# The origin of cyanide in a psychrophilic basidiomycete

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Abstract:
It has long been known that cyanide is evolved in basidiomycetes, however, the mechanism by which it is formed has not been established. Possible precursors and intermediates for cyanide were investigated by administering eight different carbon-14 labeled amino acids to cultures of a psychrophilic basidiomycete (strain W-2, J. B. Lebeau, Lethbridge, Alberta, Canada). Comparison of the dilution factors indicated that isoleucine and valine were the most effective sources of HC14N. Inasmuch as isoleucine and valine served as sources of HC14N, the cyanogenic glucosides lotaustralin and linamarin, were investigated as possible intermediates in cyanide formation in this organism. Both of these cyanogenic glucosides were isolated from the fungus and identified by paper chromatography and infrared spectrophotometry. Some evidence that the glucosides may serve as a source of cyanide was established by incubating mycelial mats with carbon-14 labeled valine and isoleucine and determining the specific activity of the glucosides formed at various times during the incubation period (3 to 48 hr.). Carbon-14 labeled lotaustralin was prepared by the administration of isoleucine-U-C14 to flax seedlings. When labeled lotaustraline was incubated with a crude enzyme preparation of the fungus, there was a decrease in the concentration of lotaustralin with a concomitant increase in HC14N as measured by liquid-scintillation counting. Heat-inactivated controls showed no activity. Inasmuch as the organism contained the necessary enzymes to release cyanide from the cyanogenic glucosides, it was desirable to purify and characterize these enzymes. A cell free extract was prepared from mycelial mats of the organism and subjected to precipitations by acetone and protamine sulfate, ammonium sulfate fractionation, dialysis and finally separation on a DEAE cellulose column. The elution pattern from the DEAE cellulose column showed two separate peaks having β-glucosidase activity against the chromogenic substrate p-nitrophenyl-β-D-glucoside. The peaks represented two different enzymes since their pH and temperature optima, energies or activation, Km's, and substrate specificities were different. The enzyme from one peak hydrolyzed linamarin to -hydroxyisobutyronitrile and glucose, the other hydrolyzed lotaustralin to methyl-ethyl-ketone cyanohydrin and glucose. That the fungus produced an oxynitrilase capable of oxidizing acetone cyanohydrin and methyl-ethyl-ketone cyanohydrin to HCN and the corresponding ketone, was demonstrated by incubating the cyanohydrins with a crude enzyme preparation and then analyzing the products in a gas chromatograph. The oxynitrilase from the fungus oxidized acetone cyanohydrin, methyl-ethyl-ketone cyanohydrin and lactonitrile but had no activity on aromatic cyanohydrins In summary, the release of cyanide by the psychrophilic basidiomycete first involves synthesis of cyanogenic glucosides from amino acid precursors followed by a two step enzymic breakdown of the cyanogenic glucosides by β β-glucosidase and a crude preparation showing oxynitrilase activity.

1078

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TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

