Cyanide resistance and cyanide utilization by a strain of Bacillus pumilus
by Boleslaw Stanislaw Skowronski

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Microbiology
Montana State University
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Abstract:
Some unidentified microorganisms have been described as capable of surviving 10-3M and less concentrations of cyanide, but none have been described which survived higher concentrations of this compound. A strain of Bacillus pumilus was isolated from a clay which had supported flax growth for 73 consecutive years. This strain differed from the Bacillus pumilus, American Type Culture Collection in only one important respect: inability to produce acetylmethylcarbinol. The isolate survived in 8 synthetic and 5 nonsynthetic media in the presence of 10-1M cyanide. Addition of vitamins to the synthetic media and depletion of sugars from the nonsynthetic media enhanced bacterial growth. When grown on trypticase soy yeast extract with 10-1M KCN, the bacteria formed long filaments irreversibly, During the first 24 hours of incubation, the cells in the filaments were connected to one another firstly by cytoplasmic bridges and secondly by strands, At about 72 hours a cross wall appeared between the cells, the shape of which were changed* The filamentous forms took up 3 times as much oxygen as the original ones when incubated on medium without cyanide and 18 times as much on medium with 10-1M KCN, Cyanide decreased rapidly in the medium during bacterial growth and when K14CN was fed, 14CO2 was produced. Sterilized bacteria accumulated cyanide in the cell but did not produce 14CO2 This may imply the existence of a specific permease system.

When KC15N was fed,production peaked at 54 hours; sterilized bacteria showed no production. The relative production of 15NH4 from cyanide nitrogen and 14CO2 from cyanide-carbon suggests a relatively greater use of cyanide nitrogen. Incorporation of labelled cyanide into the amino acid fraction was demonstrated. A quantitative and qualitative analysis of amino acids showed that the bacterial pool of free amino acids contained no heterocyclic amino acids and no arginine. When grown on cyanide, these bacteria lost all of the detectable basic, all the sulphur containing, and the aromatic (traces of phenylalanine only) amino acids and lysine and valine, but contained on the average 33 times more arginine than any of the other amino acids in the pool. There is a possibility glutamic acid is massively converted to arginine and that cysteine forms a thiocyanate. The importance of the isolate in the cyanide microcycle and its potential usefulness for mankind is discussed.

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ABSTRACT

Some unidentified microorganisms have been described as capable of surviving 10^{-3}M and less concentrations of cyanide, but none have been described which survived higher concentrations of this compound. A strain of Bacillus pumilus was isolated from a clay which had supported flax growth for 73 consecutive years. This strain differed from the Bacillus pumilus, American Type Culture Collection in only one important respect: inability to produce acetylmethylcarbinol. The isolate survived in 8 synthetic and 5 nonsynthetic media in the presence of 10^{-1}M cyanide. Addition of vitamins to the synthetic media and depletion of sugars from the nonsynthetic media enhanced bacterial growth. When grown on trypticase soy yeast extract with 10^{-1}M KCN, the bacteria formed long filaments irreversibly. During the first 24 hours of incubation, the cells in the filaments were connected to one another firstly by cytoplasmic bridges and secondly by strands. At about 72 hours a cross wall appeared between the cells, the shape of which were changed. The filamentous forms took up 3 times as much oxygen as the original ones when incubated on medium without cyanide and 18 times as much on medium with 10^{-1}M KCN. Cyanide decreased rapidly in the medium during bacterial growth and when $K^{14}CN$ was fed, $^{14}CO_2$ was produced. Sterilized bacteria accumulated cyanide in the cell but did not produce $^{14}CO_2$. This may imply the existence of a specific permease system. When $KC^{15}N$ was fed, $^{15}NH_4$ production peaked at 54 hours; sterilized bacteria showed no $^{15}NH_4$ production. The relative production of $^{15}NH_4$ from cyanide nitrogen and $^{14}CO_2$ from cyanide-carbon suggests a relatively greater use of cyanide nitrogen. Incorporation of labelled cyanide into the amino acid fraction was demonstrated. A quantitative and qualitative analysis of amino acids showed that the bacterial pool of free amino acids contained no heterocyclic amino acids and no arginine. When grown on cyanide, these bacteria lost all of the detectable basic, all the sulphur containing, and the aromatic (traces of phenylalanine only) amino acids and lysine and valine, but contained on the average 33 times more arginine than any of the other amino acids in the pool. There is a possibility glutamic acid is massively converted to arginine and that cysteine forms a thiocyanate. The importance of the isolate in the cyanide microcycle and its potential usefulness for mankind is discussed.

INTRODUCTION

The possible importance of a cyanide cycle under primitive earth conditions has been demonstrated in experiments conducted independently in several laboratories. Miller (1955) showed that aldehydes and HCN were the products of an electric discharge through mixtures of hydrogen, methane, ammonia and water. Matthews and Moser (1966) were able to synthesize peptide precursors by a polymerization reaction of HCN with NH_4. Oro (1961) proposed a mechanism of adenine synthesis from HCN under conditions assumed to have existed on the primitive earth. Kliss and Matthews (1962) proposed a mechanism in which 1, 1 biradical species of HCN reacting with HCN could yield aminoacetonitriles, precursors of amino acids. From the 1, 3 biradical form of HCN the authors proposed a pathway to the precursors of proteins and purines. Cyanide could have played an important role in the origin of life; therefore, the discovery of its metabolism by an ever growing list of living organisms might be expected.

Experimental results obtained by Allen and Strobel (1966) and Strobel (1967 a) on cyanide utilization originated the hypothesis that a cyanide cycle exists in nature. This cycle involves the production of HCN by bacteria, fungi, plants, and the fixation of this compound by microorganisms and higher plants. Conditions under which this is most likely to occur would exist in the soil and some evidence was presented for this by Strobel (1967 b).

This natural cycle consists of cyanide release and utilization. Clawson and Young (1913) described the production of HCN by *Bacillus*

pyocyaneous, Bacillus fluorescens, and Bacillus violaceus. Patty (1921) reported varying amounts of HCN from different strains of B. pyocyaneous when grown aerobically and at pH 5.4 to 5.8. Lorck (1948) experimented with nine strains of the same bacterium on various synthetic media and at several temperatures. He detected HCN only when using glycine as a nitrogen source at 26 C; the production of HCN was accelerated by addition of small amounts of yeast extract. Bügel and Müller (1962) confirmed Lorck's results, in that glycine was the precursor of HCN. Michaels and Corpe (1965) and Michaels et al. (1965) observed the production of HCN by Chromobacterium violaceum on various media. The best production of HCN was obtained when succinate was added to these media. These authors also suggested glycine as the most probable precursor.

In fungi, Bach (1948) detected HCN formation in fruiting bodies of Pholiota aurea and Clitocybe geotropa, but only 48 hours after harvesting the fruiting bodies. It was postulated that HCN was formed enzymatically by the oxidative decomposition of amino acids and that the HCN production depended upon the oxygen pressure. Bach (1956) listed HCN formation by 35 fungi. Robbins et al. (1950) were able to inhibit growth of a series of microorganisms with HCN produced by an unidentified basidiomycete. Lebeau and Dickson (1953) found that the highest yield of HCN by an unidentified basidiomycete occurred at low temperatures. Ward and Lebeau (1962) assumed that HCN production was due to autolysis, but later Ward and Thorn (1965) increased the yield of HCN from cultures using glycine as the only nitrogen source. Stevens and Strobel (1966) and Stevens (1967)

have shown that this fungus uses valine and isoleucine as precursors to the cyanogenic glucosides linamarin and lotaustralin, respectively. They suggested that at least three enzymes acting together released HCN from these glucosides.

Cyanide utilization by several fungi was shown by Allen and Strobel (1966). Working with the previously mentioned unidentified basidiomycete Strobel (1964 and 1966) showed that α-aminopropionitrile was a precursor of alanine and that α-aminopropionitrile was produced enzymatically by the condensation of HCN, acetaldehyde and ammonia. Furthermore, Strobel (1967 a) presented evidence, based on isotope labelling and enzyme experiments, for a pathway of cyanide fixation leading from succinic semialdehyde, cyanide and ammonia to 4-amino-4-cyanobutyric acid which in turn was hydrolyzed to glutamate. Murphy and Nesbitt (1964) testing biological degradation of cyanide wastes added $K^{14}CN$ and harvested an unidentified viable microbial cell mass from which radioactive glutamic acid was isolated. They assumed that $^{14}CO_2$ was formed from HCN and fixation had occurred according to the scheme of Wood and Werkman (1941). No evidence was presented for this pathway. It may have been that a system similar to the one proposed by Strobel (1966) was operating. Ware and Painter (1955) isolated an organism provisionally classed among the <u>Actinomycetaceae</u>, that grew on silica gel media containing only KCN as a source of carbon and nitrogen. This bacterium was not maintained for future study.

The mechanism of HCN formation from plant tissue has been investigated very extensively. Butler (1965) listed 23 higher plants containing cyanogenic glucosides. These glucosides are apparently hydrolyzed by a β-glucosidase; the released aglycone then decomposed to HCN and an α-ketone (Butler et al. 1965). Trelawny et al. (1956) have shown that the release of HCN from plants can be attributed to the microbiological decomposition of decaying plants rich in glycosides and nitriles. Iwanoff and Zwetkoff (1936) starved Aspergillus niger on a sugar solution without nitrogen and later supplied KCN. The enzymatic oxidation of cyanide was 100% and could be accelerated by sodium thiosulphate. They assumed that in the presence of elemental sulphur KCNS was formed which served as an energy source according to the proposed reaction:

$$2\ KCNS + 5H_2O + 3O_2 \longrightarrow K_2SO_4 + (NH_4)_2\ SO_4 + CO_2 + (CH_2O)$$

That KCNS can act as an energy source for Thiobacillus thiocyanoxidans was demonstrated by Youatt (1954).

Incorporation of cyanide has been most extensively studied in plants. Blumenthal et al. (1963) showed the incorporation of $H^{14}CN$ into asparagine by seedlings of a number of species. Probably the carbon of $H^{14}CN$ entered the amide carbon of asparagine by a 3 + 1 carbon condensation, where the 1 carbon is of HCN origin. This was supported by feeding $H^{14}CN$ to seedlings of Lathyrus odoratus, Vicia saliva and Ricinus communis and observing the immediate formation of β-cyanoalanine (Tschiersch 1964 a). Similar results were obtained by Tschiersch (1964 b) with Linum usitatissimum. According to Tschiersch (1963) plants which do

not contain cyanogenic glucosides and do not promote the release of HCN are not capable of its utilization. Floss _et al._ (1965) administered $K^{14}CN$ and $H^{14}CN$ to homogenates of _Lotus tenuis_ seedlings and found evidence for cyanoalanine synthetase. Perhaps it serves to dispose of HCN that arises from cyanogenic glucoside. It is also possible that the cyanide fixation scheme that yields β-cyanoalanine and ultimately asparagine is important as a source of asparagine for the plant. Abrol and Conn (1966) have shown that labelled asparagine isolated from the seedlings of _Lotus arabicus_ and _Lotus tenuis_ which had been fed $H^{14}CN$ derived its amide carbon from the nitrile carbon. When the plants were fed with L-valine-$U^{14}C$ the distribution of radioactivity in the labelled asparagine suggested that radioactive cyanogenic glucoside formed from valine breaks down yielding HCN which in turn is incorporated into asparagine.

Presently, there are no reports on the _in vivo_ bacterial metabolism of HCN. Most of the past studies were concerned with adaptability of bacteria to an environment containing HCN. From a mixed culture obtained from cyanide treated activated sludge pilot plant, Asslestad (1961) isolated a bacterium which he placed in the family _Corynebacteriaceae_. This organism survived up to and including 2.5×10^{-3}M KCN only when 1.6×10^{-3}M sodium acetate with either cysteine or thiosulphate was added to the experimental media. Howe (1965) reported a successful bacterial acclimatization to cyanide by daily increased doses of sodium cyanide to the media. Eventually, some of the unspecified bacteria survived in media

containing 0.001% cyanide. Howe (1965) stressed the necessity of future research in identification of these bacteria. Trelawny et al. (1956) isolated from soil an obligately anaerobic, gram-positive, motile, rod-shaped unidentified bacterium able to survive in a basal medium containing of 0.2% cyanoacetic acid adjusted to pH 7.0. They believed its adaptation to cyanide to be similar to that of Tetrahymena pyriformis as reported by McCashland and Steinacher (1955).

Cyanide tolerance has been used also in the classification of bacteria. Braun (1938) experimented with media of 0.001% KCN and perfected the identification techniques for Bacterium coli, Bacillus lactis aer., Vibrio coma, staphylococci, streptococci, enterococci and pneumococci. Möller (1954) used 0.07% KCN in media similar to those used by Braun for differentiation between Salmonella and Escherichia freundii with variable results. Edwards and Fife (1956) recommended Möller's media for the differentiation of three genera of the tribe Salmonelleae. Kauffmann and Möller (1955) examined 400 Salmonella cultures in the Möller media with KCN and were able to differentiate between Salmonella delpata and Arizona delpata previously considered one strain. Within the Arizona group a few KCN resistant strains were encountered but not further investigated.

