Ascochyta chrysanthemi Toxin: Purification and Partial Characterization

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Based on portions of a Ph.D. thesis by the senior author.

Helpful discussions with W. D. Bauer are acknowledged.

Accepted for publication 24 March 1975.

ABSTRACT

Ascochyta chrysanthemi (AC) toxin was purified from 14-day-old culture filtrates of *Ascochyta chrysanthemi* isolate L.I.-1 by sorption and desorption from activated carbon, precipitation of nontoxic material with methanol, anion exchange chromatography, paper chromatography, and gel filtration. AC toxin is soluble in water, slightly soluble in amyl alcohol, and insoluble in other organic solvents with dielectric constants from 1.9 to 32.6. On paper chromatograms, AC toxin reacted with iodine vapor and with ammonical silver nitrate, but not with reagents used to detect free monosaccharides, amino acids, organic acids, pyrimidine reduction products, phenols, nitrophenols, or indoles. During paper chromatography, AC toxin is mobile in aqueous and acidic solvents; it is not retained during anion exchange chromatography in Tris [tris(hydroxymethyl)-aminomethane-HCl] buffer at pH 8.0, but is retained in boric acid-sodium borate buffer at pH 8.0. Upon gel filtration in Bio-Gel P-2, fractions containing AC toxin gave a positive test for carbohydrate and a negative test for free reducing groups. The only sugar detected in hydrolyzed AC toxin was mannose.

AC toxin was not inactivated by tetranitromethane or by sodium borohydride, but was inactivated by periodate and by hydrolysis with trifluoroacetic acid. Infrared spectra of AC toxin had many characteristics in common with spectra of mannose; AC toxin did not absorb light between 200-450 nm.

AC toxin appears to be a low-molecular-weight mannoside. The nature of the aglycone remains to be determined.

Phytopathology 65:912-917

Additional key words: Mycosphaerella ligulicola, Ascochyta blight of florist's chrysanthemum, mannoside.

Ascochyta chrysanthemi Stevens (*Mycosphaerella ligulicola* Baker, Dimock and Davis) is the causal agent of Ascochyta blight of florist's chrysanthemum [*Chrysanthemum morifolium* (Ramat.) Hemsl.] (2). Earlier studies indicated that a translocatable toxin is involved in symptom development in this disease; the toxin was subsequently shown to be a relatively stable, nonvolatile, low-molecular-weight organic compound that was not soluble in a range of organic liquids with dielectric constants from 1.9 to 32.6 (21). Some *Ascochyta* formae speciales produce phytotoxins and/or antibiotics, but the toxin produced by *Ascochyta chrysanthemi* (hereafter referred to as AC toxin) did not appear to be one of the previously reported compounds (21).

The research reported here was undertaken to determine additional chemical and physical properties of AC toxin, with the ultimate aim of employing the data obtained to purify and to determine the structure of the toxin.

MATERIALS AND METHODS.—Maintenance of *A. chrysanthemi* isolate L. I.-1 (14) in pure culture and culture filtrate production were as described previously (21), with the exception that Watkins' medium was adjusted to pH 5.0 with 1 N HCl prior to autoclaving. The "petiole method" of bioassay and the symptom evaluation procedures were previously described (21). A dilution endpoint method was used to quantitate toxin levels. Aliquots of a given preparation were lyophilized to dryness, and the dry weight determined to the nearest 0.1 mg. The minimum dose that induced symptoms was determined by serial dilution of another aliquot of the test solution, and the dry weight of the minimum dose that produced symptoms was calculated.

Activated carbon (Norit A, Pfanstiehl Laboratories, Waukegan, Ill.) was used as received from the manufacturer. After being mixed in a given solution for 3-5 minutes with a magnetic stirrer, the carbon was collected either by centrifugation (750 *g*, 5 minutes) or by filtration of the suspension through glass fiber paper (Whatman GF/A, H. Reeve Angel Co., Clifton, N.J.). Diethylaminoethyl (DEAE) cellulose (Whatman DE 11, H. Reeve Angel Co., Clifton, N.J.) was prepared according to the manufacturer's directions (9). After preparation, the exchanger was suspended in 50 mM boric acid-sodium borate buffer at pH 8.0. Ten ml of the sample to be applied to the DEAE-cellulose were diluted to 50 ml with buffer prior to application to the exchanger. Linear NaCl gradients (150 ml total volume) from 0 to 200 mM in buffer were used to elute the toxin. Eluant flow rate was 20 ml per hour. The ion exchange columns were 1.76 × 28.0 cm, and were run at 4 C. Five-milliliter fractions were collected. Conductivity of each fraction was determined with a YSI Model 31 Conductivity Bridge (Yellow Springs Instrument Co., Yellow Springs, Ohio) for estimation of salt content.