## Abstract

It has long been known that cyanide is evolved in basidiomycetes, however, the mechanism by which it is formed has not been established. Possible precursors and intermediates for cyanide were investigated by administering eight different carbon-14 labeled amino acids to cultures of a psychrophilic basidiomycete (strain W-2, J. B. Lebeau, Lethbridge, Alberta, Canada). Comparison of the dilution factors indicated that isoleucine and valine were the most effective sources of $HC^{14}N$. Inasmuch as isoleucine and valine served as sources of $HC^{14}N$, the cyanogenic glucosides lotaustralin and linamarin, were investigated as possible intermediates in cyanide formation in this organism. Both of these cyanogenic glucosides were isolated from the fungus and identified by paper chromatography and infrared spectrophotometry. Some evidence that the glucosides may serve as a source of cyanide was established by incubating mycelial mats with carbon-14 labeled valine and isoleucine and determining the specific activity of the glucosides formed at various times during the incubation period (3 to 48 hr.). Carbon-14 labeled lotaustralin was prepared by the administration of isoleucine-U-$C^{14}$ to flax seedlings. When labeled lotaustraline was incubated with a crude enzyme preparation of the fungus, there was a decrease in the concentration of lotaustralin with a concomitant increase in $HC^{14}N$ as measured by liquid-scintillation counting. Heat-inactivated controls showed no activity. Inasmuch as the organism contained the necessary enzymes to release cyanide from the cyanogenic glucosides, it was desirable to purify and characterize these enzymes. A cell free extract was prepared from mycelial mats of the organism and subjected to precipitations by acetone and protamine sulfate, ammonium sulfate fractionation, dialysis and finally separation on a DEAE cellulose column. The elution pattern from the DEAE cellulose column showed two separate peaks having $\beta$-glucosidase activity against the chromogenic substrate p-nitrophenyl-$\beta$-D-glucoside. The peaks represented two different enzymes since their pH and temperature optima, energies or activation, Km's, and substrate specificities were different. The enzyme from one peak hydrolyzed linamarin to $\alpha$-hydroxyisobutyronitrile and glucose, the other hydrolyzed lotaustralin to methyl-ethyl-ketone cyanohydrin and glucose. That the fungus produced an oxynitrilase capable of oxidizing acetone cyanohydrin and methyl-ethyl-ketone cyanohydrin to HCN and the corresponding ketone, was demonstrated by incubating the cyanohydrins with a crude enzyme preparation and then analyzing the products in a gas chromatograph. The oxynitrilase from the fungus oxidized acetone cyanohydrin, methyl-ethyl-ketone cyanohydrin and lactonitrile but had no activity on aromatic cyanohydrins. In summary, the release of cyanide by the psychrophilic basidiomycete first involves synthesis of cyanogenic glucosides from amino acid precursors followed by a two step enzymic breakdown of the cyanogenic glucosides by $\beta$-glucosidase and a crude preparation showing oxynitrilase activity.

# INTRODUCTION

The formation of cyanide by an organism was first demonstrated in the basidiomycete Marasmius oreades by Losecke (1871). Greshoff (1909) reported a similar phenomenon in Clitocybe sp. and Collybia sp. Bach (1956) lists 31 cyanogenic species of fungi belonging mainly to the genera Clitocybe, Marasmius, and Tricholoma. Locquin (1944) obtained evidence of cyanogenesis in some 300 species of basidiomycetes and in a number of Ascomycetes. He believed that hydrogen cyanide was a normal product of metabolism in the higher fungi. Cyanogenesis occurs elsewhere in the plant kingdom as well. Cyanide evolution was detected by Butler (1965) in Amygdalis nona, Sorghum vulgare, Prunus spp., Linum spp., Lotus spp., Manihot utilissima and by Coletelo (1961) in Medicago. Michaels (1965) recently described cyanide formation in the bacterium Chromobacterium violaceum. Eisner et al. (1963) described cyanide production by millipeds as a defensive mechanism to ward off predators. Thus, the spectrum of living systems releasing cyanide includes plants, bacteria, fungi and animals.

Only in the plants has the evolution of cyanide been widely studied and a mechanism of cyanide release fully established. It is well known that different cyanogenic glycosides exist in various cyanogenic plants. Several investigators cited by Butler and Conn (1964) have shown that the amino acids phenylalanine, tyrosine, valine and isoleucine were effectively converted to the aglycones of amygdaline, dhurrin, linamarin and lotaustralin, respectively. Butler and Conn (1964) have

shown that the nitrogen atom of L-valine was essentially retained when the amino acid was converted to linamarin by flax seedlings. Uribe and Conn (1966) showed a similar case in the conversion of L-tyrosine to dhurrin in Sorghum vulgare. Efforts to detect metabolic intermediates in the biosynthesis of cyanogenic glucosides from amino acid precursors have been unsuccessful, however, Tapper et al. (1967) reported that the administration of $\alpha$-keto-isovaleric acid-U-$C^{14}$ oxime or isobutyraldoxime U-$C^{14}$ results in the incorporation of label into linamarin. This would suggest that the amino acid is decarboxylated and the amino nitrogen first dehydrogenated and then hydroxylated and the amino nitrogen first dehydrogenated and then hydroxylated to form the oxime intermediate. The hydroxyl group would then be shifted to the $\beta$ carbon and subsequently $\beta$ linked to glucose by a glucose UDP-glucose reaction. Tschiersch (1966) agrees that there is first a decarboxylation, however he has provided evidence that the $\beta$ carbon is oxidized with retention of the amino group to form an amide. Finally, the $\beta$ carbon is hydroxylated and joined to glucose as discussed previously. Much work remains to be done in this area to elaborate the complete pathway.

