Chemical and biological properties of a phytotoxic glycopeptide from Corynebacterium insidiosum
by Stephen Michael Ries

Abstract:
Phytotoxins produced by Corynebacterium spp. have been reported by several investigators. However, in the case of a toxin produced by C. insidiosum, the complete chemical, physical, and biological characterization has not been established. The present study demonstrates that the toxin of C. insidiosum is a glycopeptide with an empirical formula of $C_{108}H_{2260}O_{132}N$ based on one atom of nitrogen. Chemical analysis shows the toxin is 83.1% polysaccharide composed of mannose (1), glucose (5), galactose (5), and L-fucose (10), with trace amounts of rhamnose and an unidentified reducing sugar. An unknown organic acid comprises 8.8% of the toxin and a single peptide composed of lysine2, arginine1, aspartic acid1, threonine1, serine1, glutamic acid1, glycine2, alanine2, valine2, leucine2, and isoleucine1 accounts for 2.57% of the toxin molecule. The toxin has a blue chromophore due to copper chelation at a concentration of 75 moles copper/mole toxin. Sepharose 2B column chromatography, analytical ultracentrifugation, and light scattering data indicates that the glycopeptide has a molecular weight of 5×10^6 daltons. Electron micrographs of the glycopeptide shows it to be an amorphous globule 80.0 - 90.0 mµ in diameter. The toxin has a specific optical rotation of -166°, an intrinsic viscosity of 0.2307 dl/g,. and decomposes at 260°. The toxin causes wilt in both leaves and stems of test plants and exhibits no specificity between alfalfa or tomato seedlings. Biological activity of the compound is dependent on time and on concentration. The toxin is stable to wide fluctuations in pH and still retains biological activity after heating to 121° for 2 hours. Partial acid hydrolysis results in a drastic reduction of biological activity. The mechanism of action of the toxic glycopeptide is unknown but it may cause plugging of xylem vessels or membrane damage.

CHEMICAL AND BIOLOGICAL PROPERTIES OF A PHYTOTOXIC
GLYCOPEPTIDE FROM CORYNEBACTERIUM INSIDIOSUM

by

STEPHEN MICHAEL RIES

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fulfillment of the requirements of the degree

of

DOCTOR OF PHILOSOPHY

in

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

viii

ABSTRACT

Phytotoxins produced by <u>Corynebacterium</u> spp. have been reported by several investigators. However, in the case of a toxin produced by <u>C</u>. <u>insidiosum</u>, the complete chemical, physical, and biological characterization has not been established. The present study demonstrates that the toxin of <u>C</u>. <u>insidiosum</u> is a glycopeptide with an empirical formula of $C_{108}H_{226}O_{132}N$ based on one atom of nitrogen. Chemical analysis shows the toxin is 83.1% polysaccharide composed of mannose (1), glucose (5), galactose (5), and L-fucose (10), with trace amounts of rhamnose and an unidentified reducing sugar. An unknown organic acid comprises 8.8% of the toxin and a single peptide composed of $lysine_2$, $arginine_1$, aspartic $acid_1$, $threonine_1$, $serine_1$, glutamic $acid_1$, $glycine_2$, $alanine_2$, $valine_2$, $leucine_2$, and $isoleucine_1$ accounts for 2.57% of the toxin molecule. The toxin has a blue chromophore due to copper chelation at a concentration of 75 moles copper/mole toxin. Sepharose 2B column chromatography, analytical ultracentrifugation, and light scattering data indicates that the glycopeptide has a molecular weight of 5×10^6 daltons. Electron micrographs of the glycopeptide shows it to be an amorphous globule 80.0 - 90.0 mµ in diameter. The toxin has a specific optical rotation of -166°, an intrinsic viscosity of 0.2307 dl/g, and decomposes at 260°. The toxin causes wilt in both leaves and stems of test plants and exhibits no specificity between alfalfa or tomato seedlings. Biological activity of the compound is dependent on time and on concentration. The toxin is stable to wide fluctuations in pH and still retains biological activity after heating to 121° for 2 hours. Partial acid hydrolysis results in a drastic reduction of biological activity. The mechanism of action of the toxic glycopeptide is unknown but it may cause plugging of xylem vessels or membrane damage.

INTRODUCTION

Bacterial wilt of alfalfa (Medicago sativa L.), caused by

Corynebacterium insidiosum (McCull.) Jensen, is distributed widely in

North America and is probably the most important malady of the crop

in the United States. Alfalfa plants are killed so rapidly that

fields are unprofitable after 3 or 4 years. The typical symptoms of

this disease are reduction in vigor of the plants, yellowing and

bleaching of the leaves, and finally wilting and death of the plants.

The taproot shows a pale-brown discoloration of the outer woody

tissue.

Phytotoxic polysaccharides from some plant pathogenic bacteria

exert their effect on plants by causing wilting. Hodgson, Peterson,

and Riker (22, 23) and Feder and Ark (12) reported that certain poly-

saccharides produced by plant pathogenic bacteria, Agrobacterium

tumefaciens (Erw. Smith and Town) Conn, Xanthomonas phaseoli (Erw.

Smith) Dowson, and Erwinia carotovora (Jones) Holland, were phytotoxic

and possessed wilt-inducing properties. These toxins were non-specific

and of high molecular weight.

Further work on toxins produced by Pseudomonas solanacearum

(Erw. Smith) Erw. Smith (24), the causal agent of southern bacterial

wilt of several plants, indicated that the culture filtrate contained

pectolytic and cellulolytic enzymes. The heat-treated culture

filtrate contained material which also caused wilting in tomato and

tobacco cuttings. This non-enzymatic, heat-stable material was thought to cause plugging in the vessels. Stewart, in 1894 (10), stated that wilting symptoms result from plugging of conductive elements by the pathogen or its products. The mechanical plugging theory has been supported by many investigators working on various bacterial wilt diseases, including wilt of cucumber caused by *Erwinia tracheiphila*, (Erw. Smith) Holland, wilt of sweet corn caused by *Xanthomonas stewarti* (Erw. Smith) Dowson, wilt of carnation caused by *Pseudomonas caryophilli* (Burkholder) Starr and Burkholder (7) and several others. However, in the case of *Pseudomonas solanacearum*, Hutchinson in 1931 (7) reported that wilting was due to alteration in cellular permeability produced by a toxin.

Recent studies on *Corynebacterium* spp. indicate that they produce phytotoxic polysaccharides in culture media and in the plant. Spencer and Gorin (39) reported that *C. insidiosum* and *C. sepedonicum* (Speckerman and Kotthoff) Skaptason and Burkholder, produced viscous polysaccharide solutions in culture media. Partial purification showed that the polysaccharide isolated from *C. insidiosum* induced wilt in alfalfa cuttings at a concentration of 0.002%. They detected fucose, galactose, and glucose residues in the acid hydrolyzed polysaccharides and offered presumptive evidence that these polysaccharides were present in infected alfalfa and potato plants by demonstration of the

rare sugar L-fucose in them. They postulated that the mechanism of action was plugging of xylem vessels. The acidic nature of these polysaccharides was ascribed to 4,6-0-(1'-carboxyethylidene)-D-galactose (16). Patino-Mendez (32) isolated a polysaccharide from a culture of C. michiganense (Erw. Smith) Jensen, and detected fucose, galactose, glucose, mannose, rhamnose and an unidentified compound from the acid hydrolysate of the polysaccharide. He also found that a 0.01% solution of toxin caused wilting in tomato cuttings and postulated plugging as the mechanism of action. In both cases (32, 39) no attempt was made to characterize the toxins other than through their sugar components. Little attempt was made to study their physical properties, to demonstrate homogeneity, or to study their biological parameters. The complete physical, chemical, and biological characterization of these toxins was needed. Strobel (41, 43) reported that the toxin isolated from C. sepedonicum was glycopeptide in nature and possessed 3 sugars (glucose, mannose, L-fucose), 2 unidentified reducing compounds, an unidentified non-reducing compound, 9 amino acids, and 1 organic acid (2-keto-3-deoxygluconic acid). The purified toxin had a molecular weight of 21,400, was highly branched, and had an empirical formula of $C_{48}H_{96}O_{48}N$. He showed that the toxin lost biological activity after acid hydrolysis in 0.5N sulfuric acid, and he demonstrated the presence of the toxin in infected potato

plants by a number of methods including infrared and ultraviolet spectral analysis, X-ray crystallography, and high voltage paper and disc gel electrophoresis experiments. Strobel and Hess (42) proposed an alternate theory to mechanical plugging by demonstrating that membranes were being damaged by the toxin.

Johnson and Strobel (25) established the active site of the glycopeptide as the carboxyl group of the organic acid. Rai and Strobel (34, 35) reported the isolation of physiologically active compounds from C. michiganense. They reported that C. michiganense produces 3 biologically active compounds. These toxins also contained the sugar residues glucose, galactose, mannose, and fucose, and 10 amino acid residues. They postulated that the mechanism of action of these glycopeptides was not plugging but membrane damage. They also demonstrated the presence of the toxins in infected plants by sero-logical methods.

The purpose of this thesis is fourfold: (a) to define the homogeneity of the toxic preparation produced by C. insidiosum, (b) to clarify which chemical residues constitute the purified toxin, (c) to measure the physical properties of the toxin, and (d) to determine and measure various biological parameters of the toxin.

MATERIALS AND METHODS

Culturing: A culture of C. insidiosum (courtesy of F. I. Frosheiser,

University of Minnesota) was maintained on a medium containing 1.5%

glucose, 2.0% agar, 1.0% yeast extract dialysate, and 0.5% calcium

carbonate (43). The culture was streaked and taxonomically identified

as C. insidiosum (6). The organism produced wilt symptoms when

inoculated by stem wounding into a 2 year old alfalfa plant.

Preparation and purification of toxin: The organism was grown on

250 ml of liquid medium in 1 liter flasks. The culture was aerated

on a Psycrotherm Incubator Shaker at 20 rpm at 20° for 4 days. The

toxin was isolated in a manner similar to that employed by Spencer

and Gorin (15, 39), Strobel (41), and Rai and Strobel (34, 35). The

culture was centrifuged at 20,000 xg for 10 minutes and the pellet

discarded. The supernatant liquid was treated with 3 volumes of

acetone (-15°) and the resulting precipitate pelleted by centrifug-

ation at 10,000 xg for 10 minutes. The pellet was dissolved in 100

ml of distilled water and passed through a column of Dowex 1 (formate

form), 2.5 X 5.0 cm, 200-400 mesh, rinsed with 50 ml of water, and

the effluent passed through a column of Dowex 50 (H^{+} form), 2.5 X 5.0

cm, 200-400 mesh, and rinsed with another 50 ml of water. The efflu-

ent was collected and fractionated 2 times with ammonium sulfate

according to the procedure of Falconer and Taylor (11). Ammonium

sulfate (20-35%) precipitated the toxin which was removed by

centrifugation at 10,000 x g for 5 minutes and redissolved in 100 ml
of distilled water. The toxin was dialyzed against distilled water
for 4 days at 4° with many changes of distilled water. The solution
of purified toxin was stored at -15° until used.

Optimum toxin production: Aliquots of a growing culture were removed
at various time intervals. The turbidity due to growth was measured
with the Klett-Summerson Photoelectric Colorimeter, and the amount of
toxin production determined with the Beckman Laboratory Carbonaceous
Analyzer. Toxin solution (20 µl) was injected into the instrument
and the amount of carbon determined in ppm from a standard curve after
calibration of the instrument with acetic acid standards.

Biological assay: The assay procedure used was similar to that
employed by Johnson and Strobel (25). Tomato seedlings (Lycopersicon
esculentum Mill.) cultivar Earliana and alfalfa seedlings (Medicago
sativa L.) cultivars Ladak and Orca grown in vermiculite under
continuous illumination for 10-14 days were severed at the crown
region and placed in small test tubes containing solutions of the
toxin buffered with 0.05M phosphate to a final pH of 7.0. The test
tubes were mounted in a plexiglass holder surrounded by a transparent
plexiglass box. The top of the box served as a reservoir for water
to prevent heating by an artificial light source (100W bulb and a

circular fluorescent bulb) placed above it. . After given time intervals the cotyledons were removed with a sharp razor blade and the strength of each stem was determined using the wilt-o-meter. This instrument applies a steadily increasing force against a stem until it is no longer able to maintain an erect position. The degree of wilting in any given treatment was the average of three readings obtained from each of 5 stems.

Radioactive methods: C. insidiosum was grown on the standard liquid medium containing 25µC of D-galactose-1-^{14}C (specific activity 3.0 mC/mM). The radioactivity in the toxin was determined in the liquid scintillation spectrometer (Model 6804 Nuclear Chicago). The toxin solution (10-20 µl) was placed in a vial along with 1.5 ml of absolute methanol and 13.5 ml of scintillation solution containing 4.0 g of .2,5-diphenyl-oxazole and 100 mg of p-bis-2(5-phenyloxazolyl)-benzene per liter of toluene. The channels ratio method was used to correct for quenching. Autoradiography was performed using Kodak No-screen X-ray film, and electrophoretograms were scanned with a Packard Radio-chromatogram Scanner (Model 385). Scanning was performed at 0.5 cm/min with a colliminator width of 5.0 mm, a time constant of 10, a linear range of 300, and a gas flow rate of 350 cc/min.

<u>Molecular exclusion chromatography</u>: The toxin was taken to dryness and 1-5 mg dissolved in 1 ml of 0.05M Tris buffer at pH 7.0 in 40% sucrose. The sample was then fractionated with the buffer through columns of Sepharose 4B or Sepharose 2B. Fractions (1.5 ml) were collected with the aid of a drop counter, and radioactivity and total carbon determined in each fraction. Attempts to dissociate labelled toxin into subunits with 30% pyridine (36) or 8M urea followed by dialysis against distilled water for 48 hours was also followed by fractionation on Sepharose 2B.

<u>Electrophoresis</u>: High voltage paper electrophoresis was conducted in a manner similar to that employed by Strobel (42). One-hundred μg of toxin dissolved in 20 μl of water was distributed over 25 cm in the center of a 34 X 40 cm piece of Whatman #1 filter paper. The paper was pressed between a lower water-cooled plate and an upper surface covered by a flexible plexiglass plate. Pressure was exerted to the upper plate by a water filled rubber bag with a pressure of 6 pounds per square inch. The temperature of the water in the bag was 4°. The molarity and pH of the buffers used were varied in these experiments. All experiments were conducted with a 22.5 v/cm potential for 2 hours. The toxin was detected by the methods of Trevelyan (45), with 0.3% ninhydrin in ethanol, for radioactivity with the chromatogram scanner, and visually for the chromophore.

