MECHANISMS OF CYANIDE ASSIMILATION IN
Pseudomonas fluorescens NCIMB 11764

DISSERTATION

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

by

Olagappan Nagappan, B.E., M.S.
Denton, Texas
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Pseudomonas fluorescens NCIMB 11764 was capable of utilizing cyanide as a sole nitrogen source for growth. Cyanate (OCN⁻) and β-cyanoalanine could also serve as nitrogenous substrates, but do not appear to play a role as intermediates in cyanide metabolism. Growth of this strain on cyanate as the sole nitrogen source led to the induction of an enzyme characterized as a cyanase (EC 3.5.5.3) based on its stoichiometric conversion of cyanate to ammonia, and dependence on bicarbonate for maximal activity. However, since cyanase activity was not elevated in cyanide-grown cells it was concluded that it serves no role in cyanide metabolism. Related studies aimed at examining a possible role for β-cyanoalanine as a cyanide-assimilation intermediate showed that while this compound also serves as a nitrogen source, it also is not important in cyanide metabolism.

Studies focused on the utilization of free cyanide as a growth substrate led to the development of a fed-batch cultivation procedure greatly facilitating further experimentation aimed at the identification of cyanide
metabolites. In addition to CO$_2$ and NH$_3$ as described earlier, two additional metabolites including formamide and formate were detected by using $^{13}$C-NMR, HPLC, radioisotrappping methods and other analytical means. The formation of metabolites was shown to be induced after growth on cyanide with the relative product yields dependent on the availability of oxygen. These findings support earlier work in which an oxygen-dependent mechanism was proposed for the formation of CO$_2$ and NH$_3$. However, at least two additional oxygen-independent pathways of cyanide conversion can be elaborated by this organism. One of these involves conversion to formate and ammonia while the other leads to the formation of formamide, which is not further degraded. Thus, growth on cyanide appears to occur by several mechanisms of chemical transformation presumably serving both detoxification and nutritional roles. Since two of these mechanisms generate ammonia, which is readily assimilated, growth is presumed to proceed via ammonia as a provisional nitrogenous substrate.
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I remember with special fondness Dr. J. Silva-Avalos, Chien-sao Wang and the members of Drs. O’Donovan, Benjamin and Shanley labs for the camaraderie which made this work pleasant. None of this would have been possible without the emotional support of my parents and wife, Pavai.
# TABLE OF CONTENTS

LIST OF TABLES ................................................. v
LIST OF ILLUSTRATIONS ..................................... vi

CHAPTERS

I. INTRODUCTION .............................................. 1
   Properties of Cyanide
   Detoxification of Cyanide by Microorganisms
   Utilization of Cyanide as a Potential Growth Substrate
   Cyanide Assimilation by *Pseudomonas fluorescens* NCIMB 11764
   Biochemistry of Cyanide Assimilation
   Proposed Mechanism(s) of Cyanide Assimilation in *P. fluorescens* 11764
   Statement of the Problem

II. MATERIALS AND METHODS ................................. 15
   Bacterial Strains
   Growth Media and Culture Conditions
   Preparation of Cell Extracts and Determination of Protein Content
   Enzyme Assays
   Isolation of Cyanate Defective Mutants
   Fed-batch cultivation of cells on KCN
   Biotransformations of KCN using Intact Cell Suspensions
   Analytical Methods
   HPLC Analysis
   NMR Spectroscopy
   $^{14}$C Isotope Fractionation

III. RESULTS .................................................. 40
   A. Role of Cyanate as an Intermediate in Cyanide Metabolism
      Cyanate as a Growth Substrate
      Conversion of Cyanate to Ammonia by Crude Cell Extracts
Activities of Cyanase under Different Growth Conditions
Isolation and Characterization of Cyanate Defective Mutants
Assay of 11764 for the Induction of Putative Oxygenase Activity

B. β-Cyanoalanine as a Possible Intermediate of Cyanide Metabolism

β-Cyanoalanine as a Growth Substrate
Metabolism of β-Cyanoalanine by Crude Cell Extracts

C. Characterization of Cyanide Metabolic Products

Cultivation of Cells on Free Cyanide (KCN)
Bioconversion of Cyanide by Washed Cell Suspensions

13C-NMR Detection and Identification of Cyanide Bio-transformation products
Further Identification of Metabolites by HPLC
Recovery of Cyanide Transformation Products
Radiolabelling Experiments
Effect of Reaction Conditions on Cyanide Conversion and Product Formation
Metabolism of Formamide

IV. DISCUSSION

V. BIBLIOGRAPHY
LIST OF TABLES

1. Composition of Lennox medium ................. 16
2. Growth media reagents ......................... 17
3. Activities of cyanase in cell extracts of
   NCIMB 11764 .................................. 49
4. Growth of NCIMB 11764 on various nitrogen
   sources ......................................... 51
5. Activities of cyanase in wild type and
   mutant of NCIMB 11764 ....................... 52
6. Rate of oxygen uptake by resting cells of
   NCIMB 11764 .................................. 54
7. Activities of S-cyanoalanine hydrolase and
   asparaginase .................................. 59
8. Recovery of cyanide conversion products generated
   under aerobic conditions ..................... 77
9. Recovery of cyanide conversion products generated
   under anaerobic conditions ................. 83
LIST OF ILLUSTRATIONS

1. Ammonia assimilation in microorganisms .................. 10
2. Standard plot for analysis of KCN ....................... 24
3. Standard plot for analysis of ammonia by hypochlorite method ....................... 26
4. Standard plot for analysis of formamide ................... 29
5. Characteristic absorption spectrum in formate assays ....................... 32
6. Ultraviolet spectrum of tetracyanonickelate .................. 38
7. Growth of NCIMB 11764 on cyanate supplied at various concentrations ....................... 41
8. Conversion of cyanate to ammonia by crude extracts ....................... 44
9. Effect of bicarbonate on cyanase activity .................. 46
10. Growth plot of NCIMB 11764 on β-cyanoalanine .................. 57
11. Growth plot of NCIMB 11764 in KCN fed-batch .................. 62
12. Biotransformation of 10 mM KCN by a washed cell suspension ....................... 66
13. $^{13}$C-NMR spectrum of cyanide reaction products .................. 69
14. $^{13}$C-NMR spectrum of standard $^{13}$C-enriched CO$_2$ in buffer ....................... 71
15. HPLC elution profile of reaction products .................. 75
16. Fractionation pattern of $^{14}$C-labelled reaction products ....................... 79
17. Metabolic pathways of cyanide conversion by P. fluorescens NCIMB 11764 ....................... 90
CHAPTER I

INTRODUCTION

Large quantities of cyanide are generated in various industrial processes including metal plating, the production of synthetic fibers, steel tempering, and in the mining industry (Knowles, 1976; Knowles and Bunch, 1986; Pettet and Ware, 1955; Way, 1981; and, White et al., 1988). Cyanide arises in the environment not only by industrial activity but also by biological means. The organisms that generate cyanide include plants, algae, bacteria and fungi (Castric, 1981; Castric, 1983; Conn, 1981; Cooke and Coursey, 1981; Knowles, 1976; Nahrstedt, 1988; Nartey, 1981; and Vennesland et al., 1981). Thus in addition to representing a potential toxic industrial pollutant, cyanide can also be considered a natural product. Although cyanide formation (cyanogenesis) by bacteria was noted as early as 1913 (Emerson et al., 1913), the metabolism of this compound by microorganisms has received only moderate attention. For this reason, concentrated studies aimed at investigating the fundamental basis of cyanide metabolism in the bacterium Pseudomonas fluorescens NCIMB 11764, were undertaken.

Properties of Cyanide

a. Chemical and Physical Properties of Cyanide
Hydrogen cyanide is a colorless weak acid (pKa=9.3) which boils at 26°C, and is therefore readily volatilized from aqueous solution. Free cyanide (CN⁻ or HCN) is highly reactive. It will bind metals as a strong ligand to form complexes of variable stability and toxicity (Bjerrum et al., 1958; Sharp, 1976; and, Smith and Martell, 1976). Examples include the tetracyano complexes of divalent nickel, copper and zinc ([M(CN)₄]²⁻, where M²⁺ = metal), the hexacyano complex of iron (both Fe²⁺ [ferrocyanide] and Fe³⁺ [ferricyanide]), and related derivatives of chromium.

b. Cyanide Toxicity

Cyanide is highly toxic to most biological systems and is well known to be poisonous to man and animals (American Public Health Association, 1980; Arena, 1974; and, Sykes, 1981). Some of the effects of cyanide on microorganisms include decreased lag times for growth, altered cell morphology, decreased biological oxygen demand, and decreased motility (Skowronski and Strobel, 1969; and, Towill et al., 1978). One of the most significant effects of cyanide is the inhibition of respiration at the cytochrome oxidase level (Knowles, 1976). Inhibition of respiration is an extensively studied effect (Arima and Oka, 1965; McFeters, Wilson and Strobel, 1970; and, Niven, Collins and Knowles, 1975). Although sensitive, some biological systems can elaborate an electron transport system that is cyanide tolerant (Henry and Vignais, 1979;
Porter et al., 1983; Minagawa et al., 1991). This is thought to occur by the formation of alternative cytochrome oxidase enzymes that do not respond to the toxic effects of cyanide (Arima and Oka, 1965). Tolerance to cyanide is often used as a taxonomic tool for differentiating members of the family Enterobacteriaceae (Krieg and Holt, 1984; Moller, 1954; and Munson, 1974).

Detoxification of Cyanide by Microorganisms

The industrial importance of cyanide is well known in its use in the production of polymers, acrylonitrile, methylmethacrylate and adiponitrile (Ottinger, 1973). Cyanide wastes arise from industries involved in electroplating and mining (Hamilton and Hardy, 1974; Ottinger et al., 1973). They are also found in paint sludge and old paint residues (Hardy and Boylen, 1971). Copper, zinc, nickel, and mercury are few of the toxic metals known to accompany cyanide (Ottinger et al., 1973).

Several chemical and physical treatment processes were evaluated in pilot plant either alone or in combination to degrade cyanide compounds from waste waters prior to their discharge into surface or ground waters that served as marine or fresh water habitats (Mudder and Whitlock, 1984). These processes require large quantities of expensive chemicals, resulting in high operating costs. Therefore, research directed towards evaluation and development of a biological treatment process which produces a low toxicity
effluent simply, and cost effectively must be examined.

