# EFFECT OF CYANIDE ON THE GROWTH OF *KLEBSIELLA* SPECIES AND *BACILLUS* SPECIES

By

GEMMA LYNN BRUEGGEMANN

Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

1975

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
December, 1977

EFFECT OF CYANIDE ON THE GROWTH

OF KLEBSIELLA SPECIES AND

BACILLUS SPECIES

Thesis Approved:

Elizabeth T. Gandy
Thesis Adviser

N. F. Gandy Jr.

Norman N Durham

Norman N Durham
Dean of the Graduate College

## ACKNOWLEDGMENTS

The author sincerely appreciates the following individuals and organizations for their assistance during the course of this investigation.

Dr. Elizabeth Gaudy, for her friendliness, concern and time; her guidance and helpful suggestions as thesis adviser are truly appreciated. Dr. Anthony Gaudy, Jr., for serving on the Advisory Committee and for originally creating in me an interest in this investigation. Dr. N. N. Durham, for serving as a member of the Advisory Committee.

Yuan-Jenn Feng, for his collaboration on the investigation and his helpful discussions; his assistance with methods and with obtaining materials for this investigation are gratefully appreciated. Crosby Jones, for being a friend and for constantly taking time to discuss problems and results of this investigation. Marianne McKeeman, for carefully preparing the figures for this thesis and for her enlightening friendship. Charlene Fries, for her expert ability in typing the final draft of this thesis.

Financial aid was given by the Department of Microbiology and the School of Biological Sciences, Oklahoma State University, in the form of a graduate teaching assistantship for two years. Also, financial assistance was received in the form of a traineeship from the Environmental Protection Agency, U.S. Department of the Interior, through a graduate training grant in water supply and water pollution control

(T-900078) to Dr. Anthony F. Gaudy, Jr. This financial assistance is greatly appreciated.

My father, Mr. W. E. Brueggemann, for his genuine concern and interest and just for being there.

The author is indebted to her mother, Mrs. Deborah Brueggemann, for her confidence and encouragement at a time in her life when she most needed my encouragement and time. Although she died shortly after this investigation was undertaken, her confidence and encouragement was an inspiration to its completion.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

# CHAPTER I

## LITERATURE REVIEW

The major source of cyanide in the environment is industrial wastes. Hydrocyanic acid and cyanide salts are used as fumigants, in insecticides, for case hardening steel, and in electroplating solutions. Cyanide wastes are a particular and major concern of the plating industry.

Cyanide ion is an extremely toxic compound. Very small amounts have been reported to be fatal to man and fish. White et al. (1973) explained that the high toxicity of cyanide was due to the fact that cyanide ion in low concentrations combined rapidly with the oxidized ferric form of cytochrome oxidase. Cyanide is not specific for cytochrome oxidase, but combines rapidly with other metal-requiring enzymes such as peroxidases and catalases. The extreme reactivity of cyanide makes it potentially hazardous to many different organisms.

Most of the methods employed in determining a permissible concentration of cyanide in water have involved the toxicity of the compound to fish. From various studies with Ohio River water, the Aquatic Life Advisory Committee (1960) recommended that free cyanide not exceed 0.025 mg/ℓ. Concentrations in excess of this amount were considered unsafe for fish life. As pointed out by the Committee, it is difficult to establish a standard concentration of cyanide in water because of the varying characteristics which influence toxicity such as temperature, dissolved oxygen, pH, total alkalinity, hardness, and other dissolved

materials. Because of the obvious hazards involved in the release of wastewaters containing cyanide, a standard concentration of 0.01 mg/ℓ was recommended by the U.S. Public Health Service (1962); the maximum allowable concentration was set at 0.2 mg/ℓ.

It has been known for some time that the presence of cyanide in water has an inhibitory effect on the biological activity of the system. In studying the effect of cyanide on the inhibition of BOD, Ludzack et al. (1951) obtained variable results, but they found that concentrations as low as 0.3 mg/ℓ had inhibitory effects on the seed organisms. For reasons of this nature, industry has avoided the cheaper biological treatment of cyanide wastewater and has relied on expensive chemical methods of treatment. The methods generally employed are chemical oxidations. Three chemicals that have proven to be satisfactory for the oxidation of cyanide are ozone, chlorine and permanganate.

Dodge and Zabban (1951) found that hypochlorite oxidation of cyanide requires a pH greater than 10.0 in order to insure formation of cyanate and not the highly toxic cyanogen chloride. Weber (1972) suggested that the less toxic compound cyanate should be further oxidized to carbon dioxide and nitrogen. This is not normally practiced because of the high cost. It is thought possible that cyanate can be reduced back to the cyanide ion, but Resnick et al. (1958) found that under natural conditions no measurable amount of cyanide ion was formed from cyanate. When necessary, further oxidation requires a lower pH and, as suggested by Dodge and Zabban (1951), this oxidation can be carried out by further addition of hypochlorite or acid hydrolysis.

Ozonation is similar to chlorination in treating cyanide wastes. It completely oxidized cyanide to carbon dioxide and water.

Permanganate oxidized cyanide to cyanate at pH 12.0 to 14.0, according to Weber (1972). This method is not generally used by industry.

Chemical oxidation has its advantages and disadvantages, with the main disadvantage being the cost. Murphy and Nesbitt (1964) estimated the cost of biological treatment of industrial cyanide waste in an established plant to be half the cost of chemical treatment by alkaline chlorination.

The high cost of chemical treatment has sparked a number of investigations into the biological treatment of industrial wastes. By far, activated sludge has been the most popular method in determining the feasibility of biological methods for treating cyanide wastes. Nesbitt et al. (1959) fed two no-waste activated sludge systems 60 mg and 120 mg $CN^-$/day. These units were able to remove 99+ % of the cyanide and 97.9 % was reported as being converted to carbon dioxide. Growth was slow and erratic at times, but the suspended solids were maintained.

Ludzack et al. (1959) determined the effect of nitriles on an activated sludge unit. The sludge was easily acclimated to the various nitriles tested and the system behaved like conventional systems. Later Ludzack and Schaffer (1962) reported that, after acclimation, activated sludge successfully degraded cyanide, cyanate and thiocyanate, and was effective on cyanide concentrations below 60 mg/ℓ. Slug doses disrupted the system and small changes in concentration had a slight disruptive effect. The addition of extra nutrients as energy source improved degradation of cyanide and cyanate, and the sludge became acclimated more rapidly.

Murphy and Nesbitt (1964) successfully treated a synthetic cyanide waste by both extended aeration and sludge wasting. The maximum cyanide

loading was 50 mg $CN^-$/g MLVS/hr. Ninety-nine percent or more of the cyanide was degraded. Sludge wasting was considered by Murphy and Nesbitt (1964) to be the best of the two methods because it was not as subject to biological failure and could handle shock loads better than could extended aeration. They concluded that an activated sludge unit could handle an industrial cyanide waste adequately if a supplement of domestic sewage was added.

Winter (1962) isolated some actinomycetes from a filter that had previously been treated with cyanide. These organisms were then established on laboratory scale trickling filters and acclimated to cyanide. The organisms had a degradation efficiency of 90% or more and the filters were fed 45 to 105 mg/ℓ KCN. The same systems were able to tolerate high metal concentrations from plating wastes.

Gurnham (1955) was able to develop a trickling filter tolerant to a cyanide concentration of 200 mg/ℓ, but the tolerance had to be built up slowly. At this high concentration the filter was not efficient. A 75 to 80% reduction of cyanide was obtained when the cyanide concentration was kept below 100 mg/ℓ.

After testing the effects of nitriles on anaerobic digestion, Ludzack et al. (1959) did not recommend this method for treating organic cyanide wastes. Lacto- and acrylonitrile were very toxic to the system, while the system could become acclimated to other nitriles tested. Most researchers have reported that anaerobic treatment of cyanide waste is not as effective as aerobic treatment.

Painter and Ware (1955) were probably one of the first to describe a bacterium able to utilize cyanide as a sole source of carbon and nitrogen. They isolated this organism, which they classified as an

actinomycete from a percolating filter being fed potassium cyanide and sewage. The isolate was able to grow slowly on a silica gel medium containing potassium cyanide and traces of iron, copper and phosphate.

Youatt (1954) preceded Painter and Ware in reporting an organism capable of using a cyanogenic compound. Youatt found that Thiobacillus thiocyanoxidans used thiocyanate as a source of energy, carbon and nitrogen. In this case the thiocyanate was first hydrolyzed to cyanate and sulphide. Cyanate was further metabolized to $CO_2$ and ammonia, while the sulfide was oxidized to sulfate.

A little over a decade later, Allen and Strobel (1966) exposed mycelial mats of various fungi to 1.0 μcurie of $K^{14}CN$. Alanine was radioactive in all three species of Pholiota tested, in Rhizopus nigricans, and in Marasmius oreades. In Fusarium nivale, asparagine was the only radioactive amino acid. Of the organisms tested, Pholiota aurivella was most capable of fixing the cyanide.

Strobel (1967) presented strong evidence that the soil might contain organisms capable of converting cyanide carbon and nitrogen to carbonate and ammonia, respectively. Strobel treated soil samples with doubly labeled cyanide ($^{14}C^{15}N$) and found that those soils most able to metabolize cyanide were those coming from areas supporting plants containing cyanogenic glycosides.

From one of these types of soils, Skowronski and Strobel (1969) islated a strain of Bacillus pumilus capable of metabolizing cyanide. The organism was grown in minimal medium with $10^{-1}$M KCN as the sole source of carbon and nitrogen. Labeling studies supported evidence that the KCN was being metabolized and converted to $CO_2$ and $NH_3$.

Castric and Strobel (1969) isolated Bacillus megaterium in the same manner in which Skowronski and Strobel (1969) isolated Bacillus pumilus. Bacillus megaterium was grown in a much richer medium which contained trypticase soy broth with 1% glucose and 1mM KCN. The cyanide disappeared from the medium during active growth of the Bacillus. Labeling studies suggest that Bacillus megaterium in vivo and in vitro can produce asparagine and aspartic acid from cyanide and serine. They proposed the following pathway:

$$\text{Serine} + \text{HCN} \longrightarrow \beta\text{-cyanoalanine}$$

$$\beta\text{-cyanoalanine} + H_2O \longrightarrow \text{Asparagine}$$

$$\text{Asparagine} \xrightarrow[H_2O \quad NH_3]{} \text{Aspartic acid.}$$

There are a number of other organisms that as yet are not known to metabolize cyanide, but are resistant to cyanide. Moeller (1954) developed a KCN medium which was used to distinguish members of the Enterobacteriaceae. The medium is best suited for distinguishing Salmonella and Citrobacter. Munson (1974) made improvements in the medium and from his experiments Citrobacter, Klebsiella, Enterobacter, and Proteus seemed to be the most resistant species.

Sadasivam (1974) was able to isolate two bacteria, a Streptomyces species and two fungi, an Aspergillus species and Rhizopus nigricans, which were resistant to cyanide ion. The fungi showed tolerance at 200 mg/ℓ while the bacteria were tolerant at 50 mg/ℓ.

Some microorganisms are assumed to be tolerant to cyanide due to the fact that they produce small amounts of HCN which do not seem to affect their growth. Clawson and Young (1913) were probably the first to report such a bacterium. Bacillus pyocyaneus could produce HCN when

grown on medium containing a protein source. Besides this Bacillus, several Pseudomonas species have recently been shown to produce HCN. Homogenates of a Pseudomonas species were prepared by Wissing (1975) and tested in the presence of glycine for cyanide-producing activity. This activity was greatest in the fine particle fraction which contained mainly cytoplasmic membranes. An increase in activity was noted when phenazine methosulphate and FAD were added along with glycine to the preparations.

Castric (1975) found that Pseudomonas aeruginosa strain 9-$D_2$ produced HCN immediately after the active growth period. Protein synthesis was a prerequisite to this process. The synthetic medium in which this bacterium was grown contained glutamic acid, methionine and glycine. When glycine was lacking, there was an 80% decrease in cyanide production. Castric hypothesized that this cyanogenic process regulated glycine concentrations and avoided cell damage at the end of active growth.

Later, Meganathan and Castric (1977) found that an optimum inorganic phosphate concentration was required for biosynthesis of HCN by a strain of Ps. aeruginosa. The optimum phosphate concentration was 1-10 mM. They speculated that other concentrations favored secondary metabolites or disrupted synthesis of the enzyme involved.

Freeman et al. (1975) were able to show that Ps. fluorescens also could produce HCN.

Chromobacterium violaceum has been the subject of a number of experiments involving the organism's ability to produce and incorporate cyanide. Sneath (1955) grew a heavy culture of Chromobacterium violaceum on nutrient medium and blood agar and observed the production of HCN. A decade later Michaels and Corpe (1965) determined that cyanide ion in the

medium had no effect on viability or cell yield of Chromobacterium violaceum. They established that glycine and methionine together in the medium resulted in greater cyanide production than either amino acid alone. Glycine and methionine were believed to act as methyl donors in the production of cyanide.

Brysk et al. (1968) further investigated the production of HCN by C. violaceum by determining the fate of the cyanide. β-Cyanoalanine was the product produced by C. violaceum when incubated with serine and $K^{14}CN$. The radioactivity was localized in the cyano-carbon. The function of methionine and other methyl donors was obscure, but their presence did increase β-cyanoalanine production.

Michaels et al. (1965) and later Brysk et al. (1969) clearly demonstrated that C. violaceum, when grown with methionine and $^{14}C$ -labeled glycine, was able to produce $^{14}C$ -labeled cyanide from the methyl carbon of glycine. Methionine could be replaced by other methyl donors in this system. Brysk et al. (1969) proposed that this cyanogenic process was a means of disposing of excess amino acid.

Later, Brysk and Ressler (1970) found a new product from cyanide incorporation by C. violaceum. Young cells reinoculated into fresh glutamate salts medium were found to produce γ-cyano-α-aminobutyric acid along with the usual products, cyanide and β-cyanoalanine. When glutamate was replaced in the medium by other amino acids, serine, threonine and aspartic acid were found to be involved in the production of γ-cyano-α-aminobutyric acid. Ressler et al. (1973) tested cell-free extracts of Chromobacterium violaceum with several amino acids, including methionine and aspartic acid, and found that only homocysteine was

involved as a cosubstrate in the production of γ-cyano-α-aminobutyric acid.

Not until recently did Niven et al. (1975) propose a basis for the resistance of C. violaceum to cyanide. They explained this resistance as being due to a terminal branching of the respiratory system of the bacterium. One branch is sensitive and the other is resistant. The branch was described as coming after the antimycin A and 2-heptyl-4-hydroxyquinoline-N-oxide inhibition sites.

Mizushima and Arima (1960) worked with Achromobacter and its resistance to cyanide. Achromobacter was able to adapt physiologically to $10^{-3}$M cyanide. Cell-free preparations made from adapted and nonadapted cells were examined for their cytochrome content. Greater amounts of $a_2$ (and $a_1$) were found in the adapted cell preparation. In further studies with Achromobacter, Arima and Oka (1965) and Oka and Arima (1965) found the main respiratory pigments to be cytochromes $b_1$ and o under heavy aeration. In the presence of cyanide, the electron transport system was shown to contain cytochrome b, more cytochrome $a_1$ and $a_2$, and less cytochrome o. They explained that cytochrome $a_2$ was induced by cyanide and was resistant to high concentrations of cyanide.

The slight resistance to cyanide of Azotobacter vinelandii was attributed by Kauffman and Van Gelder (1973, 1974) to a minor respiratory pathway to oxygen. Respiratory particles of Azotobacter vinelandii in the presence of cyanide showed the disappearance of oxidized cytochrome d. They concluded that cyanide binds to the oxidized conformation of cytochrome d, but they also found a residual activity of 5% in the respiratory particles. This residual activity was due to a b-type cytochrome involved in a minor pathway to oxygen.

Several other microorganisms have cytochrome systems which provide them with resistance to cyanide. Through experiments by Mizushima et al. (1959), the ability of Aerobacter cloacae to adapt to $10^{-3}$ M cyanide was attributed to a terminal oxidase system which was insensitive to cyanide. Later, McFeters et al. (1970) described the resistance of a strain of Bacillus cereus to $10^{-3}$ M cyanide ion. In cells grown in the presence of cyanide there was a 50% increase in cytochrome b and a twofold increase in an a-type cytochrome. Cyanide also seemed to decrease the number of active electron transport chains.

Weston et al. (1974) cited evidence of a terminal branching of the respiratory system of Beneckia natriegens. One branch was resistant to 10 μM cyanide.

Escherichia coli cannot be omitted from the list of organisms resistant to cyanide. Blumenthal-Goldschmidt et al. (1965) described an enzyme found in extracts of E. coli $K_{12}$ which is capable of catalyzing the formation of β-cyanoalanine from serine and cyanide. β-Cyanoalanine could also be formed from cysteine and cyanide.

Pudek and Bragg (1974) studied the respiratory particles of E. coli. They found that cytochrome o is prevalent during log growth and that cytochrome d is predominant during the stationary phase. The respiratory particles containing more cytochrome d than cytochrome o were less sensitive to inhibition by cyanide.

Ashcroft and Haddock (1975) discovered that E. coli, when grown in the presence of low concentrations of cyanide and an oxidizable substrate, synthesized alternate membrane-bound redox carriers. They suggested that the carriers normally present are inhibited by cyanide; therefore, it was necessary for the cells to produce alternate carriers.

