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Volume 37 of Advances in Applied Microbiology is sadly dedicated to Dr. Frank K. Higson, University of California, Riverside, who passed away during the preparation of this volume to which he made two scholarly contributions.

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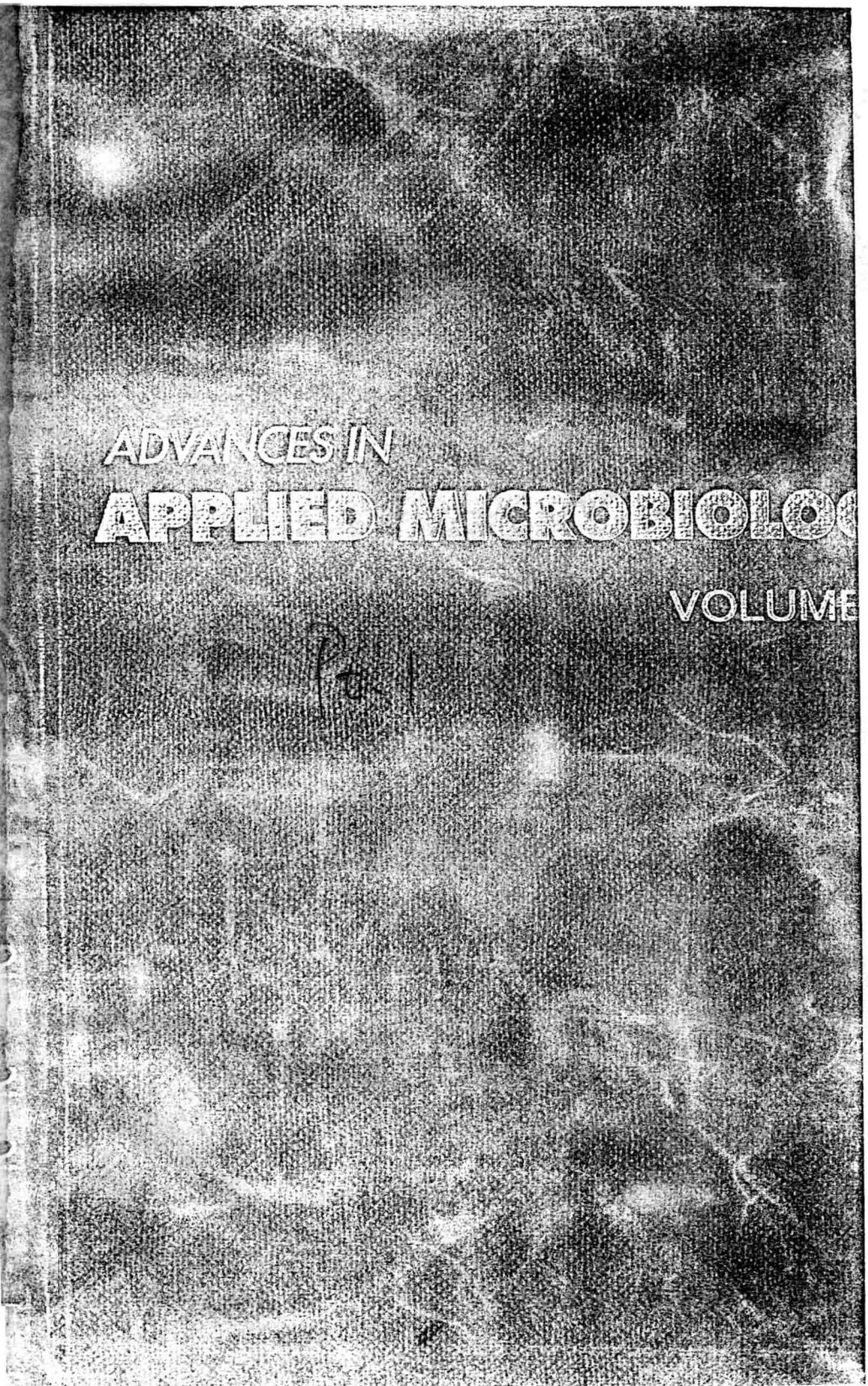
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Microbial Degradation of Nitroaromatic Compounds