Paper chromatography was carried out on 22 × 22-cm sheets of Whatman No. 3 paper (H. Reeve Angel Co., Clifton, N.J.). The solvent systems used are given in Table 1. Chromatograms were developed in ascending manner in battery jars maintained at 20 C to a height of 17.5 cm above the line of origin, and dried in a stream of warm air. Solvent systems containing butanol left a phytotoxic residue after air drying; in such cases, the chromatograms were dried in a vacuum oven at 60 C for 18-24 hours to remove the residual solvent prior to analysis. After drying, the chromatograms were observed for fluorescence under near- and far-ultraviolet light. If

chemical tests were to be performed directly on the chromatograms, 2-cm strips were cut parallel to the direction of development in the area where sample had been applied. Aniline-oxalate (6) and ninhydrin [(6), or 0.3% ninhydrin in ethanol] were employed as detecting reagents on each chromatogram. For bioassay, toxin was eluted with glass-distilled water from strips cut perpendicular to the direction of development of the chromatograms. The eluted samples were filtered through glass wool and ultraviolet and visible spectra of each sample were taken with a Bausch and Lomb Spectronic 505 spectrophotometer.

Bio-Gel P-2 and P-10 (Bio-Rad Laboratories, Richmond, Calif.) were prepared in glass-distilled water according to the manufacturer's directions (3). The columns used were 1.1 × 50.0 cm and were run at 20 C; glass-distilled water served as eluant, at a flow rate of 20 ml per hour. Samples (0.5 ml) were applied to the columns, and 5.0 ml fractions were collected. For bioassay, 0.5 ml of each fraction was diluted to 2.5 ml with distilled water. A solution containing glucose, raffinose, and dextran (mol wt 200,000-300,000, Sigma Chemical Co., St. Louis, Mo.), 1 mg/ml each, was used to calibrate the columns.

Gas-liquid chromatography of alditol acetate derivatives of neutral monosaccharides and hydrolyzed purified toxin (Table 2) was carried out following the method of Albersheim et al. (1) on a Perkin-Elmer Model 900 gas chromatograph, equipped with a glass column, 182.9 cm (6 feet) long × 2.0 mm internal diameter. The column was packed with 0.2% (by weight) polyethylene glycol adipate, 0.2% polyethylene glycol succinate, and 0.2% silicone XF-1150 (Applied Science Corp., State College, Penna.) on 149-131 μm (100/120-mesh) gas chrome Q. The column temperature was programmed to increase from 170 to 200 C at 1 C per minute during the analysis. Samples were prepared for gas chromatography by the method of Lindberg (12) as modified for samples of small volume (20). Myo-inositol served as internal standard, and a solution containing L-rhamnose, L-fucose, D-xylose, L-arabinose, D-mannose, D-galactose and D-glucose (1 mg/ml each; Sigma Chemical Co., St. Louis, Missouri) was used as standard and control for the preparation procedure and analysis.

Infrared spectra were taken with a Perkin-Elmer Model 337 grating infrared spectrophotometer, with samples as 1-2% (by weight) in a pressed pellet of KBr (infrared quality, Harshaw Chemical Co., Solon, Ohio).

