Cyanide assimilation by Bacillus megaterium
by Peter Allen Castric

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

Abstract:
A bacterium identified as Bacillus megaterium was isolated from a cyanide enrichment of Fargo Clay. This organism grew in a cyanide medium and altered its respiration in response to the presence of cyanide. Electron microscopic examination of cells grown in the presence of cyanide showed no membranous structures other than the cytoplasmic membrane while examination of cells grown in the absence of cyanide showed mesosome-like bodies.

Cyanide began to disappear from a medium inoculated with Bacillus megaterium at the same time that the growth of the organism in this medium began. Washed cells administered K^14CN converted a large part of the radioactivity into cation and carbonate fractions. The two compounds comprising the major part of the cation fraction were identified as asparagine and aspartic acid. Screening of radioactive precursors to asparagine showed thatK^14CN, serine -l4C plus KCN, and (3-cyanoalanine -l4^C were converted most efficiently of the 13 precursors tried. The analysis of asparagine isolated from the whole cell feeding of K^13C^15N and serine -l4C showed the amide C and N tp be derived from cyanide and the rest of the carbon skeleton of asparagine to be derived from serine (β-cyanoalanine -l4C was identified as a product of this experiment). Whole cells of bacillus megaterium converted asparagine -14C to aspartic acid -14C, 14CO2 and l^C ethanol insoluble material. Cell-free extracts of this organism catalyzed the formation of asparagine -14C from serine -14C and KCN or serine and K^14CN as was the conversion of asparagine -14C to aspartic acid -14C. These results as well as analyses of the labelling patterns of β-cyanoalanine -14c and asparagine -l4C isolated from the above whole cell feeding experiments support the hypothesis that a biosynthetic pathway in Bacillus megaterium begins with the condensation of serine and cyanide forming p-cyanoalanine which is hydrolyzed tp asparagine and then, in turn to aspartic acid.

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ABSTRACT

A bacterium identified as Bacillus megaterium was isolated from a cyanide enrichment of Fargo Clay. This organism grew in a cyanide medium and altered its respiration in response to the presence of cyanide. Electron microscopic examination of cells grown in the presence of cyanide showed no membranous structures other than the cytoplasmic membrane while examination of cells grown in the absence of cyanide showed mesosome-like bodies.

Cyanide began to disappear from a medium inoculated with Bacillus megaterium at the same time that the growth of the organism in this medium began. Washed cells administered $K^{14}CN$ converted a large part of the radioactivity into cation and carbonate fractions. The two compounds comprising the major part of the cation fraction were identified as asparagine and aspartic acid. Screening of radioactive precursors to asparagine showed that $K^{14}CN$, serine $-^{14}C$ plus KCN, and β-cyanoalanine $-^{14}C$ were converted most efficiently of the 13 precursors tried. The analysis of asparagine isolated from the whole cell feeding of $K^{13}C^{15}N$ and serine $-^{14}C$ showed the amide C and N to be derived from cyanide and the rest of the carbon skeleton of asparagine to be derived from serine (β-cyanoalanine $-^{14}C$ was identified as a product of this experiment). Whole cells of Bacillus megaterium converted asparagine $-^{14}C$ to aspartic acid $-^{14}C$, $^{14}CO_2$, and ^{14}C ethanol insoluble material. Cell-free extracts of this organism catalyzed the formation of asparagine $-^{14}C$ from serine $-^{14}C$ and KCN or serine and $K^{14}CN$ as was the conversion of asparagine $-^{14}C$ to aspartic acid $-^{14}C$. These results as well as analyses of the labelling patterns of β-cyanoalanine $-^{14}C$ and asparagine $-^{14}C$ isolated from the above whole cell feeding experiments support the hypothesis that a biosynthetic pathway in Bacillus megaterium begins with the condensation of serine and cyanide forming β-cyanoalanine which is hydrolyzed to asparagine and then, in turn to aspartic acid.

INTRODUCTION

Plants are the primary source of free cyanide in nature. According to Conn and Butler (14) cyanogenesis has been noted in 750 species of higher plants. The mechanism of cyanide release has been studied in several species and is due to the degradation of cyanogenic glucosides (14). The glucosidic link is broken by a glucosidase yielding an aldehyde or a ketone and free cyanide. The glucoside degradation and cyanide release by plants appears to be a defense mechanism against parasitic invasion since this molecule is not released in large amounts until the tissue is damaged.

Many species of fungi are able to produce cyanide (5). The mechanism of cyanide formation of one psychrophilic basidiomycete has been shown by Stevens and Strobel (37) to be the same as that of the cyanophoric higher plants. It is interesting to note that cyanide release by this pathogenic fungus appears to be a mechanism by which this cyanide resistant parasite can overcome a cyanide sensitive host plant.

The evolution of cyanide has been noted in the bacteria, Bacillus pyocyaneous, Bacillus fluorescens, Bacillus violaceous (15), and Chromobacterium violaceum (27, 28). The mechanism of cyanide production in bacteria is not well understood. The last two references cited postulate the conversion of glycine to cyanoformic acid and the decarboxylation of the cyanoformic acid to free cyanide and CO_2. In cyanophoric bacteria as in other cyanophoric organisms the production of cyanide offers a definite evolutionary advantage over their non-cyanophoric relatives.

While HCN is usually thought to be a highly toxic respiratory inhibitor some organisms have been observed to be resistant to this compound. The most dramatic example is the growth of a strain of Bacillus pumilus in 10^{-1}M KCN and its survival in 2.5M KCN (41). One possible explanation is that the cyanide does not get into the resistant organism or that cyanide enters as a non toxic derivative. Beppu and Arima (3, 7) have demonstrated that arsenite resistance in a Pseudomonad was due to an induced permeability barrier. Skowronski (41), however, reported that Bacillus pumilus not only allowed KCN to enter but metabolized both the carbon and the nitrogen atoms of cyanide. McFeters and Strobel (26) observed that neither disruption of whole cells of Bacillus pumilus nor treatment of whole cells with permeability barrier destroying agents increased sensitivity towards cyanide again indicating the lack of a permeability barrier.

An alternate or branching electron transport system is another possible explanation of cyanide resistance. It has been established that the spadix of Arum maculatum will show greatly increased respiration in the presence of cyanide. Bendall (8) proposed that cytochrome b_7 was an alternate and less efficient (accounting for the higher rate of respiration) electron transport system in this organism. Lips and Biale (23) also explained the stimulation of respiration in the presence of cyanide as an alternate or branching electron transport system.

A similar explanation is the induction of a resistant cytochrome oxidase. Arima and Oka (4) and Mizushima et al. (29) have evidence for

an inducible cytochrome oxidase (a_2) from a species of Achromobacter. This cytochrome binds cyanide very weakly and can become oxidized in the presence of cyanide; its presence can be induced by low O_2 tension (this can be simulated by cyanide poisoning). McFeters and Strobel (26) using B. pumilus have noted that an increase in respiration in the presence of cyanide appears with time indicating a possible inductive effect of cyanide on the respiratory apparatus.

With examples of cyanide resistance and cyanide evolution in nature it is not surprising to find the assimilation and utilization of cyanide by living organisms. Strobel (34) has observed that a basidiomycete which could produce cyanide could also incorporate the cyanide carbon into the C-1 of L-alanine. This reaction is an enzyme-mediated Strecker synthesis utilizing acetaldehyde, ammonia and cyanide as precursors and involving α-amino propionitrile as an intermediate.

A second study with this organism (40) indicated that L-glutamic acid is another product of cyanide administration. The cyanide carbon is incorporated into the C-1 position of L-glutamic acid in a similar enzyme-mediated Strecker synthesis.

Blumenthal-Goldschmidt et al. (9) while working on the mechanism of cyanogenic glucoside synthesis in the cyanophoric sorghum seedling, discovered that the administration of $H^{14}CN$ led to extensive labelling in the amide group of asparagine. They observed this occurrence in the cyanophoric flax and white clover seedlings. Tschiersch (44) independly made similar observations.

Blumenthal-Goldschmidt (9) proposed a mechanism by which serine and cyanide condense ultimately forming asparagine. Nigam and Ressler (30) further made the observation that $H^{14}CN$ or serine-^{14}C was converted by common vetch into the dipeptide γ-glutamyl-β-cyanoalanine. Degradation of this molecule showed that cyanide contributed to the C-4 position of β-cyanoalanine and serine contributed to the C-1, 2, 3 positions while glutamic acid remained unlabelled, indicating that β-cyanoalanine could be an intermediate. Fowden and Bell (19) proved this to be the case in an experiment in which the γ-glutamyl-β-cyanoalanine forming alga Chlorella pyrenoidosa was fed $Na^{14}CN$. β-Cyanoalanine became labelled first with the radioactivity moving next into the dipeptide. They obtained from sweet pea seedlings (a plant species which synthesized asparagine-^{14}C from $H^{14}CN$ but formed no dipeptide) an extract which contained a nitrilase which hydrolyzed β-cyanoalanine to asparagine but which contained a nitrilase which hydrolyzed β-cyanoalanine to asparagine but which contained no activity for dipeptide synthesis. Fowden and Bell (19) also obtained from vetch seedlings (a plant which formed γ-glutamyl-β-cyanoalanine-^{14}C but which formed no asparagine-^{14}C from $H^{14}CN$) an extract which could synthesize the dipeptide but which showed only slight nitrilase activity towards β-cyanoalanine.