Since cyanide arises in the environment it has been proposed that microorganisms presumably have mechanisms for its detoxification. Several of the proposed mechanisms for its transformation are as follows:

a. Cyanide hydratase (EC 4.2.1.66 formamide hydrolase)

Phytopathogenic fungi have been shown to elaborate a cyanide hydratase, which catalyzes the conversion of cyanide to formamide:

\[
HCN + H_2O \rightarrow HCONH_2
\]

For example, both spores and mycelia of Stemphylium loti and Gloeocercospora sorghi are induced when cultivated in the presence of cyanide suggesting that its induction has a detoxification role (Fry and Millar, 1972; Fry and Munch, 1975; Nazly and Knowles, 1981; and Nazly et al., 1983).

b. Rhodanese (EC 2.8.1.1. thiosulphate: cyanide sulfur transferase).

Rhodanese catalyzes the conversion of cyanide to thiocyanate in the presence of thiosulphate:

\[
S_2O_3^{2-} + CN^- \rightarrow SO_3^{2-} + SCN^-
\]

It is widely distributed in biological systems having been described in mammalian tissues, plants and microorganisms (Sorbo, 1953; Silver and Kelly, 1976; Ryan
and Tilton, 1977; and Alexander and Volini, 1987). In addition to cyanide detoxification, this enzyme has also been proposed to function in the transfer of sulfur atoms in oxidative metabolism (Westley, 1981).

c. β-Cyanoalanine synthase (EC 4.4.1.9, L-cysteine hydrogen sulphide-lyase).

Incorporation of cyanide into certain amino acids, particularly the sulfur-containing amino acids, by β-cyanoalanine synthase is thought to represent a major mechanism by which cyanide is detoxified in plants (Hendrickson and Conn, 1968; Ressler et al, 1969; Ting and Zschoche, 1970). The reaction catalyzed by this enzyme is as follows:

\[
\text{HCN} + \text{cysteine (or O-acetylserine)} \rightarrow \beta-\text{cyanoalanine} + \text{H}_2\text{S} \text{ (or acetate)}
\]

In addition to plants, this enzyme has been detected in fungi (Strobel, 1967; and Castric, 1981) and some bacteria including *E. coli* (Dunnill and Fowden, 1965); *Chromobacterium violaceum* (Brysk et al., 1969; Rodgers, 1978; Rodgers, 1981; and Macadam and Knowles, 1984); and, *Enterobacter* species (Sakai et al., 1981; and Yanese et al., 1982). Moreover, the further conversion of β-cyanoalanine to asparagine and its subsequent hydrolysis has also been reported (Castric and Strobel, 1969; and Yanese et al., 1982).
d. Nitrogenase (EC 1.18.6.1, reduced ferredoxin: dinitrogen oxireductase (ATP-hydrolysing).

Although not explicitly demonstrated in vivo, organisms that elaborate nitrogenase have also been proposed as capable of detoxifying cyanide. This is so because it has long been recognized that this enzyme, in addition to catalyzing the reduction of molecular nitrogen, can also reduce various substrate analogues of nitrogen such as HCN (Li et al., 1982). Products of cyanide reduction include methane and ammonia (six electron reduction). The reduction of cyanide by nitrogenase has been described for several organisms including *Rhodospirilllus gelatinosa* (Harris et al., 1987).

e. Cyanide Oxidation by Cyanide Oxygenase

In addition to the above transformations, an additional detoxification mechanism proposed by Knowles and his colleagues is the oxidative conversion of cyanide to CO$_2$ and ammonia (Harris and Knowles, 1983a).

$$\text{HCN} + \text{O}_2 \rightarrow \text{CO}_2 + \text{NH}_3$$

They further proposed that this transformation, first described in *P. fluorescens* NCIMB 11764, was mediated by an oxygenase-type enzyme.

*Utilization of Cyanide as a Potential Growth Substrate*

Since cyanide contains two of the essential elements of
life it might be hypothesized that microorganisms could utilize it for growth. But the toxicity of cyanide presents problems in isolating bacteria capable of using it as a substrate for growth. Thus, a high enough concentration of cyanide as a source of carbon and energy, to support appreciable growth might prove to be too toxic to allow growth to occur. There has been no report of organisms that could use cyanide as a nutritional source of carbon so far. Ivanoff and Zwetkoff (Ivanoff and Zwetkoff, 1936) and Rangaswami and Balasubramanian (Rangaswami et al., 1963) claim to have isolated strains of Aspergillus niger that utilize cyanide as the sole source of nitrogen. Ware and Painter (Ware and Painter, 1955) isolated a cyanide utilizing Gram-positive, filamentous bacterium from a percolating filter that had been seeded with cyanide-acclimatized sewage sludge. Furuki and coworkers (Furuki et al., 1972) have studied the conditions of optimum growth of a bacterium that utilizes cyanide as a nitrogen source. Gauthier and coworkers (White et al., 1988) reported the isolation of a pseudomonad from an inoculum of coke-plant activated sludge maintained in a chemostat fed only cyanide. Silva-Avalos and Kunz (Silva-Avalos and Kunz, 1989; Silva-Avalos et al., 1990) reported the isolation of seven Pseudomonas and three Klebsiella species that can assimilate tetracyanonickelate or KCN as the sole nitrogen source. Recently, a report (Ingvorsen et al., 1991) describing a
cyanide-metabolizing bacterium isolated from soil, identified as Alcaligenes xylosoxidans subsp. denitrificans, was published.

Cyanide Assimilation by Pseudomonas fluorescens NCIMB 11764

Pseudomonas fluorescens NCIMB 11764 was the organism of choice for this study, since it had the exceptional ability of assimilating cyanide as a sole source of nutritional nitrogen. This bacterium was isolated by enrichments on solid media on plates exposed to low repeated doses of KCN by Harris and Knowles at the University of Kent, U.K. Work done by Rollinson and coworkers (Rollinson et al., 1987) as well as in this study has shown that metal complexed cyanide, as tetracyanonickelate (TCN) can also be used as the source of nitrogen when it is added directly to the growth medium in batch cultures. TCN was used as a model compound for KCN initially, since KCN proved to be highly toxic for growth of cells in liquid culture. The use of a modified fed-batch method as described in Materials and Methods enabled the use of KCN during the later part of the research.

Biochemistry of Cyanide Assimilation

Harris and Knowles reported the successful recovery of cyanide transforming activity in cell extracts of P. fluorescens 11764. This process induced by prior growth on cyanide, required NADH and oxygen for catalytic activity.
NH₃ and CO₂ were identified as principal metabolic products and thus, it was proposed that cyanide assimilation occurs by transformation to ammonia, which can then be assimilated by well recognized mechanisms. This presumably occurs via incorporation of ammonia into α-ketoglutarate via the glutamine synthetase (EC 6.3.1.2) / glutamine 4:2-oxoglutarate aminotransferase (EC 1.4.1.13) (GS/GOGAT) and glutamate dehydrogenase (EC 1.4.1.3) pathways, as illustrated in Figure 1 (Rhee et al, 1978).

Proposed Mechanism(s) of Cyanide Assimilation in P. fluorescens 11764

a. Cyanide oxygenase / Cyanide dioxygenase

Knowles and co-workers (Harris and Knowles, 1983b; and Knowles, 1988) proposed that the initial cyanide attack in this bacterium was oxygenase-mediated based on the fact that washed cell suspensions and cell-extracts did not catalyze its conversion under anaerobic conditions. Also, cyanide turnover appeared to be correlated with simultaneous oxygen uptake, and consumption of reduced pyridine nucleotide (NADH).

The proposed reaction is as follows:

\[ \text{HCN} + \text{O}_2 + \text{NADH} + \text{H}^+ \rightarrow \text{HOCN} + \text{NAD}^+ + \text{H}_2\text{O} \]

Cyanate (cyanic acid) was proposed as a likely intermediate resulting from monooxygenative attack, and was
Figure 1. Ammonia assimilation in microorganisms. Scheme illustrates glutamate dehydrogenase pathway and glutamine synthetase/glutamate synthase pathways. (Adapted from Prescott et al., 1990).
Glutamate dehydrogenase (GDH)

\[ \begin{align*}
\text{Glutamate} & \xrightarrow{\text{GDH}} \text{α-ketoglutarate} \\
\text{NAD(P)H} & \xrightarrow{\text{GDH}} \text{Glutamate} \\
\text{H}_2\text{O} & \xrightarrow{\text{GDH}} \text{Amino acid}
\end{align*} \]

Glutamine synthetase reaction

\[ \begin{align*}
\text{Glutamic acid} + \text{NH}_3 + \text{ATP} & \xrightarrow{\text{Glutaminase}} \text{Glutamine} + \text{ADP} + \text{P}_i
\end{align*} \]
further suggested to be metabolized by an enzyme resembling cyanase (EC 3.5.5.3) as follows:

\[ \text{HOCN} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{NH}_3 \]

Alternatively, it was proposed that CO₂ and NH₃ could be formed directly by dioxygenative attack with the following stoichiometry:

\[ \text{NAD(P)H} + \text{H}^+ + \text{HCN} + \text{O}_2 \rightarrow \text{CO}_2 + \text{NH}_3 + \text{NAD(P)}' \]

b. Β-Cyanoalanine synthase

It was hypothesized in our laboratory that alternate pathways besides the oxidative pathways proposed for 11764 (Knowles, 1988) could be important in cyanide assimilation. Β-Cyanoalanine, generated by incorporation of cyanide into cysteine by a hypothetical Β-cyanoalanine synthase, might serve as a precursor for further metabolism as shown below.

\[ \text{HCN} + \text{cysteine} \rightarrow \text{Β-cyanoalanine} + \text{H}_2\text{S} \]
\[ \text{Asparagine} \rightarrow \text{Aspartate} + \text{NH}_3 \]

c. Cyanide hydratase:

The simplest route of cyanide degradation involves its conversion to formate and ammonia. This could occur either directly by a nitrilase or indirectly via formamide by cyanide hydratase and formamidase. The action of formate
dehydrogenase would then result in the release of CO₂.

