A study of a-aminobutyronitrile in Rhizoctonia solani
by Francis Hing Soon Liu

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Chemistry
Montana State University

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A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

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Bozeman, Montana

August, 1972

ACKNOWLEDGMENT

I would like to first acknowledge the Endowment and Research Foundation of Montana State University for its support of Dr. B. P. Mundy's research endeavors. I would also like to thank the Department of Chemistry at Montana State University for their financial support in the way of teaching assistantships and tuition waivers. I would like to thank Dr. Mundy for his continued help and interest not only in the area of my research, but also the overall value of my education at this institution. Without his patience and confidence, this thesis would not have been possible. I would also like to acknowledge the help of the staff members and graduate students of the department for their discussion and technical aid to my special problems, especially Dr. K. Hapner for the assistance in setting up and operating the amino acid analyzer; Drs. S. Roger and G. Julian in using the electrophoresis instrument; Dr. L. Jackson in preparing TLC plates and in the use of scintillation counting, and Dr. P. Jennings for mass spectrometry.

Especially, I would like to thank Dr. G. A. Strobel for all the help and suggestions he gave throughout the

course of this research. I would like to thank my colleagues in our research group, Mr. B. Larsen and Mr. J. Sun for their meaningful discussion.

Most importantly, I would like to thank my parents, without whom all of this would not have been possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

α-aminobutyronitrile was found in Rhizoctonia solani as a product of potassium cyanide assimilation. Labeled potassium cyanide (KC*N) was used to study the pathways.

INTRODUCTION

Cyanide assimilation occurs in various living systems. Some species of insects are known to be able to assimilate hydrogen cyanide and transform it into aspartic acid.[1] In some higher plants, it has been observed with feeding experiments that hydrogen cyanide was incorporated into asparagine to a considerable extent.[2] Other investigations have concluded that β-cyanoalanine is the intermediate of cyanide assimilation.[3] It has been proposed that hydrogen cyanide condenses with cysteine in the presence of an enzyme, cyanoalanine synthetase,[4] to produce β-cyanoalanine and hydrogen sulfide. This β-cyanoalanine can then be hydrolyzed into asparagine with a nitrilase or

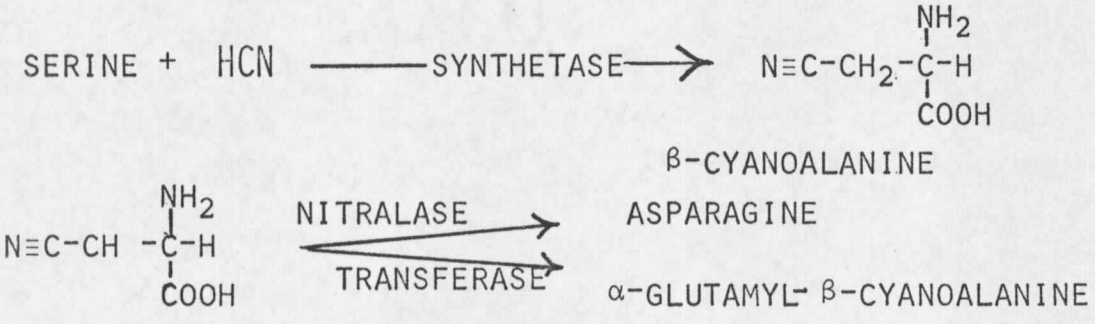


Fig. 1

Proposed Scheme of HCN Assimilation

condenses with γ-glutamyl moiety by means of a γ-glutamyl transferring enzyme to produce γ-glutamyl-β-cyanoalanine.[5]

Castric and Strobel[6] reported, in a similar study, evidence of the formation of asparagine and aspartic acid from cyanide and serine by a bacterium.

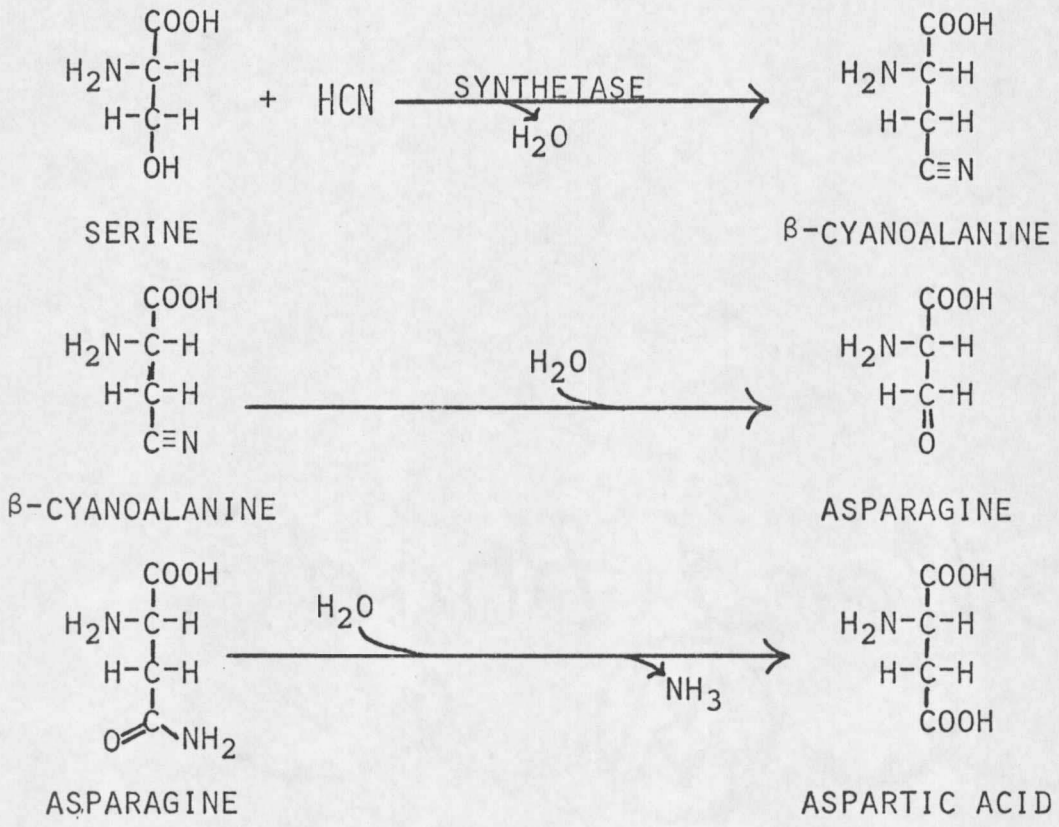
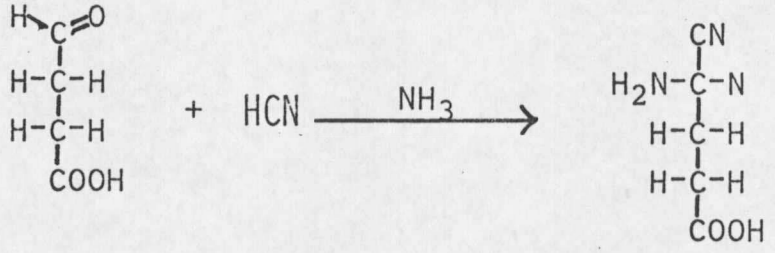


