The origin of cyanide in a psychrophilic basidiomycete

by Dennis Leroy Stevens

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:
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.

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Approved:

Head, Major Department

Chairman, Examining Committee

Graduate Dean

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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 $HC^{14}N$. 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 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 α-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-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 α-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 β carbon and subsequently β 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 β carbon is oxidized with retention of the amino group to form an amide. Finally, the β 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 β-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.

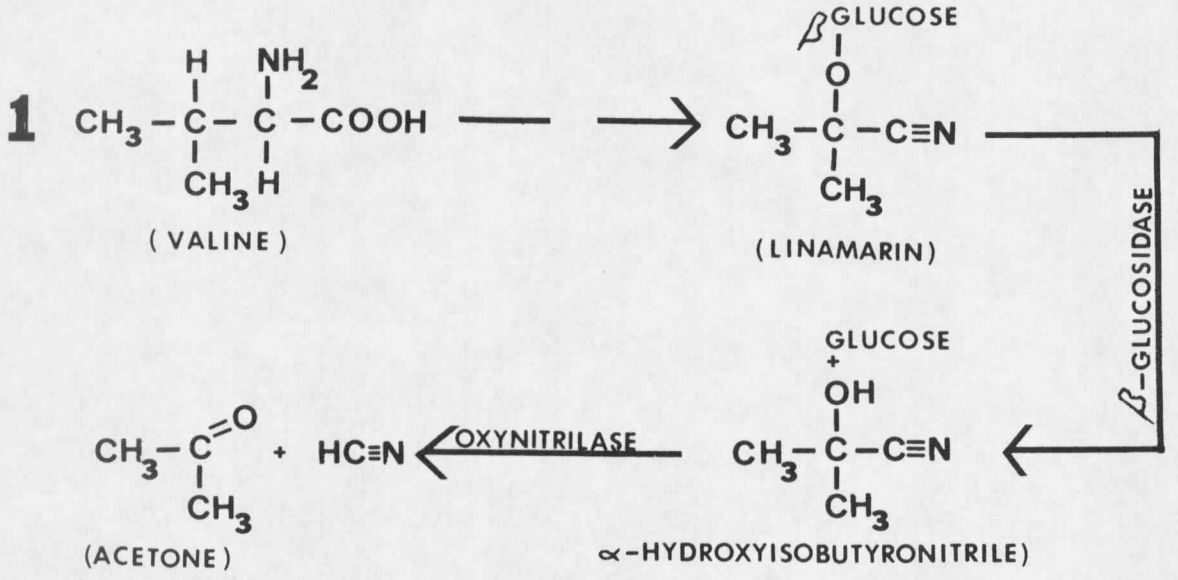
It was reported by Michaels et al. (1965) that growing cultures of Chromobacterium violaceum produced cyanide in a variety of culture media, but that the greatest cyanide yield occurred when a chemically defined medium was supplemented with glycine and methionine. Michaels and Corpe (1965) showed in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

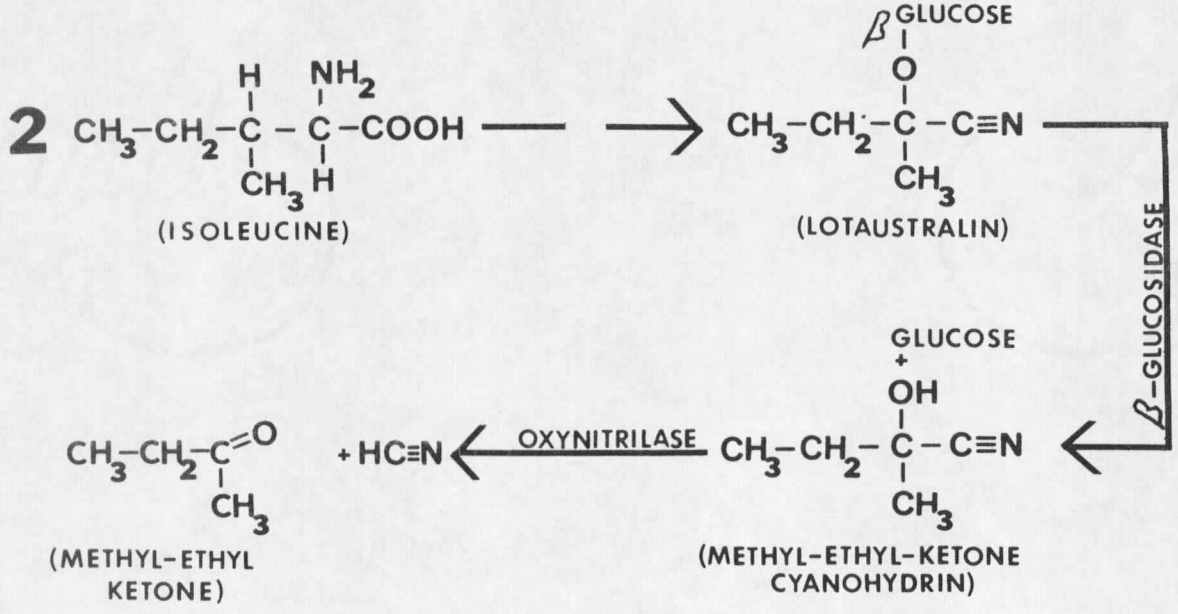
The work involving cyanide formation in the fungi, however, has largely been descriptive and has been in conjunction with a host parasite relationship such as in fairy ring of grasslands caused by Marasmius oreades, (Lebeau, 1961) or in snow mold of alfalfa and grasses caused by an unidentified psychrophilic 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 α-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 - β-D glucoside and p-nitrophenyl - β-D galactoside were purchased from Sigma. All other chemicals used were reagent grade. Uniformly labeled lotaustralin was obtained by feeding 10 μc 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 β-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.

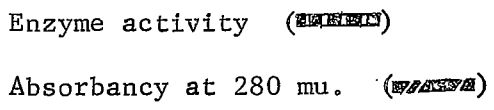
Assay of Oxynitrilase Activity. The reaction mixtures contained 46 μ moles citrate buffer, pH 5.3, 0.1 ml enzyme and .30 μ moles of the various substrates. The substrate was added to initiate the reaction which was then incubated at 10°C for 10 minutes. A diagram of the reaction vessel and cyanide trap 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.



