Review Article

Microbial cleavage of nitrate esters: defusing the environment

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Introduction

The products of condensing organic hydroxyl groups (ROH) with the mineral acids (hydrochloric, nitric, phosphoric and sulphuric) collectively constitute a major part of the output of the synthetic organic chemical industry, with a wide diversity of applications including surfactants, pesticides, herbicides, dyes, methylating agents, explosives and pharmaceuticals. Compounds containing similar or related structures also occur as natural products. Phosphate esters are, of course, exceptional for their ubiquity in the living world, ranging from the simple intermediary metabolites of glycolysis, through phospholipids, to the backbone of DNA. Sulphate esters too are abundant (Dodgson *et al.*, 1982) and naturally occurring halogenated compounds are also being detected in increasing numbers (for examples, see Strunz, 1984; Engvild, 1986; Neidleman & Geigert, 1986; Harper & Hamilton, 1988). It is therefore no surprise that living organisms have evolved phosphatase (Boyer, 1971), sulphatase (Dodgson & Rose, 1975; Dodgson *et al.*, 1982) and dehalogenase (Neilson, 1990;

Hardman, 1991) enzyme systems for initiating biodegradation of such compounds by the removal of the mineral moiety. In marked contrast, we are unable to find any examples of naturally occurring nitrate esters, so that the introduction of such compounds into the environment during their industrial production and usage constitutes a true xenobiotic challenge to micro-organisms. This raises intriguing questions about microbial capability for biotransformation/biodegradation of nitrate esters, not only from an academic viewpoint but also because of the wide industrial usage of these compounds and their likely impact on the environment.

True nitrate esters may be considered as the products of condensation of alcoholic hydroxyl groups with nitric acid, HNO₃. Indeed, such direct nitration, sometimes with sulphuric acid added as catalyst, still forms the basis for the synthesis of nitrate esters, first discovered by Sobrero (1847, 1848), and later brought to the industrial scale by Alfred Nobel (Bergengren, 1960) in the synthesis of glycerol trinitrate (GTN). The resulting ester structure, C-O-NO₂, is chemically quite distinct from nitro-organic compounds containing the C-NO₂ group; the former are converted to alcoholic ROH groups and NH₃ on reduction whereas the latter are reduced to amines (C-NH₂) without separation of the nitrogen from its organic linkage (Urbanski, 1965). Parallel distinctions in terms of chemical structure may be made between sulphate esters (C-O-SO₃⁻) and sulphonates (C-SO₃⁻), and phosphate esters (C-O-PO₃²⁻) and phosphonates (C-PO₃²⁻).

Nitrate esters find their major industrial applications as explosives and as vasodilators in pharmaceuticals. Shortly after the first successful synthesis of GTN in the mid-nineteenth century (Sobrero, 1847, 1848) nitrate esters were found to be effective for the treatment of heart disease (Murrell, 1879). Because heart disease is currently a major health problem in the Western World, this application has grown to become an important component of the modern pharmaceutical industry.

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Abbreviations: 1,2-GDN, glycerol 1,2-dinitrate (3-hydroxypropane-1,2-diyl dinitrate); 1,3-GDN, glycerol 1,3-dinitrate (2-hydroxypropane-1,3-diyl dinitrate); 1-GMN, glycerol 1-mononitrate (2,3-dihydroxypropyl nitrate); 2-GMN, glycerol 2-mononitrate (1,3-dihydroxypropane-2-yl nitrate); GSH, glutathione (γ -glutamyl-cysteinyl-glycine); GTN, glycerol trinitrate (propane-1,2,3-triyl trinitrate); EGDN, ethylene glycol dinitrate (ethane-1,2-diyl dinitrate); EGMN, ethylene glycol mononitrate (2-hydroxyethyl nitrate); ISDN, isosorbide dinitrate (1:4,3:6-dianhydro-D-glucit-2,5-diyl dinitrate); 2-ISMN, isosorbide 2-mononitrate (1:4,3:6-dianhydro-D-glucit-2-yl nitrate); 5-ISMN, isosorbide 5-mononitrate (1:4,3:6-dianhydro-D-glucit-5-yl nitrate); PEDN, pentaerythritol dinitrate [2,2-bis(hydroxymethyl)propane-1,3-diyl dinitrate]; PEMN, pentaerythritol mononitrate [3-hydroxy-2,2-bis(hydroxymethyl)propyl nitrate]; PETN, pentaerythritol tetranitrate [2,2-bis(nitrooxymethyl)propane-1,3-diyl dinitrate]; PETriN, pentaerythritol trinitrate (2-hydroxymethyl-2-nitrooxymethylpropane-1,3-diyl dinitrate).

Fig. 1. Chemical structures of some nitrate esters.

