



Chemical and biological properties of phytotoxic glycopeptides isolated from *Corynebacterium michiganense*
by Palthad Vittal Rai

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements of the degree of
DOCTOR OF PHILOSOPHY in Botany (Plant Pathology)
Montana State University
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Abstract:

Phytotoxins produced by *Corynebacterium* spp., have been reported by several investigators. However, in the case of the toxin produced by *C. michiganense* a complete chemical characterization, the mechanism by which the toxin affects plants and role of the toxin in disease symptom production had not been established. The present study demonstrated that the toxin of *C. michiganense* was antigenic, non-specific and could be classified as a vivotoxin. The chemical characterization of the toxin showed that it was a glycopeptide and contained 3 biologically active, serologically related fractions when purified by Sephadex G-200 column chromatography. The estimated molecular weights of the fractions were = 200,000; 129,700; 35,280 and the intrinsic viscosities were as follows: crude toxin, 0.2500; fraction I, 0.2250; fraction II, 0.2140 and fraction III, 0.1904. The empirical formulas were $C_{55}H_{113}$ (Formula not captured by OCR) and $C_{27}H_{52}O_{25}N$. Thin layer chromatography of cation fraction of the acid Hydrolyzed crude toxin and its fractions revealed that, the crude toxin and fractions I and III contained alanine, glycine, lysine, methionine, serine, threonine and an unknown ninhydrin positive compound, whereas fraction II contained alanine, glycine, lysine, serine and an unknown compound. Paper chromatography of neutral fraction of the crude toxin and its fractions showed that crude toxin and fraction III contained fucose, galactose, glucose, mannose and 2 unknown reducing compounds whereas fraction I and II had galactose, glucose, mannose and 2 unknown compounds. This toxin was heat resistant 14 acid labile and water soluble. Radioautography of plants treated in C-toxins showed that the C-labeled toxin was distributed throughout the plant prior to wilting. Only 40.0 to 60.0 μ g of crude toxin was required to cause wilting when determined by the radioactivity differences in the C-toxin solution prior to the treatment and after feeding the plants. Furthermore, when toxin treated and water treated plants were transferred in acid fuchsin the dye migrated to the tip of the plant at the same rate, whereas in the case of a dextran (a known plugging agent) treated plant there was a drastic reduction in the movement of the dye. An electron microscopic study showed that no occlusions were present in the vascular system of toxin treated plants. These experiments collectively indicated that the plugging phenomenon was not involved as the mechanism causing wilting. Further evidence by electron microscopy indicated that the key mechanism of wilting in plants was due to the damage in the cell membrane system. Other experimental evidences showed that this toxin had a major role in disease production.

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ISOLATED FROM CORYNEBACTERIUM MICHIGANENSE

by

PALTHAD VITTAL RAI

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

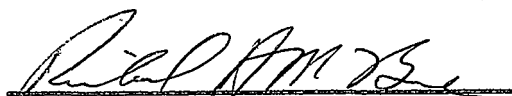
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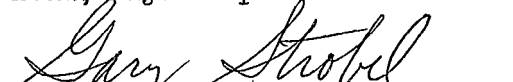
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Approved:


Head, Major Department


Chairman, Examining Committee


Graduate Dean

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ABSTRACT

Phytotoxins produced by Corynebacterium spp., have been reported by several investigators. However, in the case of the toxin produced by C. michiganense a complete chemical characterization, the mechanism by which the toxin affects plants and role of the toxin in disease symptom production had not been established. The present study demonstrated that the toxin of C. michiganense was antigenic, non-specific and could be classified as a vivotoxin. The chemical characterization of the toxin showed that it was a glycopeptide and contained 3 biologically active, serologically related fractions when purified by Sephadex G-200 column chromatography. The estimated molecular weights of the fractions were = 200,000; 129,700; 35,280 and the intrinsic viscosities were as follows: crude toxin, 0.2500; fraction I, 0.2250; fraction II, 0.2140 and fraction III, 0.1904. The empirical formulas were $C_{55}H_{113}O_{52}N$, $C_{42}H_{82}O_{40}N$, $C_{473}H_{940}O_{449}N$ and $C_{27}H_{52}O_{25}N$. Thin layer chromatography of cation fraction of the acid hydrolyzed crude toxin and its fractions revealed that, the crude toxin and fractions I and III contained alanine, glycine, lysine, methionine, serine, threonine and an unknown ninhydrin positive compound, whereas fraction II contained alanine, glycine, lysine, serine and an unknown compound. Paper chromatography of neutral fraction of the crude toxin and its fractions showed that crude toxin and fraction III contained fucose, galactose, glucose, mannose and 2 unknown reducing compounds whereas fraction I and II had galactose, glucose, mannose and 2 unknown compounds. This toxin was heat resistant, acid labile and water soluble. Radioautography of plants treated in ^{14}C -toxins showed that the ^{14}C -labeled toxin was distributed throughout the plant prior to wilting. Only 40.0 to 60.0 μg of crude toxin was required to cause wilting when determined by the radioactivity differences in the ^{14}C -toxin solution prior to the treatment and after feeding the plants. Furthermore, when toxin treated and water treated plants were transferred in acid fuchsin the dye migrated to the tip of the plant at the same rate, whereas in the case of a dextran (a known plugging agent) treated plant there was a drastic reduction in the movement of the dye. An electron microscopic study showed that no occlusions were present in the vascular system of toxin treated plants. These experiments collectively indicated that the plugging phenomenon was not involved as the mechanism causing wilting. Further evidence by electron microscopy indicated that the key mechanism of wilting in plants was due to the damage in the cell membrane system. Other experimental evidences showed that this toxin had a major role in disease production.

INTRODUCTION

Bacterial canker of tomato (Lycopersicon esculentum Mill), caused by Corynebacterium michiganense (E.F.S.) Jensen was reported from Montana by Morris and Afanasiev (1953). The typical symptom of this disease is wilting of foliage beginning with the lower leaves which turn brown and die. Setting of fruit is reduced. Stems and leaves become brittle, and birds eye spots develop on fruits.

Several polysaccharides, isolated from various phytopathogenic bacteria were demonstrated to be involved in bacterial wilts. Hodgson, Peterson and Riker (1947, 49), and Feder and Ark (1951) reported that certain polysaccharides produced by plant pathogenic bacteria, Agrobacterium tumefaciens (Smith and Town) Conn, Xanthomonas phaseoli (E.F.Sm.) Dows., and Erwinia carotovora (Jones) Holland, were phytotoxic and possessed wilt inducing properties. These toxins, were nonspecific and were of high molecular weight.

More sophisticated work has been done with toxins produced by Pseudomonas solanacearum, the causal agent of southern bacterial wilt of several plants. Husain and Kelman (1958) reported that the culture filtrate of this organism contained two pectolytic and cellulolytic enzymes. The heat treated culture filtrate had a compound which also caused wilting in tomato and tobacco cuttings. In the anatomical studies of infected plants they showed that the enzymes disintegrated the parenchymatous cells in the vascular region and this non-enzymic, heat stable compound caused plugging in the vessels. The mechanical plugging theory, proposed by Stewart in 1894, (cited in Dimond 1955);

stated that wilting symptoms result from plugging of conductive elements by the pathogen itself or its products. The mechanical plugging theory was supported by many investigators working on various bacterial wilt diseases, such as wilt of cucumber caused by Erwinia tracheiphila, wilt of sweet corn caused by Xanthomonas Stewarti (Harris 1940), wilt of carnation caused by Pseudomonas caryophylli (Holtzmann - cited in Buddenhagen and Kelman 1964) and several others. However, in the case of Pseudomonas solanacearum, Hutchinson in 1913 (cited in Buddenhagen and Kelman 1964) reported that wilting was due to the alteration in cellular permeability produced by a toxin.

Recently, studies on the toxins isolated from Corynebacterium spp. have been reported by several workers. Spencer and Gorin (1961) reported that C. insidiosum and C. sepedonicum produced phytotoxin polysaccharides in culture media. Those substances resembled the compounds isolated from C. insidiosum and C. sepedonicum infected alfalfa and potato plants, respectively. These investigators detected fucose, galactose, and glucose residues in the acid hydrolyzed polysaccharides. They also showed that the polysaccharide isolated from C. insidiosum induced wilting in alfalfa cuttings at the concentration of 0.2 percent. They further suggested that the mechanism of toxin effect in wilting was the mechanical plugging of the xylem vessels. Patino-Mendez (1964) isolated a polysaccharide from a culture of C. michiganense. He detected fucose, galactose, glucose, mannose, rhamnose and an unidentified compound from the acid hydrolyzed polysaccharide. He also found that a

solution containing 0.01% toxin caused wilting in tomato cuttings. He further explained that the mechanism of wilting was plugging of the vessels, the reasons being that the affected plants recovered when transferred to water from the toxin solution and failure to draw the tracheal fluid from the toxin affected plants by negative pressure. In both cases (Spencer and Gorin 1961, and Patino-Mendez, 1964) no attempt was made to characterize the toxins other than their sugar components. The complete physical and chemical characterization of the toxin was wanting. Furthermore, meager evidences were given to state that plugging of the xylem vessels was the mechanism of toxin effect in plants. Strobel (1967) reported that the toxin isolated from C. sepedonicum was a glycopeptide in nature and possessed 2 sugars, 2 unidentified reducing compounds, an unidentified non-reducing compound and 6 amino acids. The purified toxin, in his study, had the molecular weight 21,450, was highly branched and its empirical formula was $C_{48}H_{96}O_{48}N$. He showed that the toxin lost biological activity after acid hydrolysis in 0.5N H_2SO_4 . Strobel refuted the suggestion of Spencer and Gorin (1961) that the mechanism of wilting was mechanical plugging of vessels. Rai and Strobel (1967) reported physiologically active compounds isolated from cultures of C. insidiosum, C. michiganense and C. sepedonicum. They showed that these 3 toxins were antigenic, serologically related and non-specific. Based on serological evidence and the biological assay developed by them, they showed that the toxins were present in C. michiganense and C. sepedonicum infected plants.

The purpose of this report is threefold as follows: 1) to clarify the chemical and physical properties of the toxin produced by C. michiganense, 2) to elucidate the mechanism of toxin action in plants, and 3) to determine the role of the toxin in disease symptom production.