There has been a report of a nitrilase that hydrolyzed HCN at a low rate and formamidase is common in many soil and water bacteria (Harris et al., 1987).

\[
\text{HCN} \rightarrow \text{HCONH}_2 \rightarrow \text{HCOOH} \rightarrow \text{CO}_2
\]

Finally, it is possible that cyanide could be converted directly to formate and ammonia as recently was described for *Alcaligenes xylosoxidans* subsp. *denitrificans* DF 3 (Ingvorsen et al., 1991) and an unclassified pseudomonad (White et al., 1988).

\[
\text{HCN} + 2\text{H}_2\text{O} \rightarrow \text{HCOOH} + \text{NH}_3
\]

**Statement of the Problem**

The goal of this research was to investigate the pathway responsible for cyanide assimilation in *P. fluorescens* 11764. The research was focused on: (1) verifying the proposed oxidative pathways of Knowles and coworkers (Harris and Knowles, 1983b; and, Knowles, 1988) which may involve cyanate as a metabolic intermediate (2) determining whether ß-cyanoalanine might serve as metabolic intermediate of cyanide assimilation, and (3) evaluating the feasibility of the involvement of formamide or formate as
metabolic intermediates. Detailed studies were conducted to identify the products of cyanide breakdown at varying substrate concentrations and to determine reaction stoichiometries. Mass balance was done for all the C and N products in order to understand the patterns of product formation, which may shed light on the involvement of more than one pathway.
CHAPTER II

MATERIALS AND METHODS

Bacterial Strains

All experiments were conducted with *P. fluorescens* biotype II (NCIMB 11764), which was obtained from the National Collection of Marine and Industrial Bacteria, Torrey, Scotland. This bacterium was isolated by R.E. Harris and C.J. Knowles in the United Kingdom (Harris and Knowles, 1983a). *P. fluorescens* mt-0101 (Cnt-) was obtained as part of this work. Both strains were maintained in a medium containing dimethyl sulfoxide at -80°C and subcultured on Lennox agar (Table 1) plates every four weeks.

Growth Media and Culture Conditions

The minimal medium used for both batch and modified fed-batch growth determinations and for growing cells for enzyme assays and resting cell incubations is shown in Table 2.

For the growth curves described in Results, glucose was used as the carbon source (10-20 mM). Cells were initially cultured on 10 mM ammonium sulfate and washed three times in a buffer containing 30 mM Na₂HPO₄·7H₂O and 20 mM KH₂PO₄ (pH 7.0) before a 4% inoculum was provided to flasks containing
Table 1. Composition of Lennox medium (Lennox, 1955)

**Lennox broth medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<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>1,000 ml</td>
</tr>
</tbody>
</table>

pH 6.8, sterilize at 121°C for 20 min. For Lennox agar, add 20 g of agar.
Table 2. Growth media reagents (Kunz et al., 1981).

**Buffer phosphate (P10X)**

- $\text{KH}_2\text{PO}_4$ 91.0 g
- $\text{NaOH}$ 16.8 g
- Distilled water to complete 1,000 ml
- pH 7.0. Store in bottle.

**R-salts (R-200X)**

- 10% $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 400 ml
- 1% $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$ 100 ml
- + 2 ml conc. HCl

Mix A & B, dispense in bottle, pH 1.95. Sterilize at 121°C for 20 min. (5 ml R-salts added per 1,000 ml P1X solution)

**Minimal medium composition**

- P1X (sterile) 100 ml
- Glucose 1 M (sterile) 2 ml
- R-salts 200X (sterile) 0.5 ml

Add glucose and R-salts aseptically to sterilized P1X in a sterile flask. For minimal agar, add sterilized 2% purified agar (Difco) before adding glucose and R-salts.
either KOCN (10 mM), (NH$_4$)$_2$SO$_4$ (10 mM), β-cyanoalanine (1 mM), TCN (0.75 mM), KCN (0.75 mM) or HCONH$_2$ (0.25 to 5 mM) as the sole nitrogen source. Cultures were checked for purity before harvest.

**Preparation of Cell Extracts and Determination of Protein Content**

Cells used for the preparation of cell extracts were grown in 1 l of minimal medium as described above containing either KOCN (10 mM), NH$_4$Cl (10 mM), TCN (0.75 mM), β-cyanoalanine (1 mM) or KCN (0.75 mM, modified fed-batch) as nitrogen sources.

Cells were harvested in the log phase by centrifugation at 20,000 x g for 10 min. The cells were washed twice with 50 mM Na/K phosphate buffer (pH 7.0), and resuspended in twice the volume of the same buffer. The cells were broken in a French press (20,000 lbs per square inch). A small amount of DNAase was added to the broken cells and the preparation incubated at room temperature for 5 min before centrifugation for 30 min at 35,000 x g at 4°C. The supernatant was used as the crude extract.

The procedure as described by Lowry et al. (Lowry et al., 1951) was used routinely to determine the protein content in cell extracts. To 5 μl of an appropriately diluted protein sample 1 ml of 49:1 mixture of 2% Na$_2$CO$_3$ in 0.1N NaOH and 0.5% CuSO$_4$.5H$_2$O in 1% NaK tartrate was added. After precisely 10 min, 0.1 ml of 1N Folin-phenol reagent
was added. The absorbance of the resulting blue color was read at 750nm. Bovine serum albumin was used for the determination of the standard plot.

**Enzyme Assays**

a. Cyanase

Cyanase was assayed by measuring ammonia formation at 30°C essentially as described by Anderson (Anderson, 1980). The standard assay mixture contained the following (in 5 ml): 50 mM KH$_2$PO$_4$ (pH 7.5), 3 mM bicarbonate, 4 mM KOCN, and crude extract (0.05 to 2 mg of protein per ml). Following the addition of enzyme, 0.5 ml samples were removed at intervals and mixed with an equal volume of Nessler reagent (1:3) and the A$_{420}$ was read.

b. β-cyanoalanine Degrading and Asparaginase Activities

The β-cyanoalanine degrading activity was assayed in a reaction mixture containing 20 µmol of β-cyanoalanine, 50 µmol of Tris-HCl buffer, pH 8.5, and the enzyme solution (0.1 to 2 mg of protein), in a final volume of 1.0 ml (Yanese et al., 1983). The reaction was carried out at 30°C and the formation of ammonia was measured by Nesslerization (Anderson, 1980). For the assay of asparaginase activity, 20 µmol of asparagine was used as a substrate instead of β-cyanoalanine (Yanese et al., 1983). The reaction was carried out as described above, and the amount of ammonia formed was determined.
c. Oxygen Uptake Measurements

Suspensions of bacteria were added to Na/K phosphate buffer, pH 7.0 in a magnetically stirred chamber of an oxygen electrode (Hansatech, Great Britain) to give an \( A_{540} \) of 1.0. The rate of respiration was measured in this chamber at 30°C, which was kept constant by a temperature controlled water jacket. TCN or KCN was added at a final concentration of 0.1 to 0.2 mM and the initial increase in the oxygen consumption rate was used as a measure of oxygen uptake by cells (Dorr and Knowles, 1989).

Isolation of Cyanate Defective Mutant

Wild type cells of \( P. \text{fluorescens} \) NCIMB 11764 were grown in 25 ml of minimal medium with 10 mM \((\text{NH}_4)_2\text{SO}_4\) as nitrogen source. The cells were washed and resuspended in 20 ml of 100 mM sodium citrate buffer, pH 5.5. A standard plate count was done to estimate the total number of cells. A solution of nitrosoguanidine made in sodium citrate was added to the suspension to give a final concentration of 100 \( \mu \text{g/ml} \). The bacteria were incubated at 30°C in a gyratory shaker for precisely 30 minutes. Another standard plate count was done to estimate the percentage of killing. The cells were washed twice in 50 mM Na/K phosphate buffer and allowed to grow in minimal medium with 10 mM \((\text{NH}_4)_2\text{SO}_4\) as the nitrogen source for 12 hours. The cells were harvested and washed three times.

The cells were regrown in a 250 ml Klett flask with 5
mM KOCN as sole nitrogen source and were closely monitored for growth until an increase of 100 Klett units which was considered an appropriate time for lysis of wild type cells. Penicillin-G at a final concentration of $10^4$ U/ml and D-cycloserine at a final concentration of 0.2 mg/ml were added to the flask. The cells underwent lysis for 10 hours before harvesting and washing procedures. Minimal medium plates containing 10 mM (NH$_4$)$_2$SO$_4$ as the sole nitrogen source were used for plating the enriched bacteria. Single colonies from these plates were transferred to master plates using a 50 x 50 grid. Subsequently, these colonies were transferred onto plates containing either 5 mM KOCN or 10 mM (NH$_4$)$_2$SO$_4$ as nitrogen sources for screening mutants.

**Fed-batch cultivation of cells on KCN**

Growth of cells on KCN was achieved in minimal medium as described above with glucose (20 mM) as the carbon source and KCN was fed at subtoxic levels in a modified fed batch mode. This procedure involved the inoculation of a 36 hr old single colony from a Lennox agar plate to a 250 ml Erlenmeyer flask containing 100 ml of NH$_4$Cl (1 mM) as the nitrogen source. This culture was then used as inoculum for 1.8 liters of medium contained in an Erlenmeyer flask supplied with 0.25 mM KCN. This culture was pulsed with 0.25 mM KCN after 24 hours. The 48 hour old culture was again pulsed with 0.25 mM KCN for 24 hours before cells were harvested. The cyanide (CN$^-$) content of the culture samples
during growth was colorimetrically determined. Growth was monitored turbidimetrically at 540 nm.

**Biotransformations of KCN Using Intact Cell Suspensions.**

Cells which were grown in KCN using the modified fed batch mode procedure were harvested by centrifugation at 20,000 x g at 4°C for 10 minutes. They were washed twice with 50 mM Na/K phosphate buffer (pH 7.0) and resuspended in the same buffer at a cell concentration of 40 mg dry weight per ml. One ml of suspension was then transferred to each of the 15 ml bottles with teflon seals. The bottles were crimped and were used as reaction vessels. Following the addition of substrate to the reaction vessel by syringe, samples were removed by Hamilton syringes at time intervals and centrifuged in a microfuge for 1 min at 15,000 x g. The clear supernatant was used for analysis of cyanide and other metabolic products.