Moreover, it has spawned a wide range of scientific studies (Taylor *et al.*, 1987) on mammalian metabolism and pharmacokinetics of compounds such as GTN, pentaerythritol tetranitrate and isosorbide dinitrate (Fig. 1). The efficacy of these compounds stems from their common ability to serve as sources of nitric oxide, NO (Feelisch & Noack, 1987; Schror *et al.*, 1991), which is emerging as an important and universal chemical messenger in mammals, with roles already identified in the immune response (Hibbs *et al.*, 1987; Knowles *et al.*, 1990), vascular function and reactivity (Moncada *et al.*, 1988; Palmer *et al.*, 1988; Hutcheson *et al.*, 1990; Radomski *et al.*, 1990), endotoxin shock (Kilbourn *et al.*, 1990*a, b*) and in neurotransmission (Knowles *et al.*, 1989; Palacios *et al.*, 1989).

Alkyl nitrate esters are generally strongly explosive materials. Several possess about as many nitrate ester groups as carbon atoms which makes their internal oxygen content sufficient to convert the compound to CO_2 , H_2O and N_2 . Thus some of the compounds of therapeutic value, such as GTN, ethylene glycol dinitrate and pentaerythritol tetranitrate (Fig. 1) are also among the most powerful explosives. Because of the pharmaceutical, industrial and military importance of nitrate esters, there is considerable interest in biological means of waste disposal and bioremediation of contaminated sites. Much of the work is documented in military and navy reports which have recently been reviewed elsewhere (Kaplan, 1992, 1993; Walker & Kaplan, 1992). Whilst these studies are numerous and extensive, they are concerned primarily with characterization and optimization of waste treatment and bioremediation technologies. Consequently, still not much is known about the microbial biochemistry involved, especially in comparison with mammalian systems for which metabolic pathways, enzymology and pharmacokinetics of nitrate

ester metabolism are established already (Taylor *et al.*, 1987). The limited information available for microbial metabolism is scattered in a variety of literature sources, and is also rather diffuse among the bacteria and protists involved in the biodegradation, and in terms of the nitrate esters on which competent organisms have been found to act. This review attempts to assemble and summarize relevant information.

Glycerol trinitrate

For much of its relatively long industrial history, GTN had been regarded as either recalcitrant to microbial biodegradation or even inherently non-biodegradable (Logan, 1953), and during the mid-1970s, some reports even indicated that high concentrations of the ester (> 900 p.p.m.) were inhibitory to bacterial growth (US Army, 1973, 1974). Despite such discouragement, increasing pressures to ensure adequate, safe and cost-effective disposal of glycerol mono-, di- and tri-nitrates present in manufacturing waste-streams, have led to concerted efforts to detect and exploit microbial biodegradation (US Army, 1974, 1975).

First signs that GTN was biodegradable came from studies with mixed cultures in laboratory-scale activated sewage systems. Bacteria present were capable of achieving a significant reduction in concentration of GTN although doubt remained as to whether the ester was actually supporting microbial growth (US Army, 1973, 1974, 1975). Nevertheless, TLC and HPLC analysis of organo-solvent extracts of mixed cultures (US Army, 1974, 1975) showed partial conversion to the isomeric GDNs (Fig. 2). In a further series of experiments to determine the biodegradability of GTN, Wendt *et al.* (1978) monitored changes in the nitrate ester composition in three related microbial systems. A mixed-

Fig. 2. Possible reaction sequences for the cleavage of the nitrate ester linkages in GTN. From Wendt *et al.* (1978); published with the permission of the American Society for Microbiology.

culture in a two-stage bench-scale activated sewage sludge unit converted GTN to 1,3-GDN and 1,2-GDN in roughly comparable amounts. The final effluent was free from tri-, di- and mononitrate esters. Several pure but unidentified bacterial cultures, isolated after enrichment from an established continuous culture chemostat, and subsequently grown in batch culture, converted GTN to 1,3-GDN, 1,2-GDN and GMN (unspecified isomer) but in undetermined amounts. Collectively, these data showed sequential conversion of GTN to GDN (both isomers), then GMN (one or both isomers), according to the scheme in Fig. 2, which, in fact, includes all possible sequences for loss of nitrate ester groups. Relative contributions of the parallel pathways in Fig. 2 remain unknown but significant steady state accumulations of di- and mononitrates implied that successive steps became progressively slower (Wendt *et al.*, 1978). No further work has been published on bacterial metabolism of glycerol nitrates, so that many questions remain; some of these are formulated and addressed in the concluding section of this review.