A strain of Achromobacter was found to give adaptive growth and respiration in the presence of 10^{-3}M KCN (Mizushima and Arima 1960 a, b, c, d) and adapted cells contained more of each cytochrome, especially cytochrome oxidases. It was postulated that cyanide-resistant respiration

was possible because of the increased amount of the oxidases (cytochromes a_1 and a_2). Preliminary studies were performed on the terminal electron-transport system of this bacterium and the role of cytochromes in respiratory activity were discussed from a theoretical point of view. The mechanism of the cyanide resistance of the same bacterium was studied by Arima and Oka (1965) and Oka and Arima (1965). Dunhill and Fowden (1965) used an extract of E. coli and demonstrated formation of β-cyanoalanine from $K^{14}CN$.

The study of cyanide utilizing bacteria is important, as it relates to the possibility of detoxification of cyanide wastes from effluents produced in common industrial operations, i.e., metal hardening, finishing and plating. From such industrial waste Winter (1962) isolated a microorganism tentatively described as belonging to the genus Nocardia. This waste was diluted with tap water until it contained 0.01% of the original, not specified cyanide concentration in ionic or complexed forms and growth of bacteria occurred. Cultures of this microorganism were capable of degrading 90% of 0.0025% KCN in diluted effluents containing added phosphate. Raynaud and Bizzini (1959) working for France's Renault Auto Industry patented three bacteria isolated from factory effluents: a gram-positive coccus, a gram-positive rod and a mobile gram-negative rod, probably from the family Pseudomonadaceae. The last named organism is probably able to utilize sodium cyanide in a concentration of 250 mg/1. This quantity of cyanide is much less then is usually found in factory effluents. Furthermore, the pH range for the experimental media was

between 6.7 to 7.3. It is unlikely that the mentioned cyanide concentration could be maintained at these low pH values. Howe (1966) conducted a 15 year study of biological degradation of cyanide wastes and developed a process now protected by 3 patents in the United States, whereby unspecified concentrations of soluble cyanides from industrial effluents could be degraded by a bacterial mass isolated by the author. In other experiments of this type Nesbitt et al. (1959 and 1960), Ludzak et al. (1959), Brink and Thayer (1960) failed to isolate a specific bacterium from industrial wastes. Pettet and Mills (1954) were able to destroy 0.01% of cyanide complexed with zinc, cadmium and copper or as KCN when added to sewage being treated in laboratory scale percolating filters. A flora capable of destroying cyanide was found but no particular microorganism was identified.

Although cyanide has been used for taxonomy and for cytochrome studies in bacteriology, no report to date has been concerned with cyanide as a bacterial nutrient. Furthermore, the role of bacteria in the cyanide cycle seems to be important but has not been elucidated.

The purpose of this report is to close this gap and open new ways in research leading to better understanding of the cyanide microcycle in nature.

MATERIALS AND METHODS

Isolation of a cyanide resistant bacterium from soils. Soil samples were collected from the top 4 inches beneath the O horizon as described by Strobel (1967 b). Soils were air dried, sieved for removal of large particles and stored in plastic bags at 4C. Prior to their assay the soils were worked into a more or less friable state according to Davis (1967).

Stock solutions were prepared by mixing 10 g of each soil sample, as listed in Table I, with 90 ml of deionized, double distilled water made 10^{-3}, 10^{-2}, 10^{-1}, 1, 2, and 2, 5 M in respect to cyanide. On all subsequent experiments a standard 10^{-1}M concentration of cyanide was used. Streak plates were made from each suspension on trypticase soy yeast agar and approximately 25 colonies were transferred to nutrient agar slants for further study.

Generic identification. The morphological and physiological criteria of Breed *et al.* (1957) were used for the taxonomic description of the culture isolated from a sample of Fargo clay. Techniques and media used were primarily those described in Manual of Microbiological Methods (1957).

Bacterial growth. Viability of the isolated bacterium was tested with synthetic and nonsynthetic media with 10^{-1}M KCN, listed in Tables II and III, respectively.

Culturing. Three bacteria were used in the research. *Bacillus pumilus* (ATCC No. 7061), a second isolated from Fargo clay soil and a third filamentous type developed from the Fargo clay isolate on a medium containing cyanide. These bacteria were referred to later as BP, BPA, and BPF,

Table I

Experimental Soils

No.	Soil name	Crop	pH of solution 10g of soil in 90 ml H_2O
1	Cheyenne wheat stubble	Prune orchard	6.9
2	Huffine silt loam	Grass vegetation	6.7
3	Yolo loam	Peach orchard (7 consecutive years cultivation)	6.8
4	Bridger-clay loam	Moss in the forest area	6.6
5	Fargo clay Provided by H. H. Flor, Fargo, North Dakota	Flax (73 consecutive years of cultivation)	7.5

Table II

Synthetic Media*

No.	Medium description	Modification	Vitamins* added	References
1	Minimal nutritional requirement for genus *Bacillus*	no amino acids added, pH not adjusted	+	Proom and Knight (1955).
2	"	"	-	"
3	Mineral, with trace elements according to Vinogradsky	no ammonium sulphate and magnesium carbonate 1ml of trace elements added Trace elements in mg/1000ml H_2O 3600 $FeCl_3 \cdot 6H_2O$, 440 $ZnSO_4 \cdot 7H_2O$ 200 $CoCl_2 \cdot 6H_2O$, 20 $CuSO_4 \cdot 5H_2O$ 20 $MnCl_2 \cdot 4H_2O$	+	Salle (1954).
4	"	"	-	"
5	Synthetic medium Mg	without 0.4% glucose	+	Dulbecco (1950).
6	"	"	-	"
7	Improved minimal medium	without Na_3-citrate without 1% glucose	+	Davis and Mignoli (1950).
8	"	"	-	"
9	Tris buffer solution of minerals	pH not adjusted without 1% glucose	+	Hershey (1955).
10	"	"	-	"
11	Mineral salt solution	without sodium acetate ammonium chloride and yeast extract	+	Asslestad (1961).

Table II
(continued)

12	Mineral salt solution	without sodium acetate ammonium chloride and yeast extract	-	Asslestad (1961).
13	Minimal amounts of components	no yeast extract no pH not adjusted	+	Müller (1966) Medium Ic.
14	"	no yeast extract no pH not adjusted	+	"
15	"	Sterilized tap water only	+	

* 100 ml in 250 ml E. flasks

** 1 ml of vitamins was added each time consisting of:
1% solution of biotin
1% solution of thiamine

Table III

Nonsynthetic Media*

No.	Medium description	Modification	References
16	Trypticase Soy Broth	---	Products for Microbiological Laboratory (1964).
17	"	without glucose	"
18	Trypticase Soy Yeast Broth	---	"
19	"	without glucose	"
20	Dextrose Broth	---	Difco (1964).
21	"	without glucose	"
22	Glucose Broth	---	Salle (1954).
23	"	without glucose	"
24	Mineral with Bacto-Nutrient Broth	---	Bügel and Müller (1963).
25	"	without glucose	"

* 100 ml in 250 ml E. flasks

respectively. In all experiments unless otherwise stated, the bacteria were grown on BBL trypticase soy yeast extract broth without glucose (TSYB). The incubation was performed in a Psycro-Therm Gyrotory Shaker at 40 C and 50 RPM referred to later as "optimal growth condition" and these conditions were used in all experiments, unless otherwise stated. The bacterium (BPA) was also grown in the standard medium without cyanide adjusted to pH 8.5 with phosphate buffer. Anaerobiosis was investigated in Brewer jars with a control tube containing an oxidation-reduction indicator (Fildes-McIntosh 1921).

Isolation of the cation fraction. Bacteria were grown on 300 ml of the standard medium containing 10^{-1}M KCN at optimal conditions. They were harvested in the logarithmic phase of growth by centrifugation at 12,000 x g for 10 minutes. The pellet was washed twice and centrifuged at 12,000 x g for 5 minutes. In all these procedures the temperature was maintained at 4 to 6 C. The washed cells were disrupted in a Braun model MSK mechanical cell homogenizer as follows: cell paste:0.1M pyrophosphate buffer pH of 8.5 : 0.11-0.012 mm diameter beads (1 : 1 : 2 w/v/w) were cooled and agitated for 30 seconds. After this period of time 100% of the bacteria were disrupted as observed by light microscopy in stained and wet mounts. The beads were separated from the homogenate by centrifugation at 2000 x g for 3 minutes. Then, 20 ml of 95% ethanol was added slowly with stirring to the homogenate for protein precipitation and the suspension was centrifuged at 16,000 x g for 10 minutes. The anion, cation and neutral fractions were prepared by using Dowex 1 formate and

Dowex 50-H^+, 200-400 mesh in 15 cm x 1.5 cm colums. After elution with 6 ml of 6 N HCl the cation fraction was taken to dryness in a flash evaporator and dried over P_2O_5 and NaOH. Samples were taken up in 0.1 ml of distilled water and 10 μl quantities chromatographed in various solvents. These samples were also analyzed in an amino acid analyzer through the courtesy of Dr. Darrel Weber, Department of Biology, University of Houston, Houston, Texas.

Chromatography. The cation fraction was separated on sheets of Whatman No. 1 paper using the following solvent systems: 1) n-butanol-acetic acid-water (4 : 1 : 5 v/v), 2) phenol 88%-water (4 : 1 v/v) and also by one-and-two dimensional thin layer chromatography on plain silica gel plates in the following by 4) n-butanol-acetic acid-water (3 : 1 : 1 v/v). Nineteen known amino acids as references were also separated in these solvent systems. Amino acids in both paper and thin layer chromatography were detected by spraying 0.3% ethanolic ninhydrin on the developed chromatograms.

GENERAL METHODS

Radioactivity determination. Radioactivity was measured with a Nuclear Chicago Liquid Scintillation Counter, Model 6804. The solvent used in the vials consisted of 1.5 ml methanol and 13.5 ml toluene containing 4.0 g 2, 5 diphenyloxazole and 100 mg of p-bis-2(5-phenyloxazolyl)-benzene per liter. Radioactivity on the chromatograms was detected by a Packard Radiochromatogram Strip Counter, Model 385. Counts were converted to dpm by the quench correction method with the use of a standard curve. ^{15}N

determinations were based on 100-atom per cent excess were performed as described by Stojanovic and Broadbent (1965).

Electron microscopy. The bacteria were grown with and without 10^{-1}M KCN. The cells were harvested from their corresponding logarithmic growth phases by centrifugation at 12,000 x g for 10 minutes. Cells grown without cyanide were washed twice with 0.14 M sodium chloride and those grown with 10^{-1}M KCN with 0.1 M pyrophosphate solution of pH 8.5. Appropriate amounts of the cell suspensions were centrifuged in small plastic capsules for 5 minutes at full speed in an International Clinical Centrifuge. Subsequently the compact cells were fixed with either glutaraldehyde as proposed by Sabatini et al. (1963) or in permanganate as described by Luft (1956). Material which had been fixed with glutaraldehyde was given a secondary fixation in osmium tetroxide. After dehydration with acetone the samples were embedded in the Epoxy Casting Resin A as described by Glavert and Glavert (1958). The thin sectioning was performed with a Reichert Om U2 ultramicrotome. Sections were stained either with uranyl acetate according to Watson (1958) or in permanganate according to Lawn (1960). The examination and photography was performed with a Zeiss EM 9A electron microscope.

Cyanide determination. The picric acid text (Kolmer and Boerner, 1945) was applied to quantitatively determine cyanide. A modification of their method was applied to the following fashion:

Various quantities of a standard solution of 0.005 M KCN was added to spectrophotometer tubes with 2 ml of 2% KOH and 1 ml of picric

acid: Na_2CO_3 : H_2O (1 : 5 : 200 w/w/v) and distilled water added to give 8 ml in each tube. The tubes were incubated for 10 minutes in a 37 C water bath, cooled for 20 minutes in a refrigerator and read in a Spectronic 20 at 475 mμ wavelength. The readings permitted a drawing of a standard curve for μg KCN versus absorbancy. The sensitivity of this method was 20 μg of cyanide.

Respiration study. All three bacterial strains used in this study were examined for cyanide inhibition of resting respiration.

Experiments were performed in a Gilson Differential Respirometer Model GPR 20. For each particular experiment a single temperature barometric pressure factor was applied to reduce the gas exchange data to absolute terms according to Gilson (1964). Bacteria were harvested in the log phase, washed and centrifuged twice with buffered water and resuspended in Dulbecco's Broth without glucose and NH_4Cl. Control flasks contained 1 ml of suspension, modified Dulbecco broth in the flask and the side arm. Experimental flasks contained sufficient KCN in the side arm to give a concentration of 10^{-1}M when added to the flask contents. CO_2 was trapped in the center well with 20% NaOH. In all experiments the data are presented as an average value of two readings corrected for standard conditions.

Conversion of cyanide carbon. Bacteria were harvested in the logarithmic phase of growth, washed twice with 0.14 M sodium chloride by centrifugation at 2000 x g for 3 minutes. One ml of a uniform suspension in 0.14 M sodium chloride was mixed with 50 ml of Dulbecco Broth, without glucose

and NH_4Cl in a 250 ml Erlenmeyer flask with a center well containing 2 ml of 4N NaOH. To the medium was added KCN to the final concentration of 10^{-1}M and 11.0 μc of $K^{14}CN$, specific activity 45.7 mc/mM. For each incubation period of 6, 24, 36, 54 and 72 hours respectively, a separate experiment was set up. At the end of each incubation time, the NaOH solution was added to 4 ml of 0.3 M $Ba(OH)_2$. The resulting precipitate of $BaCO_3$ was collected by centrifugation and washed with distilled water, dried at 70 C and weighed.