**Analytical Methods**

In all experiments for spectrophotometric measurements the samples for analysis were centrifuged in a microfuge (Savant model HSC-10000) for 1 min at 15,000 x g and supernatants were analyzed spectrophotometrically.

a. Cyanide

KCN consumption was monitored by a colorimetric method (Lambert et al., 1975). Samples were centrifuged and 50 μl of supernatants were added to 1.1 ml of distilled water, 50
μl of N-chlorosuccinimide-succinimide reagent and 50 μl of barbituric acid-pyridine reagent. The absorbance at 580 nm was read 15 min after the addition of barbituric acid-pyridine reagent. A lavender color indicated the presence of cyanide. The standard plot used in the estimation of KCN concentrations in culture supernatants is shown in Figure 2.

b. Ammonia

Ammonia formation was estimated by using the colorimetric method described by Fawcett and Scott (Fawcett and Scott, 1960). One ml of reaction mixture containing 0.125 ml of supernatant (sample), 0.25 ml of sodium phenate, 0.375 ml of 0.01 % of sodium nitroprusside and 0.375 ml of 0.02 N sodium hypochlorite. After 30 min, the absorbance was measured at 630 nm at room temperature. A blue color in the reaction mixture indicated the presence of ammonia. Ammonia was measured in cyanase assays by using the method of Anderson (Anderson, 1980). The standard assay mixture contained the following (in 5 ml): 50 mM KH$_2$PO$_4$ (pH 7.5), 3 mM bicarbonate, 4 mM KOCN, and crude extract (0.1 to 2 mg of protein per ml). Following the addition of enzyme, 0.5-ml samples were removed at intervals and mixed with an equal volume of Nessler reagent (1:3) and the A$_{420}$ was read. The standard plot used for the determination of ammonia by the hypochlorite method is shown in Figure 3.
Figure 2. Standard plot for analysis of KCN (values obtained from a mean of four determinations).
Figure 3. Standard plot for analysis of ammonia by hypochlorite method (values obtained from a mean of four determinations).
c. Cyanate

Cyanate was determined by using a modification of the procedure described by a method (Guilloton and Karst, 1985) involving cyclization with anthranilic acid to give 2,4-(1H, 3H)-quinazolinedione; the latter gives a characteristic maximum at $A_{310\text{nm}}$. For this purpose, equal volumes (0.5 ml) of sample and 10 mM anthranilic acid were mixed at pH 4 to 5 and heated at 40°C for 10 minutes. The final pH of this mixture was critical since coupling at higher pH values is unfavorable and lower values result in cyanate hydrolysis prior to derivatization (Guilloton and Karst, 1985). Following the 10-minute incubation period, 1 ml of 6 N HCl was added and the samples were again heated at 100°C for 1 minute before the $A_{310\text{nm}}$ was read with a spectrophotometer.

d. Formamide

A colorimetric method described by Fry and Millar was used to determine formamide based on its conversion to ferric hydroxamate (Fry and Millar, 1972). The sample (0.1 ml) was incubated precisely for 10 minutes at 60°C with 0.2 ml of a 1:1 mixture of 2.5N NaOH and 2.3 M hydroxylamine hydrochloride. This was followed by the addition of 0.1 ml of 4N HCl and 0.12 ml FeCl₃, prepared in 0.075N HCl. The $A_{540\text{nm}}$ was read within 5 minutes after the addition of FeCl₃. The standard plot for formamide determination is shown in Figure 4.
Figure 4. Standard plot for analysis of formamide (values obtained from a mean of four determinations).
The graph shows a positive correlation between HCONH₂ concentration (mM) and absorbance at 540 nm. The absorbance increases linearly as the concentration of HCONH₂ increases.
Formate was determined enzymatically using commercial formate dehydrogenase (FDH) (Sigma Chemical Co., St. Louis, MO.) as described by Höpner and Knappe (Höpner and Knappe, 1974). Reaction mixtures contained the following in 0.4 ml: 18-20 μmol KH₂PO₄ (pH 7.5), 0.4 μmol NAD⁺, 400 mU.ml⁻¹ formate dehydrogenase, and 0.01-0.02 ml (0.1-0.2 μmol) of biological sample. Reactions were initiated by the addition of FDH and the change in absorbance at 340 nm measured over 2-5 minutes (Figure 5). Since reactions proceeded only to about 62% completion, an average extinction value for NADH of 3.85 mM⁻¹ cm⁻¹ was routinely used to calculate the formate concentration. Spectrophotometric measurements were recorded on either a LKB Ultraspec II or Perkin-Elmer Lambda-6 uv/vis instrument.

HPLC Analysis

The identification of formate and formamide in incubation mixture supernatants was further accomplished by high performance liquid chromatography (HPLC). Analysis was performed at ambient temperature on a HPX-87H ion-exchange column (200 mm x 7.8 mm; Bio-Rad Laboratories, Richmond, Ca.) using a Rainin Dynamax system. The mobile phase consisted of 0.015N H₂SO₄ containing 0.4 mM ethylenediamine tetraacetic acid (pH 2.0) maintained isocratically at an elution rate of 0.5 ml min⁻¹. Compounds were detected by uv absorption at 210 nm using a Knauer variable wavelength
Figure 5. Plot showing change of absorbance at 340 nm, indicating increase in formation of NADH after the addition of formate dehydrogenase to reaction mixtures containing biologically produced formate.
detector coupled to a Macintosh computer by a Rainin HPLC Data Acquisition System.

**NMR Spectroscopy**

The analysis of $^{13}$C-labelled conversion products by $^{13}$C-NMR was conducted with washed cell suspensions supplied K$^{13}$CN (99 atom %) at concentrations as high as 50 mM (3.26 mg ml$^{-1}$). The same general incubation procedure as described above was employed, and at various intervals following the addition of substrate, reaction mixture supernatants were transferred to NMR tubes. $^{13}$C-NMR spectra were recorded at ambient temperature on either a Varian VXR-300 spectrometer at 75 MHz, or a Varian GEM 200 instrument at 50 MHz. Chemical shift values for $^{13}$C-resonances (broad band proton decoupled) were referenced against benzene-d$_6$ as an external standard, which was assigned a chemical shift value of 128.7 ppm (Levy and Nelson, 1972; Stothers, 1972). Heteronuclear J$_{c,H}$ couplings were determined by acquiring data in the gated decoupled mode. Aqueous solutions of $^{13}$C-enriched commercial compounds were prepared in Na-K phosphate buffer (pH 7.0) at concentrations ranging from 0.8-8.0 mg.ml$^{-1}$ except for formamide which was prepared at 65 mg.ml$^{-1}$ and the resonance for natural abundance $^{13}$C recorded. The $^{13}$C-NMR spectrum for bicarbonate was obtained either by gassing phosphate buffer with commercial $^{13}$CO$_2$, or by preparation of a suspension containing Ba$^{13}$CO$_3$. At pH 7.0 these compounds equilibrate to give H$^{13}$CO$_3^-$ as the principal
species.

\(^{14}\text{C Isotope Fractionation}\)

Radiolabelled metabolites were identified and recovered as either volatile \((\text{CO}_2/\text{HCO}_3^-)\) or non-volatile (formamide and formate) products using \(\text{BaCl}_2\) as a bicarbonate trapping reagent according to the procedure described by Fallon et al. (Fallon et al., 1991). Reactions were initiated by the injection of 1-2.5 \(\mu\text{Ci}\) of \(\text{K}^{14}\text{CN}\) (47 mCi mmol\(^{-1}\)) to 1 ml of cell suspension contained in a 50 ml serum-stoppered flask fitted with a center well. Non-isotopic KCN was also added to bring the substrate concentration to the desired level (0.5-50 mM). When reactions were complete, as determined by simultaneous colorimetric assays, 0.3 ml 5N NaOH was injected into the center well to trap volatile \(\text{CO}_2\) and the flask allowed to incubate for an additional 15 minutes. At the end of this time the contents of the center well and main compartment of the flask were removed and fractionated with \(\text{BaCl}_2\) to recover volatile and non-volatile products. For this purpose, 0.5 ml of the contents from the main compartment were centrifuged for 2 minutes in a microcentrifuge. Cell-associated radioactivity (cell pellet fraction) was then determined after resuspending the pellet in 0.5 ml phosphate buffer, which was added to scintillation fluid. The supernatant (0.3 ml) was combined with 0.12 ml of 0.1N NaOH plus 0.03 ml 40% \(\text{BaCl}_2\) for five minutes and separated into fractions designated, main compartment.
alkaline-barium precipitate (containing bicarbonate), and main compartment alkaline barium-soluble (containing formamide plus formate), following centrifugation. After washing the pellet (alkaline barium-precipitate) with an additional 0.2 ml of 0.12N NaOH, which was combined with the supernatant (alkaline barium-soluble), the pellet was resuspended in 0.5 ml of 0.12N NaOH and both samples added to scintillation fluid. The contents of the center well (0.3 ml) were treated in exactly the same fashion generating fractions designated, center well alkaline barium-precipitate (containing CO$_2$/HC0$_3^-$) and center well barium-soluble (containing non-CO$_2$/HC0$_3^-$ products). All radioactive samples were added to 8 ml Aquasol scintillation fluid (NEN Research Products, DuPont, Boston, Massachusetts) and counted on a Beckman LS 7000 scintillation counter (Irvine, California).

MATERIALS

KOCN (99 %) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI), and was recrystallized before use from a water-ethanol mixture as described by Scattergood (Scattergood, 1946). The recrystallized compound was then stored under P$_2$O$_5$ in a vacuum desiccator before use. Since cyanate undergoes slow, spontaneous hydrolysis (Amell, 1956; Guilloton and Karst, 1987), stock solutions for growth tests and enzyme assays were prepared just prior to use.

TCN was prepared by D. A. Kunz as described by
Fernelius and Burbage (Fernelius and Burbage, 1946). The identity of the preparation was confirmed by UV and Fourier-Transform Infrared (FT-IR) spectroscopy. The aqueous UV spectrum (Figure 6) had absorption maxima at 267 and 286 nm; from which a molar extinction coefficient (€) at $A_{267}$ of 11,200 M$^{-1}$ cm$^{-1}$ was calculated.

