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Microbes and microbial enzymes for cyanide degradation

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Abstract

Cyanide is an important industrial chemical produced on a grand scale each year. Although extremely toxic to mammalian life, cyanide is a natural product generated by fungi and bacteria, and as a result microbial systems have evolved for the degradation of cyanide to less toxic compounds. The enzymes which utilize cyanide as a substrate can be categorized into the following reaction types: substitution/addition, hydrolysis, oxidation, and reduction. Each of these categories is reviewed with respect to the known biochemistry and feasibility for use in treatment of cyanide containing wastes.

Introduction

Hydrogen cyanide (HCN) is a colorless liquid with appreciable vapor pressure (bp = 27.5 °C) having the distinctive odor of bitter almonds. It is produced on an industrial scale of 2–3 million tons per year and used in the synthesis of polymeric precursors methyl methacrylate and adiponitrile, as well as dyes, pharmaceuticals and agricultural products (Klenk et al. 1988). Its alkali salt, sodium cyanide, is employed for the recovery of precious metals (gold, silver) from ores. Additional uses encompass the electroplating processes of brass, copper, zinc, and cadmium and the metal surface hardening of small metal parts (Palmer et al. 1988).

Cyanide is extremely toxic to living systems by inactivation of respiration due to its tight binding to cytochrome c oxidase and to a lesser extent, other metalloproteins (Solomonson 1981). Acute cyanide poisoning in humans can lead to convulsions, vomiting, coma, and death. The lethal dose rate is in the range 0.5–3.5 mg/kg body weight. Longer term effects are less well understood but include neuropathy, optical atrophy, and pernicious anemia (Way 1983). As a result, cyanide waste streams generated from organic nitrile synthesis and the above industrial processes must be reduced to considerably low levels (< 1 mg/l) before release into the environment.

Current chemical methods of waste water treatment include alkaline chlorination, ozonation, wet-air oxidation, and sulfur-based technologies (Palmer et al. 1988). Each of these technologies has its own cost and disposal considerations. Despite the notoriety of cyanide as a metabolic inhibitor, biological methods of waste treatment are feasible as cyanide is also produced naturally by a wide variety of plants and fungi and some bacteria. A number of microbial systems have thus evolved for the detoxication or degradation of cyanide. Biodegradation technologies are particularly appealing for cyanide wastes with additional organic components to serve as substrates for microbial growth.

Scattered reports of microbial degradation of cyanide wastes by mixed populations in acclimatized sludge have appeared for nearly a quarter of a

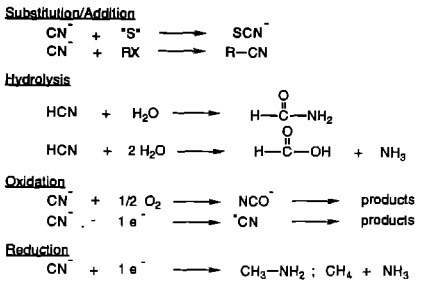


Fig. 1. Enzymatic reaction types for cyanide degradation.

century. In many cases specific microorganisms have been isolated which either degrade or assimilate cyanide, and these pathways studied in some detail. It is the aim of this review to focus on the types of microbial biochemical reactions for the detoxication of cyanide and the enzymes which catalyze these transformations. Previous reviews of microbial cyanide metabolism have appeared on a regular basis (Castric 1981; Harris et al. 1987; Knowles 1976; Knowles 1988). Aspects of cyanide production (cyanogenesis) by fungi and bacteria will not be covered, nor will the use of cyanide as a metabolic inhibitor (Solomonson 1981). The reader is referred to the above reviews for additional information not included here, and two excellent books which have appeared on cyanide compounds in biological systems (Evered & Harnett 1988; Vennesland et al. 1981).

Reaction types for cyanide degradation

The enzymatic reactions of cyanide can be loosely categorized into four types: substitution/addition, hydrolytic, oxidation, and reduction depicted in Fig. 1.

Each reaction type has its own particular consideration for treatment of cyanide contaminated wastewaters which include: cofactor requirements, cell viability for cyanide treatment, available form of cyanide utilized, and products from the enzymatic reactions which must be subjected to further degradation. In this review, each category will be developed in turn, focusing on studies with purified enzymes when possible. The biochemical reactions then serve as a basis for the discussion of more recent reports of isolates capable of cyanide detoxication.

Substitution/addition reactions

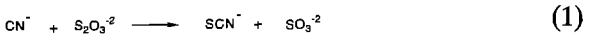
Cyanide is the conjugate base of a weak acid (HCN; pKa = 9.1) and a powerful nucleophile in substitution reactions. It also undergoes reversible addition to ketones and aldehydes and conjugate addition to α,β unsaturated carbonyl groups. In nucleophilic substitution reactions, it is an ambident nucleophile with possible attack by either nitrogen or carbon to give the isonitrile (RNC) or the nitrile (RCN). Although both isomeric products can be produced chemically, biochemically only nitrile synthesis has been observed (Ferris 1983).

Cyanide also has a great affinity for sulfur, particularly in the persulfide form ($R\text{-}S\text{-}S^-$). Thiophilicity in general parallels the nucleophilic series for carbon substitution, however cyanide is nearly equal to other thiols and far ahead of hydroxide and other common nucleophiles. As Westley (1981) has noted, the equilibrium constant for the reaction between cyanide and thiosulfite lies 10^{10} in favor of the products thiocyanate and sulfite (Eq. 1). The reverse reaction has not even been detected.

The nucleophilic and thiophilic properties of cyanide provide two avenues for its detoxication to substitution products which can then be excreted by the organism. In this light two sulfur transferases which are involved in the conversion of cyanide to thiocyanate ion will be considered first, and then two pyridoxal phosphate enzymes from the cyanogenic *Chromobacterium violaceum* which detoxify cyanide through substitution/addition reactions.