Rather more progress has been made on the biodegradation of GTN by pure fungal isolates. Of various

species of filamentous fungi tested, *Geotrichum candidum* exhibited notable capacity to cleave the nitrate ester linkages in GTN. Conversion rates were largely independent of the presence of alternative carbon or nitrogen sources in incubations of resting cells with GTN (Ducrocq *et al.*, 1989). Production of 1,2-GDN and 1,3-GDN was simultaneous and concomitant with disappearance of GTN, and most of the GMN was produced after the GTN was exhausted. Thus, as with the bacterial mixed-culture systems, these results were consistent with sequential conversion of GTN to the dinitrates, and thence to the mononitrates.

GTN contains two types of nitro group, viz. two primary nitrate esters (C1 and C3) and one secondary ester (C2). Thus formation of 1,2-GDN can occur in one of two ways (attack at C1 or C3), whereas 1,3-GDN is formed in only one way (attack at C2). If the enzyme(s) catalysing cleavage of the nitrate ester linkage lack regioselectivity, the process will occur randomly at all three nitrate groups, giving rise to a ratio of 1,3-GDN/1,2-GDN of 0.5. From the data presented by Ducrocq *et al.* (1989), the observed ratio can be estimated to lie in the range 1.2–1.8, corresponding to relatively elevated levels of the 1,3-isomer and clearly demonstrating a selectivity (about 3-fold) in favour of removal of the central (secondary) nitrate group.

If it is assumed that cleavage of the nitrate ester linkage is non-regioselective and with a starting value of 1.2 for the 1,3-GDN/1,2-GDN ratio, isomers of GMN would be produced in a 2-GMN/1-GMN ratio of $0.5/(0.5 + 1.2) = 0.29$. A starting ratio for 1,3-GDN/1,2-GDN of 1.8 would yield a 2-GMN/1-GMN ratio of $0.5/(0.5 + 1.8) = 0.22$. Thus if the cleavage of the nitrate ester linkages in dinitrates is non-regioselective, the predicted range for the 2-GMN/1-GMN ratio is 0.22–0.29, which is considerably less than the observed ratio of 0.5 derived from NMR data (Ducrocq *et al.*, 1989). Therefore the assumption of non-selectivity is invalid and there is regioselectivity in the cleavage of the nitrate ester linkages in 1,2-GDN in favour of attack at the primary nitrate group at C1. In fact, the attack on 1,2-GDN (the only source of 2-GMN) needs to be at least 2.3:1 in favour of removal of the primary nitrate from C1 to account for the NMR data.

To summarize, the cleavage of the first nitrate ester linkage in GTN occurs with regioselectivity 3:1 in favour of attack at the secondary (C2) ester, whereas in the second stage, cleavage of 1,2-GDN occurs preferentially, by about the same ratio, but now in favour of the primary (C1) ester. Thus, although the observed value of 0.5 for the ratio 2-GMN/1-GMN is equal to the ratio of secondary and primary nitrate groups in GTN, this apparent lack of overall regioselectivity (Ducrocq *et al.*, 1989) in fact masks two sequential cleavages, each with

significant but counter-balancing regioselectivity. These observations raise intriguing questions about the possible multiplicity of enzymes involved and their mechanisms of action; answers to those questions must await the relevant enzymological studies.

Comparison of the overall regioselectivity in the production of 2-GMN and 1-GMN by a number of fungi (Ducrocq *et al.*, 1990) showed that in contrast with *G. candidum*, *Sporotrichum paranense* and *Phanerochaete chrysosporium* converted GTN preferentially into 2-GMN. In addition to its academic interest, this selectivity for removal of both primary nitrates is of industrial relevance because it offers a biotransformation route to 2-GMN for which no specific chemical synthesis is currently available.

Further studies with *P. chrysosporium* (Ducrocq *et al.*, 1990; Servent *et al.*, 1991) showed that it converted GTN with 80% selectivity for production of 1,2-GDN by cleavage of the nitrate ester linkage at one of the terminal carbons. Biotransformation of 1,2-GDN was 85% selective for production of 2-GMN. The enzyme systems involved were constitutive and several enzyme activities appeared to be involved in the overall biotransformation of GTN (Servent *et al.*, 1991). Mycelia were capable of rapid conversion of nitrite to nitrate but the reverse reaction was much less effective. This, coupled with the detection of both ions during biodegradation of GTN, and the similarity in rates of nitrate production from GTN and from nitrite, suggested that the metabolic sequence was GTN → nitrite → nitrate (Servent *et al.*, 1991). Moreover, the combined rate of loss of nitrate ester groups from GTN and the dinitrates was very similar to the 'true' rate of nitrite production (calculated as the sum of the rate of nitrite appearance from GTN plus rate of oxidation of exogenous nitrite under equivalent conditions). The growing conviction that nitrite, not nitrate, was the immediate inorganic nitrogenous metabolite of GTN, was confirmed with the detection of an aerobic glutathione *S*-transferase activity in cytosolic extracts, capable of converting GTN to nitrite (Servent *et al.*, 1992).