Formamide (99+%), sodium formate (99+%), formic acid (95-97%), K$^{13}$CN (99 atom%), Ba$^{13}$CO$_3$ (98 atom%), Na formate-$^{13}$C (99 atom%), and $^{13}$CO$_2$ (99 atom%) were obtained from the Aldrich Chemical Co. (Milwaukee, WI). K$^{14}$CN was purchased from NEN Research Products. These and other chemicals from commercial sources were used without further purification.
Figure 6. Ultraviolet (A) spectrum of tetracyanonickelate II (TCN) recorded on a model 4050 UV/vis spectrophotometer.
A. Role of Cyanate as an Intermediate in Cyanide Metabolism

Cyanate as a Growth Substrate

Since cyanate was proposed as a possible intermediate in cyanide breakdown (Harris and Knowles, 1983b; Harris et al., 1987; Knowles, 1988; and, Knowles and Bunch, 1986), one of the earliest experiments performed in this research was simply to test whether this compound might also serve as a nitrogen source for growth. To accomplish this NCIMB 11764 was cultivated in glucose minimal medium and cyanate (KOCN) was supplied as the sole nitrogen source. These experiments revealed that indeed KOCN was utilized. Figure 7 shows comparative growth results for cells of NCIMB 11764 cultivated on cyanate and ammonia. Although the growth rate on cyanate was less than half that observed on ammonia (generation time \( t_{\text{gen}} \), 1.0 h), cells grew readily on cyanate (10 mM) \( t_{\text{gen}}, 3.4 \) h after an initial lag period of 6 h. Comparison of the growth results between separate cultures that were supplied various concentrations of cyanate revealed that optimal growth occurred at 10 mM. A longer lag period accompanied by a slower growth rate \( t_{\text{gen}}, 5.6 \) h) was observed on 20 mM cyanate, which is probably due
Figure 7. Growth of *P. fluorescens* NCIMB 11764 in glucose (20 mM) minimal medium supplied with various sources of nutritional nitrogen. Cells were grown on 10 mM ammonium sulfate and washed three times in 50 mM Na-K phosphate buffer (pH 7.0) before providing a 1% (v/v) inoculum to flasks containing: ■, (NH₄)₂SO₄ (10 mM); △, ▲, □, ◆, KOCN (1.0, 10, 20, 40 mM) and ◊, control (no fixed nitrogen).
to cyanate toxicity. Shorter lag periods (approximately 3 h) observed at low concentrations of cyanate (e.g. 1.0 mM), and the complete absence of growth at high concentrations (e.g. 40 mM) are consistent with this hypothesis (Kunz and Nagappan, 1989).

**Conversion of Cyanate to Ammonia by Crude Cell Extracts**

In order to determine the enzymatic basis of cyanate utilization by NCIMB 11764, crude cell-extracts from cells grown on 4 mM cyanate as the sole nitrogen source were incubated with cyanate and tested for ammonia formation. The results in Figure 8 show that cell-extracts quickly converted cyanate quantitatively into ammonia (molar ratio, 1.13 ± 0.25 [n = 3]), which is consistent with the expected stoichiometry for cyanase (Anderson, 1980; Taussig, 1960). Indeed, the rate of cyanate conversion in the presence of cell extract was significantly higher than that due to spontaneous hydrolysis (half-life = 27 h) (Guilloton and Karst, 1987), strongly indicating that an enzyme-catalyzed reaction was responsible for its breakdown. This was further suggested by the results in Figure 9, which show that the rate of cyanate conversion was bicarbonate dependent. Typically, cyanase enzymes require bicarbonate for more activity (Anderson, 1980). For example, the initial rate of conversion in the presence of NaHCO₃ (3 mM) was found to be twice that observed in its absence. These observations strongly indicate that a cyanase (EC 3.5.5.3)
Figure 8. Conversion of cyanate to ammonia by crude cell-extracts containing cyanase from *P. fluorescens* NCIMB 11764. Reaction mixtures contained 1.0 mM KOCN, crude extract (4 mg protein) and 3 mM bicarbonate in 10 ml 50 mM KH$_2$PO$_4$ buffer (pH 7.5). At the indicated times 1.0 ml samples were withdrawn and analyzed respectively for cyanate (▲) and ammonia (○) as described in the text.
Figure 9. Effect of bicarbonate on cyanase activity in crude cell-extracts from *P. fluorescens* NCIMB 11764. Enzyme activity was measured as described in Results in the presence (●) and absence (△) of 3 mM bicarbonate.
type enzyme was involved in catalysis. Inability to observe activity towards either urea or thiocyanate (KCNS) was consistent with this conclusion (Kunz and Nagappan, 1989).

Activities of Cyanase under Different Growth Conditions

The finding that cyanate could support growth and was degraded by an enzyme resembling cyanase prompted experiments to determine the conditions under which this enzyme might be elevated. It was hypothesized, for example, that any involvement of cyanate as an intermediate in cyanide breakdown would require that this enzyme be elevated in cyanide-grown cells. Table 3 shows the activities of cyanase in cells grown under various conditions. Cyanase was significantly elevated over that seen for ammonia-grown cells when 11764 was cultivated on cyanate as the sole nitrogen source. However, no elevation in activity was observed when cells were cultivated either on KCN or tetracyanonickelate (TCN). Activity was still present when cells were grown in either ammonia-containing minimal medium or L-broth supplemented with cyanate. These findings indicated that (i) ammonia does not significantly repress cyanase synthesis and (ii) that this enzyme does not appear to be involved in cyanide breakdown.

Isolation and Characterization of Cyanate Defective Mutants

The possible involvement of cyanate as a cyanide-breakdown intermediate was further explored by examining the
Table 3. Activities of cyanase\textsuperscript{a} in cell-extracts of NCIMB 11764

<table>
<thead>
<tr>
<th>Nitrogen source\textsuperscript{b} supplied for growth</th>
<th>Specific activity (nmoles min\textsuperscript{-1} mg\textsuperscript{-1} protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOCN (10 mM)</td>
<td>168</td>
</tr>
<tr>
<td>KOCN (10 mM) + (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}</td>
<td>316</td>
</tr>
<tr>
<td>KOCN (10 mM) + L-Broth</td>
<td>194</td>
</tr>
<tr>
<td>KCN (0.75 mM)</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>TCN (0.25 mM)</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}</td>
<td>&lt; 5</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Measured by ammonia formation with Nessler's reagent

\textsuperscript{b}Glucose (20 mM) served as carbon and energy source in liquid minimal medium
growth pattern of cyanate mutants; the rationale here was that if cyanate is an intermediate, then mutants unable to utilize cyanate should also be unable to grow on cyanide. The procedure for isolation of such mutants, based on penicillin-enrichment techniques, was described in the Materials and Methods. Two mutants were detected after screening 250 colonies taken from glucose plates which were supplied ammonia as the nitrogen source. Only one mutant was retained for investigation and designated as strain 0-101 since these could represent siblings. Table 4 shows the growth results when mutant 0-101 and the wild-type were tested for their growth response towards cyanide and related nitrogenous substrates. These results show that cyanide (KCN or TCN) was still utilized by 0-101 whereas cyanate was not.

In order to determine whether the negative growth response towards cyanate by mutant 0-101 could be attributed to an inability to produce cyanase, cells were tested for the ability to induce cyanase under conditions shown to do so in the wild-type. For these purposes, cells were cultivated on ammonia and then pulsed with 4 mM KOCN during logarithmic growth. The results in Table 5 show that under these conditions cyanase was induced to high levels in the wild-type, but not in the mutant. Also, like the wild-type, the mutant was still able to grow on cyanide, but elevated cyanase levels could not be detected. These results
Table 4. Growth of NCIMB 11764 on various nitrogen sources\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Strain</th>
<th>HCN</th>
<th>TCN</th>
<th>KOCN</th>
<th>(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCIMB 11764</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11764/O-101\textsuperscript{c}</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Tested on minimal salts agar (Noble, Difco) plates with glucose (10 mM) supplied as the carbon and energy source.

\textsuperscript{b}Supplied at concentrations ranging from 0.25-10 mM except for HCN which was provided as vapor.

\textsuperscript{c}Obtained by standard nitrosoguanidine/penicillin mutagenesis enrichment.
Table 5. Activities of cyanase\textsuperscript{a} in wild type and mutant of NCIMB 11764

<table>
<thead>
<tr>
<th>Nitrogen source for growth</th>
<th>Enzymatic Activity in:\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NCIMB 11764</td>
</tr>
<tr>
<td>KCN</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>TCN</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} + KOCN\textsuperscript{c}</td>
<td>1348</td>
</tr>
<tr>
<td>(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}</td>
<td>&lt; 5</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Formation of ammonia measured with modified Nessler's reagent (Sigma)

\textsuperscript{b}Expressed as nmol min\textsuperscript{-1} mg\textsuperscript{-1} protein

\textsuperscript{c}Cells were grown on (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} (10 mM) for 12 hours and then pulsed with KOCN (4 mM) for 6 hours before harvest.
therefore, strongly suggested that neither cyanate nor the enzyme cyanase was important in cyanide metabolism.

Assay of 11764 for the Induction of Putative Oxygenase Activity

As pointed out in the Introduction (page 9), it was earlier proposed that cyanate might arise by an oxygenation of cyanide. However, the inability to obtain any evidence that cyanate was an intermediate prompted additional experiments to detect oxygenase activity in NCIMB 11764 cells. For this purpose, cells were grown under various conditions as shown in Table 6 and cyanide-dependent oxygen uptake measured with an oxygen electrode. It should be noted that growth on cyanide was accomplished in two ways. Cells were cultivated either on KCN in a fed-batch mode to be described later or they were grown on the model cyanide compound tetracyanonickelate (II) (TCN). The use of the latter substrate is not discussed in detail other than to say that it was used extensively in initial experiments because (i) cells could be grown readily on it, (ii) it was less hazardous to handle, and (iii) other investigators and results from our laboratory have shown that it serves as a useful model cyanide substrate (Rollinson et al, 1987; Silva-Avalos and Kunz, 1989). The results in Table 6 show that neither KCN- nor TCN-grown cells was induced for cyanide oxygen uptake. Since other investigators had earlier shown that hypothetical oxygenase activity could
Table 6. Rate of oxygen uptake by resting cells of NCIMB 11764

<table>
<thead>
<tr>
<th>Nitrogen source for growth</th>
<th>Rate of O₂ uptake (nmols min⁻¹ mg⁻¹ dry cell wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCN (0.75 mM)</td>
<td>UD^c</td>
</tr>
<tr>
<td>KCN (0.25 mM)</td>
<td>UD</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (10 mM)</td>
<td>UD</td>
</tr>
<tr>
<td>TCN pulse^a</td>
<td>25^d</td>
</tr>
<tr>
<td>KCN pulse^b</td>
<td>UD</td>
</tr>
</tbody>
</table>

^a(NH₄)₂SO₄ (1.0 mM) grown stationary phase culture was pulsed with TCN (0.25 mM) after 24 hours

^b(NH₄)₂SO₄ (1 mM) grown stationary phase culture was pulsed with KCN (1 mM) after 24 hours

^cUndetectable

^dCorrected for endogenous activity (3 nmols min⁻¹ mg⁻¹ dry cell weight)
best be detected when stationary phase cultures were pulsed with TCN, this strategy was used. For this purpose, cells were grown on 1 mM (NH₄)₂SO₄ to stationary phase and either 0.25 mM TCN or 1 mM KCN added. Following 3-6 hours, cells were harvested and oxygen-uptake towards KCN determined. The data in Table 6 show that cells to which TCN was added did show some increase in uptake (over endogenous) when cyanide was supplied, but the KCN-pulsed culture did not. These data provided some suggestive evidence that a cyanide oxygenase was responsible for the elevated oxygen uptake rates by TCN-pulsed cells. However, the absence in elevation of activity in stationary phase cultures of either KCN or TCN grown cells, or KCN-pulsed cells made this conclusion tenuous.

