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HOST-SELECTIVE TOXINS FROM HELMINTHOSPORIUM CARBONUM: PURIFICATION, CHEMISTRY, BIOLOGICAL ACTIVITIES, AND EFFECT ON CHLOROPHYLL SYNTHESIS IN MAIZE

By

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ABSTRACT

HOST-SELECTIVE TOXINS FROM HELMINTHOSPORIUM CARBONUM: PURIFICATION, CHEMISTRY, BIOLOGICAL ACTIVITIES, AND EFFECT ON CHLOROPHYLL SYNTHESIS IN MAIZE.

By

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Helminthosporium carbonum race 1 is the causal agent of a leafspot disease that affects only certain inbred lines and hybrids of maize. The fungus produces a host-selective toxin (HC toxin) that is required for disease development. A new and simpler purification scheme was developed for the major form of toxin (HC toxin I). The procedure eliminated the need for TLC and HPLC as preparative steps and resulted in rapid accumulation of crystalline toxin I. Yields were over 80 mg toxin per liter of culture fluid.

Three analogs of toxin I were isolated from culture fluids of the fungus using the new purification scheme. The analogs had the same specificity as does the pathogen, and were designated HC toxins II, III, and IV. HPLC was required for final purification of the analogs. Spectral and other data indicated that the analogs, like the previously characterized toxin I, are cyclic tetrapeptides with one unusual epoxide-containing amino acid. HC toxin

IV differs chemically from toxin I only at the carbon adjacent to the epoxide. Toxin IV has a hydroxylated carbon and toxin I has a carbonyl carbon. This conclusion was based on amino acid analyses, fast atom bombardment mass spectroscopy, and ^{13}C and ^{1}H NMR. $NaBH_4$ reduction of the ketone in toxin I produced a compound with the same chromatographic and spectral properties as toxin IV, confirming the structure.

ED_{50} values based on inhibition of susceptible seedling root growth for toxins I, II, III, and IV were 0.2, 0.4, 2.0, and 20 ug/ml respectively. The preparation of toxin II was found to be more active than was reported previously. Resistant seedlings tolerated 100-fold higher concentrations of pure toxin I than did susceptible seedlings. Hydrolysis of the toxin I epoxide to a diol destroyed toxicity to susceptible and resistant seedlings, suggesting that the same mechanisms are affected in resistant and susceptible plants.

HC toxin I was found to have a rapid inhibitory effect on the synthesis of chlorophyll in etiolated maize leaves. Approximately 50% inhibition of chlorophyll synthesis was observed in susceptible leaves with toxin at 20 ug/ml 6 hr after the initiation of greening. There was inhibition with < 1.0 ug toxin per ml and linear increases over at least five orders of magnitude. Resistant maize tissues were similarily affected when toxin concentrations were

100-fold higher than were required for susceptible tissues. The application of δ-aminolevulinic acid (ALA), the first and limiting step of the chlorophyll synthesis pathway, prevented the toxin-induced inhibition. The data indicate that toxin somehow causes a block in the synthesis of ALA in etiolated maize leaves exposed to light. This is the most rapid inhibitory effect observed to date for HC toxin.

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LIST OF ABBREVIATIONS

ALA	δ-aminolevulinic acid
aoe	2-amino-8-oxo-9,10-epoxydecanoic acid
FAB MS	fast atom bombardment mass spectroscopy
HPLC	high pressure liquid chromatography
NBP	p-(nitrobenzyl)-pyridine
NMR	nuclear magnetic resonance
TLC	thin layer chromatography

Introduction

Helminthosporium carbonum (Ullstrup) race 1 is a pathogen of certain varieties of maize. The fungus, which causes a severe leaf blight, produces a host-selective toxin (HC toxin) as its major disease determinent (36). When this research was initiated, only one form of toxin (HC toxin I) had been purified and characterized chemically. The existing purification scheme was slow and tedious; thin layer chromatography (TLC) and analytical high pressure liquid chromatography (HPLC) were limiting preparative steps. In the first part of this research a new purification scheme was developed for toxin based on flash chromatography (46) on silica gel. The scheme eliminated the need for TLC and HPLC, thus greatly simplifying and expediating toxin purification. At least three analogs of toxin I were isolated from culture fluids. All analogs required HPLC for final purification. Two analogs (HC toxins II and III) were characterized chemically as part of another thesis (47); the structure of the third analog (HC toxin IV) was elucidated in my research using amino acid analyses and spectroscopic techniques (FAB MS, ^{13}C and ^{1}H NMR). Shortly after the research was initiated, another group reported the structure of toxin II (19). However, my preparation was much more active, suggesting higher degree of purity.

The second portion of my research was concerned with the mode-of-action of HC toxin. This has been an elusive problem for toxin researchers since all early effects of HC toxin on susceptible cells to date are stimulations or increases rather than inhibition of activities (22,57,58). I found that toxin has a rapid inhibitory effect on chlorophyll synthesis in etiolated leaves of susceptible maize. HC toxin inhibitied chlorophyll synthesis within 4 to 6 hr after exposure to light. The data are the most rapid inhibitory response to HC toxin observed to date, and may probide the basis for an improved and more rapid bioassay for toxin. The inhibition of chlorophyll synthesis could be prevented by supplying ALA, the first limiting step of the chlorophyll synthesis pathway, to toxin-treated leaves. The data provide clues to the mode-of-action of HC toxin.

Literature Review

Remarkable selectivity is involved in many plant diseases; resistance or susceptibility often is controlled by one gene pair. Also, pathogenicity or the ability to induce disease is known or hypothesized to be under single gene control in many fungal pathogens. Most genetic studies of diseases involving fungi have progressed little beyond this observation of genotype or race specificity. Little is known about gene products for susceptibility or resistance in plants, and few pathogenicity factors are known from fungi. The only known exceptions are in the diseases involving host-selective toxins (36). In these systems, a single metabolite from the fungus, the host-selective toxin, is known with confidence to be the major disease determinant. Host-selective toxins are low molecular weight compounds produced by the pathogen that have the same host specificity as does the pathogen; resistant genotypes and species are not affected or are highly tolerant (36). To date, 15 host-selective toxins are recognized (37).

The host-selective toxin from H. carbonum race 1 (HC toxin) is the subject of this study. The fungus causes a leaf spot and ear rot that affects only certain genotypes

of maize (50). Scheffer and Ullstrup (42) first demonstrated that the fungus produced a host-selective toxin in culture. That report came shortly after the discovery of the host-selective toxin from Periconia circinata (PC toxin), a pathogen of grain sorghum (39). The discovery of HC toxin was also preceded by the discovery of host-selective toxins from Alternaria alternata f. kikuchiana (AK toxin) and Helminthosprium victoriae (HV toxin), pathogens of Japanese pear cv. Nijissicki and certain cultivars of oats, respectively (29,37). The physiological effects of HC toxin have often been compared to those of PC and HV toxins. These comparisons are described below.

HC toxin is well-characterized chemically. Pringle (33) offered a partial characterization of toxin based on amino acid analyses and paper chromatography. Leisch et al. (25) provided strong spectral evidence that toxin is a cyclic peptide containing alanine, proline, and an unusual epoxide-containing amino acid, 2-amino-8-oxo-9,10-epoxy-decanoic acid (aoe), in a 2:1:1 ratio, respectively. This amino acid composition was confirmed by two other groups of researchers, (32,52), who reported an amino acid sequence different from that proposed by Leisch et al. (25). Walton et al. (52) first identified the toxin as cyclo-(2-amino-8-oxo-9,10-epoxydecanoyl-prolyl-alanyl-alanyl). Synthesis of the toxin has confirmed the structure (18). The epoxide was shown to be necessary for toxicity (6,53). Hydrolysis

of the epoxide to a diol resulted in a nontoxic compound that did not protect susceptible seedling from toxin in molar ratios of 7:1 (diol:toxin) in root growth inhibition bioassays (6). An analog of toxin that differed structurally from the major form of toxin by the substitution of glycine for alanine adjacent to aoe was found in culture fluids of the fungus (19). The analog had the same specificity, but was reported to be 35-fold less potent, on a molar basis (19).

HC toxin was used in several classic experiments that established host-selective toxins as pathogenicity factors. The teleomorph of the fungus, Cochliobolus carbonum, was crossed with C. victoriae, the producer of HV toxin, and the ascospore progeny were analyzed (38). The ascospore progeny produced either the maize toxin, the oat toxin, both toxins, or neither toxin in a 1:1:1:1 ratio. All ascospores which produced the maize toxin were pathogenic to susceptible maize, and all ascospores which did not produce the toxin were not pathogenic to maize. These findings were confirmed and extended by use of laboratory mutans and wild type isolates of the fungus from around the world (55). These data are convincing evidence for toxins as pathogenicity factors, and indicate that HC and HV toxins are produced under control of single, unlinked genes (38).

HC toxin is required for colonization of susceptible maize. Conidia of C. victoriae and of a nonpathogenic isolate of C. carbonum germinated and penetrated cell walls of susceptible maize but did not develop further; at most, a few cells were penetrated, and a hypersensitive death of the cells occurred. Resistance was evident by 16 hr post-inoculation (7). When toxin was administered exogenously to the infection court, these non-pathogenic isolates colonized susceptible tissue in the same manner as did the pathogenic isolate.

Resistance of maize to H. carbonum is inherited in a simple dominant fashion (51). The major locus controlling the disease reaction, Hm, was mapped on chromosome 1. Further work demonstrated that the Hm locus has 3 alleles. The degree of resistance in plants not carrying the dominant allele (Hm) was conditioned by a second locus, Hm_2, located on chromosome 9 (31). Resistance to the fungus was then compared with resistance to the toxin (23). These comparisons were only made with the Hm genotypes; Hm_2 was not available for tests. Genotypes that were highly susceptible to the fungus were most sensitive to toxin, whereas the genotypes that were resistant to the fungus were insensitive to toxin; genotypes intermediate in susceptibility were intermediate in sensitivity to toxin (23). Thus, resistance to the toxin equals resistance to the fungus.

Plants treated with toxin exhibit many of the same responses as do plants infected with the fungus. Infection by H. carbonum was reported to stimulate maize leaves to fix more CO_2 in the dark than do control plants (27). The level of CO_2 fixation by infected tissue eventually decreased relative to control plants, apparently because of the accumulation of inhibitors (27). HC toxin was shown to cause the same effect in susceptible leaves 4 hr after exposure to toxin, but the levels always remained above those of control plants (22). Apparently, no inhibitors accumulate after toxin treatment (22). Toxin-treated resistant leaves also fixed more CO_2 in the dark than did their water-treated controls, but the level of increase was always smaller than in toxin-treated susceptible leaves at a given toxin concentration (22). Susceptible leaves consumed about 30% more oxygen than did control leaves in response to an eight hr exposure to toxin, and respiration remained above control levels for at least 30 hr (22). Increased respiration is the usual response by plants to infection. The increased respiration of maize leaves in response to HC toxin was less in magnitude and slower to develop than that which occured in susceptible oat leaves treated with HV toxin (35,40).