Recoveries of nitrate from exogenous nitrite (Servent *et al.*, 1991), and of nitrite from GTN (Servent *et al.*, 1992), were only about 40–45%, suggesting the possible presence of other forms of nitrogen not detected by the HPLC system used. The EPR spectrum of mycelia harvested at intervals during biodegradation of GTN clearly indicated the presence of NO as one such compound. By comparison of the spectra with those for mycelia incubated with nitrite, and with others in the literature, two forms of NO were detected. The first of these, produced early in the biodegradation of GTN, contained NO bound to reduced haem. The second form, superimposed on the first in spectra of later samples, was

Fig. 3. Biochemical mechanism proposed for the biodegradation of GTN in *Phanerochaete chrysosporium*. From Servent *et al.* (1991); published with the permission of Elsevier Science Publishers.

a nitrosyl complex with a non-haem Fe-S protein, similar to that formed during degradation of nitrite by this fungus. These observations led to formulation of the reaction scheme depicted in Fig. 3. Early in the metabolism of GTN, when the nitrite concentration was still low, the haemoprotein-NO species (hP-NO in Fig. 3) dominated, whereas later in the degradation when nitrite accumulated, or when exogenous nitrite was added, the Fe-S-protein-NO complex was detected. The inclusion, in the reaction scheme, of a GSH *S*-transferase activity ('GSH-ST like') as the nitrite-liberating enzyme was based originally (Servent *et al.*, 1991) on the known involvement of such enzymes (formerly referred to as nitrate ester reductases) in mammalian metabolism (Habig *et al.*, 1975; Keen *et al.*, 1976; Keen & Jakoby, 1978; Taylor *et al.*, 1987, 1989; Jakoby, 1989). Such an enzyme, active on GTN, has recently been shown to be present in the cytosol of *P. chrysogenum* (Servent *et al.*, 1992). In addition, cytosolic and microsomal fractions also contained several haemoproteins including cytochrome P₄₅₀-like activity, which when incubated with GTN produced nitric oxide complexes detectable by EPR. No efforts have yet been reported to purify and characterize the relevant proteins, nor is it clear whether the same, similar or different enzyme systems act on the glycerol di- and mono-nitrates. Further discussion of the involvement of GSH *S*-transferases in microbial biodegradation of nitrate esters is deferred to the conclusion of this review.

Glycidyl nitrate

Direct environmental discharge of GTN-containing wastes is prohibited due to its toxicity and sensitivity to detonation by thermal and mechanical shock. GTN in industrial wastes is therefore chemically 'desensitized' by treatment with calcium hydroxide, which results in the disappearance of GTN and the isomers of GDN and GMN. During this process, however, the epoxides

Fig. 4. Intermediates in the chemical/microbial degradation of GTN following its 'desensitization' by alkaline treatment. From Kaplan *et al.* (1981, 1982); published with the permission of the American Society for Microbiology.

glycidol and glycidyl nitrate (Fig. 4) are formed, which themselves are potential pollution problems (Kaplan *et al.*, 1981). Both glycidol and glycidyl nitrate contain the reactive epoxide group which is toxic and mutagenic through its capacity for alkylating DNA (Hemminiki, 1979; Wade *et al.*, 1979).

With a view to coupling chemical desensitization to biological waste-treatment, D. L. Kaplan and colleagues (Kaplan *et al.*, 1981, 1982) tried to determine the susceptibility of glycidol and glycidyl nitrate to microbial degradation. Interpretation of data was complicated by the spontaneous (i.e. abiotic) degradation by which glycidyl nitrate was converted stoichiometrically to 1-GMN (Fig. 4). Rates of the spontaneous reaction increased significantly with temperature from 3 to 100 °C but the process was insensitive to light, indicating a simple hydrolytic cleavage of the epoxide ring. The addition of a mixed microbial culture (activated sludge from a sewage treatment plant) produced some acceleration in the disappearance of glycidyl nitrate which was complete in about 15 d. In marked contrast, the accumulating concentrations of the product 1-GMN,

which accumulated to stoichiometric amounts in sterile conditions, were markedly reduced by the presence of micro-organisms. Thus, it appears that the major contribution of micro-organisms was to accomplish biodegradation of 1-GMN formed through the (mainly) chemical degradation of glycidyl nitrate. This microbial contribution was confirmed by the absence of any effect of micro-organisms on the glycidyl nitrate or 1-GMN profiles during treatment at 3 °C, at which temperature microbial activity would be minimized. There was also some evidence (Kaplan *et al.*, 1981, 1982) for microbially enhanced degradation of glycidyl nitrate via 1-GMN under anaerobic conditions. This work indicated that no special pathway operated for glycidyl nitrate and that cleavage of the nitrate ester linkage was dependent largely on prior cleavage of the epoxide ring to 1-GMN.