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I. Overview

Nitroaromatics are produced on a massive scale in the manufacture of dyes, plastics, and explosives. Their discharge in wastewater and application as pesticides have broadened their environmental impact and called for solutions for remediation of these toxic compounds. The use of microorganisms to transform or eliminate nitroaromatics has been proposed in effluent treatment and land reclamation. While microbial strains utilizing nitrobenzoates or nitrophenols were isolated from contaminated sources several decades ago, microbial action on 2,4,6-trinitrotoluene (TNT) was largely limited to nitro group reduction and formation of azoxy derivatives which may complex with humus. However, recent work has identified organisms capable of using TNT as the sole carbon and nitrogen source.

II. Introduction

Although compounds bearing a nitro substituent are synthesized by microorganisms (Bush *et al.*, 1951; Hirata *et al.*, 1954; Cooke, 1955) and the bacterial degradation of chloramphenicol was reported to generate 4-nitromandelate, 4-nitrobenzyl alcohol, and 4-nitrobenzoate (Lingens *et al.*, 1966), by far the greatest current producer of nitroaromatics is the

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chemical industry. Nitrobenzene, nitrotoluenes, nitrophenols, and nitrobenzoates are used in the manufacture of pesticides, dyes, explosives, polyurethane foams, elastomers, and industrial solvents. The antibiotic chloramphenicol and the tranquilizer nitrazepam are examples of drugs whose primary action depends on the presence of aromatic nitro groups. The insecticides parathion and paraoxon are derived from 4-nitrophenol; a class of herbicides that provides broad-spectrum weed control in cotton and soybeans and includes Treflan, is based on 4-trifluoromethyl-2,6-dinitroaniline. 2,4,6-Trinitrotoluene (TNT) has been used extensively in explosives since 1902 and current world annual production is around 2 million pounds (Hartter, 1985).

Nitrobiphenyls are important as plasticizers for cellulose acetate and polystyrene, as textile fungicides and wood preservatives, and in the synthesis of dyes (Masse *et al.*, 1985). Nitroanilines and their derivatives occur in wastewater from the dye and pharmaceutical industries and in soils as metabolites from microbial degradation of certain herbicides (Laanio *et al.*, 1973; Golab *et al.*, 1979).

Soil and groundwater contamination by TNT has resulted from munitions manufacture, loading, assembling, and packing (Haas and von Loew, 1986). The practice during shell loading operations has been to discharge hot water saturated with residual explosive (100 ppm) into holding lagoons and allow TNT to pass gradually into local streams. Wastewater from dye production makes a considerable input of nitrotoluenes into the environment. As much as 19 million pounds of nitrobenzene are reportedly discharged into natural waters (von Loew *et al.*, 1989). Studies of Rhine water indicated nitrotoluenes present at concentrations up to 18 ppb (U.S. Environmental Protection Agency, 1978). Nitroaromatics are also present in combustion emissions and airborne particulate matter (Meijers *et al.*, 1976; Pitts *et al.*, 1982; Schuetzle, 1983).

TNT is toxic to freshwater unicellular algae (*Selenastrum capricornutum*, *Microcystis aeruginosa*, *Chlamydomonas reinhardtii*), tidepool copepods (*Tigriopus californicus*), and oyster larvae (*Crassostrea gigas*) at concentrations as low as 2.5 ppm and is a frameshift mutagen to *Salmonella typhimurium* (Won *et al.*, 1976; Wang *et al.*, 1980). Hudock and Gring (1970) and Smock *et al.* (1976) found it to be toxic to fathead minnows (*Pimephales promelas*) and bluegills (*Lepomis macrochirus*). Most fungi, yeasts, actinomycetes, and gram-positive bacteria showed severely limited growth in the presence of 50 ppm TNT (Nay *et al.*, 1974). Deaths from toxic hepatitis and aplastic anemia caused by TNT exposure were significant during the world wars [20] and subclinical effects of TNT exposure affecting survival of erythrocytes, liver func-

tion, and the lens of the eye have been described (McConnell and Flinn, 1946). Methemoglobinemia, cyanosis, anemia, and jaundice were reported in man as a result of exposure to dinitrotoluene in the workplace (Hathaway, 1985), and a dose-dependent increase in hepatocellular carcinoma was observed in rats fed technical grade dinitrotoluene (McGee *et al.*, 1942).

