be closely related to naphthalene dioxygenase, but the enzyme was not active in cell extracts and has not been characterized further.

A *Pseudomonas* strain isolated from contaminated soil by selective enrichment grows on 2-nitrotoluene as the sole source of nitrogen and carbon (52).

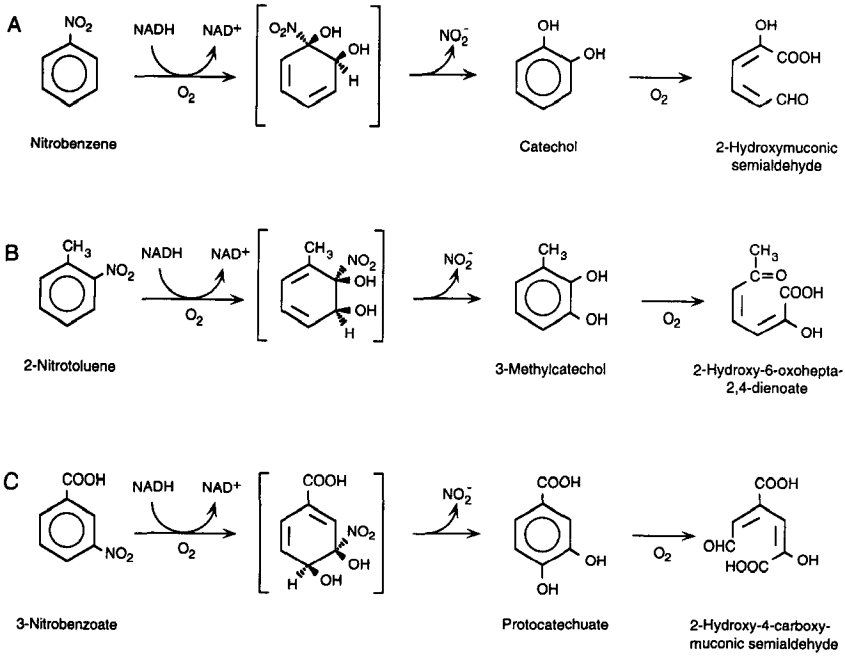

Figure 5 Pathways involving initial reactions catalyzed by dioxygenase enzymes (52, 84, 88).

The catabolic pathway (Figure 5*b*) involves an initial dioxygenase attack at the 2,3 position of the molecule to form 3-methylcatechol and release nitrite. 3-Methylcatechol is degraded by a typical *meta* cleavage pathway. The enzymes of the 2-nitrotoluene degradative pathway are constitutive, and 2-nitrotoluene dioxygenase has been purified and characterized (1). It consists of three components: an iron-sulfur protein with two subunits similar to the terminal oxygenase component of other dioxygenases, a flavo-iron-sulfur reductase, and a small ferredoxin-like protein, which can be replaced by the ferredoxin obtained from naphthalene dioxygenase. The enzyme system appears to be very similar to naphthalene dioxygenase isolated from another strain of *Pseudomonas* (34). Purification of the enzyme allowed rigorous proof that the insertion of molecular oxygen and release of nitrite involves a dioxygenase mechanism, and that the rearomatization of the ring does not require a separate enzyme.

Early work indicated that protocatechuate is an intermediate in the degradation of 3-nitrobenzoate by a *Nocardia* sp. (22). 3-Hydroxybenzoate was also oxidized by freeze-dried cells but not by resting cells, so the authors proposed

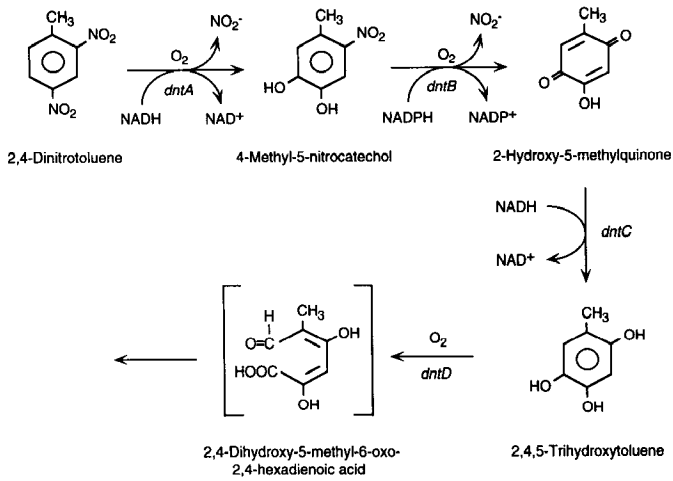

Figure 6 Biodegradation of 2,4-dinitrotoluene by *Pseudomonas* sp. strain DNT (50, 113).