Carbon dioxide evolved from 10 mg of the $BaCO_3$ was trapped in 0.5 ml of hyamine hydroxide 10-X solution, which was transferred to a vial containing 14.5 ml of scintillation solution. As a control, autoclaved bacteria were tested in the same manner.

Conversion of cyanide nitrogen. The experiments were set up in a manner similar to those described for cyanide carbon utilization. $KC^{15}N$ of 96.1 atom per cent excess was added to the medium. A curved outlet tube from the reaction flask was dipped into a beaker containing 5 ml of 2N H_2SO_4 which trapped the ammonia both during the experiment and at the termination of the experiment when 4 ml of 20% NaOH was added to the culture and distillation carried out to release residual ammonia. The sulphuric acid trap solution was made alkaline with an excess of sodium hydroxide and was then subjected to steam distillation and the ammonia nitrogen trapped in 4% boric acid quantitatively determined by titration with 0.1N HCl.

Reagents. $K^{14}CN$ was obtained from Nuclear Chicago corporation, $KC^{15}N$ from Bio-Rad Laboratories, amino acids from Sigma Chemical Company, Dowex 1-chloride and Dowex 50 H from Baker Chemical Company, Hyamine hydroxide 10-X solution from Packard Instrument Company. All other chemicals were reagent grade.

EXPERIMENTAL RESULTS

Isolation of cyanide resistant bacteria. Of the five soils examined only in the Fargo clay were live bacteria found in any of the cyanide dilutions used. Bacteria from this soil survived the cyanide concentration up to saturated KCN. The concentration of 10^{-1}M KCN contained 3 types of bacteria, whereas higher concentrations contained only one type. The soil with 10^{-1}M concentration of cyanide was chosen for further study because it offered more cultural varieties. The three types were:

1) A strain of *Bacillus pumilus*, the taxonomy of which is described later,
2) A strain equivalent to *Bacillus subtilis/pumilus* intermediate as described by Knight and Proom(1950). The description of this organism is not included, as it was not studied further,
3) A unspecified organism, tentatively identified as belonging to genus *Flavobacterium*, according to Breed *et al*. (1957). The description of this organism is not included, as it was not studied further.

However, only the first listed bacterium was able to survive the transfer from the soil dilution containing 10^{-1}M KCN to the standard medium with 10^{-1}M KCN. The second and third listed bacteria did not survive this passage, but were successfully transferred by a "soil passage" as proposed by Bassalik (1949) from the standard media without cyanide to a flax soil dilution with 10^{-1}M KCN as described in the Materials and Methods. These phenomena are unexplained.

Identification of the cyanide resistant bacterium. The unidentified bacterium, tested according to the scheme proposed by Breed et al. (1957), behaved the same as Bacillus pumilus ATCC, except that it did not produce acetylmethylcarbinol and the spore location was terminal. Nevertheless, as seen in Table IV the other physiological and morphological features of this organism justified its tentative identification as Bacillus pumilus.

Growth studies on different media. Table V shows the growth of BPA in synthetic media. In some of them KCN was probably the only source of either carbon or nitrogen for bacterial growth. In two of synthetic media (No. 1 and 3, Table V) the addition of thiamine and biotin appeared to have a protective action against cyanide. This phenomenon, was not investigated further and the effect of vitamins on less complete media was questionable. Table VI shows that the bacterial growth was more abundant in nonsynthetic media. In media with 10^{-1}M KCN, the greatest number of viable bacteria after 24 hours was observed in trypticase soy yeast broth without glucose.

Optimal growth conditions. The effect of shaking on the maximum population of BPA is presented in Fig. 1. Here, the generation time for the bacteria grown with 10^{-1}M KCN was 270 min when not shaken, and 44 min when shaken. The generation time in the medium without cyanide and without shaking was 232 min; in medium without cyanide and with aeration by shaking was 42 min. The best speed of shaking for optimal growth within 24 hours was 50 RPM as shown in Fig. 2. To a much lesser extent, the

Table IV

Identification of the Cyanide Resistant Bacterium

Features and Behavior	*B. pumilus* (BPA)	*B. pumilus* (ATCC No. 7061)
Shape	rod	rod
Size	0.72 x 2.0 μ	0.7 x 3.0 μ
Gram reaction	+	+
Spore formation	few	abundant
Spore shape	ellipsoidal	ellipsoidal
Spore swelling	not definitely	not definitely
Spore wall	thin	thin
Spore location	terminal	central
Agar colonies:	circular, convex, undulate, creamy white, butyrous	circular, flat, entire, yellow white, not adherent
1) Nutrient Agar	positive	positive
2) Glucose BCP	not fermented	not fermented
3) Sucrose BCP	not fermented	not fermented
4) Lactose BCP	not fermented	not fermented
5) 1% tryptone	indole negative	indole negative
6) Gelatin	slow liquefaction	slow liquefaction
7) Acetylmethylcarbinol	not produced	produced
8) Nutrient agar with 1% glucose	negative (no vacuolation)	negative (no vacuolation)
9) Nitrate broth	negative	negative
10) 7% NaCl in nutrient broth	positive	positive
11) Litmus milk	slight peptonization, no curds	slight peptonization
12) Ammonium glucose	no gas, acid positive	no gas, acid positive
13) Ammonium lactose	no gas, acid negative	no gas, acid negative
14) Ammonium mannitol	no gas, acid positive	no gas, acid positive
15) Glucose broth	pH after 7 days - 6.5	pH after 7 days - 6.5
16) Citrate utilization	positive	positive
17) Starch	not hydrolyzed	not hydrolyzed
18) Anaerobiosis	aerobic	aerobic
19) Lecithinase	negative	not tested

Table V

Quantitative Measurement of Bacterial Growth on Synthetic Media with and without 10^{-1}M KCN in Optimal Conditions

No. of medium*	Vitamins added **	10^{-1}M KCN added	No. of cells/ml after 6 hours	No. of cells/ml after 24 hours
1	+	+	2	1×10^2
	+	-	6	4×10^2
2	-	+	3	27
	-	-	4	2×10^2
3	+	+	9	3×10^3
	+	-	10	4×10^4
4	-	+	8	98
	-	-	12	2×10^2
5	+	+	4	48
	+	-	6	2×10^2
6	-	+	6	10
	-	-	8	81
7	+	+	3	27
	+	-	5	90
8	-	+	2	0
	-	-	10	81
9	+	+	5	30
	+	-	10	42
10	-	+	6	6
	-	-	12	36
11	+	+	6	10
	+	-	13	18
12	-	+	5	0
	-	-	14	19
13	+	+	4	0
	+	-	3	42
14	+	+	7	14
	+	-	1	18
15	+	+	3	36
	+	-	7	15

* Media No. referred to Table III: 100 ml in 250 ml E. flasks

** 1 ml added each time consisting of 1% thiamine + 1% biotin

Table VI

Quantitative Measurement of Bacterial Growth on Nonsynthetic Media with and without $10^{-1}M$ KCN

No. of medium *	Presence of sugars in media	$10^{-1}M$ KCN added	No. of cells/ml after 6 hours	No. of cells/ml after 24 hours
16	+	+	8	0
	+	-	9	27
17	-	+	3	5×10^3
	-	-	9	10
18	+	+	7	3×10^4
	+	-	15	4×10^6
19	-	+	6	9×10^5
	-	-	21	5×10^2
20	+	+	26	2×10^2
	+	-	16	3×10^2
21	-	+	27	295
	-	-	21	5×10^3
22	+	+	30	2×10^2
	+	-	25	3×10^4
23	-	+	31	1.4×10^2
	-	-	26	2×10^3
24	+	+	18	25
	+	-	17	2×10^2
25	-	+	12	35
	-	-	8	4×10^3

* Media No. referred to Table III: 100 ml in 250 ml E. flasks

Figure 1 Influence of 50 RPM of shaking on bacterial growth (BPA).

Number of viable bacteria produced with (▬ ▬ ▬) and without (▬—▬) 10^{-1}M KCN, curves A and B. No shaking applied, curves C and D.

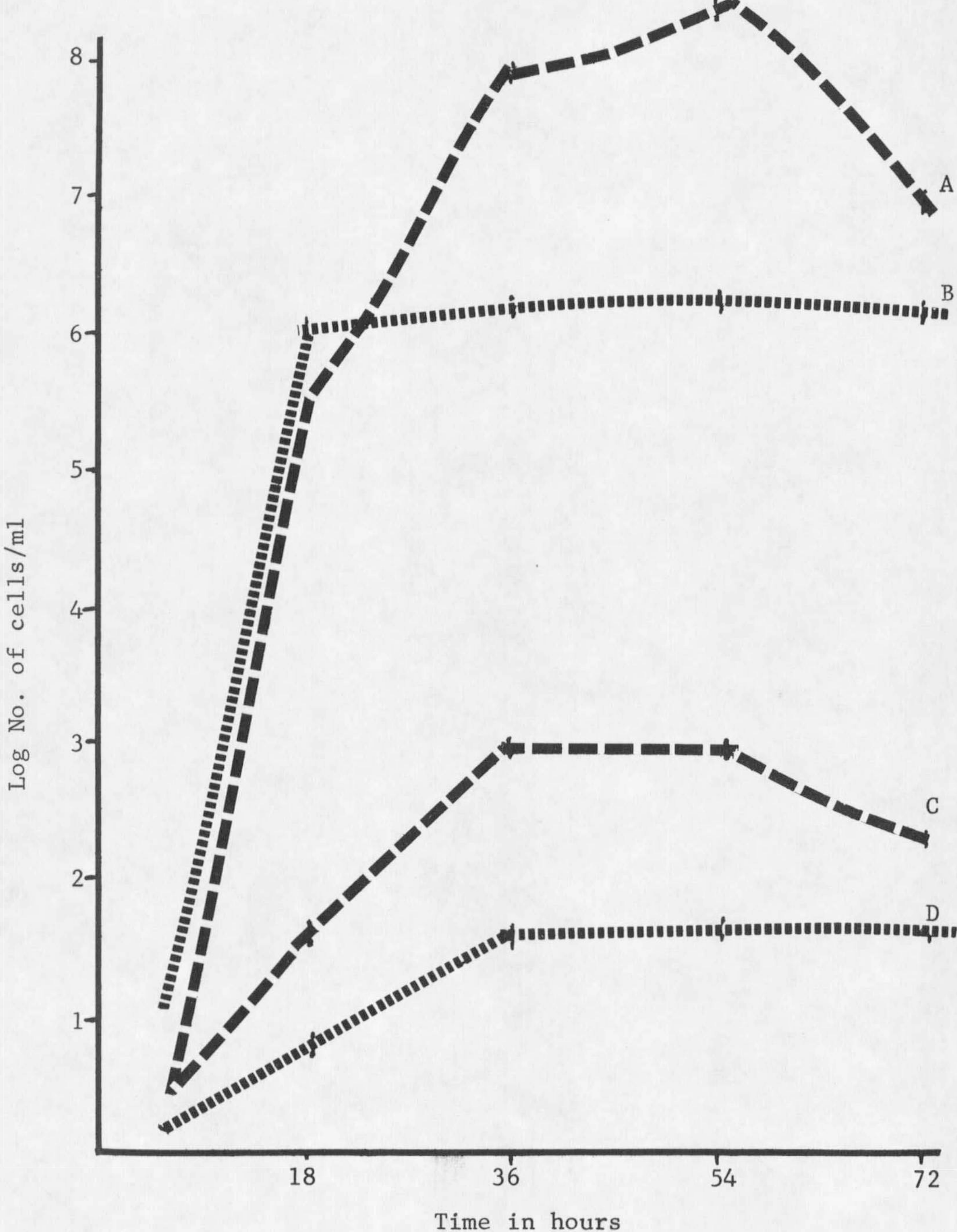
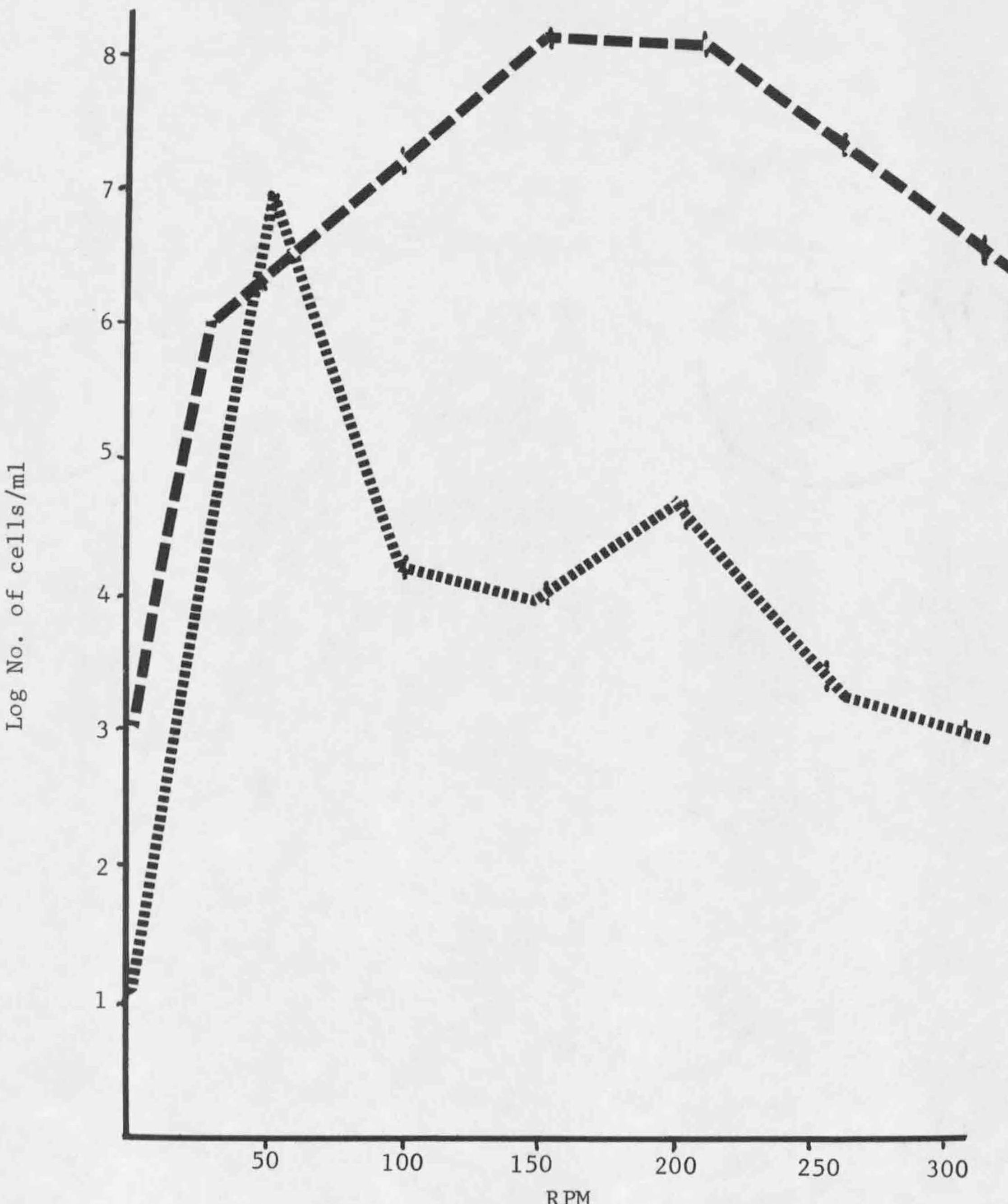