Many of the physiological effects of HC toxin differ from the effects of other host-selective toxins. A toxin preparation that had an ED_{50} of 1.0 ug/ml against susceptible seedlings stimulated root growth of the same

seedlings at 0.125 ug/ml over 48 hr (23). Higher toxin concentrations stimulated root growth of resistant seedlings (23). The same preparation was tested for inhibition of seedling root growth of various non-host plant species. All the non-host plants were much more resistant to toxin than was susceptible maize, but some species, for example tomato and radish, were not as tolerant as was resistant maize (23). Many of the non-host species were stimulated in their root growth by low toxin concentrations, suggesting that some processes were affected by toxin in all plants tested (23).

HC toxin at 5.0 ug/ml caused a rapid but transient increase in the negative electropotential across the plasmalemma of susceptible maize cells (13). The increases, 10 to 40 mv in magnitude, were evident within the first 3 minutes of toxin exposure, but the electropotential returned to initial levels within 30 min (13). Such increases were in sharp contrast to the effects of HV and PC toxins on their susceptible hosts. Those toxins gradually decreased the electropotential of susceptible cells at a rate of approximately 50 mv/hr (13). The data indicate that the effect of HC toxin on the plasmalemma differs from that of the other toxins (13).

There have been attempts to demonstrate uptake of toxin by plant tissues. Roots of seedlings were placed in toxin solutions for 12 hr, after which seedlings were

removed and another set of seedlings were exposed for 12 hr. A third set of seedlings was handled the same way. The residual toxin solution was then bioassayed and found to contain as much toxic activity as did control toxin solutions which had never been in contact with seedlings (21). The same negative results were obtained in experiments with resistant and susceptible seedlings (21). If toxin was removed from solution by either genotype, the amounts were not detectable by the bioassay (21). In another experiment, leaves took up toxin in the transpiration stream for 20 hr. The leaves were then ground, and water extracts were bioassayed. No host-selective toxicity was recovered from leaves of susceptible or resistant seedlings. These negative results are similar to those for HV toxin (41). Host-selective toxin activity, as determined by the root growth inhibition assay, was recovered from leaves of sorghum plants exposed to PC toxin (12). Equal amounts of PC toxin activity were recovered from susceptible and resistant plants in those experiments (12).

Experiments indicate that uptake or activity of HC toxin is governed by rate-limiting mechanisms, and requires metabolic energy (21). Small seedlings were placed in toxin solution for various times (4, 6, or 10 hr) under a variety of conditions. The seedlings were then rinsed and placed in water for 96 hr. The inhibition of root growth after the 96 hr incubation was used as a measure of toxin

activity during the brief exposure to toxin. Activity increased with toxin concentration, temperature, and exposure time (21). The metabolic inhibitors azide, cyanide, and 2,4-dinitrophenol decreased toxin activity when they were administered during the brief exposure to toxin (21). Similar reductions in activity were observed when anaerobic conditions were imposed during the toxin exposure time (21). The data could be interpreted in several ways, but the most simple explanation is that toxin uptake was affected by the manipulations (21).

The effect of toxin on nitrate reductase, a substrate inducible enzyme system, was determined. Toxin at 20 ug/ml increased *in vivo* nitrate reductase activity by 20 to 30% within 4 hr after exposure to the nitrate substrate (57). Toxin treatment also doubled the uptake of nitrate by susceptible maize by one hr after exposure. Nitrate reductase activity from plant extracts was not affected *in vitro*, indicating that toxin had no direct or primary effect on the enzyme (57). Other experiments indicated that toxin had no influence on the ability of cells to retain nitrate; overall, the data indicate that the increased level of nitrate reductase activity probably was the result of increased availability of substrate caused by the stimulated uptake of nitrate (57).

HC toxin similarly increased the uptake of Na, Cl, leucine, and 3-o-methylglucose in susceptible maize roots,

but had no effect on uptake of NO_2, K, Ca, phosphate ions, SO_4, and glutamic acid (58). These data indicate that HC toxin does not cause the general disruption of plasma membranes that is caused by HV and PC toxins. Rather, the increased uptake of certain solutes appears to be the result of other toxin-induced changes in the plasmalemma.

Electrolyte leakage from susceptible maize tissues is increased by toxin, but this does not occur until 10 to 16 hr after exposure to toxin (32). The slow response indicates that the plasmalemma is not disrupted in the early events of toxin action.

The data indicate that HC toxin is subtle in its action; the known physiological processes are stimulated or increased rather than disrupted. Additionally, resistant tissues respond as susceptible tissues, provided toxin concentration is approximately 100-fold higher (23,57,58). This has led to the idea that similar processes in many plants may be affected by toxin. However, the available data do not allow us to propose an initial lesion site for toxin. Because disruptive effects of toxin are slow to develop, the only suitable bioassay for toxin to date is based on inhibition of susceptible root growth. This assay is quantitatively reliable, but requires at least 48 hr exposure to toxin (6,23,38). This has limited the scope of experiments concerning the mode-of-action.

An objective of my research was to investigate physiological systems that might be used to gain clues to

the mode-of-action, and perhaps provide the basis for a more rapid bioassay. I found that the toxin has a rapid inhibitory effect on chlorophyll synthesis in etiolated corn leaves, so it is appropriate to briefly review the pertinent literature. A detailed review on chlorophyll synthesis has been published recently (5).

Chlorophyll is a porphyrin derived from a branch of the tetrapyrrole pathway. δ-aminolevulinic acid (ALA) is the first identified precursor of the pathway, which gives rise to heme synthesis in all organisms (including bacteria and animals), as well as to chlorophyll in plants (1). In plants, the pathway branches after protoporphyrin IX; insertion of Fe^{2+} into this intermediate leads to heme synthesis whereas chelation of Mg^{2+} results in chlorophyll synthesis (1).

Etiolated leaves, when placed in light, have a lag phase of about 2-4 hr before chlorophyll is synthesized (15,30). When ALA is supplied in the dark, this lag phase is eliminated; the leaves begin making chlorophyll immediately upon exposure to light (30). Leaves supplied with ALA and held in the dark accumulate protochlorophyllide, an intermediate of the pathway which requires light for the enzymatic reduction to chlorophyllide. These experiments have led to two important conclusions: 1) all of the enzymes required for chlorophyll synthesis are non-limiting in etiolated leaves,

except for the enzyme complex which makes ALA; and 2) ALA is the major controlling point of the pathway. The lag phase of chlorophyll synthesis is thought to be associated with the de novo synthesis of the unnamed enzyme complex which makes ALA (30). Inhibitors of protein synthesis prevent the formation of chlorophyll in etiolated leaves placed in the light, but the application of ALA reverses this effect (30). Other studies directly show that ALA formation in maize leaves is light-dependent (15).

Isolation and characterization of the enzyme complex which makes ALA in plants has been accomplished only recently. The system is remarkably different from that in bacteria and animals, where ALA is formed from succinyl CoA and glycine (17,43). In plants, ALA is formed from glutamic acid (2) by an enzymatic system that consists of two protein fractions and an RNA species (17). The RNA is a chloroplast glutamate tRNA that functions as a cofactor in the synthesis of ALA (43).

Methods and Materials

Toxin Production and Initial Purification.

Procedures for production and initial purification of toxins were modified from those used previoulsy (34). Single spore isolates of *H. carbonum* race 1 were maintained on potato dextrose agar slants at 4 C. For toxin production, the fungus was grown in a modified Fries solution containing yeast extract (34). Fluids (10-20 liters) from 21 day old cultures were filtered through cheesecloth, and through paper (Whatman #1), and then concentrated to approximately 0.1 original volume under reduced pressure at 40 C. An equal volume of methanol was added to the concentrated filtrate and the solution was stored overnight at -20 C; a precipitate was removed by filtration through paper (Whatman #42). Methanol was then removed under reduced pressure and the aqueous solution was extracted three times, each time with an equal volume of methylene chloride. Steps to this point were completed as quickly as possible to avoid hydrolysis of the epoxide. All subsequent preparations were dried under reduced pressure and stored under argon or nitrogen at -20 C.

The combined methylene chloride extracts were dried under reduced pressure. The remaining reddish oil (several ml) was dissolved in methanol and placed on a column (4.1 x

35 cm) of Sephadex LH-20 (Sigma Chemical Co.) previously equilibrated with methanol. The column was eluted with methanol and five ml fractions were collected. Fractions containing toxin were identified by bioassay (38) and by TLC.

Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was on .25 mm silica gel plates (Merck) developed with acetone/methylene chloride (1:1, v/v). Toxins were detected by spraying the chromatographs with the epoxide indicator 4-(p-nitrobenzyl)-pyridine (NBP) (14).

Flash Chromatography

The toxin containing fractions from the LH-20 column were combined and concentrated under reduced pressure, giving a viscous orange oil that was fractionated by flash chromatography (46) using 230 - 400 mesh flash silica. The column diameter was dictated by sample dry wieght (46), but the length of the packed silica bed was approximately 15 cm regardless of column diameter. The column was eluted with hexane:methylene chloride:acetone (1:1:1, v/v/v; solvent A) and fifty ml fractions were collected. The fractions were periodically examined by TLC, using the epoxide indicator for the presence of a reactive spot at R_f .55. This spot was shown to be toxin I. After toxin I was eluted in solvent A, the mobile phase was changed to methylene chloride:acetone (1:1, v/v; solvent B). The column was

then eluted with 1000 ml of solvent B and the eluate was collected as a single fraction. TLC of this eluate indicated epoxide-containg spots between R_f .25 and .35. These spots were shown to have host-selective toxicity, indicating that they are possible toxin analogs. The entire preparation that eluted in solvent B was refractionated by flash chromatography on silica gel using a smaller column (20-30 mm diameter) with both solvents A (300 ml) and B (500 ml) as described above. Twenty ml fractions were collected. The solvent A eluate from this step was pooled with that from the previous step, and toxin I was crystalized with diethyl ether (25). Toxin analogs were located in the solvent B fractions by TLC; the fractions were pooled to form the minor toxin preparation.