Fig. 2

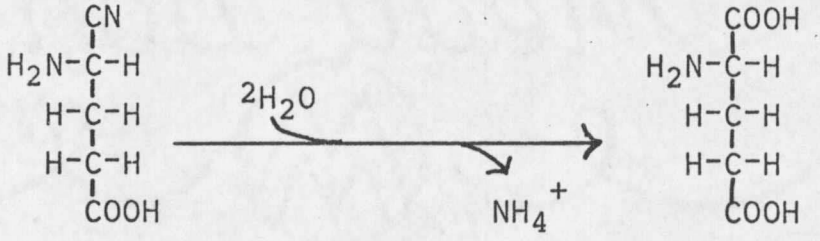
Scheme of Incorporation of
Cyanide to Aspartic Acid

In extending this line of research to fungus systems, Strobel[7] found that cyanide assimilation in fungus proceeds via a different pathway. A novel compound, 4-amino-4-cyanobutyric acid, was isolated as an intermediate from these studies. It was then found that the product apparently was derived from the cyanide, an aldehyde and ammonia



SUCCINIC SEMIALDEHYDE

4-AMINO-4-CYANOBUTYRIC



GLUTAMIC ACID

Fig. 3

Scheme of Incorporation of
Cyanide in a Fungus

Earlier, Thimann[8] had reported an enzyme with the ability to hydrolyze certain aliphatic nitriles to the corresponding carboxylic acids. He further proposed the following mechanism for this enzyme, nitrilase.[9]

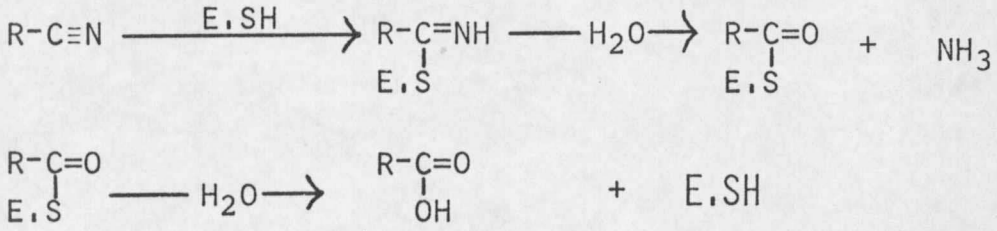


Fig. 4

Proposed Mechanism of Nitrilase

In searching for the presence of aldehydes in living systems, Quinn and Strobel[10] recently reported the existence of propanal in Rhizoctonia solani. Propanal was found to be a product of a decarboxylation reaction involving α-ketobutyric acid and a non-oxidative decarboxylase found in R. solani.

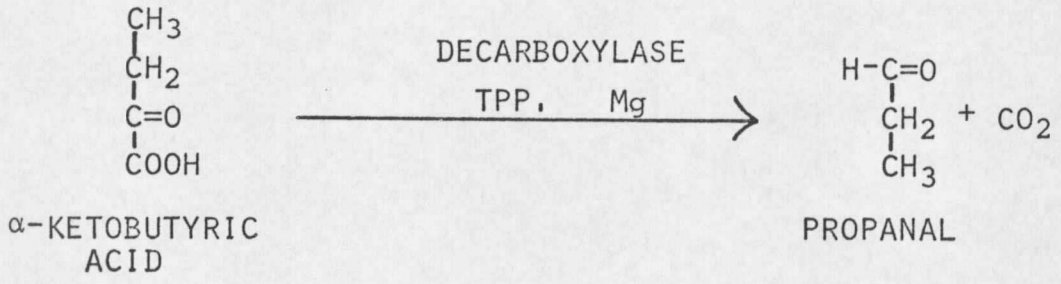


Fig. 5

Decarboxylase Reaction

Strobel's theory is that aldehyde, cyanide and ammonia condense to form an α-aminonitrile which can then hydrolyze into an amino acid. If this is correct, the corresponding α-aminonitrile that would be derived from propanal would be α-aminobutyronitrile. In order to carry out any work to justify the above supposition, one would of necessity have had to have access to samples of authentic α-aminobutyronitrile.

A literature search revealed that α-aminonitriles had a history rich in synthetic difficulties.[11] The difficulty is not, however, in the synthesis but in the preservation of the product. Due to the presence of the nitrile group adjacent to the amino group, these compounds polymerize very readily.

α-aminonitriles were postulated to be intermediates in Strecker's synthesis of α-amino acids. Therefore, one possible method of synthesis would be this process.[12]

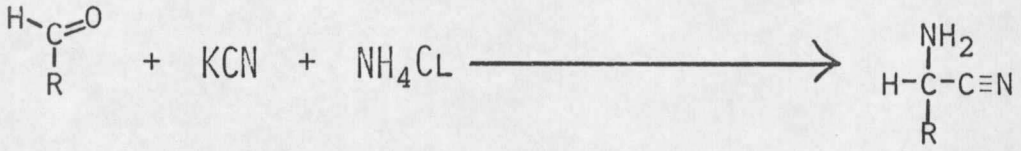


Fig. 6

Strecker's Synthesis of α-aminonitriles

Strobel[13] was able to synthesize α-aminopropionitrile in unspecified yield by a modification of the procedure of Loftfield.[14] An analogous system, α-aminocyano acid, was synthesized by Strobel[15] in 1% yield. In a recent paper by Ressler, et al.[16], a 43-63% yield was obtained for a series of α-aminocyano acids via an enzymatic pathway.

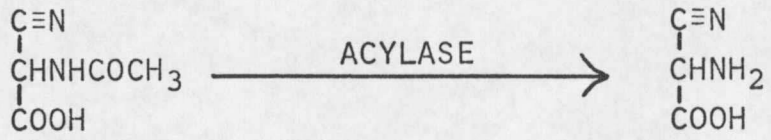


Fig. 7

Mechanism of Ressler's Synthesis

The objectives of this research are threefold. One involves finding a better means to synthesize any α-aminonitrile. Another is discovering if potassium cyanide is metabolized by the fungus R. solani. The last objective is finding the pathway of this cyanide metabolism and the means of incorporation.

DISCUSSION AND RESULTS

Synthesis of α-aminonitrile

The initial method of synthesis was Strecker's.[17] This synthesis involved addition of aqueous hydrogen cyanide onto the carbonyl functionality of aldehydes in the presence of ammonia.

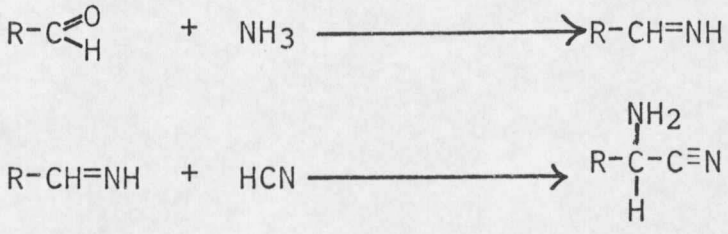


Fig. 8

Proposed Mechanism of Strecker's Synthesis of α-aminonitrile

Tiemann[18] modified this synthesis to an amination of Cyanohydrin.