The path of cyanide was further elaborated by Dunnill and Fowden (17) who showed that cell-free extracts of E. coli could catalyze the condensation of serine-^{14}C and KCN to form β-cyanoalanine. This activity was lessened by the addition of cysteine indicating a possible

competition. Dunnill and Fowden (17) proposed that β-cyanoalanine formation might be due to nonspecific activity of the enzyme serine sulfhydrase (reaction 1).

$$\text{Serine} + H_2S \longrightarrow \text{Cysteine} + H_2O \qquad (1)$$

Cyanide might substitute for hydrogen sulfide (reaction 2) and cysteine for serine (reaction 3).

$$\text{Serine} + HCN \longrightarrow \beta\text{-cyanoalanine} + H_2O \qquad (2)$$

$$\text{Cysteine} + HCN \longrightarrow \beta\text{-cyanoalanine} + H_2S \qquad (3)$$

Floss et al. (18) using acetone powders of whole seedlings and seedling mitochondria of Bird's-foot trefoil observed that while serine and $K^{14}CN$ condensed to form β-cyanoalanine, cysteine greatly increased the amount of this product. This indicated that reaction 3 might be the prefered mechanism in this organism. Support was given to reaction 3 when Hendrickson (21) purified several hundred fold an enzyme from mitochondria of Blue Lupine seedlings which catalyzed reaction 3 specifically. This enzyme was termed β-cyanoalanine synthase. Hendrickson also isolated a soluble enzyme which catalyzed the formation of cysteine from O-acetyl serine and H_2S. It was proposed that serine could be converted to cysteine via this route thus accounting for the activity of serine as a precursor in some cases.

Bryske et al. (12) indicate that serine and cyanide are condensed to form β-cyanoalanine as the primary product in whole cells of the cyanophoric bacterium Chromobacterium violaceum. Cell-free work or specificity of precursor studies have not been published.

Blumenthal et al. (10) presented further evidence for the specificity of reaction 3 in enzyme preparations of sorghum, Blue lupine, Birdsfoot trefoil and common vetch. Experiments with sorghum, Blue lupine and common vetch seedlings showed that cysteine -^{14}C was converted to asparagine -^{14}C with an increase in yield when HCN was added. The increase was least in the seedling of the cyanophoric plant sorghum. Serine -^{14}C was incorporated into asparagine -^{14}C to any extent only in sorghum seedling and this was also increased with the addition of cyanide.

Thus, in nature situations exist in which cyanide can be dissimilated or assimilated (sometimes by the same organism) and in which resistance to the toxic effects of cyanide is not uncommon. These would be the elements of what Allen and Strobel (2) call the cyanide microcycle.

This report describes the effects of KCN on the growth, cytology and respiration of Bacillus megaterium and presents evidence for the biosynthesis of aspartic acid from KCN and serine by this organism. To the author's knowledge this is the first report to provide in vivo and in vitro evidence for this pathway.

MATERIALS AND METHODS

Culturing. The culture of Bacillus megaterium used in this study was obtained from a 10^{-1}M KCN enrichment broth of Skowronski (41). This broth had been inoculated with Fargo Clay from Fargo, North Dakota, a soil which had supported flax plants for 72 years.

The morphological and physiological criteria of Breed et al. (11) were used for the taxonomic description of the bacterium. The techniques and media used for identification were primarily those described in Manual of Microbiological Methods (25). The flagella stain was that of Blenden and Goldberg (8). Tyrosine agar was prepared in either of two ways: (1) 1% tyrosine in basal salts agar; (2) 0.1% tyrosine in nutrient agar.

The media used for growth and stock culturing were trypticase soy broth with glucose (TSB) (Baltimore Biological Laboratory) and trypticase soy broth agar with glucose. Broth cultures were grown at 35°C in cotten stoppered erlenmeyer flasks on a rotary shaker. Cell mass was determined by optical density at 660 mμ in a Bausch and Lomb Spectronic 20 colorimeter or by dry weight determination. Cells were harvested at the end of their logarithmic growth phase by centrifugation at 20,000 xg at 4°C for 10 minutes, then rinsed twice and resuspended in distilled water, trypticase soy broth, or buffer.

Materials. The ^{14}C compounds used in this study were obtained from the following sources: KCN (45.2 mc/mM), L-serine (14.5 mc/mM), sodium formate (0.96 mc/mM), Nuclear Chicago Corporation; D,L-cysteine (25.0 mc/mM),

The Radiochemical Centre, Amersham; acetyl-CoA (46.2 mc/mM) and sodium pyruvate (3.16 mc/mM), New England Nuclear; sodium carbonate (10 mc/mM), Tracerlab Company; sodium acetate (48.9 mc/mM), Volk radio-chemical; Glycol aldehyde (0.74 μc/mM) was prepared by the decarboxylation and deamination of L-serine -^{14}C according to the method of Dakin (16); α-amino, β-hydroxy, propionitrile (1.47 μc/mM) was prepared by Strecker addition of cyanide -^{14}C and ammonia to glycol aldehyde; formamide (0.977 μc/mM) was prepared by heating ammonium formate -^{14}C at 120°C overnight in a sealed tube with subsequent distillation of the formamide; Ethanolamine (13.4 mc/mM) and β-cyanoalanine (10 mc/mM) were a generous gift of E. E. Conn, University of California, Davis. In addition to these ^{14}C compounds, K^{13}CN and KC^{15}N were purchased from Biorad Laboratories.

Hyamine Hydroxide 10X was purchased from Packard Instrument Company and NCS protein solubilizer was purchased from Nuclear Chicago Corporation. Aspartic decarboxylase was purchased from Sigma Chemicals. All other chemicals were reagent grade.

Analytical Techniques. The technique of Aldridge (1) was used for the quantitative determination of cyanide. Protein concentration was determined by the method of Lowry et al. (24). The Bausch and Lomb Spectronic 20 was used for all quantitative colorimetric determinations. The ninhydrin reaction products of β-cyanoalanine were formed by the method of Ressler (52) and its absorption measured in a Beckman DK-2

scanning spectrophotometer. Infrared spectra were measured with a Beckman Microspec infrared spectrophotometer utilizing KBr micro-pellets.

The N-bromosuccinimide decarboxylation technique was performed according to Blumethal-Goldschmidt et al. (9). Decarboxylation with aspartic decarboxylase (30) was carried out in a gas evolution vessel with 5N NaOH as the trapping agent.

Ammonia was determined by the micro-Kjeldahl method. Dilute sulfuric acid was added to the boric acid trap after titration with 0.1 N HCl and the solution was taken to dryness in a 60°C oven.

Chromatography. Sheets of Whatman 541 paper were used for paper chromatography and the following solvent systems were employed: (1) n-butanol-acetic acid-water (4:1:5); (2) methanol-pyridine-water (40:2:10); (3) 80% phenol in water. Plain silica gel plates and Eastman Chromatogram prepared silica gel plates were used for one and two dimensional thin layer chromatography, respectively. The following solvent systems were employed for thin layer chromatography; (4) n-butanol-acetic acid-water (3:1:1), and (5) isopropanol-ammonium hydroxide (67:33). Amino acids in both paper and thin layer chromatography were detected by spraying with 0.03% ninhydrin in ethanol. Radioactivity was detected on two dimensional thin layer chromatograms with the x-ray overlay method used by Strobel (38). Radioactivity was also detected on one-dimensional thin layer and paper chromatograms with

the Packard Radiochromatogram Scanner model 385.

Paper Electrophoresis. Sheets of Whatman No. 3MM were used for paper electrophoresis. The following buffers were used: (1) Pyridine-acetate, pH 5.2 (2% pyridine in 1% aqueous acetic acid), 16 volts/cm and (2) Borate, pH 8.8, 0.05 M, 17 volts/cm. Radioactivity and amino acids were detected as indicated previously.

Isotope Determinations. Radioactivity was quantitatively measured with a Nuclear Chicago scintillation counter model 6804. The scintillation fluid was 1.5 ml absolute methanol and 12.5 ml toluene containing 4.0 g 2,5 diphenyloxazole and 100 mg p-bis-2(5-phenyloxazolyl) benzene per liter. Cpm were converted to dpm by the quench correction method using a standard curve.