8
7
6
5
4
3
2
1
Log No. of cells/ml
A
B
C
D
18
36
54
72
Time in hours

Figure 2 <u>Influence of the shaking speed on bacterial growth (BPA).</u>

Number of viable bacteria produced with different RPM's with (▯▮▯▮▯▮▯) and without (▨▯▨▯▨) 10^{-1}M KCN.


Log No. of cells/ml
8
7
6
5
4
3
2
1
50
100
150
200
250
300
RPM

speed of 200 RPM was also effective in promoting bacterial growth. The effect of temperature upon the growth in 24 hours was measured from 10 to 60 C in 10 C increments. Maximum growth was obtained at 40 C (Fig. 3).

Morphological and cytological changes under the influence of 10^{-1}M KCN. Observations by light and electron microscopy reveal drastic changes in bacterial grouping and morphology when grown in medium with 10^{-1}M KCN.

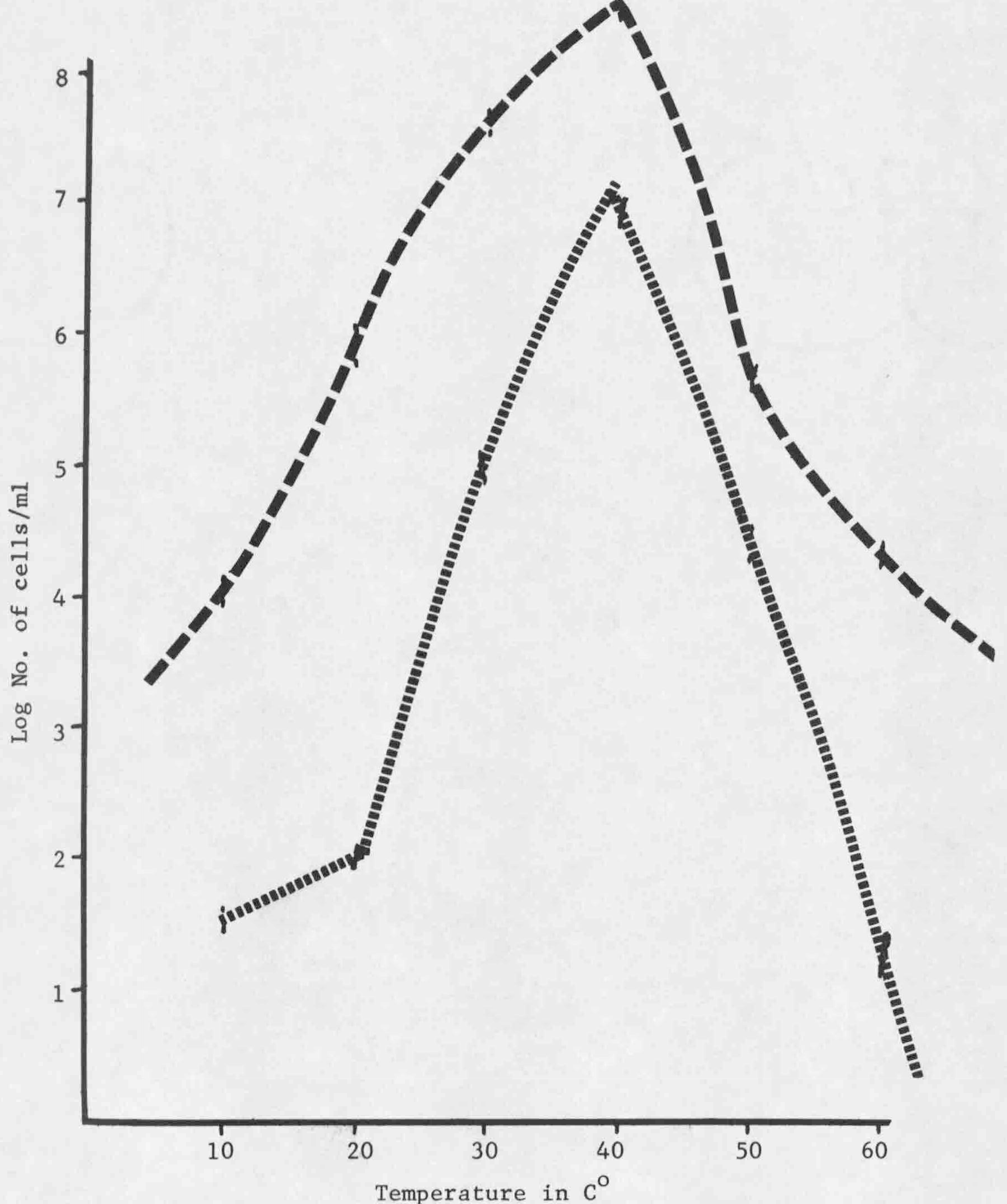
Changes in cell arrangement could be seen by comparing the bacteria grown without cyanide (Fib. 4) with those grown with 10^{-1}M KCN in the medium, Fig. 5. The bacteria grown in cyanide formed long filaments.

Once formed, the filamentous type was not reversible to the normal cell type. Plating of filamentous forms for counts showed that they were able to multiply and form colonies appearing similar to those formed by the nonfilamentous original type of bacteria. Growth on medium pH 8.5 without cyanide did not induce formation of filaments.

Electron microscopy studies of the cells revealed a uniformly thick cell wall encompassing a typically rod-shape structure as shown in Fig. 6. The appearance of the outer surface of the cell was smooth. The plasma membrane was not visible. A network of protoplasmic membranes was observed to extend throughout the entire length of the cell. Several electron dense areas were visible within the bacterial protoplasm. The average ratio of length to width was 5 to 1. A cross section of the bacterium cultivated under the same conditions is presented in Fig. 7.

Figure 3 Influence of the temperature on bacterial growth (BPA).

Number of viable bacteria produced with different temperatures with ( ) and without ( ) 10^{-1}M KCN.

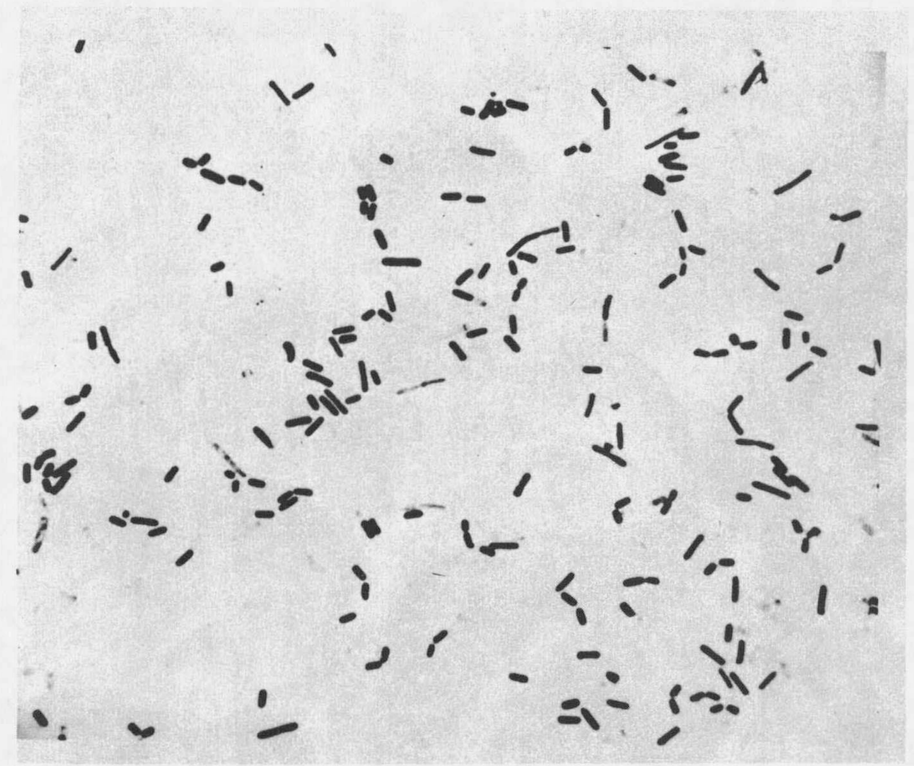


8
7
6
5
4
3
2
1
Log No. of cells/ml
10
20
30
40
50
60
Temperature in C^{o}

Figure 4 Bacillus pumilus (BPA). x 1000.

Figure 5. Bacillus pumilus (BPF) grown for 24 hours with 10^{-1}M KCN.

x 2000.



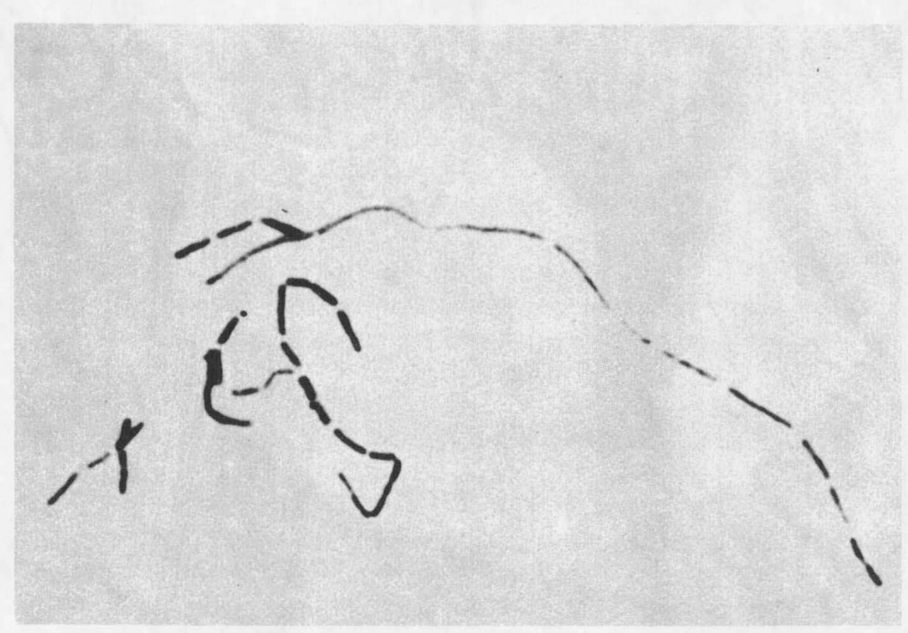


Figure 6 Longitudinal section of Bacillus pumilus (BPA) grown for 24 hours. x 105,000.