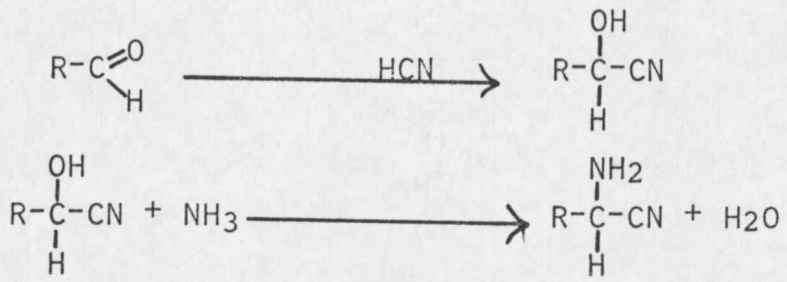


Fig. 9

Proposed Mechanism of Tiemann's Synthesis of α-aminonitrile

Finally, Steiger[19] reported the following procedure. Equal molar amounts of aldehyde and ammonium chloride were stirred at 4°C. To this mixture, potassium cyanide solution was added in a drop-wise manner. After the reaction was stirred at room temperature overnight, the mixture was extracted with several portions of methylene chloride.

The product was recovered after evaporation of the methylene chloride. The yield was very low. When the liquid from the reaction vessel was evaporated, ammonium cyanide was obtained. The possible mechanism for the reaction is as given previously.

Further literature search yielded another means of synthesis. In the procedure, cyanohydrin was synthesized

and removed from the reaction mixture. Ammonia was then added to the cyanohydrin.[20]

The procedure was modified by stirring in ether as a solvent for propanal. An equal molar amount of ammonium chloride was mixed with the aldehyde in the presence of ether. This was followed by a drop-wise addition of aqueous solution of potassium cyanide. The mixture stirred at room temperature and left to stand overnight.

It was then extracted with ether. After the ether was removed, the residue was dissolved in methanol and then saturated with dry ammonia. The reaction was left in this manner for four days. When the ammonia and methanol were removed, the crude product was collected in 80% yield.

It was found that polymerization was retarded if the product was not isolated from the methanol-ammonia mixture, but allowed to stand in it.

α-aminoacetonitrile was first synthesized by this method. Subsequently, α-aminopropionitrile and finally, the requisite α-aminobutyronitrile was synthesized.

Another means of preventing polymerization was converting the α-aminonitrile to its hydrochloric salt. After the α-aminonitrile was dissolved in anhydrous ether solution, gaseous hydrochloric acid was bubbled through the

solution. The salt crystallized out of the solution in yellowish tinted crystals. The infrared spectra of the crystal resembled that of the free α-aminonitrile with the shift in the amino group corresponding to the salts of amino compounds.

For the purpose of continuing this research, pure samples were not required. The crude product was used for the following experiment.

Fungus Selection and Culture

α-aminobutyric acid is not a natural amino acid. In other words, if by treating a biological system with some chemical, the end result is a production of α-aminobutyric acid, it can be concluded that the acid is a direct result of the chemical added.

Quinn and Strobel[21] had reported the presence of α-ketobutyric acid in the fungus R. solani. Furthermore, their group also reported the presence of a decarboxylase in the same system that was capable of converting this α-keto acid to an aldehyde with one less carbon fragment (Fig. 3).

Therefore, the choice of R. solani as the fungus in this work was made on the basis that if potassium

cyanide was incorporated and α-aminobutyric acid was the product of the reaction, then one could conclude that indeed the acid was derived on some way from the potassium cyanide added into the fungus. If one traces the possible production of α-aminobutyric acid, one would observe from our provious discussion that condensation between potassium cyanide and propanal would produce α-aminobutyronitrile. This nitrile could then be hydrolyzed to the amino acid by a nitrilase.

Identification of the intermediate and the product can be easily performed by co-chromatographing with authentic samples. This would be the first report of cyanide activity in R. solani.

The media used for this study[19] was Eckert's Modified Media, a glucose based media with Difco peptone and other inorganic ions added.

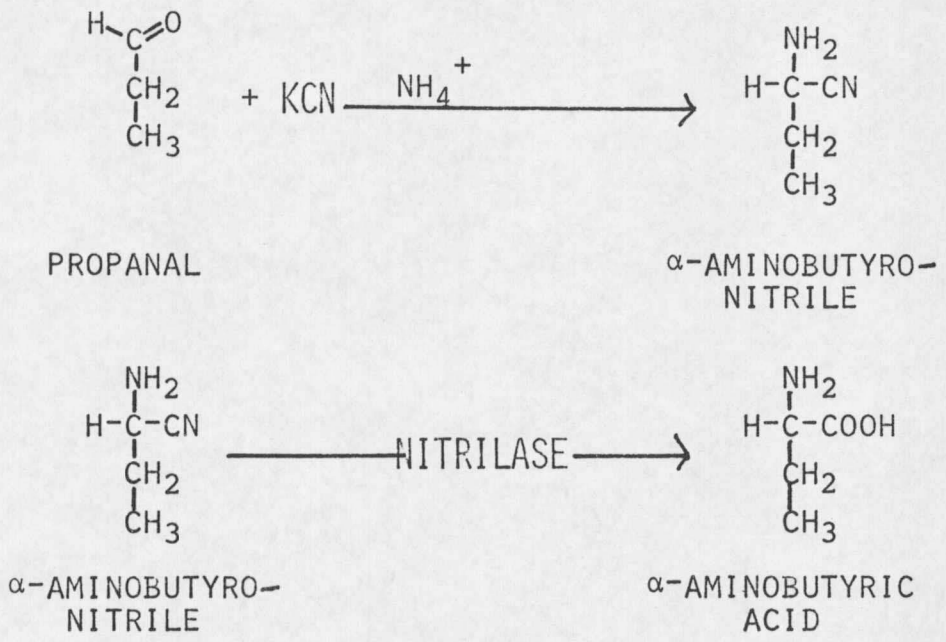


Fig. 10

Proposed Path of Enzymatic Conversion of Propanal to α-aminoacid

Incorporation of Potassium Cyanide

Since the general reaction of potassium cyanide, aldehyde and ammonium chloride proceeded at room temperature, one important fact had to be established before any experiment was conducted. If there was cyanide incorporation, it would be due to some biochemical reaction of the fungus and not just a simple chemical reaction

A mycilial mat of a ten day old growth of R. solani was killed with heat. Potassium cyanide was added to the mat in distilled water. After incubating and stirring for four days, the mat was homogenized and hot ethanol was added. The mixture was centrifuged with the pellet being discarded. After the supernat was evaporated to dryness, it was brought into solution with a small amount of water. This was co-chromatographed with an authentic sample of α-aminonitrile and α-aminobutyric acid on silica gel using a solvent system of propanol and ammonium hydroxide. A ninhydrin sensitive spot corresponding to the authentic samples was not observed. In similar experiments using live fungus, a faint visualization corresponding to authentic nitrile could be seen.

