ROLE OF α-KETO ACIDS IN CYANIDE DETOXIFICATION AND ASSIMILATION BY Pseudomonas BACTERIA

THESIS

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By

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Cyanide was rapidly removed when added to culture supernatants of seven different *Pseudomonas*. The ability to remove cyanide was correlated with the accumulation of \( \alpha \)-keto acids (pyruvate and \( \alpha \)-ketoglutarate). These compounds react with cyanide forming less toxic cyanohydrins, thus conferring a mechanism for bacterial cyanide tolerance. When added to growth media the \( \alpha \)-keto acids were shown also to serve as effective cyanide antagonists. While all bacteria tested accumulated \( \alpha \)-keto acids, only those capable of utilizing cyanide as a nutritional nitrogen source were able to metabolize cyanohydrins. In *P. fluorescens* NCIMB 11764, the same enzyme (cyanide oxygenase) shown previously to be involved in cyanide metabolism appears responsible for cyanohydrin transformation. Keto acid excretion is believed to represent a new mechanism of bacterial cyanide detoxification with further enzymatic metabolism of the cyanohydrins helping to explain how cyanide can satisfy the nitrogen requirement in cyanide-utilizing bacteria.
ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Daniel Kunz for providing the opportunity to work in his lab, for his continued encouragement, guidance, and support. I also thank the committee members of my master's program, Dr. Gerard O'Donovan and Dr. Kent Chapman, for their guidance and review of this thesis. I also thank my coworker and good friend, Jui-Lin Chen for his continued help throughout this work.
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CHAPTER I

INTRODUCTION

1. Physico-chemical properties of cyanide and its toxicity to living things

Hydrogen cyanide (HCN) is a colorless, weak acid (pKa = 9.3). Its boiling point is 26°C, and thus it can easily volatilize from aqueous solution. Free cyanide (HCN or CN⁻) is highly reactive and can complex tightly with metals, especially transition metals such as iron, copper, nickel, zinc and gold (Sharp, 1976). Because of its high reactivity, cyanide can bind tightly to some essential components of living things and thus inhibit their normal function. Its inhibitory effect on respiration has been known since the 1920's when Warburg and coworkers first demonstrated that cyanide could bind tightly with trivalent iron in cytochrome oxidase (EC1.9.3.1) (Warburg et al., 1933). Although the toxic effect of cyanide has mainly been attributed to the inhibition of cytochrome oxidase, other enzymes and cell components are also very sensitive to cyanide. Cyanide is known to react with carbonyl groups, Schiff-base intermediates, and thiol groups of enzymes leading readily to their inhibition (Solomonson, 1981).

2. Origin of cyanide in the environment

Cyanide is one of the important precursors of the abiotic synthesis of essential biologic constituents such as amino acids,
purines, and pyrimidines (Wolman, 1972). It also represents a normal part of our current biosphere since it is produced by various bacteria, fungi and plants by a process called cyanogenesis (Knowles, 1976). The processing of cyanogenic crops such as cassava and bitter almonds can also produce considerable amounts of cyanide by the decomposition of cyanogenic glucosides. In modern society, cyanide is also involved in various industrial practices such as electroplating, steel production, synthetic fiber production, ore leaching and other petrochemical processing (Pettet and Ware, 1955; Knowles, 1976; Way, 1981). Large amounts of cyanide wastes are produced in the world each year.

Cyanide pollution can lead to great damage to the environment. Traditionally, cyanide is detoxified by chemical processes which are expensive and may produce new pollutants requiring additional treatment. For example, alkaline chlorination, a widely used process to detoxify cyanide, requires careful control of the chlorine concentration and may lead to uncontrolled formation of toxic and biologically recalcitrant organochlorine compounds, thus producing effluents requiring additional treatment. Many research efforts have been devoted towards development of chlorine-free and more economical techniques, with one area of research being microbial treatment of cyanide effluents (Knowles, 1988).

3. Mechanisms of biological cyanide detoxification

In spite of its high toxicity, various enzymatic mechanisms of cyanide detoxification are thought to exist. In mammals the enzyme rhodanese(thiosulphate sulphurtransferase;EC 2.8.1.1), which
catalyzes the conversion of cyanide to less toxic thiocyanate (Lang, 1933) is believed to play a major role in cyanide detoxification (Scheme 1).

\[
\text{S}_2\text{O}_3^{2-} + \text{CN}^- \xrightarrow{\text{rhodanese}} \text{SCN}^- \\
\text{Scheme 1}
\]

In higher plants cyanoalanine synthase (EC 4.4.1.9) is thought to play a major role in cyanide detoxification (Scheme 2) (Blumenthal et al., 1968; Miller and Conn, 1980).

\[
\text{HCN} + \text{cysteine} \xrightarrow{\beta\text{-cyanoalanine synthase}} \beta\text{-cyanoalanine} + \text{H}_2\text{S} \\
\text{Scheme 2}
\]

Seely et al (1966) proposed that cyanide could also be detoxified via formation of mandelonitrile (benzaldehyde cyanohydrin) as shown in Scheme 3.

\[
\text{Benzaldehyde} + \text{HCN} \rightarrow \text{Mandelonitrile} \\
\text{Scheme 3}
\]

In microorganisms, cyanide is transformed by various enzymatic mechanisms, but whether these are important in cyanide tolerance has not generally been demonstrated. Fry and Miller (1972) showed that various phytopathogenic fungi could convert
cyanide to formamide using an enzyme described as cyanide hydratase (formamide hydrolase) (Scheme 4).

\[
\text{HCN} + \text{H}_2\text{O} \xrightarrow{\text{cyanide hydratase}} \text{HCONH}_2
\]

Scheme 4

Nazly et al (1981,1983) showed that cyanide hydratase had considerable potential for detoxification of industrial cyanide-containing effluents, since immobilized fungi (Gloeocercospora sorghi and Fusarium moniliforme) completely degraded cyanide at concentrations as high as 6500 ppm. Knowles (1980) found that the snow mold fungus also metabolized cyanide, but in this case the product of conversion CO\(_2\), was formed by an unknown mechanism.