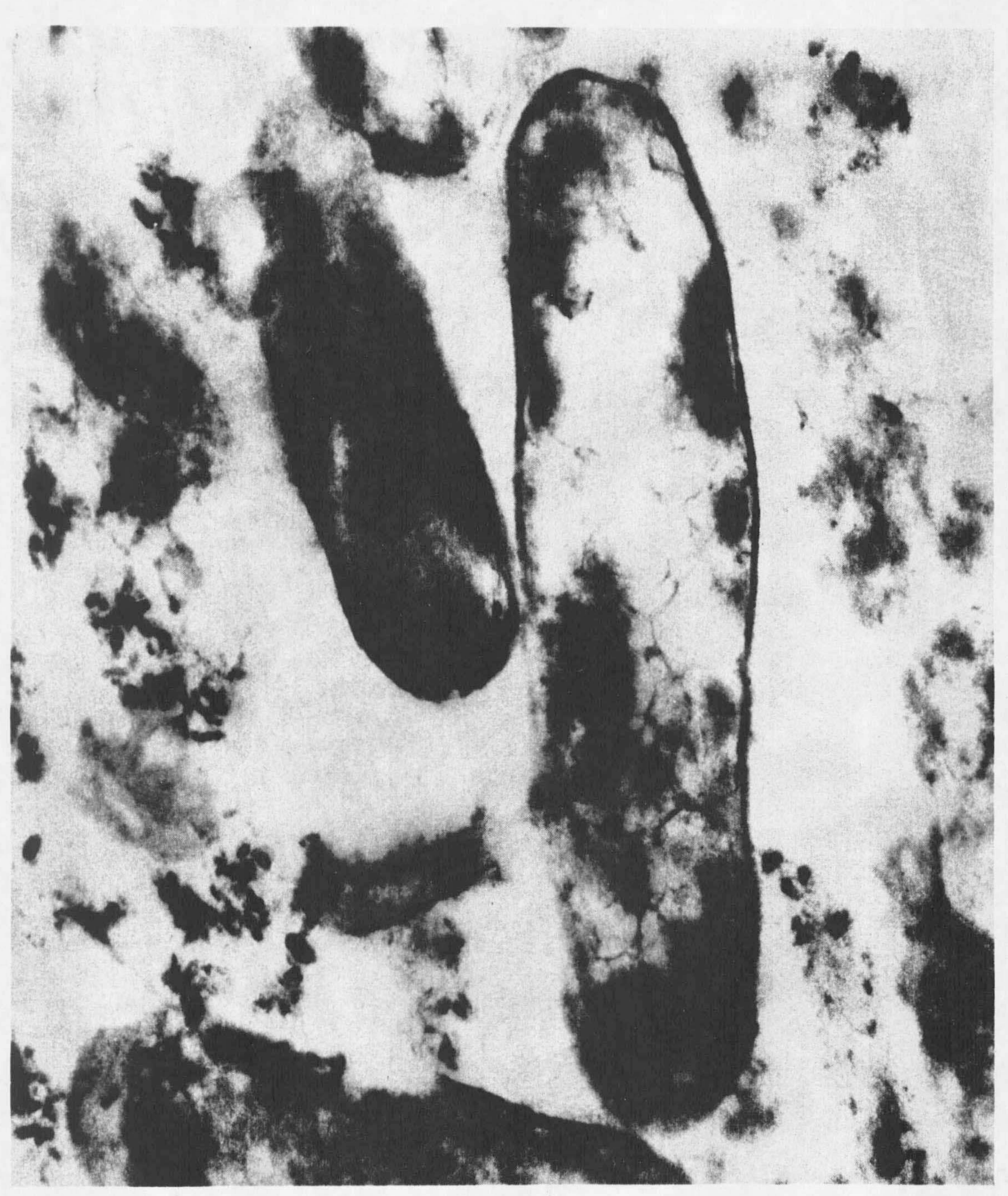
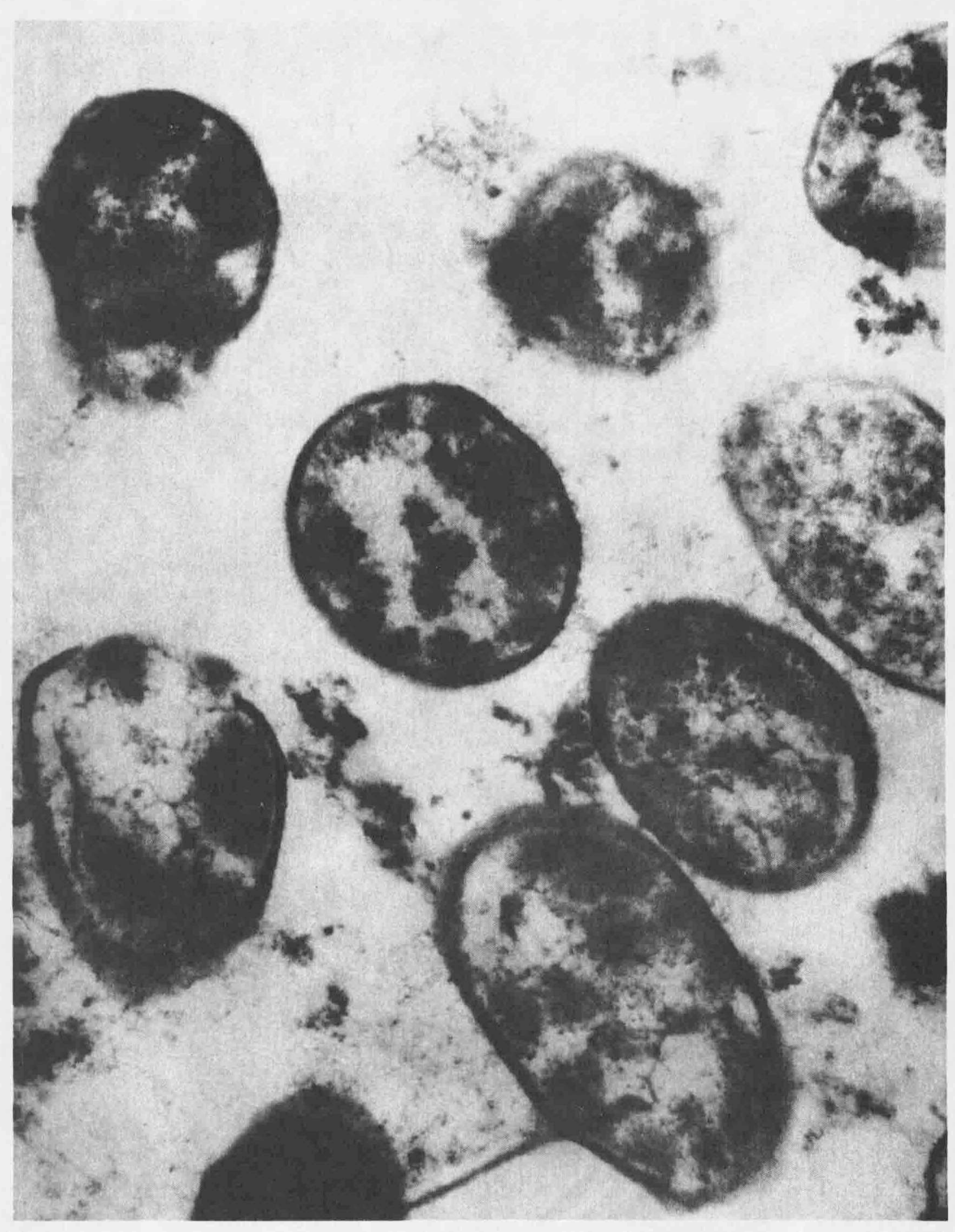


Figure 7 Cross-section of Bacillus pumilus (BPA) grown for 24 hours. x 105,000.

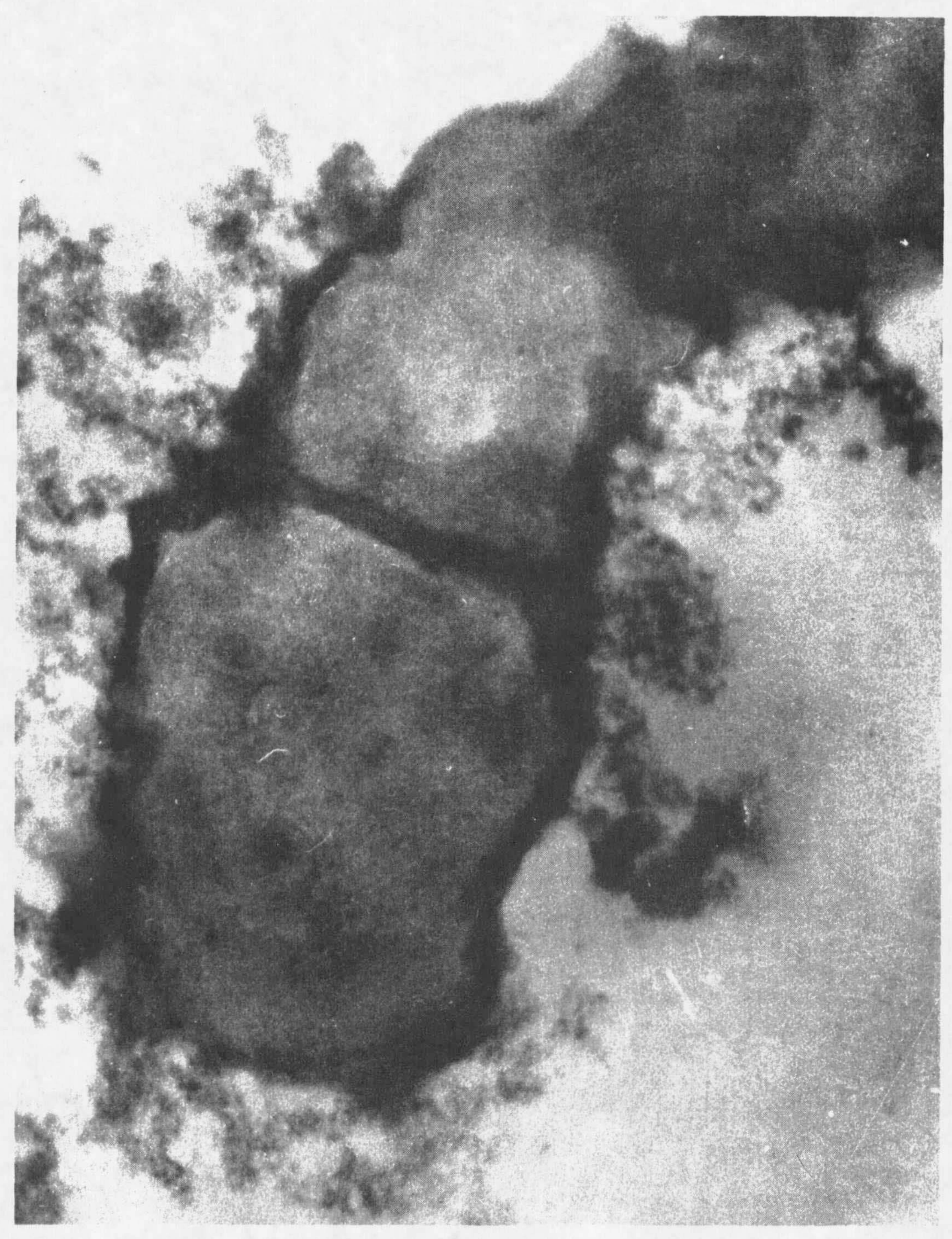


Here also the outer surfaces of uniformly thick cell walls appeared to be smooth and a plasma membrane was not discernible. Electron opaque materials were scattered throughout the cell, and a system of protoplasmic membranes crossed the entire surfaces of the cross section in various directions. Cross-sectional views of the bacterial cells revealed circular to oval outlines. Fig. 8 shows a longitudinal section of a bacterial filament fixed after 24 hours growth with 10^{-1}M KCN. Here, the individual cells appear to be connected one to another by internal strands. Septa were not visible and electron opaque material of uniform density appeared throughout all the cells. An oval morphology of each individual cell within the filament was observed. Cytoplasmic bridges between these bacteria were formed around the beginning of the 12th hour of incubation. The width of these bridges was approximately equal to 1/8 that of the bacterium. In one isolated case, a connection consisting of two cytoplasmic bridges was observed. Prolonged incubation to 72 hours in the medium with cyanide caused formation of a cross wall between the cells of a filament, as illustrated in the Fig. 9. Here no protoplasmic connections extended from one cell to another. The cell wall thickness varied from cell to cell. Protuberances appeared to extend from the surfaces of the walls outwardly. A network of protoplasmic membranes was only faintly visible, and the cells were of irregular shape.

Respiration study. The filamentous forms (BPF) took up oxygen at least 3 times more rapidly than the nonfilamentous forms (BPA) when the cells

Figure 8 Longitudinal section of the filament from Bacillus pumilus grown for 24 hours with 10^{-1}M KCN. x 94,000.

Figure 9 Longitudinal section of the filament from Bacillus pumilus (BPF) grown for 72 hours with 10^{-1}M KCN. x 200,000.



harvested from TSYB were incubated in a respirometer at 40 C and 50 oscillations per minute in modified Dulbecco Broth medium (No. 5, Table II). However, when the cells were grown under similar conditions except that the Dulbecco Broth was made 10^{-1}M with respect to KCN, the oxygen uptake was about 18 times greater in the filamentous forms (BPF) than BPA or BP, (Figures 10 and 11 respectively). The total oxygen consumed in the control flask containing complete reaction mixture without bacteria was insignificant. The weight of the cells in flasks was determined by drying a equal quantity of the cell suspension and weighing. An inadvertent second variable was introduced, because the pH of the Dulbecco Broth changed with addition of cyanide from about 7 to 8.5. The effect of this is not known.

Cyanide uptake studies. Living cells are able to decrease the cyanide concentration in medium with 10^{-1}M KCN (Fig. 12). Dead cells, however, do so to a much smaller extent (Fig. 12). The cyanide taken up by living cells is not detectable in homogenates while the small amount taken up by the dead cells is measurable (Fig. 13). This suggests that the living bacteria not only tolerate cyanide but actively metabolize it (Fig. 13). The autoclaved bacteria retained their shape as observed with light microscopy.