that sequential monooxygenase reactions convert 3-nitrobenzoate to protocatechuate via 3-hydroxybenzoate, and release the nitro group as nitrite. In contrast, strains of *Pseudomonas* and *Comamonas* do not oxidize 3-hydroxybenzoate after growth on 3-nitrobenzoate (84). These strains convert 3-nitrobenzoate to protocatechuate by means of a dioxygenase attack at the 3,4 position with subsequent elimination of nitrite (Figure 5*c*). Protocatechuate 4,5-dioxygenase catalyzes the oxidation of protocatechuate in the *Pseudomonas* strain.

2,4-Dinitrotoluene (2,4-DNT) is a by-product of the manufacture of TNT and is also used extensively as an intermediate in the synthesis of toluene diisocyanate. It has been released widely into the environment, where it seems to be relatively stable. Bacteria able to mineralize 2,4-DNT have been isolated from a variety of contaminated soils, and the biodegradation of 2,4-DNT has been studied in pure cultures. Mineralization of a nitroaromatic compound via a dioxygenase initial attack was first reported as a result of studies with *Pseudomonas* sp. strain DNT grown on 2,4-DNT. The dioxygenase enzyme that catalyzes the initial reaction is constitutive and has a broad substrate range that is very similar to that of naphthalene dioxygenase (120). It adds hydroxyl groups to the 4 and 5 positions on the ring of 2,4-DNT, and the nitro group is eliminated nonenzymatically as nitrite (Figure 6) (113). The genes that encode 2,4-DNT dioxygenase (*dntA*) are on a 180-kilobase (kb) plasmid in *Pseudomonas* sp. strain DNT (121). Cosmid cloning and restriction analysis led to

the isolation of a 6.8 kb fragment that expresses dioxygenase activity in *E. coli*. Sequence analysis (120) revealed four open reading frames whose sequences exhibit a high degree of similarity to the corresponding sequences in the naphthalene dioxygenase genes. The cloned 2,4-DNT dioxygenase expressed in *E. coli* catalyzes the oxidation of naphthalene and the other substrates of naphthalene dioxygenase. The only obvious difference in the substrate ranges of the two enzymes expressed in *E. coli* is the inability of naphthalene dioxygenase to catalyze the release of nitrite from 2,4-DNT.

4-Methyl-5-nitrocatechol (MNC) produced by 2,4-DNT dioxygenase is the substrate for a monooxygenase that catalyzes the replacement of the nitro group and elimination of nitrite (Figure 6). The constitutive enzyme, partially purified from cells of *Pseudomonas* sp. strain DNT, converts MNC to 2-hydroxy-5-methylquinone (50). The reaction mechanism is similar to that described for other enzymes that catalyze the removal of nitro groups from nitrophenols (111, 136) and the removal of other electron-withdrawing groups from substituted phenols (134) or carboxylic acids (60). Detection of the quinone as a product of the reaction provides the first clear evidence of the reaction mechanism that previously has only been postulated based on the stoichiometry of the reaction. In the earlier work, the hypothetical quinone intermediates were not detected because they were reduced rapidly either chemically or by the action of quinone reductases. 2-Hydroxy-5-methylquinone is sufficiently stable under the conditions of the reaction to allow its detection and quantitation.

The gene that encodes MNC monooxygenase (*dntB*) is located on the same large plasmid as *dntA*, but the two genes are not contiguous and seem to be regulated independently (121). It has been cloned on a 2.2 kb fragment and expressed in *E. coli*, but the sequence of the gene and the characteristics of the protein have not been reported.