Disc gel electrophoresis was performed in 5.0% polyacrylamide gels with glycine-Tris buffer, pH 8.8. Fifty μg of labelled sample in 40% sucrose was subjected to 2.5 ma in each acrylamide tube (8). After electrophoresis for 30 minutes, the toxin was detected with aniline blue black for proteins, Schiff's base reagent for carbohydrates (33), for the chromophore, and by autoradiography after drying the gel according to the procedure of Herrick and Lawrence (20). Gels developed by each technique were scanned on a Joyce Chromoscan Densitometer.

Analytical ultracentrifugation: The sedimentation coefficient of the toxin was determined with a Spinco Model E analytical ultracentrifuge using the schleiren optical system on 1.0, 2.6, and 5.0 mg/ml solutions of toxin. Centrifugation was performed at 64,000 rpm and photographs were taken at 32 min intervals with a bar angle of 50°. The diffusion coefficient was determined by creating a synthetic boundary at 8,000 rpm with solutions of the same concentrations. The partial specific volume of the toxic polysaccharide was assumed to be 0.65 (14). The sedimentation coefficient, diffusion coefficient, and molecular weight calculations were calculated by the method of Schachman (38).

Light scattering: The molecular weight of the toxin was determined with the Brice Pheonix Universal Scattering Photometer (Series 2000)

using light with a wavelength of 4358Å on a series of 6 solutions

ranging from 0.035% - 0.090%. Calculations were based on those of

Anacker (2). Double extrapolations to zero angle and zero concen-

tration from the resulting Zimm plot yielded the molecular weight.

Specific viscosity: A Cannon-Ubbelohde semi-micro viscometer, Model

75 (Cannon Instrument Co.), was used to determine the specific

viscosity at concentrations of 2.6, 1.3, 0.87, 0.65, 0.52, 0.43, and

0.22 mg/ml solutions. The intrinsic viscosity was the intercept of

the graph of specific viscosity/concentration as a function of

concentration (38).

Elemental analysis: Carbon, hydrogen, and oxygen were determined by

Schwarzkopf Microanalytical Laboratory, Woodside, New York; nitrogen

was determined by the microkjeldahl technique. From the percentages

of these the empirical formula was calculated.

Chromophore: Five ml of 0.1% solution of toxin was treated with 0.01M

ethylenediamine tetraacetic acid (EDTA) at 50° for 15 minutes. The

toxin and apotoxin were then dialyzed against distilled water for 48

hours after which the ultraviolet and visible spectrum (240-750 mμ)

were measured on a Cary Split-beam spectrophotometer using a 3 ml

cuvette with a 1 cm light path.

A 0.1% solution was assayed to determine the presence of metal ions which were measured on a Jarell-Ash atomic absorption flame emission spectrophotometer by removing the ion with EDTA and then separating the EDTA-metal-ion complex from the toxin by dialysis.

Acid hydrolysis: Twenty ml of 0.5N sulfuric acid was added to a 50 ml flask containing 50 mg of toxin. The toxin was refluxed for 8-12 hours. The mixture was cooled, diluted with 100 ml of distilled water, and neutralized with excess barium carbonate. The precipitate was removed by centrifugation and the supernatant liquid passed through a column of Dowex 50 (H^+ form), 0.5 X 2.0 cm, 200-400 mesh, followed by a 10 ml rinse of water and passage through a column of Dowex 1 (OH^- form), 0.5 X 2.0 cm, 200-400 mesh. The Dowex 1 column was rinsed with 10 ml of water and the effluent dried under vacuum desiccation over P_2O_5. This was considered the neutral fraction. The organic acid fraction was obtained by eluting the Dowex 1 column with 10 ml of 6N formic acid. The eluant was dried and stored in an evacuated desiccator over P_2O_5.

The procedure used for amino acid hydrolysis was that of Moore and Stein (30). Toxin (5-10 mg) or peptide (0.25 mg) were placed in constant boiling hydrochloric acid, a crystal of phenol was added, and the tube sealed under vacuum. The tube was heated for 20 hours at 110°. Upon cooling the contents of the tube were dried by flash

evaporation at 50° and stored under vacuum desiccation over sodium

hydroxide pellets. This preparation was taken up in 5 ml of distilled

water and passed through Dowex 50 (H^{+} form), 0.5 X 1.0 cm, 200-400

mesh. A 5 ml distilled water rinse followed, and the amino acids were

then eluted with 6N hydrochloric acid and dried as above.

Chromatography: Separation and identification of sugar residues was

done according to the method of Albersheim, et al. (1). The sugars

were reduced to their alditols with sodium borohydride in 1N ammonium

hydroxide. The reaction was stopped by the addition of a slight

excess of glacial acetic acid and borate removed by 5 methanol evap-

orations. Acetic anhydride (1 ml) was added, the tubes containing

the sugar alcohols sealed, and heated for 3 hours at 121°. The

resultant alditol acetates were identified and their amounts estimated

by gas-liquid chromatography. The best separation was attained using

Gas Chrome P (100-120 mesh) coated with 0.2% poly(ethyleneglycol

succinate), 0.2% of poly(ethyleneglycol adipate) and 0.4% of silicone

XF 1150. The F and M gas chromatograph electrometer was set at range

100 and attenuation 4. A 6 foot column was used which was heated to

120° for 10 minutes after injection and then raised 1° per minute to

190°. The detector temperature was 250°, and the carrier gas flow

rate was 30-50 cc per minute. The amount of each sugar was determined

by a comparison with standard curves established with authentic sugars.

Separation of organic acid residues was done by one-dimensional, descending, paper chromatography, on Whatman #1 filter paper in the following solvent systems: (A) 1-butanol:acetic acid:water (4:1:5) v/v, (B) 1-butanol:pyridine:0.1N hydrochloric acid (5:3:2) v/v, (C) ethyl acetate:acetic acid:water (3:1:3) v/v, (D) 2-butanone:acetic acid:water (8:1:1) v/v, (E) 80% phenol v/v, and (F) 1-propanol: cineole:formic acid (5:5:2) v/v.

Identification and quantification of amino acid residues was done on a Beckman automatic amino acid analyzer.

Peptide: The β-elimination procedure described by Anderson, et al. (3) and Tanaka and Pigman (44) to remove peptides from the glycoprotein was used. Toxin (40 mg) was placed in 20 ml of 0.5N sodium hydroxide and incubated for 216 hours at 4° in 0.3M sodium borohydride. The sodium borohydride reaction was stopped by the addition of a slight excess of acetic acid, and borate removed as methyl borate by 5 methanol evaporations. The solution was neutralized with sodium hydroxide and passed through a column of Dowex 50 (H^+ form), 1.0 X 5.0 cm, 200-400 mesh. The retained peptide was then eluted with 6N hydrochloric acid and dried over sodium hydroxide.

Peptide (1 mg) was spotted on a 34 X 40 cm piece of Whatman #1 filter paper and subjected to electrophoresis for 3 hours with 12.5 v/cm potential across the paper in formic acid (0.54M) acetic acid

(1.11M) buffer, pH 1.85 (5). This same electrophoretogram was dried
and chromatographed in solvent (A) for 10 hours in the other dimension.
The peptide was detected with a modified ó-tolidine procedure. The
electrophoretogram was passed through acetone:ethanol (1:1) solution
and while still moist suspended over a solution of 1N hydrochloric
acid and 0.5N potassium permanganate for 5 minutes on each side. The
chromatogram was passed through a solution of 0.5M potassium iodide
mixed 1:1 with saturated o-tolidine in 2N acetic acid.

<u>NH$_2$-Terminal amino acids</u>: The dansyl technique, described by Gray
and Hartley (18) was used to determine the NH$_2$-terminal amino acid
in the peptide. One mg of the peptide and 25 mg of sodium bicarbonate
were dissolved in 0.7 ml of water and the solution adjusted to pH 8.2.
One-tenth ml of a solution containing 1 mg of dansyl chloride per ml
of acetone was added and the solution was incubated for 12 hours at
room temperature. After drying, followed by acid hydrolysis in 1.0
ml of constant boiling hydrochloric acid for 12 hours, the sample was
compared against a series of standards by thin-layer chromatography
on Absorbosil #5 in (a) benzene:pyridine:acetic acid (16:4:1) v/v, and
in (b) toluene:pyridine:ethylene chlorohydrin:0.8N ammonium hydroxide
(10:3:6:6) v/v, upper phase. The dansylated amino acids were detected
by ultraviolet light.

<u>Electron microscopic study</u>: A 0.1% solution of toxin was frozen in Freon 22 in the Balzars Apparatus (BA 360M) and freeze-etched as described by Moor (29) and Moor and Muhlethaler (28). The replicas were prepared for examination in the electron microscope as described by Hess and Stocks (21) and examined on a Hitachi HS7 electron microscope.

EXPERIMENTAL RESULTS

Purification and biological activity of toxin: Figure 1 is a flow

chart illustrating the procedure used to isolate the toxin. The toxin,

represented by the vertical axis, was exocellular, precipitated with

3 volumes of cold acetone, and was not retained on Dowex 1 or 50.

The effectiveness of each step in removing contaminating materials is

shown in Table I. On a specific biological activity basis the purifi-

cation factor was 5.57X. The culture produced 1.25 g of toxin per

liter.

The tomato seedling assay developed by Johnson and Strobel (25)

was valid for use with this toxin. Tomato seedlings (Figure 2) wilted

to 100 mg stem strength in a 0.25% solution of buffered toxin in 1

hour when measured on the wilt-o-meter, while both cultivars of alfalfa

checked wilted to values of 200 mg. The resistant cultivar of alfalfa

(Ladak) wilted to the same degree as the susceptible cultivar (Orca)

which implied resistance to the disease is apparently not related

to the toxin.

Wilt was dependent on concentration (Figure 3) with measurable

wilt occurring in a 0.02% (4×10^{-8}M) solution of toxin and maximal

wilt in 0.15% (3×10^{-7}M) solution of toxin in 1 hour. Wilt of the

plant cuttings was a function of time and also concentration up to

0.25% toxin solutions (Figure 4).

The toxin still contained contaminating materials after passage

Figure 1.   Purification procedure for the toxin produced by

C. insidiosum. The toxin was isolated according to

the procedure shown. Location of biological activity

was determined using the tomato seedling assay. The

toxin is exocellular, precipitates with acetone, is

not retained on Dowex 1 or 50, and precipitates in

the 25-35% fractions with ammonium sulfate.

Growth of Culture

↓

Centrifuge out Cells
and $CaCO_3$

↓

Acetone Precipitation ————————————→ Supernatant
(dissolve ppt. in H_2O)   (discard)

↓

Dowex 1 (formate form) ————————————→ Eluate
                                                              (discard)

↓

Dowex 50 (H^+ form) ————————————→ Eluate
                                                            (discard)

↓

$(NH_4)_2SO_4$
fractionation

↓   ↓   ↓

0-25%   25-35%   35-100%
(discard)   (discard)

↓

$(NH_4)_2SO_4$
fractionation

↓   ↓   ↓

0-25%   25-35%   35-100%
(discard)   (discard)
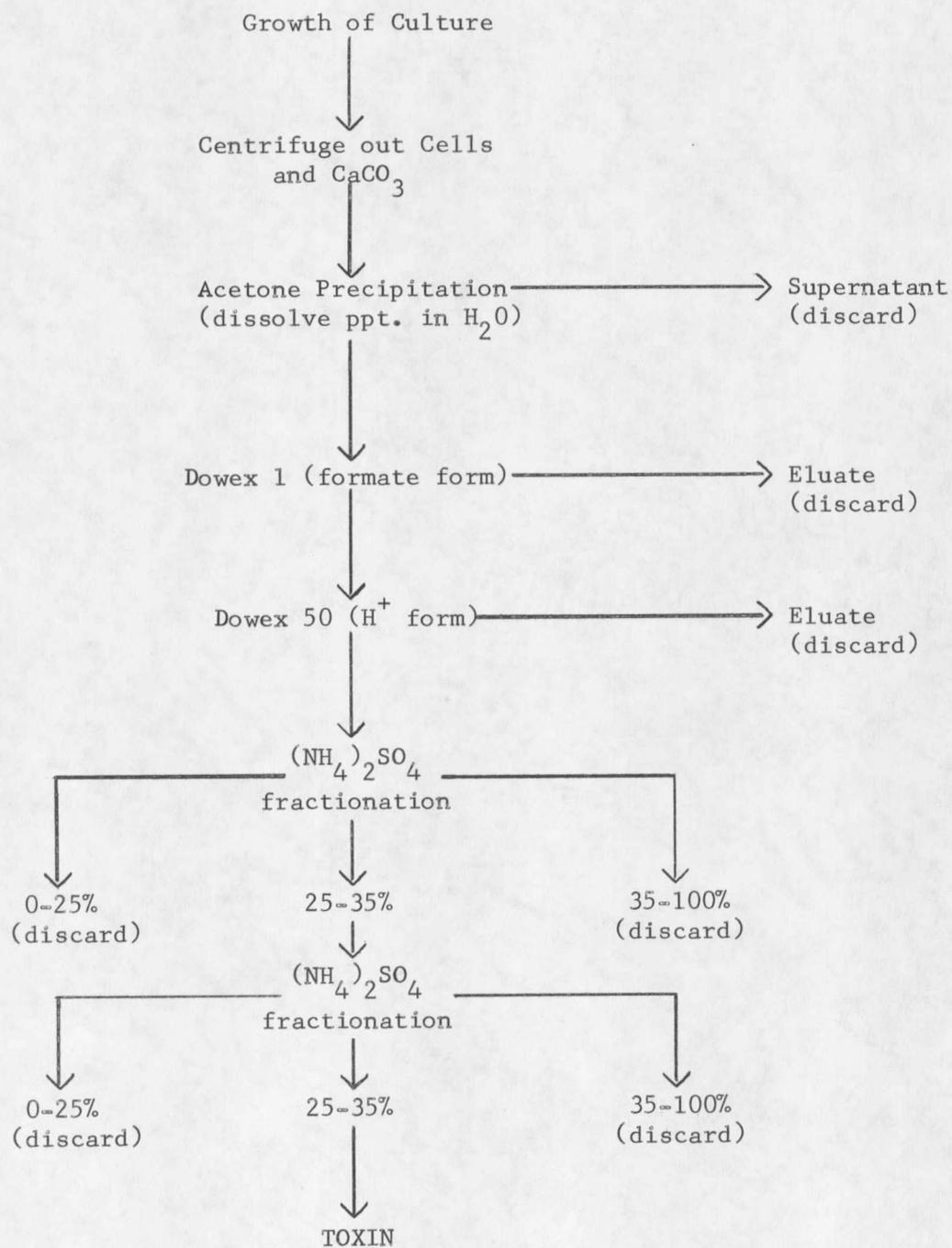
↓

TOXIN

Table I

Effectiveness of each Step in the Purification Scheme

in the Isolation of the Toxic Glycopeptide

Step in purification[a]	Dry weight	Specific Biological Activity[b]	Purification Factors
	g	mg	
Crude culture supt.	6.78	418	1.00
Acetone precipitate	2.62	285	1.47
Dowex 1 treatment	2.17	238	1.76
Dowex 50 treatment	1.93	219	1.91
1st $(NH_4)_2SO_4$ ppt.	1.39	127	3.29
2nd $(NH_4)_2SO_4$ ppt.	1.25	75	5.57

[a] For each step in the purification procedure, the total dry weight, the specific biological activity, and the purification factor based on specific biological activity were determined. The data are based on 1 liter of crude culture supernatant liquid as the starting material.