Most of the bacteria previously studied overcome inhibition to cyanide by metabolizing it or by some sort of resistant electron transport system. Nazar and Wong have discovered a different approach that E. coli uses in overcoming cyanide inhibition. Nazar and Wong (1969) were experimenting with inhibitor-induced shift-down response in E. coli. They observed a four- to fivefold decrease in cell growth and inhibition of RNA synthesis in the presence of 25 μM cyanide. Methionine was able to overcome this inhibition. Other amino acids were studied to determine whether they could overcome the cyanide inhibition. Aspartic acid, histidine and cystine had very slight effects while others showed no effect. Nazar and Wong (1973) found that cyanide results in excess accumulation rather than loss of triphosphates which may serve to control RNA metabolism. Nazar and Wong added methionine to E. coli cells treated with cyanide. The cells took up the methionine rapidly and RNA synthesis increased immediately. Methionine also increased DNA and protein synthesis and growth of the cyanide-treated cells. Small amounts of methionine had a short-lived effect as the methionine was used up. A study of the cyanide-treated cells showed a drop in intracellular methionine. Nazar and Wong observed this stimulatory effect in E. coli strains $K_{12}$ $leu^-$ and $15T^-$ and not in strains B or $K_{12}W6$. They speculated that the reason for the drop in methionine with the addition of cyanide was due to formation of a complex of cyanide with vitamin $B_{12}$. Vitamin $B_{12}$ is a cofactor of a transmethylase involved in the last step of methionine biosynthesis in E. coli. Methionine is important in the cell in the initiation of protein synthesis. It is reasonable that anything involved in inhibiting the synthesis of methionine would also inhibit important processes in cell growth.

It is interesting to note that in most pure culture studies, there was a requirement for a nutrient-rich medium. The Bacillus megaterium isolated by Castric and Strobel (1969) required a complex medium of trypticase soy broth plus 1% glucose for metabolizing the 1mM KCN present.

Mizushima and Arima (1960), and Arima and Oka (1965) both employed a rich bouillon medium containing meat extract and peptone for their studies on cyanide resistance of Achromobacter.

McFeters et al. (1970) used trypticase soy broth plus 0.3% yeast extract for growing Bacillus cereus in the presence of cyanide.

These bacteria may require rich media for growth, but possibly some of these nutrients aid bacterial resistance to cyanide. For instance, Nazar and Wong (1972) found with E. coli that the addition of methionine to a cyanide medium stimulated cell growth and protein synthesis. Only with methionine present were the cells able to grow in the presence of cyanide.

In activated sludge systems both Ludzack and Schaffer (1962) and Murphy and Nesbitt (1964) found that added nutrients enhanced the ability of the system to degrade cyanide. Ludzack and Schaffer added dextrose to their unit for quicker acclimation and improved their operation with the addition of fish food containing 40% protein. They suggested that industrial cyanide waste be supplemented with domestic sewage and treated biologically.

Many researchers encountered a lag period when they grew various microorganisms in media containing cyanide. While studying the effects of cyanide on biological oxygen demand, Ludzack et al. (1951) found that cyanide caused an initial lag before the microorganisms present could

exert a BOD. The lag period varied with cyanide concentration and the condition of the seed organisms.

Zintgraff et al. (1969) reported similar results. In their study measuring oxygen uptake by a heterogeneous population, the lag period increased with increasing cyanide concentration. They also found that increased seed concentration decreased the lag period. They strongly supported the hypothesis that the cyanide saturated the active sites in cytochrome oxidase and that the lag was a result of overcoming the inhibition of this enzyme.

Feng (1977) had a 15 hour lag period before a heterogeneous batch system containing cyanide was able to reach a substrate removal efficiency of 83% while it took only 3 hours for a system without cyanide.

Mizushima and Arima (1960) encountered a 3 hour lag before Achromobacter would start growing in $10^{-3}$ M cyanide. This lag period was quite short compared to the lag period reported by Mizushima et al. (1959) for Aerobacter cloacae and by Castric and Strobel (1969) for Bacillus megaterium. Aerobacter cloacae had a 7 to 8 hour lag period while Bacillus megaterium had a lag of 10 to 12 hours before there was evidence of growth.

Cyanide is truly a toxic compound and an inhibitor of microbial growth but, as pointed out here, this is not always the case. Nesbitt et al. (1959), Ludzack and Schaffer (1962), and Murphy and Nesbitt (1964) have all reported success in treating cyanide wastes by various activated sludge processes, as has Feng (1977). Feng operated a bench-scale extended aeration pilot plant with a "hydrolytic assist." The extended aeration process functions to purify wastewater biologically and digest sludge aerobically. According to Gaudy et al. (1971), the "hydrolytic

assist" functions as an aid to aerobic sludge digestion. The "hydrolytic assist," as applied to the pilot plant operated by Feng, consisted of withdrawing 900 ml of sludge once every week. This sludge was hydrolyzed by acidifying to pH 1.0 with concentrated $H_2SO_4$, autoclaved for five hours at 15 psi, 120°C, and then neutralized to pH 7.0 with 10 N KOH. The resulting hydrolysate was fed to the unit along with a minimal medium containing 10 mg/ℓ $CN^-$ which was later raised to 15 and then 20 mg/ℓ $CN^-$. Besides determining solids, COD, $NO_3^-$ and $NH_3$ throughout the 500 days of operation, Feng also measured the cyanide concentrations in the sludge and in the effluent. He reported excellent results for the performance of his unit. When cyanide concentrations were increased, there was a transition period where COD removal efficiency dropped and $NH_3$ in the effluent increased and then the system returned to "normal." Throughout his study the effluent contained no cyanide to less than 0.1 mg/ℓ cyanide and the sludge on the average contained only 1 mg/ℓ cyanide.

The present investigation was undertaken in an attempt to isolate organisms from Feng's unit that might metabolize $CN^-$. Since $CN^-$ was not present in the effluent and only 1 mg/ℓ was found in the sludge while the unit was being fed 10 mg/ℓ $CN^-$, it seemed reasonable that such bacteria might be present.

# CHAPTER II

# MATERIALS AND METHODS

## Media

The medium used in most experiments was a minimal medium, M-9 of Dulbecco (1950). The medium was composed of: $NH_4Cl$, 1.0 g; $KH_2PO_4$, 3.0 g; $Na_2HPO_4$, 6.0 g; NaCl, 0.5 g; $MgSO_4$, 0.1 g; glucose, 4.0 g; and distilled water, 1000 ml. At times yeast extract (Difco), 0.5 g, was added. The phosphates were autoclaved together as were the ammonium and sodium salts. All other ingredients were autoclaved separately and then aseptically mixed together.

Variations on the M-9 medium were made by the addition of extra nutrients. Besides yeast extract (Yex), hydrolysate from Feng and the Bioenvironmental Laboratories, and various amino acids and mixtures of amino acids were added. The amino acid mixture was composed of 0.01 g of each of the following amino acids: L-aspartic acid, L-asparagine·$H_2O$, L-alanine, L-arginine·HCl, cysteine hydrochloride, L-isoleucine, L-glutamic acid, glycine, L-histidine mono-HCl mono-hydrate, L-leucine, L-lysine·HCl, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine in 20 ml of distilled water. In addition to the amino acid mixture, individual solutions of each amino acid were also used. Each amino acid stock solution was made with 0.01 g amino acid per 10 ml distilled water.

Cyanide was added in solution form at the start of the experiments, unless stated otherwise. Stock solutions were made fresh before the experiment at either 1000 or 10,000 mg/ℓ $CN^-$.

Tryptic soy broth (TSB), nutrient broth (NB) and tryptic soy agar (TSA) plates and slants were prepared by rehydrating the powdered media (Difco) with distilled water. The plates contained 25 ml of medium.

The buffered salts solution used in isolation procedure was based on the M-9 medium. All ingredients were the same except that the nitrogen source, $NH_4Cl$, and the carbon source, glucose, were omitted. Potassium cyanide was added at the time of inoculation as a sole source of carbon and nitrogen.

## Cultivation of the Organisms

All test organisms were stored at room temperature and carried on TSA slants. The test organisms were transferred once every two months to fresh TSA slants.

For most experiments the test organism was grown up overnight in the appropriate medium without cyanide at 30°C on a reciprocal shaker. When cells were grown in minimal medium, they were transferred from the minimal medium to fresh medium containing 10 mg/ℓ $CN^-$, and grown up overnight. Later they were allowed to grow for a period of 14 hours.

## Identification of Organisms

For identification of the two organisms which were used in most of the experimental work, the following differential media were used: sucrose, lactose, glucose, xylose, glycerol, and mannitol fermentation broths, and nitrate, urea, Koser's citrate and tryptone broths. All the

above media contained Durham tubes for detection of gas except urea, citrate and tryptone. All fermentation media were made with Bromthymol-blue or Bromcresol purple as pH indicators. Other media used were methyl red-Voges Proskauer (MRVP), litmus milk, Kligler's iron agar slants, and plates of starch, gelatin and tributyrin. All incubations were at 30°C. All readings were taken at 24 and 48 hours except for litmus milk, nitrate, and the methyl red portion of MRVP. The litmus milk was read at 1, 2 and 7 days, while the nitrate and methyl red broths were tested at 7 days. Additional information on the organisms was obtained from Gram stains, wet mounts, spore stains, India ink wet mounts, and the catalase reaction. Difco's decarboxylase medium base plus 1% lysine, arginine or ornithine was used for identification of one of the test organisms. The eighth edition of Bergey's Manual (1974) and part A of Identification Methods for Microbiologists (Gibbs and Skinner, 1966) were consulted for identifying the two isolates.

## Growth Measurements

For measuring the growth of all organisms in this study the organism was transferred to side arm or top arm flasks containing the appropriate medium. These flasks were incubated at 30°C in a Warner-Chilcott shaking water bath at 104 cycles/minute with a 1" stroke length. Growth was followed on a Coleman Junior Spectrophotometer at 540 nm. Specific growth rate was determined by plotting absorbance vs. time on semi-logarithmic paper. The growth rate $\mu$ was calculated from the graph using the equation $\mu = 0.693/t_d$, where $t_d$ is the doubling time.

## Cyanide Determination

Three different methods were used for determining cyanide concentrations. The first method employed was Skowronski and Strobel's (1969) modification of Kolmer and Bociner's picric acid test. A fresh stock solution of KCN was measured in various quantities and added to tubes matched for the spectrophotometer. In addition to KCN, 2 ml of 2% KOH, 1 ml of picric acid: $Na_2CO_3$: $H_2O$ (1 : 5 : 200 w/w/v) and enough distilled water or media was added to bring the volume in the tubes to 8 ml. The tubes were then incubated for 10 minutes in a 37°C water bath and transferred to a 4°C refrigerator for 20 minutes. After 20 minutes the tubes were read in a Coleman Junior spectrophotometer at 475 nm. From these readings a standard curve of mg/ℓ $CN^-$ vs. absorbancy was drawn and concentrations of samples could be calculated.

A second method involved a direct measurement of $CN^-$ by an Orion $CN^-$ electrode and an Orion Research model 701 digital pH meter. The procedure was that given in the instruction manual for use of the cyanide electrode. The procedure involves preparing 100, 10, and 1 ppm $CN^-$ standards, 100 ml each and adding 1 ml of ionic strength adjuster (ISA, 400 g NaOH/1 $H_2O$). Samples also contained 1 ml ISA per 100 ml sample. The electrodes were placed in the 10 ppm standard and with the function switch on Exp. M.V., the reading was set to 000.0. With the function switch on M.V. and with constant stirring, the next two standards and samples were read. The M.V. readings were then plotted against concentration on semi-logarithmic paper. Sample concentrations were determined from the calibration curve.

A third method involved the titration method found in Standard Methods for the Examination of Water and Wastewater (1975). Several

modifications of this method were used which included using a sample size of 20 ml, 0.2 ml indicator and diluting the titrant tenfold. Also, it was not necessary to distill samples since there was no apparent interference from the medium.

# CHAPTER III

# EXPERIMENTAL RESULTS

## Isolation of Organisms

The organisms used in this study were isolated from a laboratory scale extended aeration activated sludge unit with a hydrolytic assist and recycle which was being fed 10 or 20 mg/ℓ $CN^-$. The unit was run in the Bioenvironmental Engineering Laboratories at Oklahoma State University. Several methods were used to isolate a variety of organisms from this unit.

A 0.1 ml sample from the unit was diluted in 0.15 M NaCl to concentrations of $10^{-2}$, $10^{-4}$, and $10^{-6}$; then 0.1 ml of these dilutions were transferred to plates containing tryptic soy agar (TSA) resulting in final dilutions of $10^{-3}$, $10^{-5}$, and $10^{-7}$, respectively. The microorganisms were dispersed across the plate with a sterile bent elbow glass rod and incubated at 30°C. Individual colonies were picked from the $10^{-5}$ dilution plate and streaked on TSA plates which were incubated at 30°C. Stock cultures were made from isolated colonies.

Another method involved aseptically spreading 0.5 ml of a 1000 mg/ℓ stock solution of cyanide across a TSA plate. A series of these plates were immediately inoculated with 0.1 ml, 0.05 ml, and 0.025 ml of a sample from the activated sludge unit. Control plates were made in the same manner, but did not contain cyanide. Plates were incubated at 30°C

for 24 hours. The first colonies to appear on the cyanide plates were restreaked on TSA plates and isolated.

The third method involved inoculating a series of 250 ml flasks containing 50 ml of a buffered salts solution with $10^{-3}$M, $10^{-2}$M, $10^{-1}$M, and 1M cyanide. The flasks were incubated at room temperature for 3 days. There was no visible evidence of growth, but samples taken from each flask were streaked and isolated.

All isolates were then tested for their ability to grow in M-9, M-9 + 10 mg/ℓ $CN^-$, M-9 + 500 mg/ℓ Yex, and M-9 + 500 mg/ℓ Yex + 10 mg/ℓ $CN^-$. From this and other data two bacteria were chosen from all the isolates for further study.

## Identification of Organisms

The two organisms used in this study were isolated from a "hydrolytically assisted" extended aeration unit operated by Feng (1977). Both organisms were subjected to differential tests for subsequent identification. The first isolate was a Gram-positive, large, straight rod which contained elliptical, terminal spores. Colonies were large and opague. From these characteristics and the results in Table I, the organism was identified as a member of the genus Bacillus because it was a facultative, spore-forming rod and it produced catalase.

The second isolate was a Gram-negative, nonmotile rod of variable shape depending on age and growth medium. The colonies were rounded and glistening. With time they imparted a brownish color to the agar medium. The cells were encapsulated as indicated by an India ink wet mount. From these data and the results shown in Table II, the organism was identified as Klebsiella sp. The genus Klebsiella was determined as a result

TABLE I

IDENTIFICATION OF THE FIRST ISOLATE
AS *BACILLUS* SPECIES

| Test | Results |
|---|---|
| Sucrose fermentation | Acid (48 hours) |
| Lactose fermentation | Negative |
| Xylose fermentation | Acid |
| Glucose fermentation | Acid |
| Mannitol fermentation | Acid |
| Glycerol fermentation | Negative |
| Citrate as sole carbon source | Positive |
| Nitrate reduction | Positive to nitrite |
| Urease production | Negative |
| Indole production | Negative |
| Catalase production | Positive |
| Methyl red | Negative |
| Voges-Proskauer | Negative |
| Kligler's iron agar slant | Glucose fermentation and deamination |
| Litmus milk | Alkaline, then reduction and peptonization |
| Starch agar plates | Positive |
| Tributyrin | Positive |
| Gelatin medium | Positive |
| Gram stain | Positive |
| Cell morphology | Large rod |
| Spore stain | Terminal, elliptical spores |

TABLE II

IDENTIFICATION OF THE SECOND ISOLATE
AS *KLEBSIELLA* SPECIES

| Test | Results |
|---|---|
| Sucrose fermentation | Acid and gas (48 hours) |
| Lactose fermentation | Acid (48 hours) |
| Xylose fermentation | Acid |
| Glucose fermentation | Acid and gas |
| Mannitol fermentation | Acid |
| Glycerol fermentation | Acid |
| Citrate as sole carbon source | Positive |
| Nitrate reduction | Positive to nitrite |
| Urease production | Postive (48 hours) |
| Indole production | Positive |
| Catalase production | Positive |
| Methyl red | Negative |
| Voges-Proskauer | Positive |
| Kligler's iron agar slant | Acid throughout |
| Litmus milk | Acid, no curd |
| Starch agar plates | Negative |
| Tributyrin | Negative |
| Gelatin medium | Negative |
| Gram stain | Negative |
| Cell morphology | Rod (variable size) |
| Spore stain | Negative |
| Decarboxylase for | |
| Lysine | Positive |
| Arginine | Negative |
| Ornithine | Negative |

of the three decarboxylase tests according to Gibbs and Skinner (1969), its use of citrate and glucose as sole carbon source, and its ability to produce indole. The species most similar to this isolate was pneumoniae. It was identified from Bergey's Manual as K. pneumoniae and not another species because of the results from the MRVP test.

The Bacillus was chosen for these experiments because it was isolated several times in different isolation procedures. It was one of the first colonies to grow on the plates containing $CN^-$ and it was also isolated from buffered salt solutions containing $10^{-1}$M KCN. Its frequent appearance may be due to the fact that it forms spores and therefore easily survives the effects of $CN^-$.

Klebsiella was chosen because it was isolated in almost every isolation procedure. It was usually the dominant organism, especially on the plates containing cyanide. It was also chosen because of its ability to grow well in M-9 medium and M-9 + $CN^-$.