HPLC

Solvent B eluate from the flash chromatography was dissolved in water (25 ml) and placed on a column (2.2 x 15 cm) of C_{18} (40 um, J.T. Baker Chemical Co.). Loosely absorbed compounds were removed by passing 100 ml of water through the column. Analogs of toxin I were then eluted with 30% ethanol in water (100 ml). This eluate was loaded onto a Waters uBondapac C_{18} HPLC column (.78 X 30 cm) and chromatographed with a Varian 5000 instrument. The initial solvent of 7% ethanol in water was linearly changed over 30 min to 20% ethanol in water , which was then held constant for the next 15 min. The flow rate was constant at 2.0 ml/min and absorbance was monitored at 215 nm. Final

purification of the toxic analogs was with a 0.4 X 25 cm column of Whatman Partisil 5. Elution was with an isocratic mixture of hexane/ethanol (95/5 or 93/7, v/v) at 3.0 ml/min and absorbance was monitored at 215 nm.

Hydrolysis of Epoxide

The epoxide of toxin I was hydrolyzed to a diol with 0.1% (v/v) triflouroacetic acid (TFA) in water (6). The diol was purified on the 2.2 x 15 cm column of C_{18} as described above for the minor toxins, except that elution was with 100 ml of 10% (v:v) ethanol in water. The eluate, rich in toxin I diol, was subjected to HPLC, using the Partisil 5 column and hexane/ethanol (85:15, v/v) as described above.

$NaBH_4$ Reduction of Toxin I

The carbonyl adjacent to the epoxide of toxin I was reduced with $NaBH_4$ in anhydrous methanol. $NaBH_4$ (1 to 2 M) was added to 0.2 M toxin. The reaction mixture, capped under dry N_2, was placed on ice. The reaction was stopped after one hr incubation by the addition of several ml water. Reduced toxin was immediately extracted with several volumes of methylene chloride. This extract was subjected to HPLC, using the Partisil 5 column as described above for toxin analogs.

Spectroscopic Analyses

Amino acid analysis was performed by the Macromolecular Structure Facility at Michigan State University using a Waters Associates Pico-Tag analyzer.

^{1}H- and ^{13}C-NMR spectra were collected in $CDCl_3$ at 250 and 68.9 MHz, respectively, on a Bruder WM-250 spectrometer. Chemical shifts are relative to internal $CHCl_3$.

FAB MS was performed by Michigan State University Regional Mass Spectroscopy Facility, Department of Biochemistry, on a JEOL HX 110 HF instrument. High resolution masses were relative to internal glycerol or HC toxin I.

Root Growth Inhibition Bioassay

A root growth inhibition bioassay was used to determine activity of toxins (38). Seeds were near isogenic Pr X K61 (susceptible) and Pr1 X K61 (resistant) maize. Seeds (5 per duplicate dish) were placed in 9-cm Petri dishes containing 10 ml water or dilutions of toxin in water. Root lengths were measured after incubation for 72 hr. Percent inhibition and ED_{50} values were determined as described elsewhere (38). The assay data were from the results of a single representative experiment and all assays were performed three times with similar results. Toxins used in the assays were freshly purified by HPLC to minimize the possibility that inactive toxin (6) contaminated the preparations.

Electrolyte Leakage Experiments

Electrolyte leakage from maize leaves was determined by a method modified from a procedure developed for use with other toxins (4). The terminal 2 cm of the second true leaf of plants (10 to 14 days old) grown under Sylvania Gro-Lux lights was excised and cut once to give two 1-cm pieces. Leaf pieces (200 mg per duplicate sample) were enclosed in cheesecloth bags and submersed in 10 ml of distilled water or HC toxin I solution (20 ug/ml) contained in scintilation vials. The samples were vacuum-infiltrated for 15 min, and incubated in experimental solutions was for a total of 2 hr (including the time under vacuum).

After incubation, the leaves were rinsed thoroughly in distilled water, and 10 ml of fresh distilled water was added to each vial. The conductivity of the ambient solution was measured periodically with a conductivity meter (Markson Science, Inc). Duplicate samples were used for each treatment.

Chlorophyll Synthesis in Etiolated Maize Leaves

Crystalline toxin I were used in all experiments on chlorophyll synthesis. Toxin and other experimental compounds were used in 6 ml of 0.01 M potasium phosphate buffer in 10 ml vials. ALA was purchased from Sigma Chemical Co. Protocol for experiments on chlorophyll synthesis was modified from that used in work on maize (15) and barley (30). Near isogenic susceptible (Pr x K61) and

resistant (Pr1 x K61) maize seedlings were grown in the dark for 9 to 11 days. The etiolated leaves were excised above the coleoptile while working under a green safe-light (30). The basal ends of excised leaves (2 to 4 gm fresh weight per duplicate sample) were placed in the experimental solutions and held for two hours in the dark; a small fan was used to increase transpiration and the uptake of solutions (30). In most experiments, leaves were then removed from toxin solutions and the basal ends were placed in water. The leaves were held under Sylvania Gro-Lux lights at 33 uE/m^2/sec to promote chlorophyll synthesis. In experiments involving the exogenous application of ALA, leaf bases were left in the experimental solutions while greening. Light intensity for those experiments was 1.7 uE/m^2/sec.

Determination of Chlorophyll

After exposure to light, leaves were blotted dry and weighed. Chlorophyll was extracted with acetone/0.1 M $CaCO_3$ in water (9:1, v:v) as described elsewhere (30). The leaves were ground in 30 ml of the solution; an additional 20 ml were used to rinse the blender. The extracts were filtered through paper, placed in capped vials, and allowed to clarify overnight at 4 C in the dark. Chlorophyll content was measured with a Gilford 240 spectrophotometer, using the equations of MacKinney (26). Duplicate samples were used in each treatment. Standard deviations generally were less than 10% of the mean. Each experiment was

performed at least three times with similar results. Data given are the results of a single experiment.

Formation of Heat Shock Proteins in Maize

A procedure modified after that of Cooper and Ho (8) was used to induce the synthesis of and to analyze heat shock proteins in maize. Seeds of susceptible and resistant maize were germinated between moist filter paper for three days at room temperature. The terminal 5 to 10 mm of each root tip was excised and placed in Eppindorf tubes containing 100 ul water or HC toxin I at 20 ug/ml four root tips were used per tratment. The tissues were incubated for 1 hr at room temperature, after which 50 uCi ^{35}S-methionine (specific activity 1330 Ci/mMol) was added to each treatment. The samples were capped and held at room temperature (controls) or were heat shocked at 45 C for an additional 3 hr. At the end of this incubation the root tips were rinsed thoroughly, blotted dry, placed in new Eppindorf tubes, and frozen in liquid nitrogen.

Proteins were extracted by homogenizing the frozen root tips in 100 ul of an extraction buffer with the aid of a ground glass tissue homogenizer. The extraction buffer, modified from (24), contained 140 mM tris (pH 7.5), 2mM phenylmethylsulfonyl flouride, 2% (w:v) sodium docylsulfate, and 1% (v:v) dimethylsulfoxide. The homogenate was microfuged for 15 min, and the supernatant was transferred to a new Eppindorf tube; the pellet was

discarded. Incorporation of ^{35}S-methionine into protein was determined by precipitation with 5% and 10% trichloroacetic acid in water (28). Equal cpm of incorporated ^{35}S-methionine per treatment was subjected to polyacrylamide gel electrophoresis. Samples were subjected to electrophoresis overnight in buffer, modified from (24) of 10% glycerol (v:v), 2% beta-mercaptoethanol (v:v), 2.1% SDS (w:v), 625 mM Tris (pH 6.8). The gels were stained with a solution of 0.1% coomassie brilliant blue (w:v), 7% glacial acetic acid (v:v), 50% methanol (v:v) for 1 hr, and destained with a solution of 5.4% (v:v) glacial acetic acid, 15.4% (v:v) methanol, and 2.3% (v:v) glycerol (8).

Results

Isolation and Identification of Major Toxin

Flash chromatography with solvent A gave a relatively pure toxin preparation. Toxin I was crystalized from the eluate in diethyl ether; one liter of culture filtrate yielded well over 80 mg of crystaline toxin I. HPLC on the Partisil 10 column indicated that crystaline toxin I had a retention time of 11 min; no impurities were detected (Fig 1). High resolution FAB MS indicated a molecular formula of $C_{21}H_{32}N_4O_6$, which is identical to that reported for HC toxin I (32). ^1H and ^{13}C NMR spectra (Fig 2 and 3) were also identical to those published for toxin I (32); this confirms the chemical identity of the compound (Fig 4).

ED_{50} for the crystalline toxin I was 180 to 230 ng/ml (ca. 0.4 umolar) in three assays against susceptible seedlings; resistant seedlings were not affected at concentrations up to 5.0 ug/ml (Fig 5). This specific activity matches that of the most active preparation reported for HC toxin (32). The ED_{50} value of toxin I against resistant seedlings was about 20 ug/ml, or about 100-fold higher than that for susceptbile seedlings (Fig 6). Crystals were very stable when stored for several months; no reduction in specific activity was ever observed for any preparation of crystals. Crystals were usually

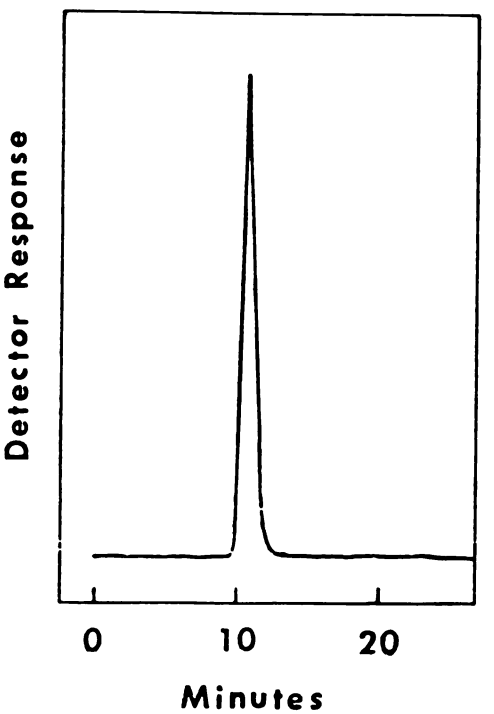
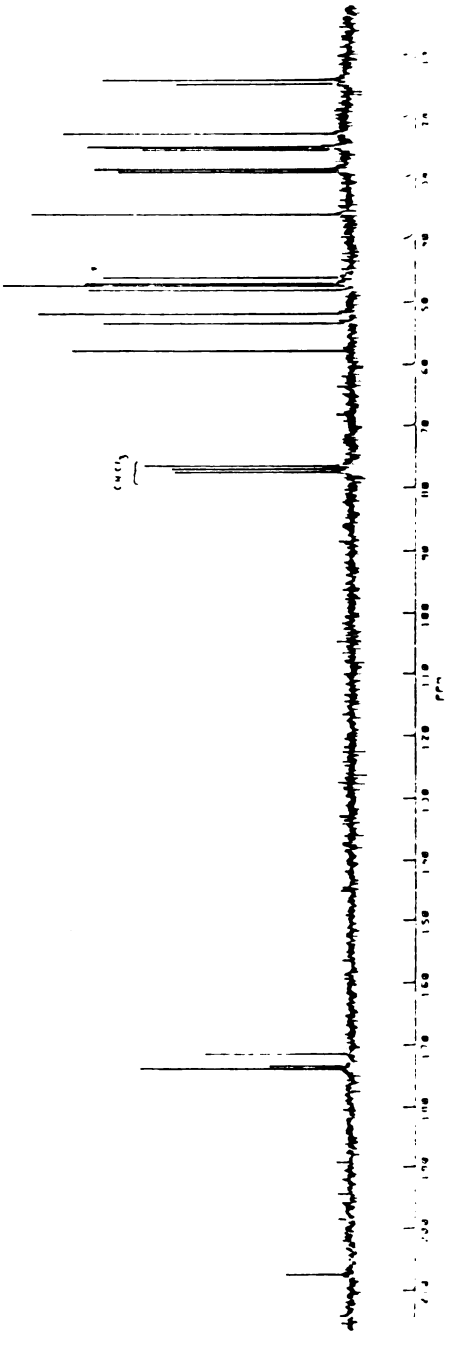