TABLE 1. Behavior of Ascochyta chrysanthemi toxin during paper chromatography[a]

	Solvent system	R_f of toxin	Notes[b]
I	Diethylether: acetic acid: water (13:3:1, v/v)	0.0	$A^+B^+C^+D^+$
II	Ethanol: ammonia: water (18:1:1, v/v)	0.0	$A^-B^-C^-D^+$
III	Ethyl acetate: methanol: acetic acid (8:1:1, v/v)	0.0	C^+D^+
IV	Isopropanol: acetic acid: water (3:1:1, v/v)	0.0-0.31 (smear)	$A^+C^+D^+$
V	Butanol: acetic acid: water (2:1:1, v/v)	0.0-0.48 (smear)	$A^+B^+D^+$
VI	Water: ethanol (9:1, v/v)	0.0-1.0 (smear)	
VII	2% Acetic acid	0.14-0.60 (smear)	
VIII	15% Acetic acid	0.0-1.0 (smear)	
IX	BAW (upper phase of butanol: acetic acid: water (4:1:5, v/v)	0.05	$A^-B^-C^+D^+$

[a]Toxin at various stages of purification was used for chromatography. No difference in R_f was observed between culture filtrate and the more purified preparations.

[b]A^+ = Aniline-oxalate reactive material coincident with toxin activity.

A^- = Aniline-oxalate reactive material not coincident with toxin activity.

B^+ = Ninhydrin-reactive material coincident with toxin activity.

B^- = Ninhydrin-reactive material not coincident with toxin activity.

C^+ = Material showing absorption peak at 260-265 nm coincident with toxin.

C^- = Material showing absorption peak at 260-265 nm not coincident with toxin.

D^+ = Fluorescence coincident with toxin.

Fluorescence spectra of aqueous toxin solutions were taken with an Aminco-Bowman Spectrophotofluorometer (American Instrument Co., Silver Spring, Md.), equipped with a 1P21 phototube (Hamamatsu TV Co., Middlesex, N.J.).

TABLE 2. Purification of Ascochyta chrysanthemi toxin

Stage of purification[a]	Total volume, (ml)	Dry weight, (mg/ml)	Minimum effective dose[b] (μg/ml)
Culture filtrate	900	19.4	39
Desorbed toxin	50	18.2	23
Deproteinated toxin	10	83.5	20
DEAE-purified toxin	2	373	125
Paper-chromatographically purified toxin	0.5	3.9	1.9
P-2 purified toxin	0.5	1.8	0.75
Purified AC toxin	5	0.14	0.23

[a]See text for procedures for each of the designated toxin preparations.

[b]Minimum amount of sample that induced symptoms on chrysanthemum plants by the petiole bioassay.

All experiments were repeated at least twice, with replicates of each treatment in each experiment.

RESULTS.—*Purification of AC toxin.*—*A. chrysanthemi* was grown for 14 days on Watkins' medium At the end of the incubation period, the culture liquid was filtered through cheesecloth and through Whatman No. 2 filter paper. Toxin preparations at this stage are hereafter referred to as culture filtrate (Table 2).

Activated carbon (10 g/liter) was added to culture filtrate, and the suspension was thoroughly mixed. The carbon was collected and washed twice by suspension in glass-distilled water (75 ml/10 g of carbon). The toxin was sorbed by the carbon, and was desorbed by suspending the carbon in 50% acetone, 50-75 ml of solvent/10 g of carbon, for a total of 7-10 times. The solvent fractions were pooled, and concentrated on a rotary evaporator at 40 C to a volume of 50 ml. Toxin at this state of purification is referred to as desorbed toxin (Table 2). Supernatant solutions, after sorption of toxin from culture filtrate, showed a positive Nelson test (17) for reducing sugars and a positive Lowry assay (13) for protein, indicating that some selectivity was involved in the sorption process.