[b] Defined as the amount of strength of a tomato hypocotyl remaining after 1 hour treatment in a 1 mg/ml solution of each preparation as measured by a wilt-o-meter. Buffer controls had a stem strength of 523 mg.

20

Figure 2.   <u>Effect of toxin solutions on alfalfa and tomato</u>

<u>seedlings</u>. The toxicity of the compound was measured

on two cultivars of alfalfa; Ladak, a resistant

cultivar; Orca, a susceptible cultivar, and one

cultivar of tomatoes (Earliana). Stem wilt was measur-

ed with the wilt-o-meter in mg of pressure and the

degree of wilt measured at time intervals of 0, 15,

30, 45, and 60 minutes. Each point represents the

average of three readings of five cut stems.

          ● - Orca cont.

          O - Orca

          ■ - Tomato cont.

          □ - Tomato

          ▲ - Ladak cont.

          △ - Ladak
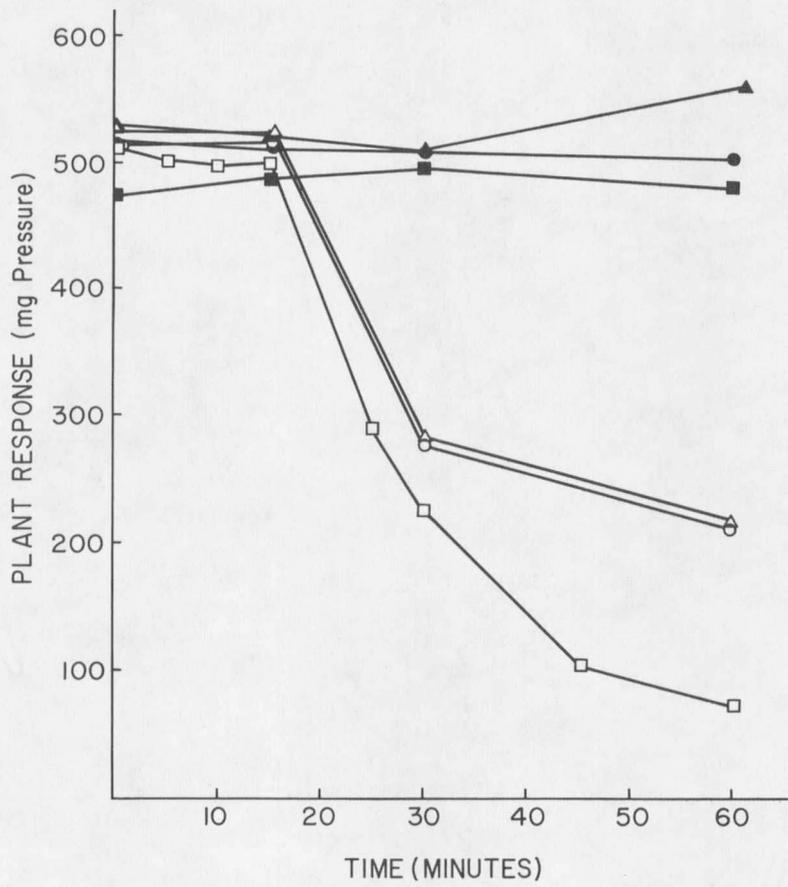
Figure 3.   Relationship of toxin concentration and the degree

of wilt. Tomato seedlings were placed in 0.005,

0.01, 0.02, 0.03, 0.04, 0.05, 0.10, 0.15, 0.20, 0.25,

and 0.50% solutions of toxin in 0.05M phosphate buffer,

pH 7.0. Loss of stem response was measured on the

wilt-o-meter after 1 hour. Buffer control excised

stems gave a wilt-o-meter value of 480 mg.

PLANT RESPONSE (mg Pressure)

TOXIN CONCENTRATION (%)

BUFFER CONTROL 0.005 0.01 0.02 0.03 0.04 0.05 0.10 0.15 0.20 0.25 0.50

24
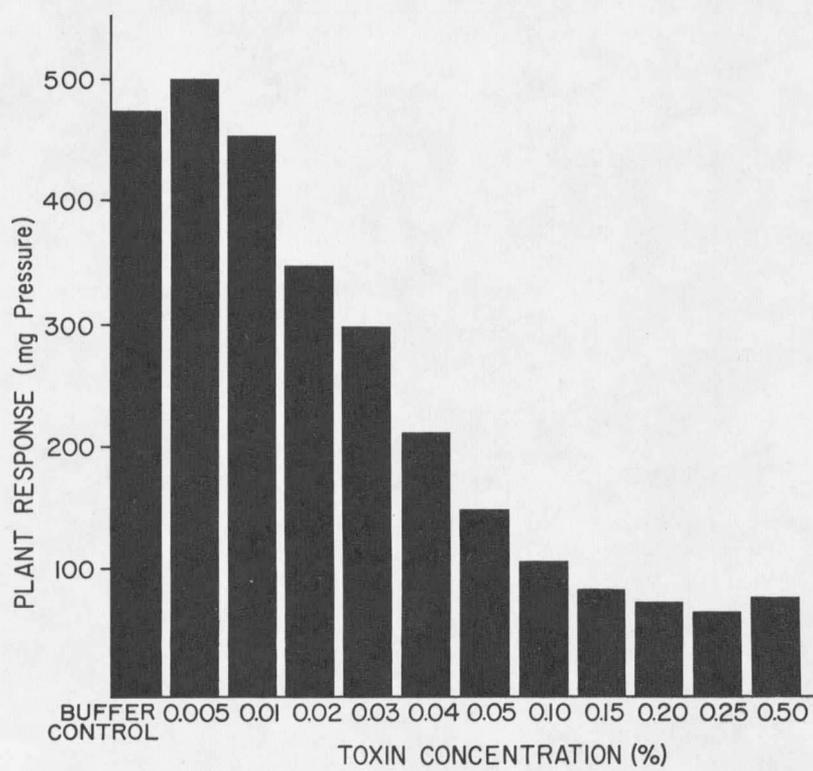
Figure 4.   <u>Effect of glycopeptide concentration as a function</u>

<u>of time</u>. Degree of wilt measured with a wilt-o-meter,

in mg pressure, of 14 day old tomato seedling stems

as a function of time in minutes.

▲ - control

○ - 0.02%

△ - 0.05%

□ - 0.10%

● - 0.25%

■ - 0.50%

through Dowex 1 and 50 ion exchange columns. Figure 5 demonstrates
that when labelled toxin with a specific activity of 3500 dpm/mg was
fractionated with ammonium sulfate, a labelled precipitate occurred
at the 25-35% fraction. The biological assay established that this
was the toxin. Considerable radioactivity, probably labelled contam-
inants, remained in the supernatant liquid. When the precipitate was
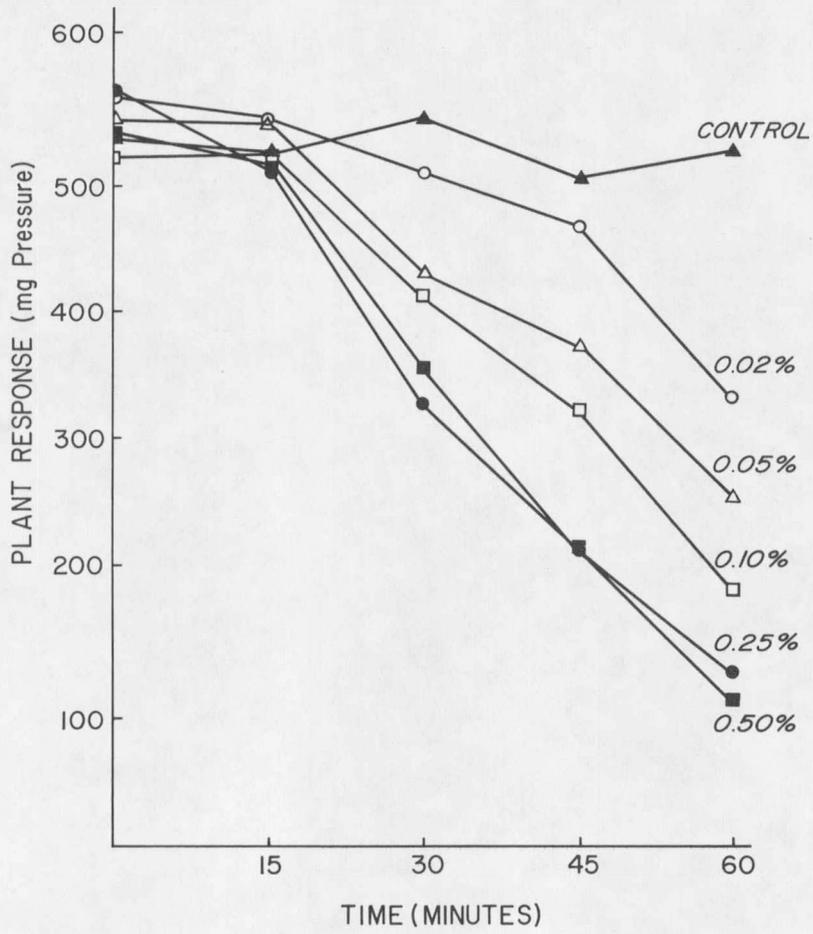redissolved in water, dialyzed, and fractionated again with ammonium
sulfate (Figure 6), all radioactivity and biological activity pre-
cipitated in the 30-40% fraction.

Optimum toxin production: A culture flask (500 ml) was inoculated
with C. insidiosum and, at time intervals of 0 through 10 days, 5 ml
aliquots were removed. After removal of calcium carbonate by settling,
bacterial growth was estimated with a Klett-Summerson photoelectric
colorimeter (Figure 7). The growth curve is typical of that exhibited
for most bacteria with maximal growth occurring at 6 days. From the
same culture 2 ml samples were taken at 0, 1, 2, 3, $4\frac{1}{2}$, 6, 7, 8, 9,
and 10 days. The toxin was obtained from these samples, dissolved in
1 ml of distilled water, and 20 μl injected into the Beckman Laboratory
carbonaceous analyzer. The total amount of toxin fluctuated with
culture age (Figure 7) reaching a peak amount at $4\frac{1}{2}$ days. When
correlated with the growth curve maximal toxin production occurred at
maximal growth. As the growth declined so did the amount of toxin.

Figure 5.   First ammonium sulfate fractionation. Labelled

toxin after Dowex treatment was fractionated with

5% of saturation increments of ammonium sulfate.

After each addition any resultant precipitate was

removed by centrifugation, checked for biological

activity, and radioactivity in the supernatant

measured.   Radioactivity in dpm/ml was plotted against

percent of saturation with ammonium sulfate.
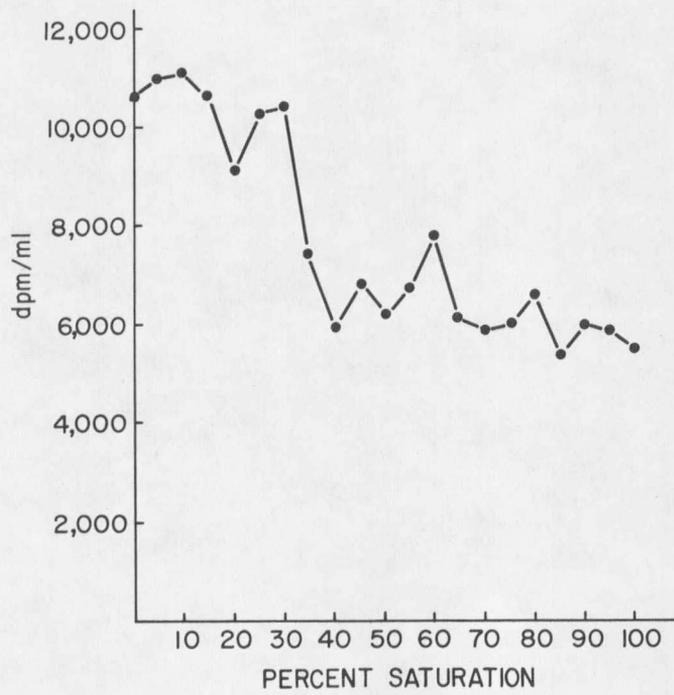
Figure 6.   Second ammonium sulfate fractionation. Labelled

toxin was treated as described in Figure 2. All

radioactivity and biological activity precipitated

in the 30-40% of saturation fraction.

31
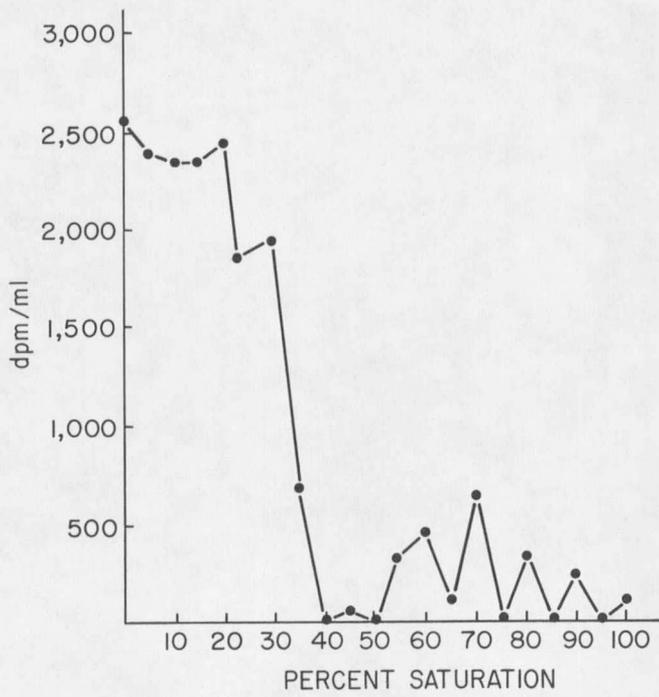
Figure 7.   Toxin production in relationship to growth of C.

insidiosum. Turbidity of a culture after removal

of calcium carbonate by settling is plotted versus

the age of culture in days after inoculation. The

amount of purified toxin in µg C/ml produced is also

plotted against the same time intervals.