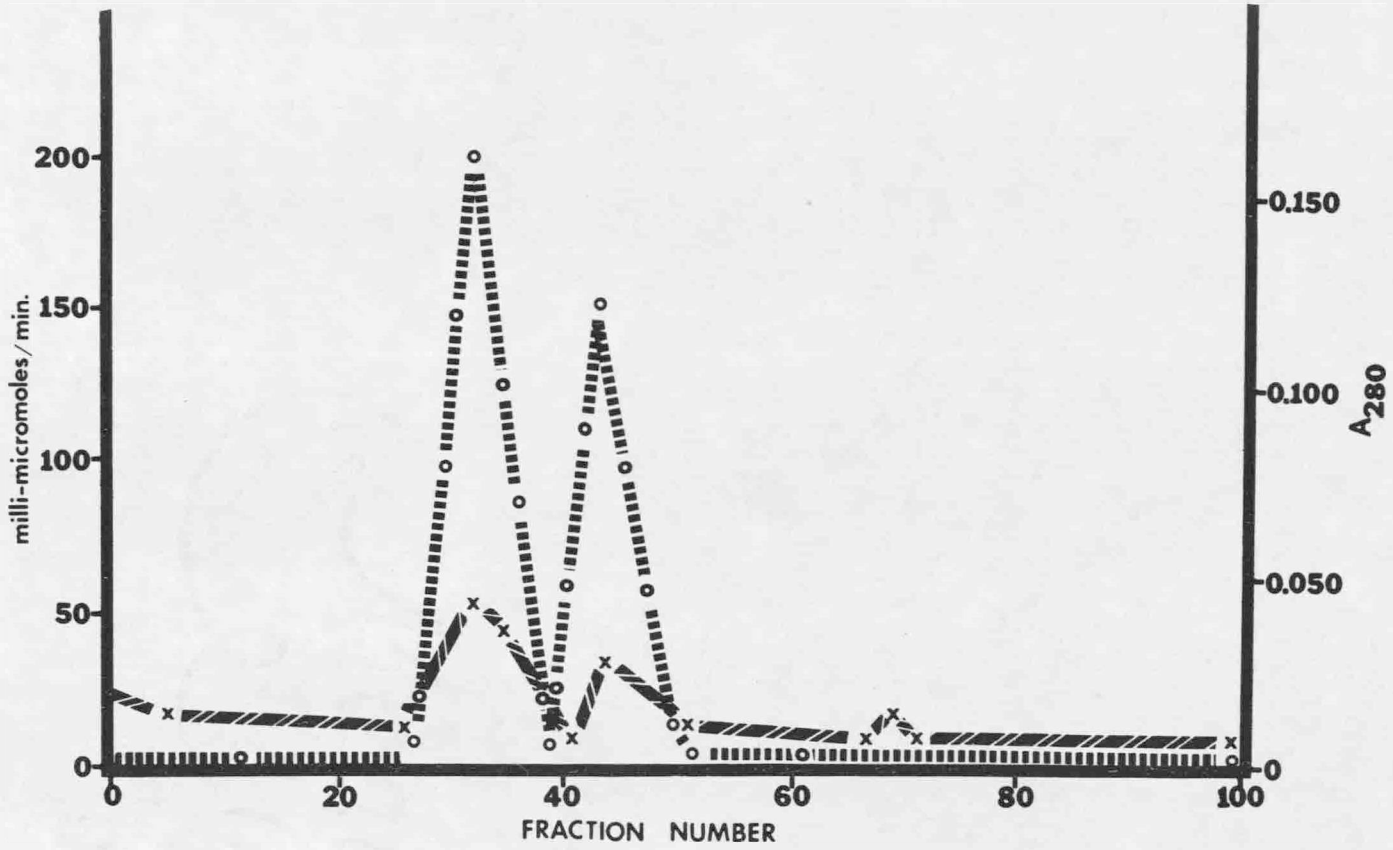
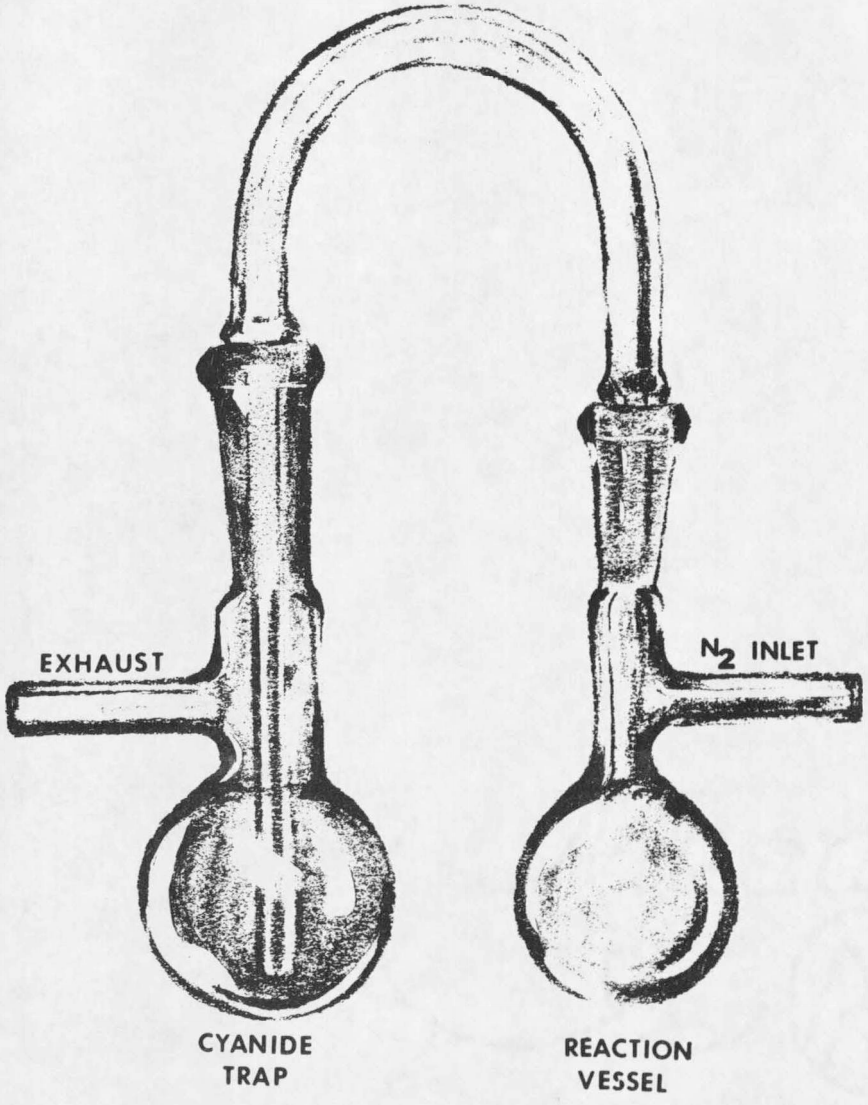

200
150
100
50
0
milli-micromoles/min.
0.150
0.100
0.050
0
A_{280}
0
20
40
60
80
100
FRACTION NUMBER

Figure 3. The reaction vessel used for determining oxynitrilase activity.


EXHAUST
N_2 INLET
CYANIDE TRAP
REACTION VESSEL

Determination of Aglycone Hydrolysis Products. Three moles of either α-hydroxyisobutyronitrile or methyl-ethyl-ketone cyanohydrin were placed in a closed reaction vessel containing 46 μ moles phosphate buffer, pH 5.3 and 0.1 ml of enzyme. The reaction vessel was incubated at 10°C for 12 hours. A 1.0 μl sample from each vessel was then analyzed in a Beckman GC-4 Gas Chromatograph using a 4 ft x 1.5 mm column packed with 100-120 mesh Porapak Q. The helium carrier gas rate was 200°C. The retention times of the reaction products were determined and compared to standards.

EXPERIMENTAL RESULTS

The Utilization of Amino Acids as Precursors to Cyanide Formation by the Psychrophilic Basidiomycete. Uniformly labeled C^{14} amino acids were fed to mats of the psychrophilic basidiomycete to ascertain the best precursors for cyanide formation. The dilution factors were determined for each amino acid fed and appear in Table II. The results show that although all the amino acids contributed to labeling in HCN, isoleucine and valine were the best precursors since their dilution factors were the lowest. (See discussion of the dilution factor technique under Materials and Methods.)

Isolation and Characterization of Linamarin and Lotaustralin. Inasmuch as isoleucine and valine appeared as good cyanide precursors in the fungus and since, as mentioned in the Introduction, isoleucine and valine are precursors for lotaustralin and linamarin biosynthesis in cyanogenic plants, it was logical to determine whether or not these cyanogenic glucosides existed in the psychrophilic basidiomycete. Mycelial mats of the fungus were extracted and the cyanogenic glucosides isolated as previously described. Table III shows that the R_f values of two compounds on the chromatographs were identical to the R_f values of the reference compounds in all solvent systems used. To further establish the identity of these 2 compounds, potassium bromide pellets were prepared and analyzed by infra-red spectrophotometry. Absorption spectra from each of the 2 compounds were compared to spectra of the authentic compounds linamarin and lotaustralin. The results indicated that the spectrum of one compound was identical to that of linamarin