## Effect of $CN^-$ on Escherichia coli B and Pseudomonas aeruginosa

To determine how two different bacteria would react to cyanide, known cultures of Escherichia coli B and Pseudomonas aeruginosa were tested for resistance to cyanide. An inoculating needle was used to transfer cells to each of a series of tubes containing 3 ml of either nutrient broth or M-9 and various concentrations of cyanide. The tubes were incubated at 30°C and checked for growth daily. Tables III and IV contain the results of two separate experiments on E. coli B and Ps. aeruginosa. In the first experiment difficulty was encountered when Ps. aeruginosa failed to grow at all in the M-9 medium and E. coli B

TABLE III

CYANIDE RESISTANCE OF ESCHERICHIA COLI B

| mg/ℓ $CN^-$ | N.B. Medium Days After Inoculation 1 | 2 | 3 | 4 | M-9 Medium Days After Inoculation 2 |
|---|---|---|---|---|---|
| 0 | +* | + | + | + | + |
| 10 | + | + | + | + | + |
| 20 | + | + | + | + | + |
| 30 | + | + | + | + | + |
| 40 | + | + | + | + | + |
| 50 | + | + | + | + | + |
| 60 | | + | + | + | + |
| 70 | | + | + | + | + |
| 80 | | + | + | + | + |
| 90 | | + | + | + | + |
| 100 | | + | + | + | + |
| 110 | | | | | + |
| 120 | | | | | + |
| 130 | | | | | |
| 140 | | | | | |
| 150 | | | | | |
| 160 | | | | | |
| 170 | | | | | |
| 180 | | | | | + |
| 190 | | | | | + |
| 200 | | | | | + |

*+ indicates evidence of growth.

TABLE IV

CYANIDE RESISTANCE OF PSEUDOMONAS AERUGINOSA

| mg/ℓ $CN^-$ | N.B. Medium Days After Inoculation 1 | 2 | 3 | M-9 Medium Days After Inoculation 1 | 3 |
|---|---|---|---|---|---|
| 0 | +* | + | + | + | + |
| 10 | + | + | + | + | + |
| 20 | + | + | + | + | + |
| 30 | + | + | + | + | + |
| 40 | + | + | + | + | + |
| 50 | + | + | + | | + |
| 60 | + | + | + | + | + |
| 70 | + | + | + | | + |
| 80 | | + | + | | + |
| 90 | | + | + | | + |
| 100 | | | + | | + |
| 110 | | | + | | + |
| 120 | | | + | | + |
| 130 | | | + | | + |
| 140 | | | + | | + |
| 150 | | | + | | + |
| 160 | | | + | | + |
| 170 | | | + | | + |
| 180 | | | + | | + |
| 190 | | | + | | + |
| 200 | | | + | | + |

*+ indicates evidence of growth.

only grew in the zero cyanide control after two days. These tubes were left in the incubator and by the sixth day E. coli B had grown in the M-9 medium containing $CN^-$ concentrations up to 80 mg/ℓ. The experiment was run again using the two test organisms and this time both showed evidence of growth in the M-9 medium.

There may be some discrepancies in these results since the inoculations were made with a needle and therefore were not consistent and may have been too heavy in some cases. Although better results may have been gained by using a small exact inoculum, the experiments do show the ability of both organisms to grow in high concentrations of cyanide. Ps. aeruginosa with its complete cytochrome system seemed to be the more resistant organism.

Since in the above situation high concentrations of cyanide did not inhibit growth, the possibility that cyanide affects the growth rate was then tested for both organisms. The growth rates of both E. coli and Ps. aeruginosa were determined in M-9 medium and M-9 plus $CN^-$. After the cells had grown overnight, 0.5 ml of the cell suspensions were transferred to 250 ml side arm flasks containing 20 ml of media. Cyanide concentrations in the flasks were 20 and 80 mg/ℓ and were made by diluting the appropriate amount of a 1000 mg/ℓ $CN^-$ stock solution with the media. As seen in Table V, the growth rate of both organisms in the presence of 20 mg/ℓ $CN^-$ was less than half the growth rate in medium without cyanide. Table V also shows that the lag period for E. coli in 20 mg/ℓ $CN^-$ was 105 hours while that for Ps. aeruginosa was 46 hours. Both organisms had extremely long lag periods in cyanide medium, but Ps. aeruginosa was able to overcome the inhibitory effects of cyanide faster than E. coli. These data show the extreme effect cyanide has on these bacteria.

TABLE V

GROWTH RATE CONSTANTS FOR *ESCHERICHIA COLI* B AND *PSEUDOMONAS AERUGINOSA* IN M-9 AND M-9 + $CN^-$

| Organism | Medium | $\mu$, $hr^{-1}$ | Lag Period |
|---|---|---|---|
| *E. coli* B | M-9 | 0.330 | 1.0 hour |
| *E. coli* B | M-9 + 20 mg/ℓ $CN^-$ | 0.131 | 105.0 hours |
| *Ps. aeruginosa* | M-9 | 0.433 | 3.0 hours |
| *Ps. aeruginosa* | M-9 + 20 mg/ℓ $CN^-$ | 0.210 | 46.0 hours |
| *Ps. aeruginosa* | M-9 + 20 mg/ℓ $CN^-$* | 0.239 | 46.5 hours |
| *Ps. aeruginosa* | M-9 + 80 mg/ℓ $CN^-$* | 0.315 | 105.0 hours |

*These flasks were inoculated with cells from the previous flask (M-9 + 20 mg/ℓ $CN^-$) after growth was complete.

Since the lag period was so long in the presence of cyanide, cells were transferred to fresh cyanide medium to determine whether those cells that did grow were mutants that could grow readily in cyanide. The results of this experiment for Ps. aeruginosa are presented in Table V. The results show that the organism when transferred to fresh medium behaved as it did in the original medium.

## Growth of Bacillus sp. and Klebsiella sp. in High Concentrations of Cyanide

Having acquired some knowledge of how bacteria behave in cyanide, the isolates from the activated sludge unit were then tested to determine whether they could metabolize cyanide or were resistant to cyanide. The two isolates were first tested for their tolerance to cyanide. Cyanide concentrations of 60, 70, 80, and 90 mg/ℓ were made by weighing the appropriate amount of KCN and adding it directly to the 250 ml side arm flasks containing TSB or M-9 + Yex. An inoculum of 0.25 ml of an overnight culture was aseptically added to each of the flasks. Growth was monitored and growth curves were drawn.

### Growth of Bacillus

Bacillus can grow in M-9 + Yex, but it does not grow well in this medium; therefore TSB was used as the basal medium for this bacterium. Growth curves for Bacillus in TSB and in TSB plus 60 and 70 mg/ℓ $CN^-$ are shown in Figure 1 and the growth curves in TSB plus 80 and 90 mg/ℓ $CN^-$ are shown in Figure 2. The growth of the Bacillus in the presence of 70, 80, and 90 mg/ℓ $CN^-$ was rather erratic for the first 5 hours, as shown in Figure 3. The optical density declined rapidly shortly after inoculation

Figure 1. Growth Curve for Bacillus sp. in TSB Medium and TSB Plus 60 mg/ℓ and 70 mg/ℓ $CN^-$

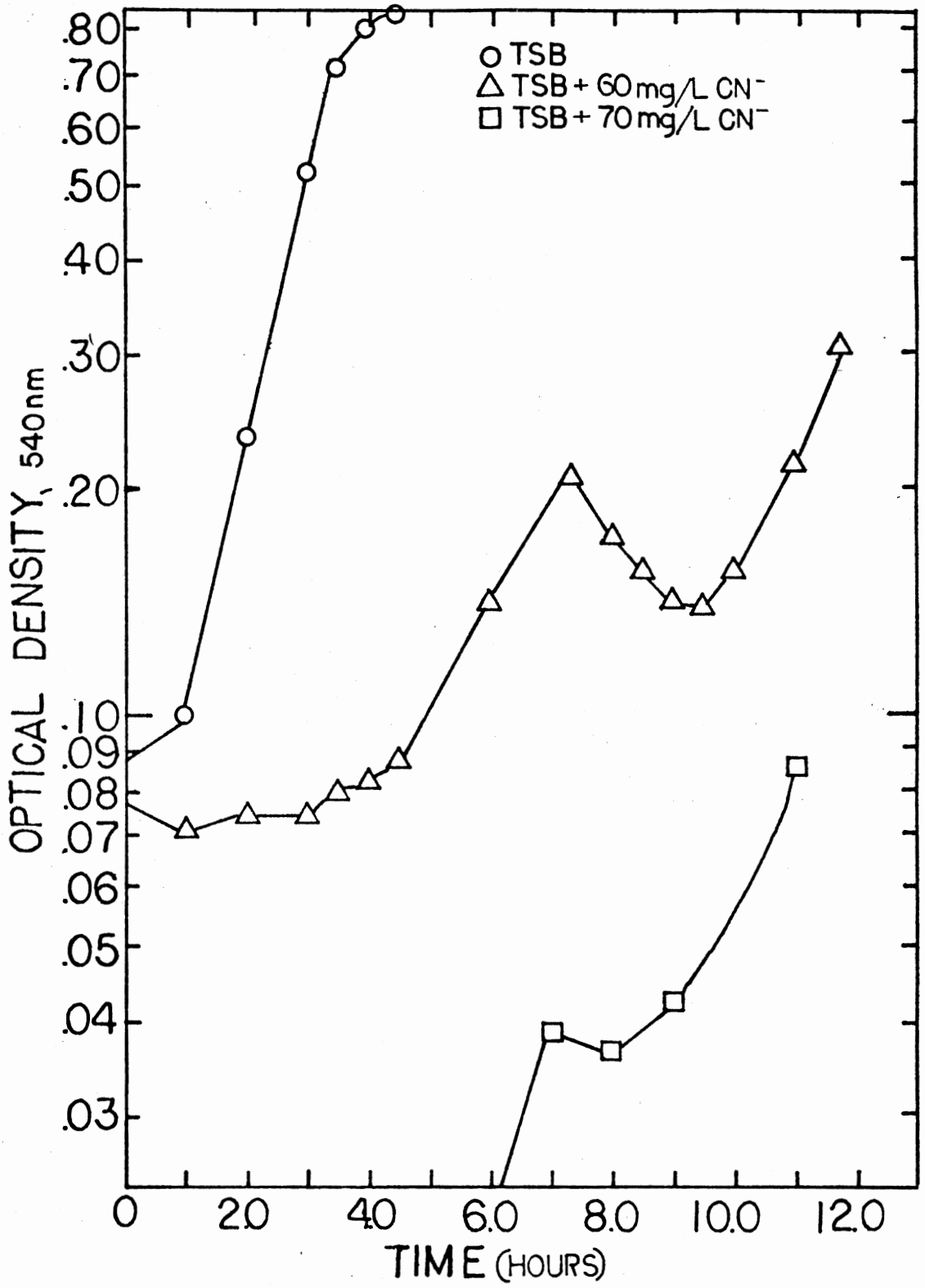

TSB
TSB + 60 mg/L CN⁻
TSB + 70 mg/L CN⁻
OPTICAL DENSITY, 540nm
.80
.70
.60
.50
.40
.30
.20
.10
.09
.08
.07
.06
.05
.04
.03
0
2.0
4.0
6.0
8.0
10.0
12.0
TIME (HOURS)

Figure 2. Growth Curve for *Bacillus* sp. in TSB Plus 80 and 90 mg/ℓ CN

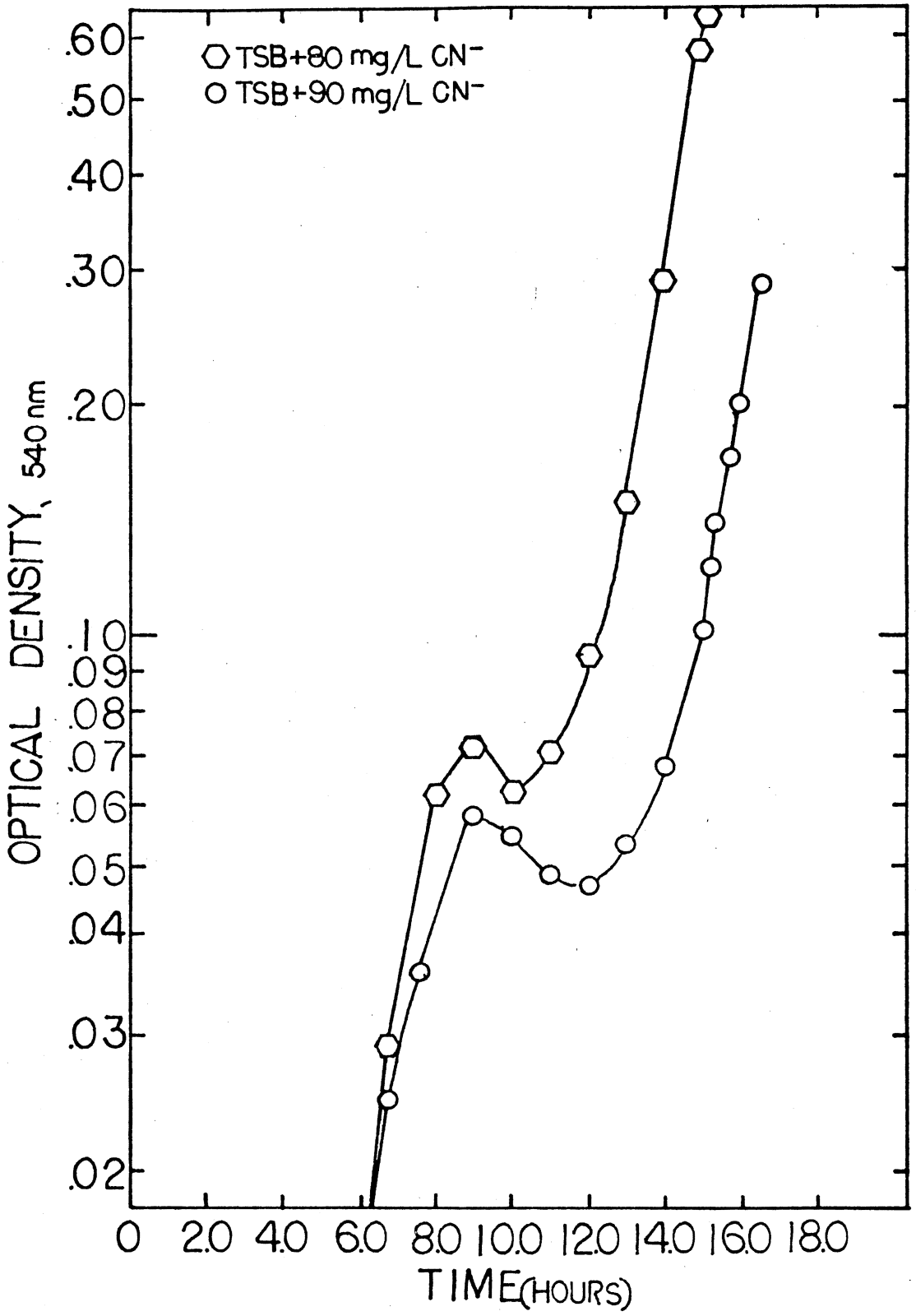
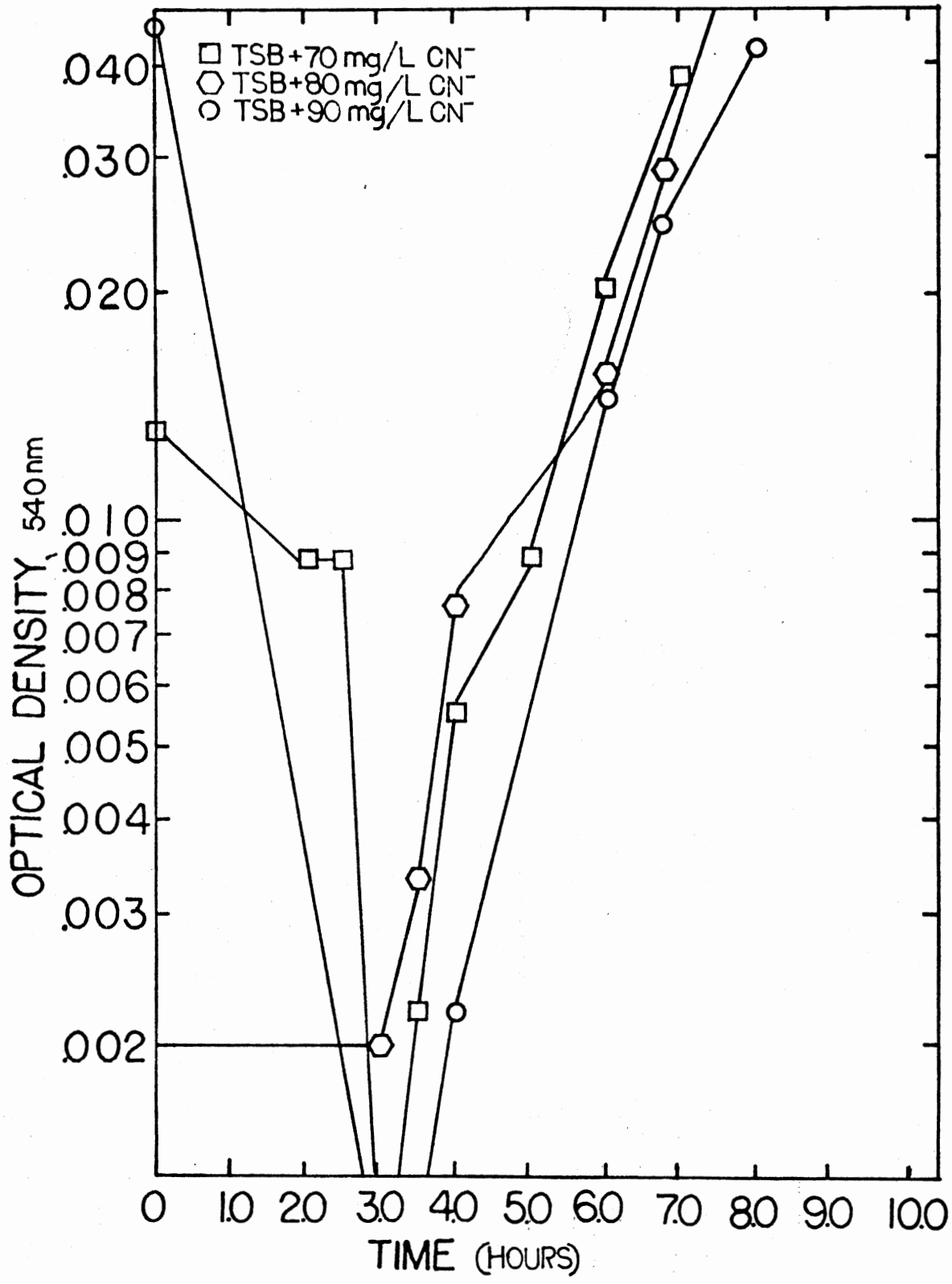