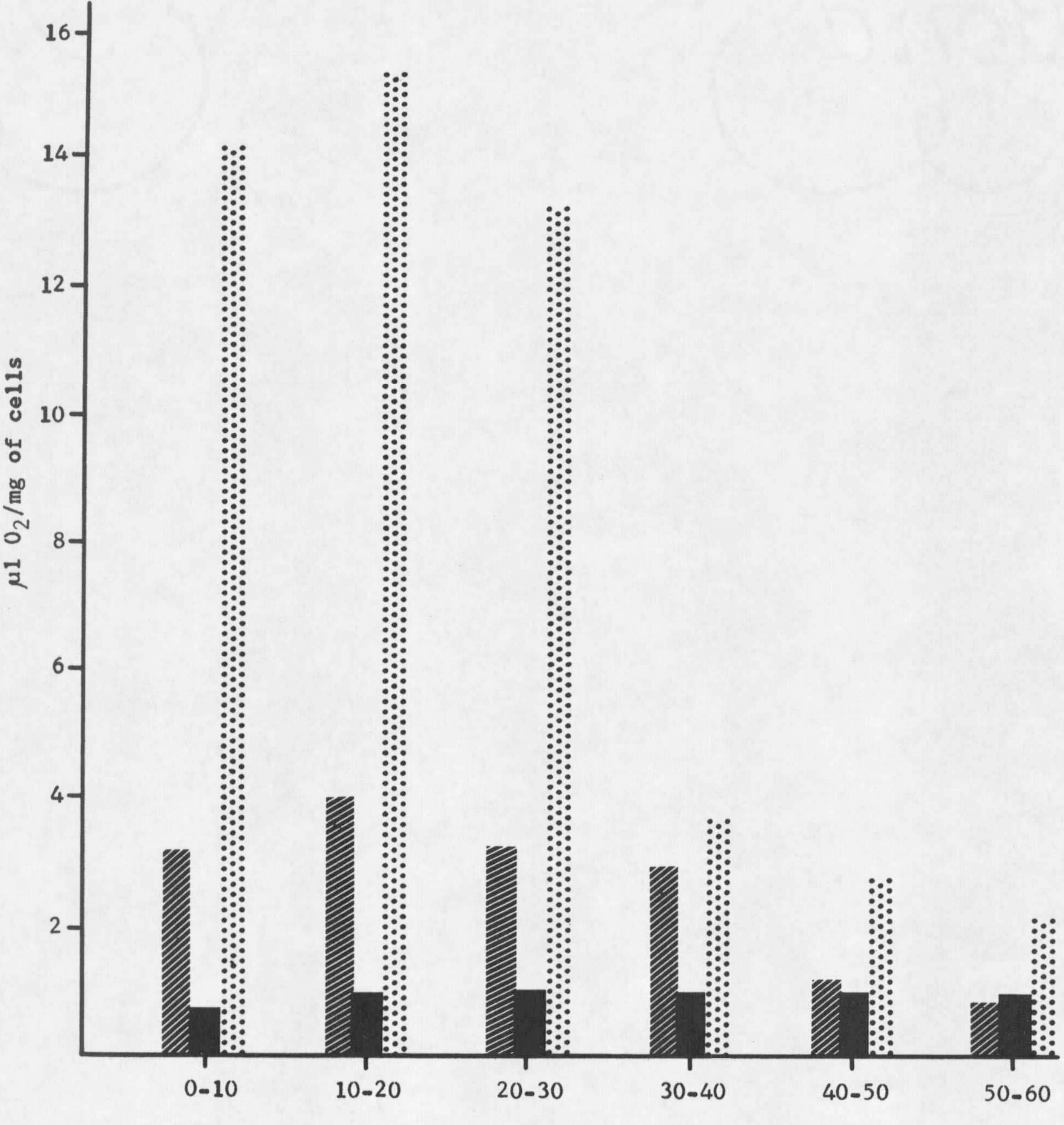
When bacteria were fed $K^{14}CN$ the $^{14}CO_2$ production ceased after 36 hours of incubation, Fig. 14. In the feeding experiment with $KC^{15}N$ the production of $^{15}NH_3$ did not attain its maximum until about the 36th hour, the rate decreased after 54 hours, Fig. 15. This curve suggests that

Figure 10 Oxygen uptake in modified Dulbecco Broth.

( ) (BPA)

( ) (BPF)

( ) (BP)



16
14
12
10
8
6
4
2
µl O_2/mg of cells
0-10
10-20
20-30
30-40
40-50
50-60
Time in minutes

Figure 11 Oxygen uptake in modified Dulbecco Broth with 10^{-1}M KCN.

( ) (BPA)

( ) (BPF)

( ) (BP)

The first appearance of the filamentous forms was between 50 and 60 minutes.

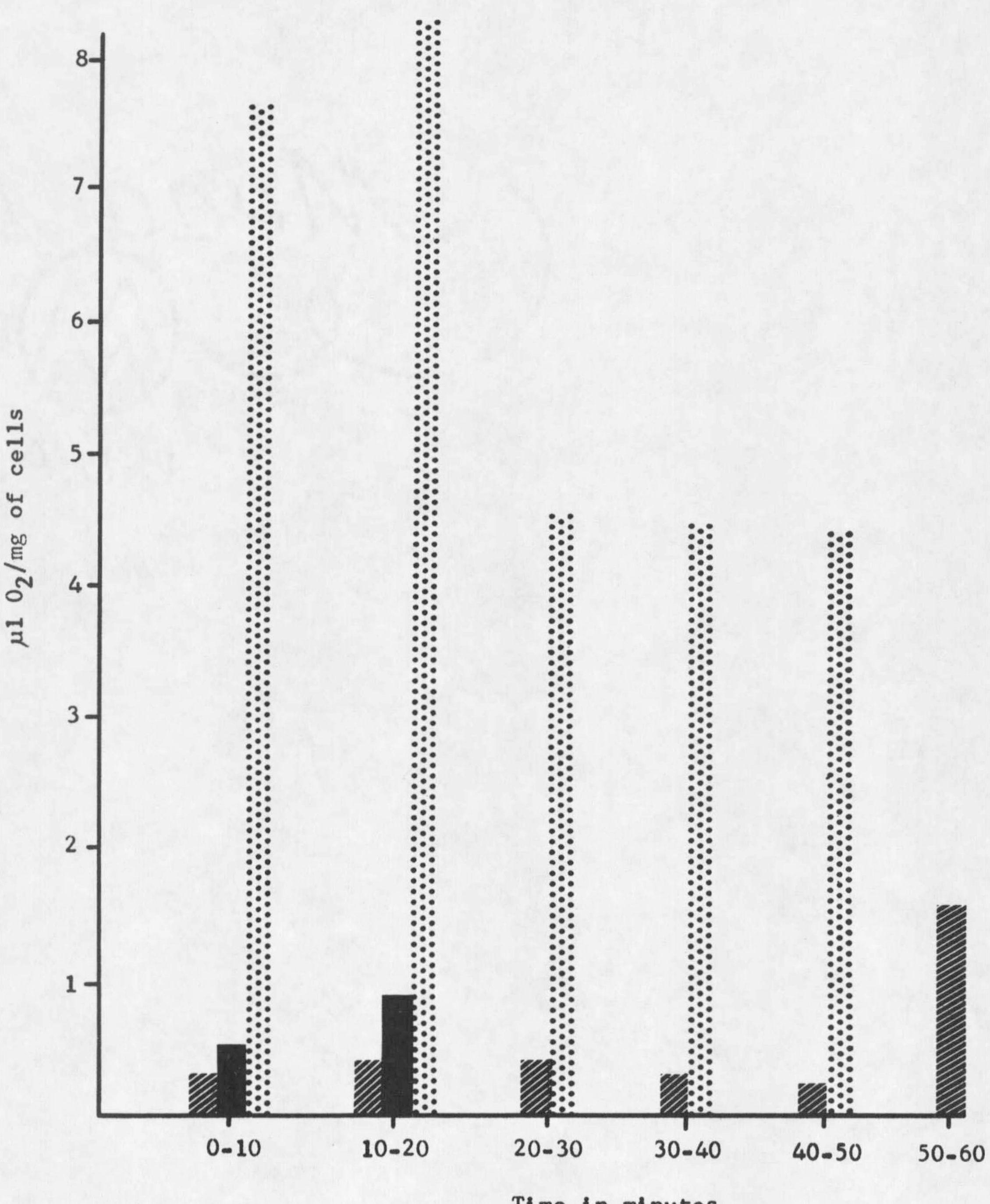

8
7
6
5
4
3
2
1
µl O_2/mg of cells
0-10
10-20
20-30
30-40
40-50
50-60
Time in minutes

Figure 12 Disappearance of cyanide from the medium with 10^{-1}M KCN as a function of time.

Live (▬ ▬ ▬) and sterilized (▬ ▬ ▬) bacteria (BPA).

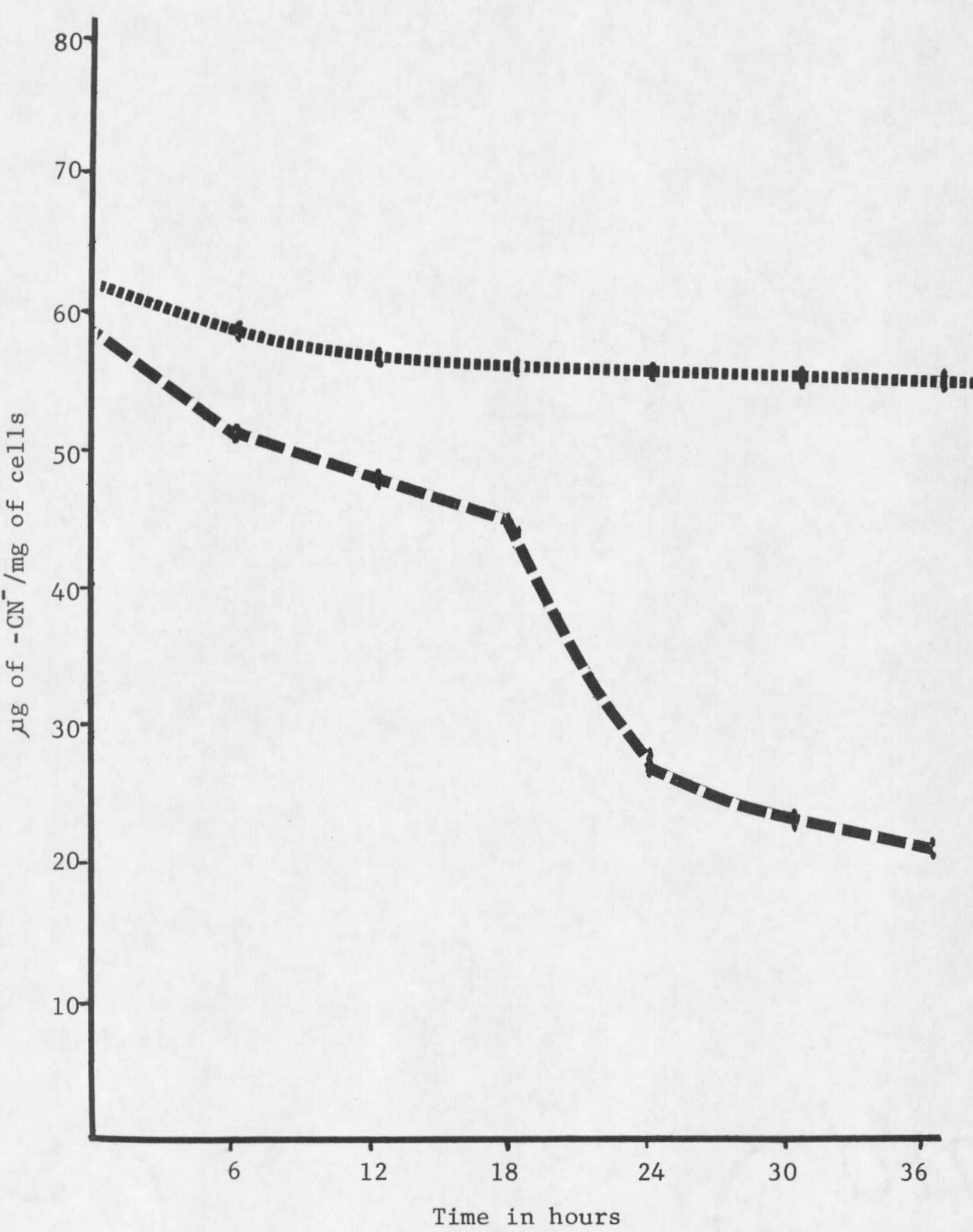
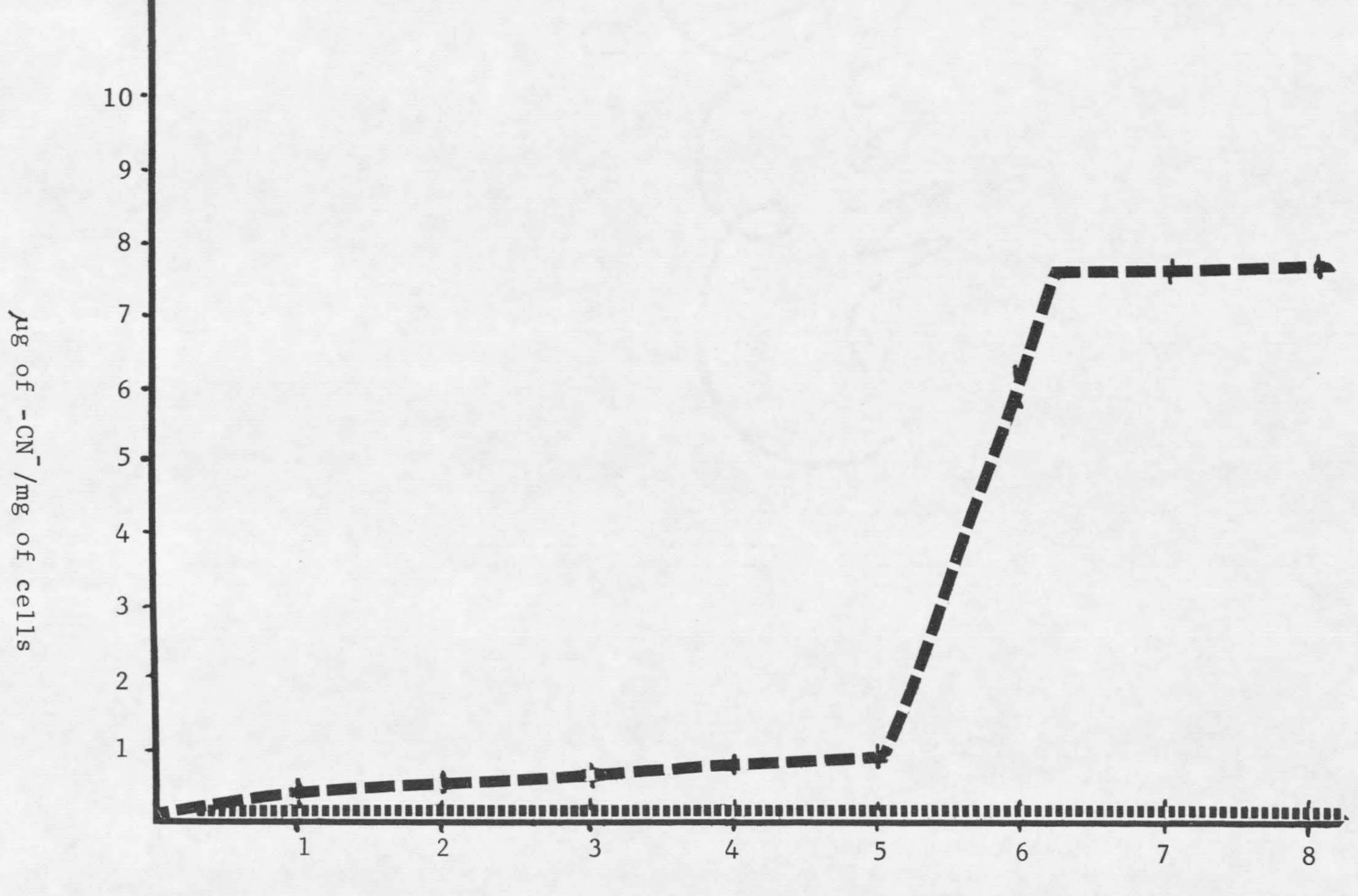