B. S-cyanoalanine as a Possible Intermediate of Cyanide Metabolism

Since the above results did not provide a strong indication that an oxygenase was induced in cyanide-grown cells, research was directed towards examining the possibility of alternative routes of metabolism. One possibility as developed in the Introduction, was that cyanide might be incorporated into an amino acid to give, for example, S-cyanoalanine as a metabolic intermediate. Thus, a set of experiments similar to those outlined earlier for cyanate was conducted to examine whether S-cyanoalanine
might be involved.

\textbf{B-Cyanoalanine as a Growth Substrate}

Cultivation of NCIMB 11764 either in liquid or solid media supplied B-cyanoalanine as the nitrogen source resulted in rapid growth. The growth kinetics in liquid culture shown in Figure 10 illustrate that the growth rate was strikingly similar to that observed for growth on ammonia (generation time \( t_{\text{gen}} \), 1.0 h).

\textbf{Metabolism of B-cyanoalanine by Crude Cell Extracts}

Since B-cyanoalanine was shown to serve as a growth substrate, experiments were conducted to determine whether cell-extracts from cyanide-grown cells might be capable of metabolizing this compound. Since there were reports in the literature that B-cyanoalanine can be degraded via asparagine, ultimately generating ammonia, cell-free incubations supplied B-cyanoalanine were assayed for the time-dependent appearance of ammonia. As a control, ammonia production from asparagine was also monitored. The enzymes catalyzing the formation of ammonia from B-cyanoalanine and asparagine respectively, are known as B-cyanoalanine hydrolase and asparaginase. The results in Table 7 show that whereas significant activity for the hydrolase enzyme could be measured in B-cyanoalanine-grown cells, this enzyme was essentially absent in TCN-grown cells. Similarly, while asparaginase activity was present regardless of the growth
Figure 10. Growth of *P. fluorescens* NCIMB 11764 in glucose (20 mM) minimal medium supplied with β-cyanoalanine as sole nitrogen. Cells were grown on 10 mM ammonium sulphate and washed three times in 50 mM Na-K phosphate buffer (pH 7.0) before providing a 1% (v/v) inoculum to flasks containing:

- ✔, (NH₄)₂SO₄ (10 mM);
- ✗, β-cyanoalanine (5 mM);
- ●, control (no fixed nitrogen)
Table 7. Activities of β-cyanoalanine hydrolase and asparaginase in cell-extracts of *P. fluorescens* NCIMB 11764

<table>
<thead>
<tr>
<th>Nitrogen source for growth</th>
<th>Enzymatic Activity (nmols min⁻¹ mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-CNAla hydrolase</td>
</tr>
<tr>
<td>TCN</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>β-CNAla</td>
<td>112</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>&lt; 5</td>
</tr>
</tbody>
</table>
conditions, no significant elevation in TCN-grown cells was observed. Thus, the lack of elevation of both enzymes after growth on cyanide suggested that β-cyanoalanine was not an intermediate.

C. Characterization of Cyanide Metabolic Products

Having demonstrated that cyanate and β-cyanoalanine were unlikely intermediates in cyanide metabolism, the focus of research was directed towards verifying earlier reports describing CO₂ and NH₃ as end products of cyanide transformation. For these experiments, a decision was made to concentrate the research effort exclusively on free cyanide (HCN or CN⁻) versus TCN. This was done to avoid complications due to the metal, and because other work in this laboratory had shown that the metabolism of TCN results in the formation of free cyanide (Silva-Avalos et al, 1990). Thus, it was hypothesized that a concentrated effort to identify the metabolites derived from free cyanide would be useful in determining how this toxic compound was assimilated.

Cultivation of Cells on Free Cyanide (KCN)

A problem encountered early on was that it was difficult to cultivate cells in batch mode because of high KCN toxicity. To avoid this KCN was added at low concentrations (0.125-0.25 mM), but this gave poor cell yields because of nitrogen limitation. In contrast, at
higher concentrations (e.g., 0.75 mM) growth was completely inhibited because of KCN toxicity. Since earlier studies had shown that a fed-batch procedure could be used to avoid excessive cyanide toxicity (Harris and Knowles, 1983), it was decided to adapt a modification of this procedure for the cultivation of cells. To accomplish this, cells were initially grown in batch mode on limiting ammonia (1 mM NH₄Cl) until all available nitrogen had been depleted (A₅40₅, 0.7; 48 h). This culture was then added (10% vol/vol) to a flask containing 1 liter of minimal medium supplied with glucose (20 mM) and KCN (0.25 mM), and the flask was incubated on a shaker at 30°C for 24 hours before a second addition of 0.25 mM KCN was made. This procedure was repeated once more (total cultivation time, 72 h, with KCN additions totaling 0.75 mM; see Figure 11) before cells were harvested. Under these conditions, the culture achieved a final turbidity of approximately 0.8 with the cell yield being about 0.3 (dry weight) grams per liter of medium.

A typical time course for the fed-batch cultivation of 11764 on KCN is shown in Figure 11. Although most of the initial KCN added was consumed in 10 hours, very little growth occurred. Growth commenced after this, until the culture appeared to become nitrogen limited (20 hours). At 24 h, 0.25 mM KCN was again added. This time, a shorter lag period (ca 5 hours) was observed before growth resumed. A similar relationship between substrate consumption and
Figure 11. Growth of *P. fluorescens* NCIMB 11764 in a modified glucose (20 mM) minimal fed-batch culture pulsed at the times indicated with 0.25 mM KCN as the sole nitrogen source. Symbols: □, growth; ▲, KCN consumption; ●, disappearance of KCN in an uninoculated control.
growth occurred when subsequent KCN additions were made (24 and 48 hours). The removal of KCN on successive additions proceeded at slightly elevated rates over that seen initially. Calculation from semi-logarithmic plots revealed a generation time of 8 hours for cells cultivated by this method.

Bioconversion of Cyanide by Washed Cell Suspensions

The approach attempted in trying to determine what the end products of cyanide breakdown might be, was to incubate washed cell suspensions with KCN and try to correlate its degradation with the appearance of metabolites. In initial experiments, reaction mixtures contained KCN supplied at 0.25-0.5 mM and cyanide-grown cells at 8 mg cell dry weight per ml. Under these conditions KCN was rapidly degraded, but ammonia, as measured by the indophenol method described in the Materials and Methods, could not be detected. When analogous incubations were performed with cells cultivated on ammonia, or with incubations made anaerobic by flushing with nitrogen, no cyanide degradation was observed. These results were found to be analogous to those reported earlier for NCIMB 11764 by Knowles (Dorr and Knowles, 1989), from which it was concluded that cyanide degradation is both an inducible and oxygen-dependent process.

Further attempts to detect ammonia as a reaction product were unsuccessful until it was discovered that raising the substrate concentration to a minimum value of 1
mM led to its accumulation. These experiments were complemented by simultaneous attempts to identify the nature of the carbon by-product from cyanide. To accomplish this, K$^{13}$CN was used as the substrate and incubation mixtures were analyzed by $^{13}$C-NMR spectroscopy. Although these experiments are discussed in more detail later, suffice it to say that at low K$^{13}$CN concentrations (e.g., 1 mM) no carbon by-products could be detected. For these reasons, it was decided to provide cell suspensions with higher KCN concentrations at which time it was discovered that cells were able to transform 10 mM KCN. Further impetus to increase the KCN concentrations even higher to characterize corresponding carbon by-products followed therefrom.

The kinetics of 10 mM KCN conversion in a reaction mixture supplied with cells at 40 mg ml$^{-1}$ (dry weight) are shown in Figure 12. The rapid removal of cyanide by KCN-grown cells was accompanied by the concomitant formation of ammonia produced in about 40% molar yield. From these kinetics it was estimated that the turnover of cyanide occurred at about 5 nmol min$^{-1}$ mg$^{-1}$ cell dry weight. At low substrate concentrations it was again observed that little or no degradation occurred when cells were cultivated on ammonia or when incubation mixtures were made anaerobic.

$^{13}$C-NMR Detection and Identification of Cyanide Bio-transformation Products

The finding that cells were capable of transforming
Figure 12. Biotransformation of 10 mM KCN by washed cell suspensions (40 mg ml\(^{-1}\) [dry weight]) of *P. fluorescens* NCIMB 11764. Symbols: □ and ▲, denote KCN consumption and ammonia formation respectively, by washed cells incubated aerobically after growth on cyanide as the sole nitrogen source; ■, KCN consumed by cyanide-grown cells when incubated anaerobically; ○, KCN consumed by ammonia-grown cells incubated aerobically.
high concentrations of KCN greatly facilitated the detection and identification of cyanide-conversion products using $^{13}$C-NMR. Initial efforts were made to confirm the identity of CO$_2$ as a metabolic end-product as suggested by previous findings (Harris and Knowles, 1983b; Harris et al., 1987; Knowles and Bunch, 1986). Various incubation mixtures supplied K$^{13}$CN at concentrations ranging from 5-50 mM were analyzed, and in all cases a chemical species identified as HCO$_3^-$ could be detected.