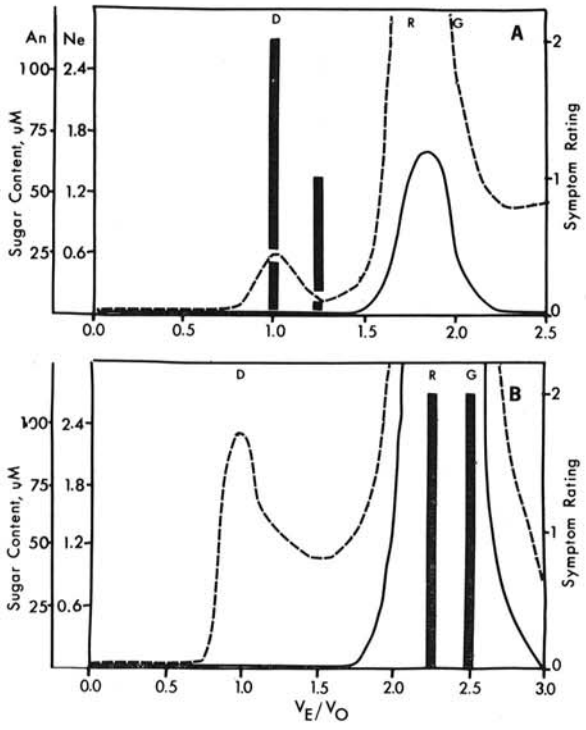

Fig. 1-(A, B). Elution patterns of desorbed toxin (Table 2) from Bio-Gel molecular sieve gel columns. **A)** P-2; **B)** P-10. Two milliliters of concentrated desorbed toxin were applied to columns of each of the molecular sieves as described in the text. Nelson's assays (Ne on the ordinate) for reducing sugars and anthrone assays (An on the ordinate) for total sugars were performed on 0.3-ml aliquots of each fraction. Solid line is reducing sugars, dashed line is total sugars, bars represent symptom ratings. In calibration, dextran (mol. wt. 200,000 to 300,000), raffinose (mol. wt. 594.5) and glucose (mol. wt. 180) eluted in fractions indicated D, R, and G, respectively. The calibration peaks for raffinose and glucose overlapped significantly. These graphs were prepared from data from two different toxin preparations.

Absolute methanol (100 ml) was added to 50 ml of desorbed toxin, and the solution thoroughly mixed. A precipitate formed immediately; after standing for 18-24 hours at 4 C, the precipitate was collected by centrifugation and discarded, since tests (21) showed that it did not contain AC toxin. The supernatant solution was reduced to a volume of 10 ml and designated deproteinated toxin (Table 2).

Deproteinated toxin (10 ml) was diluted to 50 ml with 50 mM boric acid-sodium borate buffer, pH 8.0, and was applied to a column of DEAE-cellulose in 50 mM boric acid-sodium borate buffer, pH 8.0. After a preliminary rinse with 50 ml of buffer, the toxin was eluted in fractions containing from 50-175 mM NaCl. It was previously reported (21) that when culture filtrate was diluted with 50 mM Tris [tris(hydroxymethyl)aminomethane-HCl] buffer at pH 8.0 and applied to DEAE-cellulose made up in the same buffer system, AC toxin was retained and could be eluted with 100 mM NaCl in 50 mM Tris-HCl buffer at pH 8.0; deproteinated toxin, however, was not retained by DEAE-cellulose in Tris-HCl buffer. It was necessary to include anion exchange in the purification scheme since a yellow pigment present in crude toxin preparations was not retained by DEAE-cellulose and eluted in the void volume. This pigmet could not be separated from AC toxin by any other procedure tested. Active fractions from the DEAE-cellulose column were pooled, taken to dryness on a rotary evaporator at 40 C, and the residue dissolved in 4.0 ml of glass-distilled water. This preparation was divided into four 1-ml aliquots, and treated with glacial acetic acid and methanol (20) to eliminate the phytotoxicity due to the boric acid-sodium borate buffer.

The residues in each of the four aliquots were dissolved in 0.5 ml of glass-distilled water, and streaked onto a paper chromatogram. The chromatograms were developed in ethanol:ammonia:water (18:1:1, v/v) (System II, Table 1), dried, and then developed in BAW [upper, organic phase of *n*-butanol:acetic acid:water, 4:1:5 (v/v); System IX, Table 1] for 8 hours. The dried chromatograms were observed under near- and far-ultraviolet light and a weakly fluorescent band with its lower edge approximately 4 mm above the line of origin, and its upper edge approximately 20 mm above the origin, was cut from the chromatograms. Toxin was eluted from the paper and the eluate taken to dryness and dissolved in 0.5 ml of glass-distilled water. Toxin at this stage of purification is referred to as paper-chromatographically purified toxin (Table 2). The ability of various solvent systems (Table 1) to move material away from AC toxin in paper chromatography was a factor in determining which of them would be included in the purification scheme. Further, it was observed that fluorescent bands appearing on chromatograms that had been developed first in System II and then in System IX had much better definition than those developed in System IX alone. In preliminary experiments, all chromatograms were developed to a height of 17.5 cm above the origin; in the final purifications in System IX, the chromatograms were developed for 8 hours (approximately twice the time required for the solvent front to reach 17.5 cm) in order to move the band with toxin activity away from the origin.