The breakdown of cyanogenic glucosides with concomitant cyanide evolution involves 2 enzymic processes, first hydrolysis by a $\beta$-glucosidase and then oxidation by an oxynitrilase, (Robinson, 1964). Butler et al. (1965) provided evidence that although many forms of life (rumen protozoa, plants and many bacteria and fungi) produce

non-specific β-glucosidases which will hydrolyze several glycosides (linamarin, lotaustralin, salicin, amygdalin and cellobiose), to glucose and the various aglycones, other β-glucosideases exist which are specific for only 1 or 2 glycosides. Butler (1965) described a linamarase which is highly specific for linamarin and lotaustralin but has low activity on other glucosides. Seeley et al. (1966), working on a hydroxynitrile lyase (oxynitrilase) of sorghum seedlings, showed that the enzyme preferentially catalyzed the oxidation p-hydroxymandelonitrile to p-hydroxybenzaldehyde and HCN. An almond enzyme, however, exhibited its maximum rate on mandelonitrile by oxidizing it to benzaldehyde and HCN. It appears, therefore, that each of the 2 enzymes involved in the hydrolysis of cyanogenic glycosides demonstrates very definite substrate specificities. In addition there seems to be a direct correlation between the existence of a cyanogenic glucoside and the presence of specific enzymes in the cyanogenic plant.

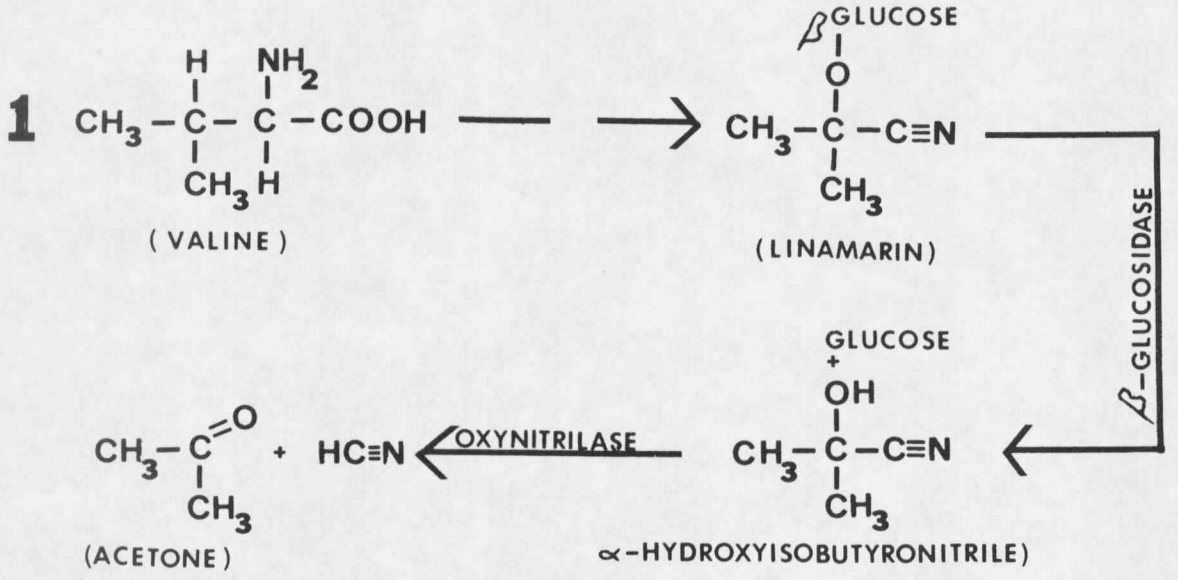
It was reported by Michaels et al. (1965) that growing cultures of Chromobacterium violaceum produced cyanide in a variety of culture media, but that the greatest cyanide yield occurred when a chemically defined medium was supplemented with glycine and methionine. Michaels and Corpe (1965) showed in addition that cells incubated in a chemically defined medium containing methionine and labeled glycine-2$C^{14}$ produced $C^{14}$ labeled cyanide. No mechanism or intermediates have yet been shown.