^{13}C and ^{15}N enrichments were determined on the Picker-AEI MS-10 mass spectrometer. ^{15}N determinations based on 100 atoms % excess were performed as described by Stojanovic and Broadbent (37). ^{13}C enrichment was measured by subtracting the ratio of mass 45 to mass 44 of naturally enriched $BaCO_3$ from the ratio of mass 45 to mass 44 of the sample carbonates. The determinations of ^{13}C were also based on 100 atoms % excess.

Electron Microscopy. Cells were grown and harvested as indicated previously. Plastic embedding capsules were filled with cell suspensions and centrifuged for 5 minutes at full speed in an International Clinical

centrifuge. The drained pellets were then fixed with glutaraldehyde according to the method of Sabatini (34). A secondary fixation was carried out for 2 hours at 4°C in the dark with 2% osmium tetroxide in potassium phosphate buffer (0.1 M, pH 7.0). The pellets were dehydrated using a graded series of acetone solutions beginning at 20% and ending with 100%. Embedding was done according to the method of Glauert and Glauert (20) using CIBA araldite (epoxy resin 6005). Sections were made using a Reichert OM U2 ultramicrotome. Post section staining was done with 2% aqueous uranyl acetate, then Reynold's lead citrate. Examination and photography were performed with a Zeiss EM 9A electron microscope.

Respiration. Cultures were grown and harvested as indicated previously. A GME model KM oxygraph (Gilson Medical Electronics) was used to measure O_2 uptake. The addition of KCN solution, trypticase soy broth or phosphate buffer (0.05M, pH 7.0) and cell suspension were used to bring the total volume in the reaction vessel up to 2.0 ml. The reaction vessel was maintained at 35°C at all times.

Administration of Labelled Compounds. Equivalent amounts of labelled compounds were administered as aqueous solutions to equivalent amounts of both live and boiled cells. Vessels with a side arm or a center well containing 5N NaOH were used when CO_2 was measured. Unless otherwise indicated the period of incubation was for 24 hours at 23°C.

Extraction of Cell Products. Cells were disrupted using the French pressure cell or the Braun MSK cell homogenizer. Either method resulted in virtually total cell disruption. Two volumes of 95% ethanol were added to the homogenate and the suspension was centrifuged at 20,000 xg at 4°C for 10 min. Where indicated, the ethanol insoluble precipitate was dried and resuspended in NCS protein solubilizer and added to the scintillation fluid in place of methanol and the radioactivity counted in the usual fashion. The supernatant solution was passed through colums (1 x 2 cm) of Dowex 50W-X8 (H+), 200-400 mesh, then Dowex 1-X8 (formate), 200-400 mesh. The cation fraction was eluted with 10 ml of 6N HCl and the anion fraction was eluted with 10 ml of 6N formic acid. All fractions including the neutral fraction were taken to dryness on a hot plate in a stream of air and stored overnight in a vacuum desiccator containing P_2O_5 and NaOH.

Carbonate Analysis. Carbon dioxide trapped as carbonate was precipitated with 0.3M $Ba(OH)_2$. The precipitate was washed twice with distilled water by centrifugation at 10,000 xg at 4°C for 10 min. Radioactivity was determined by the conversion of a portion of the $BaCO_3$ to CO_2 (by the addition of 0.5 ml of 1N H_2SO_4) in a gas trapping vessel containing 1.5 ml of hydroxide of hyamine in a sidearm. The hydroxide of hyamine was added to the scintillation fluid in place of methanol.

Purification and Quantitation of Asparagine ^{14}C. The cation fraction was further separated by paper chromatography in solvent 2. The area matching the reference asparagine was cut out and eluted with distilled water. This material was dried on a hot plate in a stream of air and further paper chromatography was carried out in solvent 1. Again the area matching asparagine was cut out, eluted and dried as indicated before. This procedure was repeated once with the asparagine isolated from the $K^{13}C^{15}N$ feeding experiment. β-cyanoalanine was isolated by these methods except that paper chromatography in solvent 3 was also used.

Asparagine was quantitatively determined using reagents described in the Technicon Research Bulletin No. 20 (43) by incubation of a portion of the isolated asparagine with these reagents at 100°C for 15 min. then measuring optical density at 570 mμ. Radioactivity of a portion of the asparagine was counted giving specific activity values for the isolated asparagine.

Preparation of Cell-free Extracts. Washed cells resuspended in appropriate buffers were disrupted in a Braun MSK cell homogenizer and centrifuged twice at 20,000 xg for 10 min at 4°C. Heated (100°C for 10 min) and non-heated portions of the supernatant solution were fed equivalent amounts of ^{14}C compounds as is indicated in the footnotes of Tables VI and VII.

RESULTS

The Effects of KCN on Bacillus megaterium

Growth. The growth curves of B. megaterium in the presence and absence of cyanide are shown in Figure 1. The cells grown in cyanide show a much longer lag period and do not attain as great an optical density as cells grown in the absence of cyanide. The arrows in the figure indicate the point at which cells are harvested in subsequent experiments.

Cytology and Morphology. The morphological differences between cells grown with and without cyanide were not striking. The cell dimensions and flagellar arrangement of cells grown under these conditions were the same (1.0-1.5 x 2.0-4.0 μ and peritrichous flagella). Both sets of cells had heavy interior fat stain-positive granulation. However, cells grown in cyanide appeared predominantly as diplo or single rod-shaped cells while cells grown without cyanide formed long chains of rods.

Electron microscopy revealed a further difference between cells grown in the presence and cells grown in the absence of cyanide. Figure 2 shows sections of cells grown without cyanide. These pictures are representative of many fields of vision viewed in that membranous structures such as cytoplasmic membranes and mesosome-like bodies are visible. Sections of cells grown in the presence of cyanide (Figure 3) show only cytoplasmic membranes.

Figure 1. The growth curves of Bacillus megaterium grown in the presence and absence of 10^{-3}M KCN.

0 - 0 Cells grown without cyanide.

X - X Cells grown with cyanide.

Arrows explained in the text.

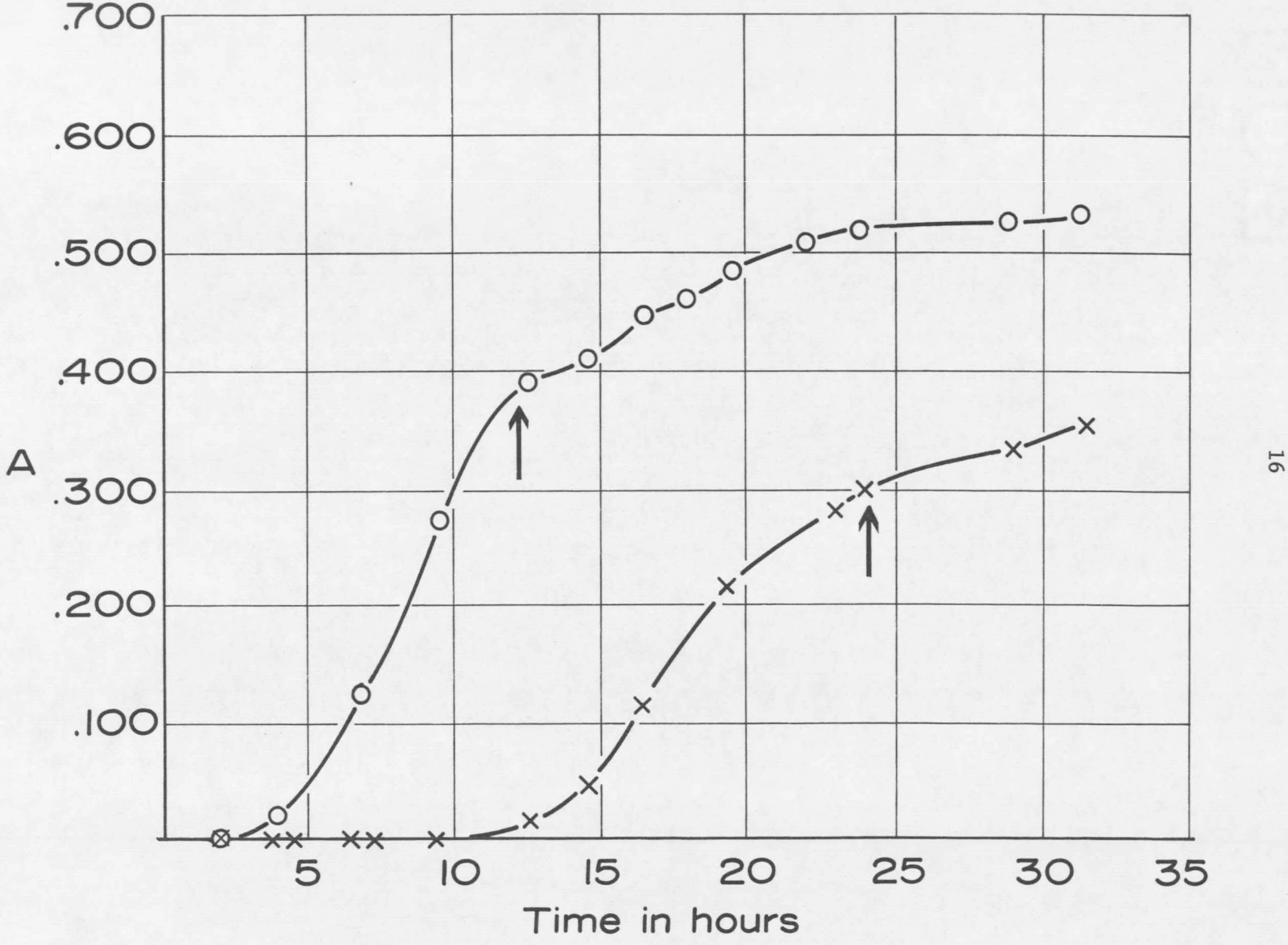
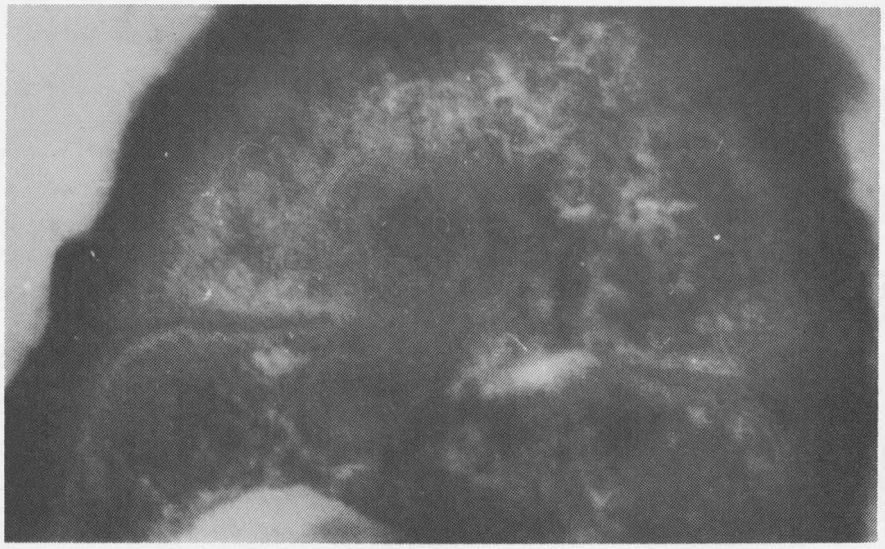