An inducible NADH-dependent quinone reductase is responsible for the conversion of 2-hydroxy-5-methylquinone to 2,4,5-trihydroxytoluene. The enzyme and the gene involved in its synthesis have not been isolated or studied in any detail. High constitutive levels of quinone reductase in both *Pseudomonas* spp. (50) and *E. coli* (121) have so far precluded determination of whether the enzyme is specific for 2-hydroxy-5-methylquinone.

2,4,5-Trihydroxytoluene serves as the substrate for a ring-fission reaction catalyzed by a dioxygenase enzyme. The mechanism of the reaction is not clear because the ring-fission product is unstable and has not been isolated (50). The gene for 2,4,5-trihydroxytoluene dioxygenase (*dntD*) exhibits a high homology with the catechol 2,3-dioxygenase gene family (54, 122), which suggests the likelihood of an extradiol cleavage as indicated in Figure 6. The evidence is circumstantial, however, and rigorous determination of the reaction mechanism awaits the identification of the ring-fission product.

A strain of *Rhodococcus* (30) and an unidentified bacterium isolated from

contaminated soil (86) biodegrade 1,3-dinitrobenzene via an initial dioxygenase mechanism similar to that described above for 2,4-DNT. 4-Nitrocatechol has been identified as the product of the initial reaction, but the details of the subsequent reactions are not known. Both of the isolates studied to date grow very slowly on 1,3-dinitrobenzene, and investigation of the biochemistry will be facilitated by the isolation of more robust strains.

The evolutionary relationships among the dioxygenases that remove nitro groups are a promising area for future study. The available preliminary evidence suggests that some of the enzyme systems are related to naphthalene dioxygenase. The structural changes that would be required to allow naphthalene dioxygenase to accept nitro-substituted molecules as substrates, however, are unknown.

Reduction of the Aromatic Ring

The electron-withdrawing properties of the nitro group cause the aromatic ring of polynitroaromatic compounds to be highly electron deficient and resistant to electrophilic attack. Lenke et al (74) discovered an alternative mechanism of transformation involving reduction of the aromatic ring. They isolated strains of *Rhodococcus erythropolis* that use 2,4-dinitrophenol as the nitrogen source for growth and later discovered that the nitro compound could also serve as a carbon and energy source. Isolation of the *R. erythropolis* strains confirmed earlier work by Hess et al (57), who isolated a *Janthinobacterium* sp. and an actinomycete able to mineralize 2,4-dinitrophenol with stoichiometric release of nitrite. The *R. erythropolis* (74) isolates released nitrite from 2,4-dinitrophenol, and significant amounts of 4,6-dinitrohexanoate transiently accu- mulated during growth. Under anaerobic conditions 2,4-dinitrophenol is converted stoichiometrically to 4,6-dinitrohexanoate and no nitrite is released. The detection of a reduced ring-fission product containing two nitro groups provides clear evidence that the *R. erythropolis* strains contain enzymes able to catalyze reduction of the aromatic ring. It is not clear whether the reduction of the ring leads to a productive catabolic pathway or is a side reaction. The accumulation of 4,6-dinitro-hexanoate suggests that the aliphatic compound is a dead-end metabolite (Figure 7).

Resting cells of *Rhodococcus erythropolis* grown on 2,4-dinitrophenol released nitrite from picric acid, and spontaneous mutants can use picric acid as the nitrogen source (73). The initial reaction in the utilization of picric acid by these strains is the addition of a hydride ion to the aromatic ring to form a hydride-Meisenheimer complex (Figure 7). The reaction has also been demonstrated in cell extracts, and rigorous proof for the structure of the hydride-Meisenheimer complex has been obtained by NMR spectroscopic analysis (103). Addition of a second hydride ion leads to the eventual formation of 2,4,6-trinitrocyclohexanone, which decomposes to form 1,3,5-trinitropentane