This demonstrates that the nitrile can be detected visually and that the living fungus is prerequisite to reaction. Whether the incorporation requires an enzyme is not established.

To show potassium cyanide incorporation, a fungus mycilial mat was exposed to labeled potassium cyanide (KC*N). After each interval of 15, 30, 45, and 60 minutes, one quarter of the mycilial mat was removed, homogenized, combined with hot ethanol and centrifuged. The supernatant

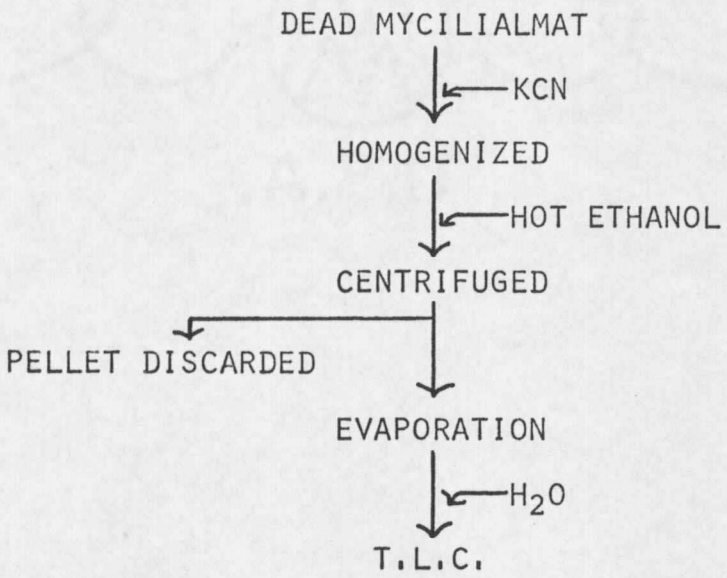


Fig. 11

Schematic Procedure for
Dead Fungus Experiment

was dried and brought into solution with one ml. of water. Aliquots of the various time runs were co-chromatographed with a sample of α-aminobutyronitrile. It was also co-chromatographed on two dimensional thin layer chromatography. The ninhydrin sensitive spots on the plates were scraped and counted. The radioactivity at these positions indicated the presence of α-aminobutyronitrile and α-aminobutyric acid.

Electrophoresis of the solution with an authentic sample as a standard was also performed. Ninhydrin sensitive spots corresponding to the nitrile was observed The result was not conclusive due to the inexperience of the researcher in this process. However, the trend indicated that electrophoresis would be a quick and easy way to perform analysis of the product and the intermediates.

The result from the thin layer chromatography plates indicated the probable presence of α-aminobutyronitrile. In order to confirm this observation, two identical volumes aliquot of the 15 minute run was chromatographed on separate plates using different solvents. Scraping of the ninhydrin sensitive region corresponding to the nitrile marker and subsequent counting gave the following data:

Table 1

Data From Two Identical Samples
Using Different Solvents on TLC

SOLVENT	CPM
N-PROPANOL/NH_4OH	136
$HOAc$/BUTANOL/H_2O	138
BACKGROUND	36

The same two solvents were used for two dimensional thin layer chromatography. Equal volume aliquots of the same time runs were used. After scraping off the region corresponding to the α-aminobutyronitrile, the scrapings were counted. The results were

Table 2

Data From Two Identical Samples Using Different Solvents on Two Dimensional TLC

SOLVENT	CPM
N-PROPANOL/NH_4OH	56
BUTANOL/HOAc/H_2O	58
BACKGROUND	36

Reaction of α-aminobutyronitrile

Labeled α-aminobutyronitrile was introduced into the fungus and was worked up in the usual manner after ten minute intervals. Chromatographing on thin layer plates and subsequent counting of the ninhydrin positive region corresponding to the authentic samples gave the following data.

Table 3

Data for Conversion of α-aminobutyronitrile to α-aminobutyric Acid

TIME (MIN)	0	10	20	30	120	BACKGROUND
CPM (NITRILE)	975	763	724	645	570	32
CPM (AMINO ACID)	155	153	147	162	147	34

α-aminobutyronitrile and α-aminobutyric acid were plotted versus time (See Table 5). The result indicated that α-aminobutyronitrile was being used up very rapidly. This was concluded from the fact that the line showed a steady decrease. If the nitrile were not being consumed rapidly, one would expect a horizontal line or one that has just a very slight slope. However, the result was not so, and one would also predict that such should not be the case. α-aminobutyronitrile should be hydrolyzed to the amino acid.

The problematic point was that of the behaviour of the α-aminobutyric acid. Since the nitrile was being used up, the concentration of the amino acid should increase, but that was not observed. Instead, the plot showed that the line maintains almost horizontal.

In order to elucidate the catabolism of the α-aminobutyric acid, R. solani was incubated with labeled α-aminobutyric acid with labeling on acid carbon in a Warburg vessel with a central well filled with sodium hydroxide. This equipment was designed to trap any gaseous carbon dioxide that might be generated.

The reaction was allowed to run for three hours and at the end of each hour, the aliquots of the fungus mixture was taken out and worked up for scintillation counting. The sodium hydroxide solution was also counted for labeled carbon dioxide that might have been generated. The sodium hyroxide data were as follows:

Table 4

Data From Carbon Dioxide Reaction

TIME	1	2	3	BACKGROUND
CPM	68	53	138	35

The data was not conclusive. However, it did indicate that carbon dioxide was given off as a by-product of the fungal cyanide reaction. The error may have been due to inaccuracies in measuring out the fungal mat such that the dry weights differ between the three runs. At any rate, the

three hour run showed an increase in radioactivity. This would not be possible unless labeled carbon dioxide was present.

Therefore, one would have to say that there was great possibility that the α-aminobutyric acid was somehow gotten rid of by the fungus. This would be expected since α-aminobutyric acid was not native to the fungus R. solani.

Furthermore, the enzymes of the fungus were extracted. α-aminobutyronitrile was then added to the enzyme preparation. An aliquot was removed at time intervals of 15, 30, 45, 60 minutes. This was analyzed on an amino acid analyzer showed that there was an increase in the concentration of ammonia with respect to reaction time.

This meant that the nitrile was being hydrolyzed to the amino acid. Ammonia should be the by-product of the hydrolysis of the nitrile to the acid and an increase in the concentration of ammonia could only indicate that such a reaction is occurring.