MATERIALS AND METHODS

Culturing: A culture of C. michiganense (courtesy of E. E. Butler, Department of Plant Pathology, University of California, Davis), was maintained on a medium containing 1.5 percent glucose, 1.3 percent agar, 1.0 percent yeast extract and 0.5 percent calcium carbonate (Spencer and Gorin 1961).

Preparation and Purification of Toxin: The isolate was grown on a liter of liquid medium (Spencer and Gorin 1961) in a 2 liter flask aerobically, for 3 weeks at room temperature. The toxin was isolated in a manner similar to those employed by Spencer and Gorin (1961), Patino-Mendez (1964) and Strobel (1967). The 3 week old culture was centrifuged at 20,000 xg for 30 minutes and the pellet containing bacterial cells was discarded. The supernatant liquid was concentrated at 45° C in a flash evaporater to approximately one fifth of its original volume. The preparation was treated with 6 to 8 volumes of 95 percent ethanol and allowed to precipitate at 4° C over night. The precipitate was collected by centrifugation 8000 xg for 10 minutes. The pellet was dissolved in distilled water and passed through a 20.0 cm x 2.5 cm column of Dowex - 50, 200 to 400 mesh (H⁺) and then Dowex - 1, 200 to 400 mesh (formate form) to remove all charged ions. The effluent was collected and dried under a stream of air. The preparation was placed in a vacuum desiccator containing P₂O₅ and NaOH. Such a preparation was termed the crude toxin.

The crude toxin was further purified by using Sephadex - G-200 column, according to the procedure of Strobel (1967). Three toxic fractions were obtained by this method and were designated as fraction-I, II and III.

Biological assay: The biological assay for the crude toxin and its 3 fractions was conducted by treating 7.5 cm long trifoliate tomato leaves (Strobel 1967) in different concentrations of the compounds. Time taken to wilt the leaves, in each suspension, was recorded. This assay was repeated 3 times and the experiments were carried out for 2 hours in each case. The controls were similar concentrations of sterilized culture medium and distilled water.

Specificity test: This test was done by treating young plant cuttings of several species, in 0.2% (wt/vol) crude toxin suspension. Wilting was recorded in each case and the assay was repeated 3 times with 3 cuttings in each case, each time.

Extraction of toxin from plant tissue: Aerial parts of the diseased plant were thoroughly washed and rinsed in distilled water. After maceration in 10 ml distilled water with a mortar and pestle, the preparation was filtered through cheese cloth and centrifuged at 8000 xg for 10 minutes. The supernatant liquid was precipitated and carried through the procedure explained for isolation of the toxin except that the Dowex columns were only 10 cm x 2.5 cm.

Preparation of antiserum: Antiserum to the crude toxin was prepared in rabbits according to the procedures of Hamilton and Ball (1966). Normal serum was collected from 2 rabbits prior to immunization. The crude toxin was dissolved in neutral phosphate buffered saline (pH 7.0, 0.01M phosphate and 0.15M NaCl), and was emulsified in Freund's complete adjuvant. One ml of the emulsion containing 10 mg of crude toxin was injected into thigh muscle followed by a similar injection of the same dosage 7 days later. The antiserum was collected 4 weeks after the second injection, by ear bleeding. Serum was isolated by low centrifugation and stored at -20° until used.

Microprecipitin test: Microprecipitin tests were conducted with periodically collected antiserum at twofold dilutions (0 to 64) with the crude toxin as antigen (0.25 mg/ml), as outlined by Hamilton (1961).

Immunodiffusion test: Antigenic analysis was also done by immunodiffusion tests (Ouchterlony 1958). The general procedure of Hamilton and Ball (1966) was followed using crude toxin as the antigen.

Radioactive methods: C. michiganense was grown in the standard medium containing either 25 µc of ^{14}C -glucose (specific activity 3.9 mc/mM) or 25 µc of ^{14}C -mannose (specific activity 4.9 mc/mM) per 50 ml of medium. The radioactivity in the isolated compounds from the bacterial culture medium was determined in the liquid Scintillation Spectrometer (model 6804 Nuclear Chicago). The sample, 10 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, 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.

Specific viscosity study: The procedure followed in determining the specific viscosity was similar to that of Schachman (1957). The intrinsic viscosity was calculated from specific viscosity.

Elemental analysis: Elemental analyses of the crude toxin and its 3 fractions were done by Schwarzkopf Microanalytical Laboratory, Woodside, New York. From the percentage of C, H, O and N, the empirical formulas of the compounds were calculated.

Acid hydrolysis: The general procedure for acid hydrolysis was similar to that of Strobel (1967) whereby 10 ml, 1.0N, sulfuric acid was added to a 50 ml flask containing 10 to 20 mg of toxin. The mixture was refluxed at 100° C for 8 to 10 hrs. Such a mixture was cooled at room temperature and neutralized with an excess barium carbonate. The supernatant, after low speed centrifugation, was taken to dryness and the preparation was called acid hydrolyzed sample.

Chromatography: Separation and identification of the sugar residues were done by one-dimensional, descending, paper chromatography, on Whatman #1 filter paper in the following solvent systems, a) n-butanol-acetic acid-water (4:1:5 v/v), b) ethyl acetate-pyridine-water (8:2:1 v/v) and c) Butanol-acetic acid-boric acid (9:1:1 v/v). The reference

sugars used were fucose, galactose, glucose, mannose and rhamnose. The sugars were detected with the reagent of Trevelyan et al. (1950). R_f and R_g values of the unknown sugars were determined. After elution from the chromatograms, reducing sugars were quantitatively estimated by the method of Nelson (1944).

Amino acid analysis was carried out by thin layer chromatography in the following solvent systems: i) isopropanol-ammonium hydroxide (67:33 v/v), ii) n-butanol-acetic acid-water (3:1:1 v/v). Reference amino acids were alanine, glycine, lysine, methionine, serine and threonine.

Electron microscopic study: The ultrastructure of toxin treated and non-treated tomato stems was studied. Two week old tomato plant cuttings were treated in 0.2 percent toxin solution for 15 minutes. Control plant cuttings were treated in distilled water. All the cuttings were fixed in 1.2 percent aqueous potassium permanganate (wt/vol) for 3 min. In general, dehydration, embedding, thin section cutting, and mounting were followed as outlined by Carroll and Shalla (1965). Sections were examined with a Zeiss EM-9 Electron Microscope. The procedure was as follows: 1) two mm long stem cuttings from 2 mm below the primary leaves were taken from both toxin treated and control plants, 2) fixed in 1.2 percent potassium permanganate for 3 minutes, 3) rinsed in neutral phosphate buffer several times, 4) dehydrated in a graded series of acetone solutions, 10 minutes in each solution,

starting from 20 percent up to 100 percent, 5) embedded in Araldite, 6) cut into thin sections on a Reichert OM U2 Ultramicrotome with glass knives, 7) mounted the sections on Collodion-coated grids, 8) post section stained in 1.2 percent aqueous potassium permanganate for 2 minutes, 9) washed in distilled water and finally 10) observed under the electron microscope.

EXPERIMENTAL RESULTS

Specificity tests using the crude toxin: Host specificity of the crude toxin was determined by treating young plant cuttings, about 5 to 6 inches in size. Cuttings of alfalfa, barley, bean, cucumber, flax, petunia, potato, sugar beet, tomato and wheat were tested in a 0.2 percent aqueous toxin solution. Three cuttings of each plant species were treated with two replications. All the plant cuttings wilted when treated in toxin solution but did not wilt in control treatments as shown in Table I. However, barley, cucumber and wheat cuttings wilted in both toxin treatment and control.

Purification of the toxin: The crude toxin was further purified by passing it through a Sephadex G-200 column of 32 x 1.4 cm as followed by Strobel (1967). Ten mg of radioactive crude toxin (sp. activity 34,500) was used in the purification procedure. Three fractions were obtained from this Sephadex column as shown in Figure 1. Fraction III consisted of 36% of the total amount of toxin placed on the column, whereas fraction I and II were 26.6% and 26.0% respectively. The total recovery of the 3 toxins from the column was 88.6% of the total amount of crude toxin applied to the column.

Toxicity of the fractions: The toxicity of each fraction and the crude toxin were determined by treating 7.5 cm tomato leaves in different concentrations of the compounds according to methods of Strobel (1967). The concentrations of the solutions tested were 0.02, 0.04, 0.06, 0.08, 0.16 and 0.20 percent. The amount of toxin and time required to wilt

Table I

Host Specificity Test of the Crude Toxin

Plant cuttings	0.2% toxin	0.2% medium	Dist. water
Alfalfa	+	-	-
Barley	?	?	?
Bean	+	-	-
Cucumber	?	?	?
Flax	+	-	-
Petunia	+	-	-
Potato	+	-	-
Sugar Beet	+	-	-
Tomato	+	-	-
Wheat	?	?	?

Plant cutting treated for half hour.

+ Showing wilting

- Non-wilting

? No conclusion

Figure 1 The separation of ^{14}C -labeled toxins by Sephadex column chromatography. Ten mg of a crude preparation with a specific activity of 34,500 dpm/mg was placed on a Sephadex G-200 column and eluted with distilled water. Fractions (1.5 ml) were collected and 0.1 ml aliquots counted by liquid scintillation spectroscopy. The total dpm in each tube is plotted against the tube number. All the three fractions were biologically active as manifested by the tomato cutting test.