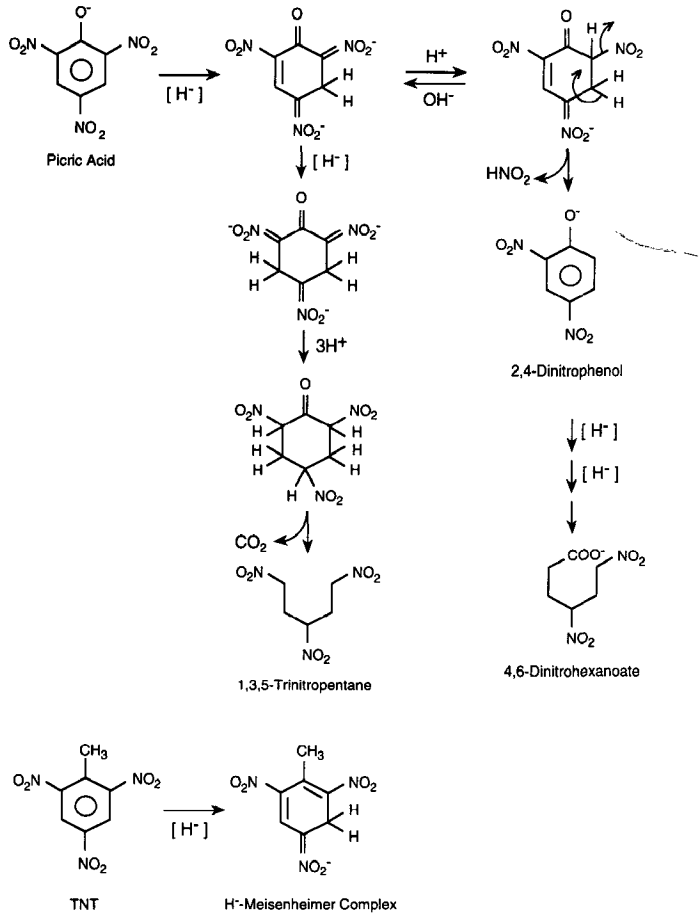

Figure 7 Reduction of the aromatic ring to form an hydride-Meisenheimer complex. Adapted from Lenke & Knackmuss (73), Lenke et al (74), Rieger et al (103), and Vorbeck et al (127).

upon acidification and extraction. This reaction sequence seems to be nonproductive. In contrast, protonation of the hydride-Meisenheimer complex leads to the enzyme-catalyzed rearomatization of the molecule and elimination of nitrite, which can be assimilated by the bacteria. The 2,4-dinitrophenol generated from picric acid is degraded by the bacteria and nitrite is eliminated, but it is not clear whether the mechanism involves formation of a second Meisenheimer complex. A preliminary report that a mixed culture grown on picric acid accumulates 2,4-dinitrophenol transiently (98) also suggests that the elimi-

nation of nitrite from the hydride-Meisenheimer complex can lead to productive metabolism of the aromatic ring.

Because the ring of TNT is also very electron deficient, it would be expected to be susceptible to reduction by a hydride ion. Duque et al (32) suggested that a hydride-Meisenheimer complex might be involved in the degradation of TNT by a constructed *Pseudomonas* strain. They initially isolated a strain able to use TNT and several other nitroaromatic compounds as the source of nitrogen. They subsequently selected a more effective strain and, based on preliminary evidence, proposed that it converted TNT to toluene via 2,4-dinitrotoluene, 2,6-dinitrotoluene, and 2-nitrotoluene. Subsequently, rigorous evidence for the conversion of TNT to the corresponding hydride-Meisenheimer complex was provided by Vorbeck et al (127) (Figure 7). Resting cells of a *Mycobacterium* sp. grown on 4-nitrotoluene accumulated several products, and the major metabolite (40%) was the hydride-Meisenheimer complex. A small amount (5%) of 4-ADNT accumulated, as did an additional, unidentified metabolite. A small amount of nitrite was released, but no dinitrotoluenes were detected.