▲ - growth curve

● - amount of toxin
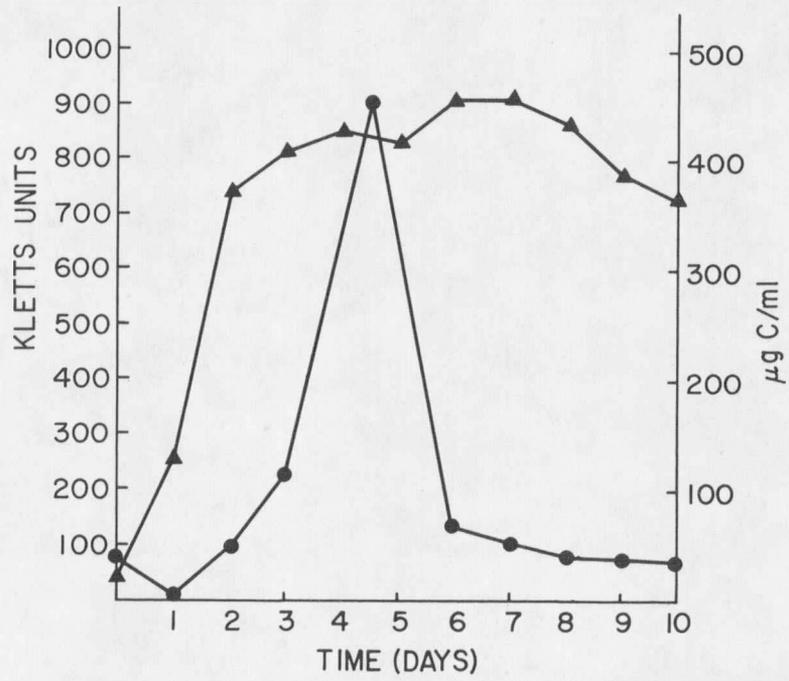
Sepharose chromatography: Molecular exclusion column chromatography

is frequently used as a criterion of homogeneity and in the estimation

of molecular sizes of compounds. A 1 mg/ml solution (3500 dpm) of

labelled toxin in 40% sucrose was layered on a Sepharose 4B column

(1.5 X 45 cm) which fractionated molecules ranging in size from 3 X

10^5 to 3 X 10^6 daltons. The toxin was eluted with 0.05M Tris buffer

at pH 7.0 and 1.5 ml fractions collected. It was located by radio-

activity determinations. A solution of Pharamacia blue dextran with

a molecular weight of 2 X 10^6 daltons was passed through the same

column and assayed by measuring absorbance at 625 mμ with a spectro-

photometer. Figure 8 demonstrates that the toxin was excluded at the

void volume, indicating its molecular weight was larger than 3 x 10^6

daltons. The blue dextran, however, exhibited an unexpected elution

profile. A broad peak appeared at fraction 35 and a sharp peak at

the void volume. This was explained in that the dextran was homo-

geneous but polydisperse and the tail of the broad peak was being

excluded at the void volume.

A 2 mg/ml solution of the labelled toxin (7,000 dpm) was layered

on Sepharose 2B which fractionates molecules ranging in size from 2 X

10^6 to 20 X 10^6 daltons. Fractionation conditions were the same as

with Sepharose 4B. Figure 9 shows a pattern similar to blue dextran

on Sepharose 4B. The two peaks, one at the void volume and the other

Figure 8.   Column chromatography of ^{14}C-labelled toxin and

blue dextran on Sepharose 4B. One mg of purified

toxin (dashed line) was layered on the column and

eluted with pH 7.0 buffer. Fractions (1.5 ml) were

collected and 0.2 ml aliquots counted by liquid

scintillation spectroscopy. The total dpm in each

tube is plotted against fraction number. Ten mg

of blue dextran (solid line) was fractionated as

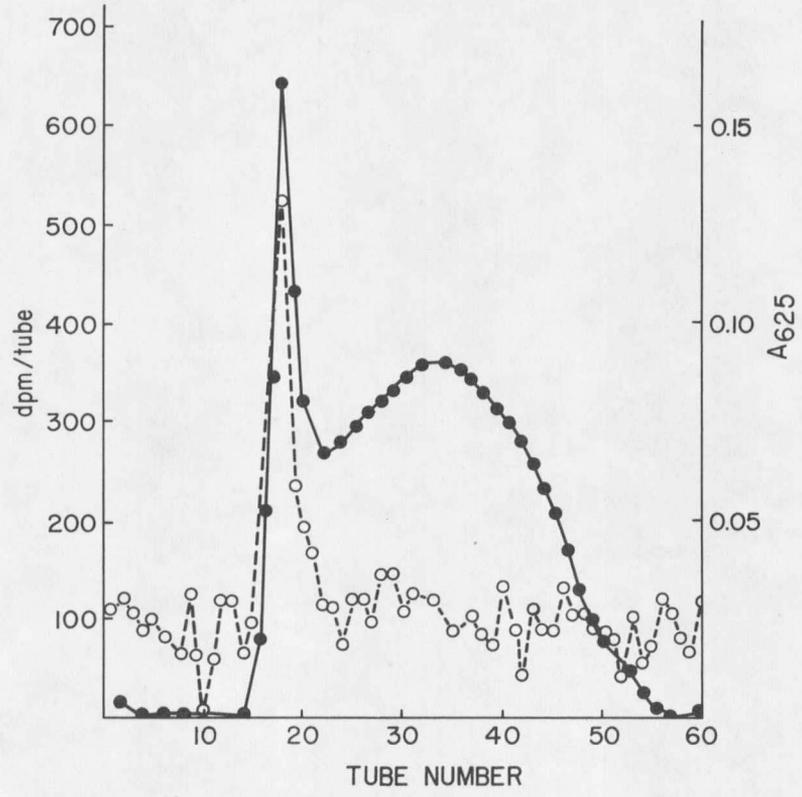above and the absorbance at 625 mμ plotted against

fraction number.

Figure 9.   Column chromatography of ^{14}C-labelled toxin on

Sepharose 2B. Toxin preparation (2 mg/ml) was

placed on the column and eluted with 0.05M Tris

buffer, pH 7.0. Fractions (1.5 ml) were collected

and 0.2 ml aliquots counted. Dpm per tube is graph-

ed versus fraction number.
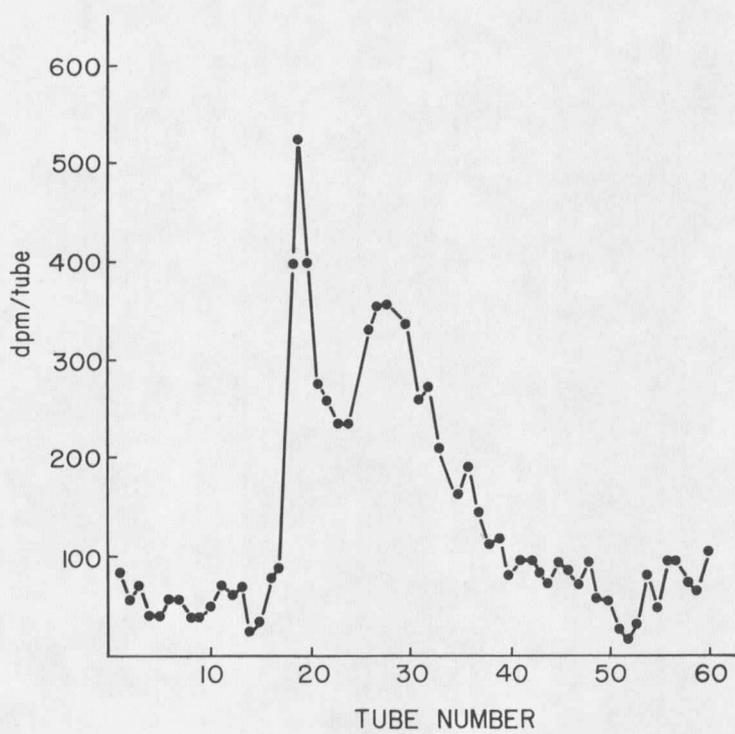
at fraction 28 probably represent the same material with the tail of the second peak being excluded at the void volume. The molecular weight of the toxin is somewhere between 2 and 20 million daltons and it is probably polydispersed as was the blue dextran.

Electrophoresis of toxin: Figure 10 demonstrates that the toxin migrated only into the top of the polyacrylamide gel. All label remained at the top, as did the chromophore, the peptide portion, and the carbohydrate moiety. Electrophoresis for longer periods of time also failed to cause migration.

High voltage paper electrophoresis of the toxin failed to move the toxin toward either pole. Figure 11 shows that at pH 7.0, 0.2M phosphate buffer, neither the chromophore, carbohydrate, protein, nor radioactivity migrated from the origin. Similar experiments at pH 2 and pH 10 failed to cause migration of the toxin.

Ultracentrifugation: Figure 12 is the ultracentrifugal pattern of a 2.6 mg/ml solution of the polysaccharide after sedimentation for 3 hours at 64,000 rpm. It indicated that the toxin was heterogeneous, a spur peak appeared to the right of the major peak. The ultra sharp characteristic of the slower sedimenting peak does not necessarily indicate homogeneity but may reflect impurity or polydispersity (14, 38).

Figure 10.   <u>Disc gel electrophoresis on toxin.</u> Labelled toxin

(50 μg) was layered on each of four polyacrylamide

gels (5%) and subjected to electrophoresis for 30

minutes at 2.5 ma/tube. Components of the toxin

were located by autoradiography, and with a densi-

tometer for the chromophore, the peptide (amido

schwartz) and the carbohydrate (Schiff base).

AUTORADIOGRAM
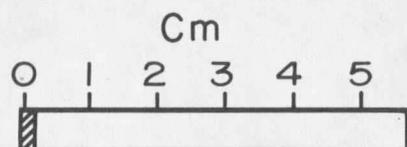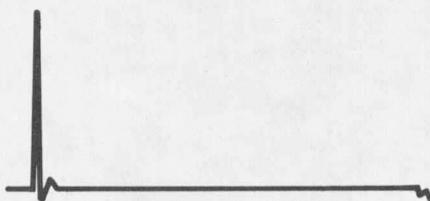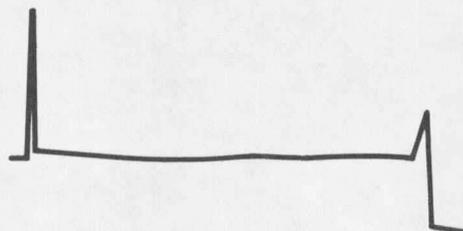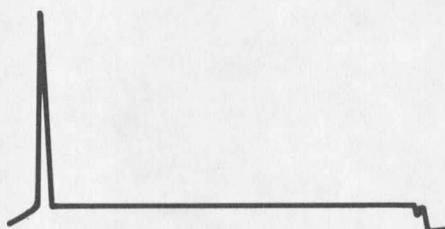
Figure 11.   High voltage paper electrophoresis on the toxin.

Toxin (100 µg) was subjected to electrophoresis

at 22.5 v/cm for 2 hours in 0.2M phosphate buffer

at pH 7.0. The electrophoretogram was developed

with basic AgNO$_3$ (A), ethanolic ninhydrin (B),

visual observation (chromophore), and radio-

chromatoscan tracing (C).

C

10   8   6   4   2   *Origin*   2   4   6   8   10

B   Cm   Cm

A   + Pole   − Pole
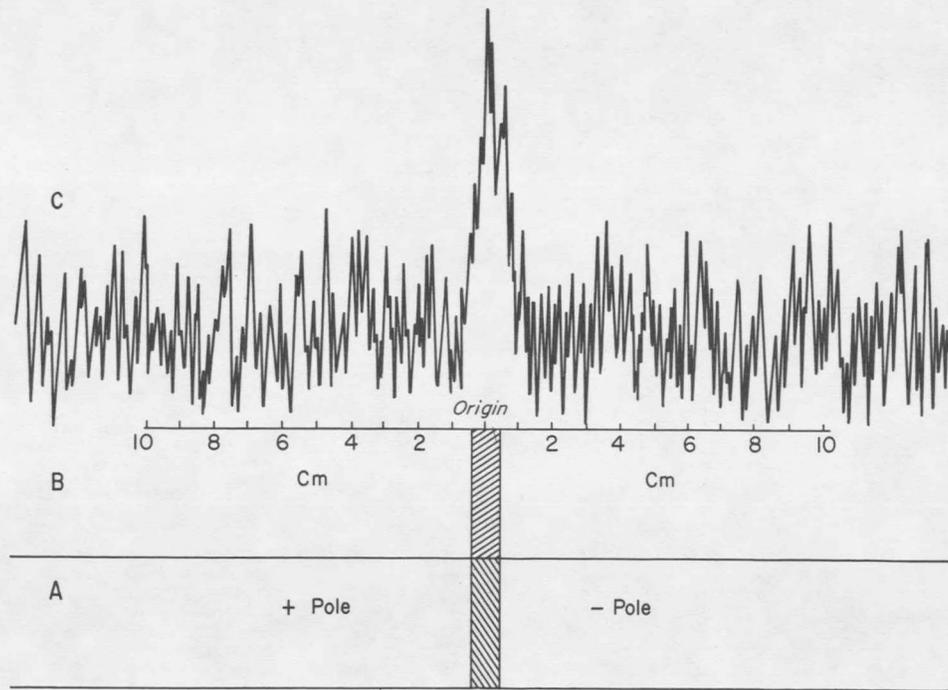
Figure 12.   Ultracentrifugation pattern of the purified glyco-