80
70
60
50
40
30
20
10
µg of -CN⁻/mg of cells
6
12
18
24
30
36
Time in hours

Figure 13   <u>Appearance of cyanide in cell extracts as a function of time</u>.

Live ( ) and sterilized ( ) bacteria (BPA) were grown on medium with 10^{-1}M KCN.



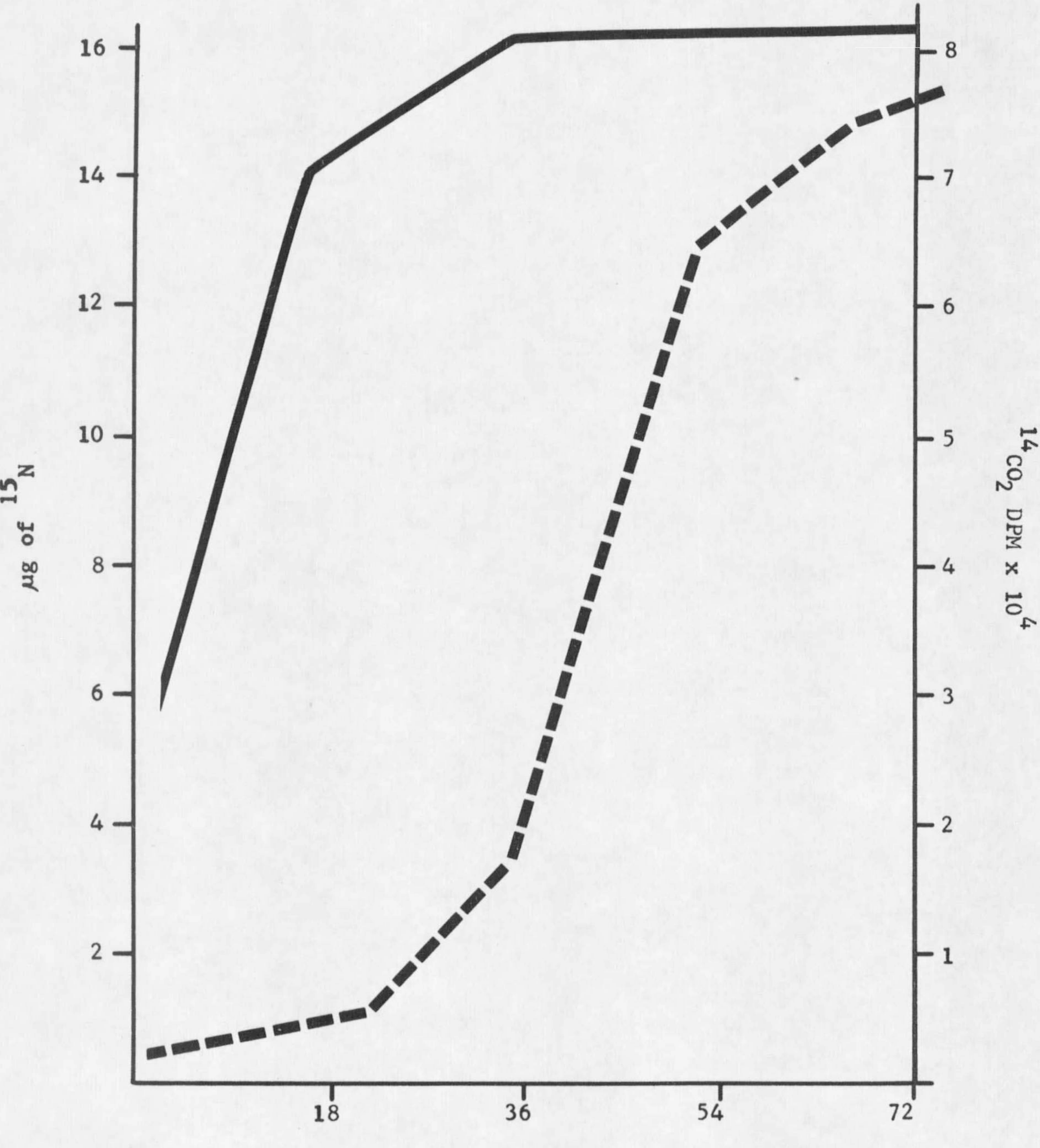
10
9
8
7
6
5
4
3
2
1
µg of -CN⁻/mg of cells
1
2
3
4
5
6
7
8
Time in days

Figure 14 Carbon-dioxide ^{14}C and ammonia ^{15}N production as the function of time in cells exposed to $K^{14}C^{15}N$.

Bacteria (BPA) were grown in modified Dulbecco Broth (Medium No. 5, Table II) with 10^{-1}M KCN. No carbon-dioxide ^{14}C and no ammonia ^{15}N production was observed in the control experiment with sterilized bacteria.

( ) ug of ^{15}N

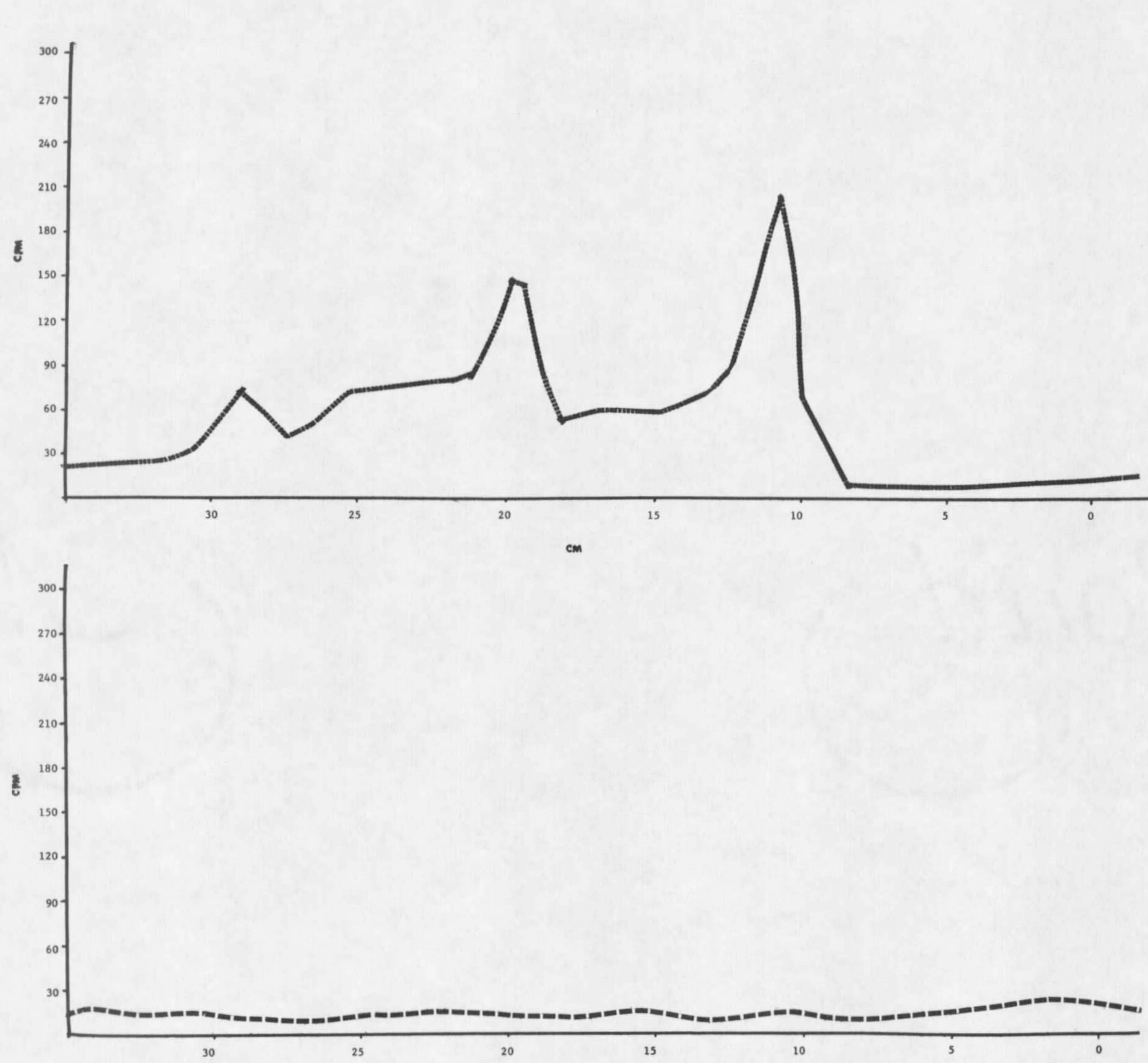
( ) $^{14}CO_2$ DPM x 10^4



16
14
12
10
8
6
4
2
μg of 15N
8
7
6
5
4
3
2
1
14CO2 DPM x 10^4
18
36
54
72
Time in hours

Figure 15 Radiochromatoscan of the cation fraction.

The live ( ) and sterilized ( ) bacteria (BPA) were fed 1 μc $K^{14}CN$ for 24 hours.



300
270
240
210
180
150
120
90
60
30
CPM
30
25
20
15
10
5
0
CM
300
270
240
210
180
150
120
90
60
30
CPM
30
25
20
15
10
5
0
CM

cyanide nitrogen is being retained when the bacteria shows the most active growth process, as it can be seen in Fig. 1, the maximum of which was reached on the 36th hour. After the cells have reached the maximum growth the cyanide nitrogen was released in form of ammonia. This may imply, that protein synthesis decreases and cells liberate ammonia due to endogenous respiration of amino acids and proteins.

Study of the cation fraction. Results of radiochromatoscanning of the paper chromatogram of the cation fraction using the bacteria exposed for 24 hours to $K^{14}CN$ are illustrated in Fig. 15. The peak with a base between 8 and 15 cm is the greatest in amplitude and corresponded to arginine or lysine according to R_f values of the standard amino acids chromatographed simultaneously. The peak with the base between 18 and 22 cm corresponded to glutamic acid, and the one between 24 and 27 cm corresponded to alanine. The peak between 27 and 32 cm did not match any of the known amino acids that were chromatographed. Radiochromatoscanning of the cation fraction from sterilized bacteria incubated under the same conditions as the live bacteria, did not show significant radioactivity. The cation fractions from the area corresponding to the peaks were eluted and chromatographed in two dimensions by thin layer chromatography simultaneously with reference amino acids. Radioautography of the thin layer chromatograms were compared to the ninhydrin positive spots on the plate. In no case did a ninhydrin positive spot match a spot on the corresponding film. That the cation fraction was the most heavily labelled, however,

is shown by the following data: cation fraction 4.4 x 10^4 dpm, anion fraction 12 dpm and neutral fraction 21 dpm.