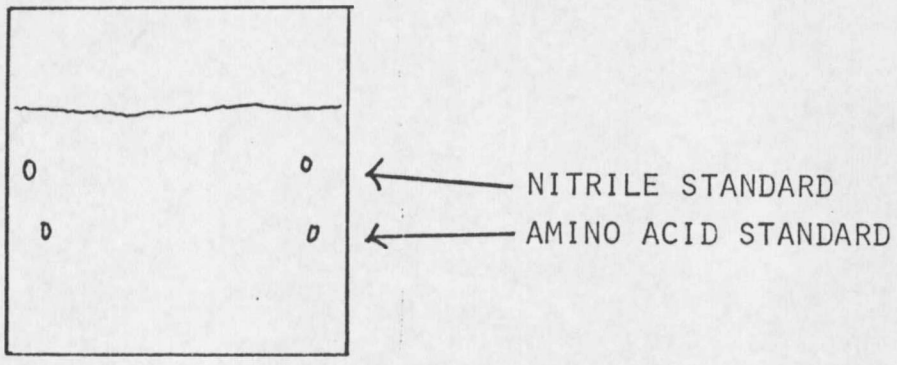


Plate of Adsorbosil-5 with n propanol/ammonium hydroxide solvent. Co-chromatographed with nitrile and amino acid standard.

Fig. 12

Typical TLC Plate

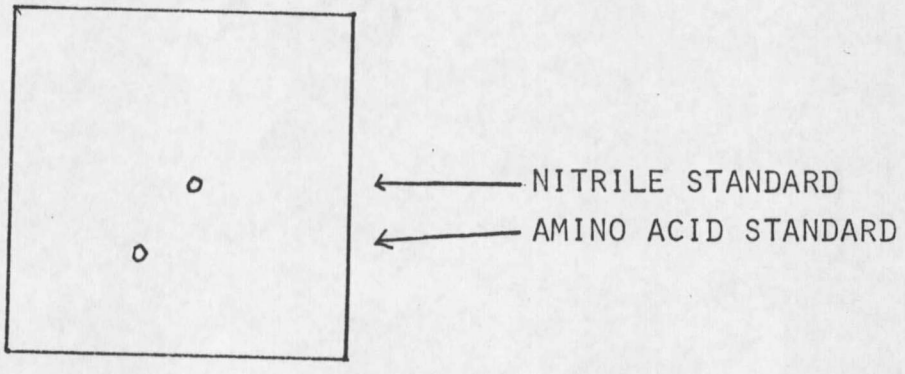


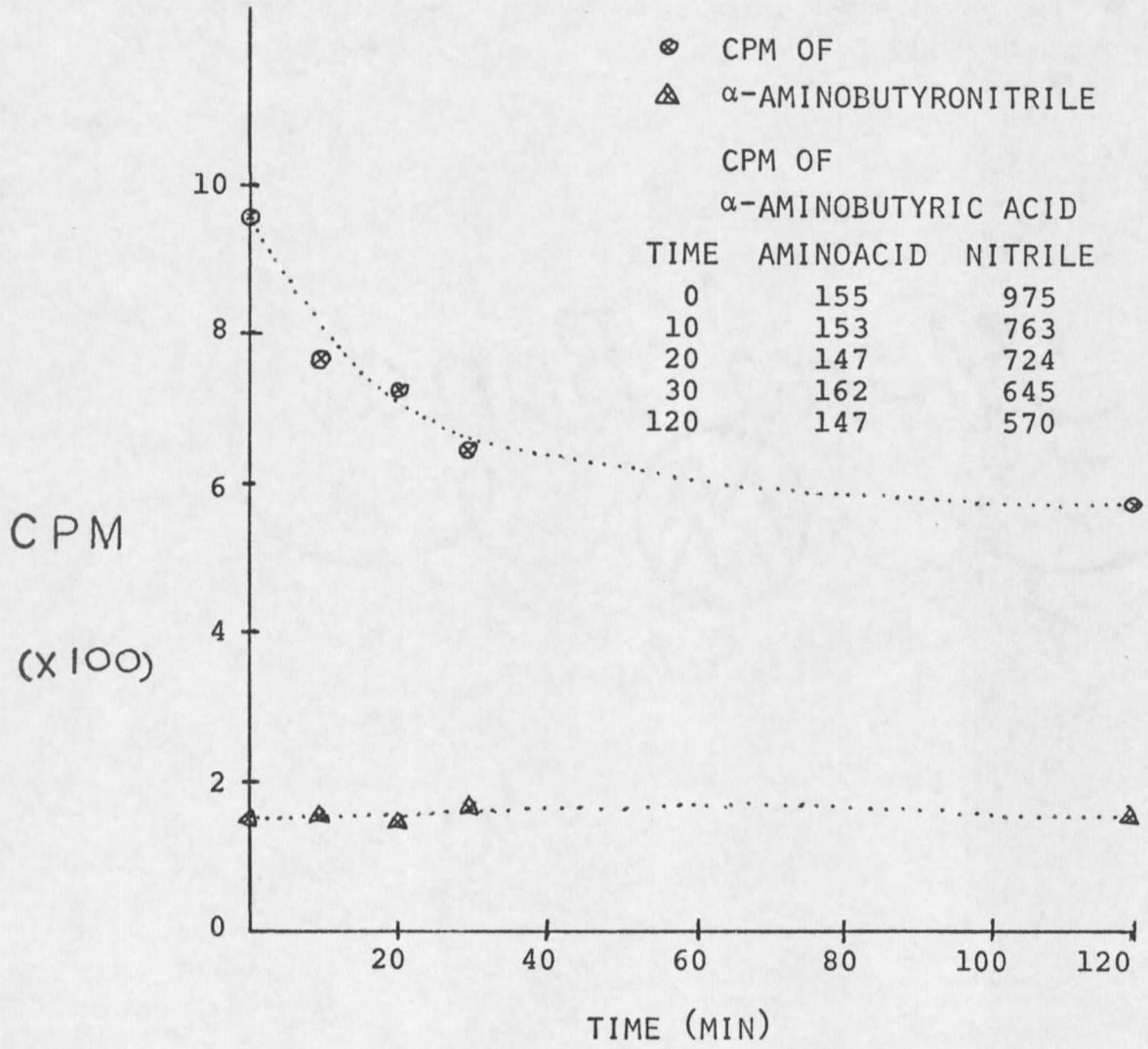
Plate of Adsorbosil-5 with n-propanol/ammonium hydroxide solvent.

Fig. 13

Typical Two Dimensional TLC Plate

Table 5

Graph of α-aminobutryonitrile and
α-aminobutyric Acid vs Time



CONCLUSION

In view of the previous discussions, one could say that α-aminobutyronitrile was definitely present in the fungus as an intermediate of cyanide assimilation. This conclusion was reached on the basis that in all the radioisotopic studies, there was always a radioactive region corresponding to the α-aminobutyronitrile. However, one must also bear in mind that this pathway was not the sole one of cyanide incorporation. Paper electrophoresis of fungal reaction fed with labeled potassium cyanide, followed by scanning by a Radio Chromatogram Scanner, showed that there are other regions of high radioactivity.

The above observation could be easily reconciled with other known facts. α-aminobutyronitrile could only be found in the fungal reaction mixture if propanal was present. However, the presence of propanal was dependent on the decarboxylation of α-ketobutyric acid. So, one would conclude that the concentration of propanal would be very minute. Therefore, the reaction product of the cyanide incorporation with the aldehyde would, of necessity, be very low.