peptide.   A 2.6 mg/ml aqueous solution of the toxin

was centrifuged at 64,000 rpm with a bar angle of

50° for 2 hours.   Sedimentation is left to right.
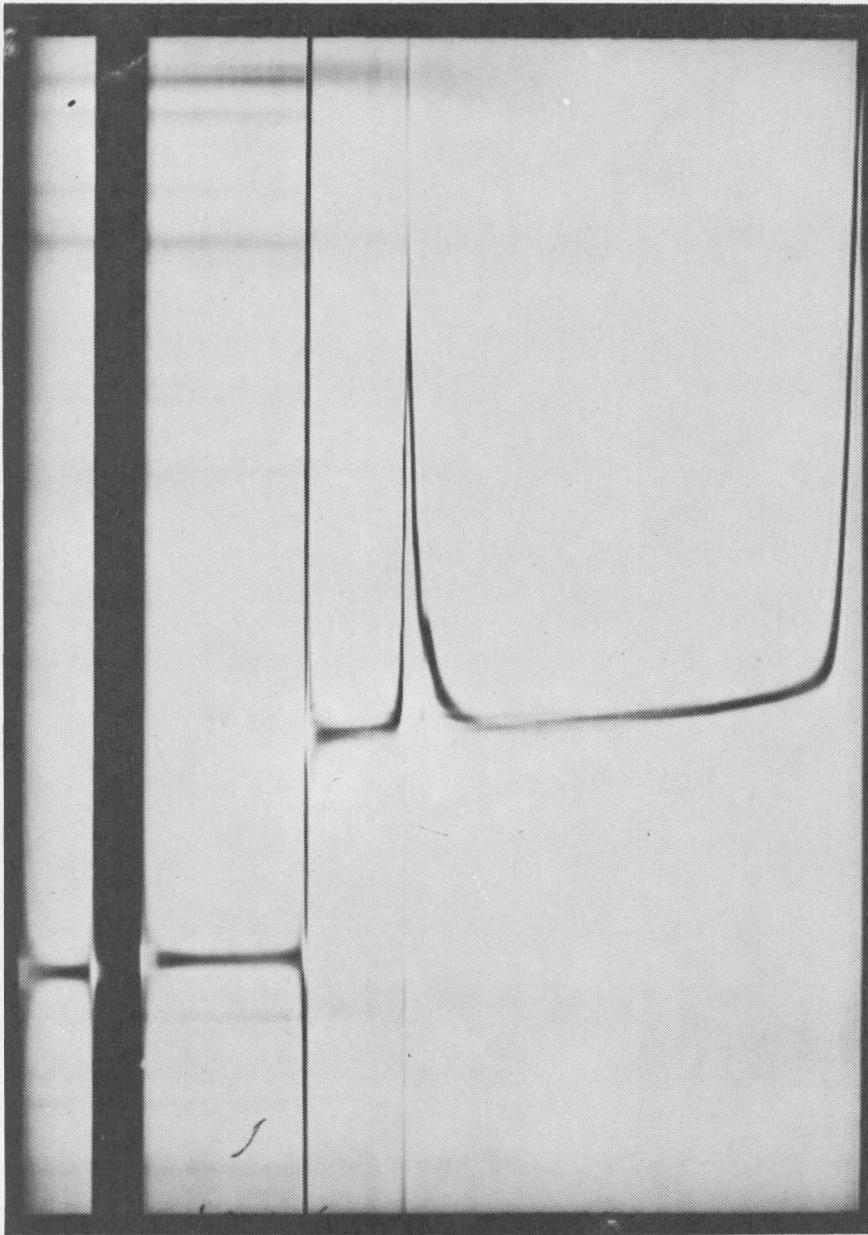
Calculations with a series of solutions (1.0, 2.6, and 5.0 mg/ml) showed that the sedimentation coefficient ($S_{20,w}$) was 1.533s. Synthetic boundary experiments at 8,000 rpm determined the diffusion coefficient ($D_{20,w}$) was 11.88 X 10^{-10} cm^2/sec. Assuming a partial specific volume of 0.65 (14), calculation of the molecular weight was 4.83 X 10^6 daltons.

Light scattering: The Zimm plot from light scattering studies is illustrated in Figure 13. Extrapolation to zero concentration (line a) and analysis by the method of least squares to determine the y intercept indicated the toxin had a molecular weight of 5.69 X 10^6 daltons. The extrapolation to zero angle (line b) and least square analysis to determine the y axis intercept indicated a molecular weight of 4.33 X 10^6 daltons.

Elemental analysis: Analysis of the compound indicated 29.52% carbon, 5.14% hydrogen, 48.02% oxygen, and 0.32% nitrogen. Therefore the empirical formula for the toxic glycopeptide was $C_{108}H_{226}O_{132}N$ based on one atom of nitrogen (Table II). The toxin decomposed when heated to 260° in a Fisher-Johns melting point apparatus. The intrinsic viscosity was 0.2307 dl/g and its specific optical rotation was $[\alpha]_{5460}^{34.5^o} = -166^o$ using a Circle Polarimeter 0.01^o with a mercury lamp.

Figure 13.   <u>Light scattering (Zimm) plots for aqueous solutions</u>

<u>of toxin.</u>

Lines a: results of extrapolation at constant

θ to $c = 0$

Lines b;   results of extrapolation at constant

c to $\theta = 0$
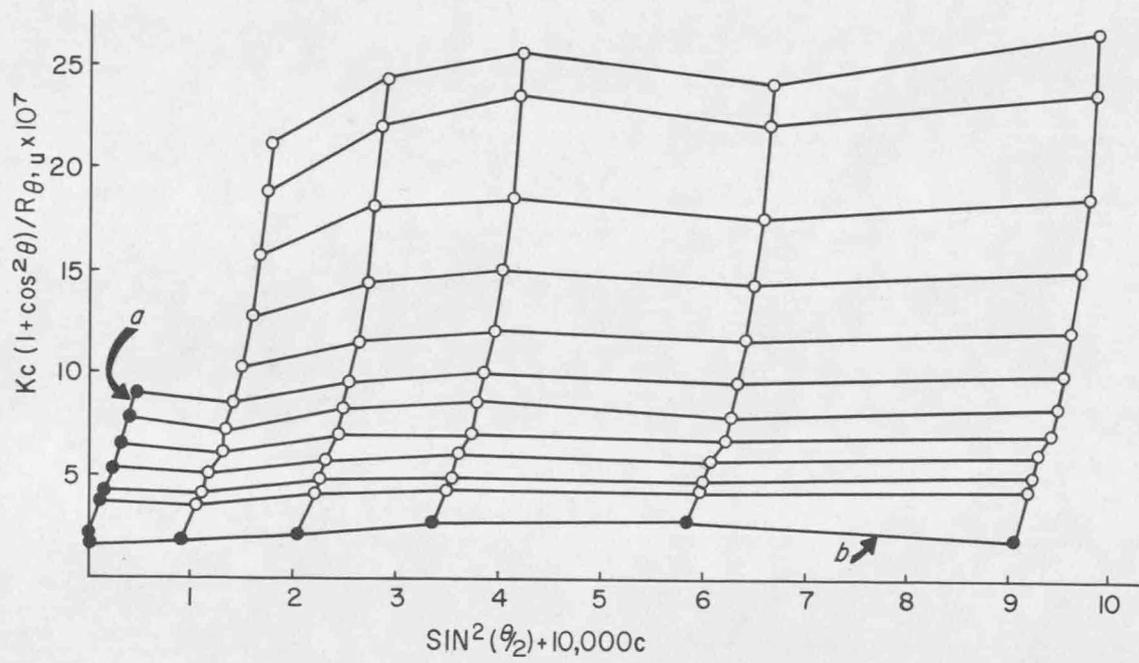
Table II

Chemical and Physical Characteristics of the
Toxin of C. insidiosum

Empirical formula	$C_{108}H_{226}O_{132}N$
Estimated molecular weight	5,000,000 daltons
Intrinsic viscosity	0.2307 dl/g
Optical rotation $[\alpha]_{5460}^{34.5°}$	$-166°$
Sedimentation coefficient	1.533s
Melting point	decomposed at 260°

Electron microscopy: Electron microscopic examination after freeze-
etching (Figure 14) demonstrates that two different size toxin mole-
cules are present. Particles (P) were amorphous globules and 80.0 -
90.0 mµ in diameter. Particles (P') appear as aggregates of the
smaller particles.

Dissociation of toxin: The large molecular weight calculated by
ultracentrifugation and light scattering data indicated the possibility
that the large compound (Figure 14) might be composed of a series of
subunits weakly bonded together. This was investigated by treating
1 mg (3500 dpm) quantities of toxin with either 30% pyridine or 8M
urea for 1 hour and dialyzing to remove the pyridine or urea. These
solutions were concentrated to 1 mg/ml and layered in 40% sucrose on
a Sepharose 2B (1.5 X 43 cm) column. Fractions (1.5 ml) were
collected using 0.05M Tris buffer, pH 7.0, and radioactivity detected.
The toxin did not dissociate into lower molecular weight subunits
with either treatment because the elution pattern from Sepharose 2B
was the same as that of non-treated toxin.

Chromophore: A 0.1% solution of toxin absorbed maximally at 280 mµ
and 635 mµ (Figure 15). The absorbance in the visible spectrum was
attributed to the blue chromophore. Treatment with ethylenediamine
tetraacetic acid (EDTA) followed by dialysis altered absorbance of

50

Figure 14.   Electron micrograph of freeze-etched replicas of

toxin. A 0.1% solution of glycopeptide was freeze-

etched and examined with the electron microscope.

Particles (P) observed and measured were 80.0 - 90.0

mμ in size. Particles (P') appeared to be aggregates

of the smaller particles (P). The field is magnified

84,000 X.
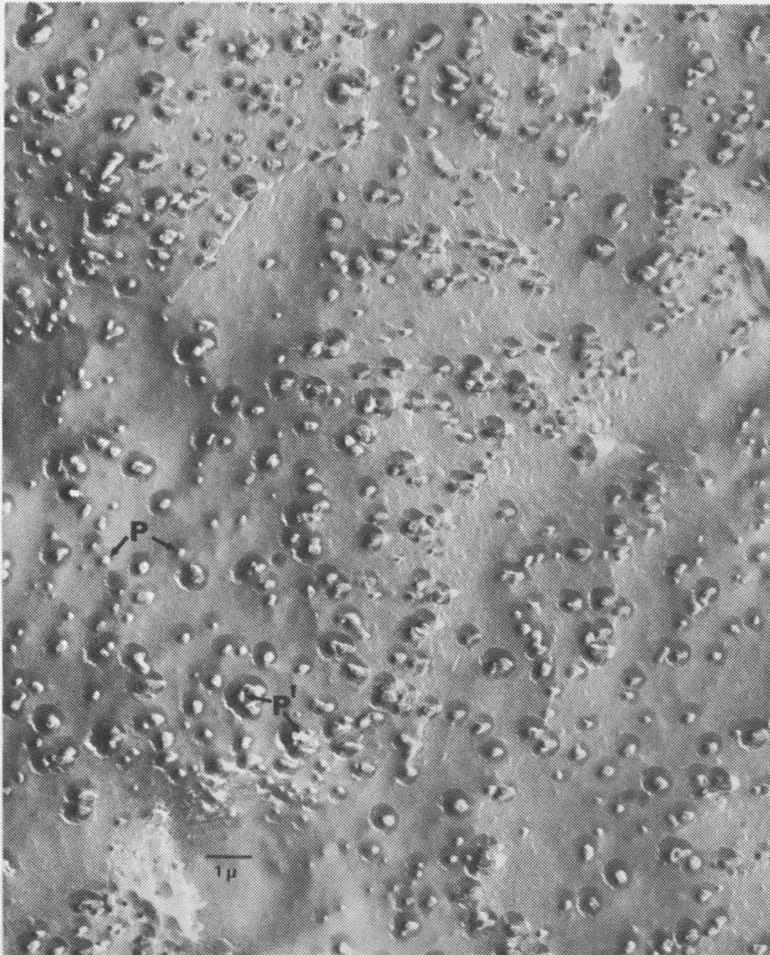
Figure 15.   Absorption spectra of the toxin and apotoxin. The

absorption spectra of the toxin (solid line) and

toxin treated with EDTA (apotoxin) (dashed line) is

plotted against wavelength in mμ.   The bar represents

the change from ultraviolet to visible spectrum.

53
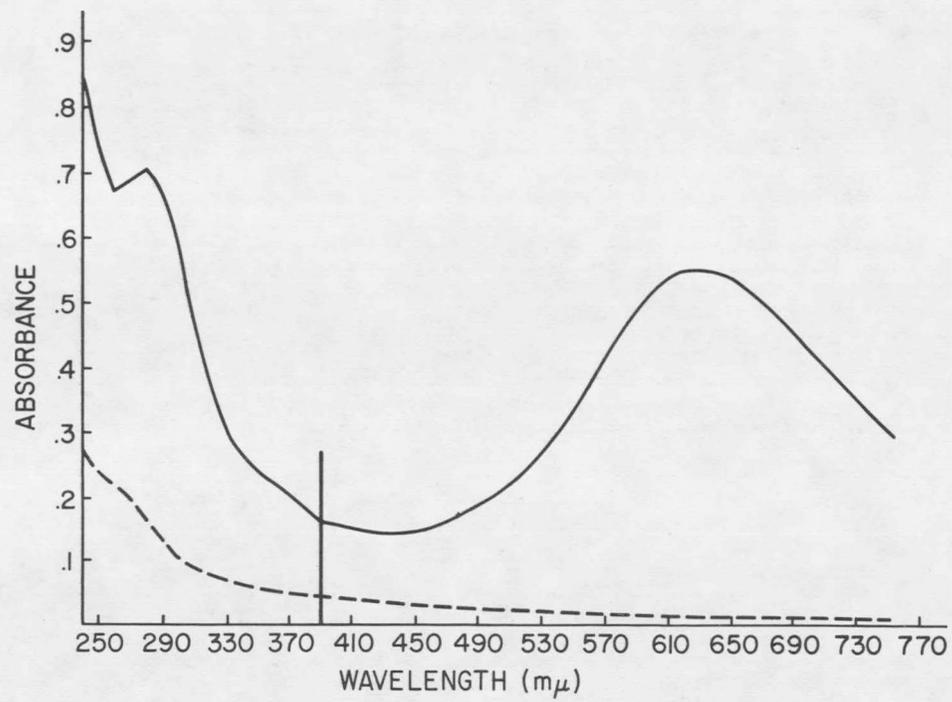
the toxin at 280 mμ and destroyed absorbance at 635 mμ. This would

indicate a metal ion was being removed by chelation from the toxin

resulting in a colorless apotoxin.

The identity of the metal ion was determined to be copper and was

measured on a Jarrell-Ash spectrophotometer (Table III). EDTA control

by itself contained no copper while the toxin had 13 moles/mole of

toxin. After treatment with EDTA the toxin (apotoxin) contained no

copper while the EDTA-Cu contained 75 moles/mole of toxin. The

difference between toxin and EDTA-Cu values was clarified by ashing

the toxin in a muffle oven at 650° for 20 hours. The ashed toxin

contained approximately the same amount of copper as did the EDTA-Cu.

Evidently the burning of the carbonaceous toxin masked much of the

copper present in it.

Fractionation of the toxin and apotoxin on Sepharose 2B and

assay with the Beckman Laboratory carbonaceous analyzer indicated

that the removal of copper did not alter the elution profile.