AC toxin was not stable to chromatography on silica gel thin-layer plates.

Paper-chromatographically purified toxin was applied to a column of Bio-Gel P-2, and developed with glass-distilled water. Although AC toxin eluted in the void volume from Bio-Gel P-2 (Fig. 1-A), toxin activity was associated with fractions that gave positive anthrone assays (16) for sugars, but a negative Nelson assay (17) for free reducing sugars. Active fractions were pooled, taken to dryness and dissolved in 0.5 ml of glass-distilled water. The toxin was then applied to a column of Bio-Gel P-10, and developed with glass-distilled water. AC toxin was retarded by Bio-Gel P-10 (Fig. 1-B). Active fractions were pooled and lyophilized. This material is designated purified AC toxin (Table 2).

Purified AC toxin is a white powder and is hydroscopic at ambient humidities; purified toxin was stored over P_2O_5 in a vacuum desiccator. The purified toxin dissolved readily in water to give a colorless solution. It did not contain free reducing sugars (17), but did contain sugar (16). One liter of culture filtrate yielded 0.5 to 0.8 mg of purified toxin (Table 2), with a minimum dose for symptom induction on chrysanthemum of 0.2 to 0.3 μg/ml.

Chemical and physical properties of AC toxin.—Observations of AC toxin in ultrafiltration (21) and molecular sieve gels suggested that the toxin has a low molecular weight. It apparently is not anionic, since partially purified toxin is not retained by DEAE-cellulose prepared in Tris-HCl buffer at pH 8.0; the toxin is retained if it and the exchanger are prepared in boric acid-sodium borate buffer at pH 8.0, suggesting that AC toxin contains carbohydrate, since carbohydrates are known to form complexes with borate ions. Yet, the toxin contained no free reducing groups, but did contain sugar. AC toxin could be a glycoside in which the free reducing group on the sugar present is bound to an otherwise unreactive aglycone. Further investigations were made to examine this possibility.

Reactions of AC toxin on paper chromatograms.—Paper chromatograms equivalent to those in Table 2 prior to elution of paper-chromatographically purified toxin were tested with a number of reagents to determine potential reactive groups on the toxin molecule. The toxin did not react with the following reagents: aniline-oxalate, for free monosaccharides (6); bromocresol green, for acids (18); *p*-dimethylaminobenzaldehyde, for pyrimidine reduction products (4); sodium hydroxide, for nitrophenols (4); Ehrlich's reagent (4) and nitrose reagent (4), for indoles; ferric chloride, for vanillin and related compounds (4), and ninhydrin [(6) or 0.3% in ethanol] for amino acids. Known standard compounds gave appropriate positive tests with each of the above reagents. AC toxin gave a positive reaction in iodine vapor (6), producing a pale-brown reaction product, and with ammonical silver nitrate (18), producing a brown to charcoal-black reaction product. It also gave weak, but positive, reactions with reagents for phenols, producing a brown reaction product with both diazotized *p*-nitroaniline (7) and sucrose in acid ethanol (4), and a blue reaction product with ferric chloride-ferricyanide (4). Purified toxin did not react with these last reagents when dissolved in water and reapplied to paper, but did react with iodine and ammonical silver nitrate as described.

TABLE 3. Degradation of Ascochyta chrysanthemi toxin by periodate

Sample	Symptom rating[d]
Toxin alone[a]	2
Toxin treated with Pb $(C_2H_6O_2)_2$[b]	2
Toxin treated with periodate, 0 time[c]	1
Toxin treated with periodate, 1 hour	0
Toxin treated with periodate, 24 hours	0

[a]Toxin preparation as used in reaction mixture, treated with water instead of periodate solution.

[b]Toxin preparation as used in reaction mixture, treated with water instead of periodate, to which 0.05 ml of 100 mM $Pb(C_2H_3O_2)_2$ was added.