A typical $^{13}$C-NMR spectrum of products detected in an incubation mixture supplied 50 mM K$^{13}$CN is shown in Figure 13. Three chemical species, designated metabolites 1-3 were detected giving resonance signals of 160.49 ± 0.04, 166.96 ± 0.10 and 171.10 ± 0.10 ppm, respectively. A single resonance corresponding to the protonated form of cyanide (H$^{13}$CN) was detected at 123.18 ppm. Prolonged incubation of cells (6 h) revealed strong resonance signals at 171.10 ± 0.10 and 166.96 ± 0.10, proving the increased accumulation of metabolites 2 and 3. The chemical shift of cyanide varied somewhat since the ratio of protonated to ionic species (H$^{13}$CN / $^{13}$CN$^-$) is concentration dependent.

Metabolite 1 (160.49 ± 0.04 ppm) was identified as H$^{13}$CO$_3^-$ by comparison with an authentic standard as described in Materials and Methods. The H$^{13}$CO$_3^-$ standard was obtained by gassing phosphate buffer with $^{13}$C-enriched CO$_2$ (Figure 14). The $^{13}$C-NMR spectra were kindly provided by
Figure 13. $^{13}$C-NMR spectrum of reaction products
(Metabolites 1-3) generated from 50 mM (3.26 mg ml$^{-1}$) K$^{13}$CN
by a washed cell suspension (40 mg ml$^{-1}$ [dry weight]) of P.
fluorescens NCIMB 11764. After a 30 minute incubation
period, cells were removed by centrifugation and the
supernatant analyzed at 50 MHz. Figure insert: $^{1}$H-$^{13}$C
coupled spectra of metabolites 2 and 3 corresponding
respectively to biologically-produced formamide and formate.
Figure 14. $^{13}$C-NMR spectrum of $H^{13}CO_3^-$ obtained by gassing phosphate buffer with $^{13}$C-enriched CO$_2$.
Dr. M. Richmond, Chemistry Department, UNT, Denton, Texas.

Chemical shift values for metabolites 2 and 3 were shown to be identical to those obtained for authentic formamide (166.96 ± 0.10) and formate (171.10 ± 0.10). These values were further shown to be the same as those reported in the literature (Levy and Nelson, 1972; Stothers, 1972; White et al., 1988). Analysis in the gated decoupled mode further revealed doublet patterns for each of metabolites 2 & 3. This is consistent with the expected coupling between a single carbon and proton nucleus found in each compound. In addition, coupling constants (J_N-H, see Figure 13) were further found to be the same as those for authentic compounds and as described previously in the literature (Dorman and Bovey, 1973; Hinton and Ladner, 1972).

Biologically produced bicarbonate (metabolite 1), as expected, showed no proton-carbon coupling. While all three Cl-metabolites were routinely observed in incubation mixtures supplied 50 mM KCN, at lower substrate concentrations (5-10 mM) bicarbonate represented the major species present.

Further Identification of Metabolites by HPLC

In addition to 13C-NMR, routine quantitation of formamide and formate were done by using either colorimetric or enzymatic methods as described in Materials and Methods. Their presence in biological reaction mixtures was further confirmed by HPLC. Samples derived from incubation mixtures
supplied 50 mM KCN showed two species on HPLC having elution times of 33.3 and 18.3 minutes (Figure 15). Comparison of these elution times with those of authentic standards chromatographed under identical conditions, confirmed these as formate and formamide. Neither compound was detected when washed-cell incubations were performed with boiled cells (10 min at 95°C), or with cells incubated in the absence of substrate.

Recovery of Cyanide Transformation Products

The results obtained when cell suspensions were supplied different concentrations of KCN and quantitated for products are shown in Table 8. The time required for conversion, as might be expected, varied with the concentration of KCN supplied, but in each instance ammonia, formamide and formate could be detected. Calculations to determine the reaction stoichiometries revealed that 62.0 to 94.6% of the cyanide-nitrogen equivalents were recovered as ammonia and formamide. However, analogous efforts to account for the total amount of cyanide-carbon recovered (molar sum of formamide and formate) fell short of 100%. It was hypothesized that this might be due to the fact that CO₂ represented an additional reaction product as already had been indicated from separate ¹³C-NMR experiments (Figure 13). To further verify this and determine the relative yield of CO₂ in comparison with other products formed, some radiolabelling experiments were performed.
Figure 15. HPLC elution profile of a culture supernatant from a resting cell incubation of 50 mM KCN which was clarified at 6.3 hours. The concentration of cells in the incubation mixture was 40 mg ml$^{-1}$ dry weight.
Table 8. Recovery of cyanide-conversion products generated under aerobic conditions by P. fluorescens NCMB 11764

<table>
<thead>
<tr>
<th>KCN supplied (mM)</th>
<th>Incubation time (h)</th>
<th>KCN consumed (mM)</th>
<th>Products Recovered (mM)b</th>
<th>Elemental Recovery (Molar %)4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NH3</td>
<td>HCONH2</td>
</tr>
<tr>
<td>5.0</td>
<td>0.6</td>
<td>5.0</td>
<td>2.2</td>
<td>0.9 (20.6)</td>
</tr>
<tr>
<td>10.0</td>
<td>1.4</td>
<td>10.0</td>
<td>5.5</td>
<td>2.2 (22.6)</td>
</tr>
<tr>
<td>20.0</td>
<td>2.4</td>
<td>18.5</td>
<td>12.5</td>
<td>5.0 (30.7)</td>
</tr>
<tr>
<td>50.0</td>
<td>6.3</td>
<td>42.5</td>
<td>16.7</td>
<td>20.9 (67.0)</td>
</tr>
</tbody>
</table>

aIncubations performed in 15 ml serum-stoppered vials containing 1 ml cell suspension (40 mg ml⁻¹ dry weight) with air in the gas phase.
bResults expressed as mean values of three separate determinations made using analytical procedures described in the Materials and Methods. Numbers in parentheses refer to relative percent of total carbon recovered for each metabolite.
CO2 determinations represent theoretical conversion estimates based on results obtained from radiolabelling experiments as shown in Figure 16.
Molar %N = Σ molar recovery NH3 + HCONH2; Molar %C = Σ molar recovery HCONH2 + HCO3 + CO2.
Radiolabelling Experiments.

Separate incubations were performed with radiolabelled cyanide as a substrate, and the amount of radioactivity recovered in volatile (14CO2) and non-volatile (14C-formamide and 14C-formate) products were determined. For this purpose, reactions were allowed to proceed to near completion as ascertained from simultaneous colorimetric measurements for cyanide, and the amount of radioactivity recovered in different incubation fractions determined as explained in Materials and Methods. The results of these experiments are summarized in Figure 16. At 5 and 10 mm KCN approximately 70% of the available radioactivity was recovered as a volatile product, presumed to be CO2. In contrast, at 20 and 50 mM KCN, less CO2 appeared to be formed and most of the radioactivity (45 and 75%, respectively) was now present in the non-volatile fraction, presumed to contain labelled formamide and formate. On the basis of the results obtained from these experiments, it was possible to estimate the amount of non-radioactive CO2 produced in separate incubations where the amount of formamide and formate (plus ammonia) had already been determined chemically. When these values were incorporated into the data shown in Table 8 approximately 73-97.4 molar% (depending on the initial substrate concentration) of the cyanide-derived carbon equivalents could be accounted for in products that included CO2, formamide and formate.
Figure 16. Fractionation pattern of $^{14}$C-labelled reaction products formed from $^{14}$CN by washed cell suspensions of *P. fluorescens* NCIMB 11764. Results shown represent the mean of three separate determinations. Symbols: [ ] , center-well barium soluble; [ ] , center-well barium precipitate ($^{14}$CO$_2$); [ ] , main-compartment barium soluble; [ ] , main-compartment barium precipitate ($^{14}$CO$_3^-$); [ ] , cell pellet.
Effect of Reaction Conditions on Cyanide Conversion and Product Formation

An examination of reaction product yields revealed a significant difference in the amount of each metabolite formed at different substrate concentrations (Table 8). This difference was of particular interest when comparisons in the yields of C1-metabolites were made. For example, at 10 mM KCN the relative product ratio of formamide : formate : CO₂ was 23:4:74 percent. By comparison, at 20 and 50 mM KCN these ratios changed to 31:20:49 and 67:18:15 percent, respectively, thus reflecting the decreased yield of CO₂ at higher substrate concentrations. These results suggested that more than a single mechanism of cyanide conversion might be operative in 11764. It was hypothesized that the formation of CO₂ might require oxygen whereas the production of formamide and formate might not. It was also noted that washed cells catalyzed a slow, but measurable disappearance of cyanide under anaerobic conditions (Figure 12). At this stage, it seemed appropriate to: (i) determine whether prolonged anaerobic incubation might lead to the accumulation of any metabolites, and (ii) compare the relative product ratios with that obtained for cells incubated aerobically. To accomplish this, cyanide-grown cells were incubated anaerobically with KCN until approximately 90% of the available substrate had been consumed at which time reaction mixtures were analyzed for
metabolic products. The results of these determinations, summarized in Table 9, show that in each experiment the time required for complete conversion of KCN was considerably longer than for comparable aerobic incubations (Table 8), but in each instance, ammonia, formamide and formate could again be detected. No significant production of CO$_2$ (determined from simultaneous incubations performed with radioactive K$^{14}$CN), was observed. Mass balance calculations further revealed that essentially 100 molar% of both carbon- and nitrogen- cyanide equivalents could be accounted as formamide and formate, and ammonia and formamide respectively. Moreover, a comparison of product ratios with those obtained under aerobic conditions revealed a significant difference, particularly with respect to carbon metabolites. In this case, only trace amounts of CO$_2$ were produced while formamide and formate were present in almost equimolar proportions. The yields of ammonia were further found to exceed those observed aerobically, presumably reflecting enhanced accumulation due to lack of metabolism under anaerobic conditions.