TSB+80 mg/L CN⁻
TSB+90 mg/L CN⁻
OPTICAL DENSITY, 540 nm
.60
.50
.40
.30
.20
.10
.09
.08
.07
.06
.05
.04
.03
.02
0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
TIME (HOURS)

Figure 3. Growth Curve for *Bacillus* sp. in TSB Plus 70, 80, and 90 mg/ℓ $CN^-$, Showing Erratic Behavior the First Few Hours After Inoculation

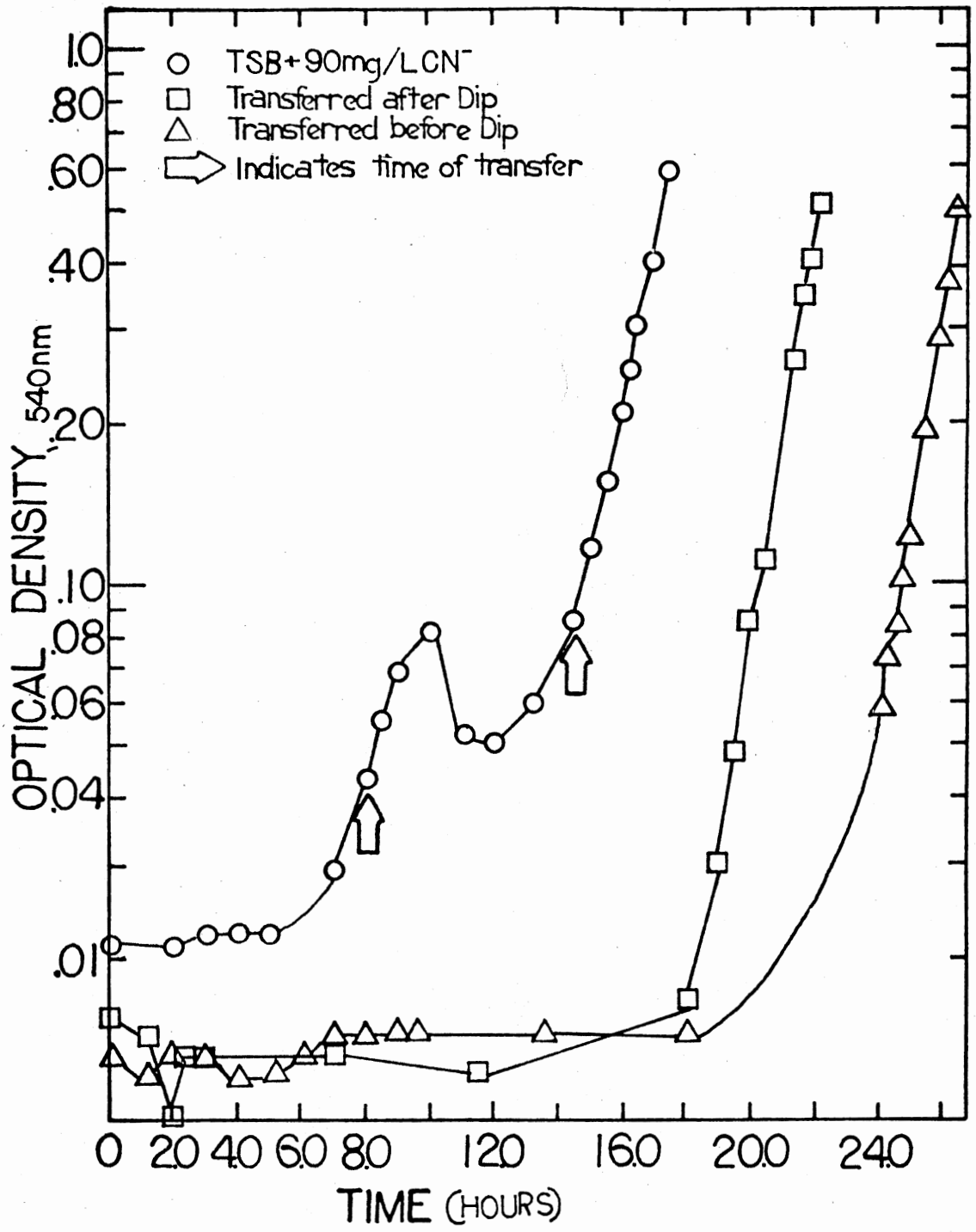

□ TSB+70 mg/L CN⁻
⬡ TSB+80 mg/L CN⁻
○ TSB+90 mg/L CN⁻
OPTICAL DENSITY, 540nm
.040
.030
.020
.010
.009
.008
.007
.006
.005
.004
.003
.002
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
TIME (HOURS)

at concentrations of 70 and 90 mg/ℓ $CN^-$. In these cases, the O.D. was zero at 3 hours. The O.D. for the flask containing 80 mg/ℓ $CN^-$ started very low and remained constant for 3 hours. The cells at all concentrations of $CN^-$ approached logarithmic growth and then the O.D. decreased, forming a dip in the curve as they began growing again. This dip is clearly demonstrated in Figures 1 and 2. The growth rate constant, $\mu$, for Bacillus in TSB without $CN^-$ was 0.815 $hr^{-1}$. After the dip in the curve, $\mu$ was 0.795 $hr^{-1}$, which is the average $\mu$ for growth at 70, 80, and 90 mg/ℓ $CN^-$. The growth rate before the dip cannot be accurately calculated, but for comparison it was roughly estimated to be 0.495 $hr^{-1}$.

In an attempt to explain the dip, 0.1 ml samples were taken before and after the dip from an actively growing culture of Bacillus in TSB + 90 mg/ℓ $CN^-$ and transferred to fresh TSB + 90 mg/ℓ $CN^-$. Figure 4 shows that the cells transferred before and after the dip experienced long lag periods of at least 18 hours. Although there were long time intervals between some readings, there was no evidence of a dip from the transferred cells.

In another experiment similar to the one above, two flasks containing TSB + 90 mg/ℓ $CN^-$ and Bacillus sp. were taken off the shaker, one before and one after the dip. The cells from each flask were harvested by centrifugation under aseptic conditions and the medium was discarded. The cells were then resuspended in fresh TSB + 90 mg/ℓ $CN^-$ and replaced on the reciprocal shaker. The results were similar to those with a smaller inoculum. In this case the lag period was approximately 16 hours for both flasks.

Figure 4. Growth Curves for Bacillus sp. in TSB Plus 90 mg/ℓ $CN^-$ and for Cells Transferred Into Fresh TSB Plus 90 mg/ℓ $CN^-$ at 8 and 15 Hours


TSB+90mg/L CN⁻
Transferred after Dip
Transferred before Dip
Indicates time of transfer
OPTICAL DENSITY, 540nm
1.0
.80
.60
.40
.20
.10
.08
.06
.04
.01
0
2.0
4.0
6.0
8.0
12.0
16.0
20.0
24.0
TIME (HOURS)

A small sample of cells was taken at the time of the dip from flasks containing 60 and 80 mg/ℓ $CN^-$. Upon microscopic examination the samples were found to contain vegetative cells and a few spores.

Growth of Klebsiella

Klebsiella sp. was also tested for its ability to grow in high concentrations of cyanide. Figures 5 and 6 show the growth curves for Klebsiella in M-9 + Yex medium and M-9 + Yex plus 60, 70, 80, and 90 mg/ℓ $CN^-$. As seen in the graphs, Klebsiella does not have a typical growth curve in M-9 + Yex. The cells in each flask except the one containing 70 mg/ℓ $CN^-$ appeared to have two phases of logarithmic growth. The first area of logarithmic growth was quite irregular in each case, but the second phase was rather constant in each case with an average $\mu$ of 0.336 $hr^{-1}$.

Klebsiella was able to grow quite well in the presence of cyanide and M-9 with Yex added. The growth of Klebsiella in M-9 + $CN^-$ was always preceded by a long lag period, e.g., the lag period of Klebsiella in M-9 + 10 mg/ℓ $CN^-$ was 7.5 to 11.5 hours, depending on the age of the cells. At first the cells used for inoculations were transferred from M-9 to M-9 + 10 mg/ℓ $CN^-$ and allowed to grow overnight. These cells had a longer lag period. After realizing the importance of the cell age, the cells were transferred at a regular time interval of 14 hours. The resulting lag periods were between 7.5 and 8.0 hours.

Effect of Medium on Cyanide Toxicity

Since cyanide is a highly reactive compound, an experiment was run to determine if cyanide might possibly be reacting with the components

Figure 5. Growth Curves for Klebsiella sp. in M-9 + Yex and M-9 + Yex Plus 60 and 70 mg/ℓ CN

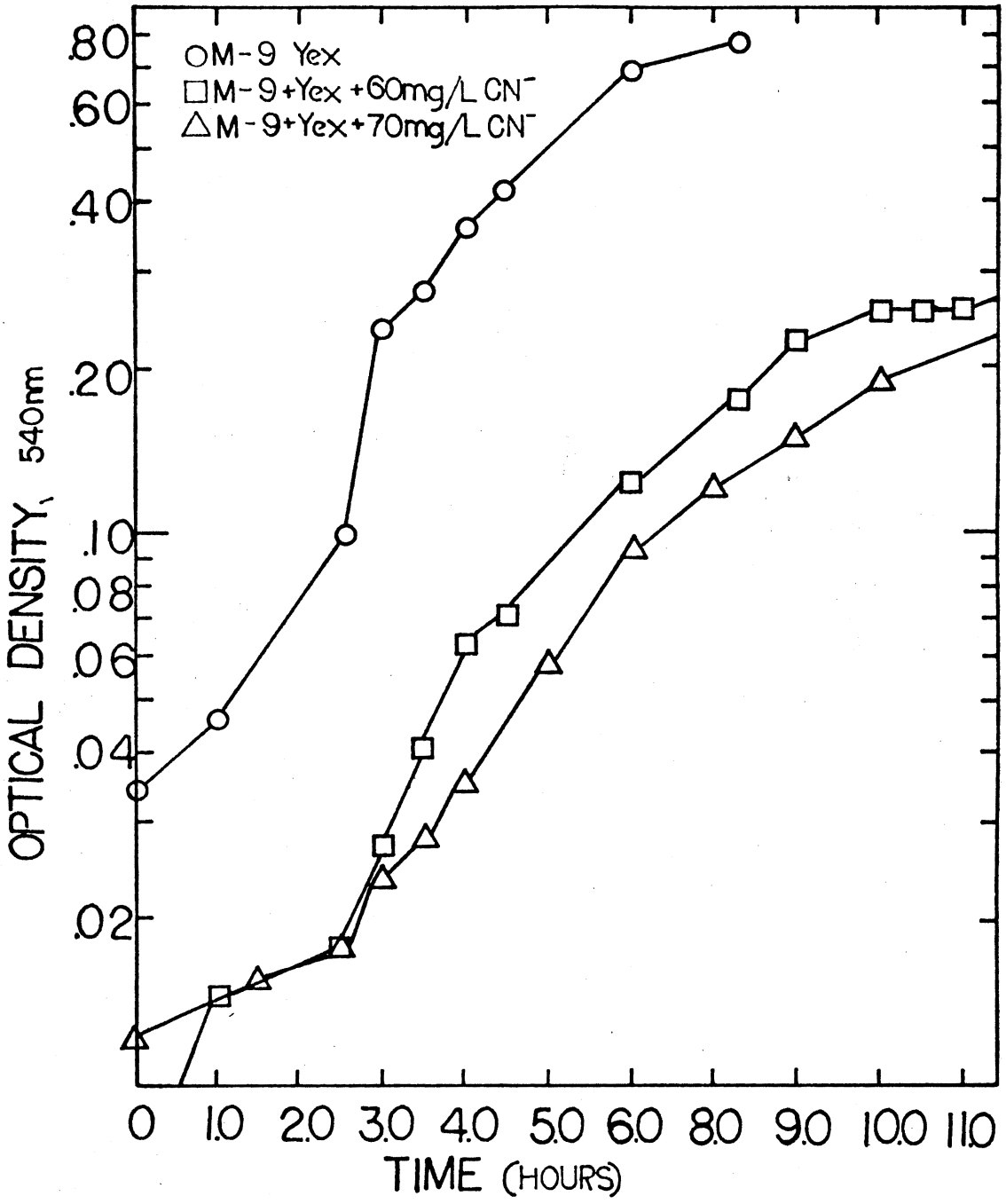
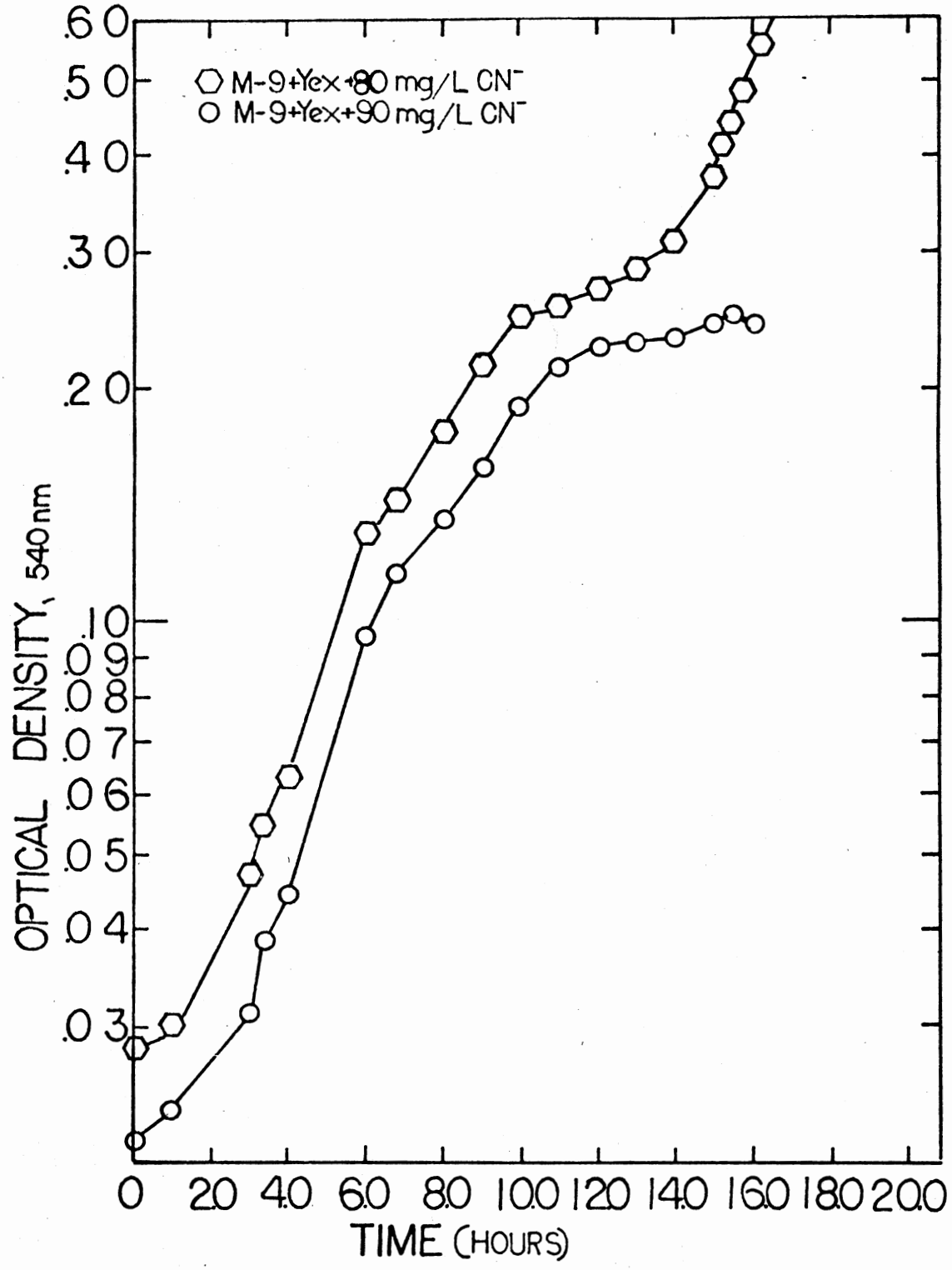

OM-9 Yex
□M-9+Yex+60mg/L CN⁻
△M-9+Yex+70mg/L CN⁻
OPTICAL DENSITY, 540nm
.80
.60
.40
.20
.10
.08
.06
.04
.02
0
1.0
2.0
3.0
4.0
5.0
6.0
8.0
9.0
10.0
11.0
TIME (HOURS)

Figure 6. Growth Curve for *Klebsiella* sp. in M-9 + Yex Plus 80 and 90 mg/ℓ $CN^-$


M-9+Yex+80 mg/L CN⁻
M-9+Yex+90 mg/L CN⁻
OPTICAL DENSITY, 540nm
.60
.50
.40
.30
.20
.10
.09
.08
.07
.06
.05
.04
.03
0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
TIME (HOURS)

in the medium and therefore becoming less toxic to the bacteria. To determine this, a series of six 250 ml top arm flasks were put on the reciprocal shaking water bath. There were two flasks of M-9 medium, two flasks of M-9 + Yex and one flask each of M-9 + 20 mg/ℓ $CN^-$ and M-9 + Yex + 20 mg/ℓ $CN^-$. Each flask contained 20 ml of medium and was allowed to incubate for 4 hours on the shaker. After the 4 hours had passed, cyanide was added to a final concentration of 10 mg/ℓ to a flask of M-9 and a flask of M-9 + Yex. At this time 0.1 ml of a culture of Klebsiella was aseptically transferred to each flask. The growth of Klebsiella was followed and from the resulting growth curves the lag periods and growth rates were determined. The results of this experiment are presented in Table VI. There was no significant difference in the inhibitory effect of cyanide when it was added 4 hours before inoculation and at the time of inoculation. It is apparent from these data that Yex or M-9 does not interfere with the toxic effect of cyanide.