To date, the formation of the hydride-Meisenheimer complexes of polynitroaromatic compounds has only been demonstrated to play a major role in the degradation of picric acid (73, 103). A number of questions remain about the enzymes that catalyze the addition of the hydride ion and those that catalyze the subsequent rearomatization and elimination of the nitro group. Three reactions of the hydride-Meisenheimer complex have been demonstrated in bacteria. The complex can (*a*) spontaneously decompose to the parent compound, (*b*) be reduced to aliphatic compounds, or (*c*) rearomatize by the addition of a proton and the elimination of nitrite. The factors that regulate the relative contributions of these competing reactions in vivo are unknown. Reduction of the nitro groups of polynitroaromatic compounds as described earlier is a competing process in aerobic bacteria that can reduce the aromatic ring by addition of a hydride ion. Thus, amino compounds are also produced from TNT by the strains that accumulate the hydride-Meisenheimer complex (32, 127, 128). Therefore, either the cells must contain competing reductase enzymes, or the same reductases can catalyze both reactions. In contrast, *Rhodococcus erythropolis* does not reduce the nitro groups of picric acid (73).

Partial Reduction of the Nitro Group Under Aerobic Conditions

Very early reports on the biodegradation of 2-nitrobenzoate (19, 20, 71) and 4-nitrobenzoate (21, 22) provided evidence that partial reduction of the nitro group might allow subsequent productive metabolism of nitroaromatic compounds. These studies indicated that the nitro groups are reduced and that the nitrogen is released from the molecule as ammonia. The participation of the

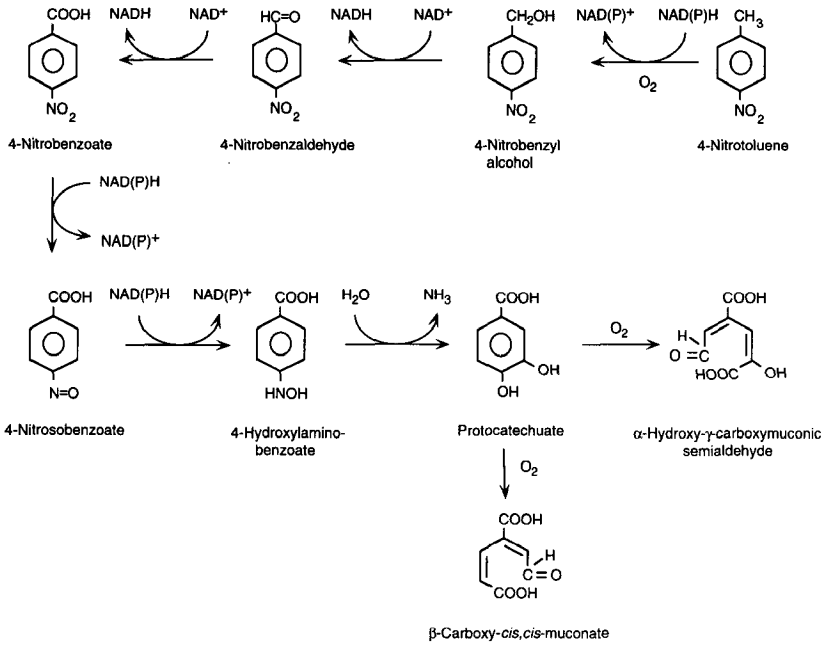

Figure 8 Pathway for catabolism of 4-nitrotoluene and 4-nitrobenzoate. The complete pathway has been found in two strains of *Pseudomonas* (51, 101). The pathway for 4-nitrobenzoate was reported first in *Comamonas acidivorans* (47, 48).

aminobenzoates in the pathway could not be demonstrated, however, and the degradation mechanism remained unclear. The mystery was solved during investigation of the degradation of 4-nitrobenzoate by a *Comamonas acidivorans* isolated from soil (47, 48). A nitroreductase purified from the isolate reduces 4-nitrobenzoate to 4-hydroxylaminobenzoate and does not catalyze further reduction of the molecule to 4-aminobenzoate (Figure 8). Another enzyme purified from 4-nitrobenzoate-grown cells catalyzes the conversion of 4-hydroxylaminobenzoate to protocatechuate without the participation of additional cofactors. The purified enzyme is stimulated by the addition of NADPH and several other reducing agents that seem to function by lowering the redox potential rather than serving as substrates in the reaction (48). The mechanism of the reaction appears to be hydrolytic but has not been studied in detail.