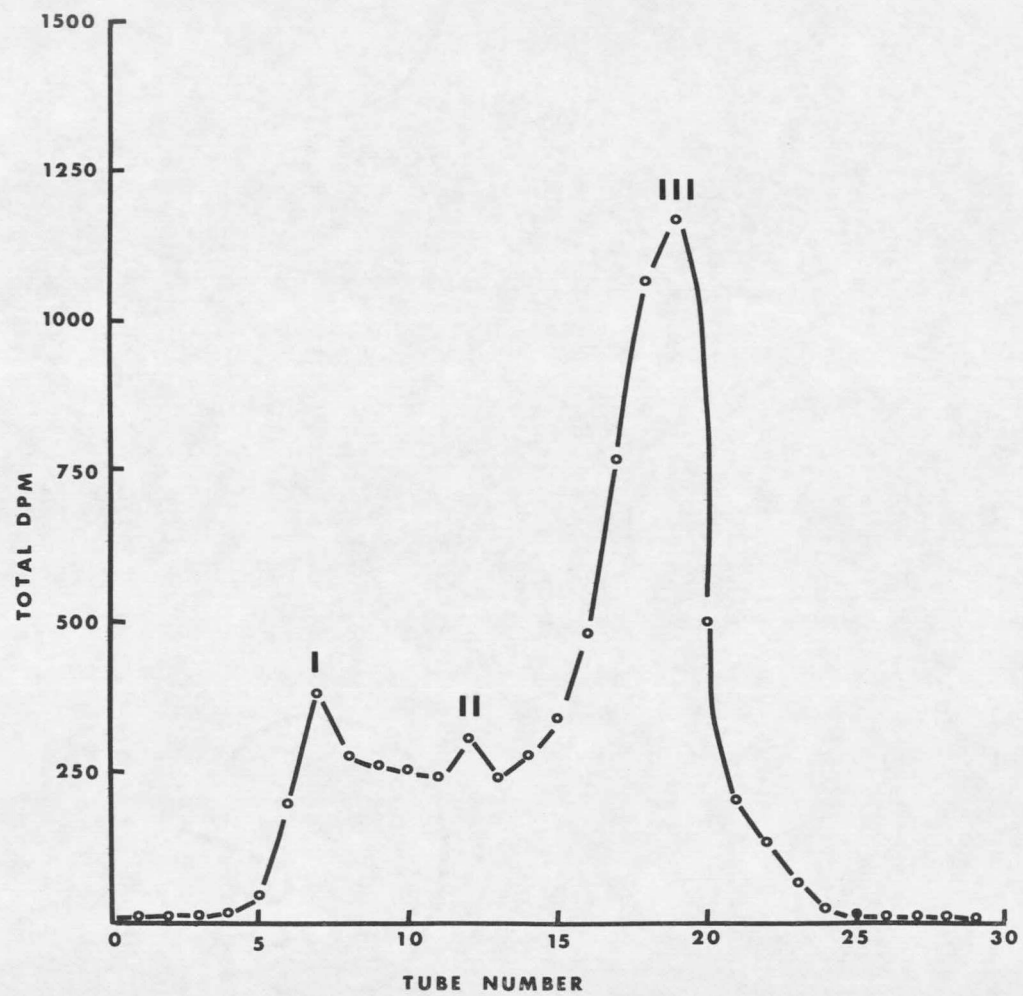
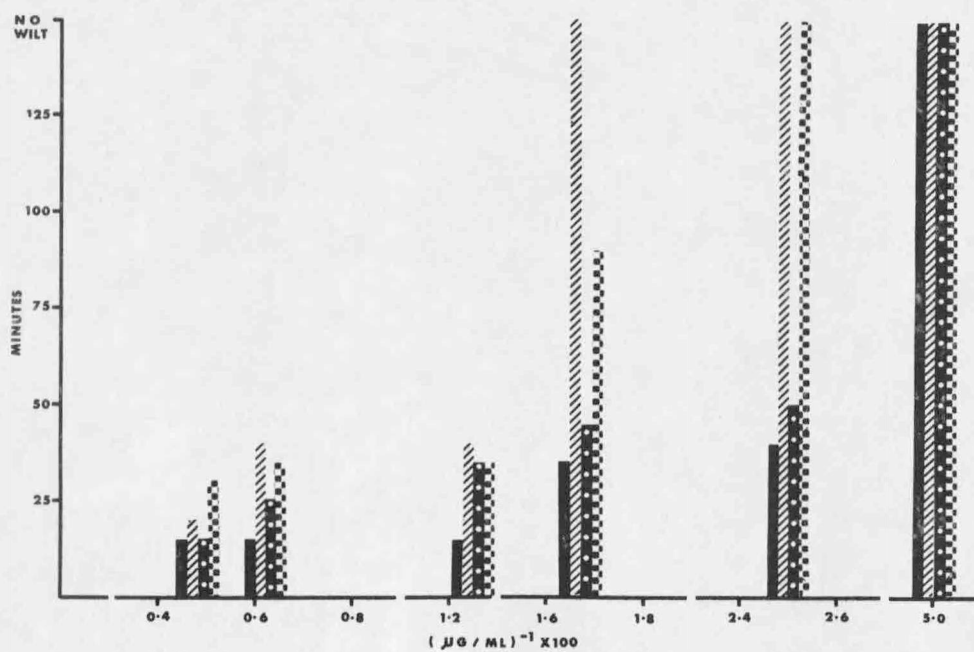


Figure 2 Toxicity comparison between the 3 fractions and the
crude toxin.

- Crude toxin.
- ▨ Fraction I.
- ▩ Fraction II.
- ▧ Fraction III.

Time, in minutes, of 7.5 cm tomato leaves to wilt as a function of toxin concentration. The concentration of the toxins are expressed as the reciprocal of $\mu\text{g/ml}$ x 100. The values on the graph (left to right) are equal to 0.20, 0.16, 0.08, 0.06, 0.04 and 0.02% solutions, respectively. Note the X axis is interrupted.



the primary leaflets of the tomato leaves in each toxin solution are shown in Figure 2. This experiment was repeated 3 times and kept under observation for 2 hours in each case. The control experiments consisting of distilled water and 0.2% culture medium did not show any effect on the leaves. Figure 2 reveals that crude toxin was most toxic causing wilting at the concentration of 0.02 percent in 40 minutes. Among the fractions, II was the most toxic since it caused wilting at the concentration of 0.02 percent in 50 minutes. Fraction I was least toxic and fraction III was intermediate. The Figure 2 also shows that the crude toxin has a linear relation between the concentration and time required to cause wilting. However, the effect levels off, at the concentration of 0.08% toxin solution up to 0.2%. Fraction I and III do not show a linear relation in their toxicity whereas fraction II shows a linear relation.

Serological relationships: Double diffusion (Ouchterlony 1958) and microprecipitin tests were done according to the procedures of Hamilton (1961) and Hamilton and Ball (1966), in order to determine the serological relationships of the crude toxin and its fractions. Twofold dilutions 0, 2, 4, 8, 16, 32 and 64 of the antiserum were tested against the toxins (0.25 mg/ml). Normal serum buffer and medium were used as controls. The tests showed that the crude toxin yielded a precipitin reaction up to a 16 fold dilution (maximum titer obtained in the periodic microprecipitin tests) of the antiserum whereas each of the fractions reacted with only concentrated or twofold diluted antiserum.

According to the immunodiffusion test, the crude toxin showed one precipitin arc, close to the antiserum well, as shown in the Figure 3. Figure 4 shows that all the 3 fractions were serologically related and identical as the precipitin lines join each other without spurs. The crude toxin was also serologically related to the fractions. The crude toxin or the fractions did not react with normal serum and buffer. 0.25 mg compound in a ml of buffer, isolated from the sterilized medium, similar to toxin isolation, did not react with the antiserum.

Molecular weight determination: Column chromatography with Sephadex has been used for molecular weight estimation of the toxins, (Granath 1965 and Strobel 1967). Using Sephadex G-200, the equation for molecular weight estimation is $\frac{V_e}{V_t} = 3.20 - 0.58 \log \text{molecular weight}$, where V_e equals the volume at which the sample is eluted from the column minus the void volume of the column and V_t equals the total volume of the column which was 32 ml. The void volume was 10.5 ml. The sample volumes were 10.5, 18, and 28.5 respective to fractions I, II and III. Fraction I eluted at void volume of the column, hence its molecular weight is 200,000 or greater (Table II).

The intrinsic viscosities of the crude toxin and its fractions were determined by the method of Schachman (1957). The concentrations used were 0, 2, 4, 6 and 8 mg per ml water at 30° C. The intrinsic viscosity was calculated by the formula Specific viscosity/Concentration (gm/deciliter) and was given in the Table II. The intrinsic viscosity

Figure 3 Semidiagrammatic drawings of immunodiffusion patterns
of crude toxin.

Central wells -

AS = Antiserum

NS = Normal serum

Peripheral wells -

T = Toxin

B = Buffer

The antigenicity of the crude toxin was determined by the immunodiffusion test. The diffusion medium consisted of 1 percent agarose melted in a buffer (neutral phosphate buffer), with 0.02 percent sodium azide as a preservative. The crude toxin (0.25 mg per ml of buffer) was used as an antigen. The diffusion systems were incubated at 20° C for 24 to 48 hours. A precipitin arc is apparent between the antiserum and toxin wells. There is no reaction between the normal serum and the toxin.

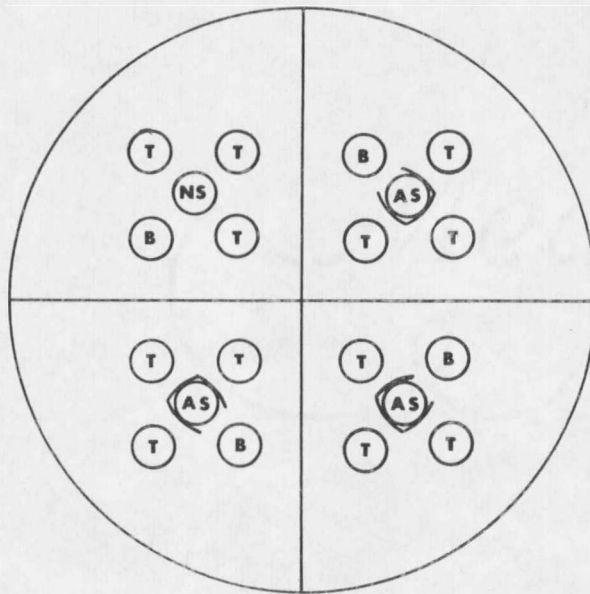


Figure 4 Semidiagrammatic drawing of immunodiffusion patterns
of the fractions I, II and III in relation to crude
toxin.

Central wells -

AS = Antiserum

NS = Normal serum

Peripheral wells -

C = Crude toxin

I, II & III = Fractions I, II & III

respectively

B = Buffer

The serological relationship between the fractions was determined by the immunodiffusion test. The crude toxin and the fractions were used as antigens (0.25 mg/ml of buffer). There is one predominant precipitin band.