This work supports the idea regarding the presence of propanal in R. solani in addition to the existence of an enzymatic pathway that condenses cyanide, aldehyde and ammonia.

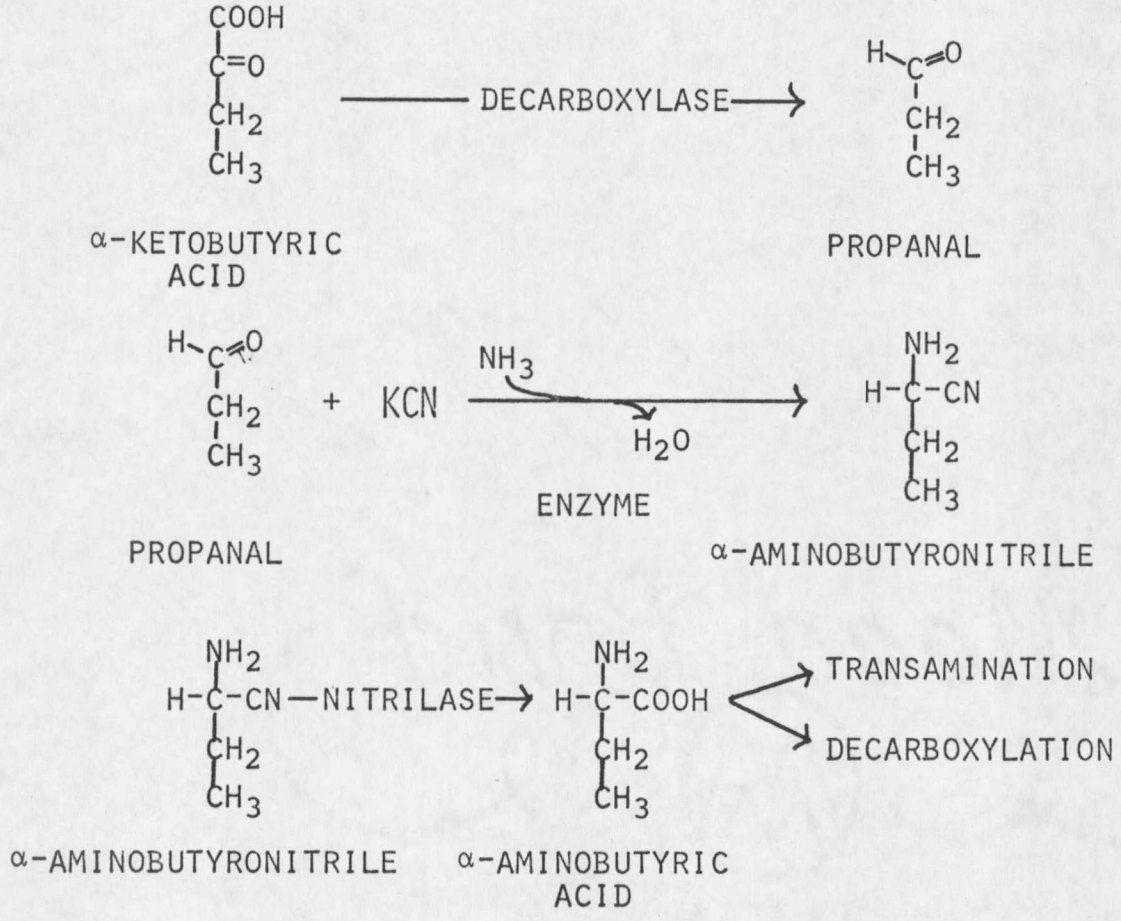


Fig. 14

Proposed KCN Pathway Yielding
α-aminobutyric Acid

One of the objectives of the research was to find a method of easy synthesis of α-aminonitriles. This was accomplished. However, stabilization of the nitriles after synthesis presented a problem that would require further work. Our means of stabilization at this moment was to convert the free aminonitrile to its corresponding hydrochloride salt. This process usually decrease the yield drastically. Moreover, a suitable solvent had to be found to accomplish this conversion. Diethyl ether was used in our experiments as the solvent, but the results were not satisfactory.

Aside from the obvious need to get quantitative data regarding cyanide metabolism, future work in the organic and biochemistry is possible.

The mechanism of the synthesis of α-aminonitrile is still unknown and warrants further research. Furthermore, if the mechanism for the polymerization can be found, methods can be used to prevent conditions for polymerization.

The enzymes extract could be purified and a study of the mechanism of the enzyme reaction will be beneficial. At present, it is not known whether a single enzyme is

responsible for the nitrile condensation of whether it is a series of reaction involving several enzymes. The specificity of the enzymes can be studied too.

METHODOLOGY

Enzymes Extract

The enzymes extract was prepared in the following manner. The cultural media in which the fungus was growing was discarded. The mycilial mat was then washed with distilled water. When the mat was thoroughly cleaned, it was transferred to a blender and homogenized. The homogenate was clarified with centrifugation. The pellet was discarded after centrifugation. To the supernatant one then added an amount of acetone that was twice the volume of the supernatant.

The acetone mixture was then centrifuged. Since the enzymes should be associated with the pellet, the supernatant was discarded. The pellet was then brought into solution with 0.01 M phosphate buffer. Undissolved materials were clarified with slow speed centrifugation. Since enzymes should be soluble in water solutions, the supernatant was saved and the pellet discarded.

At this time the solution contained materials that were not soluble in acetone but soluble in water. This would include amino acids, proteins, and nucleic acids. Nucleic acids were removed from the solution with the

addition of a 1% protamine sulfate solution. A slow speed centrifugation removed the precipitated nucleic acids.

All the above processes were performed in the cold at 4°C. to prevent the enzymes from denaturing. The enzymes were either used immediately or frozen for future use.

In order to further purify the enzymes extract, some of them were placed in a dialyzing membrane and dialyzed against a 0.01 M phosphate buffer in the cold for overnight before use.