Several enzymes catalyzing cyanide transformation have been described. Brysk et al (1969) showed that like plants, Chromobacterium violaceum produces the enzyme \(\beta\)-cyanoalanine synthase. A second enzyme produced by this organism (\(\beta\)-cyanoalanine hydrolase) catalyzes the further conversion of \(\beta\)-cyanoalanine to asparagine (Scheme 5).

\[
\text{HCN} + \text{Alanine} \rightarrow \beta\text{-Cyanoalanine} \xrightarrow{\text{H}_2\text{O}} \text{Asparagine}
\]

Scheme 5

Novo Industries (Godtfredsen,1987) described an enzyme from Alcaligenes which converts cyanide into formic acid and ammonia (Scheme 6). This enzyme, referred to as cyanidase, appears to
be made also by several other bacteria in which it has been referred to as cyanide nitrilase or cyanide dihydratase (White et al., 1988; Meyers et al., 1993).

In 1983 Knowles and coworkers (Harris and Knowles, 1983a) in Great Britain reported that *Pseudomonas* bacteria could readily be isolated from soil that used cyanide as a sole source of nitrogen. One of these isolates, *P. fluorescens* NCIMB 11764, was chosen for further investigation and shown to convert cyanide to CO$_2$ and ammonia by a unique enzymatic process that appeared to involve an oxygenase-type enzyme (Harris and Knowles, 1983b). However, direct evidence linking this enzyme to cyanide tolerance in *P. fluorescens* NCIMB 11764 and its further utilization as a nitrogen source was not provided. For these reasons, and the fact that an oxygenase-type enzyme had never before been shown to be involved in cyanide transformation, much emphasis was placed on further studying the biological role of this enzyme in cyanide assimilation in this organism.

4. Enzymatic basis of cyanide assimilation in *P. fluorescens* NCIMB 11764

Further studies of cyanide metabolism by *P. fluorescens* NCIMB 11764 showed that in addition to the production of CO$_2$ and ammonia as metabolic products, formate and formamide were also

\[
\text{HCN} + 2\text{H}_2\text{O} \xrightarrow{\text{cyanide nitrilase}} \text{HCOOH} + \text{NH}_3
\]

Scheme 6
produced (Kunz, et al., 1992). These findings suggested that in addition to the involvement of a possible oxygenase, as first proposed by Harris and Knowles (1983b), a separate cyanide nitrilase (cyanidase) and hydratase might also be elaborated. The various proposed enzymatic conversions demonstrated in NCIMB 11764 are illustrated in Scheme 7. An important question to be answered was, which of these enzymes was required for cyanide assimilation or were they all involved? Studies by J. Chen in our laboratory showed that mutants defective in the oxygenative route lost the ability to grow on cyanide and it was therefore concluded that this pathway was important for cyanide assimilation (Kunz et al., 1994; Chen, 1998).

Having established that an oxygenase was involved in cyanide metabolism, further studies focused on this enzyme. Early studies, which showed that both oxygen and NADH were required for conversion, provided the basis for suggesting that an oxygenase-type enzyme was involved (Harris and Knowles, 1983b). However, whether this enzyme functioned as a mono- or dioxygenase was not determined. As shown in Scheme 8, if a monoxygenase was involved, then an intermediate
(cyanic acid) might be formed before CO$_2$ and NH$_3$ are generated. Alternatively, direct conversion to these products by a dioxygenase was also considered (Harris and Knowles, 1983b). Although studies by Nagappan et al (Kunz and Nagappan, 1989; Nagappan, 1992) showed that NCIMB 11764 could grow on cyanate, mutants defective in its utilization could still grow on cyanide suggesting that the metabolism of the two was unrelated. Work demonstrating that the enzyme cyanase, responsible for cyanate breakdown, was uninduced in cyanide-grown cells further supported this idea (Kunz et al., 1994). To further investigate the oxygenative mechanism, studies by Wang (1995) were undertaken to follow the fate of isotopic oxygen-18 incorporated during conversion. These studies showed that one atom each from molecular oxygen and water were incorporated during conversion (Wang et al., 1996). A reaction pathway as shown in Scheme 9 was therefore proposed in which a monoxygenase catalyzes the incorporation of one atom of oxygen into cyanide forming an oxygenated intermediate of unknown identity [X-O]. A second reaction step involving hydrolysis of the oxygenated intermediate by either a chemical or enzymatic process was further proposed.
Studies in our laboratory aimed at isolating cyanide oxygenase (CNO) showed that cyanide consumption did not parallel the induction of CNO activity in cell extracts (Chen and Kunz, 1997). Further studies showed that this was because a separate non-enzymatic reaction between cyanide and an excreted metabolite was responsible for its removal (Chen and Kunz, 1997; Chen, 1998). These findings raised some interesting questions regarding the mechanism of both cyanide detoxification and assimilation. That is, it was hypothesized that perhaps cyanide was first detoxified outside of cells in a chemical reaction with biological metabolite before being further metabolized within. Given the toxic nature of cyanide this seemed to be a reasonable mechanism. Results which showed that the product of the reaction between cyanide and the unknown metabolite could be further enzymatically oxidized lent support to this hypothesis. Additional studies revealed the identity of the extracellular metabolite as an α-keto acid (both pyruvate and α-ketoglutarate were shown to be involved). The products formed when these compounds react with cyanide were identified as cyanohydrins, and were further shown to be oxidized by cell-extracts to give CO₂ and NH₃ as reaction products. These findings led to the following reaction sequence (Scheme 10) to help explain
how cyanide, an otherwise potent poison, can be utilized as a source of nitrogen for growth.