Table II

Conversion of Amino Acid-UC^{14} Precursors to HC^{14}N

Amino acid fed			HCN recovered		Dilution factor
Amino acid	mμc (x 10^{3})	Specific Activity ($\frac{\text{mμc}}{\text{umole}}$)	mμc (HC^{14}N)	Specific Activity ($\frac{\text{mμc}}{\text{μmole}}$)	$\frac{\text{Sp. Act. a.a.}}{\text{Sp. Act. HCN}}$
Glycine	3.85	1.29×10^{4}	1.29	0.53	2.44×10^{4}
Alanine	1.99	2.02×10^{4}	1.96×10^{1}	1.66×10^{1}	1.25×10^{3}
Asparagine	3.75	2.64×10^{4}	4.34	2.32	1.14×10^{4}
Valine	3.68	2.02×10^{4}	4.91×10^{2}	5.89×10^{1}	3.42×10^{2}
Isoleucine	6.05×10^{-1}	1.43×10^{4}	1.43×10^{2}	6.17×10^{1}	2.31×10^{2}
Phenylalanine	4.437	1.06×10^{3}	2.74	0.97	1.09×10^{3}
Serine	2.62	1.05×10^{5}	1.59	3.77×10^{1}	2.79×10^{3}
Tyrosine	4.65	2.98×10^{5}	8.97	2.69×10^{1}	1.08×10^{4}

Table III

Comparison of R_f Values of Unknown Compounds of Those of Linamarin and Lotaustralin in Three Different Solvent Systems

Solvent System	R_f Values			
	Linamarin	Lotaustralin	Unknown 1	Unknown 2
Methyl-ethyl-ketone-acetone-H_2O (30:10:6)	0.64	0.76	0.64	0.76
n-butanol-acetic acid-H_2O (120:30:50)	0.60	0.71	0.65	0.76
Iso-propanol-H_2O (7:3)	0.76	0.78	0.76	0.78

while the other compound was identical to lotaustralin.

Evidence for Isoleucine Incorporation into Lotaustralin. To establish that the carbon skeleton of isoleucine was incorporated into lotaustralin, 2.5 μc U-C^{14}-isoleucine was administered to mycelial mats of the fungus for 3, 6, 12, 24, and 48 hours. The specific activity of lotaustralin increased during the first 24 hours, but declined thereafter. Simultaneously, an increase in the specific activity of HCN also occured but with a more gradual decline after 24 hours. As can be seen from Table IV the moles of HCN produced are in excess of the moles of the intermediate lotaustralin except at 48 hours.

Enzyme Studies

The previous studies provided evidence that amino acids were incorporated into cyanogenic glucosides. The following enzyme studies were undertaken in order to show that the fungus had the enzyme or complement of enzymes necessary to release cyanide from lotaustralin and linamarin. Labeled lotaustralin was prepared from flax seedlings since lotaustralin comprises roughly 5% of the seedlings' dry weight. A 0.45 ml fraction of a crude mycelial extract was added to 0.24 μ moles (.013 μc) lotaustralin and 28 μ moles citrate buffer pH 6.0 and incubated at 17.5°C, 25°C, or 47.5°C for 22 hours. The released HCN was trapped in 3 ml of 2% KOH and the amount of labeled $HC^{14}N$ determined. Table V provides evidence that the breakdown of lotaustralin was enzymic and that the necessary enzymes were present in a crude extract of the fungus.

Table LV

Isoleucine U-C^{14} as a Precursor of Lotaustralin and HCN during a 48 hr study.

Time (hrs)	Lotaustralin		HCN	
	μ moles isolated	specific activity*	μ moles isolated	specific activity*
3	0.07	0.25	2.55	8.70
6	0.41	0.40	4.22	14.12
12	0.11	2.30	1.24	29.07
24	0.08	2.81	0.39	58.10
48	0.71	0.23	0.59	48.10

* Specific activity expressed as mμc/μ mole.

Table V

Evidence that the Liberation of Cyanide from Lotaustralin was an Enzymic Process.

Reaction temp. (C^{o})	DPM released as $HC^{14}N$	
	boiled crude extract	crude extract
17.5^{o}	0	1582
25.0^{o}	0	3170
35.0^{o}	0	7521
47.5^{o}	0	6791

The reaction vessel contained either 0.45 ml of crude extract or boiled crude extract and 0.24 μ moles (0.013 μc) lotaustralin and 28 μ moles citrate buffer, pH 6.0 in a total volume of 4.0 ml. The released HCN was trapped in 3 ml of 2% KOH and the amount of $HC^{14}N$ determined.

Purification of β-Glucosidase from the Psychrophilic Basidiomycete. Since the first proposed step in the release of cyanide from cyanogenic glucosides is hydrolysis at the β-glucoside linkage it was deemed important to isolate and characterize such an enzyme or enzymes. Table I shows the steps in the purification procedure of β-glucosidase activity in fungus cultures. The elution pattern on DEAE cellulose is shown in Figure 2. It should be noted that 2 peaks appeared both of which had enzyme activity on the assay substrate p-nitrophenyl-β-D-glucoside. The first peak was termed fraction 1 and the second fraction 2.

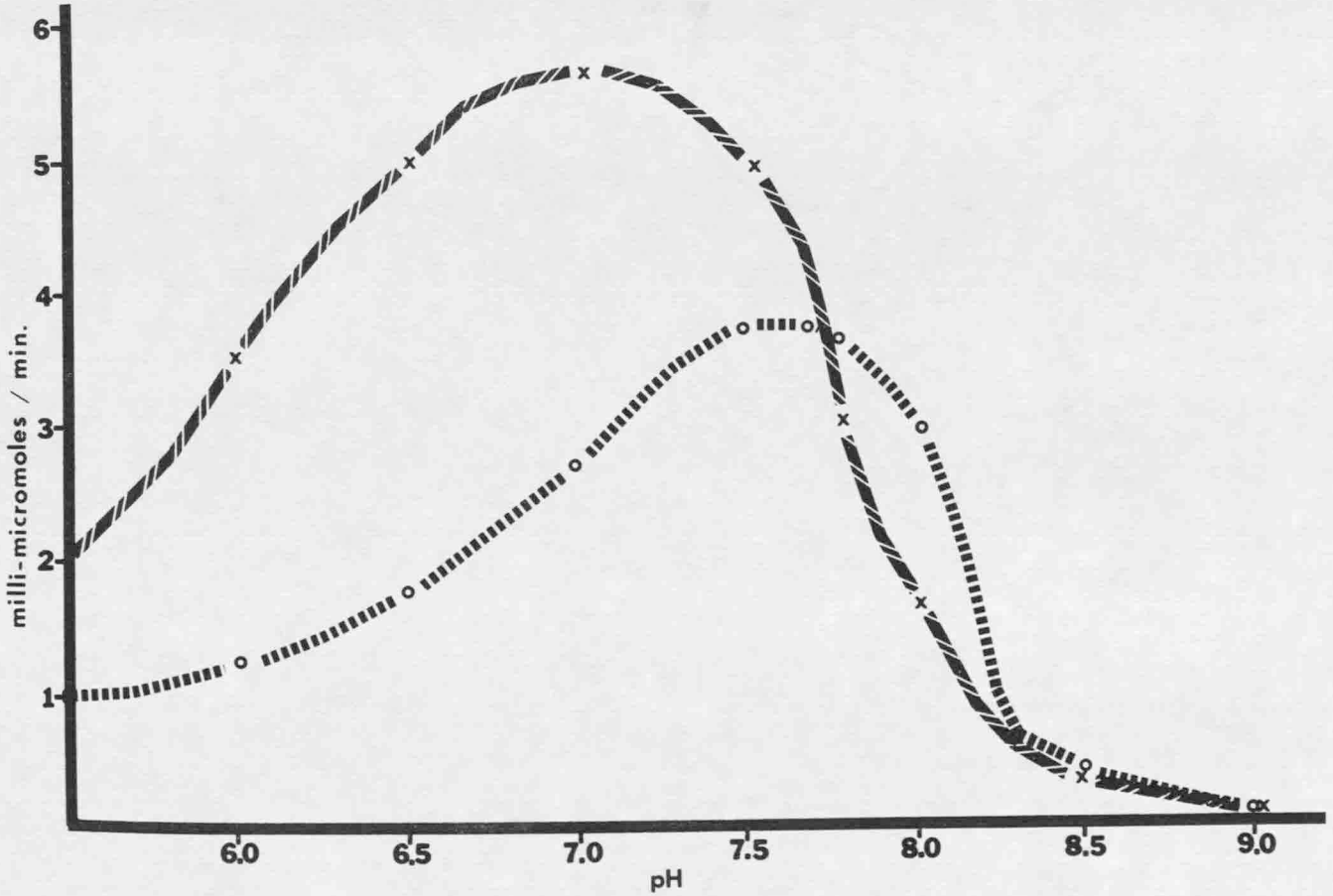
Properties of the β-Glucosidases. Fraction 1 and fraction 2 were purified 238 fold and 55 fold, respectively, from the mycelium of the psychrophilic basidiomycete as outlined in Materials and Methods. All subsequent data concerning these enzymes were obtained using aliquots of fraction 1 and fraction 2.