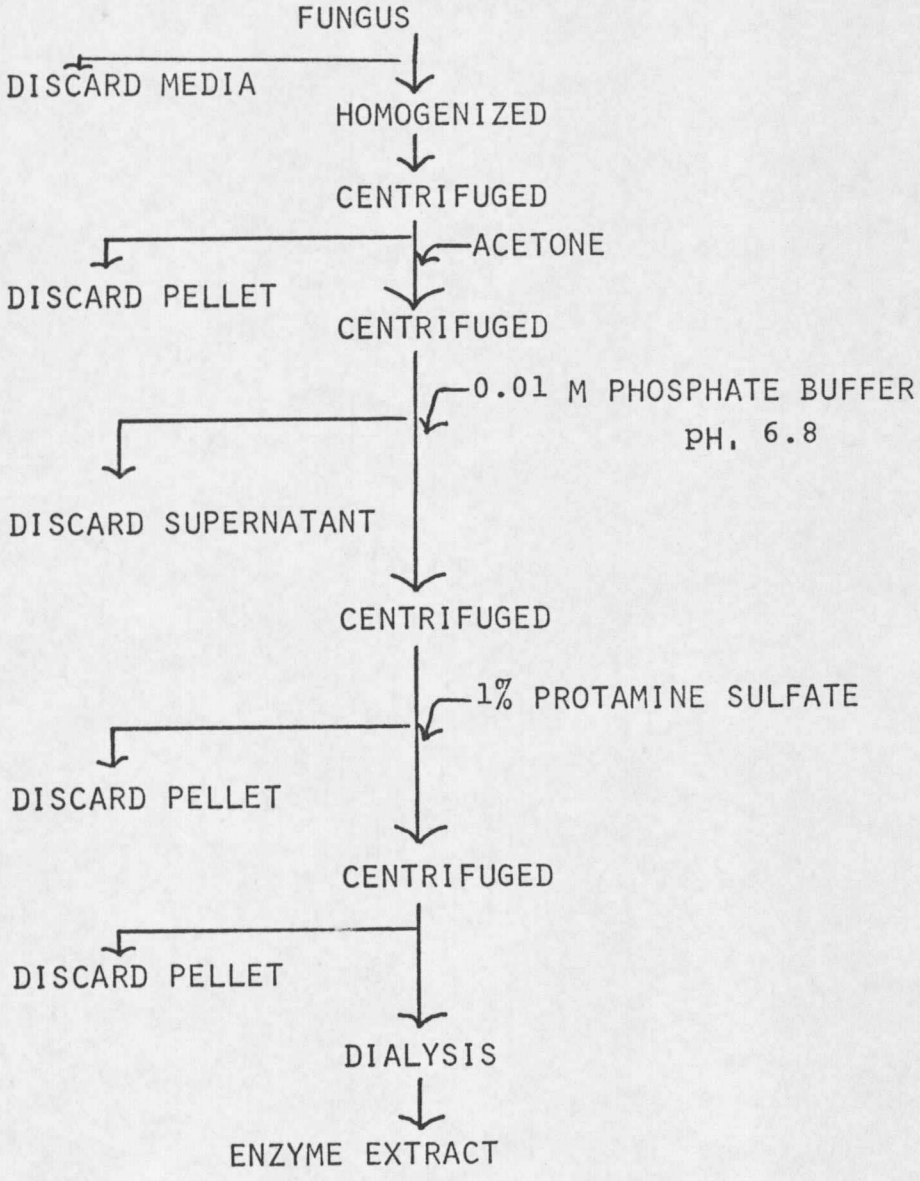


Fig. 15

Scheme for Extraction of Enzymes

Amino Acid Analyzer

Two amino acid analyzers were used. The Beckmann amino acid analyzer was a completely automated system with an attachment for print out. On the other hand, the Technicon amino acid analyzer was less well equipped.

Amino acid analyzers worked on the principle of ion Exchange chromatography. Since charged particles (compounds) would adhere to elements of opposite charges, a column that was packed with a positively charged material will retain negatively charged matters from a solution that was made up of materials of varying charges. The opposite situation presides if the column was packed with a negatively charged material. The retained materials would be released only if a stronger charged material replaces it.

In ion exchange chromatography, a column was packed with a material that was bonded into a matrix with a charged radical. Materials that were to be chromatographed were introduced into the column. After establishing equilibrium with an inert media, usually water, the retained material was eluted with materials of stronger ionic strength. If the retained materials were of a nature in which the charges varied by some degrees, the eluting solution could be of varied strength.

In the application of ion exchange chromatography to amino acid analyzer, the retained material would be amino acids. The elutant had to be of a pH that just exceed the acidity or basicity of the amino acids that was desired to be analyzed. By manipulating the pH and the ionic concentration, one could achieve separation of the amino acids.

The effluent from the column was then mixed with ninhydrin. This ninhydrin was delivered from a calibrated hose that quantitatively measures the rate of ninhydrin flow. By the usual chemical reaction of ninhydrin and amino compounds, the characteristic color developed. Colorimeters in the analyzer analyzes for the purple and yellow bands and plots out the % transmittance by means of a recorder. The area under the curve would be a quantitative representation of the amino acid present.

The Beckmann amino acid analyzer had a construction which completely isolated the instrument from outside environment. Samples were injected into the sample container and the area was flushed with nitrogen. By opening appropriate valves, the samples were placed onto different columns. The column was eluted with buffer and the effluent was allowed to react with ninhydrin. The result was

plotted out on a recorder. The area under the curve was automatically integrated and printed out on a tape simultaneously with the time by which the peak height was reached.

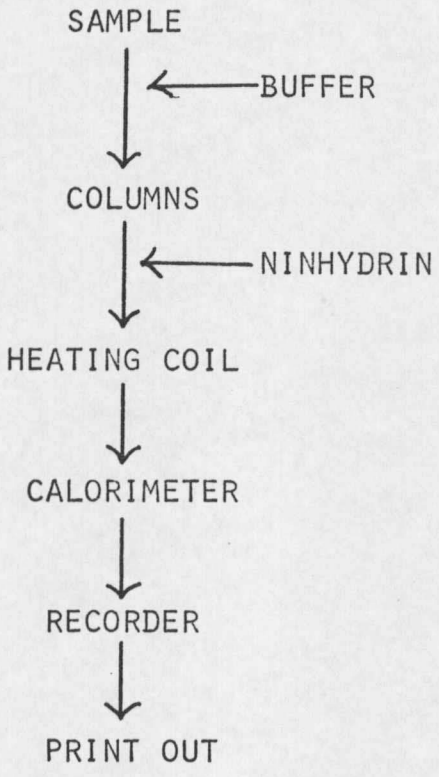


Fig. 16

Schematic of Beckman Amino Acid Analyzer

The Technicon amino acid analyzer was similar. However, this instrument was only semi-automated. The sample was introduced into the system via direct placement onto the column. The buffer solution was then pumped onto the column where ion exchange occurred. The effluent was mixed with ninhydrin and the mixture was then analyzed on two colorimeter. This result was plotted on a recorder. No integration capacity was present on this instrument and quantitization was by means of the half height method.

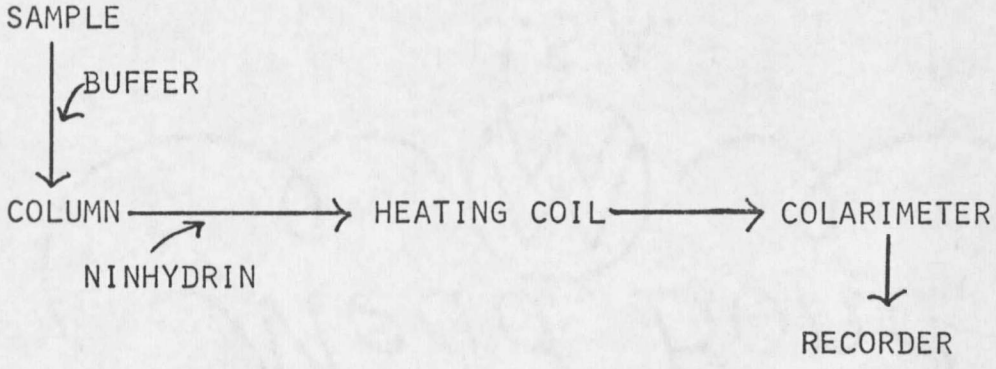


Fig. 17

Schematic of Technicon Autoanalyzer

Standards of samples to be analyzed had to be analyzed previous to any actual runs.