Ethylene glycol dinitrate

Ethylene glycol dinitrate (EGDN) is a powerful high explosive (Urbanski, 1965) used in commercial explosive compositions, often in combination with GTN. In mammalian systems, EGDN is similar to GTN in its sequential cleavage of the nitrate ester linkages to yield nitrite (Litchfield, 1971), and in its ability to serve as substrate for GSH S-transferases (Tan-Walker, 1987; Taylor *et al.*, 1987).

In her investigations on the microbial transformation of EGDN, Tan-Walker (1987) isolated four Gram-negative bacteria (two strains of *Klebsiella oxytoca*, *Enterobacter agglomerans* and *Serratia rubidaea*) by aerobic enrichment on glycerol with EGDN as sole source of nitrogen. A strain of *K. oxytoca* chosen for further studies grew exponentially on medium containing EGDN as sole source of nitrogen, with a concomitant decrease in the concentration of EGDN, and an increase in the concentration of EGMN. However, only 80% of the EGDN was recovered as EGMN. Inadequate extraction, subsequent EGMN metabolism or retention of EGMN intracellularly were considered as possible causes for the incomplete recovery of the mononitrate (Tan-Walker, 1987). Failure of different extraction procedures to improve recovery, and the inability of cells to grow on the mononitrate as source of nitrogen, pointed to intracellular accumulation as the most likely cause. However, no experiments to examine cell extracts for EGMN appear to have been made to verify this hypothesis.

Sonicated cells of *K. oxytoca* were able to convert EGDN to EGMN, which itself showed signs of subsequent degradation. The activity in crude extracts exhibited a pH optimum near neutrality, and apparent K_m and V_{max} values of 1.21 mM and 6.32 $\mu\text{mol h}^{-1} \text{mg}^{-1}$ respectively. The activity was localized in the cytoplasm,

in keeping with the suspected intracellular accumulations of EGMN, and exhibited a requirement for Mg^{2+} or Zn^{2+} .

Attempts to purify the responsible enzyme(s) were seriously hampered by the low activities and the emerging complexity of the system. Using anion-exchange column chromatography, crude extracts were fractionated into three fractions capable of producing elevated nitrite concentrations in the presence of added EGDN. Further analysis of each of these fractions by gel-permeation chromatography showed that two of the fractions achieved stoichiometric conversion of EGDN into EGMN, but with release of less-than-stoichiometric amounts of nitrite. The activities were very low as is manifest by the long incubation times employed in the assays (up to 4 d). For the third fraction, nitrite release was even slower and, moreover, the elution profiles for nitrite release and EGDN $\rightarrow$ EGMN conversion were not coincident. The data were tentatively interpreted as indicating the existence of an EGDN-degrading complex containing hydrolytic, as well as nitrate reductase, activity, although much more work is necessary before it is possible to formulate with confidence a clear notion of the number(s) and type(s) of enzyme activity involved.

Other related nitrate esters (propylene glycol dinitrate, diethylene glycol dinitrate, triethylene glycol trinitrate, trimethylolethane trinitrate) are reported (Walker & Kaplan, 1992; Kaplan, 1993) to undergo microbial transformation by a series of steps sequentially cleaving the nitrate ester groups to the parent glycols, but the biochemical events and enzymology involved remain obscure.

Isosorbide dinitrate

Among the nitrate esters used to treat cardiovascular disease are isosorbide 2,5-dinitrate (ISDN), and the corresponding mononitrates, particularly isosorbide 5-mononitrate (5-ISMN, Fig. 5). The isosorbide nitrates have the advantage over other vasodilatory nitrate esters that they are less hepatotoxic and have longer half-lives. For example, the biological half-lives of ISDN, 2-ISMN and 5-ISMN are about 1, 2.5 and 5 h respectively (Ropenga & Lenfant, 1988), whereas the value for GTN is of the order of a few minutes (Taylor *et al.*, 1987). Presently, 5-ISMN is produced chemically but the method gives a poor yield and a lack of stereoselectivity. Consequently attention is currently turning towards biological systems in the search for possible alternative synthetic routes.

In view of the well-documented involvement of GSH *S*-transferase enzymes in the mammalian metabolism of nitrate esters (*vide infra*), including cleavage of the nitrate ester linkages of ISDN (Keen *et al.*, 1976), and aware of

the presence of GSH *S*-transferases in protozoan species (Lau *et al.*, 1980), Ropenga & Lenfant (1987) explored the possibility of using the protozoan *Tetrahymena thermophila* to biotransform ISDN into the ISMN isomers.