a. Effect of pH on Activity. The standard reaction mixture was employed with the exception that the following buffer systems were used at the designated pH ranges: citrate buffer (pH 5.5 - 6.6); phosphate buffer (pH 6.5 - 8.0); and pyrophosphate buffer (pH 8.0 - 9.5). The pH optima of peak 1 and peak 2 were 7.6 and 7.0, respectively (Figure 4).

b. Effect of Substrate Concentration of Enzyme Activity. The reaction mixtures used were identical to the standard assay mixture with the exception that the substrate concentration was varied between 0.07 μ M

Figure 4. The effect of pH on the activity of fraction 1 (▭▭▭) and fraction 2 (▭▭▭) β-glucosidases.

The standard assay procedure was utilized with the exception that 28 μ moles of the following buffer systems were used at the designated pH ranges: citrate buffer, (pH 5.5 - 6.5); phosphate buffer, (pH 6.6 - 8.0); and pyrophosphate buffer, (pH 8.0 - 9.5).


milli-micromoles / min.
6
5
4
3
2
1
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

and 2.10 μ M. Figure 5 indicates the effect of substrate concentration on each fraction of β-glucosidase activity plotted according to Lineweaver and Burk (1934). The K_m value for fraction 1 was 7.26×10^{-2} M while that of fraction 2 was 4.9×10^{-1} M.

c. Substrate Specificity. The standard assay mixture was used, however, equal molar amounts of the various substrates were substituted for p-nitrophenyl- β-D-glucoside. The β-glucosidase activity on p-nitrophenyl- β-D-galactopyranoside was measured in the same manner as on the standard assay substrate, p-nitrophenyl- β-D-glucoside. Acitivity on all other substrates, however, was quantitatively determined by the glucose technique of Nelson (1944). Table VI shows that fraction 1 hydrolyzed p-nitrophenyl- β-D-glucoside, cellobiose, linamarin and dhurrin, whereas fraction 2 hydrolyzed all the β-glucosides. Neither fraction hydrolyzed p-nitrophenyl- β-D-galactopyranoside or maltose.

d. Energy of Activation. The energy of activation was determined for each fraction of the enzyme as outlined in Materials and Methods. Figure 6 is a graph of the log of the substrate hydrolyzed plotted against the reciprocal of the absolute temperature. The calculated energies of activation for fraction 1 and 2 were 6107 cal/mole and 9000 cal/mole, respectively.

e. Stability. Purified preparations of each fraction lost approximately 20% of the original activity after freezing for 2 days, but did not appreciably lose activity when stored at $4^{\circ}C$ for 4 days.

Figure 5. The effect of substrate concentration of the activity of fraction 1 (▭▭▭) and fraction 2 (▨▨▨) β-glucosidases.

The standard assay procedure was used, however, the substrate concentration was varied between 0.07 μM and 2.10 μM. In addition 28 μ moles phosphate buffer at pH 7.6 or 7.0 were used for fraction 1 and fraction 2, respectively. The units of 1/V and 1/S are 1/μ moles/min. and 1/M, respectively.