**Metabolism of Formamide**

Experiments were conducted to determine whether formamide could be further metabolized, since it was detected as a cyanide conversion product. Initial experiments to address the issue of whether formamide could be a possible intermediate involved testing cells for the
Table 9. Recovery of cyanide-conversion products generated under anaerobic conditions by
P. fluorescens NCIMB 11764

<table>
<thead>
<tr>
<th>KCN supplied (mM)</th>
<th>Incubation time (h)</th>
<th>KCN consumed (mM)</th>
<th>Products Recovered (mM)</th>
<th>Elemental Recovery (Molar %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>HCONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>0.5</td>
<td>7.0</td>
<td>0.5</td>
<td>0.26</td>
<td>0.22 (54.2)</td>
</tr>
<tr>
<td>10.0</td>
<td>12.5</td>
<td>8.9</td>
<td>4.0</td>
<td>5.0 (56.2)</td>
</tr>
</tbody>
</table>

*Incubations performed in 15 ml serum-stoppered vials containing 1 ml cell suspension (40 mg ml<sup>-1</sup> dry weight) with air atmosphere having been replaced with N<sub>2</sub>.

*Results expressed as mean values of three separate determinations. Numbers in parentheses refer to relative percent of total carbon recovered for each metabolite.

*CO<sub>2</sub> determinations represent theoretical conversion estimates based on results obtained from separate radiolabelling experiments.

*Molar %N = Σ molar recovery NH<sub>3</sub> + HCONH<sub>2</sub>; Molar %C = Σ molar recovery HCONH<sub>2</sub> + HCO<sub>2</sub>H + CO<sub>2</sub>. 
ability to grow on formamide as a nitrogen source. These tests consistently gave negative results. Further attempts to show that formamide could be degraded by either whole suspensions and cell-extracts of cyanide-grown cells performed as described in the Materials and Methods also proved to be unsuccessful, even following prolonged incubation (10 h).
P. fluorescens NCIMB 11764 was chosen for study of cyanide metabolism because of its unusual capability of assimilating cyanide as a sole nitrogen source for growth (Harris and Knowles, 1983a; 1983b). It was proposed that cyanate might serve as a possible intermediate in this process, and the results of this work show that cyanate can support growth.

Cyanase synthesis in this organism is inducible, since the specific activity in cyanate (10 mM) grown cells was 168 nmols min\(^{-1}\) mg\(^{-1}\) as compared to less than 5 nmols min\(^{-1}\) mg\(^{-1}\) in ammonia grown cells. Moreover, cyanase activity was found to be bicarbonate dependent. These findings parallel those reported previously for inducible cyanase from E. coli (Anderson, 1980) and Flavobacterium sp. (Guilloton and Hargreaves, 1972) and strongly support the hypothesis that a similar enzyme is responsible for the growth of P. fluorescens NCIMB 11764 on cyanate. Moreover, activity was still present when cells were grown in either ammonia-containing minimal medium or the broth described by Lennox (Lennox, 1955) supplemented with cyanate, suggesting that no significant repression of cyanase synthesis by ammonia occurs.
Results of this study verify the earlier observations of cyanase (Harris and Knowles, 1983) and provide the first evidence for the existence of this enzyme in a representative member of the genus *Pseudomonas*. The induction of cyanase after growth on cyanate and the conversion thereof to ammonia by crude extracts provide strong evidence that this enzyme is responsible for cyanate utilization. However, a detoxification role cannot be excluded since induction by cyanate still occurred when cells were grown in excess ammonia.

The possibility of cyanate being involved in cyanide metabolism was explored by assaying for the enzyme cyanase in TCN grown and KCN grown cells. The inability of these cells to elaborate cyanase suggested that cyanate was an unlikely intermediate. Moreover, cyanate defective mutants which were unable to utilize cyanate could still utilize cyanide. These results prove that neither cyanate nor the enzyme cyanase were important in cyanide metabolism.

The involvement of a hypothetical oxygenase in cyanide metabolism was explored by attempting to measure oxygen uptake in stationary cultures that were pulsed with TCN. Although some increase in oxygen uptake could be measured in these cells, unequivocal proof for the involvement of this enzyme could not be demonstrated.

Further investigations have revealed that β-cyanoalanine could serve as a good source of nutritional
nitrogen for 11764 and β-cyanoalanine degrading activity was shown to be responsible for this ability. Lack of this activity in cyanide grown cells eliminated the possibility of this compound occurring as an intermediate in cyanide metabolism.

Earlier studies of cyanide metabolism by *P. fluorescens* NCIMB 11764 conducted by Knowles and colleagues (Harris and Knowles, 1983a; 1983b), reveal that the cultivation of cells on cyanide as the sole nitrogen source could be achieved under fed-batch conditions in which the concentration of cyanide was not allowed to exceed toxic levels. A related fed-batch procedure for the cultivation of cells was also used in the present work as illustrated in Figure 11. This procedure, based on carefully-timed additions of KCN to batch cultures, resulted in the rapid consumption of cyanide, but growth, as measured by increases in cell density, lagged somewhat behind. These growth kinetics implied that several metabolic events may be involved in the assimilation of cyanide as a growth substrate, which deserved further investigation. Earlier reports (Harris and Knowles, 1983a; 1983b) that cyanide was converted to ammonia during growth could not be duplicated, probably because once formed, ammonia is rapidly metabolized and does not accumulate.

Although ammonia accumulation during growth was not observed, its identification as a biological reaction
product was successfully demonstrated in washed-cell experiments. Under these conditions yields of ammonia ranging from 40 to almost 70 molar % were observed (Table 8). Its formation was further shown to occur in concert with cyanide consumption as illustrated in Figure 12 for a reaction mixture supplied 10 mM KCN. These findings are therefore consistent with earlier reports describing ammonia as an end-product of cyanide conversion by strain 11764. Its identification further helps to explain how cyanide can serve as a provisional nitrogen source since ammonia is readily assimilated by this organism.

Additional experiments with washed cell suspensions revealed that cyanide-grown cells of 11764 were capable of catalyzing the conversion of cyanide at concentrations far exceeding those described in previous investigations. For example, KCN supplied at concentrations as high as 100 mM (6,510 ppm) were removed from incubation mixtures. Preliminary indications are that cyanide is degraded at rates approaching 2-5 nmol min\(^{-1}\) mg\(^{-1}\) cell dry weight, depending on the substrate concentration. Results which showed that conversion was markedly reduced under anaerobic conditions and did not occur when cells were cultivated on ammonia (Figure 12) are consistent with earlier observations (Harris and Knowles, 1983a; 1983b; and, Knowles and Bunch 1986) from which it was concluded that cyanide transformation by 11764 is both an inducible and oxygen-
dependent process. The identification of CO\textsubscript{2} as the major carbon-containing conversion product by both \textsuperscript{13}C-NMR (Figure 13) and radioisotopic trapping experiments (Figure 16) also supports previous work in which this compound was identified as a major reaction product. However, this work has now shown that CO\textsubscript{2} and ammonia are not the only products of cyanide conversion by this organism. In addition, formamide and formate were also identified by \textsuperscript{13}C-NMR spectroscopy (Figure 13), HPLC analysis (Figure 15), and other analytical methods. This discovery taken together with data on product stoichiometries determined under different reaction conditions (Tables 8 and 9), provide strong evidence that additional pathways besides the putative oxygenase-mediated mechanism proposed previously (Dorr et al., 1989; Harris et al., 1983b; and, Harris et al., 1987) can be elaborated by strain 11764. Therefore, three possible mechanisms of cyanide conversion by this organism as shown in Figure 17 are proposed. The first of these is consistent with the idea that CO\textsubscript{2} and ammonia represent reaction products formed by an oxygen-dependent pathway. Evidence in support of this comes from the finding that CO\textsubscript{2} was the major Cl-metabolite detected in aerobic incubations supplied relatively low concentrations of KCN (0.5-10 mM) (Table 8). In contrast, at higher KCN concentrations (20 & 50 mM) formamide and formate were principally formed, indicating that the route to CO\textsubscript{2} under
Figure 17. Metabolic pathways of cyanide conversion by *P. fluorescens* NCIMB 11764.
\[
\begin{align*}
2H_2O &\rightarrow HCO_2 + NH_3 \\
HCO_2 + NH_3 &\rightarrow NH_3 + CO_2 \\
O_2 &\rightarrow\text{Assimilation} \\
\text{Assimilation} &\rightarrow HCO_2 + NH_3 \\
HCO_2 + NH_3 &\rightarrow NH_3 + CO_2 \\
HCO_2 + NH_3 &\rightarrow HCONH_2
\end{align*}
\]
these conditions is less significant. The possibility that high concentrations of KCN may also inhibit the formation of CO₂ also exists.

The two alternative pathways proposed include, (i) conversion to formate and ammonia, and (ii) conversion to formamide. In addition to the detection of both formamide and formate in aerobic incubations, these compounds represented essentially the only carbon-derived products when cells were incubated with KCN anaerobically (Table 9). These findings therefore indicate that 1764 can metabolize cyanide by mechanisms that are oxygen-independent. The fact that both formamide and formate were always simultaneously present in incubation mixtures, as opposed to the detection of one metabolite over the exclusion of the other, points toward the presence of two separate pathways of conversion for each. Furthermore, inability to demonstrate that formamide could support growth or was metabolized by cell suspensions and cell-extracts of cyanide-grown cells, further suggests that it accumulates as an end-product and is not further metabolized. Thus, rather than being a precursor of formate, formamide is thought to arise by separate mechanism as depicted in Figure 17.

This work also demonstrates that bacteria may be able to degrade cyanide by several mechanisms thereby providing a possible selective advantage for both detoxification and nutritional assimilation of this otherwise toxic compound.
Subsistence on cyanide as a sole nitrogen source by *P. fluorescens* NCIMB 11764 appears to be relatively straightforward involving chemical transformation to ammonia. However, the enzymatic basis of this process is still only marginally understood. Aerobic conversion of cyanide to CO₂ and ammonia could very well be mediated by an oxygenase-type enzyme as earlier proposed, but unequivocal proof for this is yet to emerge. The pathway leading to formate could be catalyzed by an enzyme formally resembling a nitrilase (EC 3.5.5.1) involving direct cyanide hydrolysis. It is worth noting that, this conversion is similar to that recently described for *Alcaligenes xylosoxidans* subsp *denitrificans*, for which the enzyme description cyanidase was proposed (Ingvorsen et al., 1991), and that reported for the cyanide-utilizing *Pseudomonas* species isolated by White et al. (1988). Finally, conversion to formamide is analogous to cyanide transformations described in phytopathogenic fungi mediated by the enzyme cyanide hydratase (EC 4.2.1.66) (Fry and Myers, 1981; Knowles, 1988). This work shows that bacteria might also be able to carry out this transformation.
CHAPTER V

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94


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