\[
\text{KCN} + \alpha\text{-Keto acid} \rightarrow \text{Cyanohydrin} \xrightarrow{\text{cyanide oxygenase}} \text{CO}_2 + \text{NH}_3
\]

**Scheme 10**

Although it was hypothesized that the reaction between cyanide and keto acids was responsible for its detoxification, we sought to obtain direct evidence for this. The fundamental objective of this thesis was to investigate this phenomenon in NCIMB 11764 and other *Pseudomonas* bacteria. These studies revealed that biologically excreted keto acids can confer on bacteria cyanide tolerance regardless of whether they are able to grow on cyanide or not. The latter ability was further shown to be confined to those organisms able to produce the necessary enzymes (e.g., cyanide oxygenase) responsible for further cyanohydrin degradation.
CHAPTER II

MATERIALS AND METHODS

Bacterial strains. *P. fluorescens* NCIMB 11764 was obtained from the National Collection of Marine and Industrial Bacteria, Torrey, Scotland. It was isolated from soil by R. E. Harris and C. J. Knowles in the United Kingdom (Harris and Knowles, 1983a). JL102 is a mutant strain of NCIMB 11764 defective in utilizing cyanide as a nitrogen source for growth. It was isolated by nitrosoguanidine mutagenesis and penicillin-cycloserine enrichment procedures (Kunz et al., 1994; Chen, 1998). All other strains are from the stock culture in this lab (see Table 1). For long-term preservation of the bacterial strains, cells were maintained in 10% dimethyl sulfoxide and stored at -70°C.

Growth media and cultivation conditions. For routine use, cells were subcultured every 4-6 weeks on LA agar using ingredients specified in Table 2. The composition of minimal medium is given in Table 3. Unless otherwise indicated glucose (20 mM) was supplied as the carbon source and NH$_4$Cl (1 mM) as the nitrogen source (referred to as minimal GA medium). The inoculum for growth was taken from an LA plate and subcultured for 48 hours in GA medium before transferring cells (10% v/v) to a second flask containing the same medium. All cultures were incubated at 30°C on a gyratory shaker and growth monitored by absorbance measurements at 540 nm.
Table 1. Bacterial strains used in this research

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>Reference or Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. fluorescens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCIMB 11764</td>
<td>Cn⁺</td>
<td>Harris &amp; Knowles, 1983a</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JL102</td>
<td>Cn⁻</td>
<td>Kunz et al., 1994</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 13525</td>
<td>Cn⁻</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>P. putida BCN3</td>
<td>Cn⁺</td>
<td>Silva-Avalos et al., 1990</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Cn⁻</td>
<td>This lab</td>
</tr>
<tr>
<td>P. putida</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 12633</td>
<td>Cn⁻</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>P. putida BCN32</td>
<td>Cn⁺</td>
<td>Silva-Avalos et al., 1990</td>
</tr>
</tbody>
</table>

Note:

Cn⁺—— can utilize cyanide for growth
Cn⁻—— cannot utilize cyanide for growth
Table 2. Composition of LA medium (Lennox, 1955)

<table>
<thead>
<tr>
<th>Lennox Broth Medium</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>Bacto Tryptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

pH 6.8, sterilized at 121 °C for 20 min.

For Lennox agar, 20 g of Bacto-Agar was added into the broth medium.
Table 3. Composition of Glucose-Ammonia (GA) Minimal Medium

<table>
<thead>
<tr>
<th>Buffer phosphate (P10X)</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>91.0 g</td>
</tr>
<tr>
<td>NaOH</td>
<td>16.8 g</td>
</tr>
<tr>
<td>Distilled water to complete</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH 7.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>R-Salts (R-200X)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10% MgSO$_4$·7H$_2$O</td>
<td>400 ml</td>
</tr>
<tr>
<td>1% FeSO$_4$·7H$_2$O</td>
<td>100 ml</td>
</tr>
<tr>
<td>concentrated HCl</td>
<td>2 ml</td>
</tr>
<tr>
<td>Mix and sterilize at 121 °C for 20 min.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Minimal GA Medium composition*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer phosphate</td>
<td>1X</td>
</tr>
<tr>
<td>R-Salts</td>
<td>1X</td>
</tr>
<tr>
<td>Glucose (sterile)</td>
<td>20mM</td>
</tr>
<tr>
<td>Nitrogen source (sterile)</td>
<td>1mM</td>
</tr>
</tbody>
</table>

R-Salts, Glucose and Nitrogen source were added aseptically to sterilized P1X. For solid minimal medium, 2% Bacto-Agar (Difco) was added.

*Adopted from Kunz et al., (1981)
**Induction of cyanide oxygenase (CNO)** To induce cyanide oxygenase, a 200 ml starter culture cultivated as described above was transferred to 1.8 liter of GA medium. This culture was incubated for 24 hours before adding 100μM sterilized KCN to induce enzyme activity. After 3 hours, the cells were harvested by centrifugation, washed twice with Na$_2$HPO$_4$-KH$_2$PO$_4$ (Na-K) buffer (pH 7.0), and stored at -70°C before cell extracts were prepared as described below.