.700
.600
.500
.400
.300
.200
.100
A
5
10
15
20
25
30
35
Time in hours

Figure 2. Electron micrographs of Bacillus megaterium grown in the absence of cyanide.

A. Magnification x 140,000

B. Magnification x 150,000



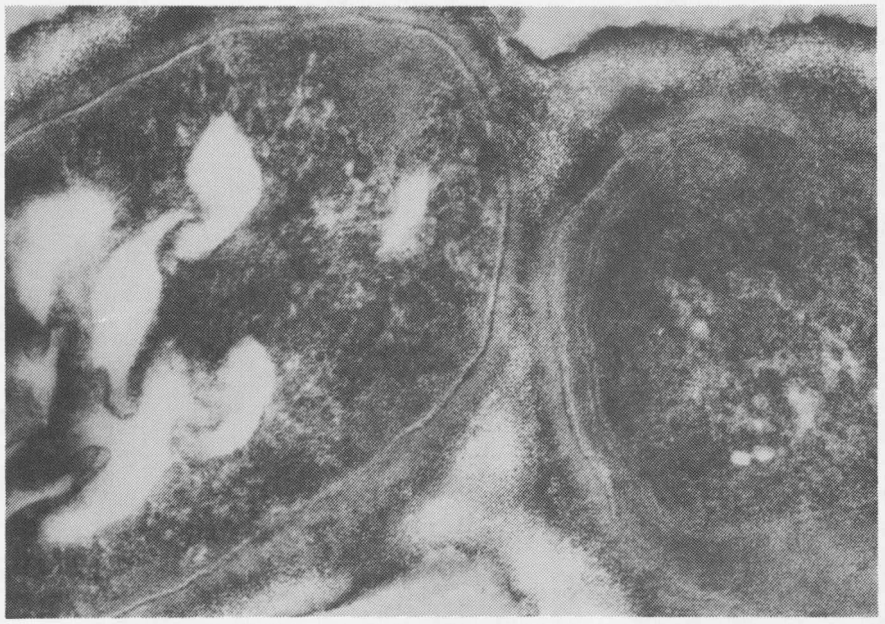
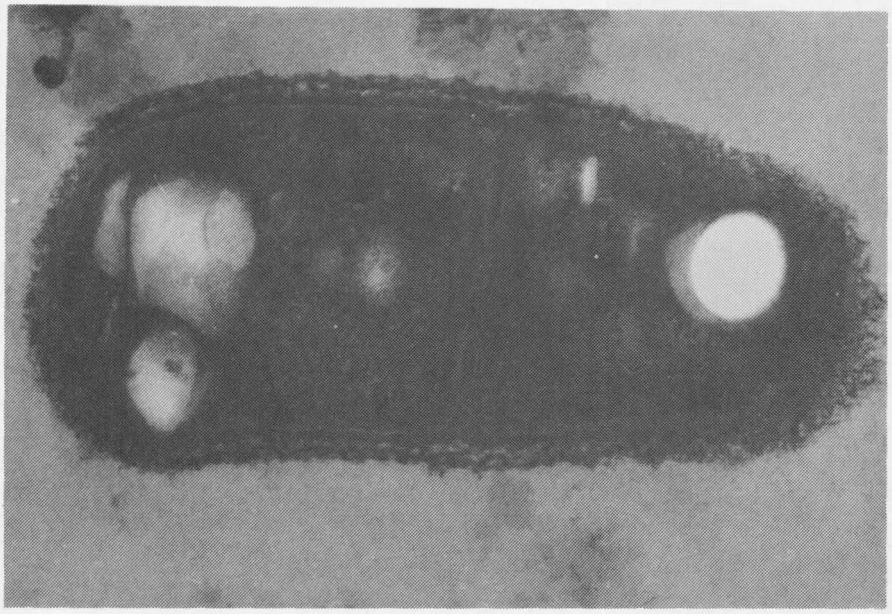
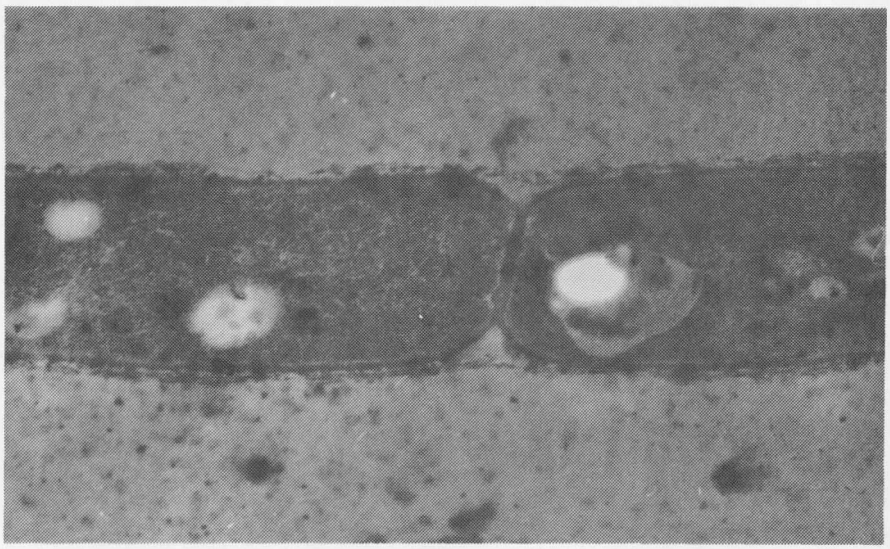


Figure 3. Electron micrographs of Bacillus megaterium grown in the presence of 10^{-3} M KCN.

A. Magnification x 70,000

B. Magnification x 60,000





Respiration. Table I shows that cells grown without cyanide and harvested and resuspended in trypticase soy broth containing no cyanide respired at twice the rate of cells grown in cyanide and harvested and resuspended in trypticase soy broth containing no cyanide. However, when the trypticase soy broth was made up to 10^{-4}M with respect to cyanide the rate of respiration of cells grown without cyanide was decreased by 85% while the rate of respiration of cells grown in cyanide was slightly increased. Increasing the cyanide concentration to 10^{-3} and 10^{-2}M virtually halted all respiration.