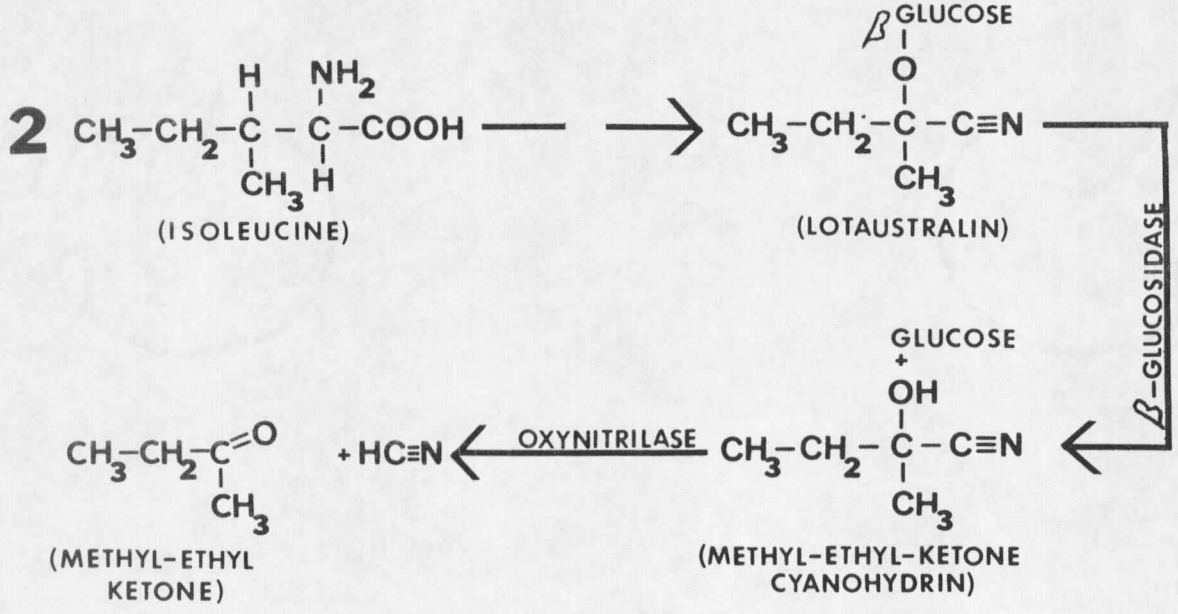
The work involving cyanide formation in the fungi, however, has largely been descriptive and has been in conjunction with a host parasite relationship such as in fairy ring of grasslands caused by Marasmius oreades, (Lebeau, 1961) or in snow mold of alfalfa and grasses caused by an unidentified psychrophilic basidiomycete, (Lebeau, 1953). In the latter case Lebeau and Dickson (1953) and (1955) reported that HCN was released by cultures growing on laboratory media and obtained evidence that this substance was produced during infection of alfalfa plants, accumulating in crown tissues in concentrations highly toxic to the host plant. In addition, Lebeau (1966) showed that every diseased plant from the field contained HCN in concentrations proportional to the damage in the host and that a close correspondence existed between the amount of cyanide produced and the degree of invasion of the host by the pathogenic mycelium. Lebeau et al. (1959) concluded that HCN production is the major factor in the etiology of diseases caused by this fungus. Ward et al. (1961) demonstrated the existence of 3 different strains of an unidentified low temperature basidiomycete on the basis of HCN formation and HCN resistance. Type A produced large amounts of HCN only in host plants and was greatly inhibited by HCN. Type B produced HCN poorly in infected alfalfa plants, but released large amounts in culture and was cyanide tolerant. Type C isolates did not liberate HCN in vivo or in vitro and were strongly inhibited by HCN.

In trying to determine the metabolic origin of HCN in the Type B strain, Ward and Thorn (1966) showed that several amino acids had a stimulatory effect on both growth and cyanide production. The greatest stimulator in each case was glycine, which was also a precursor for a cyanogenic compound that they have not yet characterized.