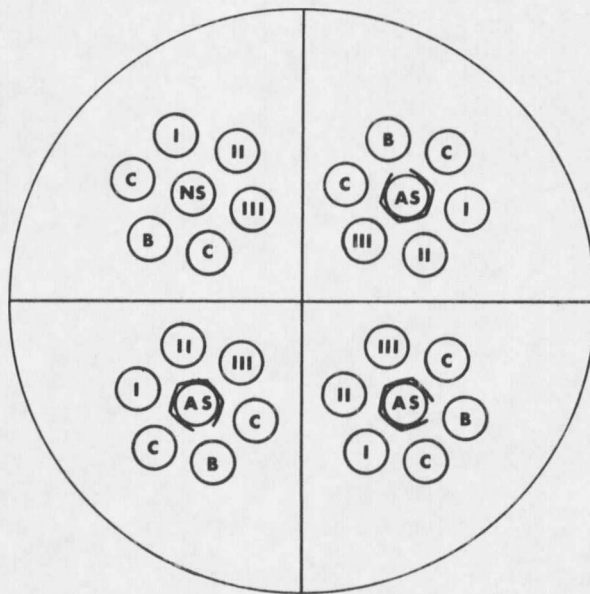


Table II

Chemical Characterization of the Toxins Isolated from C. michiganense

	Crude toxin	Fraction I	Fraction II	Fraction III
Empirical formula	$C_{55}H_{113}O_{52}N$	$C_{42}H_{82}O_{40}N$	$C_{473}H_{940}O_{449}N$	$C_{27}H_{52}O_{25}N$
Estimated molecular weight	---	$\geq 200,000$	129,700	35,280
Intrinsic viscosity	0.2500	0.2250	0.2140	0.1904
Sugar residues* and ratios	fucose, galactose, glucose, mannose 3:2:56:106	galactose, glucose, mannose 2:75:156	galactose, glucose, mannose 2:130:365	fucose, galactose, glucose, mannose 3:2:20:87
Total percent of recoverable sugars	78.9	46.9	50.2	34.4
Amino acids** and ratios	alanine, glycine, lysine, methionine, serine, threonine 1:2:1:1:1:1	alanine, glycine, lysine, methionine, serine, threonine 1:2:1:1:1:1	alanine, glycine, lysine, serine 1:2:1:1	alanine, glycine, lysine, methionine, serine, threonine 1:2:1:1:1:1

* Two unidentified reducing compounds were present in each fraction but accounted for less than 1% of the total sugars.

** One unidentified ninhydrin positive compound was present in each fraction.

values appear directly proportional to the molecular weights of the compounds.

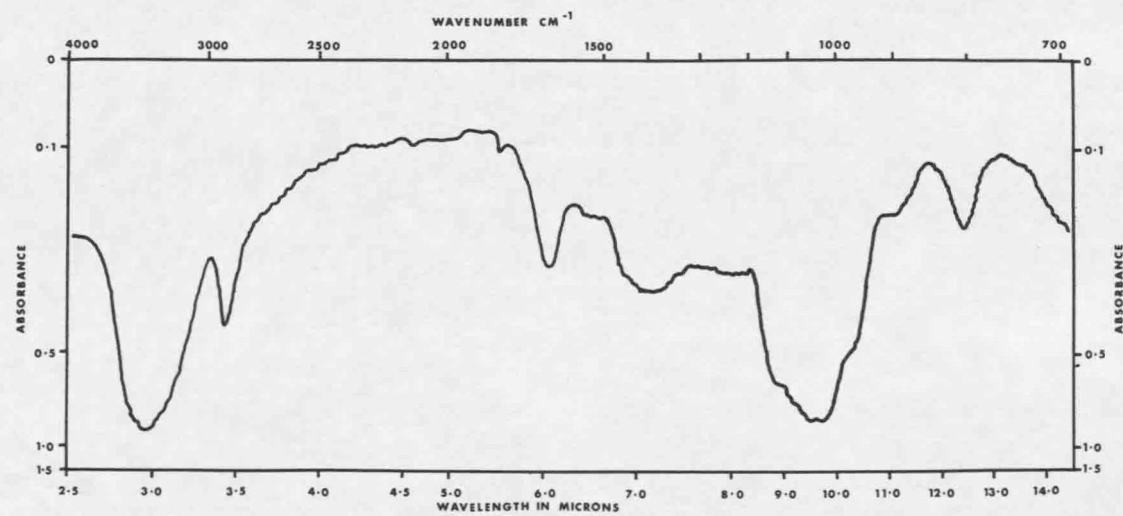
Infrared spectrum absorption study: There was no noticeable difference in the infrared spectrum between either the fractions or between the fractions and the crude toxin. A representative chart indicating the absorbance peaks in the infrared spectrum for the crude toxin is shown in Figure 5. The absorption spectrum for each fraction did not deviate from that shown in Figure 5. The spectrum appears to be the typical for that of a carbohydrate.

Elemental analysis: Percentages of carbon, hydrogen, oxygen and nitrogen varied slightly among the fractions and crude toxin. However, the nitrogen content of different compounds noticeably varied as shown in Table II. Empirical formulas of the toxins were calculated and given in the same table. Fraction II and III have empirical formulas quite different from that of crude and fraction I; this is due to the fact that fraction II contains very low nitrogen and fraction III contains comparatively high nitrogen.

Qualitative and quantitative analyses of toxins for sugar residues: Fucose, galactose, glucose, mannose and 2 unknown sugars were present in the crude toxin and fraction III whereas fractions I and II contained no fucose as shown in Table II. The quantitative analysis showed that mannose was the predominant sugar in all the fractions

Figure 5

A typical infrared spectrum absorption chart for the
crude toxin and its fractions. Micropellets were
prepared by mixing the compound in KBr and was scanned
at 0.2 inch per min with 5.5 gain.



including the crude toxin. Glucose was next in amount to mannose in quantity in all the samples. Galactose was in the smallest quantity in all the samples as shown in Table II.

Qualitative analysis of the toxins for amino acids: The crude toxin and all the fractions contained alanine, glycine, lysine, methionine, serine, threonine and an unknown ninhydrin positive compound approximately in a ratio of 1:2:1:1:1:1, except in fraction II, which lacked methionine and threonine as shown in Table II.

Stability of the crude toxin on hydrolysis: A 0.2 percent solution of the crude toxin was refluxed in 1.0N H_2SO_4 for various time periods. After each exposure time, an aliquot of the acid solution was removed and neutralized with an excess of BaCO_3 . After the removal of the precipitate the solution was taken to dryness and redissolved in an amount of water resulting in a 0.2% solution of this partially hydrolyzed toxin. A water reflux control was simultaneously conducted. Acid hydrolyzed crude toxin solution lost its toxicity within 1 minute after refluxing. When the toxin was refluxed with water for 5 minutes the toxicity was not lost.

Radioactive toxin study: In a time sequency experiment, the incorporation of ^{14}C glucose was lower in comparison to that of ^{14}C -mannose as shown in Table III. The highest percentage of ^{14}C -glucose incorporation was 0.176% in 8 day old culture whereas the highest incorporation of ^{14}C -mannose was 9.242% in the 20 day old culture. Furthermore, the

Table III

Comparison of Incorporation of ^{14}C -Glucose and ^{14}C -Mannose into the Toxin

^{14}C -compound used.	Treatment in days.	Total amount of toxin produced in mg.	Dpm recovered in toxin.	Specific activity dpm/mg.	Percentage Incorporation.
Glucose	2	2.0	854	427	0.010
8,250,000	4	5.1	5,400	1,059	0.065
DPM/treat.	8	9.6	14,510	1,512	0.176
	12	10.0	3,379	338	0.041
	16	9.3	2,028	218	0.025
	20	10.8	2,331	216	0.028
Mannose	2	2.2	36,680	16,673	0.570
6,427,000	4	4.0	98,100	24,525	1.524
DPM/treat.	8	5.8	172,645	29,766	2.682
	12	10.2	340,612	33,393	5.291
	16	14.5	491,387	33,889	7.634
	20	17.3	596,894	34,502	9.242
	24	18.3	462,845	25,292	7.190

maximum specific activity of the toxin obtained in case of ^{14}C -glucose fed culture was 1512 (dpm/mg), whereas the ^{14}C -mannose fed culture produced a radioactive toxin with highest specific activity of 34,502. The toxin production continued to increase up to 24 days as shown in Figure 6. At the end of 24 days the total weight of the recovered crude toxin was 18.3 mg whereas the culture yielded 2.2 mg toxin at the end of 2 days. There was an approximate linear relation between the age of the culture and the amount of toxin produced. The specific activity of the crude toxin approximately followed the same pattern as the toxin production. However, the specific activity curve reached to its peak in 20 days and then declined as shown in Figure 7. The crude toxin varied in labeled sugar composition according to the age of the culture as shown in the Figure 8. In the beginning of the experiment the toxin contained mannose with a high specific activity but after 6 days fucose had the highest radioactivity. After 16 days, both fucose and mannose declined in total radioactivity whereas glucose increased. These observations initially suggested that more than one high molecular weight substance was present in the crude toxin preparation.

Radioautography of plants fed labeled toxin: This was done to determine the location of the toxin in the plant prior to wilting and after wilting. The experiments were done with 2 week old tomato plant cuttings, or leaves, after feeding 0.2 percent ^{14}C -crude toxin with specific activity

Figure 6

The histogram showing the amount of toxin produced in
mg by *C. michiganense* at different intervals, in days,
of growth. Fifty ml of medium was mixed with ^{14}C -
mannose and transferred to 10, 25 ml flasks, 5 ml in
each flask, and sterilized. Approximately 3.3×10^8
cells of *C. michiganense* were inoculated into each
flask. The control flasks were immediately sterilized
after introducing the bacteria. The time intervals
followed were 2, 4, 8, 12, 16, 20 and 24 days. The
culture was incubated aerobically at room temperature.
At the end of each treatment the toxin was isolated
and the weight was determined. The weight of toxin
in mg is plotted against the intervals in days.