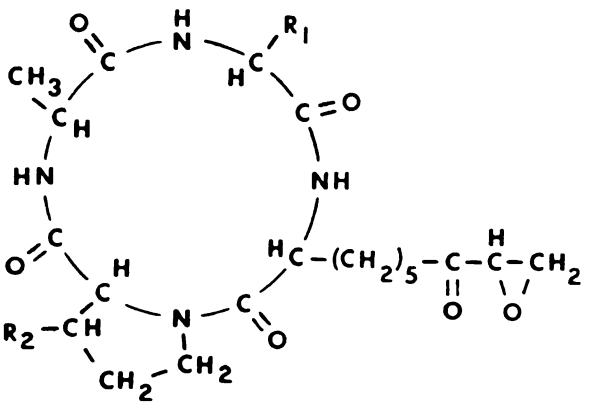
Figure 1. HPLC of HC toxin I crystals. Chromatography was on a column (0.4 x 25 cm) of Partisil 10. Elution was with hexane/ethanol (95/5, v:v) at 4.0 ml/min.

Figure 2. ^{1}H NMR spectrum of crystalline HC toxin I. Spectrum was collected on a Bruder WM-250 spectrometer at 250 MHz. Chemical shifts are relative to internal $CHCl_3$.

7 0
6 5
6 0
5 5
5 0
4 5
4 0
3 5
3 0
2 5
2 0
1 5
1 0
5
PPM

Figure 3. ^{13}C NMR spectrum of crystalline HC toxin I. Spectrum was collected on a Bruder WM-250 spectrometer at 68.9 MHz. Chemical shifts are relative to internal $CHCl_3$.





TOXIN I $R_1 = CH_3$ $R_2 = H$
TOXIN II $R_1 = H$ $R_2 = H$
TOXIN III $R_1 = CH_3$ $R_2 = OH$

Figure 4. Structures of HC toxins I, II, and III.

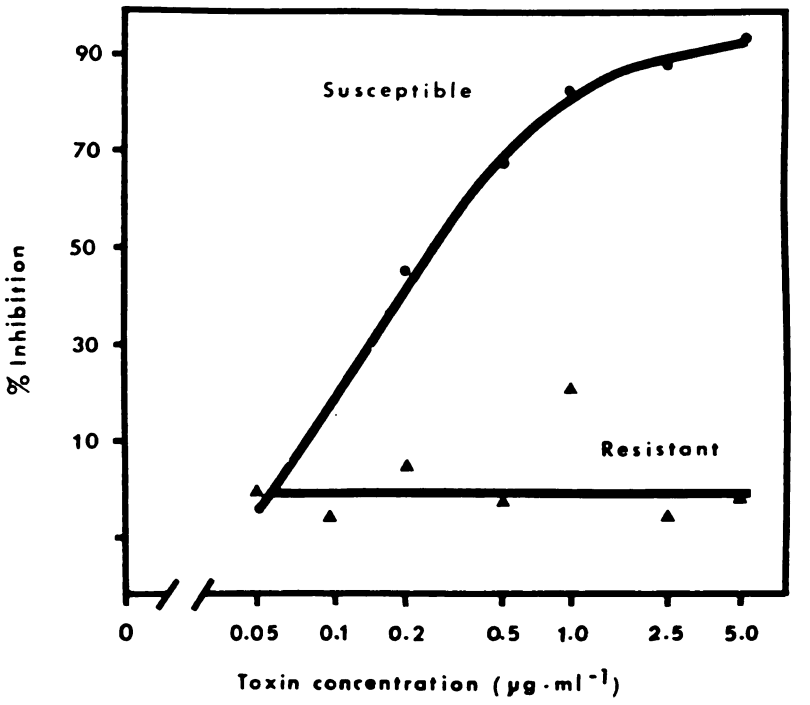


Figure 5. Effect of crystalline HC toxin I on seedling root growth. Susceptible (●) and resistant (▲) seedlings (5 per duplicate 9-cm Petri dish containing 10 ml solution) were incubated in water or in toxin solutions for 72 hr. An average was calculated for each treatment based on the longest root of each seedling after the incubation time. Percent inhibition is relative to control seedlings in water.

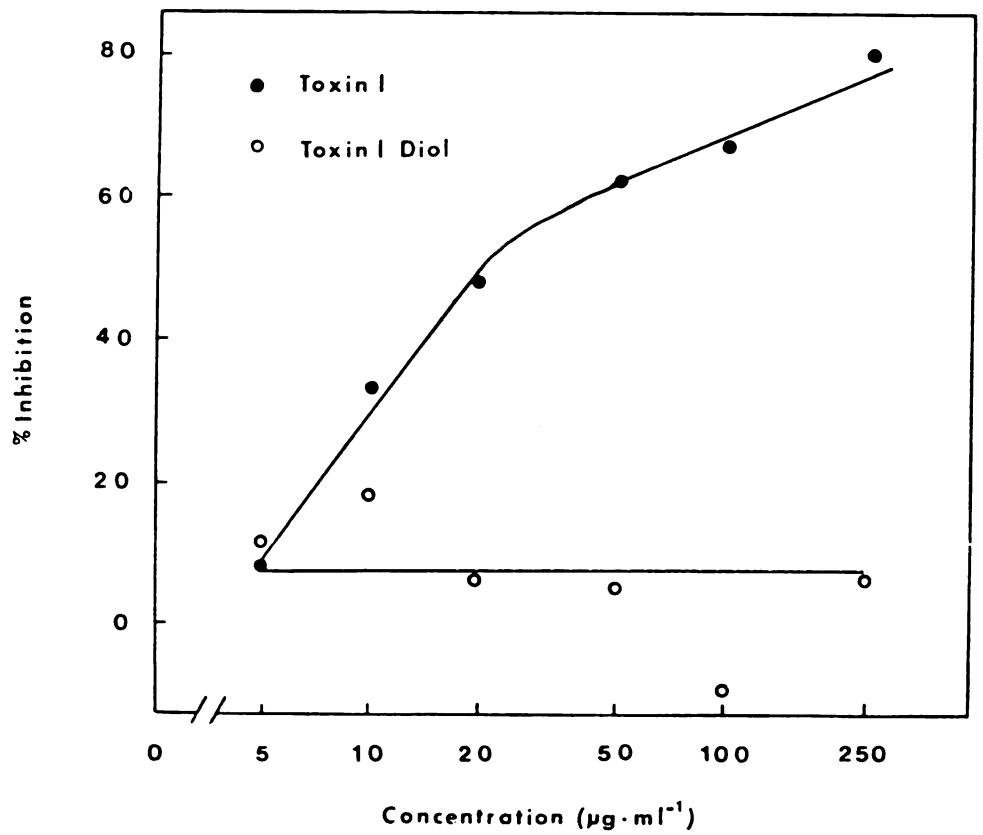


Figure 6. Effect of HC toxin I (●) and its diol (O) on root growth of resistant seedlings. Five seedlings per duplicate 9-cm Petri dish were incubated in water or in dilutions of each compound for 72 hr; 10 ml solution was used in each dish. An average was calculated for each treatment based on the longest root of each seedling after the incubation time. Percent inhibition is relative to control seedlings in water.