1,3-Dinitrobenzene is toxic to humans following occupational exposure (Clark and Paul, 1935; Chemical Industry Institute of Toxicology, 1979), and to fish (Ishihara *et al.*, 1976) and several bacterial and fungal species (Higgins, 1958; Wentsel *et al.*, 1979). It can be generated from the munitions by-product 2,4-dinitrotoluene through photoconversion (Bringman and Keuhn, 1976). Nitrated polycyclic aromatic hydrocarbons such as 1-nitropyrene, which have been detected in carbon black toners (Kitchens *et al.*, 1978), and nitrobiphenyls (Rosenkranz *et al.*, 1980) are mutagenic (McCann *et al.*, 1975; Schuetzle, 1983) owing to their conversion to *N*-hydroxyarylamines by mammalian enzymes such as microsomal cytochrome *P*-450 reductase (Mermelstein *et al.*, 1981). There are bacterial nitroreductases that can also activate nitrated polycyclics to potent mutagens (Harada and Omura, 1980).

The U.S. Environmental Protection Agency's list of 129 priority pollutants includes seven nitroaromatics: nitrobenzene, 2,4- and 2,6-dinitrotoluene, 2- and 4-nitrophenol, 2,4-dinitrophenol, and 4,6-dinitro-2-methylphenol (McCoy *et al.*, 1981). Bioremediation has been proposed for a number of recalcitrant compounds, including polychlorinated biphenyls (Keith and Telliard, 1979), and a microbial approach might also be appropriate for sites contaminated with nitroaromatics.

III. Microbial Reduction of the Nitro Group

The microbial reduction of the nitro substituent has been established for several classes of nitroaromatics. A cyanide-sensitive NADH-dependent conversion of nitrobenzoate to aminobenzoate was displayed by growing cultures and cell-free extracts of a *Nocardia* sp. and a strain of *Pseudomonas fluorescens* (Furukawa, 1982). Enzyme preparations of the strict anaerobe *Veillonella alkalescens* catalyzed reduction of 30 mono-, di-, and trinitroaromatics by hydrogen in a three-step process, via R-NO and R-NHOH (Cartwright and Cain, 1959). McCormick *et al.* (1976) demonstrated formation of aminonitrotoluenes and 3-azoxy compounds from 2,4-dinitrotoluene by the fungus *Mucrosporium*. Reduction of 2,6-dinitro-4-(trifluoromethyl)aniline, from which several herbicides are derived, was reported (McCormick *et al.*, 1978) for a *Streptomyces* isolated from soil. Naumova *et al.* (1986) observed se-

quential reduction of the nitro groups of TNT by aerobically growing *Escherichia coli*; aminodinitrotoluenes represented 70% of nitroaryl losses at the end of the exponential phase. Azoxy derivatives were again generated by coupling reactions.

More recently, Wenzhong *et al.* (1987) investigated the reductase in *Citrobacter freundii* that degraded TNT aerobically. The K_m was estimated at 0.05 m*M* and the optimum pH and temperature were 7.2 and 30°C, respectively; the addition of 1,3- and 1,4-dinitrobenzenes and 4-nitro- and 2,4-dinitrophenols each approximately halved the rate of TNT clearance. When Parrish (1977) screened 190 fungi representing 98 genera for the ability to transform TNT, 183 were active in partial reduction, but surprisingly few (five organisms) were able to transform 2,4-dinitrotoluene.