Assimilation of Cyanide by Bacillus megaterium

Disappearance of Cyanide From a Growing Culture. Figure 4 shows that in the inoculated medium (initially 10^{-3}M KCN) the rate of cyanide disappearance increased at the same time that growth in this medium began (Figure 1). The uninoculated medium did not show this increased rate of cyanide disappearance.

Incorporation of $K^{14}CN$ into Asparagine and Aspartic Acid. To establish that cyanide was being metabolized by Bacillus megaterium 4.14 µc $K^{14}CN$ (45.2 µc/um) was administered to washed cells (previously grown in 10^{-3}M KCN and the equivalent of 125 mg dry weight) suspended in 20 ml sterile distilled water. Table II shows that after 24 hours incubation the radioactivity was incorporated primarily into the cation fraction and the carbonate obtained from the live cell suspension.

Table I

The effect of cyanide on the rate of oxygen uptake of *Bacillus megaterium* grown in the presence and absence of 10^{-3}M KCN.

	Cells grown in presence of cyanide		Cells grown in absence of cyanide	
Molarity KCN	Oxygen disappearance (mμ moles O_2/sec)*	Cyanide effect as % inhibition	Oxygen disappearance (mμ moles O_2/sec)*	Cyanide effect as % inhibition
0	2.89	0.0	5.98	0.0
10^{-4}	3.20	-11.0	0.95	84.1
10^{-3}	0.17	94.1	0.06	99.0
10^{-2}	0.13	95.5	0.23	96.1

* All measurements are on a per mg dry weight basis.

Figure 4. The disappearance of cyanide from an uninoculated medium and a medium containing growing Bacillus megaterium.

0 - 0 Uninoculated TSB initially containing 10^{-3}M KCN.

X - X Inoculated TSB initially containing 10^{-3}M KCN.

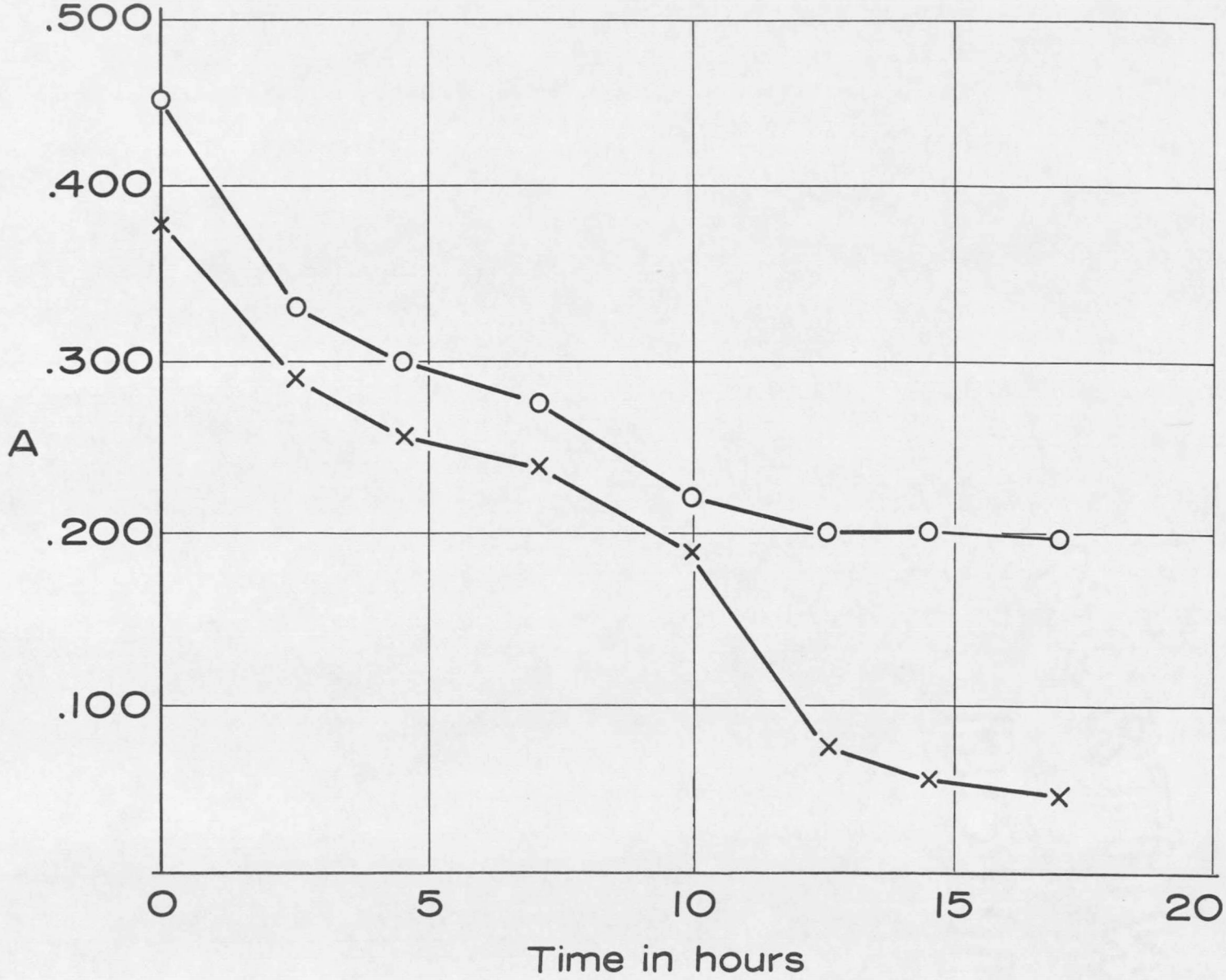

.500
.400
.300
.200
.100
A
0
5
10
15
20
Time in hours

Table II

The incorporation of radioactivity into fractions of Bacillus megaterium fed $K^{14}CN$.

Source	Radioactivity ($dpm/10^{-4}$) Fraction			
	Cation	Anion	Neutral	Carbonate
Live cells suspension	380.0	42.2	22.2	2.3
Boiled cell suspension	90.0	46.2	26.2	0.7

The cation fraction was examined by paper and thin layer chromatography and by paper electrophoresis to ascertain which compound contained radioactivity. The two compounds containing nearly all of the radioactivity (designated as unknowns 1 and 2) were later identified as asparagine and aspartic acid, respectively, as is indicated in Table III. As a further substantiation of its identity, unknown 1 was refluxed for 1½ hours in 6N HCl then dried in a stream of air on a hot plate and stored overnight in a vacuum desiccator containing P_2O_5 and NaOH. The product was then identified by paper and thin layer chromatography in solvents 1-5. The R_f of radioactive product matched that of authentic aspartic acid in all cases. Further evidence for the identify of unknowns 1 and 2 and the acid hydrolysis product of unknown 1 was obtained using two dimensional thin layer cochromatography (solvents 4 and 5) using authentic compounds with subsequent x-ray overlay. The exposed spots on the films coincided exactly to the reference compounds.

The specific location of the ^{14}C in the asparagine isolated and its acid hydrolysis product aspartic acid was determined. Table IV shows that nearly all the radioactivity resided in the amide carbon of asparagine. The radioactive aspartic acid was at least 96% in the L-configuration since it reacted to this extent with the L-specific aspartic acid decarboxylase (one of the two methods used for determining C-4 radioactivity).

Table III

Comparison of unknown compounds to asparagine and aspartic acid using paper and thin layer chromatography and paper electrophoresis.

	R_f values			
	Apn	Unknown #1	Ap	Unknown #2
Paper chromatography: Solvent System				
(1) n-butanol-acetic acid-water (4:1:5)	0.18	0.19	0.20	0.20
(2) methanol-pyridine-water (40:7:10)	0.27	0.27	0.60	0.60
(3) 80% phenol in water	0.42	0.42	0.26	0.26
Thin layer chromatography: Solvent System				
(4) iso-propanol ammonium hydroxide (67:33)	0.42	0.43	0.38	0.38
(5) n-butanol-acetic acid-water (3:1:1)	0.34	0.34	0.31	0.31
Paper electrophoresis: Buffer		cm^2/volt hr		
(1) 2% pyridine in 1% acetic acid pH 5.2	0.038	0.038		
(2) 0.05M borate pH 8.8	0.112	0.106		

Table IV

The ^{14}C labelling patterns of asparagine and β-cyanoalanine isolated from whole cell suspensions of Bacillus megaterium which had been administered various ^{14}C compounds.

Radioactive Compound assayed	Radioactive precursor	% total radioactivity per C-atom C-1	C-2, 3	C-4
Asparagine - ^{14}C	$K^{14}CN$ [a]	1.7	4.2	94.1
	$K^{14}CN$ [b]	2.9	0.2	96.7
	Serine - ^{14}C-U [b]	32.0	65.4	2.6
	β-cyanoalanine - ^{14}C-4 [b]	2.0	4.0	94.0
β-cyanoalanine - ^{14}C	Serine - ^{14}C-U [c]	35.0	62.7	2.3

[a] Data from section entitled "Incorporation of $K^{14}CN$ into asparagine and aspartic acid."