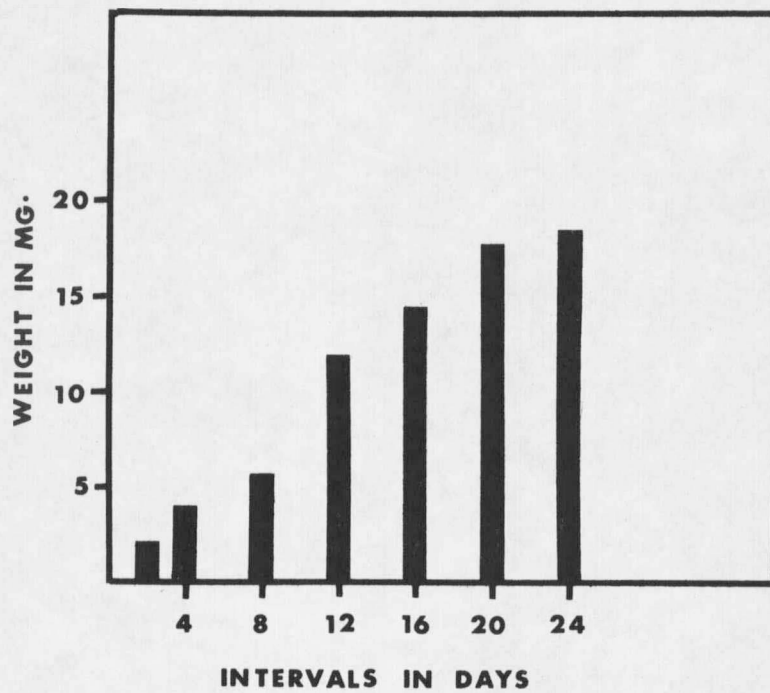


Figure 7 Specific activity curve of the toxin. Specific activity was defined as the dpm per mg of toxin. At the end of each treatment, of the time sequence experiment, the toxin was isolated from the culture. The dpm of the ^{14}C -toxin was determined and the specific activity was calculated. The specific activity is plotted against the intervals in days. The relation between the age of the culture and the specific activity is not perfectly linear.

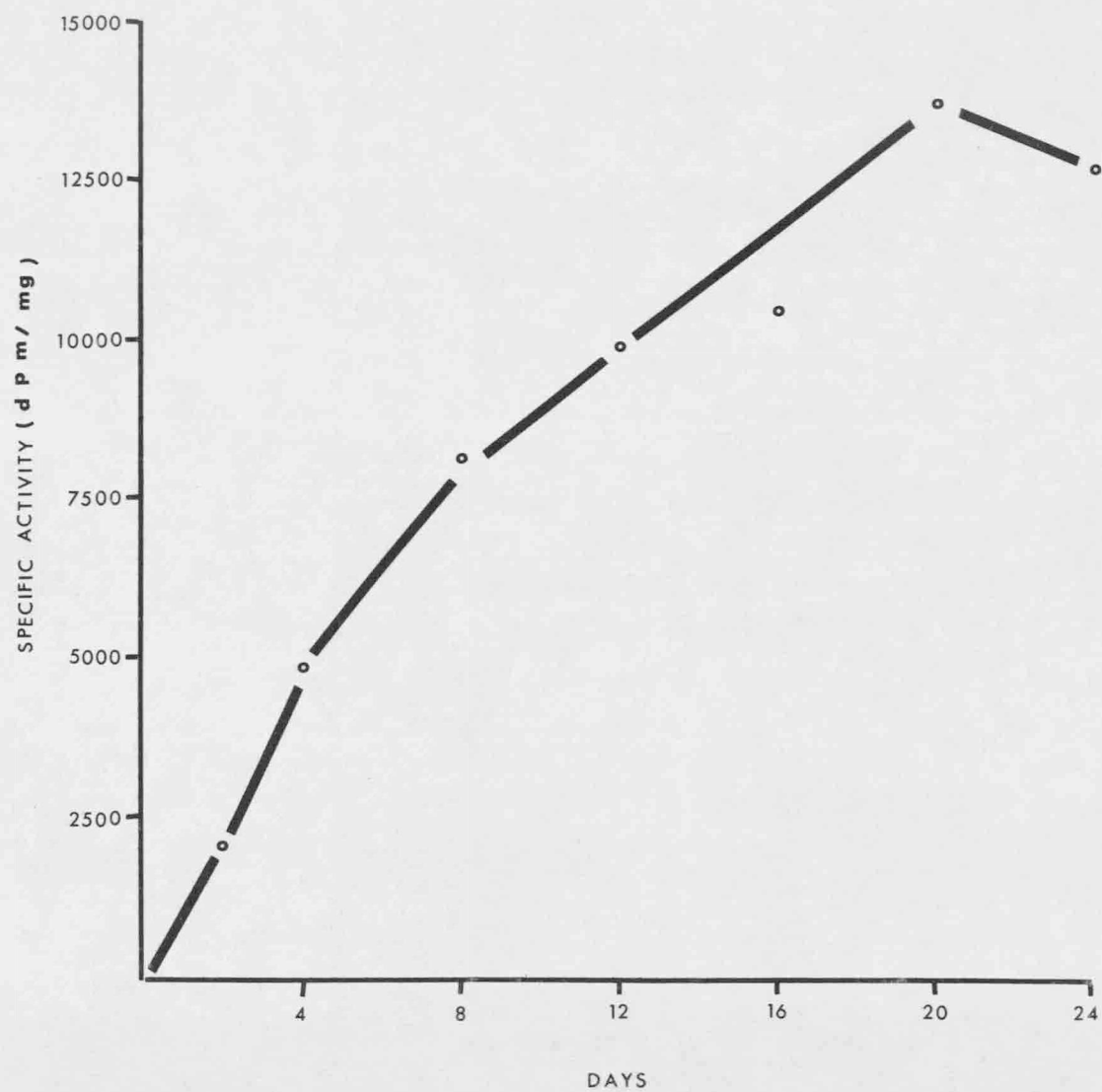
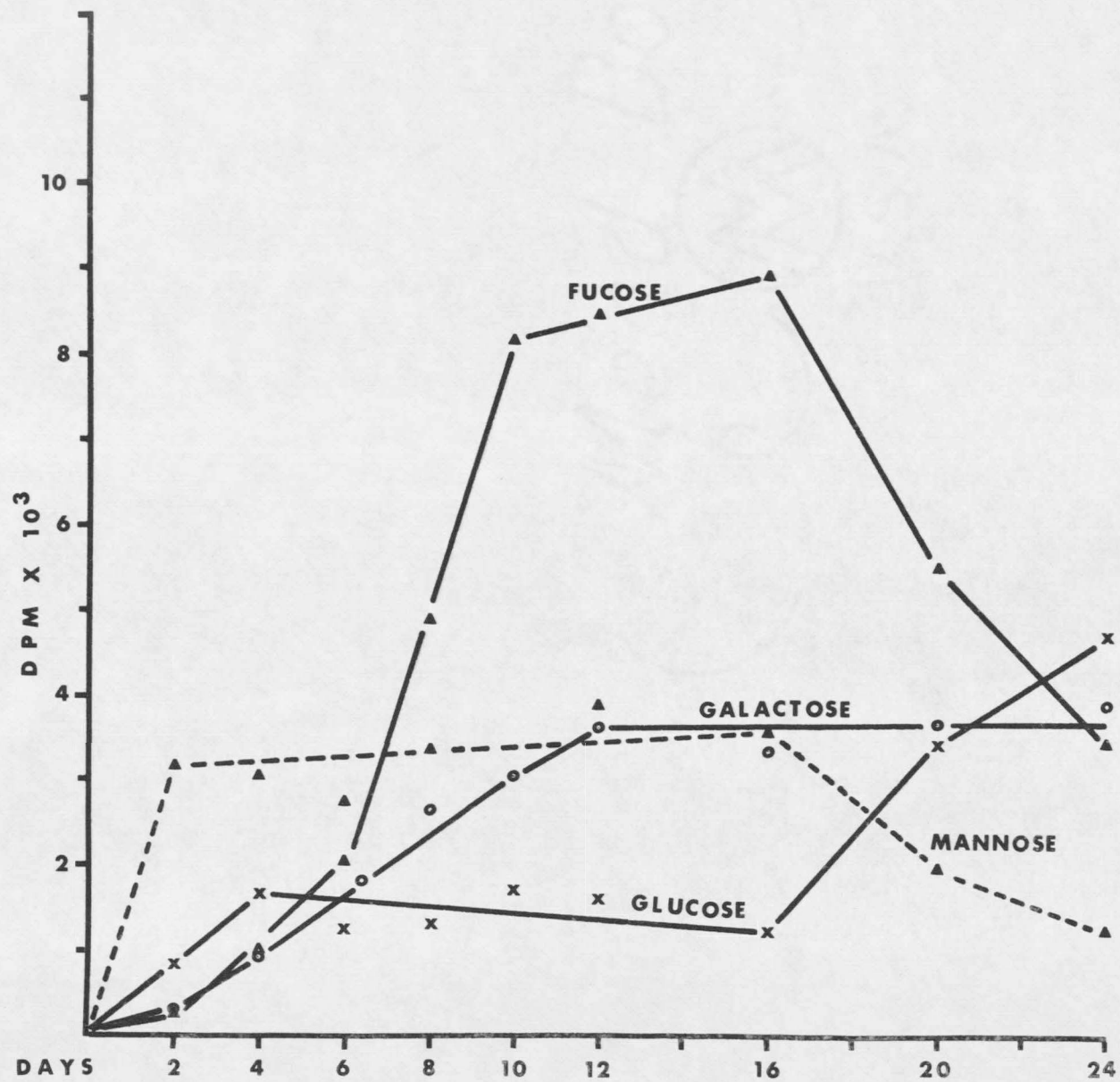


Figure 8

¹⁴C-mannose incorporation into sugar residues of the toxin at different time intervals. The toxin was isolated at the end of each treatment, acid hydrolyzed, chromatographed and each sugar residue was eluted from the chromatogram. The radioactivity of each sugar-residue was checked (in DPM) and plotted against the intervals in days.



25,000 (dpm/mg), for 10 to 30 minutes, according to the general procedures of Crafts and Yamaguchi (1963) and Strobel (1967). The plant (or leaf) cuttings were exposed to a Kodak No-screen x-ray film and incubated at 0° C for 4 weeks. In all the cases the radioactive toxin was noticed in stems, petioles, leaves, leaf veins and leaf margins. However, the greatest amount of ^{14}C -toxin was noticed in the stem as shown in the Figure 9-a and petiole and leaf margins in case of leaves as shown in the Figure 9-b. Similar procedures were followed by feeding the plants or leaves with ^{14}C -labeled fractions I, II and III individually and similar results were found in all the cases. The time variation in feeding the plants did not make difference except that the intensity of exposed marks on the film were greater, when fed for a longer time.