Chemical composition: Hydrolysis of the toxin with sulfuric acid

followed by formation of the alditol acetates of the sugar residues

and gas liquid chromatography indicated that mannose, glucose,

galactose, and fucose (1:5:5:10) were the major sugar residues present

in the toxin (Table IV). Two other reducing compounds were present

in trace amounts, rhamnose, and an unknown. These carbohydrates

Table III

Metal Ion Quantification[a]

	moles copper/mole toxin
EDTA Control	0
Toxin	13
Apotoxin	0
EDTA-Cu	75
Ashed Toxin	89

[a] Quantification of the amount of copper in the toxin, apotoxin, and EDTA after treatment of the toxin with 0.01M EDTA at 50° for 15 minutes followed by removal of EDTA-Cu by dialysis.

Table IV

Chemical Constituents of the Toxic Glycopeptide from C. insidiosum

Compound	% total toxin	Ratio number[a]
Sugar residues		
L-fucose	38.7	10
mannose	3.8	1
glucose	20.0	5
galactose	20.6	5
rhamnose[b]	Tr.	-
unknown	Tr.	-
Organic acid		
unknown	8.8	-
Amino acids	2.6	
Lys_2^c, Arg_1, Asp_1, Glu_1, Gly_2, Ser_1, Thr_1, Ala_2, Val_2, Leu_2, $Ileu_1$		
Cations		
Copper	0.1	
Total = 94.6		

[a] refers to the ratio of sugar residues in the total glycopeptide

[b] rhamnose and the unidentified reducing compound accounted for less than 1% of the total sugars

[c] refers to the number of residues of each amino acid in the peptide

accounted for 83.1% of the toxin.

The organic acid content of the toxin was determined by weighing dried eluate of the Dowex 1 column on a Cahn electrobalance. Table IV indicates the percentage of the organic acid which gave a positive test with acid-base indicator (4) and also was weakly reducing (45). The anion fraction consisted of one compound which failed to co-chromatograph with any of the organic acids checked (Table V). An infrared absorption spectrum of the compound (Figure 16) with the Beckman Microspec showed a strong broad absorption band between 2.9 and 3.3 μ, a smaller band at 3.7 to 4.0 μ, and other bands at 5.75, 6.0, 6.5, and 7.1 μ. Analysis of the acid by the thiobarbituric acid method (46) produced an alkali-labile chromophore with an absorption maximum at 549 mμ. This reaction is characteristic of β-formylpyruvate which is formed from 2-keto-3-deoxyaldonic acids.

Amino acid analysis of the toxin with the Beckman automatic amino acid analyzer and integration of the peak areas of each amino acid indicated the composition of the glycopeptide to be Lys_2, Arg_1, Asp_1, Thr_1, Ser_1, Glu_1, Gly_2, Ala_2, Val_2, Leu_2, and $Ileu_1$ (Table IV). These residues accounted for 0.4% of the total weight of the toxin.

Peptide: β-elimination effectively removed the peptide from the toxin. Acid hydrolysis and analysis of the peptide indicated the same amino acids as were present in the intact toxin. No significant decrease in

Table V

R_f Values of some Sugar Acids Relative to the Carboxylic

Acid from the Toxic Glycopeptide of C. insidiosum

Compound	Solvent Systems[a]					
	A	B	C	D	E	F
2-keto-3-deoxygluconic acid	0.21	0.25	0.11	0.19	-[b]	0.09
5-ketogluconic acid	0.33	0.11	0.14	0.22	0.24	0.04
2-ketogluconic acid	0.31	0.12	0.13	0.18	0.42	0.02
gluconic acid	0.26	0.05	0.11	0.19	0.10	0.03
glucuronic acid	0.21	0.05	0.01	0.13	0.06	0.01
galacturonic acid	0.24	0.07	0.07	0.11	0.05	0.01
unknown acid	0.07	0.03	0.01	0.07	0.24	0.00

[a] chromatography systems given in text

[b] no determination

Figure 16.   The infrared absorption spectrum for the purified

organic acid. Micropellets were prepared by mixing

the organic acid in KBr and were scanned at 0.2 inch

per minute with 6.8 gain.
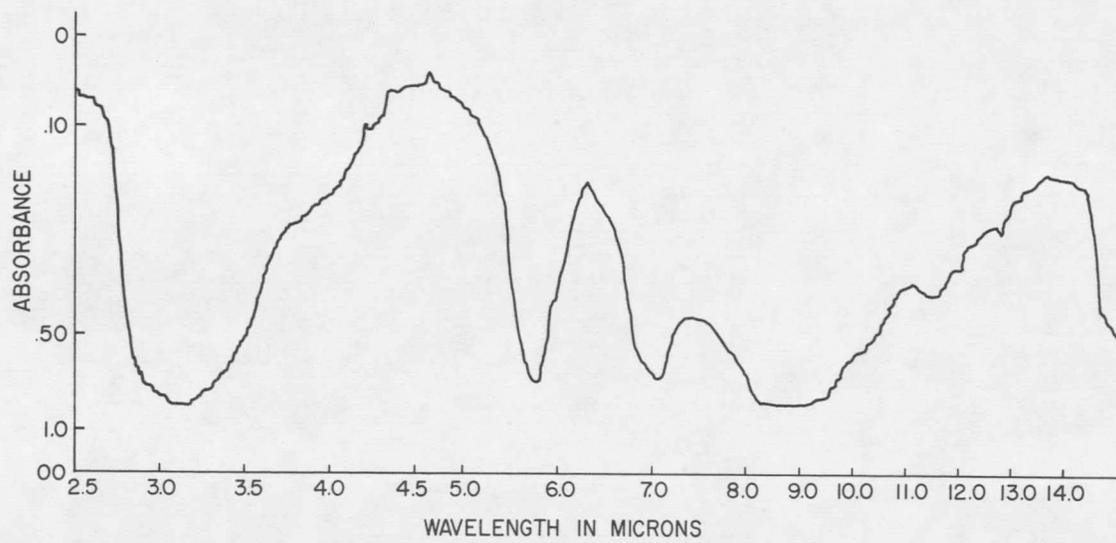
the amount of serine or threonine nor increase in valine or α-amino-butyric acid occurred after elimination and reduction. Electrophoresis of the peptide in pH 1.85 formic-acetic acid buffer (5) at 12.5 v/cm for 3 hours caused the peptide to migrate 6.9 cm toward the negative pole. Descending paper chromatography in the opposite dimension in solvent (A) for 10 hours indicated the peptide had an R_f = 0.238. Two-dimensional electrophoresis-chromatography indicated a single peptide was present in the toxin (Figure 17).

Chromatographic analysis of the dansylated peptide after acid hydrolysis revealed that glycine occurred as the NH_2-terminal amino acid.

Four hundred mg of toxin treated with 0.5N sodium hydroxide was passed through Dowex 1 (formate form), 0.5 X 1 cm, 200-400 mesh, and the peptide eluted with 6N formic acid. After drying, salt was removed by washing the dried residue with 10 ml of acetone containing 1% of concentrated aqueous hydrochloric acid. Undissolved material was removed by centrifugation, and the pellet washed 3 times with acetone-hydrochloric acid, centrifuged and discarded. The combined supernatant liquids were dried (40) and weighed on a Metler balance. The peptide represented 2.57% of the toxin on a dry weight basis. Kjeldahl nitrogen analysis indicated 0.32% of the toxin was nitrogen. Since the peptide is 16.6% nitrogen and the peptide was 2.57% of the

Figure 17. Two-dimensional electrophoresis-chromatography of

the peptide moiety of the toxin. The peptide was

subjected to electrophoresis in formic-acetic acid

buffer, pH 1.85, at 12.5 v/cm for 3 hours at 10

mamp. After drying, the electrophoretogram was

chromatographed in the other dimension in 1-butanol:

acetic acid:water (4:1:5) for 10 hours. The electro-

phoretogram was developed with o-tolidine. The

peptide moved 6.9 cm towards the negative pole and

had an R_f of 0.238.
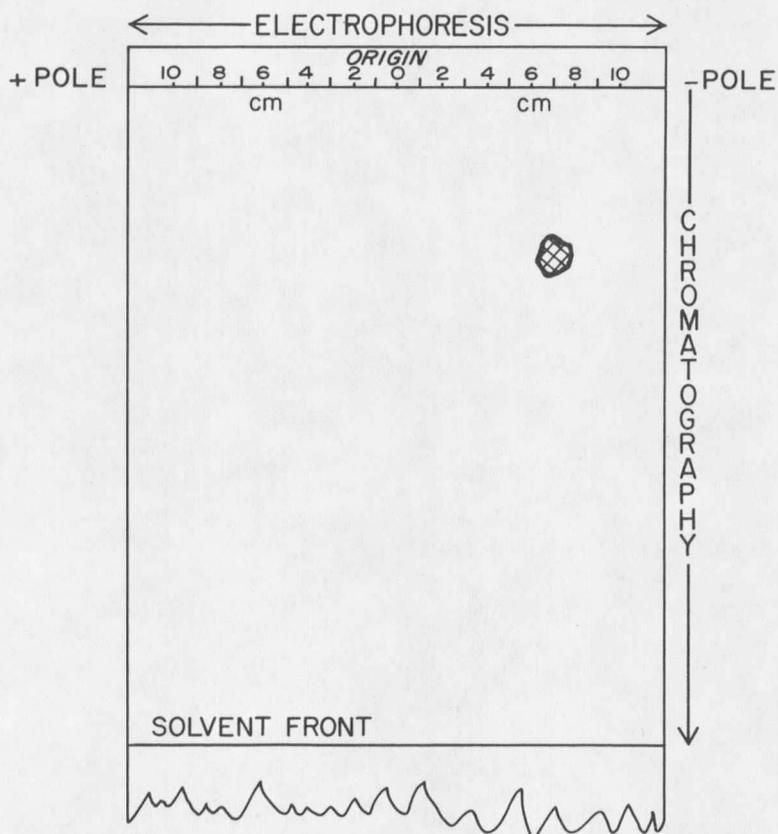
toxin, there was 0.42% nitrogen in the toxin due to the peptide, thus all nitrogen in the toxin was present in amino acids. A peptide with the residues listed in Table IV would have a molecular weight of 1683 daltons. Considering the molecular weight of the toxin there would be 76 peptides per molecule of toxin.

Stability of biological activity: The toxin was stable to great fluctuations in temperature and pH. Figure 18 indicates that heating toxin solutions (0.25%) to 121° for 10 minutes and allowing them to cool did not affect biological activity in any way. Toxin which had been autoclaved for 2 hours at 121° demonstrated a lower specific biological activity but still produced measurable wilt symptoms. The toxin was also stable to fluctuations in pH. Toxin solutions (0.25%) in phosphate buffers at pH 2, 7, and 12 for 24 hours and then adjusted to pH 7.0 still possessed unaltered ability to wilt tomato seedlings. Partial acid hydrolysis of the polysaccharide resulted in a drastic reduction of biological activity (Figure 19). All of the biological activity was lost after 5 minutes refluxing in 0.5N sulfuric acid.

Dextran (2.5 X 10^5 daltons), blue dextran (2 X 10^6 daltons), toxin (5 X 10^6 daltons), and soluble starch (70 X 10^6 daltons) were made up to 0.25% solutions. When checked for stem wilt inducing abilities only the toxin significantly possessed this property (Figure 20).

Figure 18.　　Heat stability of toxin. Toxin solutions (0.25%)

were heated for 10 minutes at 20°, 30°, 40°, 50°,

60°, 70°, 80°, 90°, and 121°, allowed to cool, and

checked for biological activity. A toxin solution

was also heated for 2 hours at 121°. Heated buffer

solutions failed to induce wilt symptoms.
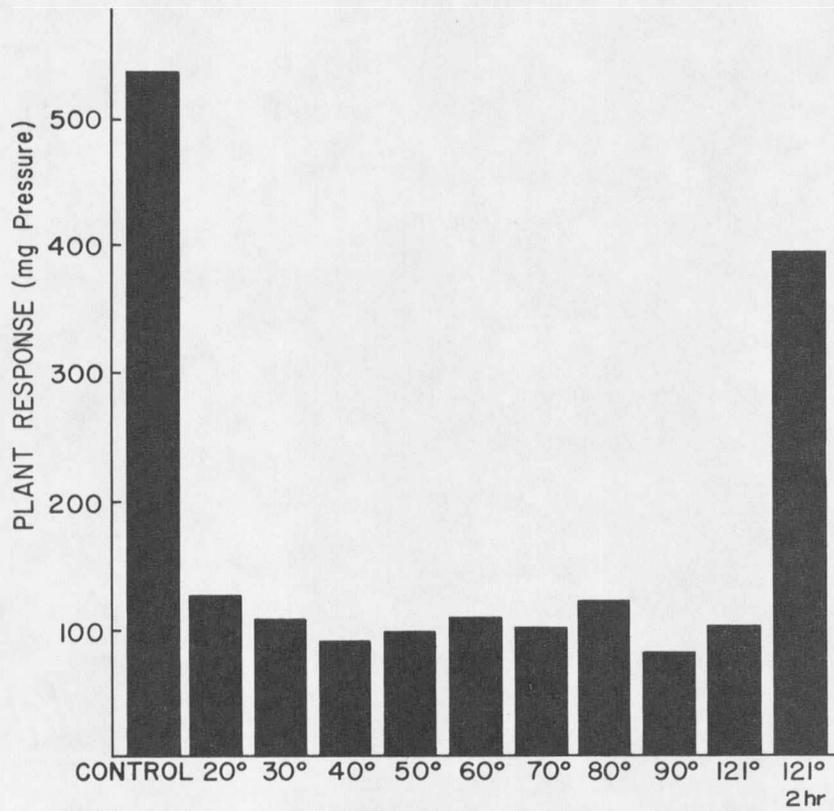
Figure 19.   Stability of toxin to acid hydrolysis. Minutes of

exposure of a 0.25% solution of glycopeptide to re-

fluxing in 0.5N sulfuric acid (X axis) is plotted

against mg stem strength of tomato stems after 1

hour (Y axis). After each exposure time and aliquot

of the acid solution was removed and neutralized with

an excess of barium carbonate. After removal of the

precipitate the solution was dried and redissolved

in a 0.25% solution of the partially hydrolyzed glyco-

peptide.
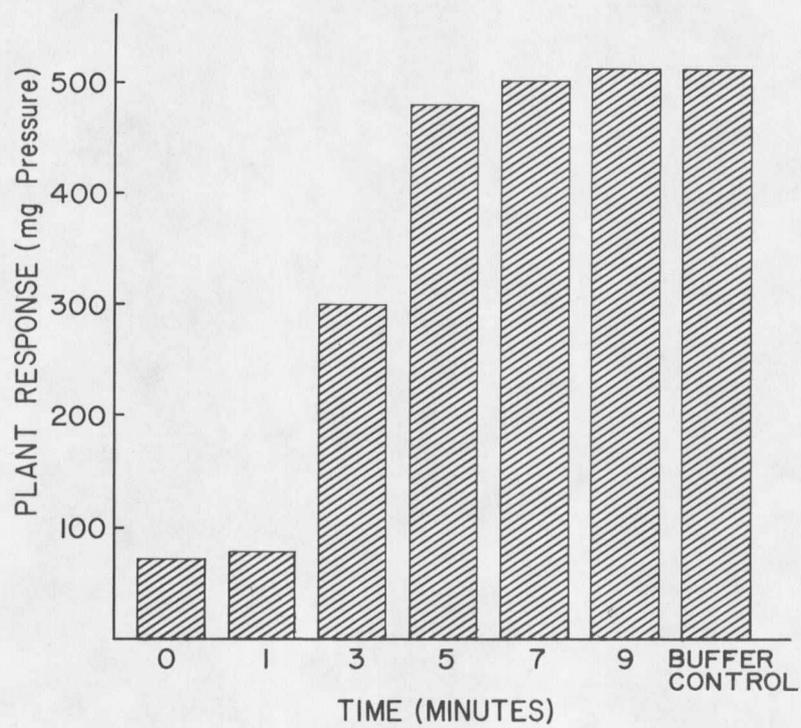
Figure 20. The histogram showing the effect of various size