[c]Reaction mixture consisted of 5.0 ml of deproteinated toxin and 5.0 ml of 200 mM sodium meta-periodate. Aliquots (0.5 ml) were taken at the times indicated, and added to 0.6 ml of 100 mM $Pb(C_2H_3O_2)_2$ to stop the reaction. Excess lead was precipitated with 6.0 N HCl; the solution was adjusted to pH 5.0 with 1.0 N NaOH prior to dilution with water and subsequent bioassay.

[d]Rating of symptoms induced on chrysanthemum in the petiole bioassay (21).

Neutral sugar analysis of AC toxin.—Purified toxin was hydrolyzed and analyzed by gas chromatography for its neutral sugar content. The toxin contained only mannose (retention time of mannose standard, 25.7 minutes; retention time of toxin hydrolysate, 25.7 minutes). Toxin activity was lost after hydrolysis in trifluoroacetic acid.

Solubility tests in amyl alcohol.—Previously described methods (21) were used to determine the solubility of the toxin in amyl alcohol (2-methyl-1-butanol, Mallinckrodt Chemical Works, St. Louis, Mo.). Amyl alcohol extracts of culture filtrates adjusted to pH 3 and 7 gave symptom ratings of 2, while the extracts from filtrates at pH 1, 10, and 13 gave symptom ratings of 1; residual aqueous phases gave symptom ratings of 2 or 3, unextracted filtrate gave a symptom rating of 3 or 4.

Fluorescent properties of AC toxin.—The fluorescent properties of the toxin were investigated, since purified AC toxin showed weak fluorescence on chromatography paper. Purified AC toxin showed a weak excitation peak at 350-370 nm, and a weak emission peak at 410-430 nm. Tetranitromethane (Aldrich Chemical Co., Milwaukee, Wis.), which forms complexes with alkenes and is a test

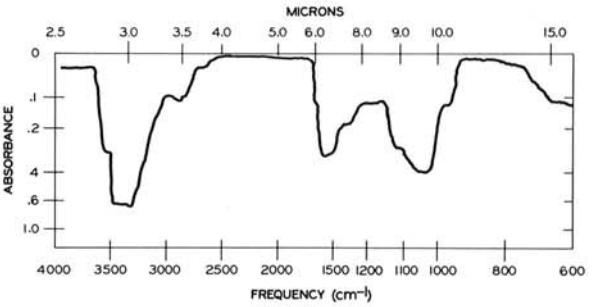

Fig. 2. Infrared spectrum of purified AC toxin. Sample was incorporated (approximately 2%) in a pressed KBr pellet. The spectrum was taken on a Perkin-Elmer Model 337 Grating Infrared Spectrophotometer.

reagent for carbon-carbon unsaturation (8), did not inactivate or reduce the activity of the toxin.

Effects of oxidizing and reducing agents on AC toxin.—Degradation of AC toxin by periodate was followed in reaction mixtures consisting of 5.0 ml of deproteinated toxin and 5.0 ml of 200 mM sodium metaperiodate (Mallinckrodt Chemical Works, St. Louis, Mo.). All reactions were run at 21 C. At given times, 0.5-ml aliquots of the reaction mixture were withdrawn and added to 0.6 ml of 100 mM $Pb(C_2H_3O_2)_2$ (Mallinckrodt Chemical Works). A precipitate of lead iodate and lead periodate formed immediately, stopping the reaction. The precipitate was settled by centrifugation at 750 *g* for 5 minutes and discarded; 6 N HCl was added dropwise to the supernatant solution, to precipitate the excess lead. The precipitate of lead chloride was collected by centrifugation and discarded; the supernatant solution was adjusted to pH 5.0 with 1 N NaOH, diluted to 2.5 ml with distilled water and bioassayed. As shown in Table 3, the toxin was completely destroyed within 1 hour by the periodate. No visible precipitate formed when toxin was treated with 100 mM $Pb(C_2H_3O_2)_2$; the toxin was not inactivated by $Pb(C_2H_3O_2)_2$ alone, but excess lead was phytotoxic to chrysanthemum in the bioassay.

AC toxin was not inactivated by treatment with $NaBH_4$ (5 mg/ml of toxin).