## Addition of Nutrients

The growth of Klebsiella sp. was severely inhibited by a concentration of only 10 mg/ℓ $CN^-$ in M-9 medium, while the same concentration had little or no effect on growth in M-9 with Yex. These data are shown in Figure 7. A series of experiments was undertaken to determine whether other complex nutrient mixtures would have a similar effect. Concentrated stock solutions of hydrolyzed extended aeration activated sludge (hydrolysate) and of a mixture containing 19 amino acids were prepared so that addition of 1 ml of concentrated solution would yield a final concentration of approximately 500 mg/ℓ of the added nutrients. The experiments were also designed to determine whether addition of Yex or

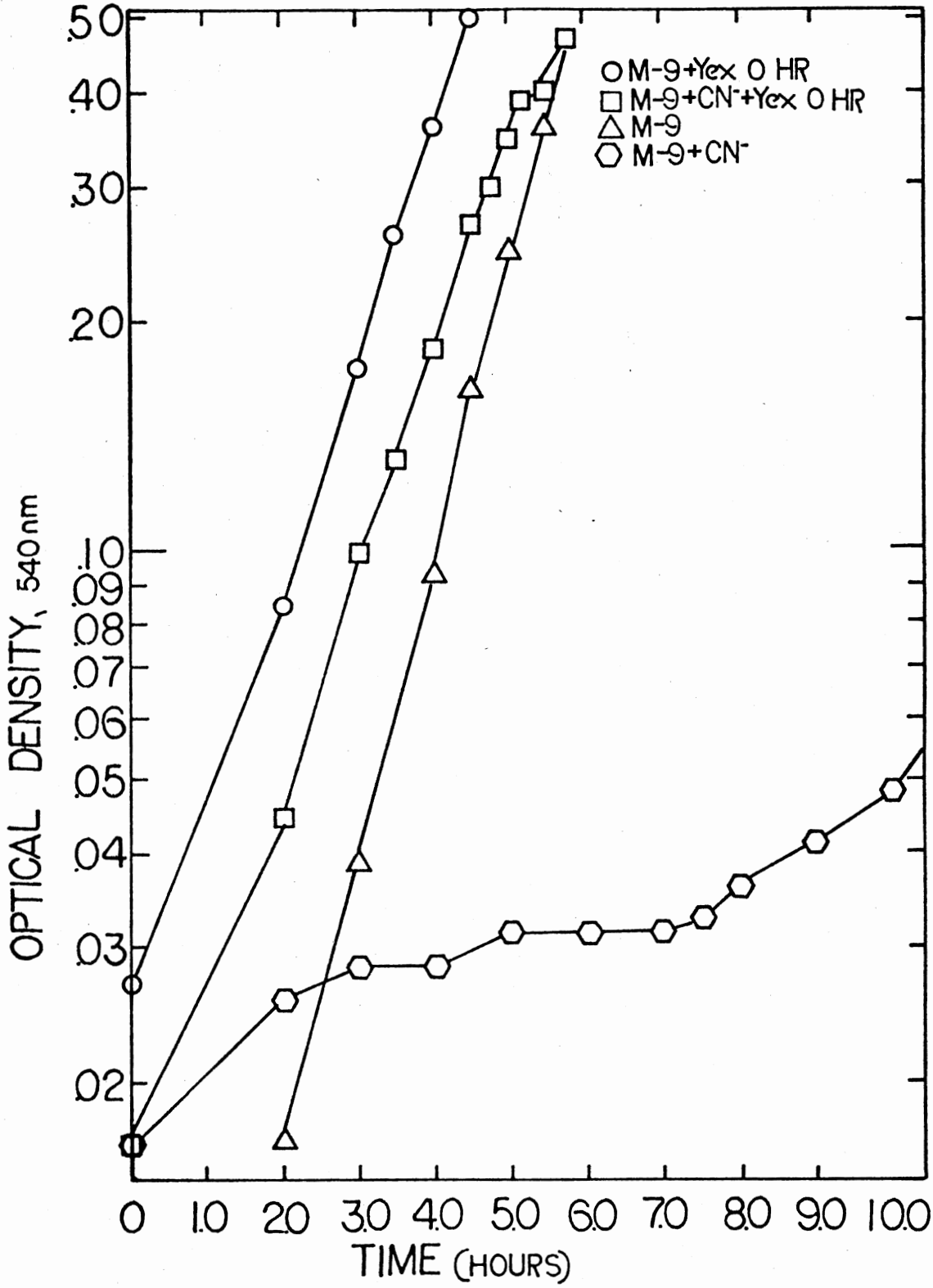
TABLE VI

GROWTH RATE CONSTANTS AND LAG PERIODS FOR KLEBSIELLA DEMONSTRATING THE EFFECT OF MEDIUM ON CYANIDE TOXICITY

| Medium | $\mu$, $hr^{-1}$ | Lag |
|---|---|---|
| M-9 | .711 | 3.5 hours |
| M-9 + Yex | .630 | 2.0 hours |
| M-9 + 20 mg/ℓ $CN^-$ | .578 | 33.0 hours |
| M-9 + Yex + 20 mg/ℓ $CN^-$ | .578 | 3.5 hours |
| M-9 + 20 mg/ℓ $CN^{-*}$ | .770 | 36.0 hours |
| M-9 + Yex + 20 mg/ℓ $CN^{-*}$ | .630 | 3.0 hours |

*$CN^-$ added after four hours of incubation.

Figure 7. Growth Curves for *Klebsiella* sp. in M-9 Plus 10 mg/ℓ $CN^-$ With Yex Added at the Time of Inoculation


OPTICAL DENSITY, 540 nm
.50
.40
.30
.20
.10
.09
.08
.07
.06
.05
.04
.03
.02
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
TIME (HOURS)
M-9+Yex 0 HR
M-9+CN⁻+Yex 0 HR
M-9
M-9+CN⁻

the other nutrients could relieve an inhibition already established by exposure to cyanide.

The first series of experiments was undertaken to learn the effects of adding Yex at different time intervals to M-9 + 10 mg/ℓ $CN^-$ inoculated with Klebsiella. Times of 0, 3, and 6 hours were chosen for the addition of Yex since they were within the interval of the lag period. The results of these experiments are shown in Figures 7 and 8. The changes in O.D. at the time of addition resulted from changing from an M-9 blank to a blank containing Yex. Upon the addition of Yex the cells in the M-9 + $CN^-$ medium began growing and soon reached a growth rate which approached the growth rate of the cells in M-9 + Yex medium without $CN^-$. This same experiment was repeated two more times, but in one of these experiments the Yex was replaced by hydrolysate obtained from Feng and the Bioenvironmental Engineering Laboratories, and in the other experiment the amino acid (aa) mixture was substituted for the Yex. As can be seen in Figures 9 and 10 and in Figures 11 and 12, the effect of adding the hydrolysate or the amino acid mixture was similar to the effect produced by the addition of Yex. In all cases the cells began to grow in the cyanide medium and their growth rate approached or equalled the growth rate of the cells in the same medium without cyanide.

## Addition of Individual Amino Acids and Mixtures of Amino Acids

Since the amino acid mixture supported the growth of Klebsiella in the presence of cyanide, the next series of experiments was designed to find a single amino acid or a combination of amino acids which would relieve the ihnibition by cyanide. All the amino acids in the original

Figure 8. Growth Curves for *Klebsiella* sp. in M-9 Plus 10 mg/ℓ $CN^-$ With Yex Added at 3 and 6 Hours After Inoculation

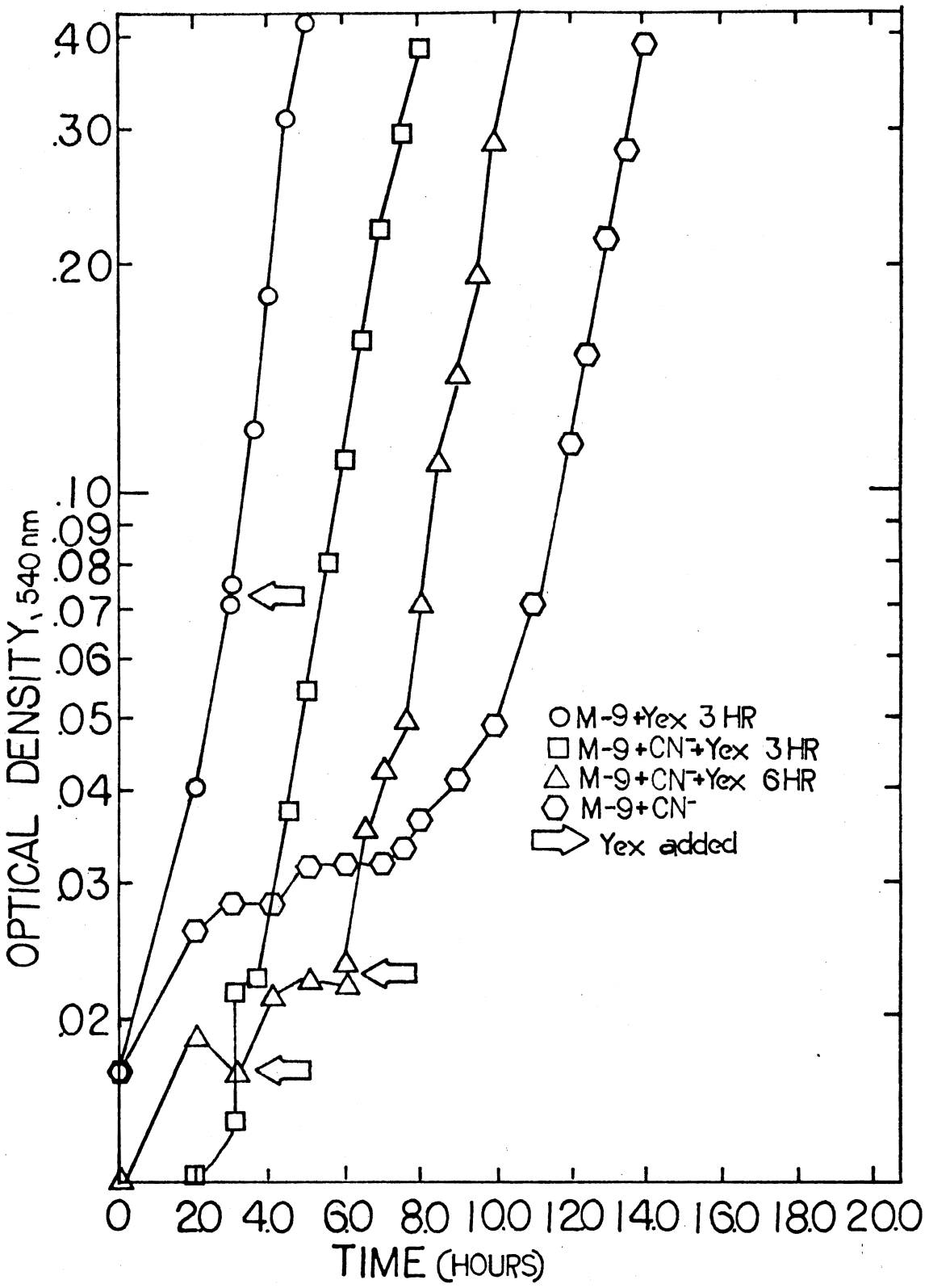

OPTICAL DENSITY, 540 nm
.40
.30
.20
.10
.09
.08
.07
.06
.05
.04
.03
.02
0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
TIME (HOURS)
M-9+Yex 3 HR
M-9+CN⁻+Yex 3 HR
M-9+CN⁻+Yex 6 HR
M-9+CN⁻
Yex added

Figure 9. Growth Curves for Klebsiella sp. in M-9 Plus 10 mg/ℓ $CN^-$ With Hydrolysate Added at the Time of Inoculation

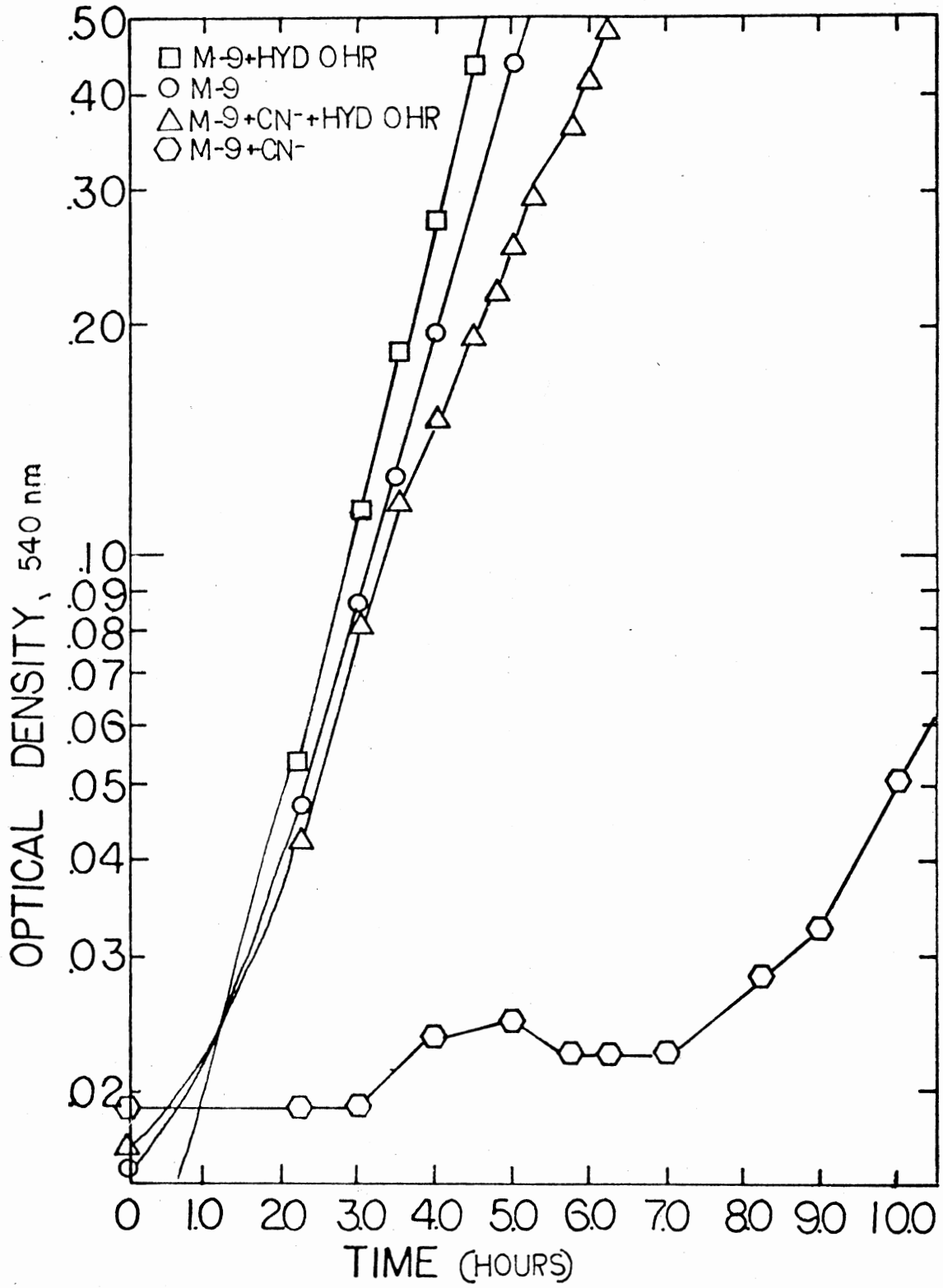

M-9+HYD 0 HR
M-9
M-9+CN⁻+HYD 0 HR
M-9+CN⁻
OPTICAL DENSITY, 540 nm
.50
.40
.30
.20
.10
.09
.08
.07
.06
.05
.04
.03
.02
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
TIME (HOURS)

Figure 10. Growth Curves for Klebsiella sp. in M-9 Plus 10 mg/ℓ $CN^-$ With Hydrolysate Added at 3 and 6 Hours After Inoculation