Although previous investigators have shown that some fungi produce quantities of cyanide, no metabolic pathways and the corresponding enzymes involved have been elucidated in cyanide formation. This report presents evidence for at least one general mechanism of cyanide production in the Type B psychrophilic basidiomycete, Figure 1.

Figure 1. Two proposed mechanisms of cyanide production in the psychrophilic basidiomycete.


1
(VALINE)
β GLUCOSE
(LINAMARIN)
β-GLUCOSIDASE
GLUCOSE
(α-HYDROXYISOBUTYRONITRILE)
OXYNITRILASE
HC≡N
(ACETONE)


2
(ISOLEUCINE)
β GLUCOSE
(LOTAUSTRALIN)
β-GLUCOSIDASE
GLUCOSE
(METHYL-ETHYL-KETONE CYANOHYDRIN)
OXYNITRILASE
+HC≡N
(METHYL-ETHYL KETONE)

## MATERIALS AND METHODS

Culturing. The organism used in the research (designated W-2) was a cyanide producing strain of Type B of an unidentified psychrophilic basidiomycete and was supplied by J. B. Lebeau, Research Station, Canada Department of Agriculture, Lethbridge, Alberta. The fungus was grown either on a synthetic medium (Ward and Lebeau, 1962) or a complex medium (Ward, 1964) for 3 to 4 weeks at 15°C. Mycelial mats grown on the synthetic medium were used for $C^{14}$ labeling experiments, whereas mats from the complex medium were used for enzyme experiments. The stock culture was maintained on PDA agar slants at 10°C.

Materials. Linamarin, lotaustralin, dhurrin, p-hydroxymandelonitrile, vanillin cyanohydrin, and iso-vanillin cyanohydrin in addition to the uniformly labeled $C^{14}$ amino acids, serine and tyrosine, were kindly supplied by E. E. Conn, University of California, Davis. All other labeled amino acids were purchased from Nuclear Chicago Corp.. Methyl-ethyl-ketone cyanohydrin was purchased from Aldrich Chemical Co., Milwaukee. Mandelonitrile and $\alpha$-hydroxyisobutyronitrile were obtained from K and K Laboratories, Plainview, New York. Lactonitrile was purchased from Eastman Organic Chemicals, Rochester; phloridizin was obtained from Nutritional Biochemicals Corporation, Cleveland; and p-nitrophenyl - $\beta$-D glucoside and p-nitrophenyl - $\beta$-D galactoside were purchased from Sigma. All other chemicals used were reagent grade. Uniformly labeled lotaustralin was obtained by feeding 10 $\mu$c L-isoleucine-U-$C^{14}$ to the stems of 20 week old flax seedlings. The ends of the seedlings were immersed in 0.4 ml $H_2O$ containing the

L-isoleucine-U-$C^{14}$. Successive 0.1 ml portions of water were added as required during a 7 hr absorption period in continuous light. Lotaustralin was then extracted as described later in Isolation and Characterization of Cyanogenic Compounds.

## General Methods

Analytical methods. Cyanide concentration was determined by the picric acid technique of Boyd (1935). Protein was quantitatively determined by the method of Lowry et al. (1951). The method of Nelson (1944) was used to determine glucose concentration. All colorimetric determinations were made on a Bausch and Lomb Spectronic 20 Colorimeter. Cyanogenic glucosides were located on chromatograms by the acetone-silver nitrate technique of Trevelyan (1950. The glucosides were quantitatively analyzed by chromatographing standard amounts of the glucosides, developing as described below and scanning the spots in a Joyce Densitometer. Since the area under the curve of the chart was proportional concentration, the unknown concentrations could be calculated from areas.

Radioactivity determinations. Radioactive samples were counted using a Nuclear Chicago Liquid Scintillation Counter. The solvent used in each vial consisted of 1.5 ml methanol and 13.5 ml of toluene containing 4.0 g 2, 5 diphenyloxazole and 100 mg of p-bis-2(5-phenyloxazolyl)-benzene per liter. Radioactive areas on chromatograms were detected

using a Packard Radiochromatogram Strip Counter. After location, these radioactive areas were cut out, eluted with 10% isopropanol, and counted in the liquid scintillation counter. In all cases counts were converted to dpm by the quench correction method using a standard curve.