Bielaszczyk *et al.* (1967) found aerobic reduction of 4-chloronitrobenzene by organisms such as an *Arthrobacter* sp. obtained from contaminated soil. A basidiomycetous yeast of the genus *Rhodosporidium* was shown by Corbett and Corbett (1981) to transform 4-chloronitrobenzene by a reductive pathway (Fig. 1). In addition to producing 4-chloroaniline, the intermediate hydroxylamine was proposed to undergo a Bamberger rearrangement, in which the hydroxyl group migrated from N to C. This generated 2-amino-5-chlorophenol and 4-aminophenol by, respectively, ortho and para attack. Acetylation of these amino compounds was reported, but no azo or azoxy derivatives, perhaps because these workers avoided the solvent extraction and vacuum evaporation stages which favor the production of these metabolites by bimolecular reactions. Reduction of 4-chloronitrobenzene was also observed by Russel (1980) in *Azotobacter agilis*, the amino group then being subjected to acetylation or propionylation. An unusual replacement of the parachlorine of 2,4-dichloronitrobenzene with a methylthio group by *Mucor javavicus* was reported by Tahara *et al.* (1981).

Hallas and Alexander (1983) reported reduction of nitrobenzene, dinitrobenzenes, nitrotoluenes, and nitrobenzoates in sewage effluent both in the presence and absence of oxygen; gas chromatographic–mass spectroscopic (GC-MS) analysis indicated the formation of acetanilide and 2-methylquinoline from the intermediate aniline and 2-methylbenzimidazole from 2-nitroaniline (Fig. 2). That these multiple-ring teratogenic compounds are not simply artifacts of high-temperature-mediated ring closure during analysis is suggested by the formation of benzimidazoles from dinitroaniline herbicides in soil (Kearney *et al.*, 1976). Uchimura (1987) showed that polypeptone supplementation enhanced dinitrotoluene reduction by seawater microorganisms at a rate dependent on the configuration of the nitro groups.

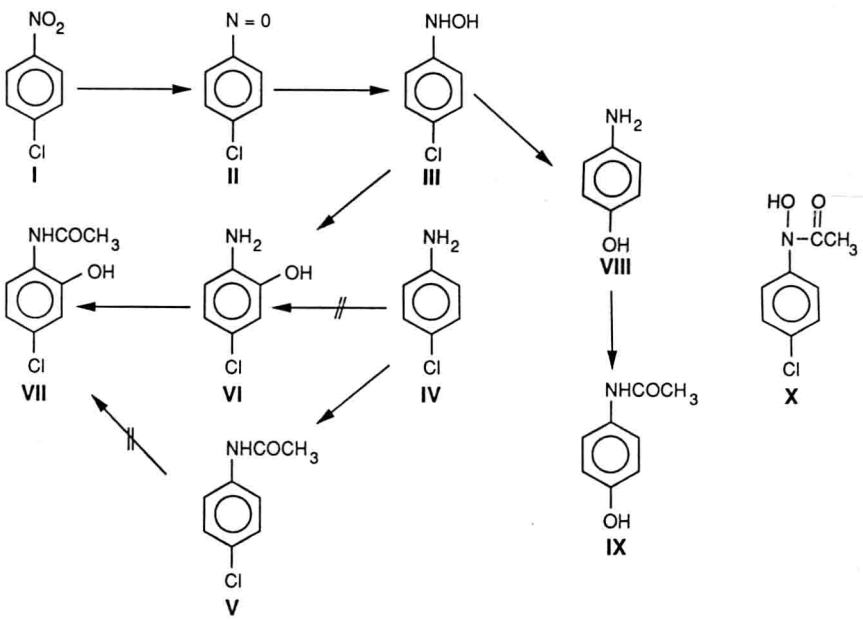

FIG. 1. The degradation of 4-chloronitrobenzene (**I**) by *Rhodosporidium* sp. (Corbett and Corbett, 1981) with the generation of nitroso (**II**) and hydroxylamine (**III**) intermediates. Also shown are 4-chloroaniline (**IV**), 4-chloroacetanilide (**V**), and their 2-hydroxy derivatives (**VI, VII**), 4-aminophenol (**VIII**), 4-hydroxyacetanilide (*IX*), and a hydroxamic acid metabolite (**X**), perhaps produced by acetylation of **III**.