**Cyanide removal from culture supernatants** The rate of cyanide removal from culture supernatants was determined by the addition of 2 mM KCN to 0.25 ml of culture supernatant contained in 2 ml screw-cap HPLC vials. At various intervals after KCN addition samples were removed with a syringe and the remaining cyanide concentration determined.

**Preparation of cell extracts** Washed cells were resuspended in Na-K buffer at a ratio of 2 ml of buffer per gram of cells. The cells were broken in a French Press at 20,000 p.s.i.(138 MPa). A small amount of deoxyribonuclease (20 mg ml$^{-1}$) was added to the resultant viscous liquid to digest DNA molecules and reduce the viscosity before centrifuging the preparation at 30,000 x g for 30 minutes. The resultant supernatant liquid, designated crude extract, was then subjected to ultracentrifugation at 150,000 x g for 90 minutes to generate high speed supernatant (HSS, cytosolic fraction) fraction.
Cyanide antagonism by α-keto acids  Protection of bacteria against cyanide toxicity by α-keto acids (or culture supernatant containing these compounds) was determined by adding α-keto acids to GA medium supplied with 9 mM of (NH₄)₂SO₄ as the nitrogen source and KCN as a growth inhibitor. KCN was injected to 10 ml of minimal medium contained in 40 ml capacity crimp-seal bottles. The bottles were allowed to incubate for 30 minutes before adding cells (1% inoculum) and growth determined by measuring the absorbance at 540 nm after 24 h incubation. The inoculum for growth was cultivated in GA medium for 48 h before cells were harvested, washed twice in sterile Na-K phosphate buffer (pH 7.0), and suspended in the original volume of sterile minimal medium. When culture supernatants containing keto acids were supplied as antagonistic agents these were first lyophilized in a Speed-Vac and filter sterilized before being added to the medium.

Analytical methods

Cyanide. Cyanide was quantitated colorimetrically by the method of Lambert et al. (Lambert J. L. et al.,1975). In a test tube containing 1.14 ml of distilled water, 50μl of N-chlorosuccinimide-succinimide oxidizing reagent was added and vortexed. After that, 10 μl of sample was added and mixed completely. After mixing, 50 μl of barbituric acid-pyridine reagent was added to the mixture, and the absorbance at 580nm was read in spectrophotometer after 15 min at room temperature. Cyanide concentration was determined by extrapolating the OD reading to a standard curve as shown in Figure 1. The N-chlorosuccinimide-succinimide oxidizing reagent was prepared by dissolving 1g of succinimide and 0.1 g of
Figure 1. Standard curve for the determination of cyanide. 10 µl of sample in each concentration was measured. The linear relationship of absorbance at 580 nm with the concentration of cyanide was plotted.
$y = 0.916x + 0.013$
N-chlorosuccinimide in a final volume of 100 ml water, which is stable for at least 6 months. To prepare the barbituric acid-pyridine reagent, 0.6 gm of the barbituric acid was dissolved in 5 ml of water and 3 ml of pyridine and the final volume was adjusted to be 10 ml. This reagent should be kept at refrigerator in the dark.

α-Keto acids. Pyruvate and α-ketoglutarate in bacterial culture supernatants were determined by HPLC. Culture supernatants containing α-keto acids were adjusted to pH2.0 with concentrated sulphic acid and then filtered by 0.22μm filter(Millepore). HPLC was then performed in a Rainin liquid chromatograph equipped with a BioRad® HPX-87H strong cation-exchange column (300mmX7.8mm) and UV detector. The mobile phase consisted of 0.015 N H2SO4 and 0.34 mM EDTA. The column was eluted isocratically with the mobile phase at a flow rate of 0.6ml/min at the ambient temperature. The concentration of α-keto acids were quantified by comparison of the peak areas to the eluted peaks to those of the standard peaks of authentic α-keto acids.

**Enzymatic conversion of cyanohydrins by cell-extracts**

Cell-free biotransformation of cyanohydrin substrates by cyanide-induced enzymes in *Pseudomonas* bacteria was determined by measuring the formation of 14CO2 from 14CN-labelled pyruvate cyanohydrin. The radiolabelled cyanohydrins were prepared by incubating pyruvate with K14CN as described below under Chemicals. 14CO2 was trapped as Ba14CO3 as described further below. Reactions were conducted in a vial assembly composed of a 2 ml HPLC vial placed inside a 15 ml sealed crimp-sealed bottle. All reaction components were placed into the inner HPLC vial that contained the
following components in a total volume of 0.25 ml: pyruvate cyanohydrin 2 mM, $^{14}$C-labelled pyruvate-cyanohydrin 1μCi, NADH 4 mM, Na-K buffer (50mM, pH 7.0). Reactions were initiated by the injection of labelled and unlabelled cyanohydrin and the vial assembly allowed to incubate for 40 minutes at 30 °C on a rotary shaker (250 rpm). After this 0.4 ml of 4N NaOH was injected into the outer crimp-seal vial and and the whole reaction unit allowed to incubate for an additional 30 minutes to trap volatile $^{14}$CO2. The vial assembly was then disassembled and the contents of both the inner and outer vials transferred to a microfuge tube and treated with 0.1 ml of 0.1N NaOH followed by 35μl of 40% BaCl₂. After 5 minutes the tubes were microfuged and the respective pellets (containing Ba$^{14}$CO₃) washed with 0.4 ml of 0.12 NaOH before being resuspended in 0.5 ml of 0.12N NaOH and added to 8 ml of scintillation cocktail (Scinti Verse I, Fisher Scientific). Radioactivity was measured with an LS7000 Scintillation Counter (Beckman) and the percent conversion of $^{14}$CN-labelled cyanohydrin to $^{14}$CO2 determined.