Rhodanese

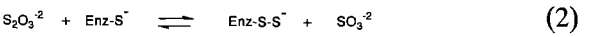
Rhodanese (thiosulfate: cyanide sulfur transferase; EC 2.8.1.1) catalyzes the following reaction:



(1)

The enzyme has been observed in animals, plants, fungi and other microorganisms. Bacterial sources include *Escherichia coli, Pseudomonas aeruginosa, Thiobacillus denitrificans, Rhodospirillum palustris, Klebsiella pneumoniae, and Azotobacter vinelandii* (Westley 1988). The physiological function of the enzyme is not well defined, but it appears to have a multi-purposeful role in living systems for the maintenance of the sulfane sulfur pool in organisms and the incorporation of reduced sulfur for iron/sulfur centers of redox proteins (Cerletti 1986). The role of rhodanese in cyanide detoxication is argued from the high concentrations of the protein found in mammalian liver mitochondria where it is correctly positioned to defend cytochrome c oxidase against inactivation. An important antidote for the treatment of cyanide poisoning is infusion of sodium thiosulfate to serve as a cosubstrate for rhodanese, promoting conversion to thiocyanate. The less toxic thiocyanate is then excreted (Way 1983).

Rhodanese has been purified from a number of sources, but the enzyme from bovine kidney has been the most studied. It is comprised of a single polypeptide chain of 32,900 daltons which contains no metals nor additional cofactors. Rhodanese is one of the best understood enzymes. The mechanism of rhodanese catalysis has been elucidated and extensively reviewed by Westley (Westley 1977; Westley 1981; Westley 1988; Westley et al. 1983). The three dimensional structure of the enzyme has been determined by diffraction methods (Hol et al. 1983). Kinetic studies support a scheme where overall catalysis occurs in two steps. In the first step, thiosulfate donates a sulfur to a cysteine sulfhydryl on the protein to form the enzyme persulfide intermediate (Enz-S-S) (Eq. 2). The Km for thiosulfate is roughly 6 mM, and the formation of the intermediate is the overall rate limiting step (Mintel & Westley 1966). Cyanide then attacks this intermediate in a second, diffusion limited step to afford thiocyanate and regenerate the free enzyme (Eq. 3). The rhodanese persulfide is surprisingly stable and is the actual form in which the enzyme is isolated and crystallized. X-ray studies on the bovine liver enzyme have also verified important aspects of the mechanism. The protein consists of two domains, similar in structure, but very dissimilar in amino acid sequence. The active site is close to the interface of the two domains. A hydrophobic pocket encloses the active site sulfhydryl Cys 247 which is transformed into the persulfide. Anion substrate binding is facilitated by cationic side chains provided by Arg 186 and Lys 249.

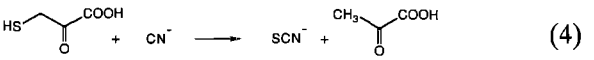


(2)

(3)

Mercaptopyruvate sulfurtransferase

Another enzyme responsible for maintaining the sulfane sulfur pool is 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) which, like rhodanese, forms thiocyanate from cyanide ion. The co-substrate mercaptopyruvate arises biochemically from the transamination of cysteine.



(4)

This enzyme has been demonstrated in animal, fungal and bacterial species such as *E. coli* and *A. vinelandii* (Jarabak 1981; Pagani et al. 1991; Vachek & Wood 1972). The enzyme has been purified from rat liver, *E. coli* and bovine kidney. The enzyme from bovine kidney is a single polypeptide chain of 33 KDa with no metals nor cofactors, although the presence of copper was once debated (Jarabak 1981). Mercaptopyruvate is the only known sulfur donor substrate for the enzyme but a variety of thiophiles can act as acceptors. Cyanide is most commonly used in enzyme assays, but mercaptopyruvate is also a suitable acceptor. This complication as well as rapid cyanohydrin formation between cyanide and mercaptopyruvate results in substantial deviation from standard Michaelis-Menten kinetics. Detailed kinetic analyses by Westley indicate the mechanism of catalysis is considerably different from rhodanese in that no sulfur enzyme intermediate is formed (Westley et al. 1983). Instead the enzyme requires both substrates to be bound in a ternary complex before the sulfur

transfer occurs. Little is known about the chemical mechanism; it has been postulated that the carbonyl group may help polarize the carbon-sulfur bond for cleavage upon attack by cyanide ion.

Use in treatment systems

Whereas the sulfurtransferases appear to be a primary route for detoxication of cyanide in mammals, the utility of these proteins for microbial biodegradation of cyanide wastes remains an open question. Rhodanese is quite prevalent among bacterial species and is induced in *Chromobacterium violaceum* after exposure to cyanide (Rodgers & Knowles 1978). Mercaptopyruvate sulfurtransferase may not be as wide spread. No evidence has been presented that the primary role of sulfurtransferases in these bacteria is indeed cyanide detoxication; sulfane sulfur metabolism and iron/sulfur synthesis is equally if not more likely.

The use of rhodanese in whole cells of *Bacillus stearothermophilus* for cyanide detoxication has been reported (Atkinson 1975). A 65 ml ultrafiltration cell was used as a small continuous reactor. 50 ml of cells (8 mg dry wt/ ml) were fed a continuous mixture of thiosulfate and cyanide; the optimal ratio was determined to be 1.5–2.5 to 1. High cyanide levels (5 mM to 50 mM) could be tolerated, however the half life of the cells was only 12 to 18 days after which appreciable levels of cyanide were detected in the filtrate.

Both sulfur transferases convert cyanide to the less toxic thiocyanate for excretion. In adapting these systems for cyanide detoxification the fate of thiocyanate in the environment must be considered. The biochemistry of thiocyanate ion has not been as extensively explored. The largest concentrations of thiocyanate (300–1000 mg/l) occur in waste waters generated from coal gasification and coking facilities which also contain high concentrations of cyanide, sulphide and phenol (Paruchuri et al. 1990). Under oxidizing conditions, the action of hydrogen peroxide and peroxidases serves only to oxidize the sulfur to sulfate and regenerate cyanide in a thiocyanate/cyanide cycle (Wood 1975).

$$3\,H_2O_2 + SCN^- \longrightarrow HSO_4^- + HCN + 2\,H_2O \qquad (5)$$

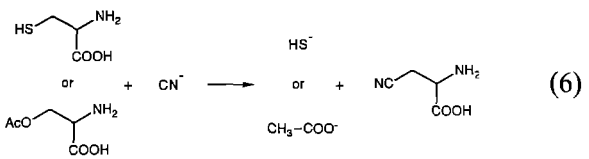
Microbial utilization of thiocyanate has been reported for *Thiobacillus, Arthrobacter*, and *Pseudomonas* species (Paruchuri et al. 1990). Several strains can grow on the ion as a source of energy and nitrogen. Conversion of cyanide to thiocyanate is a formal oxidation of carbon to its highest oxidation state. A hydrolytic pathway is used to convert thiocyanate to carbon dioxide and ammonia via cyanate, as substantiated in studies with *Thiobacillus thiocyanooxidans* (Youatt 1954). The sulfur is oxidized to sulfate to provide energy. Continuous culture systems have shown complete conversion of thiocyanate even at influent levels of 1000 mg/l. These systems may provide a route for the overall total degradation of cyanide. Potential problems include the cyanide toxicity to *Thiobacillus* strains, and growth inhibition at high thiocyanate concentrations as thiosulfate is the preferred energy source.