Toxin break down study: Experiments were performed to determine if the toxin was broken down in the plant during treatment. Such experiments were conducted by feeding tomato cuttings ^{14}C -crude toxin and ^{14}C -labeled toxin fractions individually for 15 minutes with 0.2% (wt/vol) solution. Extracts of plants were made according to the procedures given in the materials and methods and precipitated with 4 volumes of 95% ethanol. The radioactivity was checked in both the precipitate and supernatant liquid after centrifugation. Radioactivity was recovered from the precipitate in all cases and in the case of fraction I, the supernatant liquid also had radioactivity (Table IV). The supernatant liquid of fraction I treatment was passed through

Figure 9 Radioautographs of ^{14}C -toxin fed tomato plant and a leaf.

A = A representative radioautograph of ^{14}C -toxin fed tomato plant cutting.

B = A representative of radioautograph of ^{14}C -toxin fed tomato leaf.

A 2 week old tomato plant cutting (or leaf) was placed in a 0.2 percent ^{14}C -toxin (sp. act. 25000, dpm/mg) solution for 10 minutes and then exposed to a Kodak No-screen x-ray film for 4 weeks. The location of ^{14}C in the plant is represented by the exposed marks on the x-ray film which is in the stem, petiole, leaf-veins and leaf margins. Reisolation of the toxin from this plant or the leaf showed that antigenicity and toxicity were retained.

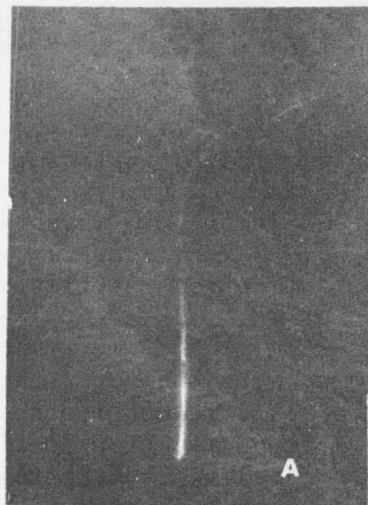


Table IV

Determination of the Toxin Breakdown in Plants

Fraction	Total radio- activity in 0.2% toxin solution DPM.	Total radio- activity taken up by 2 plants DPM.	Toxin taken up by 1 plant prior to wilt- ing in μg^* .	Total radio- activity re- covered from plant extracts DPM.	Distribution of radio- activity in plant extracts in percent.	
					PPT	SL
Crude toxin	26,116	1,471	58.0	213	100	0
Fraction I	27,000	1,480	55.0	688	73	27
Fraction II	26,000	1,205	46.5	398	100	0
Fraction III	76,413	4,128	54.0	496	100	0

* determined on the basis of radioactivity.

PPT = Precipitate ethanol

SL = Supernatant liquid

appropriate resins and the anion, cation and neutral fractions were obtained. Each of these were checked for radioactivity. The anion and neutral fractions showed radioactivity. Table IV shows the total radioactivity present in the 0.2 percent toxin solutions administered to two plants. Total radioactivity taken up by the plants prior to wilting was determined by checking the radioactivity in the toxin solutions after feeding. The amount of toxin taken up by the plants was calculated on the basis of radioactivity. Total radioactivity recovered from the crude plant extract and percentages of radioactivity in precipitate and the supernatant liquid were also given in the Table IV. Table IV also shows that the amount of toxin required to cause wilting varied from 46.5 to 58.0 μ g in different fractions. The toxin molecule in general is not broken down in the plant.

Toxin effect on water uptake: Terminal and lateral movement of water in toxin affected plants were both studied. Dextran, (Molecular weight 40,000) being the known plugging material of xylem vessels similar to pectin, xylan and carbowax (Goodman et al. 1967) was used as a control, to compare with the toxin which also believed to be a plugging agent (Patino-Mendez 1964). A 4 week old tomato cutting was treated in 0.2% toxin until the leaves wilted (15 minutes). Similar plants were placed in dextran (0.2% solution) until the leaves wilted (1 hr). The wilting pattern of leaves in the case of toxin affected plant cuttings started from the leaf margins and was accompanied by curling whereas the dextran

caused uniform wilting in leaflets. Control plant cuttings were also placed in distilled water. All the plant cuttings were transferred to 0.1% solution of acid fuchsin. The time taken by the dye to move up to the tip of the primary leaf in each case is given in Table V. The dye moved to the tip of the leaf in 5 to 6 minutes in case of toxin treated plant cuttings whereas it was 35 minutes in the case of dextran treated plants.

The toxin effect on the lateral movement of water in plants was also studied by use of the dye. This was done to detect whether the toxin plugs the pits in the vessels (Corden 1966). Tomato cuttings were split as shown in the Figure 10, and each half was given a different treatment of either water or toxin. The time required for the dye to reach the tip of the toxin affected leaf was less than that for the water treated leaves (Table V).

Electron microscopic study: Electron microscopy of the toxin treated plant sections revealed that the (plasma) membrane in the parenchymatous cells of vascular tissue were pulled away from the cell wall, ruptured considerably and large numbers of vesicles were formed as shown in Figure 11. The inner membrane of the plastids was also pulled away from the outer membrane in a few regions. In the case of the plant sections that served as the control, the plasma membrane was intact, close to the cell wall and normal (Figure 12).

Table V

Lateral and Terminal Movement of Water in Toxin Affected Plants

Compound	Time taken to wilt in minutes	Terminal movement of water	Lateral movement of water
		Time taken the dye to reach tip of primary leaf in minutes	Time taken the dye to reach tip of primary leaf in hr
Toxin - 1 0.2%	10	3 to 6	2
Dextran 0.2%	60	35	
Acid fuchsin 0.1%	--		
Distilled water	--	5 to 6	5

Figure 10. A diagrammatic representation of a tomato stem used in the study of toxin effect on lateral movement of water in plants. Tomato cuttings were split into two equal halves at the base. Top of the plant was cut by leaving one leaf at the left side of the plant to show the lateral movement of the dye after the treatment. Each split was given a different treatment as shown below and the time taken by the dye to reach the tip of the leaf was recorded.

Treatments -

Control - i) beaker A contained
distilled H_2O

Treat - ii) beaker A contained 0.2%
toxin solution
beaker B contained dye.

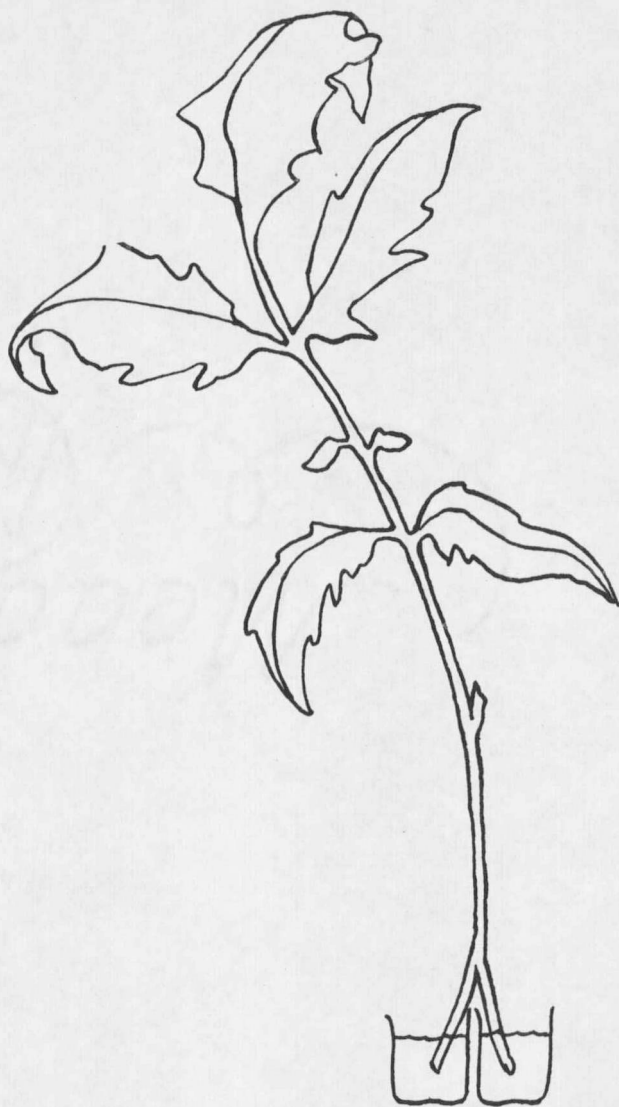


Figure 11 Electron micrograph of a tomato stem section treated with
toxin.

MB = Membrane

CW = Cell wall

PL = Plastid

VES = Vesicles

INS = Intercellular space

The tomato stem section were stained in KMnO_4 . The
magnification was 20,000 X.