[b] Data from section entitled "Screening of ^{14}C precursors of asparagine."

[c] Data from section entitled "The incorporation of $K^{13}C^{15}N$ into the amide group of asparagine."

The Screening of ^{14}C Precursors of Asparagine. Since the cyanide carbon appeared to be going into the amide group of asparagine it was necessary to determine: a) what precursors might provide the remainder of the carbon skeleton of asparagine, and b) what other precursor could provide the amide carbon of asparagine. Washed cells (the equivalent of 125 mg dry weight) suspended in 20 ml sterile distilled water were fed quantities of ^{14}C compounds thought to be likely precursors. The specific activities of the precursors fed and the asparagine isolated as well as the dilution factors are cited in Table V. These results showed that cyanide, serine, α-amino, β-hydroxy propionitrile and β-cyanoalanine were the best precursors to asparagine synthesis as their dilution factors were the lowest. The patterns of ^{14}C labelling in the asparagine derived from KCN, serine or β-cyanoalanine are given in Table IV. These results indicate that serine provided a three carbon precursor and cyanide a one carbon precursor to asparagine formation.

The Incorporation of $K^{13}C^{15}N$ into the Amide Group of Asparagine. In order to determine whether the cyanide nitrogen as well as cyanide carbon becomes incorporated into the amide group of asparagine, $K^{13}C^{15}N$ (32.5 atoms % excess ^{15}N, 36.5 atoms % excess ^{13}C) was fed to washed cells (previously grown in 10^{-3}M KCN and equivalent to 750 mg dry weight) resuspended in 200 ml sterile distilled water at a final cyanide concentration of 10^{-3}M. Also serine -^{14}C (0.9 μc) was administered as

Table V

The efficiency of various ^{14}C-compounds as precursors of asparagine.

Precursors	Precursor fed Specific activity (μc/umole)	Asparagine Isolated[a] Specific activity (μc/umole)	Dilution factor (μc/um fed) / (μc/um isolated)
One carbon			
KCN	45.20	1.46	3.09×10^{1}
Na_2CO_3	10.00	1.40×10^{-2}	7.24×10^{2}
NaCOOH	0.96	NCAB[b]	---
$HCONH_2$	0.98	NCAB	---
Two carbon			
Acetate	48.90	3.37×10^{-3}	1.45×10^{4}
Ethanolamine	13.40	NCAB	---
Glycol aldehyde	0.74×10^{-3}	NCAB	---
Acetyl-CoA	46.20	NCAB	---
Three carbon[c]			
Serine	14.50	3.71×10^{-1}	4.02×10^{1}
Cysteine	25.00	NCAB	---
α-amino, β-hydroxy propionitrile	1.47×10^{-2}	1.64×10^{-4}	8.99×10^{1}
Pyruvate	3.16	7.98×10^{-3}	3.96×10^{2}
Four carbon			
β-cyanoalanine	10.00	6.56×10^{-1}	1.53×10^{1}

[a] All counts treated on a per umole precursor fed basis.

[b] No counts above background.

[c] When the two and three carbon precursors were fed, $K^{12}CN$ was added in the amount of 1.5 μmoles.

the three carbon precursor and marker for asparagine isolation. After an incubation of 18 hours the amide and α-amino groups of asparagine isolated were analyzed for the incorporation of the isotopes ^{13}C and ^{15}N.

Table VI shows that while the amide group is enriched with ^{15}N, the α-amino group shows no detectable enrichment. The ratio of ^{15}N to ^{13}C (0.76) in the amide group of the asparagine isolated is quite similar to the ratio of ^{15}N to ^{13}C (1.03) in the cyanide administered these cells. The ^{14}C labelling pattern of asparagine showed that the first three carbons contained over 90% of the total radioactivity of the molecule which agrees with previous experiments.

During the isolation of asparagine a portion of the radioactivity was found in a compound which was subsequently identified as β-cyanoalanine by one dimensional paper and thin layer chromatography (Table VII). Two dimensional thin layer cochromatography (in solvents 4 and 5, and with authentic β-cyanoalanine) with an x-ray film overlay showed that the radioactive area exactly matched the green ninhydrin spot of authentic β-cyanoalanine on the thin layer plate. The visible absorption spectrum of the ninhydrin reaction product gave a spectrum identical to that of authentic β-cyanoalanine with a large absorption band at 645 mμ. The infra-red absorption of the compound (using a KBr micropellet) showed the distinctive nitrile band at 2250 cm^{-1}.

Table VI

The incorporation of $K^{13}C^{15}N$ into the amide group of asparagine by whole cells of Bacillus megaterium *

Asparagine isolated	ug ^{13}C	ug ^{15}N	% ^{13}C incorp.	% ^{15}N incorp.	$\frac{ug\ ^{15}N/ug\ ^{13}C\ Amide\ group}{ug\ ^{15}N/ug\ ^{13}C\ K^{13}C^{15}N\ fed}$
α-amino group	--	0.00	--	0.00	--
Amide group	18.74	14.00	1.34	1.00	0.74

* Isolated asparagine was hydrolyzed for 1½ hr in 3 ml 6N HCl. The ammonia released was evolved in an ammonia still and trapped in a 10% boric acid solution and then titrated. The hydrolysate was dried in a rotary evaporator and the aspartic acid extracted with three treatments with absolute methanol. This material was put through a column (1 x 2 cm) of Dowex 1-X8, (Formate) 200-400 mesh. The aspartic acid was eluted with 5 ml of 6N Formic acid then dried on a hot plate in a stream of air, resuspended in distilled water and passed through a column (1 x 2 cm) of Dowex 50-X8 (H+), 200-400 mesh. The aspartic acid was eluted with 5 ml of 6N HCl then dried on a hot plate in a stream of air and stored overnight in a vacuum desiccator containing P_2O_5 and NaOH. One half of the aspartic acid was then treated with aspartic decarboxylase and the CO_2 evolved from the C-4 of the compound trapped in 5N NaOH. The carbonate was precipitated with 0.3M $Ba(OH)_2$ and the precipitate washed with distilled water. The other half of the aspartic acid was digested and the ammonia treated as indicated above.

Table VII

Comparison of an unknown compound to authentic β-cyanoalanine using paper and thin layer chromatography.

Paper Chromatography Solvent Systems	R_f's β-cyanoalanine	Unknown #3
(1) n-butanol-acetic acid H_2O (4:1:5)	0.24	0.24
(2) methanol-pyridine-H_2O (40:2:10)	0.69	0.70
(3) 80% phenol	0.30	0.30

Thin Layer Chromatography Solvent Systems	β-cyanoalanine	Unknown #3
(1) iso propanol-NH_4OH (67:33)	0.50	0.49
(2) n-butanol-acetic acid H_2O (3:1:1)	0.33	0.33

The ^{14}C labelling pattern of β-cyanoalanine (Table IV) indicated that the majority of the radioactivity resided in the first three carbons of the molecule indicating that serine is a likely precursor of the portion of the molecule.

The Conversion of $K^{14}CN$ or Serine -^{14}C into Asparagine -^{14}C by a Cell-free Extract of Bacillus megaterium. Table VIII illustrates the synthesis of asparagine from KCN and serine. Under the conditions of assay all values reported are above the values of the boiled control. Asparagine was identified by chromatography of portions of the reaction mixture in solvents 1 and 3. The radioactive product chromatographed at the same R_f as authentic asparagine in each case. No radioactive asparagine could be demonstrated when these extracts were fed cysteine -^{14}C and KCN as precursors. Furthermore, the presence of β-cyanoalanine could not be demonstrated in any of the reaction mixtures. Radioactive aspartic acid was observed as a product when either $K^{14}CN$ or serine -^{14}C were used as precursors. The aspartic acid of the reaction mixture was isolated and assayed according to the procedure in Table IX.

Utilization of Asparagine -^{14}C by Bacillus megaterium. The above results indicate that β-cyanoalanine, asparagine, aspartic acid and CO_2 are products of cyanide metabolism in Bacillus megaterium. In order to place the metabolism of the asparagine formed in a better perspective, whole cell suspensions of Bacillus megaterium (the equivalent of 65.0 mg dry weight of cells suspended in 5.0 ml of trypticase soy broth) were

Table VIII

The conversion of serine-^{14}C and $K^{14}CN$ to asparagine-^{14}C by cell-free extracts of Bacillus megaterium.*

Precursor	Time of incubation (hours)	^{14}C-Compound isolated (mμmoles) Asparagine
Serine -^{14}C + KCN	0	0.00
	6	2.39
	16	1.83
Serine + $K^{14}CN$	0	0.00
	6	2.48
	16	3.53

* The reaction mixture contained 0.3 m moles sodium carbonate buffer (pH 9.5), 0.3 μmoles mercaptoethanol, 5.0 μmoles KCN (when fed as radioactive isotope, 4.95 μc fed at an overall specific activity of 0.97 μc/μm), 10.0 μmole DL serine (when fed as a radioactive isotope, 0.76 μc L-^{14}C-serine fed at an overall specific activity of 0.15 μc/μm L-serine), 9.4 mg protein and distilled water in a total volume of 3.0 ml. Incubation was carried out at 23°C, and the reaction was stopped by the addition of 2 ml 95% ethanol to samples of the reaction mixture which were then dried on a hot plate in a stream of air. Portions of these samples were assayed using paper chromatography (solvents 1 and 3). Areas matching the reference were cut out, and added to scintillation vials and the radioactivity counted.

fed 27.7 muc of asparagine -^{14}C (0.3 μc/μm) isolated from whole cells fed K^{14}CN.