Pyridoxal phosphate enzymes

Two pyridoxal phosphate enzymes are known which aid in cyanide assimilation by cyanogenic microorganisms to form nitrile derivatives of α-amino acids in an overall substitution reaction. β-Cyanoalanine synthase and γ-cyano-α-aminobutyric acid synthase are both present in the cyanogenic *Chromobacterium violaceum*. Rodgers & Knowles (1978) found cyanide production by this organism occurred near the end of exponential growth on glutamate/glycine. At this point induction of both PLP enzymes and rhodanese resulted in the decrease of cyanide concentration in the media. Studies with ^{14}C-cyanide determined β-cyanoalanine to be the major end product for cyanide detoxication. No incorporation of radioactivity was found in the hydrolysis products asparagine or aspartic acid, suggesting that the organism was not using the pathway for growth (Rodgers 1981).

β-Cyanoalanine synthase

β-Cyanoalanine synthase (EC 4.4.1.9) catalyzes the substitution of a three carbon amino acid with cyanide. This activity has been detected in a number of bacterial species such as *Escherichia coli*, *Bacillus megaterium* and *C. violaceum* and additionally in algae and higher plants (Akopyan et al. 1975; Ikegami et al. 1988a; Ikegami et al. 1988b). The three carbon substrate is either cysteine or O-acetyl serine.



(6)

Hendrickson & Conn (1969) first noted that two distinct proteins are able to catalyze β-cyanoalanine formation in the plant blue lupine. Cysteine synthase (O-acetyl-L-serine sulfhydrylase EC 4.2.99.8) normally produces L-cysteine from O-acetyl-L-serine and H_2S, but is able to use cyanide as an alternate nucleophile to give β-cyanoalanine, albeit at a rate of 10%. Blue lupine also has a true β-cyanoalanine synthase which utilizes O-acetyl serine; cysteine synthase activity of the latter protein occurs at less than 5% the rate of cyanoalanine formation. The two enzymes have much in common, being PLP enzymes with similar pH optima which utilize O-acetyl serine in a β-replacement reaction. The key difference appears to be the selectivity of the active site for the incoming nucleophile – H_2S or CN^-. Thus it should be noted that the detection of β-cyanoalanine synthase activity in bacterial systems is in many cases due to an ancillary reaction of cysteine synthase upon exposure of the organism to cyanide. Activity measurements on the enzyme are needed to determine if a true β-cyanoalanine synthase is present.

Macadam & Knowles (1984) showed the presence of a true synthase was responsible for cyanide detoxification in *C. violaceum*. The purified protein has a molecular weight of 70,000 and has two subunits with 1.7 moles of pyridoxal phosphate per dimer. The preferred amino donor is L-cysteine

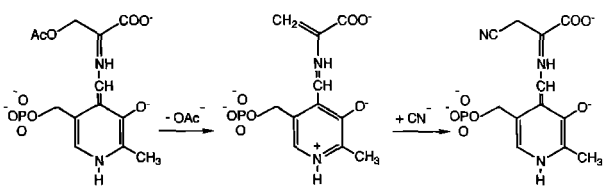
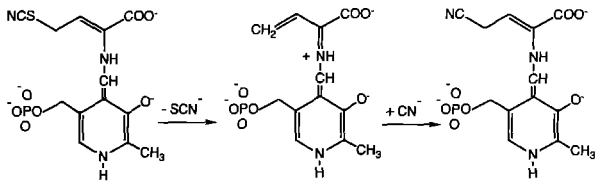


Fig. 2. Proposed intermediates during catalysis by pyridoxal phosphate-containing β-cyanoalanine synthase.

with O-acetyl serine giving 84% of activity. The pH optimum was 9.1 and the Km for cyanide 3.2 mM. Other thiols could serve as nucleophiles but at lower reaction rate and 10 fold higher Km.

The formation of β-cyanoalanine is typical of enzymatic pyridoxal phosphate catalyzed β-replacement reactions. The mechanism of these reactions has recently been reviewed by Miles (1986). Key features are shown in Fig. 2. Standard imine formation between PLP and the amino acid is followed by base catalyzed removal of the alpha proton to generate intermediate I. Braunstein & Goryachenkova (1984) have argued that cyanide attack on intermediate I then occurs in a direct substitution to give intermediate III. Kinetic studies which demand a ternary complex and failure to trap or observe putative intermediate II lend support to that argument. However Tsai et al. (1978) determined the stereochemistry of β-cyanoalanine formation occurs with retention of configuration at C-3. This stereochemical result argues against direct nucleophilic attack, as acetate and cyanide must leave and approach from the same direction. A compromise has been suggested in which the formation of intermediate II occurs only after a ternary complex has formed (Miles 1986). Upon the departure of acetate, cyanide adds to the unsaturated system to form III. Reprotonation gives the new imine which undergoes hydrolysis resulting in β-cyanoalanine as the observed product. It is therefore likely that the overall substitution reaction actually occurs via an addition mechanism.

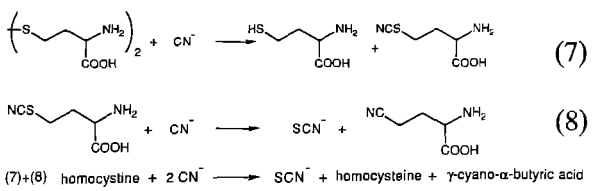


IV V VI

Fig. 3. Proposed intermediates during catalysis by pyridoxal phosphate-containing γ-cyano-α-aminobutyric acid synthase.