The facile degradation of nitrobenzoates raises the possibility that strains able to degrade the isomeric nitrotoluenes might be constructed if the methyl groups could be oxidized to carboxyl groups. Accordingly, several strains of

bacteria have been examined for their ability to transform nitrotoluenes. The enzymes of the TOL pathway involved in the degradation of toluene and xylenes can also oxidize the methyl groups of 3-nitrotoluene and 4-nitrotoluene (28). 2-Nitrotoluene is not a substrate for the initial enzyme in the sequence, toluene monooxygenase. Of the isomeric nitrotoluenes only 4-nitrotoluene can serve as an inducer for the genes of the TOL pathway. The specificity of the effector can be altered by mutagenesis, however, so that all three isomers of nitrotoluene can serve as inducers of the TOL upper pathway (27). This alteration allows pseudomonads containing the TOL plasmid and the mutant regulator to convert 3-nitrotoluene and 4-nitrotoluene to the corresponding nitrobenzoates.

Natural strains of *Pseudomonas* able to grow on 4-nitrotoluene under aerobic conditions have been isolated separately and studied by two groups (51, 101). The catabolic pathways are identical and the initial steps are analogous to those of the TOL pathway. A monooxygenase enzyme oxidizes the methyl group to the corresponding alcohol (Figure 8). Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase convert 4-nitrobenzyl alcohol to 4-nitrobenzoate. The only obvious difference in the initial steps in the pathways for 4-nitrotoluene degradation of the two *Pseudomonas* isolates is in the properties of their respective 4-nitrobenzyl alcohol dehydrogenases. The strain studied by Haigler & Spain (51) contains an NAD-dependent, membrane-bound enzyme, whereas the strain studied by Rhys-Williams et al (101) contains a soluble enzyme for which the physiological electron acceptor could not be determined. Although the first three reactions in the pathway for 4-nitrotoluene are identical to those in the TOL plasmid-encoded pathway, the genes that encode the enzymes do not seem to be closely related to the TOL genes. Attempts to detect cross-hybridization between a TOL DNA probe and genomic DNA from the 4-nitrotoluene degrader failed (101).

Both of the isolates that grow on 4-nitrotoluene use the pathway described by Groenewegen et al for the degradation of 4-nitrobenzoate (47, 48). The only discernable difference is in the mode of ring fission of protocatechuate. A protocatechuate 3,4-dioxygenase is active in the strain studied by Rhys-Williams et al (101), whereas a 4,5-dioxygenase operates in the strain studied by Haigler & Spain (51). The mechanism of ring fission was not determined for the strain in which the 4-nitrobenzoate pathway was discovered.

Bacteria able to grow on 3-nitrophenol have been isolated (41, 135), and the initial steps in the degradation pathway seem to be reductive rather than oxidative (A Schenzle & H-J Knackmuss, personal communication). Details of the catabolic pathway have not been published, however.

Bacteria containing the catabolic pathway described above for degradation of nitrobenzene via a dioxygenase mechanism (Figure 5*a*) seem to be unusual. When a range of aerobic nitrobenzene-degrading strains isolated from six