Chromatography. Sheets of Whatman No. 1 paper were used for paper chromatography and the following solvent systems employed: (1) methyl-ethyl-ketone-acetone-$H_2O$ (30:10:6); (2) n-butanol-acetic acid-$H_2O$ (120:30:50); and (3) isopropanol-$H_2O$ (7:3).

## Isolation and Characterization of Cyanogenic Compounds

Mycelial mats of the fungus were ground in a Sorvall Omnimixer at 16,000 rpm for 2 min, then 20 ml of 95% alcohol was added to the homogenate and the suspension was centrifuged at 16,000 x g for 12 minutes. The supernatant solution was passed through a column (1 x 2 cm) of Dowex 50-$H^+$, 200-400 mesh, and then through a column of Dowex 1 (formate form). The effluent was taken to dryness in a flash evaporator. Samples were then taken up in 0.2 ml of 10% isopropyl alcohol and 15-25 µl quantities were chromatographed in solvents (1), (2), or (3). The locations of cyanogenic glucosides were determined by comparing Rf values with reference compounds simultaneously chromatographed. For infrared identification unknown compounds were rechromatographed three times in solvent 1, eluted with 10% isopropyl alcohol, and dried in a desiccator. Potassium bromide was added to both authentic and unknown

cyanogenic glucosides, the mixture was pressed to form a pellet and then analyzed in a Beckman IR-4 Infrared Spectrophotometer.

## $C^{14}$ Feeding Experiments

Feeding U-$C^{14}$ Amino Acids to the Fungus to Determine Dilution Factors. The mycelial mat of the fungus grown on synthetic media was drained and transferred aseptically to a distillation flask. Uniformly labeled $C^{14}$ amino acids were introduced and the flask sealed and incubated at 21°C. After 12 hrs incubation, 10 ml of 0.5N $H_2SO_4$ were added to the flask to stop the reactions and to free cyanide. The reaction mixture was then steam distilled until 60 ml of distillate were collected in the 2% KOH trap. The volume was reduced to 5 or 6 ml in a flash evaporator, BaOH was added to remove $CO_2$, and the precipitate was removed by filtering through sintered glass. Aliquots of the filtrate were then placed in a scintillation vial, dried, and the radioactivity determined. An additional aliquot of the filtrate was also used to colorimetrically determine cyanide concentration. The dilution factor is defined as the ratio of the specific activity of the precursor fed to the specific activity of the compound isolated. This factor can easily be used to establish the relationship of a precursor compound in the biosynthesis of a second compound. Thus, if several amino acids were fed a system to determine their incorporation into compound X, the best precursor would have a dilution factor of 1 if all the precursor label were incorporated into compound X. Correspondingly, an amino acid with a

higher dilution factor indicates that (1) the label was not incorporated into compound X, e.g., some label was utilized in other pathways, or (2) the amino acid was not a direct precursor of compound X.

## β-glucosidase Studies

Assay of β-glucosidase. Since the assay substrate p-nitro-phenyl-β-D-glucoside yields p-nitro-phenal up hydrolysis the activity of β-glucosidase was followed during purification by measuring the increase in absorbance at 400 mμ (p-nitrophenyl) in a Beckman D.U. Spectrophotometer with a 1 cm light path according to the method of Schaeffer et al. (1960). The reaction mixture contained 4.2 μ moles, p-nitro-phenyl- β-D-glucoside, 0.1 ml enzyme, and 28 μ moles phosphate buffer, pH 7.87, in a total volume of 3 ml. The reaction was initiated by the addition of p-nitrophenyl- β-D-glucoside. The change in absorbance during one minute intervals was used to calculate enzyme activity. A unit of enzyme activity was defined as the amount of enzyme which hydrolyzed .104 μ moles/min.