γ-Cyano-α-aminobutyric acid synthase

The enzyme responsible for production of γ-cyano-α-aminobutyric acid in *Chromobacterium violaceum* was isolated and purified by Ressler et al. (1973). Overall synthesis of the cyano amino acid occurs in two steps from homocysteine:



(7)+(8) homocystine + 2 CN^- → SCN^- + homocysteine + γ-cyano-α-butyric acid

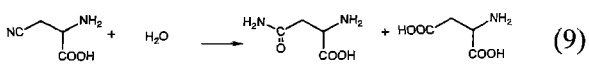
The first step in the sequence occurs nonenzymatically to generate S-cyano homocysteine which then serves as a substrate for the protein catalyzed replacement of SCN. This protein has a molecular weight of 130 K with an optimal pH between 8.5 and 10.5 The Km for cyanide is 30 mM; the enzyme requires pyridoxal phosphate for activity. As the enzyme catalyzed reaction is functionally of the pyridoxal phosphate γ-replacement type, the authors probed as to whether the activity was a secondary function of a known PLP enzyme. The protein showed no homocysteine sulfhydrylase nor O-acetyl homoserine sulfhydrylase activities and only 8% cystathionine activity suggesting its true function is the synthesis of this unusual amino acid.

No studies have appeared on the mechanism of action of this enzyme. It is probably similar to other pyridoxal phosphate dependent enzymes such as cystathionine γ synthase which catalyze reactions of the γ-replacement type (Churchich 1986). A plausible scenario (shown in Fig. 3) would comprise initial imine formation followed by deprotonation of both alpha and beta protons to give the allylic thiocyanate IV. Expulsion of the leaving group results in the unsaturated imine V to which cyanide then adds. Reprotonation of VI and hydrolysis gives the observed product. Again it is likely that the overall substitution reaction is in fact an addition of cyanide to an unsaturated intermediate.

Metabolic fate of nitriles

Little has been done in using these pyridoxal phosphate enzymes systems to treat cyanide contaminated wastewaters. The distribution of these enzymes is not as ubiquitous as the sulfur transferases. γ-Cyano-α-aminobutyric acid synthase has only been purified from *C. violaceum*, and as no other reports of either enzyme activity nor product formation have appeared, may be an isolated case. β-Cyanoalanine synthesis has been found in more species and may be potentially more useful. Both PLP substitution reactions generate nitrile derivatives of α-amino acids. Therefore the pathways for metabolism of the resulting nitriles must be considered for use in the detoxication of cyanide.

B. megaterium, *E. coli*, and *C. violaceum* have been reported to convert β-cyanoalanine via hydrolysis into asparagine or aspartate. The activity in *E. coli* was shown to be an ancillary function of asparaginase by Lauinger & Ressler (1970). True β-cyanoalanine hydratases have been purified from plant and bacterial sources. The enzyme from a *Pseudomonas* species is a large 100,000 Da protein which is specific for β-cyanoalanine and gives a mixture of asparagine and aspartate in the ratio of 2.2 to 1 (Yanase et al. 1983). It shows no activity in direct asparagine hydrolysis.



The enzyme catalyzed hydrolysis of organic nitriles is of current interest for use in biotechnology and the degradation of nitrile wastes. A number of enzymes have been characterized from bacterial sources which hydrolyze nitriles with broad sub-

strate specificity. This growing topic has also been recently reviewed and will only be briefly mentioned here (Harris et al. 1987, Ingvorsen et al. 1988, Wyatt & Linton 1988, Yamada & Nagasawa 1990). Two main pathways are available for the general hydrolysis of nitriles. Nitrilases catalyze the complete hydrolysis of a nitrile to the corresponding acid and ammonia, without formation of the free amide. Nitrile hydratases catalyze the hydration of nitriles to amides. The secondary action of an amidase is then used to generate the carboxylic acid and ammonia to support microbial growth. The substrate specificities of these enzymes are unusual in that aliphatic nitriles are normally processed by nitrile hydratase, whereas aromatic and heterocyclic nitriles are hydrolyzed via the nitrilase pathway. This trend is by no means absolute. Both pathways are potentially applicable for further metabolism of the nitrile products generated by substitution reactions. This tandem approach of PLP catalyzed substitution/addition followed by hydrolysis of the resulting nitrile has potential for the overall detoxication of cyanide.

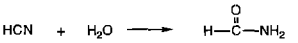
Hydrolytic pathways

Hydrolytic pathways offer several advantages over substitution for the detoxication of cyanide. First, direct enzymatic cleavage of the carbon nitrogen triple bond of hydrogen cyanide serves to destroy the cyano unit, eliminating the possibility of further reactivity. The products of hydration (formamide) and hydrolysis (formic acid and ammonia) are considerably less toxic than cyanide and can serve as growth substances in certain cases. Finally biological treatment of cyanide wastes via hydrolytic enzymes need not rely on viable, actively metabolizing cells for detoxication. No additional cofactors are necessary, the only requirement for catalysis being the presence of a functional enzyme. Hydrolytic systems therefore lend themselves to enzyme stabilization and immobilization technology, which can result in the treatment of higher concentrations of cyanide ion than could be tolerated by the living cell.

In direct analogy to the nitrile biochemistry, two systems have evolved for the enzyme catalyzed addition of water to hydrogen cyanide. Cyanide hydratase catalyzes the hydration of HCN to formamide, and cyanidase cleaves HCN in a hydrolytic fashion to formic acid and ammonia.

Cyanide hydratase

Cyanide degradation in phytopathogenic fungi occurs via the enzyme cyanide hydratase (formamide hydrolyase; EC 4.2.1.66) which produces formamide.



(10)

Such fungi utilize this enzyme to combat the toxicity of HCN produced from cyanogenic glucosides upon invasion of the plant. Fry & Millar (1972) first showed the presence of the activity in *Stemphylium loti* and attempted a partial purification of the enzyme on DEAE cellulose and Sephadex G-200, where the activity eluted in the void volume. Maximal activity occurred in the pH range 7.0 to 9.0. The enzyme was not inhibited by EDTA, ammonium chloride, potassium chloride, magnesium chloride or calcium chloride. The Km for cyanide was estimated at 15 to 20 mM. Fry & Evans (1977) then tested 31 species of fungi for cyanide hydratase activity. Enzyme activity was found in one of six fungi not pathogenic to plants, nine of fourteen pathogens of non-cyanogenic plants, and eleven pathogens of cyanogenic plants. Maximal activity was observed at 12 to 18 hours after exposure to hydrogen cyanide. High levels of specific activity were observed in *Gloeocercospora sorghi* and *Helminthosporium turcicum*.