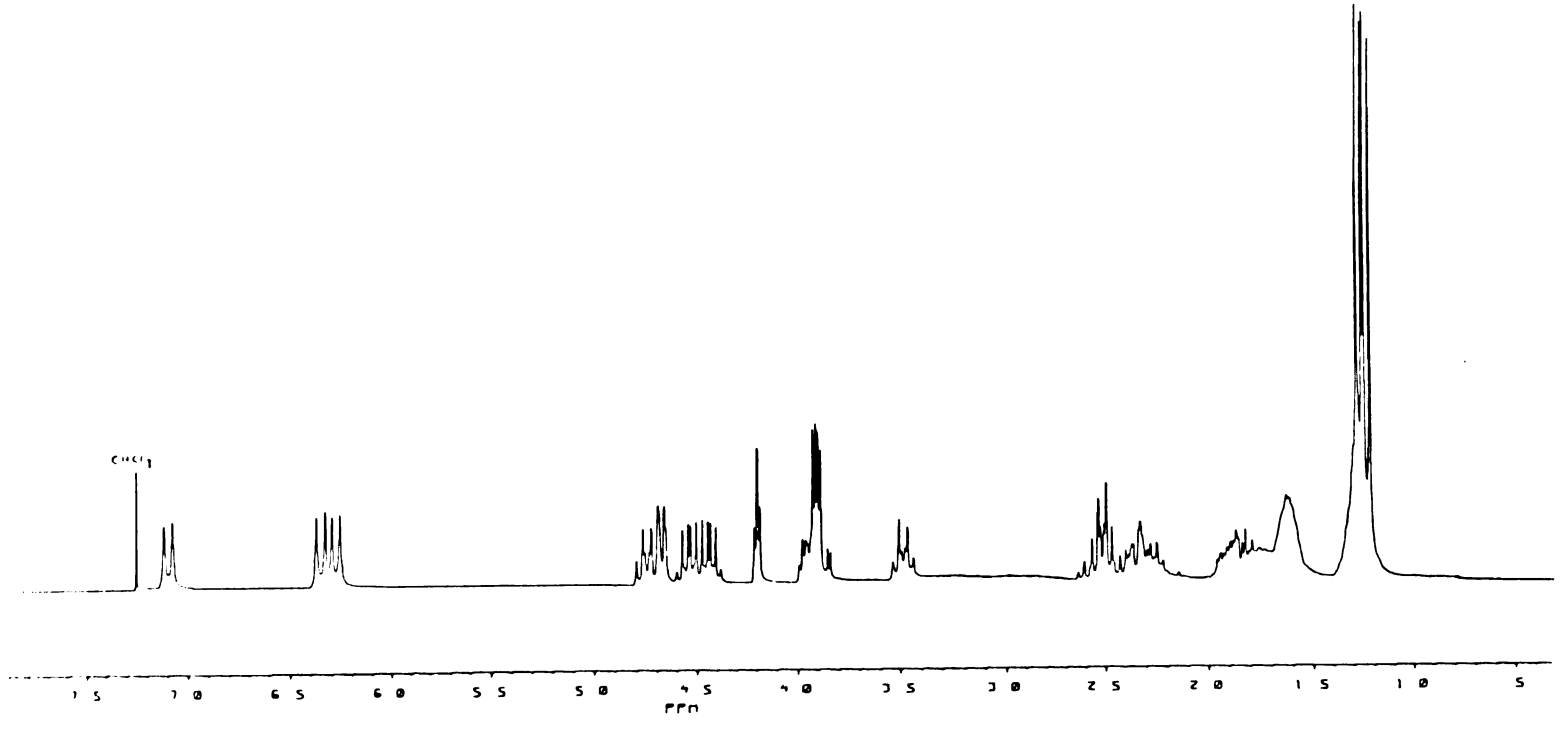
stored at -20 C, although they appeared to be equally stable at room temperature.

The epoxide of toxin I was hydrolyzed to a diol by use of 0.5% (v/v) triflouroacetic acid in water (6). The diol was purified by column chromatography and HPLC. ^{1}H NMR (Fig 7) and FAB MS data matched those previously published for toxin I diol (6), confirming the chemical identity of the compound. Toxicity against susceptible and resistant seedlings was abolished by hydrolysis of the epoxide to a diol (Fig 6); root growth of resistant seedlings was not inhibited by diol concentrations up to 250 ug/ml (Fig 6).

Isolation and Identification of Toxin Analogs

HPLC was required for purification of toxin analogs in the solvent B eluate from flash chromatagraphy. Co-chromatography of compounds was a serious problem on HPLC with C_{18} sorbent; satisfactory resolution of toxins was never achieved with that column. Three major peaks, designated RP1, RP2, and RP3 in order of elution, were obtained in a gradient of water and ethanol on the C_{18} column (Fig 8). Peaks RP1 and RP2 contained epoxides as indicated by reactivity with NBP and were selectively toxic in root growth inhibition bioassays; peak RP3 lacked an epoxide and did not inhibit the growth of maize roots. RP1 was difficult to purify further on C_{18}, but was easily separated into two major components by use of the Partisil 5 column (Fig 9).

Figure 7. ^{1}H NMR spectrum of HC toxin I diol. Spectrum was collected on a Bruder WM-250 spectrometer at 250 MHz. Chemical shifts are relative to internal $CHCl_3$.


7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
PPM

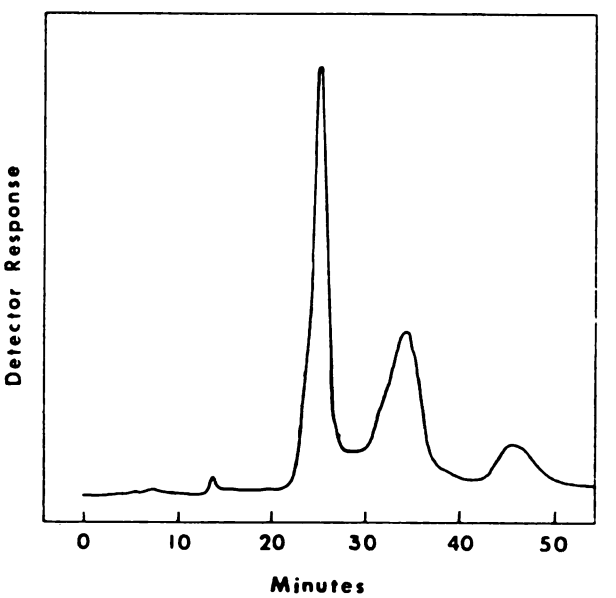


Figure 8. HPLC of a preparation containing minor HC toxins. Chromatography was on a reverse phase column (C_{18}) (2.2 x 30 cm) of Waters Bondupak. Elution was with a gradient; the initial solvent of 7% ethanol in water was changed linearly to 20% ethanol in water over 30 min. The solvent composition was then held constant at 20% ethanol in water for the next 15 min. The flow rate was constant at 2.0 ml/min.

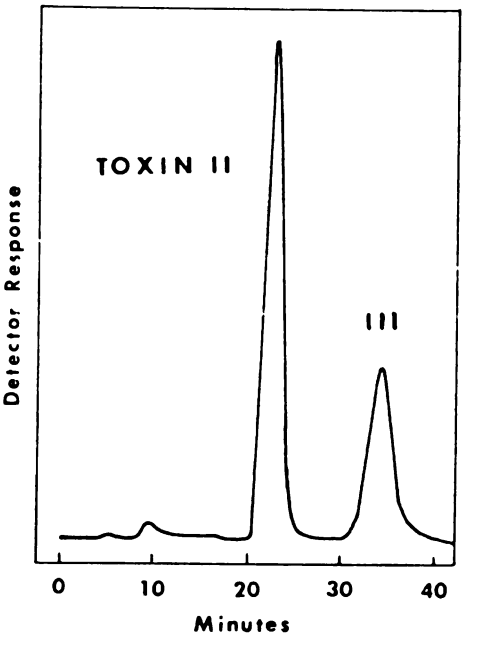


Figure 9. Partisil 10 HPLC of peak RP1 (from Figure 8), separating HC toxins II and III. Column dimensions were 0.4 x 25 cm. Elution was with hexane/ethanol (95/5, v:v) at 4.0 ml/min.

Both peaks from the Partisil 5 column had host selective toxicity; they were designated HC toxins II and III in order of elution (Fig 9). Toxin II had an ED_{50} value of 370 to 400 ng/ml (ca. 0.8 umolar) in three assays against susceptible seedlings (Fig 10). The ED_{50} for toxin III was 1800 to 2100 ng/ml (ca. 4.4 umolar) in three assays (Fig 11). Growth of resistant seedling roots was not inhibited by toxins II and III at concentrations up to 10.0 and 20.0 ug/ml, respectively (Fig 10 and 11).

Spectral data for toxins II and III were collected and published as part of another thesis (47). HC toxin II was shown to be the glycine-containing analog first described from culture fluids of H. carbonum by Kim et al. (19) (Fig 4). Toxin III was shown to contain trans-3-hydroxyproline rather than proline as its only difference from toxin I (Fig 4). Toxin III had not been reported previously. Kim et al. (19) reported an ED_{50} of 7.0 ug/ml for toxin II in a root growth inhibition bioassay that involves 96 hr incubation in 15 ml solutions. My preparation of the same compound was considerably more active in a root growth inhibition assay that used 10 ml solutions and 72 hr incubations (Fig 10). My preparation of toxin II was tested simultaneously under both assay conditions, and gave nearly the same specific activity in both assays (Table 1). Toxin II gave an ED_{50} of 0.4 ug/ml in the 72 hr assay using 10 ml solutions; an ED_{50} of about 0.3 ug/ml was observed with the assay conditions of Kim et al. (6,19) (Table 1).

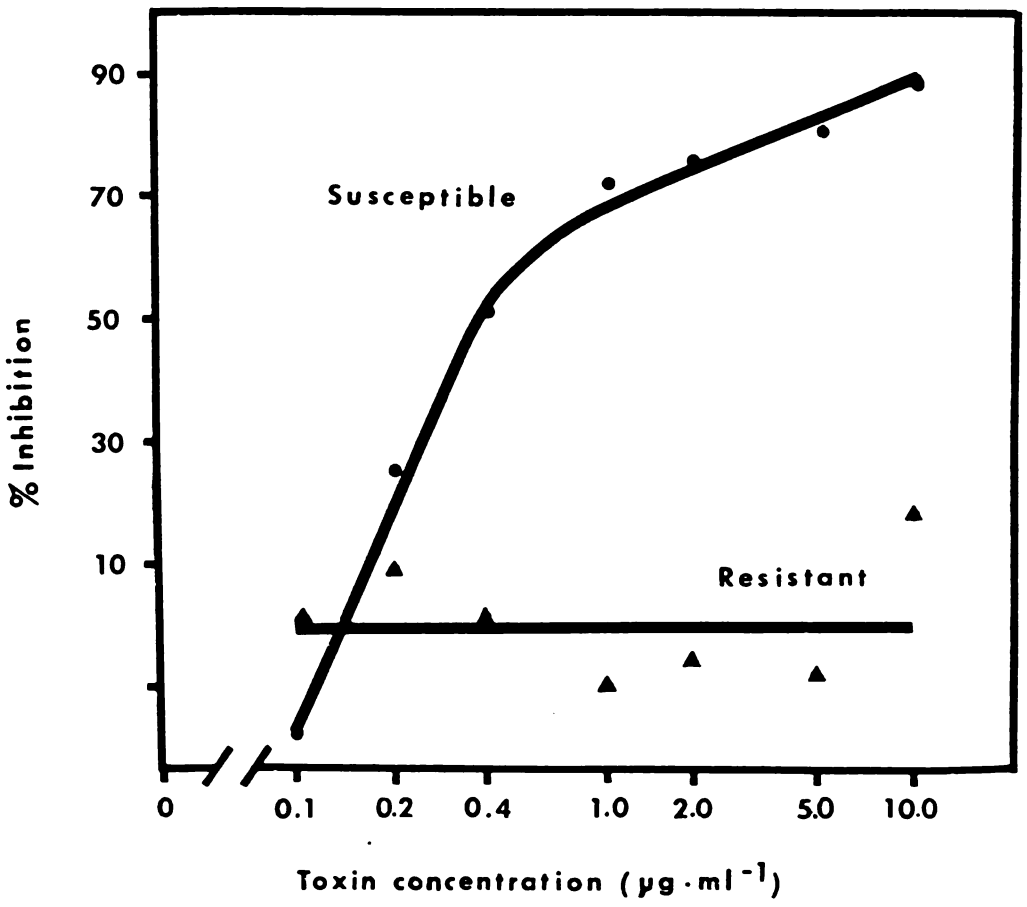


Figure 10. Effect of HC toxin II on seedling root growth. Susceptible (●) and resistant (▲) seedlings (5 per duplicate 9-cm Petri dish containing 10 ml solution) were incubated in water or in toxin solutions for 72 hr. An average was calculated for each treatment based on the longest root of each seedling after the incubation time. Percent inhibition is relative to control seedlings in water.