compounds on wilt of tomato seedlings. Solutions

(0.25%) of dextran (2.5 X 10^5 daltons), blue dextran

(2 X 10^6 daltons), toxin (5 X 10^6 daltons), and

amylopectin (70 X 10^6 daltons) were checked for

ability to induce stem wilt in 1 hour in tomato

seedlings.
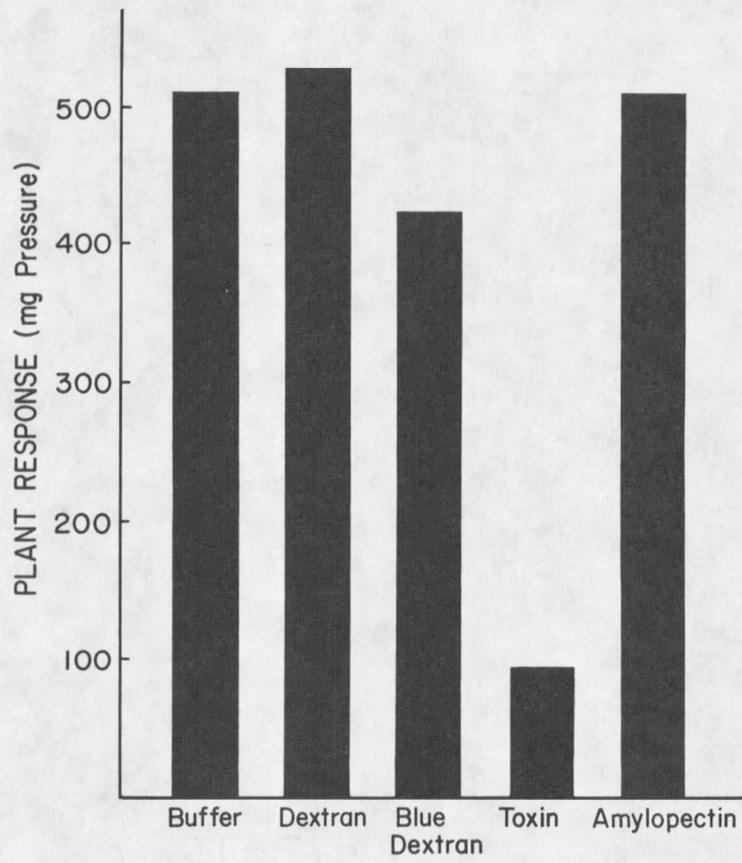
DISCUSSION

After purification 1.25 g of toxin was isolated from a 1 liter

culture of C. insidiosum. This yeild was in sharp contrast to the 15

mg toxin/1 attained fcom C. sepedonicum (42) and the 18 mg toxin/1

from C. michiganense (34, 35) and in the same range as that reported

by Spencer and Gorin (2 g toxin/1) (39). The final step in purifi-

cation (Figure 5) implied that contaminating radioactive substances

were present after ion-exchange chromatography. Based on this inform-

ation the toxin isolated by Spencer and Gorin (15, 39) from C.

insidiosum was probably a heterogeneous mixture of compounds. The

purification attained (5.57X) indicated that most of the exocellular

material (18%) produced by the organism was toxin (Table I).

The optimum incubation time to obtain maximal amount of toxin

($4\frac{1}{2}$ days) corresponded to maximal growth of the bacterium (Figure 7).

The organism evidently manufactured toxin during exponential growth

and then utilized it as either an energy or carbon source during

maximal growth. As the toxin decreased the culture entered death

phase.

Four experimental tests indicated that the substance after the

final step in purification was homogeneous; (1) all labelled material

precipitated during the last ammonium sulfate fractionation (Figure

6); (2) all markers of the toxin (radioactivity, chromophore, carbo-

hydrate, protein) migrated into the top of the gel and did not

penetrate further during disc gel electrophoresis (Figure 10), and no

other compounds were seen migrating through the gel; (3) in high

voltage paper electrophoresis experiments the toxin did not migrate

from the origin when checked in a wide range of buffers (Figure 11),

no other radioactive, reducing, or ninhydrin positive compounds

migrated; and (4) Sepharose 2B chromatography demonstrated 2 peaks in

the toxin after elution (Figure 9). This did not necessarily indicate

2 different size compounds. The elution profile of blue dextran on

Sepharose 4B demonstrated a similar pattern (Figure 8). This peak

pattern may be explained by assuming the later peak was tailing on

the column during elution. Thus, this later peak represented the

major portion of the compound being fractionated while the peak at

the void volume was the tail being grouped and excluded.

One criterion of heterogeneity in the final preparation was

shown by ultracentrifugation (Figure 12) in that a minor peak appeared

indicating two substances. The slower sedimenting peak represented

approximately 10 times more substance than the faster moving peak.

These two peaks were never completely resolved even after centrifug-

ation at 64,000 rpm for 7 hours. Further purification was not attempt-

ed. The two substances were probably both extremely large, relatively

uncharged, and seemed to differ only slightly in densities. Asy-

metric or flexible molecules interact hydrodynamically and with

large flexible molecules, extensive physical interaction, i.e., inter-

twining and entanglement of the threads can result in an exceedingly

sharp (hypersharp) boundary which may conceal extensive polydispersity

or in these experiments actual heterogeneity (14, 38).

The heterogeneity indicated by ultracentrifugation might be

explained by reexamination of the electron micrograph of the freeze-

etched toxin (Figure 14). Two particles were observed; P, a particle

80.0 - 90.0 mμ in size and P', larger in size than P but appearing to

be an aggregate of the smaller particles. If this was the case the

faster sedimenting peak could be the larger particles (P'). The

"hypersharp" boundary of the first peak probably reflects poly-

dispersity, common in large polysaccharide substances.

The freeze-etched preparation of the toxin presents an interesting

view of a polysaccharide. The structure of each particle observed in

Figure 14 closely approximates the shape while in solution. Artifact

formation due to fixing and staining is minimal. The shape of the

glycopeptide differs considerably from the shape of another protein

polysaccharide obtained by Rosenberg, Hellman, and Kleinschmidt (37).

Their preparations were dried and stained with uranyl acetate. These

protein polysaccharides then appeared as rigid filaments with numerous

branching filaments. Freeze-etching employs mild conditions and thus

results in an accurate appearance of the polysaccharide.

The toxin appeared to be an extremely high molecular weight compound. Estimation of its molecular weight by fractionation on Sepharose 2B implied it was between 2×10^6 and 20×10^6 daltons. Lack of sufficiently large molecular weight standards failed to permit an estimation of molecular weight with the gel sieving technique. A molecular weight of 4.83×10^6 daltons was estimated by ultracentrifugation studies and a molecular weight of approximately 5×10^6 daltons by light scattering. These weight average molecular weights differ considerably from those reported earlier by Strobel (41) for C. sepedonicum toxin (2×10^5 daltons) and Rai and Strobel (34, 35) for the three toxic fractions of C. michiganense after Sephadex G-200 chromatography (Fraction I $\geq 2 \times 10^5$, Fraction II = 1.29 $\times 10^5$, and Fraction III = 3.53×10^5 daltons). The toxic Fraction I may be a compound related to this toxin. Its molecular weight estimation was made from Sephadex G-200 which excluded molecules larger than 200,000. Thus, Fraction I may be as large as the toxin reported here.

Considering the large molecular size of the toxin, dissociation of the compound into lower molecular weight subunits was attempted. Treatments with 8M urea or with 30% pyridine, or removal of copper, failed to reveal subunit formation when fractionated on Sepharose 2B. The molecule appeared to be one large covalently linked polymer and

not a series of smaller polymers.

The specific viscosity of the toxin (0.2307 dl/g) compared favorably with Fraction I (0.225 dl/g) for C. michiganense while differing from the toxin of C. sepedonicum (0.125 dl/g). Intrinsic viscosity values for these polysaccharides are frequently proportioned to molecular weight implying that this toxin was larger than Fraction I reported by Rai and Strobel (34, 35). The optical activity of the toxin differed significantly from that reported by Gorin and Spencer (15) for C. insidiosum (-98°). The heterogeneity of the toxin prepared by Gorin and Spencer undoubtedly altered their value.

The broad absorption peak at 635 mμ obtained by ultraviolet and visible absorption experiments (Figure 15) was the visible chromophore of the toxin. Treatment with EDTA indicated a metal ion chelated into the toxin while quantification (Table III) of the metal showed copper was present at a concentration of 75 moles copper/mole toxin. These data sharply contrasted with those reported by Kuhn et al. (26, 27) who found and chemically characterized a low molecular weight pigment of C. insidiosum which was also blue. Since this incompatability of results exists, there may be two pigments present in C. insidiosum cultures, a large molecular weight substance containing copper and a small non-copper containing compound. Another possible explanation may be that the pigment described by Kuhn et al. was an

integral portion of the polysaccharide and the chromophore of the toxin resulted from this small pigment. This, however, is not consistent with the presence of copper in the toxin or the loss of color with EDTA treatment (Table III).

Chemical characterization of the toxin suggested this compound was chemically closely related to the phytotoxic glycopeptides from other Corynebacterium spp. Patino-Mendez (32) reported fucose, galactose, glucose, mannose, rhamnose, and an unidentified sugar residue from a toxin isolated from C. michiganense. These same sugars were reported in preparations from C. michiganense (34) and from C. sepedonicum (41, 43). Gorin and Spencer (15) working with C. insidiosum reported the constituent sugars as galactose (32%), glucose (21%), and fucose (46%). In the present study the toxin of C. insidiosum contained galactose (21%), glucose (20%), fucose (39%), and mannose (4%), with rhamnose and an unidentified sugar residue present in trace amounts. Another similar characteristic of these toxins was that fucose was present in the L-form, a fact reported by Spencer and Gorin (39) and Strobel (43).

An organic acid from the toxin preparation of C. insidiosum described by Gorin and Spencer (16) was 4,6-0-(1'-carboxyethylidene)-D-galactose. Conditions of acid hydrolysis on the purified toxin employed in the present study should have hydrolyzed this compound to

pyruvic acid and galactose, but pyruvic acid was not identified as a

constituent acid of the purified toxin. This is based on the fact

that infrared spectral analysis of pyruvic acid and the unknown organic

acid were not similar as pyruvic acid has strong absorption bands at

6.1 and 7.35 μ while the organic acid did not (Figure 16). Similarly,

the unknown organic acid possess -CH and -OH stretch which pyruvate

lacked. Reaction of each organic acid with p-anisidine (4) was

different since pyruvate gave a bright yellow spot while the unknown

organic acid gave a dull brown product. Strobel (43) described 2-

keto-3-deoxygluconic acid (KDG) from the toxic glycopeptide of C.

sepedonicum and Ghalambor, Levine, and Heath described 3-deoxy-D-

manno-octulosonic acid (KDO) from cell wall lipopolysaccharides of

Escherichia coli (13). The unknown organic acid appeared similar to

KDG and KDO because it produced an alkali-labile chromophore by the

thiobarbituric acid method and because of its infrared spectrum, but

it did not co-chromatograph with KDG or any of the oother organic

acids (Table V). Comparison of the R_f values listed for KDO (43)

and those obtained for the unknown organic acid (Table V) indicated

that they were not the same compound. The presence of an organic

acid (8.8%) represented another chemical similarity with the toxins

produced by other corynebacteria and it appears to be a keto-deoxy

acid.

The isolation and demonstration of a single peptide from the glycopeptide is shown in Figure 17. This indicates that the amino acids of the toxin are linked together by peptide bonds. Further evidence that the amino acids are covalently bonded together is that dansylation data indicated a sole NH_2-terminal amino acid, glycine. Acid hydrolysis of the peptide indicated the same amino acid residues were present as those in the untreated toxin. These data are also consistent with the concept that all of the amino acids are covalently bonded to one peptide. After β-elimination and reduction, the amounts of either serine, threonine, aspartic acid, or glutamic acid should have decreased in amount with a concomittant increase in alanine, α-amino butyric acid, homoserine, or α, δ-aminohydroxy-n-valeric acid (3, 31), but in these experiments they did not. A possible explanation for this inconsistency is that sample sizes were small and the peptide represented such a small percentage of the toxin the accuracy of the amino acid analyzer may have suffered. $Lysine_2$, $arginine_1$, $aspartic acid_1$, $threonine_1$, $serine_1$, $glutamic acid_1$, $glycine_2$, $alanine_2$, $valine_2$, $leucine_2$, and $isoleucine_1$ were present in the acid hydrolysate of the toxin (Table IV). Integration of peak areas after amino acid analysis of the intact glycopeptide and quantification of the total weight of peptide showed the peptide represented 0.4% of the total glycopeptide. Dry weight determinations of sodium hydroxide

treated toxin suggested the peptide was 2.57% of the toxin. Amino acid hydrolysis in the presence of large amounts of carbohydrate leads to humin formation and a loss of amino acids, a Maillard reaction (17, 40). This is the best explanation for the disparity of the two peptide values. Therefore the estimation utilizing the dry weight is probably the more reasonable value. This value is substantiated when compared with the amount of nitrogen in the toxin. Kjeldahl analysis of the toxin (0.32% N) with a peptide which is 16.6% N indicated that 1.9% of the toxin was peptide. A peptide comprising approximately 1.89 - 2.57% of the toxin and composed of the amino acids listed in Table IV would imply that there would be 56 - 76 peptides per molecule of toxin.

The presence of amino acids confirms the observations of Strobel (43) and Rai and Strobel (34) with the glycopeptides of C. sepedonicum and C. michiganense. Gorin and Spencer (15) failed to report that amino acids were present in their preparations but indicated 0.3% of their preparation was nitrogen, virtually the same percentage obtained in this work (0.32%).