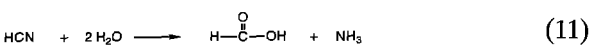
The mechanism of action of this enzyme remains obscure as attempts at enzyme purification have not been successful to date. The enzyme from *G. sorghi* is partially excluded in Biogel A1 5M with an exclusion limit of fifteen million daltons leading Fry & Myers to conclude the enzyme is associated with a membrane or particle; solubilization with detergents did not allow for further purification (Fry & Myers 1981). The activity is quite stable when frozen or at 4 °C, and was unaffected by

EDTA, mercaptoethanol, thiocyanate but inhibited at 10 mM flouride and azide.

Nazly & Knowles (1981) first showed that *S. loti* could be immobilized and used in a continuous reactor system to degrade cyanide wastes to formamide. In a second study a range of fungi were selected for immobilization after induction of cyanide hydratase with exposure to 0.5 mM KCN 10 to 12 hours before harvesting (Nazly et al. 1983). The pH profiles of the immobilized enzymes were similar to that of the free enzyme, however the apparent Km for cyanide doubled to 40–70 mM. Enzyme stability was dramatically increased to 30 days using *G. sorghi*. Addition of 1 mM glucose in the feed stocks gave a further increase to 40 days. ICI has engineered the immobilization of a non-phytopathogenic strain of *Fusarium* which has high levels of cyanide hydratase activity for the degradation of cyanide wastes in batch and fluidized bed reactors that is commercially marketed as Cyclear (Richardson 1987; Richardson & Clarke 1987). The fungi also express some nitrilase activity which may expand future versions to include nitrile wastes (Clarke 1986). The treatment is most effective at high (200–10,000 mg/l) cyanide concentrations, as a consequence of the reasonably high Km value of 50 mM. Levels of under 20 mg/l cyanide can be reached using the immobilized fungi system, however it does not degrade metal-cyanide complexed wastes.

Cyanidase

Several reports have appeared on the microbial production of ammonia from cyanide by isolated strains, mixed microbial systems and activated sludge (Howe 1965; Painter & Ware 1955). The pathways for ammonia production have not been elucidated in these cases and may occur by a number of enzymes acting in concert. In two cases direct hydrolysis of cyanide to formic acid and ammonia has been demonstrated, and in parallel with the nitrile-hydrolyzing enzyme nitrilase, these enzymes have been called cyanidases.



$$HCN + 2H_2O \longrightarrow H{-}\overset{O}{\overset{\|}{C}}{-}OH + NH_3 \qquad (11)$$

White et al. (1988) isolated a *Pseudomonas* strain by selective enrichment in a chemostat with coke-plant activated sludge using a feed of 10 mg/l cyanide. The isolate degraded the cyanide to formate and ammonia which could then be used as a nitrogen source. The organism could grow on methanol and methyl amine as a carbon source, but not formate. It adapted to increasing concentrations of cyanide with a lag time of up to 40 h. Unadapted cells began to degrade cyanide after 72 h. Respiration increased when adapted cells were exposed to cyanide, but was repressed when unadapted cells were exposed under similar conditions. The authors suggested this adaptation was consistent with the development of respiratory resistance and the induction of cyanide degrading enzymes. The *Pseudomonas* strain was able to degrade 100 mg/l cyanide when lactate was provided as a carbon source.

Upon exposure of cell free extracts to ^{13}C cyanide, formic acid was identified as the sole product by the diagnostic ^{13}CMR spectrum. Formamide was not detected as an intermediate product, neither was it degraded by cell free extracts in the presence or absence of cyanide. These observations argue for the direct hydrolysis of cyanide to formic acid, although formamide could be an enzyme bound intermediate. Enzyme activity in cell free extracts did not require oxygen and was lost upon dialysis, but could be regenerated by less than one equivalent of NAD(P)H or ascorbate.

Novo Industries reported the presence of cyanidase in a strain of *Alcaligenes xylosoxidans subsp. denitrificans* which utilized cyanide as a nitrogen source (Ingvorsen & Godtfredsen 1988). Formate and ammonia were produced upon exposure of cell suspensions to cyanide. Formamide could not be detected by HPLC. However formamide hydrolysis was observed at 30% the rate of cyanide degradation. Cyanide hydrolase activity in whole cells was maximal in the pH range of 7.5 to 8.2 and cells did not hydrolyze cyanate nor organic nitriles. Enzyme activity was inhibited at cyanide concentrations of 100 mM and greater, but first order kinetics for cyanide degradation were found even at high cyanide concentrations of 1000 mM. Although the

hypochlorite generates nitrogen and carbon dioxide.

$$CN^- + OCl^- \longrightarrow OCN^- + Cl^- \quad (12)$$

$$2\,OCN^- + 3\,OCl^- + H_2O \longrightarrow N_2 + 2\,CO_2 + 2\,OH^- + 3\,Cl^- \quad (13)$$

Such chemical precedent serves as background for an oxidative pathway found in a strain of *Pseudomonas fluorescens* biotype II isolated by Harris & Knowles (1983). The isolation was carried out on agar plates with glucose as the carbon source and cyanide as the nitrogen source supplied as hydrogen cyanide vapor. Fed-batch culture chemostats used KCN as the limiting nutrient to prevent the build up of toxic level of the ion. Cyanide grown cells degraded pulses of KCN to ammonia. Cells grown on ammonia could not degrade cyanide, suggesting that the system was inducible. A requirement for oxygen was noted in that anaerobic suspensions could not degrade cyanide. Further studies on cell free extracts localized the cyanide activity to the supernatant fraction (Harris & Knowles 1983). Cyanide degradation to ammonia required the stoichiometric addition of NAD(P)H and oxygen. NADH was the preferred reductant; NADPH could be used, however the rate dropped to 40%. $^{14}CO_2$ was trapped from extracts exposed to $K^{14}CN$. A recovery of 67% was noted; boiled extracts produced minimal $^{14}CO_2$. The overall stoichiometry of oxidative cyanide degradation by *Pseudomonas fluorescens* was thus determined to be:

$$NADH + H^+ + HCN + O_2 \longrightarrow CO_2 + NH_3 + NAD^+ \quad (14)$$

The authors postulated this process could occur in two steps. Utilization of an oxygenase could produce cyanate as an intermediate which would then be hydrolyzed by the enzyme cyanase to carbon dioxide and ammonia.