**Chemicals** $^{14}$C-labelled cyanohydrin was prepared by mixing two volumes of 100 mM pyruvate prepared in Na-K phosphate buffer (pH7.0), with one volume of K$^{14}$CN solution (1mCi/ml). The reaction was carried out in a sealed vial at 30 °C until no free cyanide could be detected. The unlabelled cyanohydrins were made by mixing pyruvate or α-ketoglutarate with KCN(80:20 mM) and incubating at 30 °C until no free cyanide could be detected and the cyanohydrin concentration was estimated to be 20 mM.
KCN(97%) was purchased from Aldrich (Milwaukee, WI). K$^{14}$CN (54 mCi mmol$^{-1}$, 2.0 GBq mmol$^{-1}$) was purchased from DuPont (NEN Research Products, Boston, MA). All other chemicals were obtained from commercial sources and were of the highest purity available.
CHAPTER III

RESULTS

1. Biologically excreted \( \alpha \)-keto acids serve as cyanide scavenging agents

   Earlier studies by Chen et al (Chen and Kunz, 1997; Chen, 1998) showed that cyanide was rapidly removed when added to cells of NCIMB 11764 grown to late stationary phase in minimal GA medium. Further studies showed that the responsible cyanide removing factor was located in the culture supernatant and was identified as an \( \alpha \)-keto acid, namely pyruvate and \( \alpha \)-ketoglutarate. Both compounds were shown to be excreted into the medium under conditions of carbon excess and nitrogen limitation which occurs in stationary growth on GA medium. These findings stimulated us to examine other Pseudomonas strains for this unique ability and whether it was also correlated with the excretion of keto acids. For these experiments, cells were cultivated in GA medium for 24 h at which time 2 mM KCN was added to the medium and the remaining cyanide concentration determined at intervals thereafter. Figure 2 shows that cyanide was removed from the cultures of all bacteria although rates of removal were markedly better in some strains compared with others. The most rapid rate of cyanide removal was observed for the following organisms: NCIMB 11764, a mutant thereof defective in cyanide oxygenase (JL102), P. fluorescens ATCC 13525 and P. aeruginosa PA01. Interestingly, the remaining three strains,
Figure 2. Cyanide disappearance from cultures of *Pseudomonas* bacteria grown in GA minimal medium. The graph shows the time-course of cyanide disappearance after 2 mM KCN was added to separate 24 h-old cultures of the bacteria indicated. Samples were removed from the cultures at the times indicated and the remaining cyanide concentration determined after cells were removed in a microfuge.
2.5
2.0
1.5
1.0
0.5
0.0
0 5 10 15 20 25 30 35
Time (min)

Cyanide (mM)

Control (Na-K buffer, pH 7.0)  P. putida BCN3
P. putida ATCC 12633  P. putida BCN32
P. fluorescens ATCC 13525  P. fluorescens JL102
P. fluorescens NCIMB 11764  P. aeruginosa PAO1

P. putida
P. fluorescens
P. aeruginosa
which all showed slower rates of removal, were strains of *P. putida* (ATCC 12633, strains BCN3, BCN32). The significance of the slower rates of removal by these strains compared with others including *P. fluorescens* and *P. aeruginosa* is unknown at this time.

In order to determine whether the ability to remove cyanide was correlated with keto acids excreted into the medium, culture filtrates were analyzed by ion-exclusion HPLC. Figure 3A shows the HPLC profile for the culture supernatant from PA01. By comparing the elution times with authentic compounds, peaks at 9.50 and 10.35 were identified as α-ketoglutarate and pyruvate, respectively (the peak at 8.82 is an additional compound of unknown identity). Figure 3B shows the results obtained when KCN was added to the culture supernatant. Peaks associated with pyruvate and α-ketoglutarate disappeared with two new peaks at 7.38 and 8.11 appearing. These results are consistent with a reaction between cyanide and the two keto acids forming the respective pyruvate and α-ketoglutarate cyanohydrins, as previously demonstrated also in related studies in our laboratory using the authentic keto acids as cyanide scavenging agents (Kunz et al., 1994).

In order to determine whether the relative rates of cyanide removal as shown in Figure 2 were in any way related to the amount of keto acids excreted into the medium their concentrations were quantitated by HPLC. Table 4 shows that the highest accumulation of keto acids (7.8 mM) occurred with strain PA01. The amount of keto acid accumulated by *P. fluorescens* NCIMB 11764, strain JL102 thereof, and *P. fluorescens* ATCC 13525 were slightly lower than that of PA01, but almost ten-fold higher than the accumulated
Figure 3. Ion-exclusion HPLC analysis of culture supernatants from *P. aeruginosa* PAO1 before (A) and after (B) the addition of KCN. 10 microliters was injected in each case. In (A) 20 μl of DD H$_2$O was added to 80 μl of culture supernatant. In (B) 20 μl of 100 mM KCN were added to 80 μl of culture supernatant and incubated at 30 °C for 10 minutes before injection.
Table 4. Relationship between accumulated α-keto acids and rates of cyanide removal in culture supernatants of *Pseudomonas* bacteria grown in minimal GA medium

<table>
<thead>
<tr>
<th>Bacterial strains*</th>
<th>Total keto acids (mM)</th>
<th>KCN removed in 30 minutes (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em> NCIMB 11764</td>
<td>6.0</td>
<td>1.8</td>
</tr>
<tr>
<td><em>P. fluorescens</em> JL 102</td>
<td>4.7</td>
<td>1.8</td>
</tr>
<tr>
<td><em>P. fluorescens</em> ATCC 13525</td>
<td>5.6</td>
<td>1.8</td>
</tr>
<tr>
<td><em>P. putida</em> BCN 3</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td><em>P. putida</em> BCN 32</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td><em>P. putida</em> ATCC 12633</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAO1</td>
<td>7.8</td>
<td>1.9</td>
</tr>
</tbody>
</table>

*Cultivated for 24h in minimal GA medium*
cencentrations for P. putida strains. It can be seen that these results closely parallel the relative rates of cyanide removal from cultures.