McCormick and co-workers (1985) showed reduction of 2-nitrodiphenylmethane, a by-product of ball powder production that is discharged from manufacturing plants in waste effluents, and the formation of annelated structures (*N*-phenylbenzimidazole and phenazine) by sewage cultures, especially under anaerobic conditions. A reduction of the fungicide pentachloronitrobenzene by *Streptomyces aureofaciens* was reported by Chacko *et al.* (1966), while Nakanishi and Oku (1969) identified pentachloromethylthiobenzene, pentachlorothiophenol, and bispentachlorophenyl disulfide as additional metabolites from the culture broth of *Fusarium oxysporum*.

E. coli isolated from human intestine reduced dinitrotoluenes to aminonitrotoluenes via the hydroxylamino compounds (Mori *et al.*, 1984), at a rate dependent on the position of the nitro group relative to the methyl. The intestinal microflora may therefore be involved in induction of methemoglobinemia or cancer (Reddy *et al.*, 1976).

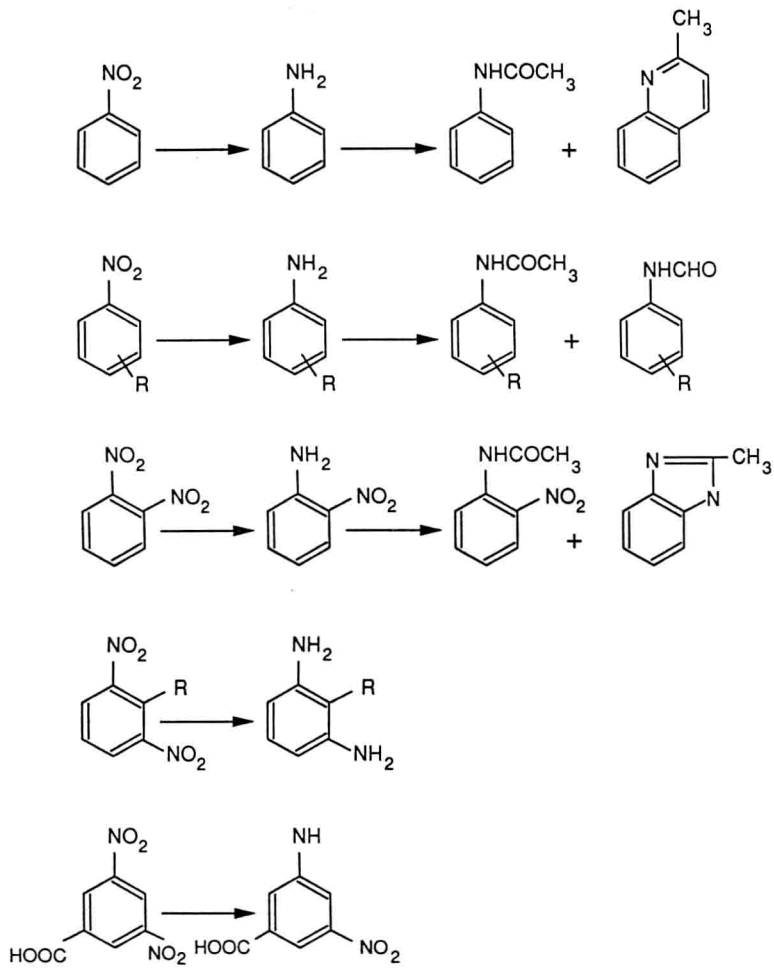

FIG. 2. Products of the metabolism of mono- and dinitroaromatic compounds in sewage (Hallas and Alexander, 1983). The nitro group is reduced, and acetylated and annelated structures are additionally produced.