$$NADH + H^+ + HCN + O_2 \longrightarrow HNCO + H_2O + NAD^+ \quad (15)$$

$$HNCO + H_2O \longrightarrow CO_2 + NH_3 \quad (16)$$

Cyanase (cyanate amidohydrolyase; EC 3.5.5.3) has been detected in *E. coli, Flavobacterium* sp. and plants. The physiological role of this enzyme is uncertain, but it is thought to protect against cyanate poisoning. Cyanate is not a known metabolic product but can arise from the decomposition of carbamoyl compounds in the cell. It was demonstrated that *Ps. fluorescens* also has an inducible cyanase when grown on KOCN as sole nitrogen source (Kunz & Nagappan 1989). However cyanase and cyanide oxygenase activity were never co-induced either during growth (on CN^- or OCN^-) or in stationary phase (Dorr & Knowles 1989). Thus a separate cyanase enzyme is not likely to be involved in the degradation of cyanide by *Ps. fluorescens*, although a form of cyanate could be an enzyme bound intermediate.

Rollinson et al. (1987) found that large amounts of cyanide oxygenase induced cells of Ps. fluorescens could be grown if nickel cyanide [$Ni(CN)_4^{-2}$] was used as a nitrogen source in batch and continuous culture systems. Copper cyanide [$Cu(CN)_4^{-2}$] could also serve as a nitrogen source, but with less growth and a considerable lag time. Cyanide oxygenase activity could also be induced by providing KCN or $Ni(CN)_4^{-2}$ to ammonia grown cells in stationary phase. It is not known whether the enzyme system is using the nickel complex directly as a substrate or if the complex serves to provide a small but continuous source of cyanide for growth governed by the dissociation constant $Kd < 10^{-10}$. This is the first report of culturing a microbial isolate on metal cyanide complexes as a nitrogen source. Such complexes are common from ore refining and electroplating wastes and are notoriously recalcitrant to degradation.

Metal-cyanide degraders may be more common than originally thought. Silva-Avilos et al. (1990) reported the isolation of ten bacterial species on [$Ni(CN)_4^{-2}$] as a nitrogen source from sites having no previous history of cyanide contamination. Seven of the ten were *pseudomonads* and three were classified as *Klebsiella* species, which are considered to be cyanide resistant. One strain, *Pseudomonas putida*, was chosen for further study. KCN could also serve as a growth substrate, inducing the cyanide utilizing enzyme system. Adapted cells completely consumed [$Ni(CN)_4^{-2}$] at concentra-

activity did not fit simple Michaelis-Menten kinetics, the Km for cyanide was estimated at between 1.5 and 10 mM cyanide.

To show that the cyanide hydrolase activity was due to a single enzyme and not a sequential pathway involving formamide hydrolysis, the enzyme was purified via protamine sulfate, DEAE-Sepharose, and Sephacryl S-300 chromatography (Ingvorsen et al. 1991). The enzyme eluted in the void volume of the Sephacryl S-300 column suggesting its native molecular weight was greater than 300,000 daltons. Only two bands of equal intensity and molecular weights of 39 and 40 Kda were observed by SDS-page. N-terminal sequencing revealed the N termini of the two bands to be identical. It is possible the two bands result from proteolytic degradation, or incomplete denaturation of the protein. Purified cyanidase (greater than 97% homogeneous) carried out the stoichiometric hydrolysis of cyanide to formate and ammonia. Formamide was not detected as a free intermediate, nor could the enzyme catalyze any formamide hydrolase activity.

The strain appears to be very useful in the detoxication of cyanide wastes and is being marketed as CYANIDASE (Ingvorsen & Godtfredsen 1988). Whole cells function at very high levels (1000 mM) of cyanide and are resistant to inactivation by organic nitriles, alcohols, phenol and metal cyanide complexes. Levels of less than 0.03 ppm cyanide are routinely reached even in the presence of 4 mM Zn^{+2}, Ni^{+2}, Fe^{+2}, and Fe^{+3}. Basheer et al. (1992) explored the utility of the biocatalyst in a fixed bed reactor. Both granulated and powered forms of the immobilized preparation were used. Michaelis-Menten kinetics were observed up to cyanide concentrations of 12 mM. The reported Km value was 3 mM. The catalyst was effective for decomposition of cyanide to levels of 0.01–0.02 mg/l and was not inhibited by chloride, sulfate, iodide, nor Fe^{+2}, Zn^{+2}, Ni^{+2} at concentrations of 70 mg/l. The lower Km value accentuates the promise for this product in effective cyanide removal. The granular catalyst was not very resistant to attrition arguing against its use in a stirred reactor.

These hydrolytic systems are some of the most promising for cyanide detoxification. They show good activity at high cyanide concentrations to produce either formamide or formic acid as products. Some potential drawbacks are due to the high Km values for the proteins (particularly upon immobilization) which result in lower efficiency at low cyanide concentrations. Long retention times are needed to reach the lower acceptable limits for release of wastewater streams into the environment.

Little is known about the mechanism of action of these proteins. Cyanide hydratase has only been preliminarily characterized from fungi and the two reports of cyanidase have only recently appeared. All considerations point to these proteins as being large oligomeric structures whose reaction pathways may not be as straight forward to decipher. On the surface, one could anticipate to find some similarities between these enzymes and the nitrile hydrolyzing proteins. This analogy would suggest that the acid HCN is the actual substrate for hydrolysis and not free cyanide ion. However, nitrile-hydrolyzing enzymes have not been reported to accept cyanide as a substrate. In fact, the highest acting nitrile hydratases are iron proteins which are inhibited by cyanide ion. Nitrilases are believed to act via a catalytic sulfhydryl group which is inactivated in the presence of metal ions. More studies are needed on the cyanide-hydrolases to determine the protein features necessary for catalysis given the additional reactivity of cyanide ion relative to the nitrile congener.