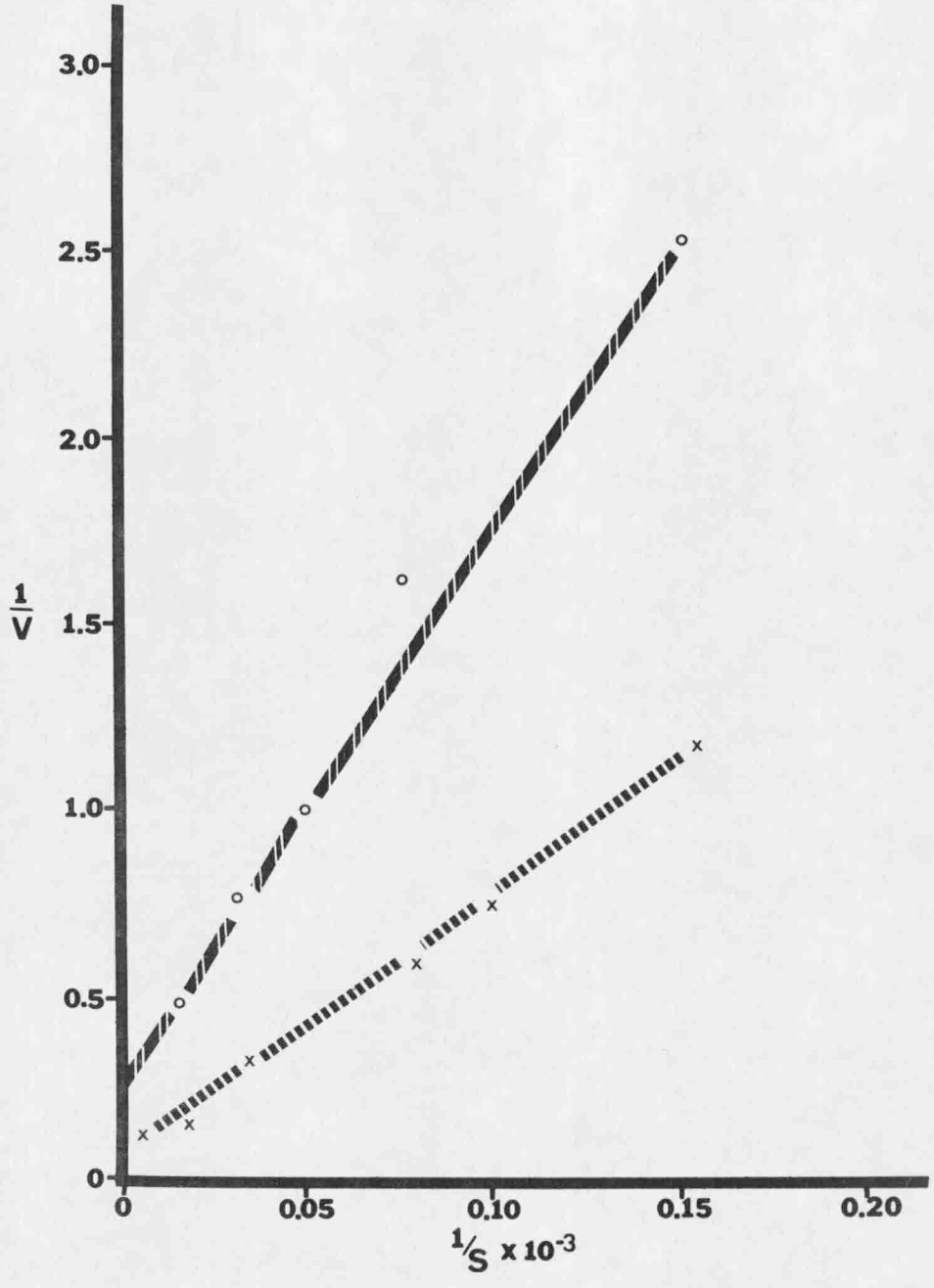

3.0
2.5
2.0
1.5
1.0
0.5
0
1/V
0
0.05
0.10
0.15
0.20
1/S x 10-3

Table VI

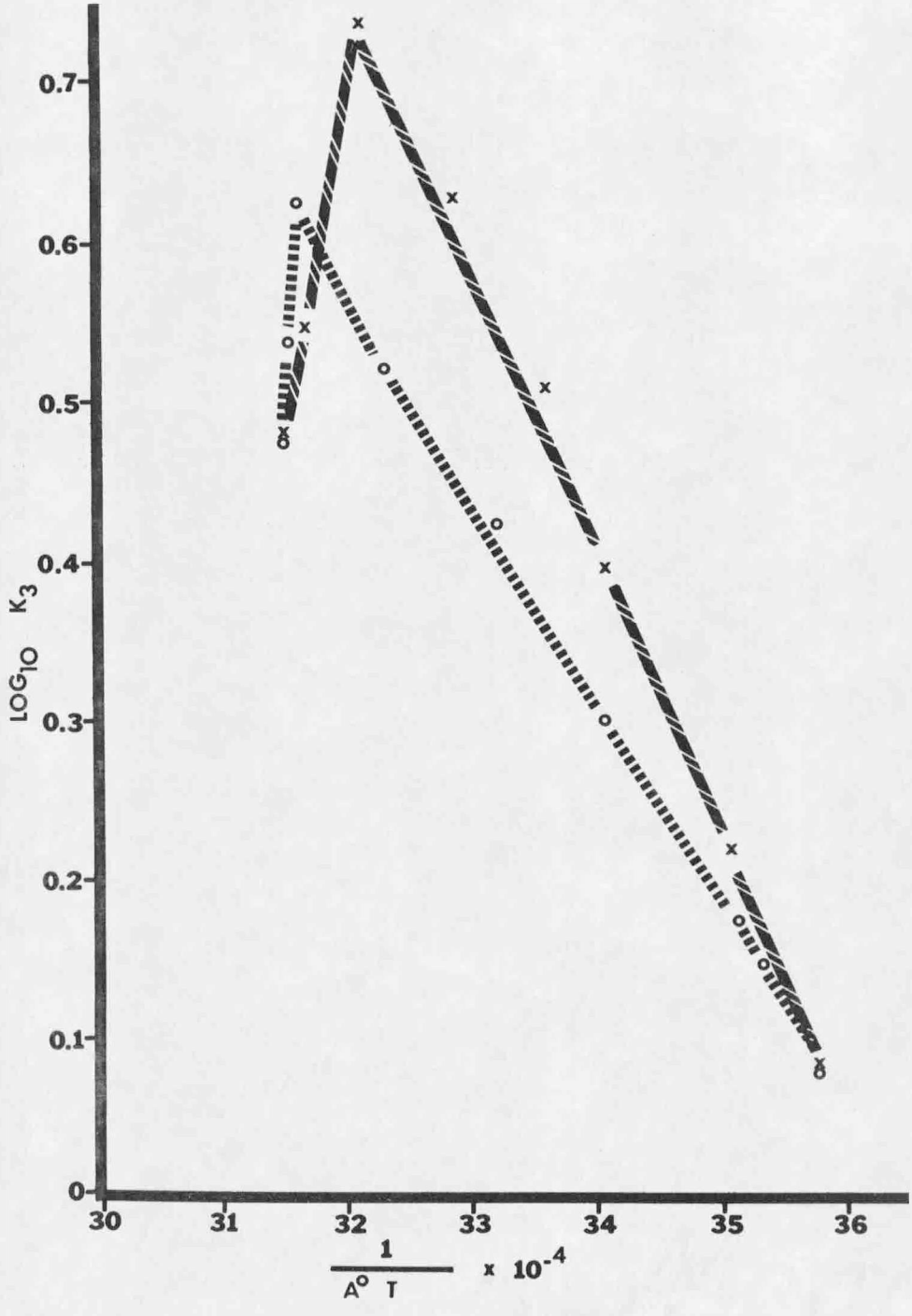
Relative Reaction Rates of the 2 β-Glucosidases on Several Substrates.

Substrate	percent relative hydrolysis	
	fraction 1	fraction 2
p-nitrophenyl- β-D-glucoside	100.00	100.00
p-nitrophenyl- β-D-galactoside	0.00	0.00
maltose	0.00	0.00
cellobiose	26.00	10.20
linamarin	2.19	2.39
lotaustralin	0.00	9.56
dhurrin	5.19	14.90
phlorizin	0.00	48.20

The reaction mixture contained 4.2 μ moles of the various substrates, 0.1 ml enzyme, 28 μ moles phosphate buffer, pH 7.6 (fraction 1 enzyme) or pH 7.0 (fraction 2 enzyme) in a total volume of 3 ml. The reactions rates were determined by measuring glucose released and the enzymic rate of hydrolysis on p-nitrophenyl- β-D-glucoside was arbitrarily set at 100%.

Figure 6. The effect of temperature on the rate of hydrolysis (K_3) of p-nitrophenyl- β-D-glucoside by fraction 1 (□□□□) and fraction 2 (▨▨▨) β-glucosidases.

The data are plotted as $\log_{10} K_3$ vs. the reciprocal of the absolute temperature. The units of K_3 are m μ moles/min.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
LOG10 K3
30
31
32
33
34
35
36
1
A° T
x 10-4

Fraction 1 lost 12% of its activity and fraction 2 lost 44% of its activity after heating in a water bath at 45°C for 20 minutes. All activity was lost after a similar treatment at 55°C.

f. Effect of Inhibitors. Since the fraction 2 enzyme hydrolyzed many glucosides but not the galactoside, or maltose, the specificity must reside primarily with the β linkage and the glucose portion of the substrate. It was of interest, therefore, to determine something about the enzyme binding site by studying the effect of various structural analogs of glucose as competitive inhibitors. The standard assay procedure was utilized, however the concentration of p-nitrophenyl- β-D-glucoside was varied between 33 μ M and 830 μ M while the inhibitor concentration was maintained at 1.4 m M. Figure 7 illustrates that the inhibition was competitive since each of the inhibitor lines on the Linneweave-Burke double reciprocal plot has the same intercept (V max) as the uninhibited reaction.

Table VII shows that the best competitive inhibitors in descending order were cellobiose, glucose, galactose and xylose.

g. Effect of Cellobiose as an Inducer of Enzyme Synthesis. In an effort to determine the inducibility of this enzyme in the classical sense, the fungus was grown on a complex medium, a synthetic medium with glucose as the sole source of carbon, and a synthetic medium with cellobiose as the carbon source. Acetone preparations from cultures grown on the three different media were prepared in the same manner as the first two purification steps in Table II. The enzyme activity from

Figure 7. The effect of several competitive inhibitors on the reaction rate of β-glucosidase.

The standard assay procedure was utilized, however the concentration of p-nitrophenyl- β-D-glucoside was varied between 33 μM while the inhibitor concentration was maintained at 1.4 μM. The units of V^{-1} are $\left(\frac{\mu \text{ Moles}}{\text{min}}\right)^{-1}$.