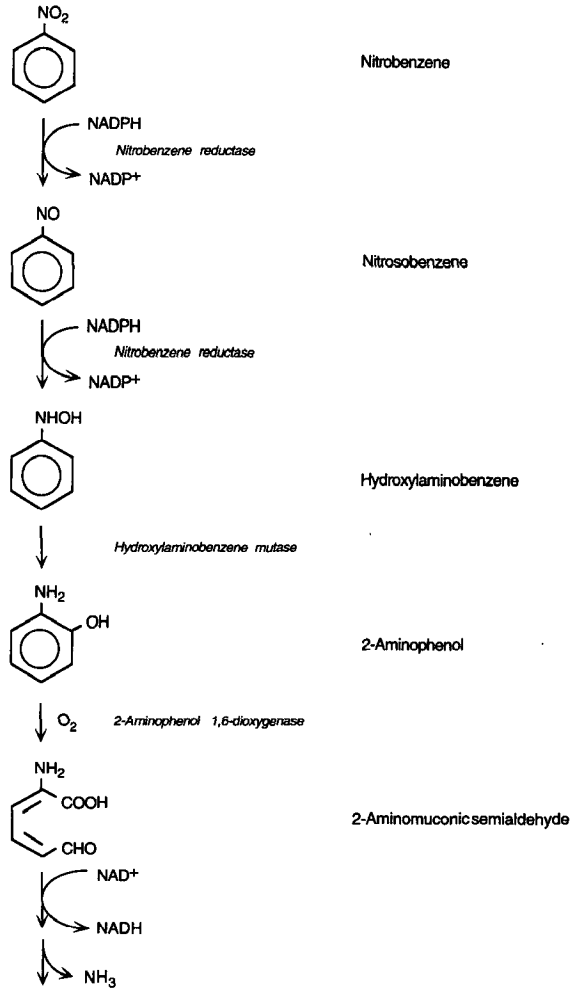

Figure 9 Partial reduction and subsequent oxidation reactions for the degradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* (87).

widely separated ecosystems were examined, only one strain out of nine used the dioxygenase pathway (SF Nishino & JC Spain, unpublished). The other strains use a pathway involving reduction of the nitro group (Figure 9) (87). The enzyme responsible for the NADPH-dependent reduction of nitrobenzene to hydroxylaminobenzene has been purified to homogeneity from a strain of *Pseudomonas pseudoalcaligenes* (108a). It is a flavoprotein with a molecular weight of 30,000, and it is active as a monomer. It is similar to the nitroreduc-

tase isolated from *Comamonas acidovorans* (47) in that it does not catalyze the reduction of the hydroxylamino compound to the corresponding amine. Both of these enzymes are unusual in this respect, because most bacterial nitroreductases convert aromatic nitro groups to the amines (5, 10, 17, 43, 72, 94, 97, 126).

A novel enzyme, hydroxylaminobenzene mutase, catalyzes the conversion of hydroxylaminobenzene to 2-aminophenol in the absence of oxygen or any added cofactors. The reaction is analogous to the Bamberger rearrangement (107), in which hydroxylamino aromatic compounds rearrange to aminophenols under mildly acidic conditions. The nonenzymatic rearrangement yields predominantly the 4-aminophenol, whereas the enzyme directs the production of predominantly (>99%) the 2-aminophenol. Analogous enzyme-catalyzed reactions have been reported in animals (117) and in yeast (23) but not in bacteria. The implications of the Bamberger-like rearrangement in biochemistry have been discussed extensively by Corbett & Corbett (24). The relationship between the hydroxylaminobenzene mutase isolated from cells grown on nitrobenzene and the hydroxylaminobenzoate lyase isolated from the *C. acidovorans* grown on 4-nitrobenzoate is not clear. It is possible to postulate similar mechanisms for at least the first steps in both reactions, but the enzyme-catalyzed reactions seem to be specific and show no cross-reactivity (JAM deBont, personal communication). Sequencing of the corresponding genes and additional characterization of the enzymes will be necessary before the relationships can be clarified.

2-Aminophenol produced by the initial steps in the pathway is degraded by a dioxygenase that catalyzes the opening of the ring at the 1,6-position to produce 2-aminomuconic semialdehyde. Only a few examples of enzymes that catalyze ring-fission reactions in the absence of two hydroxyl groups are known. Catechol 1,2-dioxygenases from *Pseudomonas arvilla* (96) and *Pseudomonas aeruginosa* (70) oxidize 2-aminophenol at a rate 1000-fold lower than the rate of oxidation of catechol, their physiological substrate. In contrast, the enzyme from *Pseudomonas pseudoalcaligenes* oxidizes 2-aminophenol, its physiological substrate, 50-fold faster than catechol. An enzyme that catalyzes a similar reaction has also been found in a strain of *Pseudomonas* grown on 5-aminosalicylate (118).

The mechanism for the degradation of 2-aminomuconic semialdehyde by *Pseudomonas pseudoalcaligenes* is not known. Enzymes in crude extracts from cells grown on nitrobenzene catalyze the degradation of the ring-fission product and release of ammonia. The requirement for NAD suggests that the first reaction is an oxidation of the aldehyde, but no clear evidence is available.