Spectral properties of AC toxin.—Ultraviolet-visible spectra of aqueous solutions (2 mg/ml) of purified AC toxin showed no absorption peaks between 200 nm and 450 nm. Infrared spectra of purified AC toxin (1-2% in a pressed KBr pellet) were similar in many respects to that of mannose (Fig. 2).

Mannose, sucrose, raffinose, mannan and dextran (2 and 10 mg/ml) did not induce symptoms on chrysanthemum when bioassayed.

DISCUSSION.—Several lines of evidence indicate that AC toxin is glycosidic in nature. The solubility properties of the toxin (21), particularly its solubility in water and insolubility in diethyl ether, place it in a large group of moderate to low molecular weight compounds with two or more polar groups, including polyhydroxy compounds and simple carbohydrates (5). Many glycosides are soluble in amyl alcohol (10); AC toxin showed definite solubility in amyl alcohol. The band containing AC toxin on paper chromatograms reacted strongly only with iodine vapor and ammonical silver nitrate, and not at all with reagents for free monosaccharides, amino acids, organic acids, pyrimidine reduction products, phenols, nitrophenols, and indoles. Lack of reactions with these reagents indicate that the reactive groups on the toxin molecule are not free. During paper chromatography, glycosides are mobile in aqueous or acidic developing solvents, while aglycones often are stationary (11). AC toxin was very mobile when paper chromatograms were developed with water, 2% or 15% acetic acid (Table 1), solvents commonly used for chromatography of glycosides. During ion-exchange chromatography, the toxin was not retained if columns of DEAE-cellulose were prepared and developed in Tris-HCl buffer. In boric acid-sodium borate buffer, the toxin was retained by DEAE-cellulose, and could be eluted with a gradient of NaCl. These observations suggest that a complex forms between the toxin and the borate ion; carbohydrates are known to form such complexes with borate and anion exchange in the presence of borate is routine for analysis of mono- and oligosaccharides. In Bio-Gel P-2 molecular sieve columns, the toxin eluted in fractions that gave a negative Nelson test for free reducing sugars (17), but a positive reaction for total sugars with anthrone (16) (Fig. 1), suggesting that the hemiacetal of the sugar was bound, as occurs with many common glycosides. When purified AC toxin was hydrolyzed, reduced, and acetylated and the products analyzed by gas-liquid chromatography (1, 12), a peak corresponding to mannose was observed. Further, glycosides are known to be highly mobile within plants, and so is AC toxin (21). AC toxin appears to be a glycoside; the sugar involved is mannose. The precise nature of the aglycone remains to be determined. The toxin is dialyzable and is not retained by an ultrafilter with retentivity for compounds with a molecular weight greater than 1,000 daltons (21). The fluorescence of the purified toxin may be a property of the aglycone, but the fluorescence levels observed were too low to be useful for characterization of the toxin. The infrared spectrum of purified AC toxin is similar to that of mannose (Fig. 2). It should be noted that the infrared spectrum of helminthosporoside (2-hydroxycyclopropyl-α-*D*-galactopyranoside) (22) is similar to that of galactose (19). The spectral properties of AC toxin do eliminate some possible structures for this toxin. For example, the lack of a strong band around 6.0 μ in the infrared (Fig. 2) assures the absence of a carbonyl group either as an acid or an aldehyde. With no absorption above 210 nm in the ultraviolet, the presence of aromatic and conjugated systems in the molecule is eliminated. Since solutions of pure mannose and related compounds did not induce symptoms when bioassayed, the toxicity of the molecule seems to reside in the compound as a whole or in the aglycone alone. Toxicity was not destroyed by treatment of toxin solutions with $NaBH_4$ or with tetranitromethane. Toxic activity was destroyed by treating the toxin with periodate; this may have been due to oxidation of the mannose moiety. The toxin was inactivated by hydrolysis with trifluoroacetic acid, HCl or NaOH (21).

Observations by McCoy and Dimock (15) of apparent toxin production by *A. chrysanthemi* are confirmed and expanded by the data presented here. The role of AC toxin in symptom expression is documented (21), but the role of the toxin in pathogenesis remains to be determined.

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