The quantitative and qualitative analysis of the amino acids from BPA grown with and without 10^{-1}M KCN and BPF grown without cyanide are listed in the Table VII. In the BPA the pool of free amino acids contained no measurable amounts heterocyclic compounds and no arginine. When this bacterium was exposed to 10^{-1}M KCN for 24 hours the free amino acid pool was diminished by all the basic, all sulphur containing and all of the aromatic (with exception traces of phenylalanine) amino acids as well as lysine and valine. On the other hand, the arginine concentration was approximately 33 times that of the average amount of the other amino acids. When these bacteria mostly in filamentous-like form were transferred to the standard medium and grown under optimal conditions for 24 hours all amino acids found in BPA appeared again, and in addition arginine was present. The average amount of any amino acid was twice as great as in the original culture.

Table VII

Free Amino Acids in the Bacterial Homogenates

Amino Acids	BPA grown on standard medium at optimal conditions	BPA 24 hours grown on standard medium 10^{-1}M KCN at optimal conditions	BPF filaments grown in standard medium at optimal conditions
Asp. A	0.08	0.03	0.13
Thre	0.10	0.03	0.23
Ser	0.12	0.05	0.32
Glu A	0.85	0.05	1.49
Gly	0.08	0.02	0.17
Ala	0.46	0.05	0.66
Cyst	0.61		1.57
Val	0.08		0.11
Meth	0.19		0.39
Ileu	0.10	0.02	0.38
Leu	0.15	0.09	0.09
Tyr	0.06		0.12
Phe-ala	0.05	0.03	0.12
Lys	0.44		0.70
His	0.50		0.07
Arg		1.65	0.10

DISCUSSION

The first phase of this research was the finding of a bacterium resistant to a high concentration of cyanide. A sample of Fargo clay, a soil which had supported flax for 73 consecutive years, was found to be an excellent source of a bacterium of this type. Flax is rich in cyanogenic glucosides, which presumably had been degraded for 73 consecutive years in this soil, releasing cyanide and thus creating a favorable environment for adaptation of a microorganism able to survive or utilize higher concentrations of cyanide. The approach of artifically enriching soil with cyanide, was not used, since it would not contribute to better understanding of a cyanide microcycle in nature.

The bacterium isolated from Fargo clay was probably a strain of *Bacillus pumilus* as confirmed by morphological and physiological tests (Fig. 4).

The main discrepancy, when this strain was compared to the *Bacillus pumilus* ATCC No. 7061 was its inability to produce acetylmethylcarbinol and the spore location tended to be more nearly terminal, then central. No reasonable relationship between these characteristics and utilization of cyanide could be established.

Optimal growth conditions for this bacterium were determined. Shaking caused more abundant growth but the rapidity of movement (RPM) was important (Fig. 2) as optimal growth was obtained at 50 RPM. At 200 RPM, another peak in bacterial production was observed and was due to unexplained factors. The cultures were strictly aerobic. Cyanide

inhibited growth in the standard medium in the presence of glucose (Table VI).

A possibility exists that glucose, behaving as a aldehyde combines with cyanide to form an addition compound to which the cell which is impermeable or which cannot be phosphorylated.

Jacobs (1960) pointed out that to fully understand the influence of a compound upon a bacterium it is necessary to study it in a variety of conditions. The tests of 10^{-1}M KCN on bacterial growth in a variety of synthetic and nonsynthetic media (Table V and VI) and a variety of physical conditions (Fig. 2, 3) essentially satisfied the criteria of Jacobs. The growth of bacteria in a medium containing only tap water with 10^{-1}M KCN can be explained by the remote possibility that the hard glass of laboratory flasks served as a source of minerals and that dissolved gases from the atmosphere in the medium also served as a source of carbon or nitrogen. Alternatively and more probably, it can be taken as evidence of the ability of the organism to utilize cyanide at least for the 72 hours tested.

After 24 hours of incubation with 10^{-1}M KCN, long filaments appeared consisting of 10 to 20 original bacterial units. There is considerable information on bacterial filaments in the literature which indicates that they may be induced by a variety of environmental conditions, such as poisons and metabolic inhibitors, nutritional deficiencies, and physical conditions. Nonseptate filaments, similar to those seen in the strain of <u>Bacillus pumilus</u> (BPF) have been reported by Adler and Hardigree

(1965) in a strain of *E. coli B* after exposure to ionizing irradiation. According to Howard-Flanders *et al.* (1964) a single gene is responsible for the formation of filaments. It seems that the elongation of a BPB filament stops when the filament reaches a length of about 20 bacteria. However, some of the filaments described in other species stop growing when they reach a length 100 times those of normal cells (Lea *et al.* 1937). The sensitivity of BPF filaments toward cyanide seems not a change as it proceeds from 10 to 20 normal bacterial cell lengths. According to Braun (1965) a bacterial filament is mostly a long cell with many nuclei. This is probably not true of BPF, as the morphology does not reveal a common bacterial cell wall (Figures 9 and 10). Also in contrast to most filamentous forms which have been studied, BPF is irreversibly changed. The formation of the filamentous BPF can hardly be explained in terms of repressor inactivation (Witkin 1967), but would seem to call for some other explanation; possibly the delayed multiplication of an episome as compared to the "chromosome". This could result in the filament becoming permanent. Other cases of a filament formation but of a non-permanent character are caused e.g., by irradiation or by prophage induction and explained by inactivation of a repressor and induction of a specific operon which may be part of an episome or part of the bacterial chromosome (Witkin 1967). In the Durham *et al.* (1966) experiment, *Bacillus subtilis* formed chains consisting of 2 to 4 bacteria connected by faintly visible bridges; similar bridges were formed

in the early incubation period of BPF in media with 10^{-1}M KCN. The size and shape of these bridges are also similar to the unusual structure connecting Bacteroides reported by Bladen (1962) designated as conjugatory bridges. In his electron microscopic observations, electron dense material can be seen near the site of attachment of these bridges; they were identified as nuclear material. Such electron dense materials are also seen near the site of attachment of one bacterium to another in BPF filaments (Fig. 8). It is possible that the survival of filaments grown at higher concentrations of cyanide may be influenced by a "neighbor restoration", (Adler et al. 1966). The BPF filament (Fig. 8) closely resemble the filaments of E. coli obtained after 3 hours growth in nutrient broth containing 13 μg/ml of platinum, described by Rosenberg et al. (1967). The bacterial connections observed in the electron microscope in BPF (Fig. 8) were similar to those encountered in the manganeze oxidizing bacteria as illustrated in Mose and Bratner (1963). That the drastic changes in bacterial morphology observed do not impair the formation of colonies, implies an unusual survival ability of BPF. Electron microscopic observations of BPF (Figures 8 and 9) did reveal faintly visible membranes as seen in BPA (Figures 6 and 7).

Observations on the respiration of BPA, BPF and BP in the presence of cyanide led to the conclusion that for the first 40 minutes BPF probably utilized endogenous substrates and thereafter cyanide as the principal energy source (Figures 10 and 11). It is possible that a

genetic control exists which can greatly modify the composition of the bacterial electron transport system. The abundant growth of the strain of Bacillus pumilus on a medium with 10^{-1}M KCN might be attributed to a cytochromeless state as postulated by White (1962) in a species of Haemophilus which was grown on proteose peptone medium with a 5 x 10^{-3}M concentration of KCN. However, Mizushima et al. (1958) have grown a strain of Aerobacter cloacae in a medium with 10^{-3}M KCN and suggested an existence of an insensitive terminal oxidase system which can be coupled with an energy generating system. This insensitivity was apparently lost within 3 generations when Aerobacter cloacae was transferred in cyanide free medium. Another example of the reversibility of acquired resistance to toxic substances is the experimental result of Arima and Beppu (1964). They treated Pseudomonas pseudomallei with 2 x 10^{-2}M arsenite. The resistance of this bacterium was retained for only 2 generations. In contrast to the phenomena just cited, the cyanide resistance of the strain BPA seems to be hereditary and not due to temporary physiological states. Some authors (Camerino and King 1966) questioned the validity of the results in certain manometric measurements of oxygen uptake; however, in this study all the requirements forwarded by Robbie (1946), Laties (1949) and Gillar (1962) were fulfilled. The survival of the strain of BPA in KCN could be explained also in terms of physical characteristics of media. The organic and inorganic matter in the bacterial environment frequently have a protective action and might react with cyanide and thereby reduce its

active concentration or it might form a protective film on the surface of the cell. According to Solomon (1960), pores in bacterial cell membranes are lined with molecules bearing positive charged ions, which hinder the enterance of positively charged ions without affecting ions like $-CN^-$. According to Danielli and Dawson (1952) the permeability of membranes is influenced by temperature. These authors emphasized the importance of activation energy. The temperature tested ranged between 10 to 60 C (Fig. 3). Testing at this temperature range fulfilled the requirement advocated by Scheuplein (1966) for aqueous biological systems.

That cyanide was actually taken up by the cells is shown in Figures 14 and 15. Labelling studies using ^{14}C and ^{15}N KCN contributed to an understanding of cyanide conversion. That the cyanide carbon and nitrogen was metabolized to CO_2 and NH_3 is shown in Fig. 14. This might show that the cyanide carbon and nitrogen make their way through a common metabolic intermediate. In the experiments with ^{14}C labelled cyanide, the greatest incorporation was observed in the cation fraction of the homogenate. None of the compounds in this fraction were identified. The finding of cyanide in the homogenate of sterilized bacteria when grown with $10^{-1}M$ KCN (Fig. 13) and absence of cyanide in the live bacterial homogenate (Fig. 13) with a concomitant disappearance of cyanide from medium (Fig. 12) suggest the existence of a cyanide permease system. Also the appearance of the cyanide is sterilized bacterial homogenates (Fig. 13) suggests that either the membranes

remain intact or cyanide becomes bound into the cell, otherwise cyanide would be washed off prior to homogenization.

Studies on the amino acid pools showed the appearance of arginine in KCN treated cells (Table VII). It can be postulated that the ability to utilize the cyanide radical which presents the organism with two of the most important elements of life, carbon and nitrogen packaged in the simplest possible form, requires a more comprehensive amino acid pool.

The disappearance of the sulphur containing amino acids (Table VII) could be explained by the formation of thiocyanate as mentioned by Catsimpoolas and Wood (1966) and utilization by the bacterium as suggested by Youatt (1954). It is possible that the genes which govern the synthesis of some enzymes involved in sequential steps in metabolic pathways are clustered in operons in this bacterial strain. This could help explain the trace amounts of glutamic acid in the free amino acid pool of the homogenate when the bacterium was grown in 10^{-1}M KCN with a simultaneous increase of arginine. Arginine might originate from glutamic acid in a way similar to that demonstrated in _E. coli_ and discussed by Jukes (1966). If the strain of BPA is cryptic toward cyanide and adaptation would require protein synthesis and the creation of an appropriate permease system and would require the utilization of free amino acids from the bacterial pool as an example of "suppression of crypticity" as described by Cohen and Monod (1957). Because the entry of cyanide may be controlled by permease the cytochrome system may never be confronted with

a inhibitory concentration of cyanide. Disappearance of histidine with the concomitant formation of arginine could suggest a massive conversion of histidine into arginine in the presence of cyanide. The remarkable small amounts of the main free amino acids in the pool of BPA grown with cyanide agreed with the findings of Pedersen (1965) on another strain of Bacillus pumilus. She investigated the free amino acids in the cells of genus Bacillus by two dimensional thin layer chromatography and noticed that Bacillus pumilus was unique. Her bacterium had the same physiological characteristics as the one from the American Type Culture Collection and had only 4 amino acids which were arginine, lysine, aspartic acid and glutamic acid, forming a small free amino acid pool.

The importance of finding a strain of Bacillus pumilus able to utilize cyanide may prove useful to mankind in the following ways: 1) The ever growing contamination of rivers and streams located close to the industrial centers having cyanide wastes creates problems of decontamination (Klein 1962). As pointed out by Murphy and Nesbitt (1964) a biological treatment unit for detoxification of cyanide load could be handled at a minimal cost as compared to pure chemical treatment. 2) Studying this organism we might learn more about the origin of life since cyanide is thought to be important in primordial conditions (Steinman and Cole 1967, Calvin 1961). 3) The CN radical corresponding to the parent molecule HCN has been detected in a number of stars as reported by Swings (1959) and in interstellar space according to Bates and Spitzer (1951).

Therefore, the isolated organism might prove useful in studies conducted on other celestial bodies, because of its unusual ability to tolerate and metabolize cyanide.

SUMMARY

A strain of Bacillus pumilus was isolated from Fargo clay in flax field near Fargo, North Dakota which had been in flax 73 consecutive years. This bacterium had an unusual ability to survive high concentrations of cyanide. It was different from American Type Culture Collection Bacillus pumilus in that it did not produce acetylmethylcarbinol. The optimal growth conditions of this strain of Bacillus pumilus as well as its morphological changes under the influence of cyanide were established with light and electron microscopy. Labelling experiments with C^{14}, N^{15} labelled KCN supported the idea of cyanide utilization by this bacterium. The influence of 10^{-1}M concentration of cyanide on the respiration of this strain of Bacillus pumilus was minimal.

When this bacterium was exposed to $K^{14}CN$, the greatest incorporation of ^{14}C appeared in the cation fraction. When the cells were grown in 10^{-1}M KCN the formation of arginine in considerable amounts in the free amino acid pool was observed.

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