Figure 5 shows that the asparagine -^{14}C was rapidly broken down by whole cells of Bacillus megaterium. Approximately 90% of the asparagine -^{14}C fed was gone after 6 hours indicating that it is rapidly metabolized by this organism. The first product of asparagine metabolism to appear with time is aspartic acid which was identified by paper and thin layer chromatography in solvents 2, 3, and 4. The aspartic acid is then metabolized to CO_2 and ethanol insoluble material (the composition of which is unknown).

The Conversion of Asparagine to Aspartic Acid by Cell-free Extracts of Bacillus megaterium. The demonstration of the cell-free conversion of asparagine to aspartic acid is presented in Table IX. Under the conditions of assay noted in Table IX the disappearance of substrate and appearance of product are linear with relations to time and protein concentration. The product was identified as aspartic acid by means of two dimensional thin layer cochromatography with authentic aspartic acid (solvents 4 and 5) followed by x-ray overlay of the thin layer plate. The exposed spot on the film exactly matched the ninhydrin positive spot on the plate.

Figure 5. Utilization and conversion of asparagine -^{14}C by whole cells of Bacillus megaterium.

△ - △ Asparagine -^{14}C

X - X Aspartic acid -^{14}C

0 - 0 $^{14}CO_2$

□ - □ ^{14}C-ethanol insoluble material

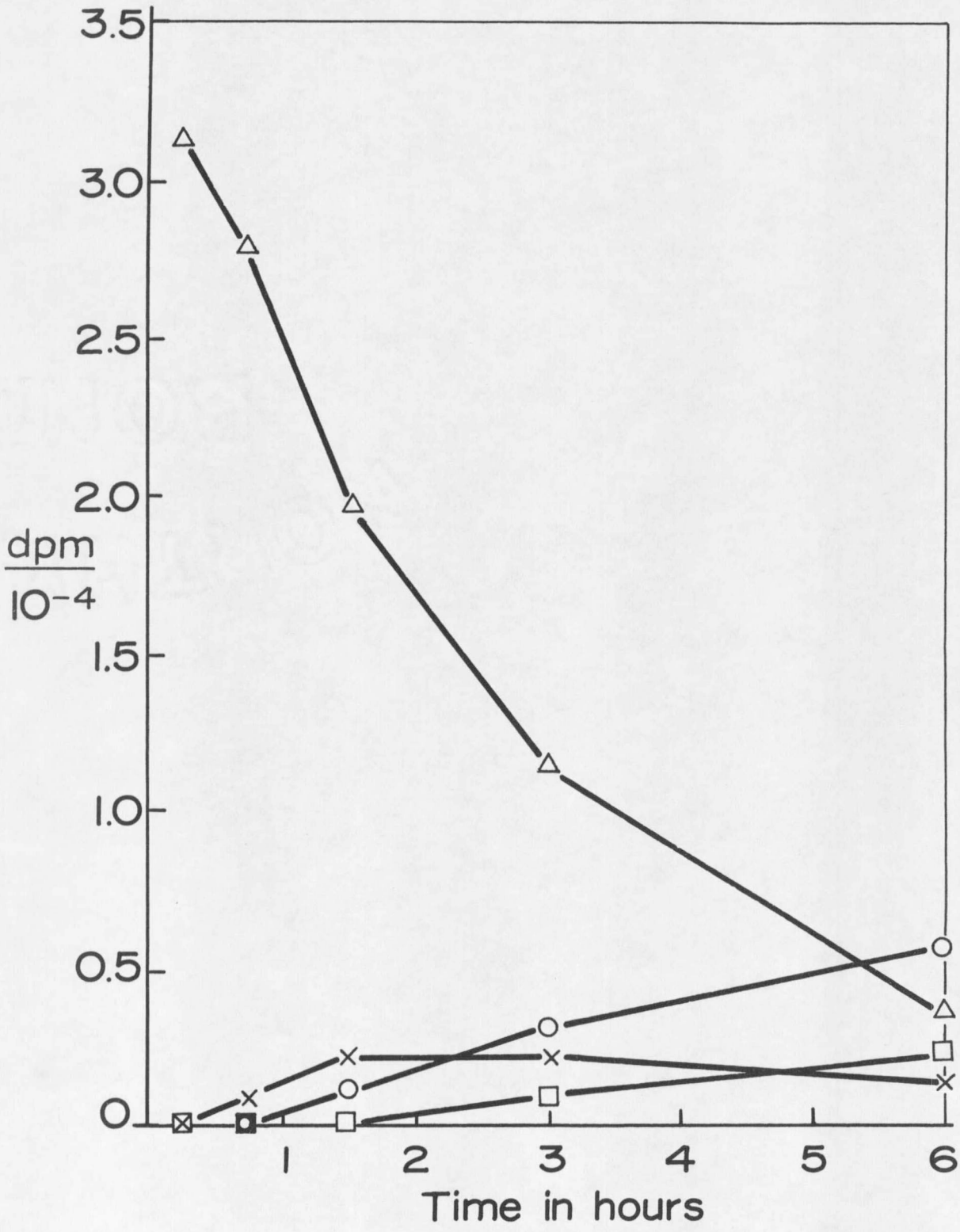

3.5
3.0
2.5
2.0
dpm
10⁻⁴
1.5
1.0
0.5
0
1
2
3
4
5
6
Time in hours

Table IX

The conversion of asparagine-^{14}C to aspartic acid-^{14}C by a cell-free extract.*

Time in hours	dpm Asparagine	Aspartic Acid
0	6,100	0
3	3,600	1,250
6	2,500	2,000

* The reaction mixture contained 25 μmoles of potassium phosphate buffer (pH 7.2), 1.1 mμc -asparagine-^{14}C (36.5 mμmoles), and 16.4 mg protein in a total volume of 2.0 ml of distilled water. Incubation was carried out at 23°C; the reaction was stopped by passing portions of the mixture through columns (0.5 x 2 cm) of Dowex 1-X8 (formate). After elution of the column with 3 ml of 6N formic acid both fractions were dried on a hot plate under a stream of air. Portions of these samples were assayed using thin layer chromatography solvent 4. The area matching the reference was scraped into scintillation vials and the radioactivity counted.

DISCUSSION

Whole cells of Bacillus megaterium grown in the presence of cyanide showed no mesosome-like bodies when examined under the electron microscope (Fig. 3). By contrast, mesosome-like structures were seen in cells of this organism grown in the absence of cyanide (Fig. 2). If the mesosomes of Bacillus megaterium contain many of the cells cytochromes as appears to be the case in Bacillus subtilus (35) then the respiration of cells lacking mesosomes whould be expected to be altered. The respiration rate of Bacillus megaterium (on a per mg dry weight of cells basis) is about 50% of the respiration rate of this organism grown without cyanide (Table I). However the respiration rate of this organism grown in cyanide was not inhibited but somewhat stimulated by the presence of 10^{-4}M KCN (Table I). This would indicate an induction of resistant respiration (as has been previously reported in bacteria (4, 26, 29) or a selection of resistant organism caused by the presence of cyanide. The extended shape of the growth curve and the longer lag phase of Bacillus megaterium grown in the presence of cyanide (Fig. 1) are probably a result of the decreased respiration rate. These data are inconclusive and represent only preliminary work. Further knowledge would be gained by a) ascertaining whether the altered respiration rate is inducible in non-dividing cells and if new protein needs to be formed for this effect using transcription and translation inhibitors, b) comparing phosphorylating efficiency of induced with original ETS (P/O ratios), c) finding the limits of cyanide resistance and surveying other ETS inhibitors for the induction effect, d) ascertaining

spectrophotometrically if new cytochromes are present in the induced system and measuring the oxidation-reduction abilities of them in the presence of cyanide, and e) doing more comprehensive electron microscopy.