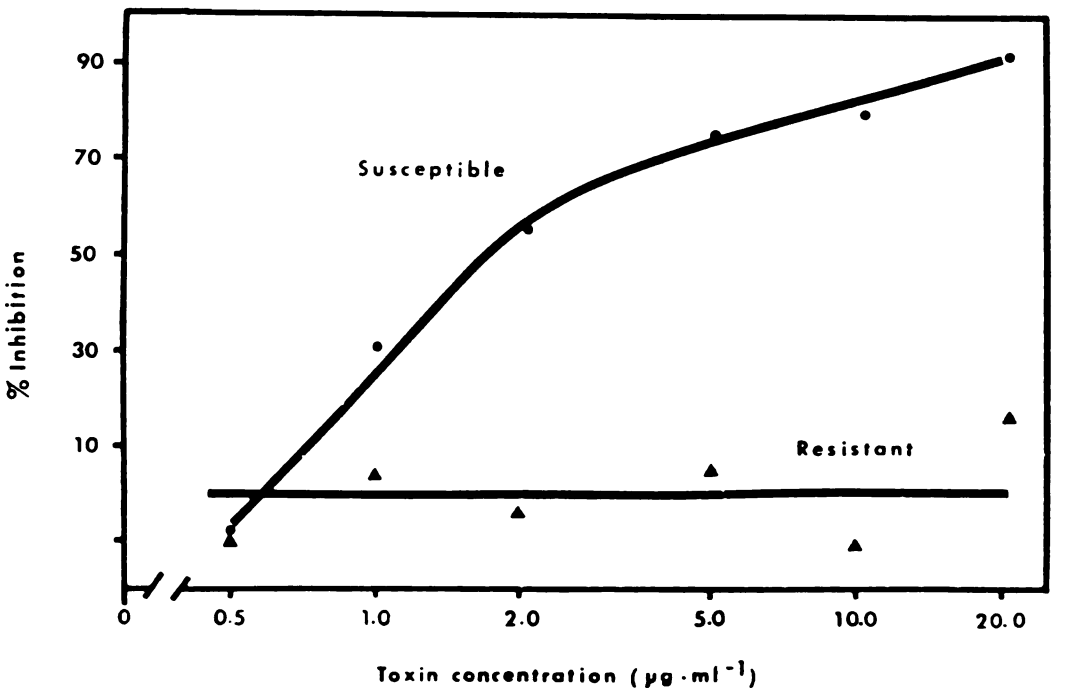


Figure 11. Effect of HC toxin III on seeling root growth. Susceptible (●) and resistant (▲) seedlings (5 per duplicate 9-cm Petri dish containing 10 ml solution) were incubated in water or in toxin solutions 72 hr. An average was calculated for each treatment based on the longest root of each seedling after the incubation time. Percent inhibition is relative to control seedlings in water.

Table 1. Inhibition of susceptible seedling root growth by HC toxin II as determined by two different assays.

HC Toxin II (ug/ml)	% Inhibition[a]	
	72 hr assay[b]	96 hr assay[c]
10.0	84	86
5.0	82	84
2.0	76	86
1.0	71	80
0.4	50	59
0.2	39	46
0.1	8	14

[a]Ten seedlings (5 per duplicate plate) were incubated in each treatment. Percent inhibition is relative to appropriate water control.

[b]Incubation was in water or in toxin (10 ml per duplicate plate) for 72 hr. This is my standard assay.

[c]Incubation was in water or in toxin (15 ml per duplicate plate) for 96 hr. This is the assay of Kim *et al.* (15).

Yields of toxin II were generally 1.6 to 1.7 times greater than the yields for toxin III; yields of each were less than 5% of those for crystalline toxin I. Another isolate of H. carbonum race 1 was examined and was found to produce the three forms of toxin in the same ratios as the original isolate.

Peak RP2 (Fig 8) was resolved into several components when chromatographed on Partisil 5; many of these components gave a positive reaction with the epoxide indicator on TLC. The retention time of the major component was 17 min; TLC indicated the compound, which had an R_f of .33, possessed an epoxide. Root growth inhibition bioassays indicated host-selective toxicity, and the compound was designated HC toxin IV. To use the HPLC more efficiently, peak RP2 was subjected to flash chromatography on a column (1.0 X 15 cm) of silica prior to further purification of toxin IV by HPLC. Toxin IV-containing fractions were identified by TLC. Final purification was by HPLC (Fig 12). The compound gave an ED_{50} of 20 ug/ml against susceptible seedlings in the root growth inhbition assay; resistant seedlings were unaffected by toxin concentrations up to 100 ug/ml, the higherst concentration assayed (Fig 13).

Structure of HC Toxin IV

Spectral data were collected for toxin IV. High resolution FAB MS established the empirical formula as $C_{21}H_{34}O_6N_4$, or toxin I plus two hydrogen atoms (calculated

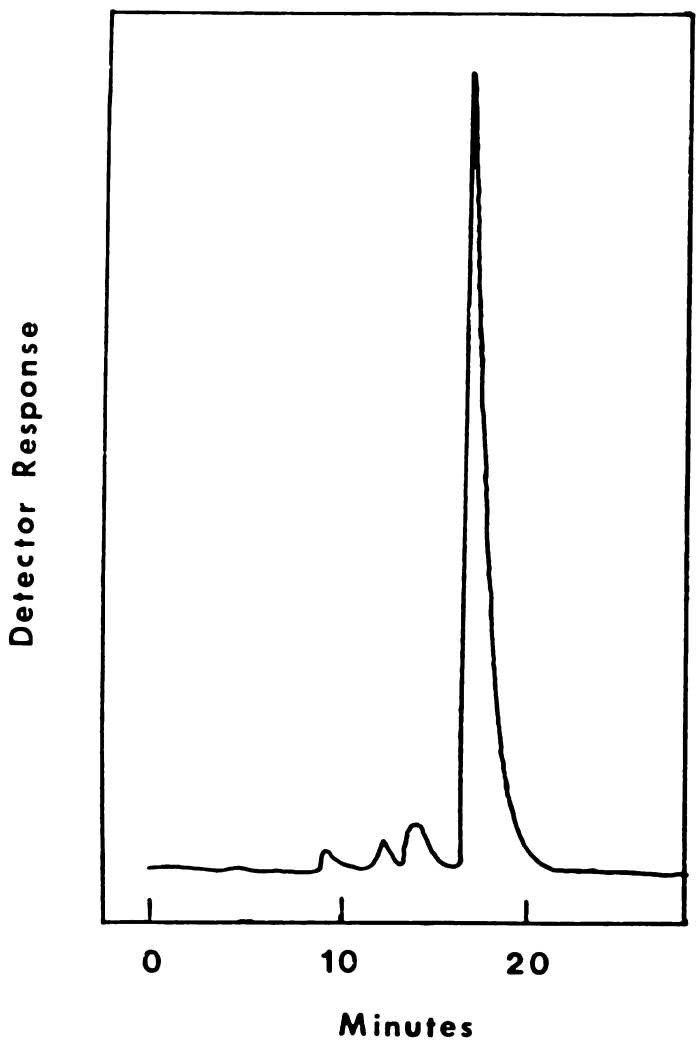


Figure 12. Final purification of HC toxin IV by HPLC. Chromatography was on a column (0.4 x 25 cm) of Partisil 5. Elution was with hexane/ethanol (93/7, v:v) at 3.0 ml/min.

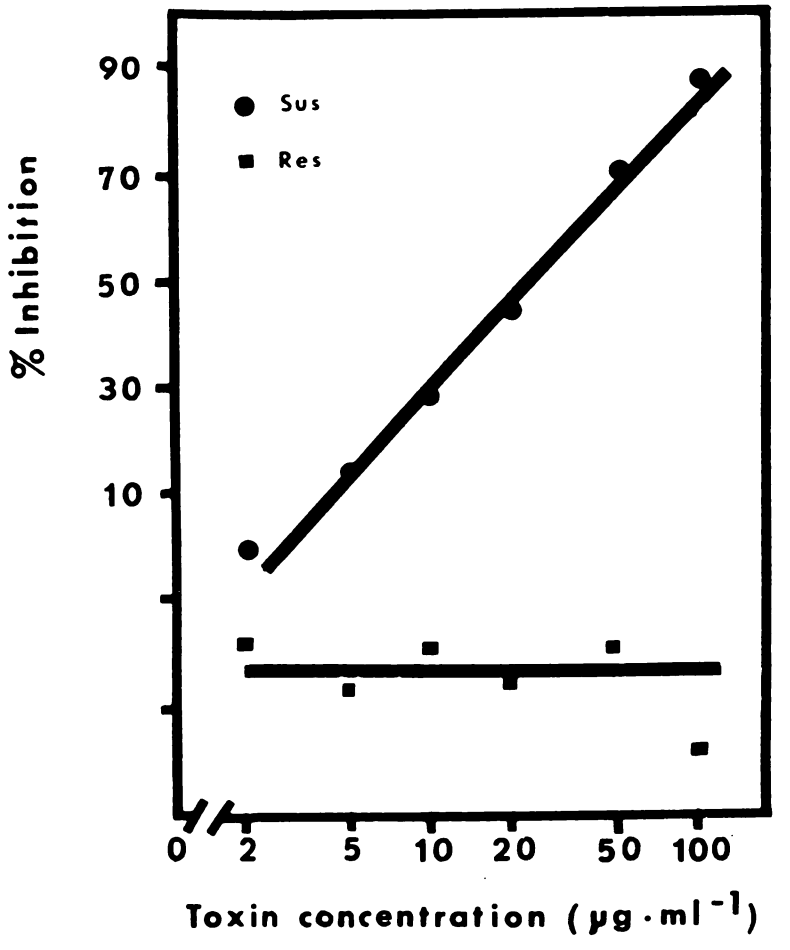


Figure 13. Effect of HC toxin IV on growth of seedling roots. Susceptible (●) and resistant (■) seedlings (5 per duplicate 9-cm Petri dish containing 10 ml solution) were incubated in water or in toxin solutions 72 hr. An average was calculated for each treatment based on the longest root of each seedling after the incubation time. Percent inhibition is relative to control seedlings in water.