2. \(\alpha\)-Keto acids serve to antagonize cyanide inhibition

The removal of cyanide from culture supernatants of \textit{Pseudomonas} bacteria suggested that this process might serve to detoxify cyanide. To test this hypothesis culture supernatant from cells of NCIMB 11764 grown in minimal GA medium was collected and added to cells cultivated in the same medium, but supplied excess nitrogen (9 mM (NH\(_4\))\(_2\)SO\(_4\)) and KCN as a growth inhibitor. Figure 4 shows that the addition of culture supernatant protected cells from KCN-induced growth inhibition in a concentration-dependent manner. The results show no growth at 2 mM KCN in the absence of culture supernatant, but with increasing amounts of culture supernatant provided the cell density increased.

Having demonstrated that culture supernatants known to contain the biologically-formed keto acids could protect cells against cyanide, related experiments were with authentic compounds were performed. Figure 5 shows the results obtained when growth of NCIMB 11764 in the presence of 2 mM KCN was determined at varying concentrations of either pyruvate or \(\alpha\)-ketoglutarate. At less than 6 mM keto acid no significant growth was observed. This is presumably, because not all of the available cyanide was titrated out by keto acid. Good growth at 6 mM keto acid or above further confirmed that at these concentrations no cyanide was apparently available to cause growth inhibition. These data also suggested that the reaction stoichiometry required
Figure 4. Protection of cells of *P. fluorescens* NCIMB 11764 against cyanide inhibition by culture supernatant obtained from prior growth of cells in minimal GA medium. Different amounts (0X, 0.25X, 0.5X, 1X, 1.5X, 2X) of concentrated culture supernatant were provided to growth medium supplied 2mM KCN and culture incubated at 30°C on a gyratory shaker (220 rpm). Bottles were photographed after 24 hours of incubation. Culture supernatant was concentrated by lyophilization in a Speed-Vac and the concentrated residue redissolved in various amount of water before filter sterilization and addition to separate cultures.
for cyanide neutralization was about 3:1 (keto acid:CN⁻), respectively.

To further demonstrate that keto acids could serve as cyanide antagonistic agents their effect on the minimal inhibitory concentration (MIC) was determined. Figure 6 shows that in the absence of added pyruvate the MIC was about 0.3 mM. This value is consistent with that shown earlier by Silva-Avalos et al. (1990), also. However, in the presence of 1 mM pyruvate or α-ketoglutarate (or an equivalent amount of keto acid in culture supernatant) the MIC increased to 0.6 mM, thus indicating that significant protection by the keto acids was conferred.

3. Cyanohydrins serve as nitrogenous substrates for growth in cyanide-utilizing bacteria

Concurrent studies demonstrated that cell-extracts from *P. fluorescens* NCIMB 11764 induced for cyanide oxygenase appeared to convert the cyanohydrins made chemically when cyanide is incubated with α-keto acids to CO₂ and NH₃ (Chen and Kunz, 1997; Kunz et al., 1998). Since NH₃ is readily assimilated by NCIMB 11764 these findings suggested that cells might grow on the cyanohydrins as sole nitrogen sources provided they could be transported into cells. To test this hypothesis, the putative cyanohydrins formed when cyanide was incubated with biologically-formed keto acids in culture supernatants were provided as sole nitrogen sources to *P. fluorescens* NCIMB 11764 and growth determined. The growth results shown in Figure 7 demonstrated that indeed these compounds (represented by X-CN for pyruvate and α-ketoglutarate cyanohydrin)
Figure 5. Effect of increasing keto acids on cyanide growth inhibition. Washed cells of *P. fluorescens* NCIMB 11764 (1% inoculum) were grown in GA minimal medium containing 2 mM KCN and various keto acid concentrations as shown. Growth was monitored after 48 h incubation at 30°C by absorbance measurements at 540 nm.
Figure 6. Effect of added pyruvate and α-ketoglutarate on growth inhibition by KCN on *Pseudomonas fluorescens* NCIMB 11764. Bacteria were grown with shaking at 30°C in sealed bottles containing GA medium but provided excess nitrogen as (NH₄)₂SO₄ (9 mM) with KCN added as a growth inhibitor. Absorbance values represent the mean from at least two separate experiments after 24 h incubation.
Absorbance (540nm)

KCN (mM)