The disappearance of cyanide from a growing culture of Bacillus megaterium (Fig. 4) was the first indication that this organism might metabolize this toxic compound. The administration of $K^{14}CN$ to a washed whole cell suspension of this organism showed that the cyanide was metabolized as was evidenced by the presence of radioactive asparagine, aspartic acid and carbonate in the cell suspension after incubation (Tables II and III). Cyanide and not one of its hydrolysis or oxidation products appears to be the primary one carbon precursor of asparagine (Table V) in experiments with whole cells although CO_2 was incorporated slightly. No two carbon precursor gave significant incorporation into asparagine (Table V). Among the three carbon precursors (other than serine) only α-amino, β-hydroxy propionitrile was significantly converted into asparagine which could be accounted for by a non-specific nitrilase hydrolyzing this compound to serine (Table V). Factors that would contribute to the order of dilution factors of KCN, serine and β-cyanoalanine are: a) proximity of the compound to the final product, and b) participation of the compound in other enzymatic reactions.

The conversion of cyanide and either serine or cysteine into β-cyanoalanine and asparagine has been documented in higher plants in vivo

(9, 19, 22, 33, 44) and *in vitro* (10, 18, 22, 45). Asparagine in plants appears to be metabolized no further (22) except into proteins. In bacteria cyanide metabolism is less well known. Bryske et al. (12) have suggested the *in vivo* incorporation of cyanide into β-cyanoalanine, asparagine and aspartic acid in *Chromobacterium violaceum*. Dunnill and Fowden (17) have shown *in vitro* that *E. coli* converts cyanide and serine or possibly cysteine to β-cyanoalanine.

This report presents the following evidence for the conversion of cyanide and serine to form β-cyanoalanine which is metabolized to asparagine then aspartic acid and then CO_2 and ethanol insoluble material: a) whole cell precursor screening indicated that serine, KCN and β-cyanoalanine are the best precursors of asparagine of the compounds tested (Table V); b) analysis of the carbon skeleton of asparagine derived from serine and KCN indicated that serine donates carbon to the C-1, 2, 3 of asparagine and KCN to the C-4 (Table IV) while analysis of asparagine derived from β-cyanoalanine gave the same labelling pattern as the β-cyanoalanine indicating a simple hydrolysis step (Table IV); c) in the $K^{13}C^{15}N$, serine -^{14}C feeding of whole cells the ^{13}C enriched the amide carbon of asparagine to about the same extent that the ^{15}N enriched the amide nitrogen while no ^{15}N enrichment was seen in the α-amino group of asparagine; d) β-cyanoalanine -^{14}C was a product of the previous (Table IV) experiment (analysis of the carbon skeleton of this compound indicated that serine carbons contributed to the C-1, 2, 3 of β-cyanoalanine); e) whole cells converted

asparagine to aspartic acid, CO_2 and ethanol insoluble material; f) cell-free extracts catalyzed the conversion of KCN and serine to asparagine and the conversion of asparagine to aspartic acid (Table VII and VIII).

The ratio of ^{15}N:^{13}C of the amide group of asparagine isolated from the $K^{13}C^{15}N$, serine - ^{14}C feeding of whole cells to ^{15}N:^{13}C of the cyanide fed indicates that the ^{13}C of cyanide appears to be going into the amide group of asparagine to a slightly greater extent than the ^{15}N of cyanide. This discrepancy could be a result of: a) experimental error or b) the hydrolysis of a portion of the asparagine to aspartic acid in which the amide nitrogen is converted to ammonia and the reverse step of asparagine synthesis (reported in bacteria 13, 31) from aspartic acid and ammonia slightly, if at all, enriched with ^{15}N.

The question of why β-cyanoalanine was isolated during the $K^{13}C^{15}N$, serine - ^{14}C feeding experiment and in no others deserves comment. While $K^{14}CN$ was ordinarily fed in the concentration of 0.1 to 1.0 mμmole per mg dry weight of cells the $K^{13}C^{15}N$ was administered in the concentration of 440 mμmole per mg dry weight of cells. Assuming that serine would be generated by the cell, the precursors of this pathway of asparagine biosynthesis would be in abundance leading to an increase in intermediates. Alternately, the asparagine produced might tend to regulate the activity of the nitrilase needed to convert β-cyanoalanine to asparagine.

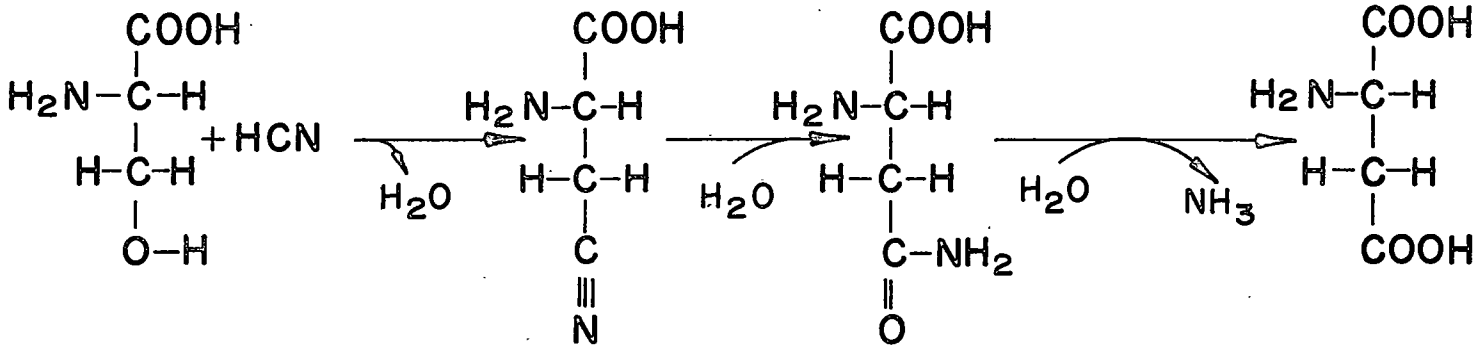
The results of the cell-free experiments (Table VII) show that in aliquots of the same extract, $K^{14}CN$ and serine were converted to

asparagine to the same extent as KCN and serine -^{14}C indicating a single pathway. β-cyanoalanine could not be demonstrated in the reaction mixtures. Either one or both of the following possibilities could give the results seen: a) at the times of assay the enzyme which condenses serine and cyanide had become denatured and the β-cyanoalanine formed converted to asparagine and aspartic acid or b) nitrilase activity, necessary to convert β-cyanoalanine to asparagine, is of the magnitude as not to allow the accumulation of β-cyanoalanine under the usual experimental conditions.

The assimilation of cyanide by Bacillus megaterium raises the question concerning the significance of this pathway. This organism, isolated from a soil which had supported the growth of flax (a cyanophoric plant) for 72 consecutive years, adapted its respiration to the presence of cyanide and converted this toxic molecule into common metabolites indicating a detoxification process. Cyanide has been postulated to be an intermediate in the prebiotic synthesis of such molecules as purine and amino acids (such as asparagine and aspartic acid, 36). The metabolic activity noted in the present report might have been acquired early in evolution and retained up the the present.

One interpretation of the significance of the pathway presented in this report is that the condensation of serine and cyanide (Fig. 6) is catalyzed by an enzyme such as serine sulfhydrase which is specific for reaction 1 of the Introduction. The product, β-cyanoalanine might then be hydrolyzed by a non-specific nitrilase to asparagine and so on.

Figure 6. A pathway of aspartic acid biosynthesis in *Bacillus megaterium*.



Serine β Cyanoalanine Asparagine Aspartic Acid

Serine sulfhydrase is ATP dependent for its normal function and the presence of ATP in the cell-free extract discussed in this report seems unlikely without an ATP generating system. Dunnill and Fowden (17) showed the activity of their bacterial enzyme preparation which was capable of condensing serine and cyanide to be stimulated by the presence of ATP. Purification of this enzyme and a survey study of substrates and cofactors involved would clarify this point.

Reports have indicated that asparagine in Lactobacillus arabinosus (31) and Streptococcus bovis (13) is formed from aspartic acid and ammonia by an enzyme termed asparagine synthetase. Bacillus megaterium, however, appeared to catalyse asparagine to aspartic acid irreversibly (Fig. 5, Table VIII). The pathway demonstrated in this report (Fig. 6) could well be a natural mechanism of asparagine synthesis in Bacillus megaterium. Although asparagine is present in this organism when no cyanide is administered the possibility exists that cyanide could be evolved (as in cyanophoric plants and bacteria) which could condense with serine to form asparagine. This point would warrant further research.

SUMMARY

Bacillus megaterium altered its morphology, cytology, respiration and growth characteristics in the presence of cyanide. This organism could condense serine and cyanide to form β-cyanoalanine which could he hydrolyzed to asparagine. In this reaction the cyanide carbon and nitrogen form the amide carbon and nitrogen of asparagine and the serine supplies the remainder of the carbon skeleton. This organism could hydrolyze the asparagine formed to aspartic acid which could be further metabolized to CO_2 and ethanol insoluble material.

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