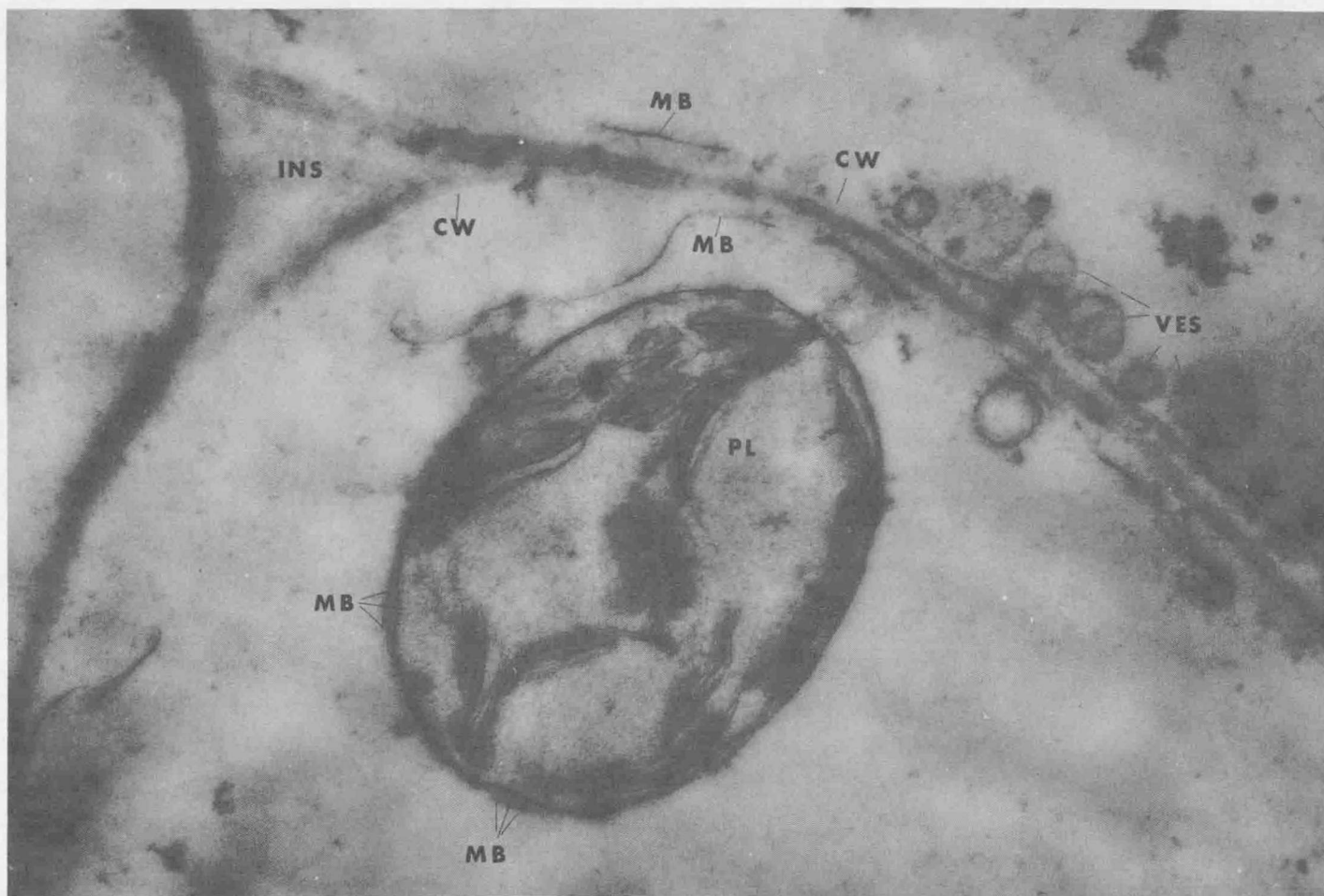


Figure 12 Electron micrograph of a non-treated tomato stem section.

MB = Membrane

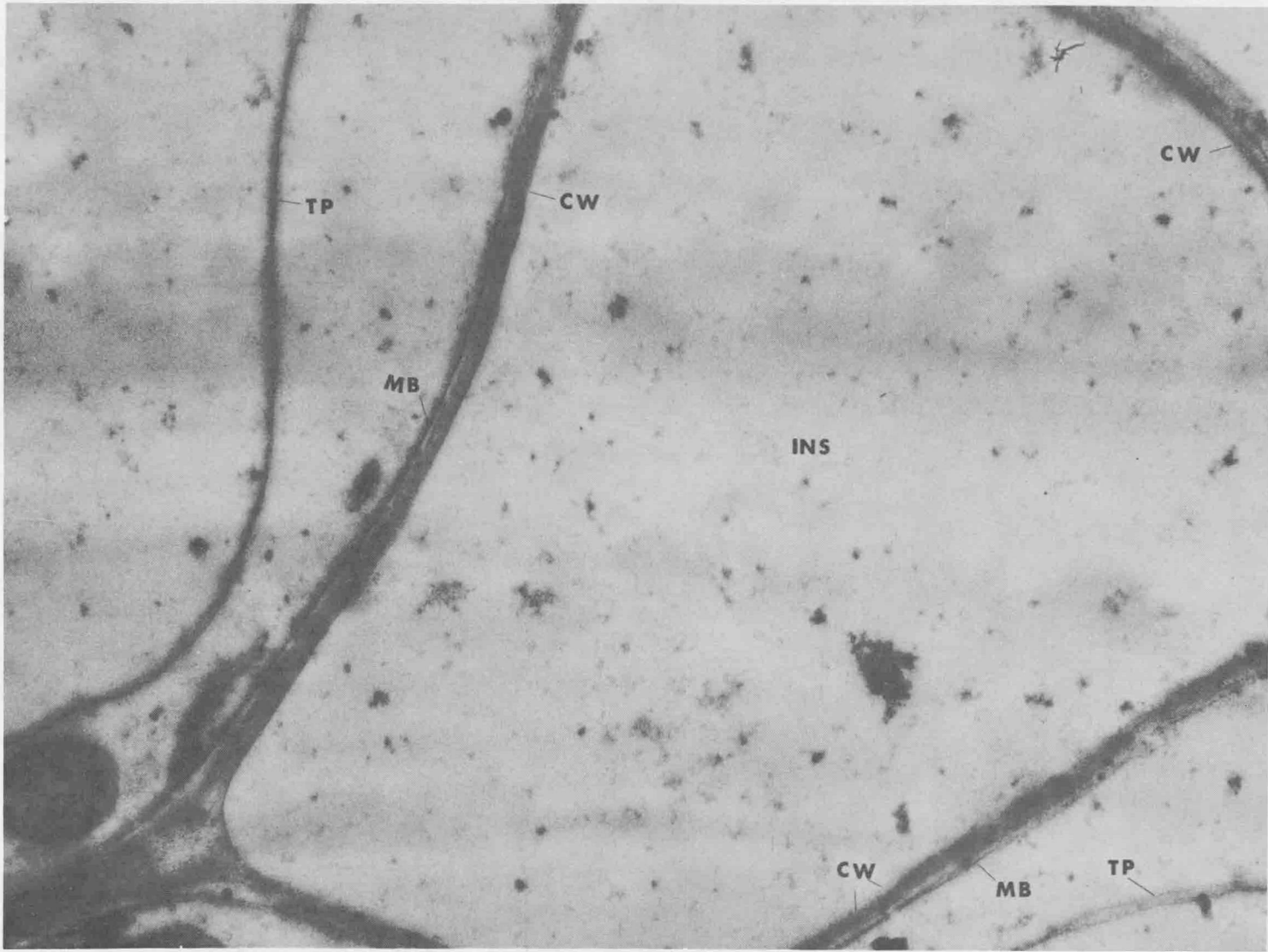
CW = Cell wall

INS = Intercellular space

TP = Tonoplast

The normal tomato stem sections were stained in

KMnO_4 . The magnification was 25,000 X.



Role of toxin in the disease production: A determination of the primary role of toxin in the symptom expression was done by isolating bacteria and toxin from different regions of the infected plants, at a different time intervals after inoculation. Six week old tomato plants were inoculated with a week old C. michiganense culture as described in Figure 13. Ten plants were inoculated and 10 plants were kept as control, where no inoculation was done. Samples were assayed at 1, 2, 4, 6 and 8 days after inoculation. At the end of each treatment 2 inoculated and 2 control plants were tested for presence of the bacteria and the toxin at different regions of the plants which contained a node, an internode and a leaf (approximately 1" stem). The first sample number was started from the primary leaf region. The presence of bacteria was determined by isolation on nutrient agar, by study of the morphological characters of colony and by the gram stain test. The presence of toxin was determined by the biological assay and serologically after isolation. Serological reaction was tested by mixing a drop of toxin, isolated from the test plant samples as described in materials and methods, with an undiluted antiserum - drop in a test tube. The reaction mixture was observed under microscope. Similar tests were carried out with normal serum, but with negative results. Control plants were observed through similar tests and found no reaction was observed.

The above test revealed that the toxin produced by the bacteria diffused throughout the plant within a day and the bacteria took at least 2 days to spread throughout the plant, as shown in Table VI. The

Table VI

Role of Toxin in the Disease Production

Intervals in days	Location of bacteria in plants										Location of toxin in plants									
	Control					Treatment					Control					Treatment				
	1	2	3	4	5*	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+	+	+	+
2	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+
4	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+

* Numbers 1 to 5 indicate 1" plant samples, starting from the primary leaf to the base of the stem, respectively.

experiment was terminated after 4 days, as bacteria were found throughout the plant parts. Control plants did not reveal either presence of the bacteria or the toxin.

Presence of toxin in infected plants: The toxin was isolated from artificially and naturally infected¹ plants by taking the plant extracts and following through the toxin isolation procedure. A typical C. michiganense infected plant is shown in Figure 13. The extract from infected plants (extraction procedure is given in materials and methods) was biologically positive in the tomato assay test; and serologically reactive. A similar extract from non-infected plants was biologically inactive and serologically negative. All the extracts showed negative reaction with normal serum. The extract of the test plants originally treated with the extract from diseased tomato plants, were also biologically active and serologically positive (Figure 14), whereas the controls (plants treated in extracts from non-infective plants) were negative. Figure 14 shows that the extracts isolated (procedure is given in the materials and methods) from the toxin affected plants had a precipitin arc similar to the crude toxin isolated from the bacterial culture.

¹ Collected from a home garden at Missoula, Montana by Dr. Strobel.

Figure 13 A 2 month old normal tomato plant at the left and a
C. michiganense infected plant of the same age at the
right. A five day old C. michiganense culture was
used to inoculate a 4 week old tomato plant. Inocul-
ation was done by cutting the petiole with a sterilized
razor blade and applying the bacterial culture to the
cut end of the petiole. In the control plant a cut
was made in the petiole, as shown in the figure.



Figure 14 Semidiagrammatic drawings of immunodiffusion patterns
of the plant extracts.