- Control
- Pyr
- αKG
- Culture fluid
supported growth. It can be seen that the growth yields were related to the amount of putative cyanohydrin supplied although an estimated concentration of 1 mM of the cyanohydrins in culture supernatant gave slightly lower growth than that obtained with 1 mM NH₄Cl. The finding that cyanohydrins could support the growth of NCIMB 11764 stimulated us to test whether these compounds could support the growth of other Pseudomonas bacteria also. For these experiments, authentic pyruvate cyanohydrin prepared as described in the Materials and Methods was supplied as the nitrogen source. Table 5 shows that of six separate strains tested only three organisms were able to grow. Interestingly, these three strains are also able to grow on cyanide, thus indicating that the ability to grow on the pyruvate cyanohydrins and cyanide are linked. Additional evidence indicating that the metabolism of cyanide and cyanohydrins is related was provided by the growth results observed when the mutant strain JL102, known to be defective in the production of cyanide oxygenase (Kunz et al., 1994) was tested for growth on pyruvate cyanohydrin. As shown in Figure 8, this strain was unable to grow thus suggesting strongly that cyanohydrin breakdown is cyanide oxygenase catalyzed.
Figure 7. Utilization of putative cyanohydrin (X-CN) reaction products formed between cyanide and biologically keto acids present in culture supernatants of P. fluorescens NCIMB 11764. Culture supernatants were concentrated fifty-fold by lyophilization and reacted with 50 mM KCN for 30 minutes at 30°C until no free cyanide could be detected. The reaction mixture was filter sterilized and appropriate amounts of the putative cyanohydrins added to give the concentrations shown before using. C. S., refers to unreacted culture supernatant control.
NH₄Cl (1mM)
X-CN (1mM)
X-CN (0.5mM)
X-CN (0.25mM)
X (C.S)
N-free
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>$A_{540\text{nm}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em> NCIMB 11764</td>
<td>0.41</td>
</tr>
<tr>
<td><em>P. fluorescens</em> ATCC 13525</td>
<td>0.01</td>
</tr>
<tr>
<td><em>P. putida</em> BCN3</td>
<td>0.41</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAO1</td>
<td>0.04</td>
</tr>
<tr>
<td><em>P. putida</em> ATCC12633</td>
<td>0.04</td>
</tr>
<tr>
<td><em>P. putida</em> BCN32</td>
<td>0.38</td>
</tr>
</tbody>
</table>

*All strains were grown in minimal GA medium but substituting 1 mM pyruvate cyanohydrin for NH$_4$Cl and the $A_{540}$ determined after 24 hours.*
Figure 8. Growth response of wild strain and mutant strain JL102(CNO-defective) of *P. fluorescens* NCIMB 11764 towards pyruvate cyanohydrin and α-ketoglutarate cyanohydrin as sole nitrogen sources. The inocula (1%) were pregrown in GA minimal medium for 24 hours and collected by centrifugation and washed for twice. The cyanohydrin was supplied at 1 mM. The growth was monitored by reading the absorbance value at 540 nm at different time intervals.
Absorbance (540nm)

Time (hr)

- Pyr-CN (wild-type)
- KG-CN (wild-type)
- Pyr-CN (JL102)
- KG-CN (JL102)
4. Bioconversion of cyanohydrins by cyanide-induced cell extracts

This study demonstrated that bacteria are able to acquire cyanide tolerance by excreting α-keto acids into the extracellular medium which react with cyanide giving the less toxic cyanohydrins as reaction products. Growth on the cyanohydrins by bacteria able to utilize cyanide, but not by strains unable to utilize cyanide, suggested that (1) cyanide utilization proceeds via the cyanohydrin as an intermediate, and(2) further metabolism of the cyanohydrin is dependent on the induction of specific enzymes for its breakdown. Studies with NCIMB 11764 indicated that cyanide oxygenase is responsible for catalyzing cyanohydrin oxidation to ammonia (and CO2)(Chen, 1998; Kunz et al., 1998). Since ammonia is readily assimilated, this helped to explain how growth on cyanide could be accomplished. In order to investigate the enzymatic basis of cyanohydrin conversion in other bacteria, all strains were grown under similar conditions as those used to induce cyanide oxygenase in NCIMB 11764. Cell-extracts were prepared and tested for the ability to convert radiolabelled pyruvate cyanohydrin into $^{14}\text{CO}_2$ under reaction conditions similar to those used provided for cyanide oxygenase (aerobic and NADH supplied). The results in Table 6 show that $^{14}\text{CN}$-pyruvate cyanohydrin was converted in 43-69% yield to $^{14}\text{CO}_2$ by extracts from the cyanide-utilizing strains, but negligible conversion was observed for the non-cyanide utilizing strains. These data are similar to the growth results shown in Table 6 demonstrating that only the cyanide-utilizing bacteria were able to grow when provided the cyanohydins as growth substrates. It was therefore concluded that only the cyanide-utilizing strains are
capable of enzymatically converting the cyanohydrins, and since $^{14}$CO$_2$ was formed under identical reaction conditions known to favor the activity of cyanide oxygenase, it may be inferred that related enzyme systems for oxidizing the cyanohydrins in the cyanide-utilizing P. putida BCN3 and BCN32 are present.
Table 6. Bioconversion of $^{14}$CN-labelled pyruvate cyanohydrin
by cyanide-induced cell extracts

<table>
<thead>
<tr>
<th>Strain</th>
<th>% conversion of $^{14}$CN-pyruvate to $^{14}$CO$_2$ by cell-extracts$^a$</th>
<th>Ability to utilize KCN for growth$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em> NCIMB 11764</td>
<td>65</td>
<td>+</td>
</tr>
<tr>
<td><em>P. fluorescens</em> JL 102</td>
<td>3.3</td>
<td>-</td>
</tr>
<tr>
<td><em>P. fluorescens</em> ATCC 13525</td>
<td>2.9</td>
<td>-</td>
</tr>
<tr>
<td><em>P. putida</em> BCN3</td>
<td>69</td>
<td>+</td>
</tr>
<tr>
<td><em>P. putida</em> BCN32</td>
<td>43</td>
<td>+</td>
</tr>
<tr>
<td><em>P. putida</em> ATCC 12633</td>
<td>3.8</td>
<td>-</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAO1</td>
<td>4.2</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ $^{14}$CO$_2$ trapped with BaCl$_2$ as described in the Materials and Methods