The protozoan converted ISDN into the mononitrates with preferential attack at the 2-position (which is sterically the less occluded, see Fig. 5) to yield 5-ISMN/2-ISMN in the ratio 2.6:1. Biotransformation was slow for the first 48 h of growth but accelerated in the stationary phase, with continuous accumulation of the ISMNs. After 72 h, the concentration of 5-ISMN began to decrease, presumably as the result of cleavage of the remaining nitrate ester linkage to yield isosorbide (although no supporting data were presented), and this may account for the failure to recover stoichiometric amounts of the mononitrates. Extracts of cells harvested during metabolism of ISDN contained GSH *S*-transferase(s) active towards ISDN, 1-chloro-2,4-dinitrobenzene and 1,2-dinitrobenzene. Differences in the activity profiles for GSH *S*-transferases measured across the batch growth-cycle for each of these substrates indicated the presence of more than one GSH *S*-transferase system with different specificities. Cultures containing a supplement of ISDN yielded slightly higher specific activities towards all substrates, suggesting inductive regulation of synthesis (Ropenga & Lenfant, 1987).

To evaluate the capacity of a wider variety of micro-organisms to biotransform ISDN, Ropenga & Lenfant (1988) screened a total of 19 strains of yeast, fungi and bacteria, of which 18 converted ISDN preferentially to 5-ISMN; these included the filamentous fungi *Cunninghamella echinulata*, *Cunninghamella elegans*, *Geotrichum candidum*, *Phanerochaete chrysosporium* and a *Pseudomonas* species. Only the yeast *Rhodotorula glutinis* converted ISDN preferentially to 2-ISMN. The two species of *Cunninghamella* gave the most rapid conversion, three times faster than observed with *Tetrahymena thermophila* (Ropenga & Lenfant, 1987). Improvement of the bioconversion efficiency was achieved by incorporating methanol in the phosphate-buffer medium to elevate the solubility of ISDN. Alternatively, replacing the phosphate buffer by a complex culture medium provided a sufficient energy source to allow cofactor regeneration, essential for GSH *S*-transferase activity, and this increased ISDN conversion to 74% for *C. elegans* and 88% for *C. echinulata*, the latter with a 5-ISMN/2-ISMN ratio of 2.5. This yield and stereoselectivity were significant improvements on the chemical synthesis (Ropenga & Lenfant, 1988).

In a more recent report (Ropenga *et al.*, 1989), *Beauveria* spp. were claimed to be among the most efficient fungi for biotransformation of ISDN, although

Fig. 5. Biotransformation of ISDN to isomeric ISMNs.

these were not included in the report cited (Ropenga & Lenfant, 1988), and comparative data have not been presented. Moreover, interpretation of GSH *S*-transferase activities measured during batch culture of *B. tenella* was made difficult by failure to control the pH adequately. Consequently, no direct correlations between enzyme activities and biotransformation of ISDN could be made.

Pentaerythritol tetranitrate

Pentaerythritol tetranitrate (PETN, Fig. 1) features with GTN and ISDN among the most commonly used treatments for angina pectoris and chronic congestive heart failure. It is also an explosive of considerable utility because of its high explosive strength and chemical stability (Urbanski, 1965). However, the only published information relating to its microbial biodegradation has arisen from interests in its therapeutic applications. Several metabolic studies have generally shown negligible amounts of PETN and other nitrate esters in the urine and faeces of treated animals (DiCarlo *et al.*, 1967; Davidson *et al.*, 1971), whereas others have demonstrated significant amounts of intact PETN in rat excreta after oral administration (Carter & Goldman, 1976). To resolve this discrepancy, King & Fung (1984) examined the possibility that microbial metabolism might be involved.

By incubating ^{14}C -labelled PETN with urine, and analysing samples at intervals using radio-TLC, PETN was shown to be extensively degraded. At low initial concentrations of PETN (below about 1 p.p.m.), residual

PETN, and its tri-, di- or mononitrated analogues (PETriN, PEDN or PEMN) were undetectable after 24 h; at higher initial concentrations of PETN, all four components were present. Kinetic analysis of metabolite production showed an initial lag in PETN degradation, consistent with growth of competent microbial populations, followed by rapid and complete disappearance of the substrate. PETriN, PEDN and PEMN appeared in sequence indicating a precursor-product relationship. The degradation was inhibited by addition of NaF, and completely prevented by reducing the incubation temperature to 0 °C or removing bacteria from the urine by filtration before incubation. Four different bacterial species were subsequently detected in the urine but not identified. Collectively, these results pointed firmly towards microbial degradation of the PETN and its metabolites. Attempts to eliminate the biodegradation by collecting urine without contamination by faecal organisms were partly successful; PETN and PETriN were still totally degraded but higher concentrations of PEDN accumulated. Homogenized and resuspended faecal pellets were also capable of biodegrading PETN completely and this capacity was eliminated by autoclaving the cell suspension.