$C_{21}H_{35}O_6N_4$ 439.2556, found 439.2561, +1.0 ppm). Amino acid analysis indicated alanine and proline in a molar ratio of 2:1 (identical with toxin I) suggesting that the two additional hydrogens were on the epoxide-containing amino acid. Allowing for an intact epoxide, as indicated by reactivity with NBP, the data were best explained by a hydroxylated carbon rather than a carbonyl adjacent to the epoxide (Fig 14). ^{13}C NMR spectra for toxins I (Fig 3) and IV (Fig 15) were very similar. Assignments of carbon resonances were made with assistance of published spectra for toxins I, II, and III (32,47), and are presented in Table 2. The spectrum for toxin I indicated 21 total carbons with carbonyl carbons at 171.2, 173.1, 173.5, and 173.6 ppm, assigned to each of the four amino acids, and at 207.3 ppm, in accordance with published data assigned to carbon η of aoe (32,47). Toxin IV contained 21 carbons, agreeing with the calculated empirical formula, and showed only the four amino acid carbonyls; the resonance at 207.3 ppm was absent and a new signal was observed at 71.5 ppm. This is consistent with hydroxylation of the carbon adjacent to the epoxide, therefore, the resonance at 71.5 ppm was assigned to that carbon (carbon η). No other differences were observed between the two ^{13}C-NMR spectra (Table 2).

^{1}H NMR data for toxins I and IV were also very similar (Fig 2 and 16). The only difference was in protons assigned to the epoxide. Toxin I appears as three double

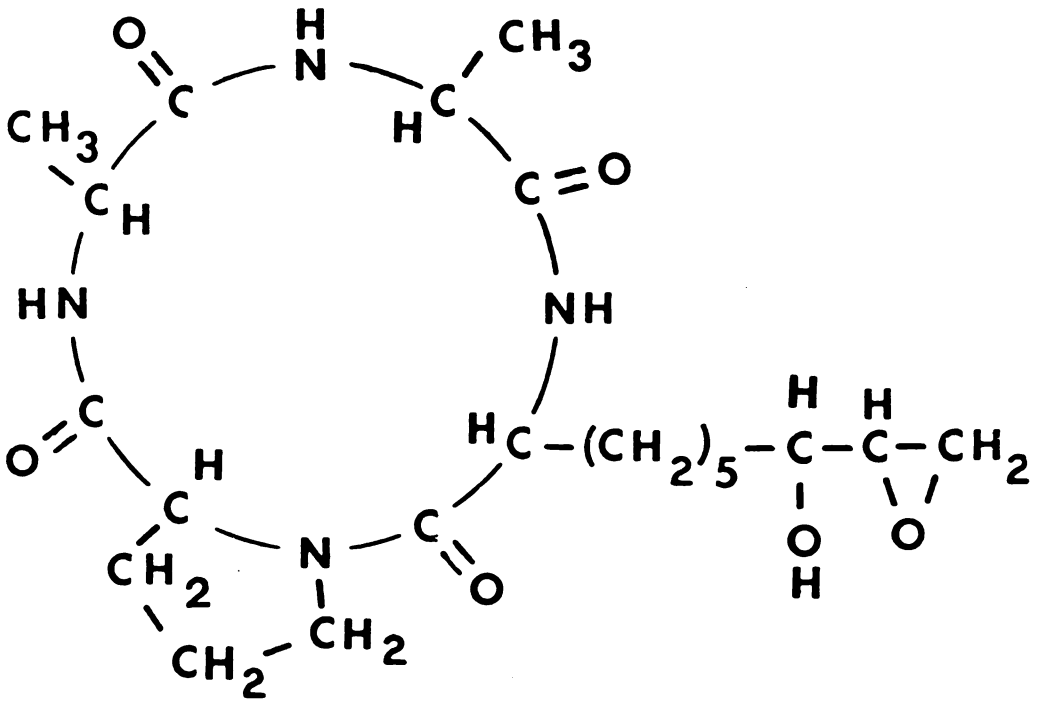
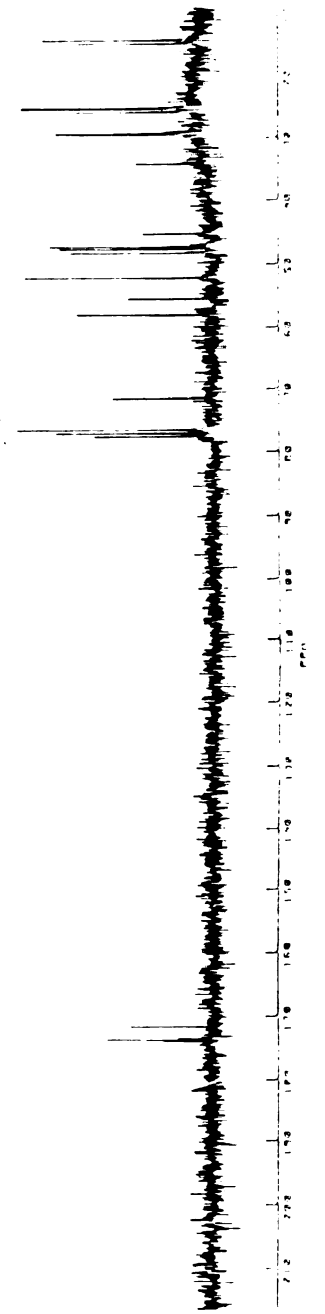


Figure 14. Proposed structure for HC toxin IV.

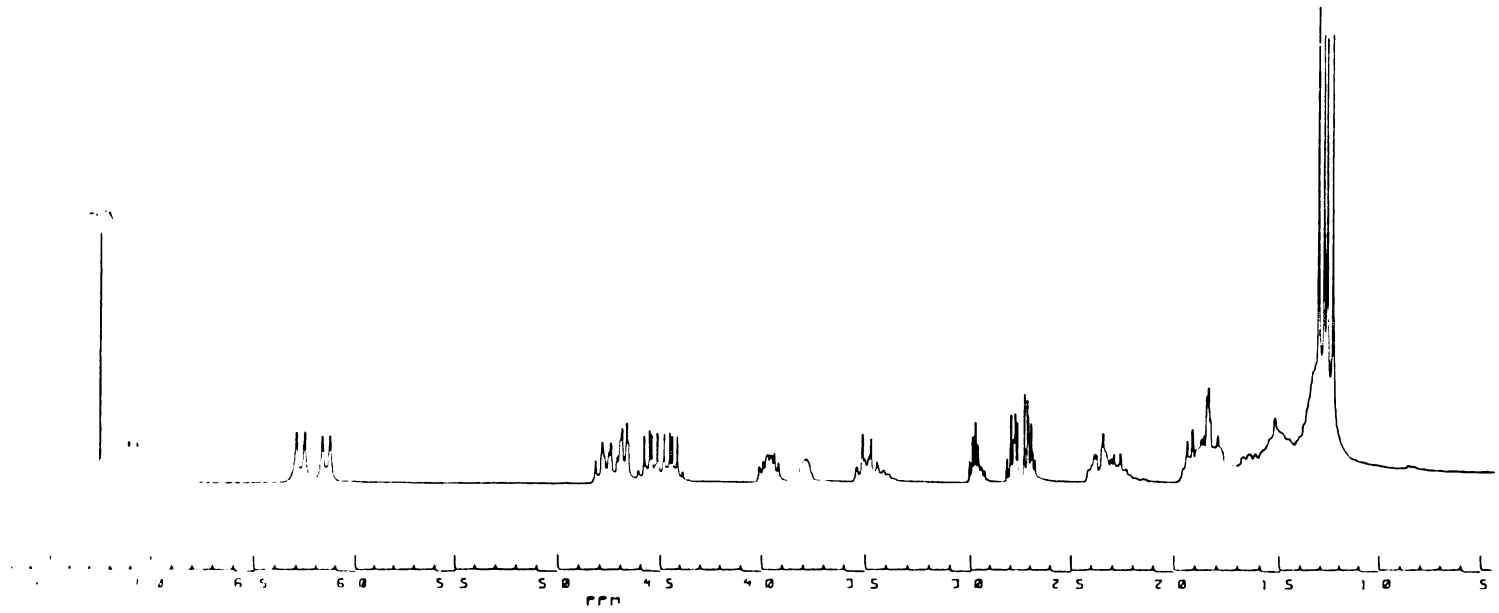
Figure 15. ^{13}C NMR spectrum of HC toxin IV. Spectrum was collected on a Bruder WM-250 spectrometer at 68.9 MHz. Chemical shifts are relative to internal $CHCl_3$.



Assignments of ^{13}C resonances for HC toxins I and IV.

Amino Acid	Carbon	Toxin I	Toxin IV
Ala_1	α	45.9	45.1
	β	14.0	14.1
Ala_2	α	47.3	47.4
	β	14.6	14.6
Pro	α	57.7	57.8
	β	28.6	29.2
	γ	22.6	24.9
	δ	48.0	48.1
Epoxide-containing Amino Acid	α	51.8	52.0
	β	29.0	29.2
	γ	24.8	25.0
	δ	24.9	25.1
	ε	25.4	25.6
	ζ	36.1	34.2
	η	207.3	71.5
	θ	46.9	47.1
	ι	53.2	55.3
	Amide C=O	173.6	173.6
		173.5	173.5
		173.1	173.2
		173.3	171.4

Figure 16. ^{1}H NMR spectrum of HC toxin IV. Spectrum was collected on a Bruder WM-250 spectrometer at 250 MHz. Chemical shifts are relative to internal $CHCl_3$.


6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
PPM

of doublets at 2.9 ppm (1H, C ι), 3.0 ppm (1H, C ι) and 3.4 ppm (1H, C θ). Toxin IV gave multiplets at 3.45 ppm (1H, C η) and at 3.05 ppm (1H, C θ), and two double of doublets at 2.75 ppm and 2.83 ppm (2H, C ι). Reduction of the carbonyl adjacent to the epoxide in toxin I with $NaBH_4$ gave a compound with the same chromatographic and spectroscopic properties as toxin IV, supporting the proposed structure (Fig 14). The compound is identified as cyclo[alanyl-alanyl-prolyl-2-amino-8-hydroxy-9,10-epoxy-decanoyl].

Yields of toxin IV were very low relative to the other forms of toxin; usually about 100 ug of toxin IV were recovered per liter of culture fluid. Thus, in terms of yield, toxin I> toxin II> toxin III> toxin IV. Based on their chromatography on C_{18} and silica gel, the order of toxins in terms of increasing polarity is I, IV, II, III.