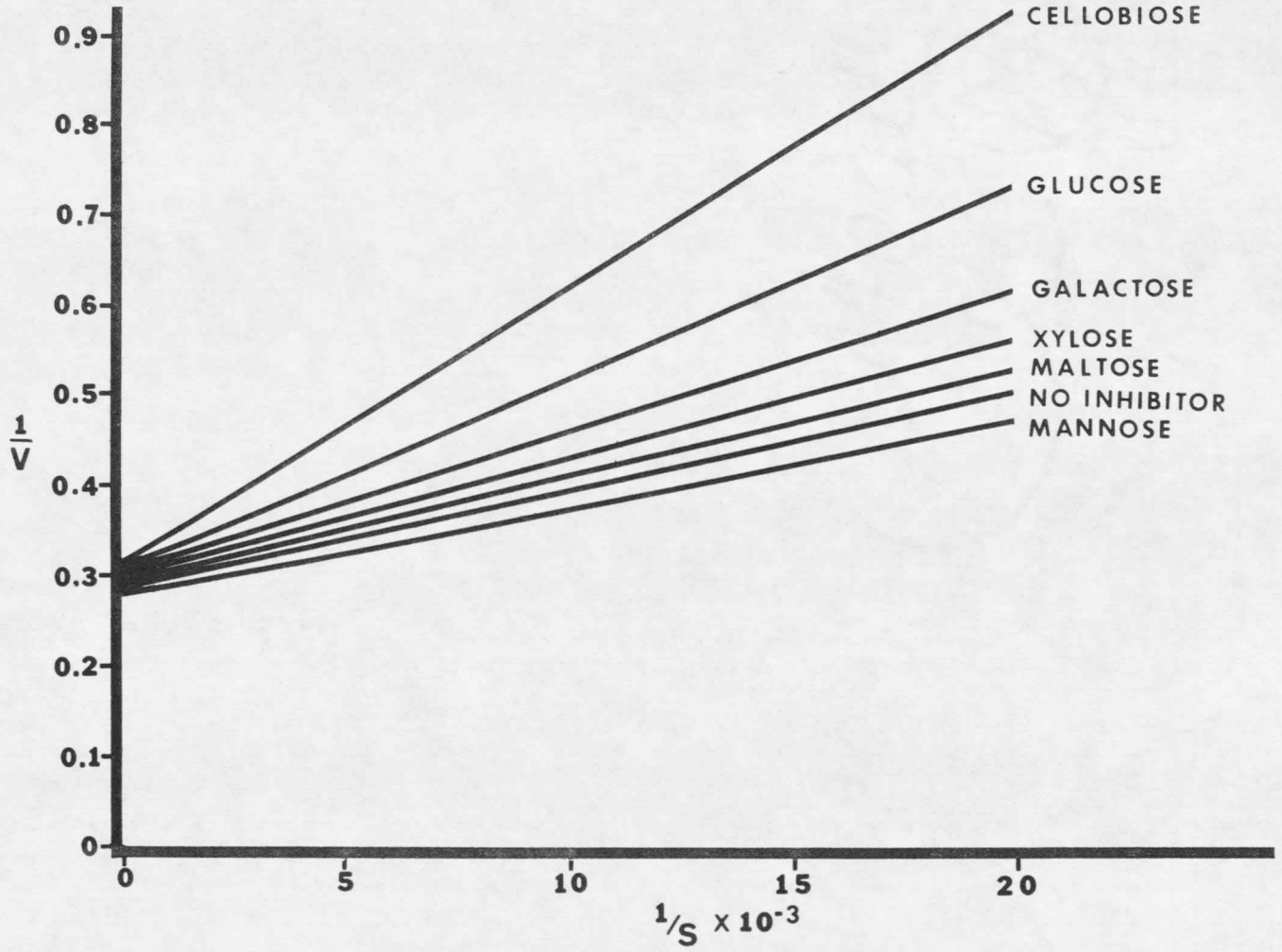

CELLOBIOSE
GLUCOSE
GALACTOSE
XYLOSE
MALTOSE
NO INHIBITOR
MANNOSE
1/V
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
1/S x 10-3

Table VII

The Effect of Competitive Inhibitors on the Reaction Rate of β-Glucosidase.

Inhibitor	% Inhibition
no inhibitor*	0
mannose	0
maltose	0
xylose	6
galactose	17
glucose	29
cellobiose	43

* The reaction vessel contained 0.132 u moles p-nitrophenyl-β-D-glucoside, 28 μ moles phosphate buffer pH 7.87 and 0.1 ml of fraction 2 enzyme in a total volume of 3.0 ml. To test the inhibitor, 1.4 μ moles of the various inhibitors were added and the standard assay procedure was used to follow the reaction.

each source was determined using the standard reaction mixture. Table VIII shows: (1) that the amount of β-glucosidase did not vary greatly from one medium to another; (2) that cellobiose was not an inducer; and (3) that glucose was not a repressor of enzyme synthesis.

Oxynitrilase Studies. From the preceding data it was established that linamarin and lotaustralin were hydrolyzed by β-glucosidases isolated from the psychrophilic basidiomycete. Butler (1965) demonstrated that the hydrolysis products of linamarin and lotaustralin from flax were glucose and α-hydroxy-isobutyronitrile and glucose and methyl-ethyl-ketone cyanohydrin, respectively. It was of interest therefore, to investigate the breakdown products of these aglycones and simultaneously, to determine the substrate specificity of the enzyme involved.

a. Products of Aglycone Oxidation. Samples of the reaction mixtures containing either α-hydroxyisobutyronitrile or methyl-ethyl-ketone cyanohydrin were injected into the gas chromatograph and retention times determined. Table IX shows the retention times of the products and various standards. The results demonstrated that acetone had the same retention time as the major component in the α-hydroxyisobutyronitrile reaction sample. In the same manner, methyl-ethyl-ketone was correlated to the methyl-ethyl-ketone cyanohydrin breakdown product. To further substantiate these results, an acetone standard and a sample of the α-hydroxyisobutyronitrile reaction mixture were simultaneously injected into the gas chromatograph. Methyl-ethyl-ketone and methyl-ethyl-ketone cyanohydrin were similarly treated.

Table VIII

β-Glucosidase Levels after Growth in Various Carbohydrate Media.

Carbon source	total units[a]	total protein (mg)	specific activity[b]
cellobiose	36.0	545.4	0.066
glucose	42.0	677.4	0.062
complex medium	43.0	729.6	0.059

a. A unit of enzyme activity was defined as the amount of enzyme which hydrolyzed 0.104 μ moles/min.

b. Specific activity was expressed as units/mg protein.

The reaction mixture contained 4.2 μ moles p-nitrophenyl-β-D-glucoside, 0.1 ml enzyme, and 28 μ moles phosphate buffer, pH 7.87 in a total volume of 3.0 ml.

Table IX

Comparison of Retention Times of Standards

with Aglycone Breakdown Products.

Compound	Retention time (min.)
acetone	1.22
methyl-ethyl-ketone	3.10
reaction sample 1*	1.18
reaction sample 2*	3.10

* Reaction sample 1 and 2 were 1 ul. samples of a reaction vessel containing 46 μ moles phosphate buffer pH 5.5, 0.1 ml. crude enzyme preparation and either 300 μ moles α-hydroxyisobutyronitrile or 300 μ moles methyl-ethyl-ketone cyanohydrin, respectively.

The results showed that; (1) there was only one major peak in each case; (2) the peak was a singlet and had no shoulders; and (3) the amplitude of the peak was larger than either the standard of the sample alone. In each case 2 other very small peaks were detected on the sample charts. The peak with the shortest retention time closely followed the CO_2 peak and was thought to be HCN. The other peak had a very long retention time and was most probably the cyanohydrin.

b. Substrate Specificity. Seven cyanohydrins were used to determine the substrate specificity of the oxynitrilase from the psychrophilic basidiomycete. Table X shows that none of the aromatic cyanohydrins were hydrolyzed. It should also be noted that all the substrates decomposed non-enzymically above pH 5.5, consequently, the system was buffered at pH 5.3. Substantial decomposition occured even at pH 5.3 when the reaction temperature was 21^{o}C. When a reaction temperature of 10^{o}C and a buffer system at pH 5.3 were used together the rate of decomposition was drastically reduced. Table X also shows that the aromatic cyanohydrin controls decomposed slightly faster than the active enzyme preparations.

Table X

Substrate Specificity of Oxynitrilase

from the Psychrophilic Basidiomycete.

Substrate	μ moles aldehyde*	μ moles HCN
p-hydroxymandelonitrile	-0.038	-0.020
mandelonitrile	-0.047	-0.030
vanillin cyanohydrin	0	0
isovanillin cyanohydrin	0	0
lactonitrile	-	0.360
methyl-ethyl-ketone cyanohydrin	-	1.850
α-hydroxyisobutyronitrile	-	1.350

* The first four substrates were aromatic cyanohydrins which dissociated to yield the corresponding aromatic aldehyde and HCN. The appearance of these aldehydes was followed spectrophotometrically.

All the substrates decomposed slowly non-enzymically. Consequently, the data represents the enzyme induced hydrolysis minus the boiled enzyme control. Negative values for p-hydroxymandelonitrile and mandelonitrile resulted in this manner.