IV. Removal of the Nitro Group

Masse *et al.* (1985) observed growth of gram-negative strain B206 on 4-nitrobiphenyl, and Takase *et al.* (1986) reported growth of *Pseudomonas cruciviae* S93B1 on 2- and 3-nitrobiphenyls. In both cases, nitrobenzoate accumulates unused in pure culture. The utilization of single-ring nitroaromatics, however, normally requires removal of the nitro group and two systems have been described that accomplish this.

One uses a nitroreductase to generate an amine and then ammonium.

In the other, nitrite is liberated in an oxidative reaction. The alternatives coexisted in the strain of *Pseudomonas putida* isolated by Zeyer and Kearney (1984), which utilized 2-nitrophenol by the formation of catechol and nitrite, and 3-nitrophenol with the release of ammonium.

While some exposure to nitroaromatic compounds has occurred over a time span that would allow the evolution of microbial degradation systems, the application of nitroaromatic pesticides such as parathion (*O,O*-diethyl-*O*-4-nitrophenyl phosphorothioate) and the discharge of nitrobenzene derivatives from manufacturing plants have much increased the selection pressure for the emergence of competent strains. The situation is analogous to the shift from a low-level exposure to biosynthetic haloaromatics such as 2,4-dichlorophenol isolated from a soil fungus (Ando *et al.*, 1970) or brominated phenols produced by red algae (Suida and de Bernardis, 1973) to a widespread distribution as pesticides, solvents, surfactants, and as a result of water prechlorination. Microbes have been observed to degrade partially (cometabolize) these xenobiotics (such as polychlorinated biphenyls), while growing on another substrate, when enzymes involved in major pathways (such as biphenyl degradation) display a relaxed specificity (Slater and Bull, 1982).

Nitroreduction is presumably carried out by enzymes recruited from normal metabolism, for nitroreductases were identified in liver (Egami and Itahishi, 1951), *Neurospora crassa* (Little, 1951), and peas (Little, 1957) having no prior contact with environmental nitro compounds. Westfall (1943) found that even TNT underwent single reduction by a succinate dehydrogenase preparation from beef heart.

An example of a nitroaromatic-degrading strain derived from a source that had received considerable anthropogenic pesticide exposure was presented by Siddaramappa and co-workers (Siddaramappa *et al.*, 1973). A strain of *Pseudomonas* sp. was isolated from Indian soil that had been repeatedly sprayed with parathion. The organism hydrolyzed the pesticide and then released nitrite from the 4-nitrophenol produced.

The following section looks at a number of nitroaromatic series for which microbial mineralization has been demonstrated.

V. Nitroaromatic Growth Substrates

A. Nitrobenzene

Moore (1949) described two strains of *Nocardia* that grew on nitrobenzene (and also aniline, nicotinate, and pyridine) as the sole carbon and nitrogen source.

B. Nitrophenols

Simpson and Evans (1953) isolated strains of microbes from sewage that could use either 2- or 4-nitrophenol but not both; nitrite was released and the organisms were induced, respectively, to form catechol and hydroquinone. A strain of *Arthrobacter* isolated by Gunderson and Jensen (1956) grew on the herbicide 3,5-dinitro-2-methylphenol as the sole carbon and nitrogen source, nitrite being detected in their culture. The soil pseudomonad isolated by Tewfik and Evans (1966) grew on the herbicide by an initial reduction to form 3-amino-5-nitro-2-methylphenol, in turn giving rise to 3-methyl-5-aminocatechol, rather than liberating nitrite. The strain of Raymond and Alexander (1971) grew on 4-nitrophenol with the liberation of nitrite and cometabolized the meta isomer to nitrohydroquinone. Sudhakar-Barik *et al.* (1976) showed mineralization of 4-nitro[^{14}C]phenol by a *Pseudomonas* sp. with the formation of labeled carbon dioxide. Spain *et al.* (1979) obtained an enzyme preparation from a *Moraxella* sp. isolated from sewage by 4-nitrophenol enrichment that oxidized the growth substrate to hydroquinone and nitrite. Activity was dependent on NAD(P)H and oxygen and stimulated by the addition of FAD. Experiments with $^{18}O_2$ showed that the incoming hydroxyl group was derived from molecular oxygen. Zeyer and Kearney (1984) obtained a cell-free nitrophenol-degradation system only for the ortho isomer from their soil isolate of *Pseudomonas putida*, which grew on 2- and 3-nitrophenols by different mechanisms.