Oxidative pathways

Few biological systems have been reported that are capable of the direct oxidation or reduction of cyanide. Many oxidoreductases are in fact metalloenzymes which show substantial if not complete inhibition by cyanide (Solomonson 1981). In contrast, current technologies exploit chemical methods for the oxidation of cyanide wastes. A common treatment involves the addition of alkaline hypochlorite which oxidizes cyanide to cyanate as an initial product (Clarke 1986). The action of further

tions of 0.25 to 1.0 mM. However at higher levels of 16 mM only 20% was degraded and a green precipitate accumulated in the medium. This biological metabolite was collected and dried; subsequent FT-IR analysis revealed it to be identical to Ni $(CN)_2$. The presence of an inducible enzyme system upon exposure to low levels of $[Ni(CN)_4^{-2}]$ or KCN to provide nitrogen for growth suggests some similarities between *Ps. fluorescens* and *Ps. putida*. It remains to be proven if this *Ps. putida* strain has the same cyanide oxygenase activity. No studies on cell free extracts have yet appeared. Oxygen dependent cyanide degradation has not yet been shown nor has ammonia been identified as a product. Nevertheless, the cyanide oxygenase system could be quite common and provide a new technology for the detoxication of metal cyanide wastes.

Mudder & Whitlock (1984) at the Homestake Mining Company have utilized a strain of *Pseudomonas paucimobilis* to degrade wastewater containing cyanide, thiocyanate, and metal cyanide complexes of iron, copper, nickel, and zinc. Oxidation products of nitrates and sulfates are produced, the only chemical requirements being trace phosphorous and inorganic carbon supplied as phosphoric acid and bicarbonate. This process was commercialized as a 5.5 million gal/day plant which employs 48 rotating biological contactors (Palmer et al. 1988). Typical influent concentrations of cyanide are 5 to 15 mg/l; thiocyanate is present in higher concentrations of 20 to 70 mg/l. Degradation proceeds to greater than 95% completion even during the winter months with the temperature as low as 11 °C. No studies on the pathway of this oxidation have appeared.

One electron oxidation of cyanide to the cyanyl radical has been observed in electrochemical and chemical oxidizing systems using spin trapping techniques. Moreno et al. (1988) screened a variety of peroxidases: horseradish peroxidase, lactoperoxidase, chloroperoxidase, NADH peroxidase and methemoglobin with hydrogen peroxide for the ability to oxidize cyanide. In all cases the cyanyl radical could be observed by spin trapping with 5,5-dimethyl-1-pyrroline-N-oxide or N-*tert*-butyl-α-phenyl nitrone. Using the nitroso spin trap t-nitrosobutane at higher pH of 9.8, the formamide radical could also be detected growing in at the expense of the cyanyl radical. The final products that arise from the one electron oxidation of cyanide have not been determined, but the authors suggest a hydrolytic pathway via the formamide radical is plausible (Stolze et al. 1989). In an application of this technology, Shah et al. (1991) have used the lignin peroxidase system of *Phanerochaete chrysosporium* to detoxify cyanide. Studies with purified lignin peroxidase verified the formation of the cyanyl radical when treated with peroxide and cyanide. Spores of *P. chrysosporium* were particularly susceptible to cyanide inhibition of glucose metabolism with I_{50} = 0.1 mM. However, six-day-old cultures could tolerate much higher concentrations, I_{50} = 5 mM. Six-day-old cultures were effective in the mineralization of ^{14}CN to $^{14}CO_2$ over a three day period with a saturation point between 2 and 10 mM cyanide. The intermediate products from the one electron cyanide oxidation have not been identified, nor has the mechanism of the complete oxidation of CN^- to CO_2 by whole cells been elucidated for this system.

Reductive pathways

In contrast to the extensive work done with aerobic microorganisms, anaerobic conditions for the reduction of cyanide wastes are not as common. Anaerobes are particularly sensitive to cyanide, containing many key metalloproteins which serve as sites for inhibition. Methanogens have been successfully employed in mixed culture anaerobic degradations of organics but are notorious for their sensitivity to cyanide. Studies by Fedorak et al. have concentrated on degradation of phenolics in coal-coking wastewater by mixed anaerobic cultures (Fedorak & Hrudey 1987; Fedorak & Hrudey 1989; Fedorak et al. 1986). Phenol is degraded to acetate which can then be utilized by methanogenic bacteria for growth and methane production. The presence of cyanide in the feed results in a long lag time before methanogenesis resumes as the cells adapt to the presence of the toxicant. The continuation of methanogenesis is accompanied by

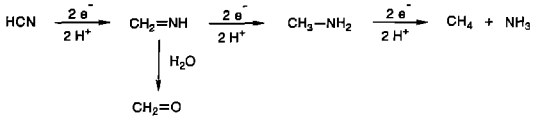


Fig. 4. Products from cyanide reduction catalyzed by nitrogenase.

a decrease in the concentration of cyanide, suggesting possible metabolic conversion. However the major product of cyanide transformation could not be identified using ^{14}CN studies.

Fallon et al. (1991) at Dupont have recently shown the anaerobic biodegradation of cyanide can occur under methanogenic conditions. Anaerobic sludge from nitrile waste was used as a starter culture for a fixed bed, activated charcoal treatment column. Cyanide concentrations of greater than 100 mg/liter were treated under methanogenic conditions using ethanol, phenol, or methanol as reduced carbon sources. Cyanide removal was accompanied by an increase in ammonia production. Test cultures produced ^{14}C bicarbonate when exposed to ^{14}CN. However attempts at isolating pure cultures capable of degrading cyanide directly from the system were not successful. The authors suggested that even under the reducing conditions employed, hydrolytic pathways were most likely which would produce formate and ammonia as products.

Nitrogenase

Expectation for the existence of reductive pathways for cyanide degradation is based partly on observations that HCN serves as an alternate substrate for the enzyme nitrogenase *in vitro* (Hardy & Knight Jr. 1967). Nitrogenase catalyzes the reduction of dinitrogen using reducing equivalents and Mg ATP to form two moles of ammonia (Orme-Johnson 1985). The enzyme consists of two metalloproteins, the molybdenum iron protein (MoFe protein) and an iron protein (Fe protein). It is commonly assayed by measuring the rate of reduction of acetylene, an *in vitro* substrate which contains a carbon-carbon triple bond. In the absence of a suitable substrate, nitrogenase catalyzes the reduction of protons to H_2. However, triply bonded HCN is isoelectronic with N_2, and can also serve as an alternate substrate. The products of nitrogenase catalyzed HCN reduction can arise from the passage of two, four, or six electrons to give respectively CH_2NH, CH_3NH_2 or CH_4 and NH_3. CH_2NH has not been observed directly, but is hydrolyzed to yield H_2CO and ammonia as in Fig. 4 (Li et al. 1982).