The reductive pathway for degradation of nitrobenzene (Figure 9) seems much more complex than the oxidative pathway (Figure 5*a*). Recruitment of the genes for several unusual enzymes was required for the operation of the

pathway. It will be interesting to determine whether the various isolates that use this pathway are derived from a common ancestor or arose separately. In either case, they seem to be more widely distributed than the strains using the simpler oxidative pathway. An explanation for this apparent conundrum lies in the cofactor and oxygen requirements of the pathways. The reductive pathway requires one mole of oxygen and one mole of NADH to convert nitrobenzene to central metabolic intermediates and release ammonia. In contrast, the oxidative pathway requires two moles of oxygen and one mole of NADH that can be regained if the 2-hydroxymuconic semialdehyde undergoes an NAD-dependent oxidation to oxalocrotonate (88). If the isolate is to use the nitrite released by the oxygenolytic reaction as its nitrogen source, three additional moles of NAD(P)H would be required for the reduction of nitrite to ammonia. In nitrobenzene-contaminated subsurface ecosystems, where all of the strains that use the reductive pathway were isolated, oxygen is limiting. In contrast, the strain that uses the oxidative pathway was isolated from an aerobic waste-treatment system. Thus, each pathway seems appropriate to the ecosystem in which it was discovered. The more complex, reductive pathway seems to be well adapted to exploit the conditions of an oxygen-limited ecosystem.

APPLICATIONS IN BIOREMEDIATION

Much of the recent interest in biodegradation of nitroaromatic compounds has been motivated by an increased awareness of the extent of environmental contamination by explosives such as TNT. Therefore, a considerable amount of work has been done on development of treatment systems based on biodegradation. Composting has been used for field-scale cleanup at several TNT-contaminated sites (131) and has been studied on a smaller scale by a number of investigators (61, 69, 83, 133). Composting, however, increases the volume of the waste material and requires a considerable amount of materials handling, and the ultimate products of the process are not well characterized chemically. There is also some evidence of residual toxicity and mutagenicity after composting of explosives-contaminated soil (46). Several groups have worked with aerobic slurry-phase bioreactors (15, 49), and one preliminary report suggests an initial anaerobic conversion of TNT to TAT with subsequent humification under aerobic conditions (75, 102). A similar approach has shown that nitrobenzene can be converted to aniline under anaerobic conditions and that the aniline can be degraded under aerobic conditions (29). Studies with the white rot fungi show considerable promise for use in bioremediation of munitions if problems with toxicity can be overcome (8, 114, 116, 119). Bioremediation under anaerobic conditions seems to be the most favored approach for removal of TNT at present. A process involving methanogenic cultures has been developed and demonstrated for the treatment of soil contaminated with TNT and RDX (26, 40). Other

processes employing sulfate-reducing bacteria have been suggested by several groups (14, 94). All of the approaches listed above involve transformation of TNT by microorganisms that use another primary carbon source for growth. Therefore, the processes are more difficult to optimize and control than they would be if the bacteria used TNT as a growth substrate. Unfortunately, reports of microorganisms able to grow on TNT are rare (32).

CONCLUSIONS

Dramatic and rapid progress has been made recently in understanding the biodegradation of nitroaromatic compounds. In addition, a number of exciting questions have been raised as a result of the recent discoveries. The potential for reduction of TNT by anaerobes is well established, yet intriguing areas of uncertainty remain about the metabolism of triaminotoluene. The initial steps in the pathways catalyzed by white rot fungi are clear and the mechanism of toxicity is better understood, but nothing is known about how the reduced metabolites of TNT are mineralized by the fungus. Aerobic bacteria have a hitherto unexpected capacity to convert nitroaromatic compounds into intermediates that can serve as growth substrates. The mechanisms of the reactions, their regulation, and the structure of the enzymes will provide fertile areas for research. Virtually nothing is known about the molecular biology of the systems. Understanding of the molecular basis for the catabolic sequences will allow their capabilities to be enhanced and exploited for practical purposes. Potential applications include not only biodegradation of environmental contaminants, but also the use of the novel enzyme reactions for biocatalysis and synthesis of valuable organic molecules.

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Annual Review of Microbiology
Volume 49, 1995

CONTENTS

vi (*continued*)