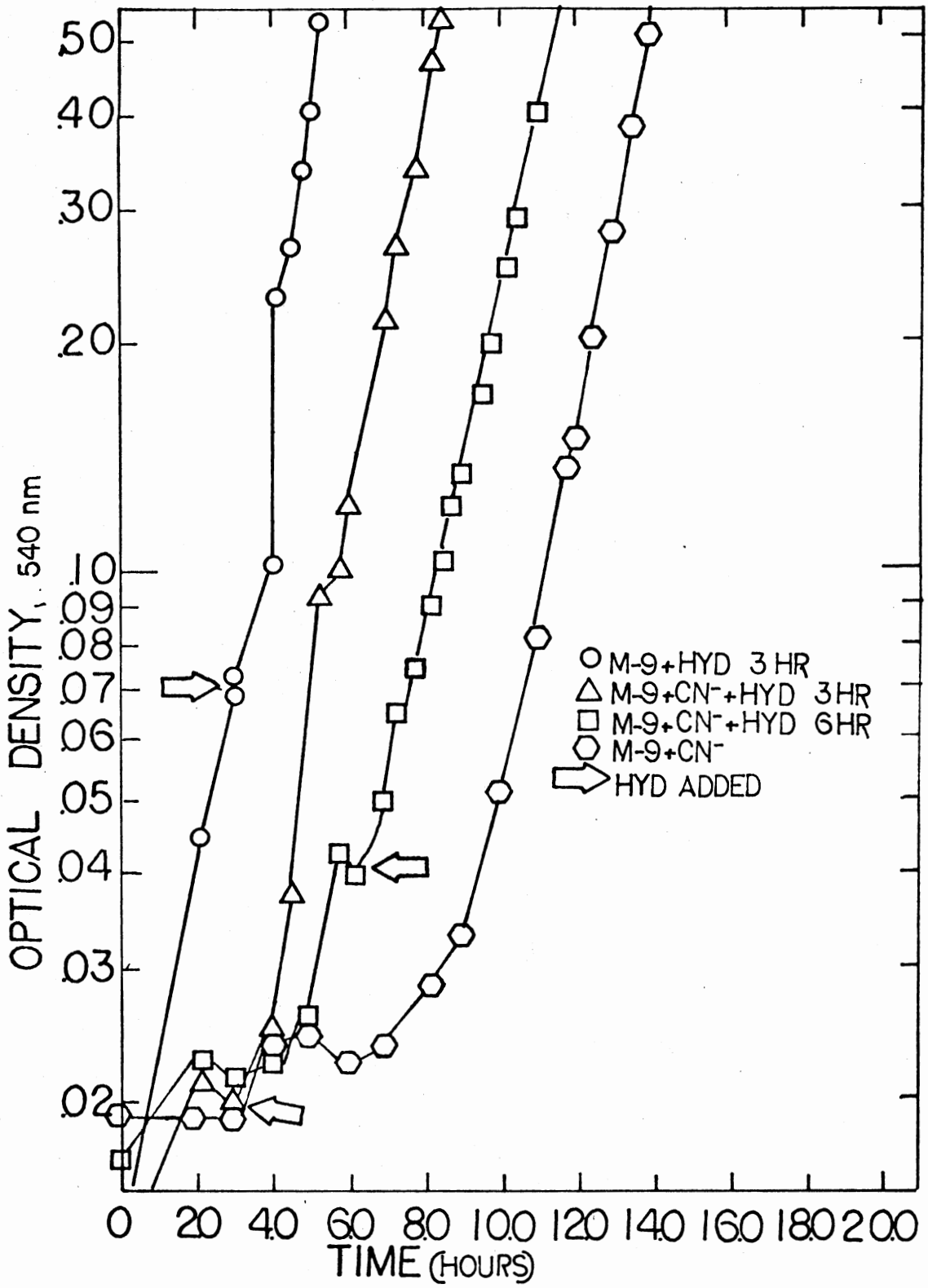

OPTICAL DENSITY, 540 nm
.50
.40
.30
.20
.10
.09
.08
.07
.06
.05
.04
.03
.02
0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
TIME (HOURS)
M-9+HYD 3 HR
M-9+CN⁻+HYD 3 HR
M-9+CN⁻+HYD 6 HR
M-9+CN⁻
HYD ADDED

Figure 11. Growth Curves for Klebsiella sp. in M-9 Plus 10 mg/ℓ $CN^-$ With a Mixture of Amino Acids Added at the Time of Inoculation

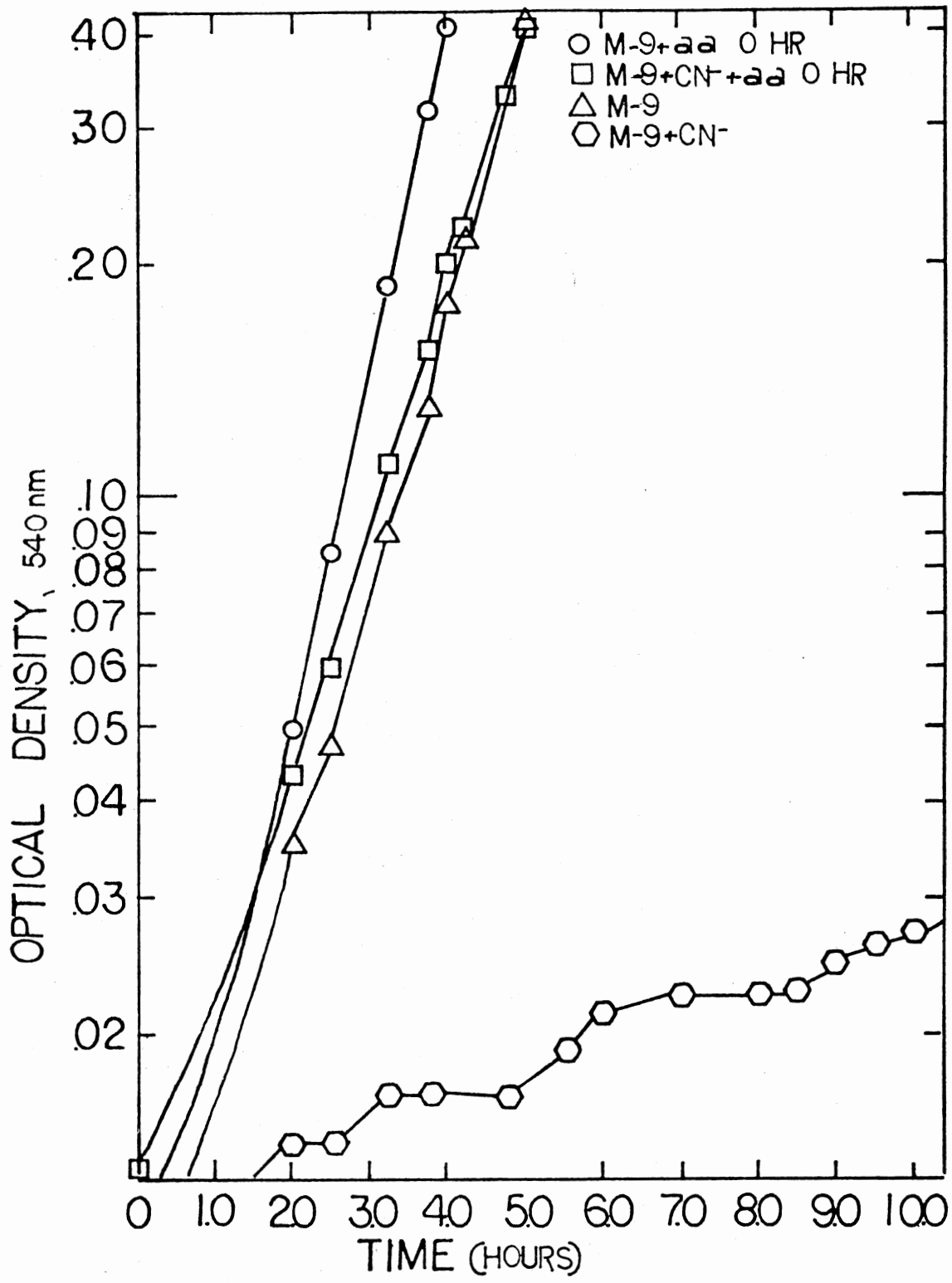
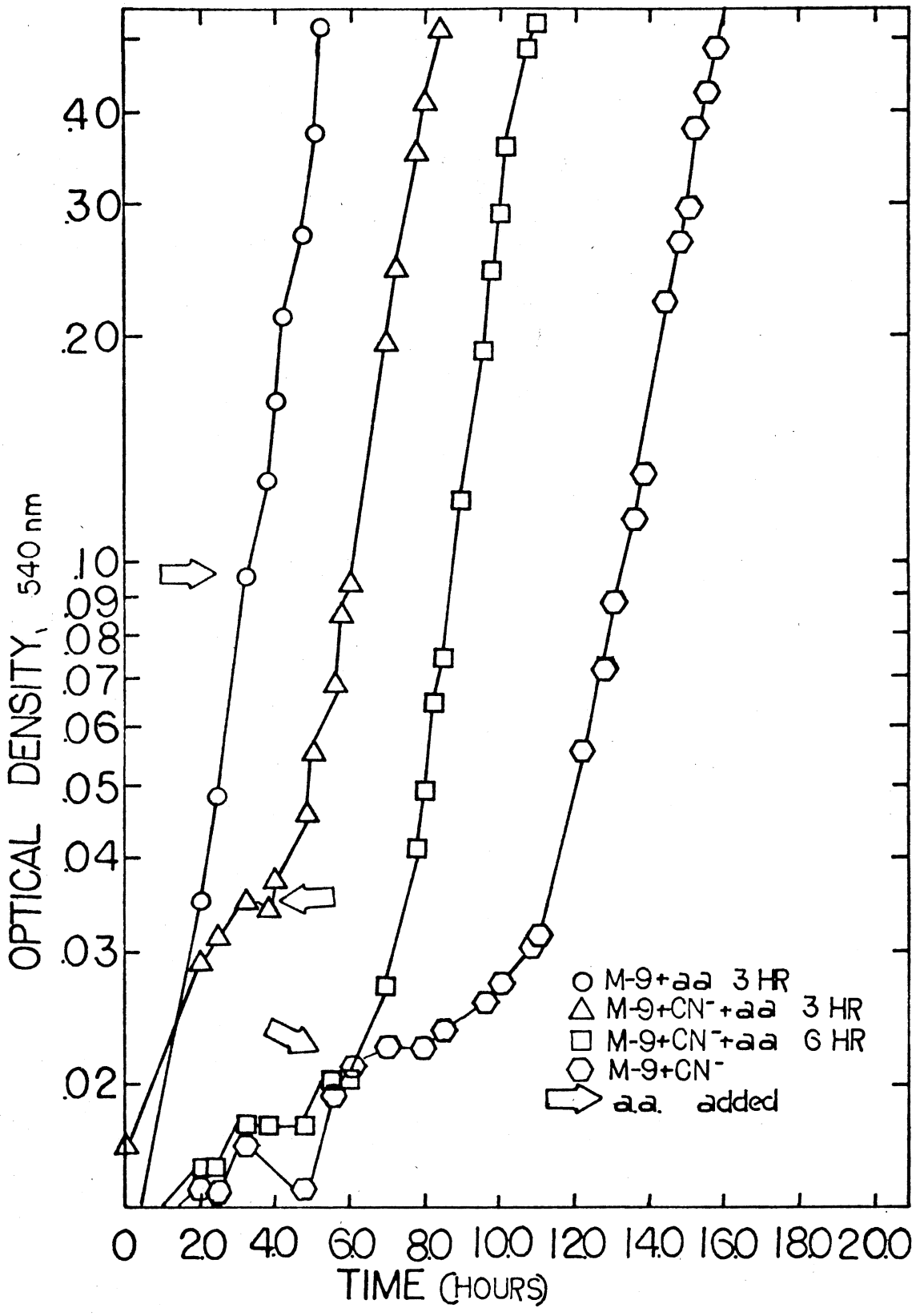

M-9+aa 0 HR
M-9+CN⁻+aa 0 HR
M-9
M-9+CN⁻
OPTICAL DENSITY, 540 nm
.40
.30
.20
.10
.09
.08
.07
.06
.05
.04
.03
.02
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
TIME (HOURS)

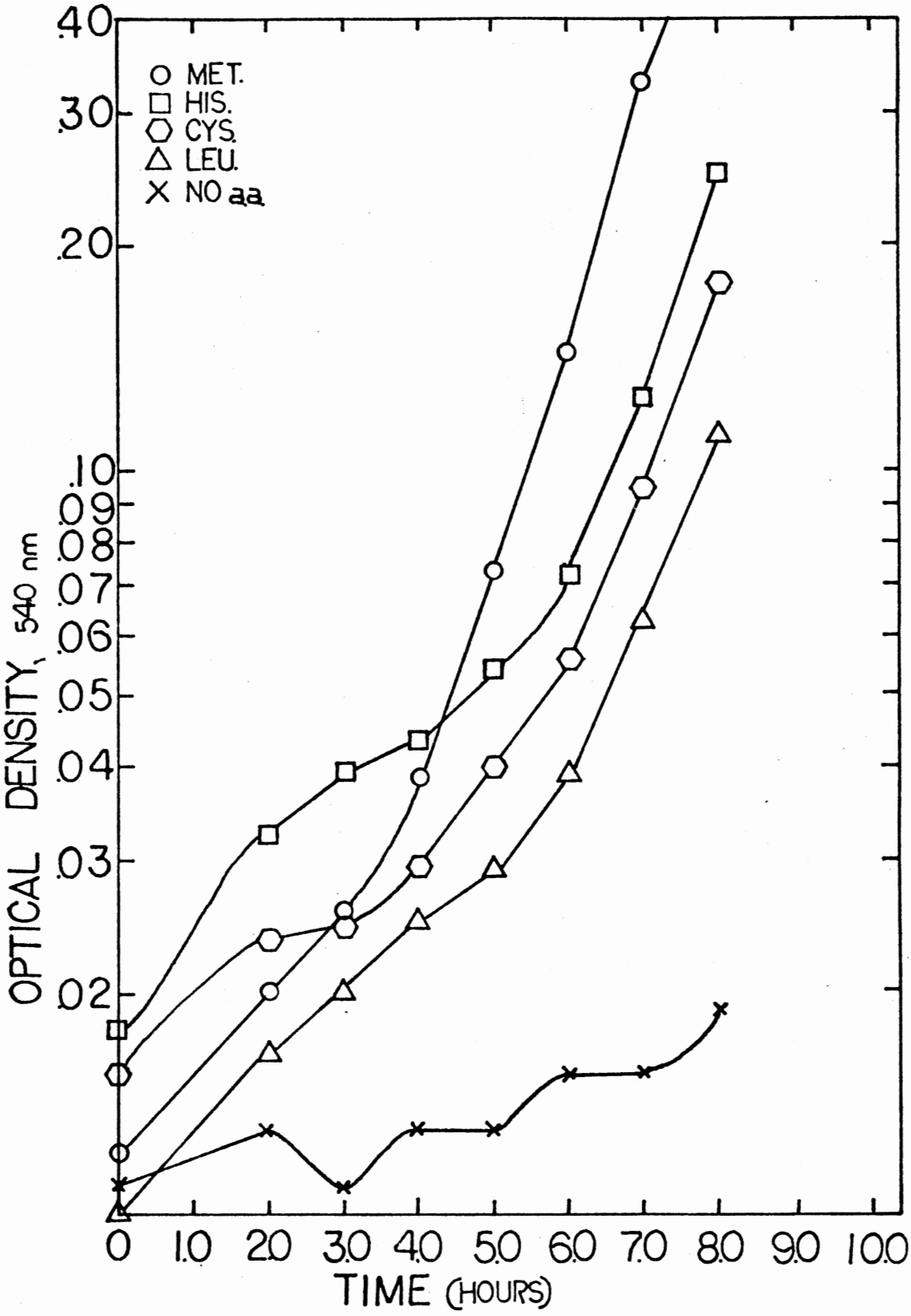
Figure 12. Growth Curves for Klebsiella sp. in M-9 Plus 10 mg/ℓ $CN^-$ With a Mixture of Amino Acids Added at 3 and 6 Hours After Inoculation


OPTICAL DENSITY, 540 nm
.40
.30
.20
.10
.09
.08
.07
.06
.05
.04
.03
.02
0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
TIME (HOURS)
M-9+aa 3 HR
M-9+CN⁻+aa 3 HR
M-9+CN⁻+aa 6 HR
M-9+CN⁻
a.a. added

mixture were tested individually by adding 1 ml of the amino acids at the time of inoculation to flasks containing 19 ml of M-9 + 10 mg/ℓ $CN^-$. The final amino acid concentration was 50 mg/ℓ. Optical density was followed to see which amino acid might stimulate growth. Those that supported growth within the 8 hour incubation period are shown in Figure 13. Methionine had the greatest effect by shortening the lag period to 4 hours. The response to histidine, cysteine, and leucine was similar, with all three having lag periods of approximately 6 hours. All other amino acids tested had no effect on the lag period within 8 hours of monitoring, so they were eliminated from further tests. The effective amino acids were mixed together in various combinations and tested in the same manner. Since methionine elicited the greatest response from the cells, it was a member of each combination. All mixtures of amino acids were made by adding equal amounts of each amino acid to a final concentration of 50 mg/ℓ. When mixtures of methionine and histidine, methionine and leucine, and methionine and cysteine were added to flasks containing M-9 + 10 mg/ℓ $CN^-$ and Klebsiella, the results were identical. Growth curves were drawn for each combination and the lines coincided, indicating similar growth rates. The lag period was 3.25 hours. These same results were obtained when the following mixtures were tested: methionine, histidine and cysteine; methionine, histidine and leucine; and methionine, cysteine and leucine. The lag period was shortened to nearly 3 hours in all three cases, and the growth rates were nearly the same.

Different concentrations of methionine were also tested. In this experiment 100, 250 and 500 mg/ℓ methionine all had the same effect on the growth of Klebsiella.

Figure 13. Growth Curves for Klebsiella sp. in M-9 Plus 10 mg/ℓ $CN^-$ With Methionine, Histidine, Cysteine, Leucine, and Without Amino Acids Added


.40
.30
.20
.10
.09
.08
.07
.06
.05
.04
.03
.02
O MET.
□ HIS.
⬡ CYS.
△ LEU.
X NO aa
OPTICAL DENSITY, 540 nm
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
TIME (HOURS)

In all experiments involving the addition of amino acids, the pH of the medium was checked to insure that there was not a drop in pH.