The data suggested that PETN was subject to extensive microbial biodegradation in untreated or unpreserved urine and faeces. Although this conclusion has important implications for experimental techniques employed in studies of drug metabolism and distribution in mammalian systems, no further studies appear to have been made on the microbial degradation *per se*. This is unfortunate, because these results apparently contradict

those of Carter & Goldman (1976) who concluded, from a comparison of PETN metabolism in conventional and germ-free rats, that intestinal flora play no role in the release of nitrite from PETN.

Cellulose nitrate

Cellulose nitrate (Fig. 1) is widely used in smokeless powders, gelatins, dynamites, varnishes, transparent films and pharmaceuticals. Disposal of cellulose nitrate wastes including 'fines' produced during manufacture presents problems; combustion yields unacceptable air pollution and land-fill is prohibited because of the explosion hazard. The consequent search for biological methods has revealed some very limited information about biodegradability.

Cellulose is the most abundant natural polymer on Earth, and the capacity of micro-organisms to biodegrade it has been extensively studied (see reviews by Ghosh & Ghosh, 1992 and Malburg *et al.*, 1992). Yet for many years it has been known that even small changes in the molecular structure of cellulose render it resistant to attack by cellulolytic micro-organisms (Siu, 1951; Selby 1968; Kaplan *et al.*, 1970; Greathouse & Wessel, 1954). It is therefore not surprising that cellulose nitrate, which is relatively highly substituted (two or three esterified OH groups per glucose residue), is very recalcitrant to microbial degradation (Wendt & Kaplan, 1976; Kaplan, 1993).

Studies to date on the biodegradation of cellulose nitrate (see Walker & Kaplan, 1992; Kaplan, 1993; and references therein) have been concerned mainly with a search for strategies to overcome its recalcitrance, and as a result they invariably involve the use of mixed-culture rather than pure microbial systems. For example, chemical pretreatment (alkaline hydrolysis) of cellulose nitrate produced a modified material which was sufficiently depolymerized and/or freed from nitrate ester groups to facilitate microbial biodegradation in an activated sludge treatment system when supplemented with an additional carbon source (Wendt & Kaplan, 1976). A combined chemical/biological treatment process was subsequently developed in which alkaline pretreatment, followed sequentially by denitrification, then activated sludge treatment, then further denitrification, yielded an effluent of satisfactory quality. More recently, composting has been used successfully in the bioremediation of soils contaminated with cellulose nitrate (Williams *et al.*, 1992). However, none of these mixed-culture studies has addressed fundamental questions such as the relative contributions of chemical, microbial anaerobic and microbial aerobic processes to the cleavage of nitrate ester linkages, sequence of depolymerization and cleavage of nitrate ester linkages, the

mechanism(s) of the cleavage or the nature of the immediate nitrogenous products.

Evidence that cellulose nitrate does succumb to microbial cleavage of its nitrate ester linkages comes from studies with *Aspergillus fumigatus* (Brodman & Devine, 1981). This fungus was able to grow utilizing cellulose nitrate as sole source of nitrogen, provided that an alternative carbon source was supplied (glucose). Analysis of growth yields suggested that *A. fumigatus* enhanced the spontaneous rate of cleavage of nitrate ester groups, but once again no analysis of the biochemical events was made.

Conclusion

The brevity and fragmentary nature of the foregoing review shows that the study of the biotransformation and biodegradation of nitrate esters in micro-organisms is in its infancy. Nevertheless, some interesting features and points of intrigue are beginning to emerge and these are now highlighted.

It is well-established that catabolism of sulphate and phosphate esters is initiated by sulphatase- and phosphatase-catalysed hydrolysis of the C-O-S and C-O-P linkages (Boyer, 1971; Dodgson *et al.*, 1982). Although it might have seemed reasonable to anticipate the occurrence of analogous 'nitrataase' (nitrohydrolase) enzymes to catalyse hydrolytic fission of the C-O-NO₂ nitrate ester bonds, the information accumulated to date appears to contradict this notion. Even in the absence of enzymes, nitrate esters in aqueous solution are resistant to simple hydrolysis (Urbanski, 1965). Part of the reason for this may be that nitric acid, in contrast with sulphuric and phosphoric acids, is monobasic and its esters are therefore uncharged. This will reduce polarity, solubility and extent of solvation in aqueous solution and may thereby affect propensity for hydrolysis compared with sulphate and phosphate esters. In all these features, nitrate esters are much closer to halogenated aliphatic compounds, and this point is developed below.