C. Chloronitrophenols

An alternative to enrichment developed by Knackmuss and co-workers is the *in vivo* assembly of partial catabolic sequences to create a complete pathway. This approach was employed (Bruhn *et al.*, 1988) in the transfer of haloaromatic-degrading sequences from chlorobenzoate-degraders to *Pseudomonas* sp. N31, a strain expressing a nitrophenol oxygenase that allows the organism to use 4-chloro-2-nitrophenol as its sole nitrogen source by the liberation of nitrite. In the transconjugant, the 4-chlorocatechol that normally accumulates from 4-chloro-2-nitrophenol was consumed, so the latter then acted as both carbon and nitrogen source.

D. Nitroanilines

Zeyer and Kearney (1983) were the first to describe an organism that utilized a nitroaniline. *Pseudomonas* P6 grew slowly on 4-nitroaniline (but not the 2- or 3- isomers) as sole carbon source; nitroaniline degra-

dation was much enhanced by the addition of yeast extract. They failed to identify metabolites by HPLC but, by analogy to the degradation of aniline (a *Nocardia* sp. forms catechol; Bachofer *et al.*, 1975) and 3-chloroaniline [which is converted to 4-chlorocatechol by strains of *Pseudomonas multivorans* (Reber *et al.*, 1979) and *Alcaligenes faecalis* (Surovtseva *et al.*, 1980)], a nitrocatechol route was proposed.

E. Nitrobenzoates

Cain (1958) obtained the first nitrobenzoate-degraders from soil and polluted streams. *Nocardia opaca* grew on 2-nitrobenzoate and *N. erythropolis* on 4-nitrobenzoate as sole carbon and nitrogen sources; 3-nitrobenzoate competitively inhibited growth on either substrate. After enrichments lasting 2 years, the *Nocardia* sp. M1 was obtained that grew on the meta isomer (Cain, 1966). 4-Nitrocatechol was isolated from cultures of *N. erythropolis* growing on 4-nitrobenzoate, and 4-hydroxybenzoate transiently accumulated under conditions of restricted aeration. 3-Hydroxybenzoate was found in cultures of strain M1 growing on 3-nitrobenzoate. Cells from both cultures were induced for protocatechuate oxidation. A scheme was presented (Fig. 3) in which the nitro group was either replaced by a hydroxyl and a second hydroxyl added in a second step, or 4-nitrobenzoate was acted on by a dioxygenase to produce the nitrocatechol. The pathway of 2-nitrobenzoate degradation was not clarified, but, from inhibition studies, did not appear to involve anthranilate (Cain, 1958).

Ke *et al.* (1959) obtained a *Flavobacterium* growing on 2-nitrobenzoate as sole carbon and nitrogen source; cells were simultaneously induced for 2-nitroso- and 2-hydroxylaminobenzoate but not anthranilate, suggesting that the nitrobenzoate was only partially reduced.

F. 1,3-Dinitrobenzene

Tennessee River water taken downstream from a munitions production facility yielded a nonaxenic culture growing on 1,3-dinitrobenzene as sole carbon source (Mitchell and Dennis, 1982); the organisms were not concomitantly induced for the oxidation of 1,2- or 1,4-dinitrobenzenes, 1,3,5-trinitrobenzene, or 3,5-dinitroaniline, which also occur in munitions discharges.

G. 2,4,6-Trinitrotoluene

Whereas nitrophenol-degraders are rather readily obtained from enrichments, the three nitro groups in TNT make it difficult for micro-