Energy of activation. The standard reaction mixture was used, and the temperature was varied from 7.0° to 45°C. The contents of the reaction vessel, minus the substrate, were equilibrated at the designated temperature for 10 min. The substrate was then added to start the reaction and the log of the substrate hydrolyzed/min was determined and plotted against $\frac{1}{T}$. The slope of the line could then be measured and the energy of activation (Ea) could be calculated from the Arrhenius equation:

$2.3 \log \frac{K_1}{K_2} = \frac{-Ea}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)$. $\frac{K_1}{K_2}$ = slope, R = gas constant, T = absolute temperature.

Purification of $\beta$-glucosidase. Twelve mycelial mats from cultures 3 weeks old were collected, drained, rinsed with cold distilled water and ground in a pre-chilled Sorvall Omnimixer for 2 minutes. The homogenate was centrifuged at 20,000 x g for 12 min. The precipitate was discarded and acetone at -15°C was slowly poured into the supernatant liquid until 2 volumes had been added. The precipitate was removed by centrifugation at 10,000 x g for 10 min and taken up in 50 ml of 0.01M phosphate buffer, pH 7.87. Three ml of 1% protamine sulfate at pH 6.0 was added to the supernatant and again centrifuged at slow speed to remove the precipitate. The supernatant was then fractionated with 20%, 40% and 60% saturated ammonium sulfate. Precipitates from the first 2 fractions were discarded. The precipitate from the 60% fraction was taken up in 25 ml 0.01M phosphate buffer, pH 7.87 and dialyzed over night against 10 liters of distilled water and finally against 3 liters of 0.001M phosphate buffer pH 7.87. The solution was passed through a column (1 cm x 5 cm) of DEAE cellulose, Cl- which had been washed and equilibrated with 0.01M phosphate buffer, pH 7.87. The column was then washed with 25 ml of phosphate buffer 0.01M, pH 7.87. The flow rate of the column was 0.50 ml/min. A continuous gradient elution was used with 0.3 M KCl in 0.01 M phosphate buffer, pH 7.87 and five ml fractions were collected. Enzyme activity

and protein concentration were determined for each fraction as previously described. All preparations were carried out at 4°C. Table I summarizes the purification procedure and Figure 2 illustrates the elution pattern of β-glucosidase from the DEAE cellulose column.

## Oxynitrilase Studies.

Preparation of Oxynitrilase from the Psychrophilic Basidiomycete. Six mycelial mats from cultures 3-4 weeks old were collected, and ground in a pre-chilled Sorvall Omnimixer for 2 minutes. The homogenate was centrifuged at 20,000 x g for 12 minutes. The precipitate was discarded and acetone at -15°C was slowly poured into the supernatant liquid until 2 volumes had been added. The precipitate was removed by centrifugation at 10,000 x g for 10 minutes, and taken up in 20 ml 0.05 M citrate buffer, pH 5.3. This preparation was then used for all subsequent reactions.

Assay of Oxynitrilase Activity. The reaction mixtures contained 46 μ moles citrate buffer, pH 5.3, 0.1 ml enzyme and .30 μ moles of the various substrates. The substrate was added to initiate the reaction which was then incubated at 10°C for 10 minutes. A diagram of the reaction vessel and cyanide trap appears in Figure 3. The dissociation of the 4 aromatic cyanohydrins to their corresponding aromatic aldehyde and HCN was followed spectrophotometrically, according to the method of Seeley et al. (1966).

Table I

Purification of β-Glucosidase from the Psychrophilic Basidiomycete

| | Total units | Total protein (mg) | Specific activity | Yield | X purified |
|---|---|---|---|---|---|
| Crude | 51.78 | 1375.00 | .04 | 100.00% | 0.00 |
| Acetone | 43.04 | 729.60 | .05 | 82.90% | 1.70 |
| Prot. $SO_4$ | 35.03 | 241.50 | .14 | 62.70% | 3.86 |
| Amm. $SO_4$ | 18.75 | 29.00 | .65 | 36.30% | 17.20 |
| Dialysis | 3.17 | 29.00 | .11 | 6.12% | 2.66 |
| DEAE tube 32 | .86 | .10 | 8.98 | 1.66% | 238.80 |
| tube 42 | .70 | .33 | 2.09 | 1.35% | 55.60 |

Figure 2. The elution pattern of β-glucosidase on a DEAE cellulose column.