Radioisotopic Technique

Radioisotopic counting was achieved by means of liquid scintillation counting. Liquid scintillation counting is a method of detecting radioactivity by means of a solution of fluors and a photomultiplier tube. The fluor dissolved in a solvent or solvent mixture and at the same time acts as a solvent for the sample.

The scintillation solution converts to light the energy of the primary particle emitted by the radioactive sample. A secondary fluor is used to shift the wave length of the emitted light to the range of about 4100 Å, which is the most efficient wavelength for detection by the photomultiplier. The photo tube responds to this light energy by producing a charge pulse which can be amplified and counted by a scaling circuit.

In our work, since the sample was in a water solution, Bray's solution for scintillation counting was used. The solution has the ability to take up to 20% of its volume in water.

For scintillation counting of paper chromatograms, a radio Chromatogram Scanner was used. The strip of paper was passed through the Geiger-Muller counter of the system and the result plotted on a recorder.

EXPERIMENTAL

Nuclear magnetic resonance spectra were run on a Varian A-60 Nuclear Magnetic Resonance Spectrophotometer using deuterated water as a solvent and TMS as an internal standard.

All boiling points are uncorrected.

Ammonia was analyzed on a Beckman Model 120 C Amino Acid Analyzer. It was also analyzed on a Technicon Auto analyzer.

Radioisotopic counting was counted on a Beckman LS 100 Liquid Scintillation System and also on a Packard Model 7201 Radio Chromatogram Scanner System.

Infrared spectra were run on a Beckman IR 5 A Infrared Spectrophotometer.

Solvents for Thin Layer Chromatography

Two solvent systems were used. One contained n-propanol and ammonium hydroxide in the ratio of 66:33. The other solvent system was made up of 1-butanol, acetic acid and water in the ratio of 3:1:1.

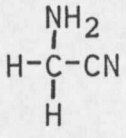
Buffer System for Electrophoresis

Electrophoresis was performed with a 2% formic acid buffer.

Synthesis of α-aminonitrile by Teimann's method[18]

Equal molar amount of potassium cyanide, ammonium chloride and ammonium hydroxide in water solutions were stirred and cooled. After the components were equilibrated the aldehyde was added. The reaction was stirred and refluxed for six hours, and the product collected by evaporation.

Preparation of α-aminoacetonitrile



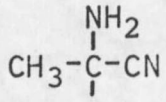
6.5 gms (0.1 mole) of potassium cyanide was dissolved in 10 ml of water. 5.9 gms (0.11 mole) of ammonium chloride in 14 ml of water was added and finally 6.7 ml of 28% ammonia ammonium hydroxide (0.1 mole) was stirred in and the whole reaction mixture cooled in an ice bath.

10 ml of formaldehyde in 16 ml of methanol was added and the reaction was stirred at 100°C. for six hours.

The product was collected by distillation at aspirator pressure. bp_{15} 34°C. Yield was 2.5%.

The α-aminoacetonitrile was converted to hydrochloride salt with gaseous hydrochloric acid. The crystals were compared with authentic samples of α-aminoacetonitrile hydrochloride. The infrared spectra of the two matched.

Preparation of α-aminopropionitrile



25 gm (0.5 mole) of sodium cyanide was dissolved in 50 ml of water. 29.5 gm (0.5 mole) of ammonium chloride in 70 ml of water was added followed by 33.5 ml of 38% ammonium hydroxide.

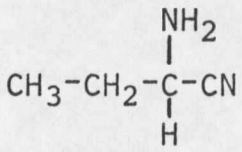
After stirring in an ice water bath, 72.5 ml of acetaldehyde was added. The reaction was allowed to react for six hours at 100°C. Distillation did not affect the desired product but instead left an intractable mass.

Synthesis of α-aminonitrile with Lowy's method[20]

Equal molar amount of ammonium chloride, aldehyde and potassium nitrile was stirred in a water solution for two hours at room temperature. The mixture was then allowed

to stand overnight. It was then extracted with 30 ml of ether six times. The ether was removed by distillation in vacuo. The residue was dissolved in 80 ml of methanol and saturated with dry ammonia in the cold. It was then allowed to stand for four days at room temperature. The product was collected after the removal of ammonia and methanol by distillation in vacuo.

Preparation of α-aminobutyronitrile



12.7 gms (0.24 mole) of ammonium chloride was dissolved in 32 ml of water at 0-5°C. and stirred while 12.2 gms (0.21 mole) of propanal in 50 ml of ether was added. This was followed by a solution of 14.3 gm (0.22 mole) of potassium cyanide in 22 ml of water added drop-wise.

This was allowed to stir for two hours at room temperature and then left to stand overnight. The reaction mixture was extracted six times with 30 ml portions of ether. The ether was removed and the residue was dissolved in 80 ml of methanol. Dry ammonia was added to the

methanol solution till saturation as indicated by no change in temperature. This was allowed to stand for 4 days at room temperature.

Excess ammonia was removed with dry nitrogen and the solvent evaporated to leave to leave α-aminobutyronitrile. The crude yield was 78%. Any attempt of purification met with polymerization. The crude produce ranged in color from brown to light yellow. Confirmation of structure was obtained by hydrolyzing the crude product with 6N hydrochloric acid to its corresponding amino acid. The amino acid compared identical with authentic α-aminobutyric acid.

Enzymatic Reactions

Reaction with Enzymes Extract

0.5 ml of enzymes extract was added to 1 ml of 0.05 M phosphate buffer, pH 7. To this was added 1 ml of α-aminobutyronitrile. The reaction was allowed to proceed for two hours at 37°C. with aliquots taken out at ten minutes intervals.

Reaction with Whole Fungus

The fungal mycilial mat was washed with distilled water and then was cut into four equal portions. 100 ml of water was introduced into the container and followed by potassium cyanide in a water solution. The reaction was allowed to proceed for one hour with one portion taken out each 15 minutes.

The quarter was homogenized and equal volume of hot ethanol was added to the homogenate. The mixture was centrifuged and the pellet discarded. The supernatant was evaporated to dryness, and one ml of water was added to the residue. If precipitate was still present, it was clarified and evaporated to dryness again. When the solution was finally free of precipitates, a 0.1 ml aliquot was taken and chromatographed on thin layer plates.

Reaction with Homogenized Fungus

As an alternative to the above procedure which did not insure equal portions of fungus in either quantity or age, the following procedure was also tried.

Fungal mycilial mat was initially homogenized. Equal aliquots of the homogenate was introduced into four

flasks and at fifteen minute intervals, one flask was removed and worked up as in previous section.

Medium for R. solani

Eckert's modified medium was used for culturing the fungus.

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