## Cyanide Determinations

The next series of experiments was designed to determine the cyanide concentration in the media and therefore determine if the cells might be degrading it. Three different methods were tried. The first method was a colorimetric method of Skowronski and Strobel (1969) involving formation of a color complex with picric acid. Color development varied with the media. M-9 medium resulted in a darker color development. The resulting standard curve was only straight through concentrations of 15 to 70 mg/ℓ $CN^-$. This method was abandoned due to inaccuracy at lower cyanide concentrations.

The direct method of measurement using the cyanide electrode was attempted. The results from this were erratic at times, giving incorrect high and low concentrations. Various experiments with the electrode indicated that none or very little of the cyanide was being stripped from the media. Since cyanide is known to be strippable, this method was eventually abandoned.

In all the experiments that follow, cyanide was measured by the titration method. The distillation procedure recommended before using the titration method was not practical for the small samples involved in this study, so care was taken to check for possible interferences from the medium. Standard samples of $H_2O + CN^-$, M-9 + $CN^-$, and M-9 + Yex + $CN^-$ were all titrated and $CN^-$ concentrations were determined. On the average, the media titered 0.58 mg/ℓ lower than the distilled water, with the largest difference between the water and the media being 0.95

mg/ℓ. Another method of checking for interference involved titrating standard $H_2O + CN^-$, M-9 + $CN^-$, M-9 + Yex + $CN^-$, and a half-and-half mixture of the distilled water sample and the media sample. The average of the water and media titers were compared to the titers of the mixtures. On the average the difference between the two was 0.648 mg/ℓ $CN^-$, with the mixture always being slightly higher than the average of the two.

To determine cyanide concentrations at various stages of growth, Klebsiella was inoculated into 20 ml of M-9 + Yex plus 5, 20 and 50 mg/ℓ $CN^-$. Cyanide was determined during the log and stationary growth phases. The results are shown in Table VII. There is no apparent correlation between the different concentrations, but the results show that cyanide was present during the growth cycle.

The rate of cyanide stripping from M-9 + Yex medium was the subject of the next experiment. Two liters of uninoculated M-9 + Yex plus 10 mg/ℓ $CN^-$ contained in a cylindrical growth chamber were incubated at 30°C in a Fisher Scientific water bath and aerated at a rate of 1 LPM. A 20 ml sample was taken from the system every 15 minutes and titrated to determine the cyanide concentrations. The original concentration was 8.6 mg/ℓ $CN^-$ and after 9.75 hours the concentration had been reduced to 4.3 mg/ℓ $CN^-$.

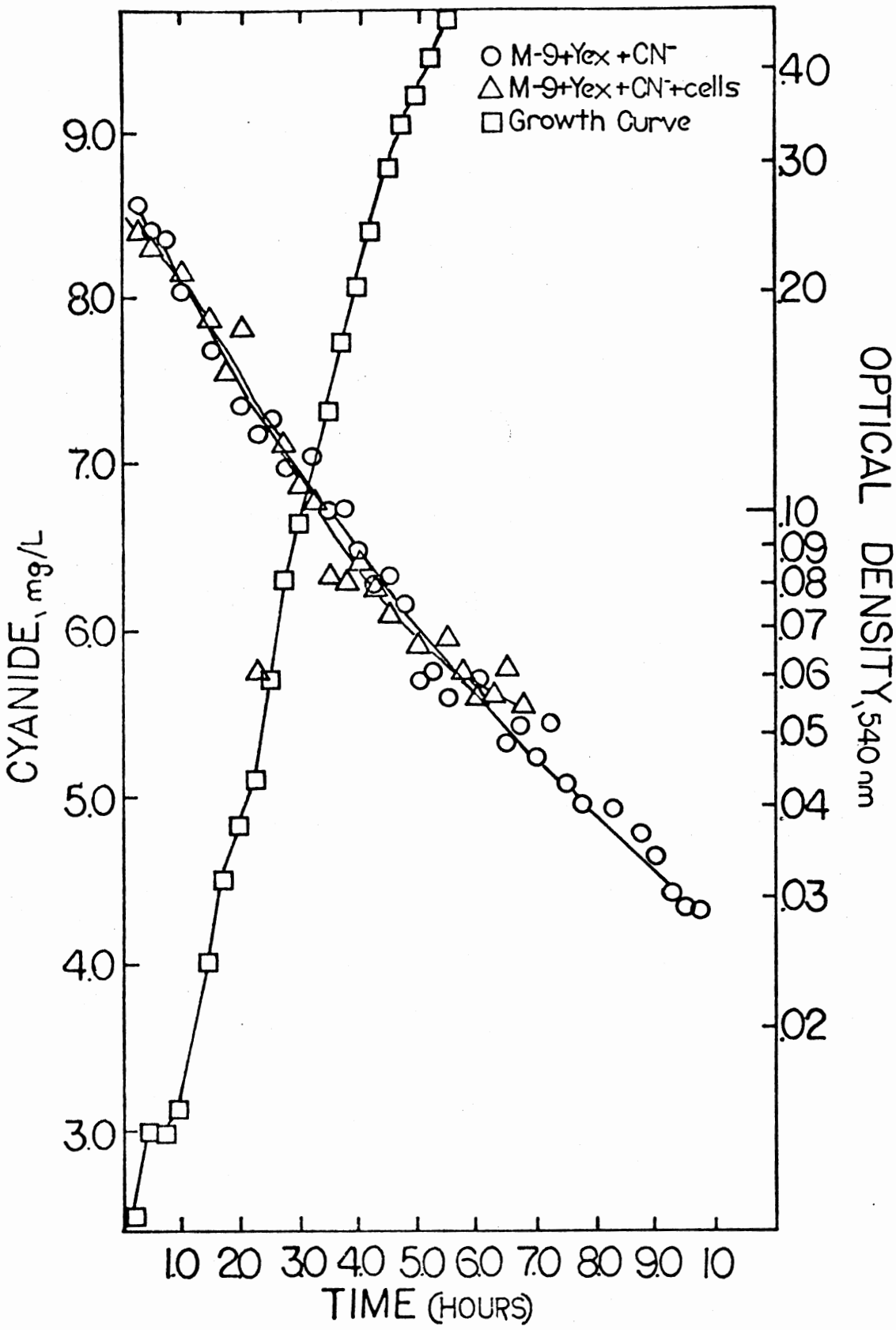
The same system was set up again and inoculated with a suspension of Klebsiella sp. in an attempt to see if Klebsiella reduced the cyanide concentration. A 20 ml sample was taken from the system every 15 minutes to determine growth rate and cyanide concentration. As seen in Figure 14, the concentration of cyanide in the inoculated system decreased at nearly the same rate as the cyanide from the uninoculated system. Cell growth and metabolism had no effect on cyanide concentration. The cells started

TABLE VII

CONCENTRATIONS OF CYANIDE AT DIFFERENT PHASES OF GROWTH OF KLEBSIELLA IN M-9 + YEAST EXTRACT

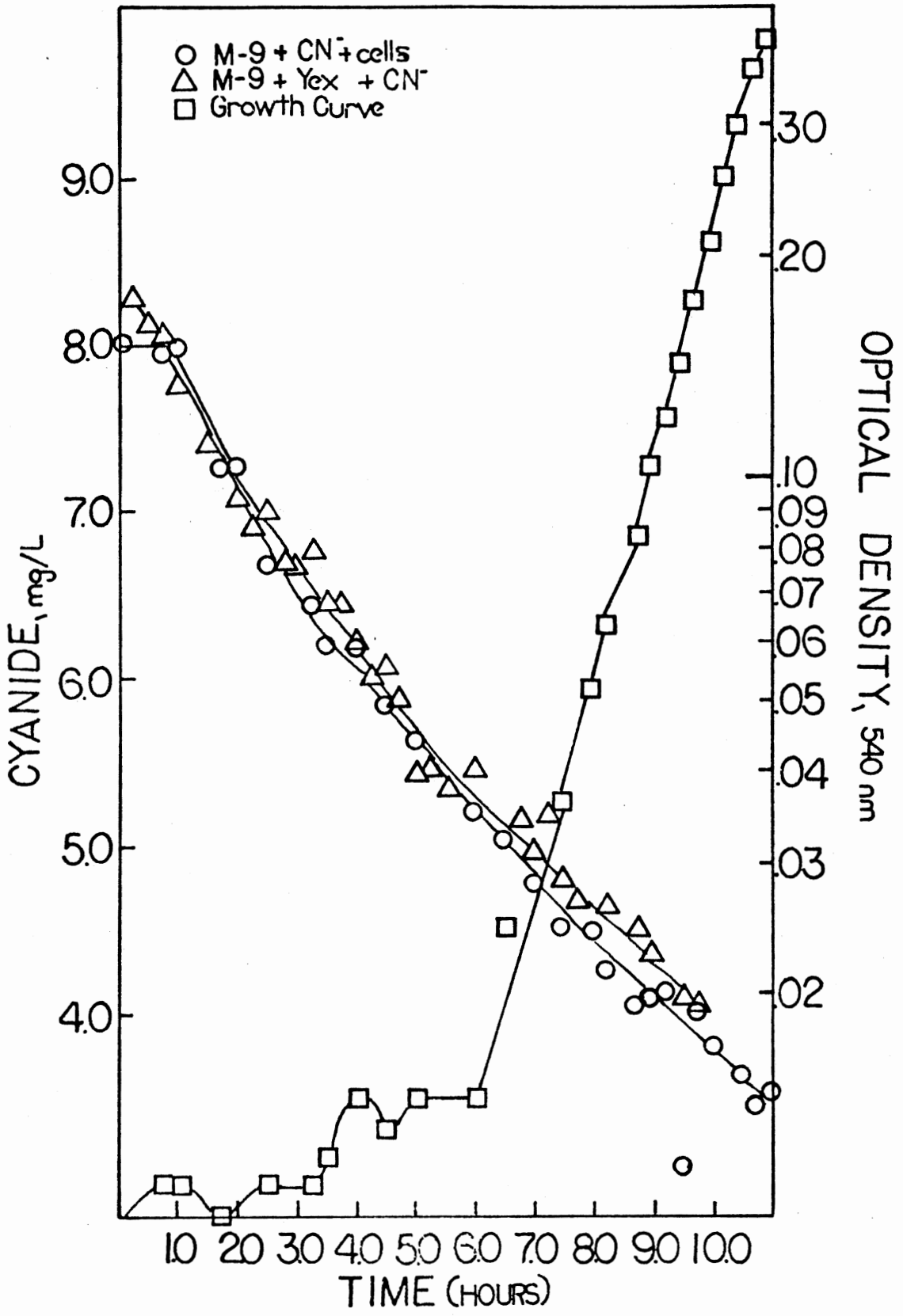
| Original mg/ℓ $CN^-$ | Concentration at Log Phase | Concentration at Stationary Phase |
|---|---|---|
| 5 | 3.56 mg/ℓ | 4.77 mg/ℓ |
| 20 | 13.62 mg/ℓ | 6.38 mg/ℓ |
| 50 | 28.48 mg/ℓ | 26.30 mg/ℓ |

Figure 14. Growth Curve for Klebsiella sp. in M-9 + Yex With $CN^-$ and the Disappearance of $CN^-$ From Inoculated and Uninoculated Medium


O M-9+Yex +CN⁻
△ M-9+Yex +CN⁻+cells
□ Growth Curve
CYANIDE, mg/L
9.0
8.0
7.0
6.0
5.0
4.0
3.0
OPTICAL DENSITY, 540 nm
.40
.30
.20
.10
.09
.08
.07
.06
.05
.04
.03
.02
1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10
TIME (HOURS)

growing while the cyanide concentration was high. In a third experiment the medium was changed to M-9 + $CN^-$ and it was also inoculated with Klebsiella. Figure 15 shows that cyanide disappeared from the inoculated medium at the same rate as that from the uninoculated medium. After 11 hours there was 3.45 mg/ℓ $CN^-$ remaining in the inoculated medium. The cells started logarithmic growth when the concentration was reduced to 5.2 mg/ℓ $CN^-$.

Figure 15. Growth Curve for Klebsiella sp. in M-9 With $CN^-$ and the Disappearance of $CN^-$ From Inoculated Medium and Uninoculated M-9 + Yex With $CN^-$


M-9 + CN⁻ + cells
M-9 + Yex + CN⁻
Growth Curve
CYANIDE, mg/L
9.0
8.0
7.0
6.0
5.0
4.0
OPTICAL DENSITY, 540 nm
.30
.20
.10
.09
.08
.07
.06
.05
.04
.03
.02
1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0
TIME (HOURS)

# CHAPTER IV

# DISCUSSION

Cyanide, with its extreme toxicity to most forms of life, has been a significant problem of industrial wastewater treatment. Several investigators have attempted to find a means other than the expensive chemical methods for treating cyanide wastes. Biological treatment would be a less expensive and more conventional means of treating cyanide wastes. Since activated sludge systems have been increasingly employed for biological treatment of wastewater, it seems fitting that the system has been examined as a means of treating cyanide wastes. Nesbitt et al. (1959) reported an activated sludge system that metabolized up to 99% of its cyanide feed. Cyanide in this system was the sole source of carbon and nitrogen. Similar results were obtained by Ludzack and Schaffer (1962). Their activated sludge unit effectively degraded cyanide up to a concentration of 60 mg/ℓ $CN^-$. Murphy and Nesbitt (1964) also reported success in treating cyanide waste by an extended aeration method.

Feng (1977) undertook an investigation to see how effective a "hydrolytically assisted" extended aeration unit would be for treating cyanide wastes. The heterogeneous population in his unit was continuously fed 10 mg/ℓ cyanide with the feed. While the unit was being fed 10 mg/ℓ cyanide, there was no evidence of cyanide in the effluent and there was less than 1.0 mg/ℓ cyanide in the sludge. Although at this time the data were not conclusive, it became apparent that possibly one

or more organisms in the unit might be metabolizing the cyanide. This possibility inspired the present study.

It has been known for a long time that cyanide has an inhibitory effect on bacteria. The early part of this study with E. coli B and Ps. aeruginosa exemplified this inhibitory action. It took E. coli 105 hours and Ps. aeruginosa 46 hours to grow in M-9 plus 20 mg/ℓ $CN^-$. On the other hand, many organisms have been reported to be resistant in some way to cyanide and a number of investigators (Painter and Ware, 1955; Castric and Strobel, 1969; Skowronski and Strobel, 1969) have reported organisms capable of metabolizing cyanide.

Twenty-five apparently different bacteria were isolated from Feng's unit. Of these 25, 2 were chosen as the most likely to metabolize cyanide. Skowronski and Strobel's (1969) method for isolating bacteria capable of metabolizing cyanide was employed as an isolation procedure. This method, which involved various molar concentrations of KCN in a buffered salts solution, yielded only the Bacillus sp. used in this study. It may have been the only survivor due to its ability to form spores.

It became apparent that the isolate Klebsiella pneumoniae was unable to metabolize cyanide under the test conditions. When the organism was grown in M-9 + 10 mg/ℓ $CN^-$, the cyanide disappeared from the medium at a rate that corresponded almost exactly with cyanide stripping from the uninoculated medium. The same results were obtained from a similar experiment involving M-9 + Yex as the growth medium. Under these conditions it can only be concluded that Klebsiella cannot metabolize or degrade $CN^-$.

No conclusive evidence was gathered to determine whether the Bacillus isolate could utilize $CN^-$. No effective means of measuring the $CN^-$

in TSB was found. The dark color of the medium interfered with color development in the picric acid method and with the color change of the titration method. One can only speculate from the data on stripping of $CN^-$ from M-9 + Yex that there was a significant amount of cyanide present during the growth of the Bacillus and that the Bacillus is resistant to $CN^-$ under the test conditions.

The behavior of the Bacillus in the presence of cyanide is still a puzzling phenomenon. After being inoculated into cyanide medium, the O.D. of the cells remained constant or dropped, indicating no growth, death, or possible spore formation. After a period of time, the cells began to grow and approached logarithmic growth. Then suddenly, about 7.5 to 9.0 hours after inoculation, the O.D. decreased, showing a dip in the growth curve. The O.D. reached its previous level after about 2.0 to 5.5 hours depending on the concentration of cyanide. With no further disturbances the cells then began growing at a rate near the original growth rate. The cause of the dip is a subject of speculation. It was thought that it might possibly be caused by spore formation. A sample taken at the time of the dip and examined under the microscope showed that there were some spores present, but mainly there were vegetative cells. Another possibility is that the cyanide causes the cells to switch from one metabolic pathway to another or from one electron transport system to another. Those cells unable to switch die and the others continue growing.

If the latter possibility were the case, then it seems that when the cells were transferred after the dip to fresh cyanide medium they would immediately start growing or have a very short lag period. As was seen in Figure 4, this was not the case. A very long lag period resulted

from the transfer of cells made both before and after the dip. Also, one might expect cells transferred before the dip to behave as they did originally, but that was not the case. These unusual characteristics warrant further study.