The reaction mixture contained 46 μ moles citrate buffer, pH 5.3, 0.1 ml enzyme and 30 μ moles of the various substrates in a total volume of 3.0 ml.

DISCUSSION

Michaels and Corpe (1965) and Ward and Thorn (1966) showed in Chromobacterium violaceum and a psychrophilic basidiomycete, respectively, that the greatest cyanide yield occurred when a chemically defined medium was supplemented with glycine and that labeled glycine-$2C^{14}$ produced C^{14} labeled cyanide. Both groups of workers, however, failed to test the effect of any other labeled amino acids on production of labeled HCN. Investigations on amino acid metabolism in the psychrophilic basidiomycete showed that glycine, alanine, phenylalanine, tyrosine, isoleucine, valine and serine were all precursors to cyanide formation, however, isoleucine and valine were incorporated into cyanide to the greatest extent (Table II).

In cyanogenic plants e.g., flax, isoleucine and valine were incorporated into the cyanogenic glucosides, lotaustralin and linamarin, respectively, (Butler and Conn, 1964). Investigation into the existence of these 2 compounds in the psychrophilic basidiomycete provided evidence that a pair of compounds isolated and purified from the fungus had identical R_f values to the authentic compounds linamarin and lotaustralin, (Table III). A comparison between the infrared spectra of authentic linamarin and lotaustralin and the spectra of the two fungal compounds, identified them as linamarin and lotaustralin. To the author's knowledge this is the first report of these compounds in microorganisms.

Table IV shows that U-C^{14} isoleucine was incorporated into lotaustralin during a 48 hr. feeding experiment. The verification of isoleucine as a precursor for lotaustralin biosynthesis was in keeping with work on three other cyanogenic glucosides; dhurrin (Koukol et al., 1962), and (Gander, 1962); prunasin (Ben-Yehoshua and Conn, 1964); and linamarin (Butler and Conn, 1964); where the three corresponding amino acids (tyrosine, phenylalanine and linamarin) were the best precursors.

Table IV shows that isoleucine was incorporated into lotaustralin, however, it also demonstrated that the moles of lotaustralin isolated were insufficient to account for the moles of cyanide released. Two possibilities could account for this apparent discrepancy; (1) another cyanogenic mechanism was operating simultaneously; or (2) the turnover rate of lotaustralin was so great that little lotaustralin existed at a given time. The first possibility was confirmed by the fact that another cyanogenic glucoside linamarin was also isolated from the fungus. In addition other cyanogenic mechanisms may also be operative.

If labeled cyanide were arising strictly from lotaustralin the ratio of their specific activities should be 1. Any additional mechanism producing $HC^{14}N$ would make the ratio higher. Table IV shows that the specified activity of HCN was much higher than the specific activity of lotaustralin. This is best explained by an observation made during the experiment, that when labeled isoleucine was fed to the psychrophilic basidiomycete, labeling appeared in both lotaustralin and linamarin. Thus, there appeared to be an isoleucine to valine interconversion in

the fungus. No attempt was made to determine the extent of this amino acid interaction.

Another explanation might be that in the $HC^{14}N$ trapping procedure some extraneous counts were carried over as organic acids. This would make the specific activity of HCN higher than it should be. This factor may have also caused the dilution factors in Table II to be somewhat higher; however, the most likely explanation would be that much of the labeled amino acid fed was detoured into protein synthesis.

The biosynthesis of linamarin and lotaustralin has not been fully established, however, Figure 8 represents the present hypothesized mechanism[1].

According to the proposed scheme of cyanogenesis in plants, the cyanogenic glucosides are hydrolyzed by β-glucosidase to glucose and the aglycone. This aglycone is next oxidized by oxynitrilase to yield HCN and the corresponding aldehyde or ketone (Robinson, 1964).

The β-glucosidase was detected, isolated and purified from the psychrophilic basidiomycete (Table I). The last step of the purification procedure (elution from DEAE cellulose columns) yielded 2 separate fractions which had β-glucosidase activity on the assay substrate p-nitrophenyl- β-D-glucoside, Figure 2. The enzymic characteristics of fraction 1 and fraction 2 β-glucosidases appear in Table XI and show

[1] E. E. Conn, personal communications.

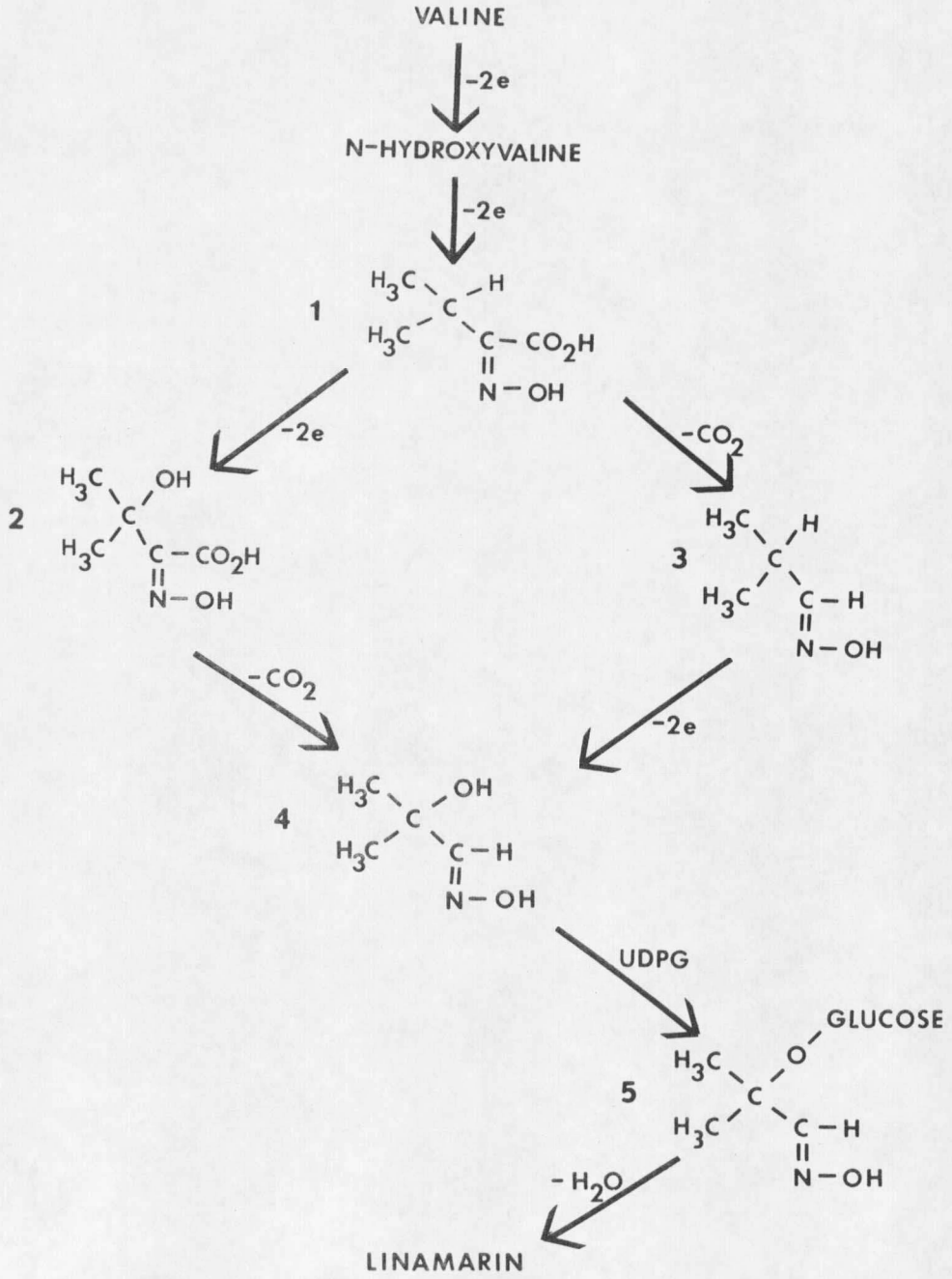
Table XI

A Comparison of the Enzymic Characteristics of Fraction 1 and Fraction 2 β-Glucosidases from the Psychrophilic Basidiomycete.