Whereas HCN serves as an alternate substrate for this metalloprotein, CN^- is an inhibitor, blocking total electron flow through the system. Thus the rate of HCN reduction is a function of the pH of the assay, being favored by less basic conditions. Detailed kinetic studies are consistent with a model in which HCN binds to the enzyme in a redox state more oxidized than that reactive toward H_2 evolution and N_2 reduction (Li et al. 1982; Lowe et al. 1989). Experimentally, dinitrogen reduction does not interfere or compete with HCN reduction by nitrogenase to CH_4 and NH_3.

Whole cell studies on nitrogen fixing bacteria have confirmed the ability to degrade cyanide via nitrogenase. Jaeger & Dotterweich (1986) studied sediment from the Alster in Hamburg noting inhibition of nitrogenase activity (commonly measured as acetylene reduction activity) in the presence of high cyanide concentrations [100 mg CN/ kg sludge]. At low concentrations of cyanide rates of acetylene reduction corresponded to high rates of HCN reduction reaching a maximum of 5mg CN/kg sludge. Increasing the cyanide concentration to 150 mg/kg sludge resulted in an increasing inhibition in both acetylene and cyanide reduction. Although hydrogen cyanide reduction is observed, the cells appear to be sensitive to inhibition by larger concentrations of cyanide.

Stam et al. (1985) demonstrated the *Rhizobium ORS* 571 was capable of cyanide assimilation upon growth on succinate. The cyanide was added gradually to a nitrogen fixing culture up to a concentration of 7 mM. An increase in growth yield was observed relative to the nitrogen grown cells and was accompanied by a decrease in hydrogen production. The respiratory chain of such cells was still inhibited by low concentrations of cyanide.

Growth in the presence of 7 mM cyanide was explained by the removal of cyanide via nitrogenase. Presence of the protein was confirmed by SDS-PAGE of crude extracts from ammonia-assimilating, nitrogen-fixing, and cyanide assimilation cells. The major bands of nitrogen-fixing and cyanide-assimilating cells were polypeptide of 62, 60, and 35 kDa, similar to the molecular weights of the nitrogenase components. These bands were not observed in the crude extracts from ammonia-assimilating cells. Cyanide degradation by nitrogen fixing cultures appears to be a possible option once the cells have converted to the synthesis of cell nitrogen from cyanide. This process requires a more oxidized state of nitrogenase than is utilized for fixing nitrogen, and less ATP.

Abiotic processes

Abiotic processes are possible in the anaerobic conversion of cyanide as shown by Hope & Knowles (1991). In the course of isolating a strain of *Klebsiella* using cyanide (5 mM) as nitrogen source and glucose (70 mM) as the source of carbon and energy, the authors noticed that considerable growth lag times were accompanied by the transformation of cyanide to ammonia. However, control experiments determined that the degradation of cyanide (which took up to 50 hours) occurred in the absence of the microorganism. The reducing sugars galactose, mannose, fructose and ribose all possessed the ability to convert cyanide to ammonia. Phenol and non-reducing polyols sorbitol, mannitol, glycerol did not. In this instance, cyanide hydrolysis is being assisted non-microbially by the sugar. The reaction between sugars and cyanide is well documented as the Kiliani reaction and involves initial cyanohydrin formation between the carbonyl group of the reducing sugar and cyanide ion (Militzer 1949). Hydrolysis of the cyanohydrin is accelerated by an intramolecular hydroxyl group to give ammonia (Serianni et al. 1980; Varma & French 1972). The sugar now possesses a carboxylic acid group and has been extended by one carbon atom. After the depletion of cyanide to suitable levels, microbial growth can commence on the remaining sugar and newly formed ammonia. Given the limited understanding on routes for anaerobic cyanide degradation at the present time, these observations highlight the need for careful controls, especially in mixed culture systems, in order to dissect the mechanisms of microbial and abiotic cyanide detoxication.

Concluding remarks

A variety of biochemical pathways are available for the degradation of cyanide despite its toxicity to many life forms. Microorganisms containing cyanide hydrolases have shown the most promise to date for the industrial treatment of cyanide containing wastes. These enzymes are the key catalysts in ICI's Cyclear process and the CYANIDASE preparation by Novo Industries. Substitution pathways result in the formation of nitriles or thiocyanate which must be further degraded in a subsequent step, and as a result have not been extensively utilized. The oxidative and reductive pathways have only recently begun to be uncovered, and the complete picture of intermediates and the enzymes involved has not yet been elucidated. The effective treatment of cyanide wastes by the Homestake mine in South Dakota relies on microbial oxidation by *Pseudomonas paucimobilis*; the details of the degradation pathway are unknown. It is clear that a wide range of microbes can utilize cyanide as a nitrogen source. A recent paper by Meyers et al. reports the isolation of twelve such strains (Meyers et al. 1991). One strain *Bacillus pumilus CI* possesses an inducible cyanide detoxification system which works efficiently on cyanide concentrations of 100 mg/l This intracellular cyanide degrading activity is noteworthy in its unusual requirement for manganese.

Cyanide in waste streams from ore refining, coal coking, and electroplating industries commonly forms complexes with transition metals which are recalcitrant to degradation. The reports by Knowles & Silva-Avalos et al. that bacteria can utilize metal cyanide complexes as a nitrogen source are important contributions which should allow for the detoxication of these species. Finnegan et al. (1991)

have recently reported an *Acinetobacter* species which is capable of degrading a wide range of cyano-metal complexes, cyanide salts and organic nitrile compounds. This activity resides in an extracellular lipid complex estimated at 90 kDa with fatty acids of chain length C3 to C23.

The limits of the spectrum of pathways and enzymes for microbial cyanide detoxication have not yet been reached. With the exception of substitution/addition reactions, the understanding of how proteins catalyze cyanide degradation is quite limited at this time. Future work will continue to center on the isolation and characterization of microbes and pathways for cyanide wastewater treatment. The isolation and purification of the enzymes involved will hopefully allow for the elucidation of key protein features necessary for catalysis of cyanide and metal-cyanide degradation.

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