Both isolates when grown in cyanide medium had a long lag phase. For Klebsiella this was only true when it was grown in M-9 and cyanide. This inhibitory action has been encountered by a number of other investigators. Mizushima et al. (1959) and Castric and Strobel (1969) reported lag periods of 7 to 8 hours and 10 to 12 hours, respectively, which corresponds closely to the lag periods found in the present study for Klebsiella in M-9 + 10 mg/ℓ cyanide. Mizushima and Arima (1960) reported a much shorter 3 hour lag period before Achromobacter would grow. Both Mizushima and Arima and Castric and Strobel used rich media of boullion plus meat extract and TSB plus glucose, respectively. In both cases it was evident that the rich media did not result in a short lag period. The Bacillus grown in TSB in this study had a lag period of about 4 hours before there was evidence of growth. On the other hand, the Klebsiella sp. experienced a short lag period when grown in M-9 + Yex with cyanide concentrations above 50 mg/ℓ. As was seen in Figure 7, the lag period in M-9 and M-9 + Yex with 10 mg/ℓ $CN^-$ was only one hour, while the lag in M-9 + 10 mg/ℓ $CN^-$ was approximately 10 hours. When Yex was added to a culture of Klebsiella in M-9 + 10 mg/ℓ $CN^-$, the cells immediately started growing and went quickly into log growth. Yex was added at 3 and 6 hours after inoculation, so these times were well within the usual lag period. These data clearly demonstrate that Yex assists Klebsiella in overcoming the inhibitory effect of $CN^-$. A somewhat similar response was encountered in activated sludge systems where Ludzack and

Schaffer (1962) and Murphy and Nesbitt (1964) found that added nutrients improved the operation of the system. Ludzack and Schaffer proposed adding domestic sewage to industrial cyanide wastes for better biological treatment of the cyanide wastes.

Since Feng's (1977) extended aeration unit functioned well with the "hydrolytic assist," some hydrolysate was obtained from Feng and was tested to see if it enhanced cell growth in cyanide. As was seen in Figures 9 and 10, the hydrolysate did assist Klebsiella in overcoming cyanide inhibition in much the same way as the Yex did. Although the addition of these nutrients do not help the cells degrade $CN^-$, it is reasonable to believe that the addition of nutrients would aid and improve the operation of a biological treatment for cyanide. According to Feng (1977) cyanide does not accumulate in large proportions in the sludge, so a hydrolytic assist would be a reasonable method of adding extra nutrients to the system.

Amino acids were tested in an attempt to narrow down the components in Yex that enhanced cell growth in the presence of cyanide. A mixture of 19 amino acids had the same effect that Yex had. Of the 19 amino acids tested, 4 were found to be capable of enhancing cell growth in the presence of cyanide. These four were methionine, histidine, cysteine, and leucine, and of these four, methionine had the greatest effect. None of these amino acids singly or in various combinations were as effective as the 19 amino acids together or the Yex or hydrolysate. The fact that methionine stimulates the growth of Klebsiella in the presence of cyanide corresponds to reports by Nazar and Wong (1969, 1973) that methionine, when added to E. coli cells treated with cyanide, stimulated protein synthesis and cell growth. The fact that methionine was depleted from

cyanide-treated cells led Nazar and Wong to suggest that cyanide forms a complex with a $B_{12}$-transmethylase involved in methionine biosynthesis. The addition of methionine would overcome this inhibition. This proposal by Nazar and Wong may partially explain why Klebsiella and possibly the Bacillus isolate were able to grow in high cyanide concentrations.

Another possibility must not be overlooked. Niven et al. (1975) and Weston et al. (1974) provided evidence that Chromobacterium violaceum and Beneckia natriegens were resistant to cyanide due to a branching of the respiratory pathway. One branch was said to be sensitive and the other resistant to cyanide. A number of other investigators (Mizushima and Arima, 1960; Arima and Oka, 1965; Kauffman and Van Gelder, 1973, 1974; and McFeters et al., 1970) have cited evidence that various changes and conditions in the electron transport system render some bacteria resistant to cyanide inhibition. This remains an uninvestigated possibility for the two isolates Bacillus and Klebsiella.

Raef et al. (1977) reported that glucose and cyanide react at an optimum pH around 11.0. They warned that the reaction above pH 8.0 is rapid enough to cause significant error in cyanide determinations. They also found the glucose-cyanide products to be biodegradable by a heterogeneous population. In addition, Raef et al. proposed combining, at a high pH, cyanide waste with a waste containing aldoses and biologically treating the resulting products. The M-9 medium in this study contains a large amount of glucose, but the pH of the medium was 7.1 to 7.2, well below pH 11.0 or 8.0. There may have been some cyanide reacting with glucose in the medium, but it would have to be a minimal amount since the titration method for determining $CN^-$ concentration only measures free ions and not cyanide complexes. The titrations showed that there was a

significant amount of $CN^-$ in the medium. Experiments designed to check for interference showed that interference from other components of the medium amounted to less than 1 mg/ℓ cyanide.

As mentioned before, biological treatment on a laboratory scale has been successful in treating cyanide wastewater. Both Murphy and Nesbitt (1964) and Feng (1977) reported success treating cyanide wastes by the extended aeration process. Cyanide stripping from the system could be a problem, but Feng reported that high solids concentration reduced stripping of cyanide. In this study the addition of extra nutrients aided cells in overcoming inhibition by cyanide. When using a "hydrolytically assisted" extended aeration process for treating cyanide wastes, the hydrolysate would have a dual function. It would not only help the autodigestion processes of extended aeration, but it would also allow cells to grow better in the presence of cyanide. The addition of hydrolysate cannot be said to help the system metabolize cyanide, but it could help cells that could remove other sources of COD to grow in the presence of $CN^-$.

# CHAPTER V

## SUMMARY AND CONCLUSIONS

In the search for an organism in Feng's extended aeration unit that could metabolize cyanide, this investigation was inconclusive. Although the two isolates, Bacillus sp. and Klebsiella sp., were shown to be resistant to high concentrations of cyanide, there was no evidence that they could metabolize cyanide.

The strange behavior of the Bacillus in the presence of cyanide is unexplained. One can only speculate that the dip was caused by the sudden formation of spores (although there was no microscopic evidence of this) or by a change to some resistant pathway in some, but not all, of the cells. This interesting behavior warrants further study.

The inhibition of growth of Klebsiella sp. was overcome by the addition of yeast extract, hydrolysate, or amino acids. Methionine was the most effective single compound in antagonizing the effect of cyanide upon growth of Klebsiella sp.

The mechanism of resistance for both isolates may be due to the fact that the medium contains a product like methionine, which is the end product of a pathway blocked by cyanide. If the cells are able to take up this product, then they could overcome the inhibition by cyanide.

Since Klebsiella eventually grows in a minimal medium containing cyanide, the resistance cannot be explained by the aforementioned mechanism. It must be theorized that this resistance is due to a branching

electron transport chain or the production of resistant cytochromes. This theory needs further investigation. Another possibility for this eventual growth in M-9 + $CN^-$ may be due to cyanide stripping to a threshold level at which the bacteria are able to grow.

## A SELECTED BIBLIOGRAPHY


Allen, J., and G. A. Strobel. 1966. The assimilation of $H^{14}CN$ by a variety of fungi. Can. J. Microbiol. 12: 414-416.

American Public Health Association. 1975. Standard Methods for the Examination of Water and Wastewater, 14th ed. New York.

Aquatic Life Advisory Committee of the Ohio River Valley Water Sanitation Commission. 1960. Stream pollution: aquatic life water quality criteria. J. Water Pollution Control Fed. 32: 65-82.

Arima, K., and T. Oka. 1965. Cyanide resistance in Achromobacter. I. Induced formation of cytochrome $a_2$ and its role in cyanide-resistant respiration. J. Bacteriol. 90: 734-743.

Ashcroft, J. R., and B. A. Haddock. 1975. Synthesis of alternative membrane-bound redox carriers during aerobic growth of Escherichia coli in the presence of potassium cyanide. Biochem. J. 148: 349-352.

Blumenthal-Goldschmidt, S., G. W. Butler, and E. E. Conn. 1965. Enzymatic formation of β-cyanoalanine from cyanide by Escherichia coli extracts. Nature 208: 1206-1207.

Brysk, M. M., W. A. Corpe, and L. U. Hanks. 1968. β-Cyanoalanine formation by Chromobacterium violaceum. J. Bacteriol. 97: 322-327.

Brysk, M. M., C. Lauinger, and C. Ressler. 1969. Biosynthesis of cyanide from (2-$^{14}C^{15}N$) glycine in Chromobacterium violaceum. Biochim. Biophys. Acta. 184: 583-588.

Brysk, M. M., and C. Ressler. 1970. γ-Cyano-α-L-aminobutyric acid, a new product of cyanide fixation in Chromobacterium violaceum. J. Biol. Chem. 245: 1156-1160.

Buchanan, R. E., and N. E. Gibbons (eds.). 1974. Bergey's Manual of Determinative Bacteriology, 8th ed. Williams and Wilkins Company, Baltimore.

Castric, P. A. 1975. Hydrogen cyanide, a secondary metabolite of Pseudomonas aeruginosa. Can. J. Microbiol. 21: 613-618.

Castric, P. A., and G. A. Strobel. 1969. Cyanide metabolism by Bacillus megaterium. J. Biol. Chem. 244: 4089-4094.

Clawson, B. J., and C. C. Young. 1913. Preliminary report on the production of hydrocyanic acid by bacteria. J. Biol. Chem. 15: 419-422.

Dodge, B. F., and W. Zabban. 1951. Disposal of plating room wastes. III. Cyanide wastes, treatment with hypochlorites and removal of cyanates. Plating 38: 561. In Weber, W. J. 1972. Physiochemical Processes for Water Quality Control. Wiley-Interscience. New York.

Dulbecco, R. 1950. Experiments on photoreactivation of bacteriophages inactivated with ultraviolet radiation. J. Bacteriol. 59: 329-347.

Feng, Yuan-Jenn. 1977. Studies on the "hydrolytically-assisted" extended aeration process treatment of wastes containing cyanide. (Unpub. M. S. thesis, Oklahoma State University.)

Freeman, L. R., G. J. Silverman, and C. Merritt, Jr. 1975. Production of hydrogen cyanide by Pseudomonas fluorescens. Appl. Microbiol. 29: 560-561.

Gaudy, A. F., Jr., P. Y. Yang, and A. W. Obayashi. 1971. Studies on the total oxidation of activated sludge with and without hydrolytic pretreatment. J. Water Pollution Control Fed. 43: 40-54.

Gibbs, B. M., and F. A. Skinner. 1966. Identification Methods for Microbiologists. Part A. Academic Press, New York.

Gurnham, C. F. 1955. Cyanide destruction on trickling filters. Purdue Univ. Eng. Ext. Dept. Bull. 89: 186-193.

Kauffman, H. F., and B. F. Van Gelder. 1973. The respiratory chain of Azotobacter vinelandii. II. The effect of cyanide on cytochromes. Biochim. Biophys. Acta. 314: 276-283.

Kauffman, H. F., and B. F. Van Gelder. 1974. The respiratory chain of Azotobacter vinelandii. III. The effect of cyanide in the presence of substrates. Biochim. Biophys. Acta. 333: 218-227.

Ludzack, F. J., W. A. Moore, H. L. Krieger, and C. C. Ruchhoft. 1951. Effect of cyanide on biochemical oxidation in sewage and polluted water. Sew. Ind. Wastes. 23: 1298-1307.

Ludzack, F. J., and R. B. Schaffer. 1962. Activated sludge treatment of cyanide, cyanate and thiocyanate. Water Pollution Control Fed. 34: 320-341.

Ludzack, F. J., R. B. Schaffer, R. N. Bloomhuff, and R. A. Taft. 1959. Experimental treatment of organic cyanides by conventional sewage disposal processes. Purdue Univ. Eng. Ext. Dept. Bull. 104: 547-565.

McFeters, G. A., D. F. Wilson, and G. A. Strobel. 1970. Cytochromes in a cyanide-resistant strain of Bacillus cereus. Can. J. Microbiol. 114: 51-54.

Meganathan, R., and P. A. Castric. 1977. The effect of inorganic phosphates on cyanogenesis by Pseudomonas aeruginosa. Arch. Microbiol. 114: 51-54.

Michaels, R., and W. A. Corpe. 1965. Cyanide formation by Chromobacterium violaceum. J. Bacteriol. 89: 106-112.

Michaels, R., L. V. Hankes, and W. A. Corpe. 1965. Cyanide formation from glycine by nonproliferating cells of Chromobacterium violaceum. Arch. Biochem. Biophys. 111: 121-125.

Mizushima, S., and K. Arima. 1960. Mechanism of cyanide resistance in Achromobacter. I. Adaptive formation of cyanide resistant respiratory system in growing cells. J. Biochem. 47: 351-360.

Mizushima, S., M. Nakano, and K. Sakaguchi. 1959. Cyanide-insensitive terminal respiratory system in Aerobacter cloacae. J. Biochem. 46: 373-381.

Moeller, V. 1954. Diagnostic use of the Braun KCN test within the Enterobacteriaceae. Acta Pathol. Microbiol. Scand. 3: 115. In Munson, T. E. 1974. Improved KCN medium. Appl. Microbiol. 27: 262-263.

Munson, T. E. 1974. Improved KCN medium. Appl. Microbiol. 27: 262-263.

Murphy, R. S., and J. B. Nesbitt. 1964. Biological treatment of cyanide wastes. Pennsylvania State Univ. Eng. Res. Bull. No. B-88.

Nazar, R. N., and J. T. Wong. 1969. Inhibitor-induced shift-downs in Escherichia coli. J. Bacteriol. 100: 956-961.

Nazar, R. N., and J. T. Wong. 1972. Nucleotide changes and the regulation of ribonucleic acid accumulation during growth rate shifts in Escherichia coli. J. Biol. Chem. 247: 790-797.

Nazar, R. N., and J. T. Wong. 1973. Azide- or cyanide-induced methionine starvation in Escherichia coli. Biochim. Biophys. Acta. 324: 218-225.

Nesbitt, J. B., H. R. Kohl, and E. L. Wagner, Jr. 1959. The aerobic metabolism of potassium cyanide. Purdue Univ. Eng. Ext. Dept. Bull. 104: 518-534.

Niven, D. F., P. A. Collins, and C. J. Knowles. 1975. The respiratory system of Chromobacterium violaceum grown under conditions of high and low cyanide evolution. J. Gen. Microbiol. 90: 271-285.

Oka, T., and K. Arima. 1965. Cyanide resistance in Achromobacter II. Mechanism of cyanide resistance. J. Bacteriol. 90: 744-747.

Painter, H. A., and G. C. Ware. 1955. Bacterial utilization of cyanide. Nature 175: 900.

Pudek, M. R., and P. D. Bragg. 1974. Inhibition by cyanide of the respiratory chain oxidases of Escherichia coli. Arch. Biochem. Biophys. 164: 682-693.

Raef, S. F., W. G. Characklis, M. A. Kessick, and C. H. Ward. 1977. Fate of cyanide and related compounds in aerobic microbial systems. II. Microbial degradation. Water Research 11: 485-492.

Resnick, J. D., W. A. Moore, and M. B. Ettinger. 1958. Behavior of cyanates in polluted water. Ind. Eng. Chem. 50: 71-72.

Ressler, C., O. Abe, Y. Kondo, B. Cottrell, and K. Abe. 1973. Purification and characterization from Chromobacterium violaceum of an enzyme catalyzing the synthesis of γ-cyano-α-aminobutyric acid and thiocyanate. Biochem. 12: 5369-5377.

Sadasivam, K. V. 1974. Cyanide-tolerant microorganisms in the rhizosphere of tapioca. Soil Biol. Biochem. 6: 203.

Skowronski, B., and G. A. Strobel. 1969. Cyanide resistance and cyanide utilization by a strain of Bacillus pumilus. Can. J. Microbiol. 15: 93-98.

Sneath, P. H. A. 1955. Cultural and biochemical characteristics of the genus Chromobacterium. J. Gen. Microbiol. 15: 70-98.

Strobel, G. A. 1967. Cyanide utilization in soil. Soil Science 103: 299-302.

U.S. Department of Health, Education, and Welfare. 1962. Public Health Service Drinking Water Standards. Public Health Service, Washington, D. C.

Weber, W. J. 1972. Physiochemical Processes for Water Quality Control. Wiley-Interscience. New York.

Weston, J. A., P. A. Collins, and C. J. Knowles. 1974. The respiratory system of the marine bacterium Beneckea natriegens II. Terminal branching of respiration to oxygen and resistance to inhibition by cyanide. Biochim. Biophys. Acta. 368: 148-157.

White, A., P. Handler, and E. L. Smith. 1973. Principles of Biochemistry, 5th ed. McGraw-Hill Book Co. New York.

Winter, J. A. 1962. The use of a specific actinomycete to degrade cyanide wastes. Purdue Univ. Eng. Ext. Dept. Bull. 112: 703-716.

Wissing, F. 1975. Cyanide production from glycine by a homogenate from a Pseudomonas species. J. Bacteriol. 121: 695-699.

Youatt, J. B. 1954. Studies on the metabolism of Thiobacillus thiocyanoxidans. J. Gen. Microbiol. 11: 139-149.

Zintgraff, G. D., C. H. Ward, and A. W. Busch. 1969. Cyanide inhibition of mixed microbial populations. Developments in Ind. Microbiol. 10: 253-270.

# VITA[2]

Gemma Lynn Brueggemann

Candidate for the Degree of

Master of Science

Thesis: EFFECT OF CYANIDE ON THE GROWTH OF KLEBSIELLA SPECIES AND BACILLUS SPECIES

Major Field: Microbiology

Biographical:

Personal Data: Born in Enid, Oklahoma, on December 9, 1952, the daughter of Warren E. and Deborah Ann Brueggemann.

Education: Graduated from Enid High School, Enid, Oklahoma, in 1971; received the Bachelor of Science degree in Microbiology from Oklahoma State University, Stillwater, Oklahoma, in May, 1975; completed the requirements for the Master of Science degree in December, 1977.

Professional Experience: Graduate Teaching Assistant, Department of Microbiology, Oklahoma State University, from September, 1975, to May, 1977; Environmental Protection Agency Bioengineer Trainee, School of Civil Engineering, Oklahoma State University, August, 1975, to present.