$^b$ Determined in this and previous studies (Silva Avalos et al., 1990; Kunz et al., 1994)
A number of microorganisms capable of detoxifying cyanide have been described (Brysk et al., 1969; Knowles, 1976; Bunch and Knowles, 1980; Harris et al., 1983; Silva-Avalos et al., 1990; Finneagan et al., 1991). All of these reports involve various enzymatic mechanisms of cyanide transformation, which are presumed to have detoxification roles. Our studies on cyanide detoxification and assimilation in \textit{P. fluorescens} NCIMB 11764 describe a new mechanism of cyanide detoxification which involves the extracellular scavenging and neutralization of cyanide by excreted \(\alpha\)-keto acids (Chen, 1998; Kunz et al., 1998). Both pyruvate and \(\alpha\)-ketoglutarate were detected in the culture supernatant of \textit{P. fluorescens} NCIMB 11764 cells when cultivated on growth limiting amounts of ammonia or cyanide, and were found to react rapidly with cyanide to form the cyanohydrins as reaction products. These compounds are far less toxic than cyanide and appear to serve as substrates for further nitrogen acquisition by the enzyme cyanide oxygenase known to be induced by cyanide in this organism.

In addition to the cyanide-utilizer \textit{P. fluorescens} NCIMB 11764, we discovered that other \textit{Pseudomonas} bacteria regardless of whether they are able to grow on cyanide excreted keto acids when cultivated under nitrogen-limiting conditions (Fig.2, Table 4). These observations suggested that keto acid excretion could confer on cells cyanide tolerance and this hypothesis was confirmed by
detailed studies conducted with NCIMB 11764. These studies showed that when culture supernatants containing the biologically-formed keto acids, or when authentic pyruvate and \( \alpha \)-ketoglutarate were added exogenously to cells exposed to inhibiting concentrations of KCN (2 mM) that the toxic effect could be overcome (Fig 4, Fig 5). This was also reflected in the higher MIC determined for cyanide when cultures were supplemented with the keto acids. The presence of keto acids in the medium therefore, appears to have an antagonistic effect on cyanide toxicity. This previously has not been demonstrated in bacteria, but does find analogy with previous reports documenting similar antagonistic effects on cyanide toxicity in mice and ascite tumor cells (Cittadini et al., 1971, 1972).

Although all *Pseudomonas* bacteria tested are assumed to achieve some level of cyanide tolerance because they can excrete keto acids, only those bacteria shown previously to grow on cyanide were found to be capable of further metabolizing the cyanohydrins formed from cyanide detoxification. This indicates that the ability to grow on cyanide must be dependent on the ability to produce enzymes for cyanohydrin oxidation such as cyanide oxygenase in the case of NCIMB 11764. Evidence for this is provided by results demonstrating that a mutant strain of this organism defective in the ability to grow on cyanide was also unable to grow when provided the cyanohydrin and was unable to oxidize the radiolabelled substrate to \(^{14}\text{CO}_2\). Interestingly, other cyanide-utilizing *Pseudomonas* strains also converted the \(^{14}\text{CN}\)-labelled pyruvate cyanohydrin to \(^{14}\text{CO}_2\) suggesting that related cyanide oxygenase may possibly be also produced by these organisms.
Thus cyanide utilization is believed to involve at least two steps, as shown in Fig. 9, one being its initial detoxification in a rapid scavenging reaction with α-keto acids that occurs extracellularly, and the other involving the uptake and enzymatic conversion of the cyanohydrin. This mechanism presumes that rather than free cyanide, the cyanohydrin is in fact the true substrate for cyanide oxygenase, an interpretation seems reasonable given the highly inhibitory nature of free cyanide on metalloenzymes, which cyanide oxygenase is likely also to be.

The protective effect of α-keto acids on cyanide could represent a common phenomenon among bacterial populations since α-keto acids are common metabolites formed by bacteria. Perhaps this mechanism of cyanide detoxification is important in nature where cyanide represents a normal metabolite generated by cyanogenic plants, fungi and bacteria. This would confer on cells an important resistance mechanism. The further ability to utilize the cyanohydrin as substrates for enzymatic attack could then confer an additional selective advantage by providing a means for surviving on cyanide as a nutritional nitrogen source. The latter ability is presumably dependent on the genetic capacity to elaborate cyanohydrins degradative enzymes such as cyanide oxygenase. Because of the importance of this enzyme, continued studies in our laboratory are directed at its purification and characterization. These investigations are sure to shed additional light on the unique ways microorganisms have adapted for surviving in the presence of one of the most potent poisons formed as a natural product in the environment.
Figure 9. Proposed pathway for cyanide detoxification and assimilation by *Pseudomonas* bacteria. $R = \text{CH}_3$ or $\text{CH}_2\text{CH}_2\text{COOH}$ for pyruvate cyanohydrin or $\alpha$-ketoglutarate cyanohydrin, respectively.
Extracellular Cyanide Detoxification

\[ \text{HCN} + \text{R-C-COOH} \rightarrow \text{CO}_2 + \text{NH}_3 + \text{R-C-COOH} \]

Intracellular Enzymatic Conversion
REFERENCES