The metabolic processing so far described for nitrate esters is characterized by its reductive theme. Thus formation of nitrite, NO and a nitrosyl haemoprotein from GTN, and of nitrite from EGDN, are reductive steps. Underlying mechanistic details are beginning to emerge, at least for biodegradation of GTN in eukaryotic microbes. The partial involvement of GSH and GSH *S*-transferase points to some links with parallel mechanisms for GTN metabolism in higher eukaryotes. In mammals, GTN is metabolized by reaction with GSH catalysed by GSH *S*-transferases, in which the electrophilic -NO₂ group undergoes nucleophilic attack by GSH, probably as the thiolate anion, to generate a GSH-sulphenylnitrite intermediate (-S-NO₂), which reacts with a second GSH

molecule to yield nitrite and oxidized (S-S dimeric) glutathione (Taylor *et al.*, 1987), which is in turn reduced by NADPH to regenerate GSH.

The mechanism(s) used in prokaryotes for the cleavage of nitrate ester linkages is at present unknown, but in the light of the known involvement of GSH in eukaryotes, it may be relevant that GSH (Fahey *et al.*, 1978) and GSH S-transferase enzymes (Dillio *et al.*, 1988) are also present in bacteria. Moreover, GSH is involved in dehalogenation of halogenated aliphatic compounds (Stucki *et al.*, 1981; Kohler-Staube & Leisinger, 1985), which may be regarded as analogues of nitrate esters because both are condensation products of monobasic acids (H-Cl or H-ONO₂) with alcoholic hydroxyl groups to yield uncharged Cl- or NO₃-substituted compounds. Sequence analysis has shown that the GSH-dependent microbial dehalogenase system belongs to the GSH S-transferase supergene family in eukaryotes (La Roche & Leisinger, 1990). In addition, some of the mammalian GSH S-transferase systems that metabolize nitrate esters also act on halogenated compounds (Ahmed & Anders, 1978; Taylor *et al.*, 1987). Collectively, these various factors indicate that the bacterial GSH S-transferase system, which achieves dehalogenation in prokaryotes, might also act on nitrate esters. Whether or not this is the case must await the outcome of further research, a contribution to which is currently in progress in our laboratory. To avoid prejudging the issue at this stage, it should also be noted that microbial degradation of halogenated aliphatic compounds occurs through a variety of biochemical pathways (Neilson, 1990; Hardman, 1991) including not only via a GSH-dependent pathway, but also by hydrolytic (for example, Janssen *et al.*, 1987; Sallis *et al.*, 1990), oxidative (Yokota *et al.*, 1986; Kaska *et al.*, 1991) and reductive (Vogel & McCarty, 1985; Fathepure *et al.*, 1987) processes. Biodegradation mechanisms for nitrate esters may prove to be equally diverse.

Several of the nitrate esters considered in this review contain multiple nitrate groups (Fig. 1) but only two, GTN and ISDN, present opportunities for regio- and stereoselectivity in the cleavage of the multiple nitrate ester linkages present. In both cases, such selectivity has already been observed, and this differential activity for different positional and stereoisomers raises questions about enzyme specificity and multiplicity. For example, does the regioselectivity for C1 and C2 nitrogroups in GTN reflect different enzymes for each position or a single system with differential activity? Are the enzymes that attack GTN also responsible for cleavage of the nitrate ester linkages in GDN and GMN? Moreover, 1,2-GDN and 1-GMN are chiral molecules at C2, raising questions about stereospecificity. For example, are the relevant enzyme systems stereospecific for enantiomeric

forms of their substrates, as are sulphatases acting on secondary alkyl sulphates (Dodgson & White, 1983)? Do the catalytic steps result in retention of configuration as observed in some sulphatase and dehalogenase reactions, or inversion as seen in others (Weightman *et al.*, 1982; Dodgson & White, 1983; Crescenzi *et al.*, 1984).

Finally, it is quite possible that the microbial metabolism of nitrate esters is closely linked to assimilatory and/or dissimilatory (denitrifying) reduction of nitrate by micro-organisms. Both involve reduction of nitrate to nitrite, albeit using distinct enzyme systems, but denitrification involves subsequent reduction of nitrite to NO, a pivotal reaction which commits the nitrogen to dinitrogen gas (and a bit of nitrous oxide) (Payne, 1981; Hochstein & Tomlinson, 1988). Nitrite and NO have already featured in this review as nitrate ester metabolites, and the anaerobic nature of denitrification would also be consistent with the recurrence of the reductive theme in nitrate ester metabolism noted hitherto. It will be interesting to determine whether or not known denitrifying organisms are able to utilize nitrate esters as sole sources of carbon and/or energy, and also whether they can use intact nitrate esters or their degradation intermediates as terminal electron acceptors.

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