Biologically the toxin appeared non-specific, affecting tomatoes as well as alfalfa (Figure 12). Other plants such as sanfoin, milk-vetch, and potatoes were also wilted by the toxin. A similar non-specificity was reported by Rai and Strobel (35) with the toxin from

C. michiganense and by Hodgson, Peterson, and Riker (22, 23) using

Agrobacterium tumefaciens, Xanthomonas phaseoli, and Erwinia caroto-

vora. The non-specificity of these toxins may show that the effect of

the toxins was a mechanical or physical disruption of host tissue.

Similarly, the non-specificity of this toxin may suggest that resis-

tance to bacterial wilt of alfalfa is not resistance to the toxin.

Disease resistance is probably due to other factors such as hardier

roots, which undergo less winter damage and thus deny the bacterium

a portal of entry, or that bacterial infection may be walled off.

Wilt induced by the toxin was dependent on concentration and on

time. Rai and Strobel (35), Strobel (41), and Johnson and Strobel

(25) demonstrated these dependencies with the toxins of other plant

pathogenic corynebacteria. The toxins of other corynebacteria were

also heat resistant and biological activity was rapidly destroyed by

partial acid hydrolysis. The toxin from C. insidiosum demonstrated

these parameters while also being stable to wide fluctuations of pH.

A covalently-linked structure with little tertiary or quaternary

structure necessary for biological activity appeared reasonable.

The mode of action of this toxin is unknown. Figure 20 demon-

strates that neither smaller nor larger substances, bracketing the

molecular weight of the toxin, caused stem wilt. The indication was

that plugging did not occur, as amylopectin (70×10^6 daltons) had

no effect on stem wilt. Similar size substances such as blue dextran (2×10^6 daltons) did not wilt stems suggesting that the toxin had an active site or sites involved in the wilt process. Electron microscopic studies, electrolyte measurement, and dye transport studies by Rai and Strobel (35) and Strobel and Hess (42) demonstrated with phytotoxins from C. michiganense and C. sepedonicum that membrane damage occurred. The active site was associated with carboxyl groups of 2-keto-3-deoxygluconic acid on the toxic glycopeptide of C. sepedonicum (25). C. insidiosum also contained a significant percentage (8.8%) of an unknown acid which may be related to the toxicity of the glycopeptide in the same manner as 2-keto-3-deoxygluconic acid is to C. sepedonicum. The chemical similarities of this toxin with the other phytotoxins of C. sepedonicum and C. michiganense may indicate a similar mode of action.

The size of the glycopeptide (250 X the molecular weight of C. sepedonicum toxin) may in fact cause plugging of the vascular bundles as has been postulated by several researchers with many examples (7, 10, 19, 24, 32, 39).

The electron micrograph of the freeze-etch preparation indicated the toxin molecules were 80.0 - 90.0 mμ in diameter which may plug xylem vessels. From these data (chemical structure and size measurements) it is impossible to ascribe a mechanism of action.

The biological significance of this compound in vivo was not investigated, but other phytotoxins reported from other corynebacteria were chemically closely related. They contained essentially the same sugar, amino acid, and organic acid residues and produced similar symptoms in vivo. The isolation of L-fucose from infected alfalfa plants by Spencer and Gorin (39) is presumptive evidence that such a compound is biologically important in the disease process.

SUMMARY

A single exotoxin possessing the capacity to wilt excised stem
cuttings was isolated from cultures of <u>Corynebacterium</u> <u>insidiosum</u> and
purified, and the physical, chemical, and biological properties
measured. The organism produced 1.25 g toxin/l of culture and
approximately 18% of the exocellular material produced was toxin.
Optimal toxin production occurred after $4\frac{1}{2}$ days of incubation coincid-
ing with maximal bacterial growth. The toxin wilted alfalfa as well
as tomato stems; stem wilt was dependent upon time and upon concen-
tration with measurable wilt occurring in 4 X 10^{-8}M solutions in 1
hour. The toxin appeared homogeneous during Sepharose 2B chromato-
graphy, ammonium sulfate fractionation, disc gel and high voltage
paper electrophoresis. Heterogeneity was indicated by analytical
ultracentrifugation.

The molecular weight as estimated by ultracentrifugation, light
scattering, and column gel chromatography was approximately 5 X 10^{6}
daltons. Electron micrographs of freeze-etched replicas of the toxin
showed an amorphous globule with a diameter of 80.0 - 90.0 mμ .
The toxin did not dissociate into small subunits when treated with 8M
urea or 30% pyridine. The empirical formula was $C_{108}H_{226}O_{132}N$,
intrinsic viscosity 0.2307 dl/g, and the specific rotation -166°.

Strong absorbance of the purified toxin at 635 mμ gave the toxin
a blue chromophore, due to the chelation of copper at a concentration

of 75 moles of copper/mole of toxin. Acid hydrolysis and chromato-graphic analysis of the purified toxin revealed that mannose, glucose, galactose, rhamnose, fucose (L-form), and an unidentified reducing compound comprised 83% of the toxin. An unknown organic acid which appeared chemically similar to keto-deoxy organic acids comprised 8.8% of the toxin. Lysine, arginine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, leucine, and isoleucine were bonded together forming a single peptide with glycine as the sole NH_2-terminal amino acid. This single peptide composed 2.57% of the toxin indicating there were 76 peptides per molecule of purified glycopeptide.

The toxin was heat resistant, acid labile and water soluble, and in vitro experiments simulated in vivo disease symptomology. An explanation of the mechanism of action was not postulated. Wilt symptoms may be due to plugging of xylem vessels or to membrane damage caused by an active site(s) on the glycopeptide.

LITERATURE CITED

1. Albersheim, P., D. J. Nevins, P. D. English, and A. Karr. 1967.
   A method for the analysis of sugars in plant cell-wall poly-
   saccharides by gas-liquid chromatography. Carbohyd. Res. 5:
   340-345.

2. Anacker, E. W. 1970. Micelle formation of cationic surfactants
   in aqueous media. In: Cationic Surfactants. E. Jungermann,
   ed., Marcel Dekker, Inc., New York. pp. 225-240.

3. Anderson, B., N. Seno, P. Sampson, J. G. Riley, P. Hoffman, and
   K. Meyer. 1964. Threonine and serine linkages in mucopoly-
   saccharides and glycoproteins. J. Biol. Chem. 239: 2716-2717.

4. Aronoff, S. 1961. Techniques of Radiobiochemistry. Iowa State
   University Press, Ames, Iowa. pp. 94-148.

5. Atfield, G. N. and C. J. O. R. Morris. 1961. Analytical separ-
   ations by high voltage paper electrophoresis. Biochem. J. 81:
   606-614.

6. Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. Coryne-
   bacterium insidiosum (McCull.) Jensen, 1934. Bergey's Manual
   of Determinative Bacteriology. The Williams and Wilkins
   Company, Baltimore. p. 591.

7. Buddenhagen, I. and A. Kelman. 1964. Biological and physiolog-
   ical aspects of bacterial wilt caused by Pseudomonas solan-
   acearum. Ann. Rev. Phytopathol. 2: 203-230.

8. Davis, B. J. 1964. Disc electrophoresis-II. Method and
   application to human serum proteins. Ann. N.Y. Acad. Sci.
   121: 404-427.

9. Dimond, A. E. and P. E. Waggonner. 1953. On the nature and role
   of vivotoxins in plant disease. Phytopathology 43: 229-235.

10. Dimond, A. E. 1955. Pathogenesis in the wilt diseases. Ann.
    Rev. Plant Physiol. 6: 329-350.

11. Falconer, J. S. and D. B. Taylor. 1945. A new type of phase
    rule solubility test for enzyme purity. Nature 155:303-304.

12. Feder, W. A. and P. A. Ark. 1951. Wilt inducing polysaccharides
    derived from crown gall, bean blight, and soft rot bacteria.

Phytopathology 41: 804-808.

13. Ghalambor, M. A., E. M. Levine, and E. C. Heath. 1966. The biosynthesis of cell wall lipopolysaccharide in Escherichia coli. III. The isolation and characterization of 3-deoxy-octulosonic acid. J. Biol. Chem. 241: 3207-3215.

14. Gibbons, R. A. 1966. Physico-chemical methods for the determination of the purity, molecular size, and shape of glycoproteins. BBA Library Vol. V. A.Gottschalk, ed., Elsevier Publishing Co., Amsterdam-London-New York. pp. 29-90.

15. Gorin, P. A. G. and J. F. T. Spencer. 1961. Extracellular acidic polysaccharides from C. insidiosum and other Corynebacterium spp. Can. J. Chem. 39: 2274-2280.

16. Gorin, P. A. G. and J. F. T. Spencer. 1964. Isolation of 4,6-0-(1'-carboxyethylidene)-D-galactose from the exocellular polysaccharide of Corynebacterium insidiosum. Can. J. Bot. 42: 1230-1232.

17. Gottschalk, A. 1966. Interaction between raducing sugars and amino acids under neutral and acidic conditions. BBA Library Vol. V. A. Gottschalk, ed., Elsevier Publishing Company, Amsterdam-London-New York. pp. 96-110.

18. Gray, W. R. and B. S. Hartley. 1963. The structure of a chymotryptic peptide from Pseudomonas cytochrome c-551. Biochem. J. 89: 379-380.

19. Harris, H. A. 1940. Comparative wilt induction by Erwinia tracheiphila and Phytomonas stewarti. Phytopathology 30: 629-638.

20. Herrick, H. E. and J. M. Lawrence. 1965. Method for drying polyacrylamide gels following electrophoresis. Analytical Biochemistry 12: 400-402.

21. Hess, W. M. and D. L. Stocks. 1969. Surface characteristics of Aspergillus conidia. Mycologia 61: 560-571.

22. Hodgson, R., A. J. Riker, and W. H. Peterson. 1947. A wilt-inducing toxic substance from crown-gall bacteria. Phytopathology 37: 301-318.

23. Hodgson, R., W. H. Peterson, and A. J. Riker. 1949. The toxicity of polysaccharides and other large molecular molecules to tomato cuttings. Phytopathology 39: 47-62.

24. Hussain, A. and A. Kelman. 1958. Relation to slime production to mechanism of wilting and pathogenicity of *Pseudomonas solanacearum*. Phytopathology 48: 155-165.

25. Johnson, T. B. and G. A. Strobel. 1970. The active site on the phytotoxin of *Corynebacterium sepedonicum*. Plant physiol. 45: 761-764.

26. Kuhn, R., W. Blau, H. Bauer, H. J. Knackmuss, D. A. Kuhn, and M. P. Starr. 1964. Uber das blaue pigment von *Corynebacterium insidiosum*. Naturwissenschaften 51: 194.

27. Kuhn, R., H. Bauer, H J. Knackmuss, D. A. Kuhn, and M. P. Starr. 1964. Die struktur der blauem pigmente von *Corynebacterium insidiosum*, *Arthrobacter atrocyaneus*, *Pseudomonas indigofera* und *Arthrobacter crystallopoieties*. Naturwissenschaften 51: 409.

28. Moor, H. and K. Muhlethaler. 1963. Fine structure of frozen-etched yeast cells. J. Cell. Biol. 17: 609-628.

29. Moor, H. 1964. Die gefrier-fixation lebender zellen und ihre unwendung in der elektronenmikroskopie. Z. Zellforsch. 62: 546-580.

30. Moore, S. and W. H. Stein. 1963. Chromatographic determination of amino acids by the use of automatic recording equipment. In: Methods in Enzymology, Vol. VI. S.P. Colowick and N. O. Kaplan, eds., Academic Press, Inc., New York. pp. 819-831.

31. Murphy, W. H. and A. Gottschalk. 1961. Studies on mucoproteins. VII. The linkage of the prosthetic group to aspartic and glutamic acid residues in bovine submaxillary gland muco-protein. Biochem. Biophys. Acta. 52: 349-360.

32. Patino-Mendez, G. 1964. Studies on the pathogenicity of *Coryne-bacterium michiganense* (E. E. Sm.) Jensen, and its transmission in tomato seed. (Thesis) University of California, Davis.

33. Price, W. H., H. Harrison, and S. H. Terebee. 1964. Disc

electrophoresis on polyacrylamide gels of serum mucoids of individuals with selected chronic diseases. Ann. N.Y. Acad. Sci. 121: 460-469.

34. Rai, P. V. and G. A. Strobel. 1969. Phytotoxic glycopeptides produced by Corynebacterium michiganense. I. Methods of preparation, physical and chemical characterization. Phytopathology 59: 47-52.

35. Rai, P. V. and G. A. Strobel. 1969. Phytotoxic glycopeptides produced by Corynebacterium michiganense. II. Biological properties. Phytopathology 59: 53-57.

36. Reichmann, M. E. 1960. Degradation of potato virus X. J. Biol. Chem. 235: 2959-2963.

37. Rosenberg, L., W. Hellmann, and A. K. Kleinschmidt. 1970. Macromolecular models of protein polysaccharides from bovine nasal cartilage based on electron microscopic studies. J. Biol. Chem. 245: 4123-4130.

38. Schachman, H. K. 1957. Ultracentrifugation, diffusion, and viscometry. Methods in Enzymology, Vol. IV. S.P. Colowick and N. O. Kaplan, eds., Academic Press, Inc., New York. pp. 32-103.

39. Spencer, J. F. T. and P. A. G. Gorin. 1961. The occurrence in the host plant of physiologically active gums produced by Corynebacterium insidiosum and Corynebacterium sepedonicum. Can. J. Microbiol. 7: 185-188.

40. Stahl, E. 1965. Thin-layer chromatography. Academic Press, Inc., New York-London. pp. 395-397.

41. Strobel, G. A. 1967. Purification and properties of a phytotoxic polysaccharide produced by Corynebacterium sepedonicum. Plant Physiol. 42: 1433-1441.

42. Strobel, G. A. and W. M. Hess. 1968. Biological activity of a phytotoxic glycopeptide produced by Corynebacterium sepedonicum. Plant Physiol. 43: 1674-1688.

43. Strobel, G. A. 1970. A phytotoxin glycopeptide from potato plants infected with Corynebacterium sepedonicum. J. Biol.

Chem. <u>245</u>: 32-38.

44. Tanaka, K. and W. Pigman. 1965. Improvements in hydrogenation
    procedure for demonstration of o-threonine glycosidic linkages
    in bovine submaxillary mucin. J. Biol. Chem. <u>240</u>: 1487-1488.

45. Trevelyan, W. E., D. P. Proctor, and J. S. Harrison. 1950.
    Detection of sugars on paper chromatography. Nature <u>166</u>: 444-
    445.

46. Warren, L. 1959. The thiobarbituric acid assay of sialic acids.
    J. Biol. Chem. <u>234</u>: 1971-1975.