Central wells -

AS = Antiserum

NS = Normal serum

Peripheral wells -

DP = Extract from diseased plants

(1 mg toxin/ml buffer)

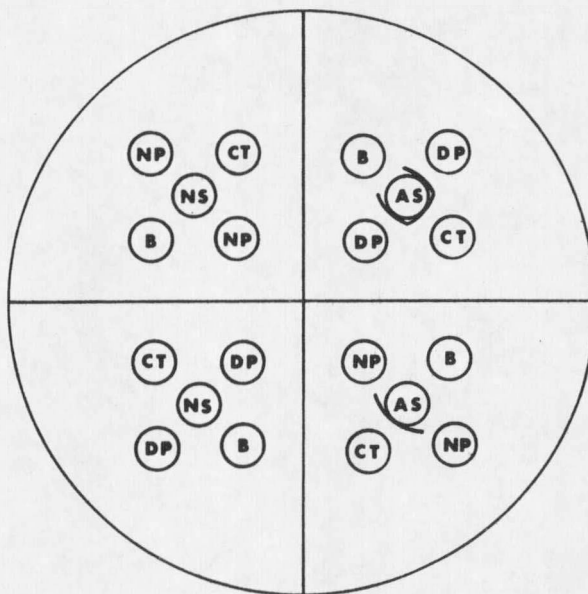
CT = Crude toxin (0.25 mg/ml buffer)

NP = Extract from normal plants

(1 mg/ml buffer)

B = Buffer

The presence of the toxin in the diseased plants was determined by gel diffusion procedures (Ouchterlony 1958). Concentrated normal and anti-serum were used in the central wells. Compounds isolated from the plants were used as antigens in the peripheral wells as well as crude toxin and buffer as controls. A precipitin arc appeared between AS and DP, similarly between CT and AS. The precipitin arcs of DP and CT against AS joined together indicating that they were serologically related.



DISCUSSION

The toxin isolated from C. michiganense was non-specific (Table I). However, 3 plant species, namely barley, cucumber and wheat, wilted in both the toxin treatment and control. This may be due to the wound shock encountered while cuttings were made or to the specific structural characteristics of the plants. The non-specificity of phytotoxins of bacterial origin was reported by Hodgson, Peterson, and Riker (1947, 1949) using Agrobacterium tumefaciens, Xanthomonas phaseoli, and Erwinia carotovora. The non-specificity of a toxin may be one indication showing that the effect of the toxin is mechanical or physical disruption of host tissue.

On purification of the crude toxin preparation by Sephadex G-200 3 phytotoxic fractions were obtained (Figure 1). Strobel (1967) reported 2 fractions in a phytotoxin isolated from C. sepedonicum, but only one fraction was phytotoxic. The crude toxin preparation from C. michiganense, was most potent as manifested by the tomato cutting assay test (Figure 2). Among the individual fractions, fraction II was most toxic. The crude toxin and the fraction II showed a linear relation to concentration vs time required to wilt the tomato cuttings, whereas in fractions I and III this was not true. The reason for the differences in the toxicity is not known.

On comparison of the crude toxin with its fractions by serological methods the crude toxin had titer count >16 whereas the fractions had titer counts only 0 or 2, which is comparatively low. This may be due to the presence of a high proportion of carbohydrates which are poor in

antigenicity, or to the impurities present in the toxin. The low titer of the fractions I, II and III are probably due to the fact that the antiserum was prepared against the crude toxin and hence may not be very sensitive to the fractions. It may be also due to antigen excess in the fractions. Figure 3 shows that all the 3 fractions and the crude toxin are serologically related, as the precipitin lines join each other without forming spurs. The serological methods could be employed as effective diagnostic tools, for an example, Guthrie et al. (1965) used serological methods in diagnosing halo blight of bean.

Patino-Mendez (1964) reported fucose, galactose, glucose, mannose, rhamnose and an unidentified sugar residue from a toxin isolated from C. michiganense. In the present study the crude toxin and its fractions contained, fucose, galactose, glucose, mannose and 2 unidentified reducing sugar residues and however, fraction I and II had no fucose (Table II). The differences in the sugar composition of the toxins might be due to the culture, age, environment, etc.

Alanine, glycine, lysine, methionine, serine, threonine and an unknown ninhydrin positive compound were present in the acid hydrolyzed cation fraction of the crude toxin in the approximate proportion of 1:2:1:1:1:1 respectively. In fraction II methionine and threonine were absent. The role of this peptide moiety in the biologically active molecule is not known. Strobel (1967) appropriately named a toxic compound of a similar nature isolated from C. sepedonicum as a

glycopeptide. He showed that methionine was in larger quantities in the toxin system whereas glycine was found in larger quantities in the present study. This could be possible as the toxins isolated in these two studies were from two different organisms.

The toxin lost its toxicity on acid hydrolysis within a minute whereas it did not lose its potency in water refluxed for 5 minutes. This indicates that the toxin is heat stable but acid labile.

The radioactive toxin study showed that ^{14}C -mannose was a better precursor than ^{14}C -glucose in the synthesis of the ^{14}C -toxin (Table III). The reason for this difference cannot be explained in this study. Initially the toxin contained mannose with a high specific activity but after 6 days fucose had the highest radioactivity (Figure 8). After 16 days both fucose and mannose declined in total radioactivity whereas glucose increased indicating that the toxin varied in composition depending upon the age of the culture.

The plugging hypothesis of toxic action has been shown to be the mechanism of toxin effect in plants by several researchers with many examples, (Harris 1940, Holtzmann 1953, Dimond 1955, Husain and Kelman 1958, Spencer and Gorin 1961, Patino-Mendez 1964, Buddenhagen and Kelman 1964, and Beckman, Holmos, Chambers, Gorden (cited in Buddenhagen and Kelman 1964)). Stobel (1967), showed in the case of C. sepedonicum -toxin, that the mechanism of wilting was other than plugging of the vascular vessels, with an evidence; the amount of toxin taken up by a 7.5 cm tomato leaf prior to wilting was only 50 μg which

seems to be too small an amount to cause plugging. In the present study, radioautography experiments showed that the ^{14}C -labeled toxin was distributed all over the plant prior to wilting (Figure 9), suggesting that plugging of the petiole is not involved in causing wilting. Furthermore, the amount of toxin required to cause wilting was 46.5 to 58.0 μg (Table IV) and seems to be too small a quantity to plug all the vessels in the vascular system of the plant. Further, a toxin treated and a water treated plant were transferred to acid fuchsin solution. In each case the dye migrated to the tip of the plant at the same rate. However, a dextran (a known plugging material like xylan, carbowax and pectin - Goodman *et al.* (1967)), treated plant obstructed the dye movement (Table V); another direct indication that the mechanism of wilting in the system of present study is not plugging. Electron microscopy of the vascular tissue of the toxin treated tomato stem revealed no indication of occlusions in the vessels or in any other cells of the vascular region; one more evidence against the plugging hypothesis. Patino-Mendez (1964) indicated that the wilting caused by the C. michiganense toxin was due to mechanical blocking of the xylem tissue with the following evidences, i) the toxin effect was reversible in tomato cuttings and ii) tracheal fluid could not be obtained from the toxin affected tomato stems. The former factor might not be a valid evidence for mechanical blocking as reversibility of wilting might be due to the recovery of permeability of the membranes. The latter phenomenon could be due to the fact that the toxin induced membrane-damage in the

cells when under high negative pressures (in the experimental set-up) accounted for vascular occlusion.

The electron microscopic study also showed that the toxin affected the plasma membrane of the parenchymatous cells of the vascular tissue of the plants (Figure 11 and 12). As the membrane systems regulate the water balance and the ion concentration in the cells, the rupturing of them causes damage to the cells, tissues and to the plant system. Similar effects have been reported by Wheeler and Luke (1963), Wheeler and Black (1962, 1963) in the case of oat plant section, affected with victorin.

Klement and Goodman (1967) indicated that the toxin production was responsible for the induction of the symptom of wild fire disease of tobacco. They explained that the toxin had limited role for the initiation of the infecting process. However, in the present study with C. michiganense, the toxin moved and diffused throughout the plant faster than the bacteria, (Table VI); of course this was a different system. However, if they meant the initiation of infection process was the penetration of the host there would not be any argument. Furthermore, the field symptoms and the green house symptoms of bacterial canker of tomato both lead one to believe that the main symptoms expressed by the plants are due to toxin rather than the presence of the bacteria. The infected plants recover during cool and humid nights when there is little transpiration. The uptake of toxin is limited in those conditions, hence the plants recover. A similar

situation was noticed in the toxin treated plants. When the plant cuttings were treated in toxin and kept in a humid chamber in dark there was no wilting (Rai and Strobel, unpublished). Also when the toxin affected plant cuttings treated in water they recovered. These observations indicate that at least in this system the toxin is playing a key role in the disease. The importance of toxins in the disease production was also positively demonstrated by Thimann and Sachs (1966) in "fasciation" of sweet peas. Furthermore, Braun (1955) and Wolley, Schaffner and Braun (cited in Goodman et al. 1967) showed with evidence the mechanism of the toxin effect in "wild fire" of tobacco that the toxin acted as a competitive inhibitor. This is again a direct indication of the main role of a toxin in production of disease.

SUMMARY

An exotoxin possessing a capacity to wilt plant cuttings has been isolated, purified and characterized from cultures of Corynebacterium michiganense. It is an antigenic, non-specific vivotoxin and is glycopeptide in nature. It has 3 biologically active, serologically related fractions after separation by Sephadex G-200 column chromatography. The estimated molecular weights of the fractions are $> 200,000$; 129,700, 35,280 and intrinsic viscosities of the crude toxin and the fractions I, II and III are 0.2500, 0.2250, 0.2140 and 0.1904, respectively. The empirical formulas of the crude toxin and its fractions are as follows: -crude toxin - $C_{55}H_{114}O_{52}N$; Fraction I - $C_{42}H_{82}O_{40}N$; Fraction II - $C_{473}H_{940}O_{449}N$ and Fraction III - $C_{27}H_{52}O_{25}N$. Chromatographic analysis of the crude toxin and its fractions revealed that crude toxin and fraction I and III contain alanine, glycine, lysine, methionine, serine, threonine, and an unknown ninhydrin positive compound, whereas fraction II contains alanine, glycine, lysine, serine and an unknown compound. The sugar residues of the crude toxin and fraction III are fucose, galactose, glucose, mannose and 2 unknown reducing compounds whereas fraction I and II contain galactose, glucose, mannose and 2 unknown compounds.

This toxin is heat resistant, acid labile, water soluble and has a major role in disease production. An explanation of a mechanism other than plugging, responsible for producing the wilt symptom was put forth as a result of the following investigations: i) radioautography, ii) determination of the amount of toxin taken up by a plant prior to wilting,

iii) water transport by toxin affected plant and iv) electron microscopic observations.

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