	K_m (10^{-2}M)	Ea (K cal/mole)	pH optimal	Thermal denaturation temp.
Fraction 1	7.26	9.0	7.6	43.5°C
Fraction 2	0.49	6.1	7.0	35.5°C

Figure 8. Present proposed mechanism of linamarin biosynthesis.

1. α-keto-isovaleric acid oxime
2. β-hydroxy- α-keto-isovaleric acid oxime
3. isobutyraldoxime
4. 2-hydroxy-isobutyraldoxime
5. isobutyraldoxime-2- β-D-glucoside


VALINE
-2e
N-HYDROXYVALINE
-2e
1
-2e
-CO2
2
3
-CO2
-2e
4
UDPG
GLUCOSE
5
-H2O
LINAMARIN

that the 2 enzymes behave differently. In addition to these differences the fraction 1 enzyme hydrolyzed only p-nitrophenyl- β-D-glucoside, cellobiose, linamarin, and dhurrin, whereas fraction 2 enzyme hydrolyzed all these plus lotaustralin and phlorizin. Neither enzyme hydrolyzed maltose or p-nitrophenyl- β-D-galactoside, but both enzymes had the greatest relative reaction rates on p-nitrophenyl- β-D-glucoside, Table VI. The above data verified the initial observation during purification (Figure 2) that 2 separate and distinct β-glucosidases existed in the psychrophilic basidiomycete.

In applying these results to cyanogenesis in the fungus, it was apparent that 2 enzymes were present both of which hydrolyzed linamarin whereas only 1 enzyme (fraction 2) hydrolyzed lotaustralin. Since the fraction 2 enzyme hydrolyzed all of the β-glucosides, but not maltose or the β-galactoside, (Table VI), the enzyme specificity is dependent on the β-linkage and the glucose portion of the substrate. The effect of various structural analogs of glucose as competitive inhibitors elucidated which portions of the glucose molecule were important. The data in Figure 7 verified that the analogs did act competitively, since the intercept ($\frac{1}{V}$ max) was the same for the inhibitors as for the uninhibited reaction. Table VII showed that mannose did not inhibit the reaction, therefore the position of the hydroxyl group on carbon 2 of glucose must be critical. Xylose, which existed as a pyranose ring was nevertheless capable of inhibiting the reaction by 6%. The hydroxyl on carbon-6, therefore, must be unimportant for binding to the enzyme.

Galactose did inhibit the reaction but since the β-galactoside (Table VI) was not hydrolyzed, the enzyme apparently cannot bind strongly enough to this substrate, whereas it could bind at least weakly to galactose. Therefore, configuration of the OH on carbon^4 appeared to be quite important.

The importance of the β-linkage was verified by the fact that cellobiose was a better inhibitor than glucose, and that maltose did not inhibit the reaction at all. It appeared, therefore, from the inhibitors studied, that the position of the hydroxyl groups in carbons 2 and 4 and the nature of the acetal linkage on carbon 1 are extremely important for substrate enzyme binding and that the nature of the moiety across the β acetal linkage from glucose was also important as evidenced by the different reaction rates on 6 β glucosides, (Table VI). These observations are in keeping with the results of Butler et al. (1965) who showed different degrees of hydrolysis with β-glucosidases from plants on the substrates linamarin, lotaustralin, salicin, arbutin, amygdalin and cellobiose.

The levels of crude β-glucosidase do not appear to vary greatly from one medium to another, (Table VIII). This was evidenced in that β-glucosidase in the psychrophilic basidiomycete appeared to be constitutive and its synthesis was not subject to substrate (cellobiose) induction or product (glucose) repression in the classical sense of the β-galactosidase model (Jacob and Monod, 1961).

Butler, (1965) demonstrated that the hydrolysis products of glucosidase activity on linamarin and lotaustralin from flax were glucose and α-hydroxyisobutyronitrile and glucose and methyl-ethyl-ketone cyanohydrin, respectively. That these products were also produced by fungal β-glucosidases was at least evidenced by the production of glucose. The results indicated (Table IX) that the further enzymic breakdown of α-hydroxyisobutyronitrile and methyl-ethyl-ketone cyanohydrin yielded acetone and methyl-ethyl-ketone, respectively. The gas chromatographic data from both samples also suggests that a compound retained slightly longer than CO_2 was most likely cyanide. This fact was confirmed by the presence of quantities of cyanide in both samples as determined by the picric acid technique of Boyd (1935) which is specific for cyanide. Thus, cyanide together with acetone and methyl-ethyl-ketone were the enzymic oxidation products of α-hydroxyisobutyronitrile and methyl-ethyl-ketone cyanohydrin, respectively.

The data from Table X provides evidence that the psychrophilic basidiomycete contains an oxynitrilase capable of oxidizing both methyl-ethyl-ketone cyanohydrin and α-hydroxyisobutyronitrile. Lactonitrile was similarly oxidized, however the aromatic cyanohydrins were not attacked. Seeley et al. (1966) described a hydroxynitrile lyase (oxynitrilase) of sorghum seedlings which preferentially catalyzed the oxidation of p-hydroxymandelonitrile and an almond enzyme which exhibited its maximum oxidation rate on mandelonitrile. No attempt was made by these workers,

however, to determine enzyme activity on aliphatic cyanohydrins. Becker and Pfeil (1966) describe an oxynitrilase from Prunaceae which reacts HCN with a great number of aliphatic, aromatic and heterocyclic aldehydes to yield α-hydroxynitriles. These workers have not attempted to study the reverse reaction.

The type B strain of the psychrophilic basidiomycete used in this study has been isolated in Western Canada in conjunction with winter crown rot of legumes and snow mold of turf grasses, (Ward et al. 1961). The pathological picture involves both production of HCN and invasion of the host by mycelium from the psychrophilic basidiomycete. However, mass invasion and disorganization of the growing point of alfalfa were not observed until 2½ months after the first positive test for HCN in the tissues (Lebeau et al. 1959). Mass invasion of the host by the mycelium and the highest HCN concentration in the crown tissues occurred simultaneously during March (Lebeau et al. 1959).

In light of the results of this thesis a proposed hypothetical basis of pathogenicity seems apparent. The alfalfa plant appears to be resistant to invasion by the mycelium but susceptible to cyanide poisoning.

Thus the psychrophilic basidiomycete growing in close proximity to the host plant could produce cyanide according to the schemes in Table I. The released cyanide would be absorbed by the crown tissues of alfalfa decreasing its resistance to invasion by the mycelium. Mass invasion of the host would result followed by even greater cyanide

production. Cyanide would originate from the hydrolysis of the fungal cyanogenic glucosides by fungal β-glucosidases, (Table I), but could also originate from the hydrolysis of alfalfa cyanogenic glucosides by the fungal enzymes. This latter point was substantiated by the fact that there was a rapid increase in cyanide concentration in the alfalfa crown tissue during the period of mass invasion of the host by the fungal mycelium, (Lebeau et al. 1959). The concentration found in crown tissues at this time was 500 ppm. (Lebeau et al. 1959) which was 5 times greater than the cyanide produced by the fungus in vitro (100 ppm) during a 24 hr trapping experiment, (Ward and Lebeau, 1962).

SUMMARY

The psychrophilic basidiomycete was found to use valine and isoleucine as precursors to linamarin and lotaustralin, respectively. The fungus contains 2 β-glucosidase and an oxynitrilase which acting together were capable of releasing cyanide from both linamarin and lotaustralin. The 2 β-glucosidases were purified and compared as to pH optima, K_m's, Ea's, thermal stabilities, and substrate specificities. The products of methyl-ethyl-ketone cyanohydrin and acetone cyanohydrin oxidation by the oxynitrilase were shown to be HCN together with methyl-ethyl-ketone and acetone, respectively. The oxynitrilase attacked aliphatic hydroxynitriles but showed no activity on aromatic hydroxynitriles.

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