At least three other constituents of peak RP2 possess an epoxide as determined by NBP, and therefore may be toxin analogs. None of these additional constituents has been characterized further, either chemically or biologically.

Effect of Toxin on Chlorophyll Synthesis in Etiolated Leaves

Etiolated control leaves in buffer solutions without toxin made very little chlorophyll in the first 2 to 4 hr after exposure to light; thereafter, chlorophyll levels increased rapidly (Fig 17). This response is typical of chlorophyll synthesis in maize and other higher plants

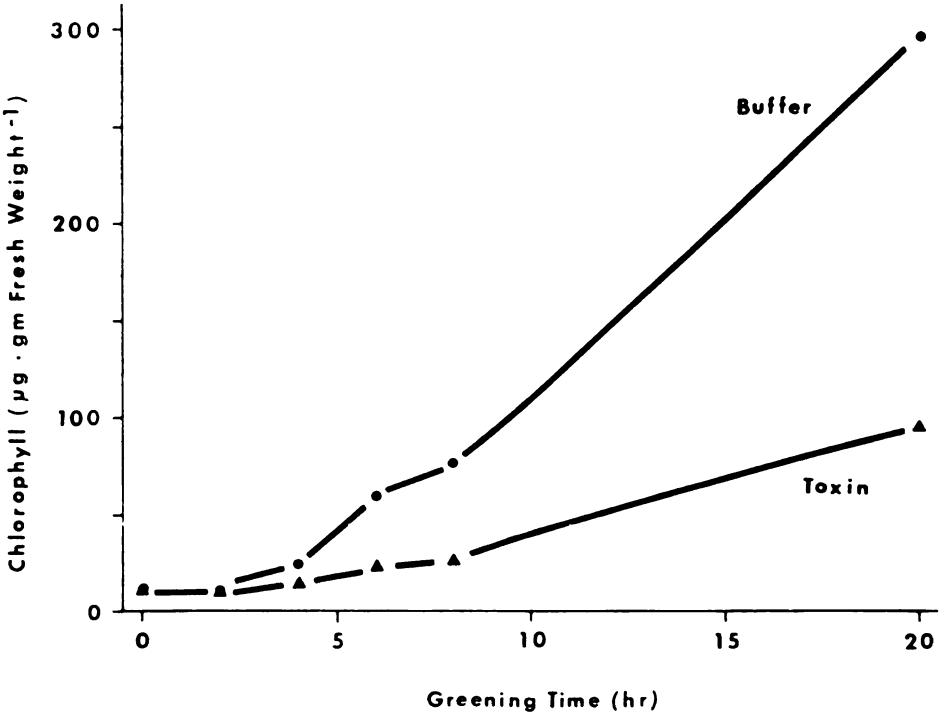


Figure 17. Effect of HC toxin (20 ug/ml) on chlorophyll synthesis in susceptible maize. Leaves accumulated solutions of buffer (●) or toxin (▲) for 2 hr in the dark (through the transpiration stream) prior to exposure to light (33 uE/m^2/sec).

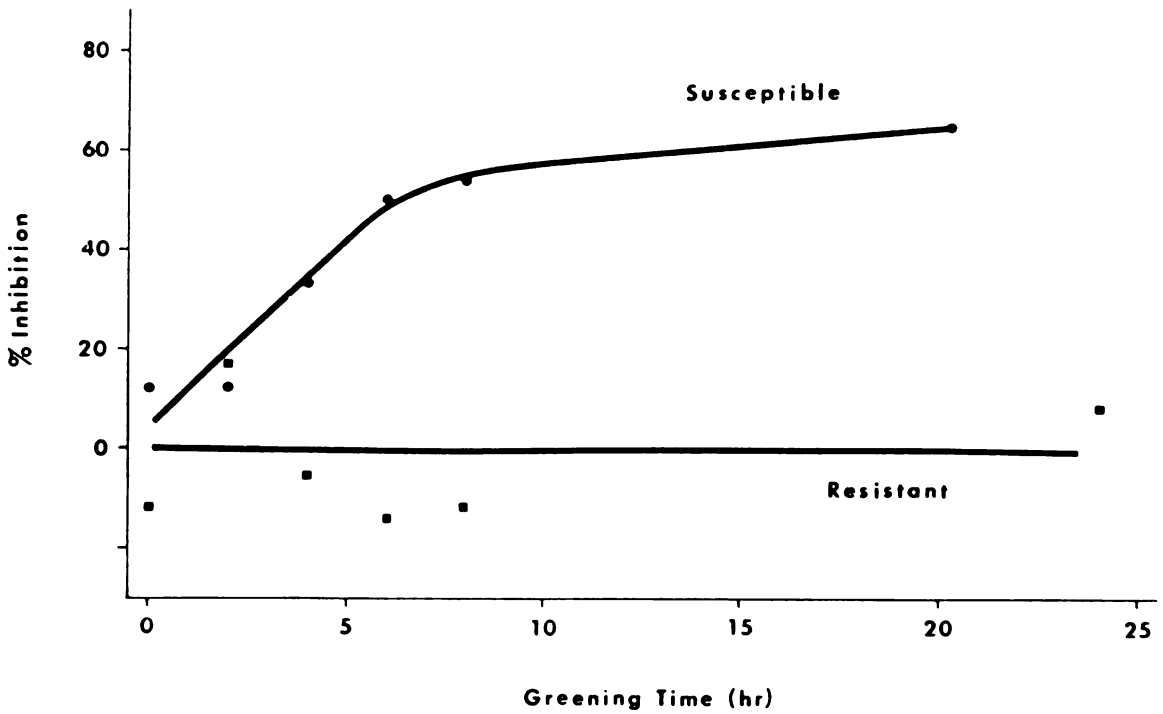


Figure 18. Effect of toxin (20 ug/ml) on susceptible and resistant leaves, expressed as % inhibition of chlorophyll synthesis. These include data given in Figure 17, plus data with resistant tissues.

(15,30). The initial lag phase of a few hours is associated with the de novo synthesis of the enzyme complex which makes ALA (30). ALA is the first recognized step in the chlorophyll synthesis pathway (1,5). Leaves treated with toxin (20 ug/ml) for 2 hr prior to light exposure made substantially less chlorophyll than did control leaves in buffer solutions (Fig 17); the reduced levels were always evident between 4 and 6 hr after exposure to light. The data from the experiment shown in Fig 18, when expressed as percent inhibition, shows that the effect was specific; resistant leaves were not inhibited in their ability to make chlorophyll, and that most of the inhibition in susceptible seedlings was evident in the first 6 hr of light exposure (Fig 18). Toxin at 20 ug/ml gave approximately 50% inhibition of chlorophyll synthesis after 6 hr in light, as compared to controls in buffer solutions (Fig 18).

Dosage/response data from an experiment using 6 hr light exposure for greening showed that the inhibition increased linearly with increases in toxin concentration (Fig 19). Inhibition for susceptible leaves was evident at toxin concentrations <1.0 ug/ml; the increase in inhibition occured over at least 5 orders of magnitude. Chlorophyll synthesis in resistant leaves was inhibited as much as was synthesis in susceptible leaves, provided the toxin levels were 100-fold higher (Fig 19). Inhibition of chlorophyll synthesis in resistant leaves was evident with about 50 ug

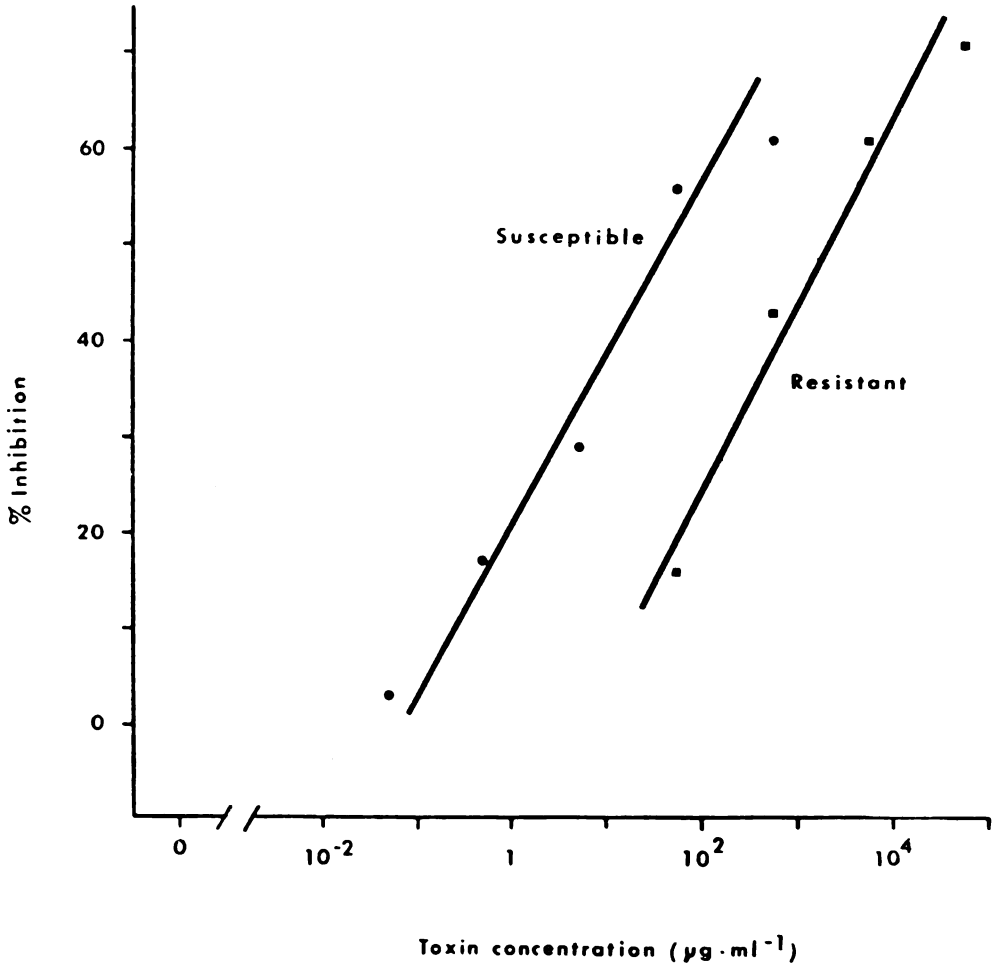


Figure 19. Toxin concentration effects on chlorophyll synthesis in susceptible and resistant leaves. Toxin was administered for 2 hr in the dark; leaves were then placed in water and in the light (6 hr) to induce greening.

toxin per ml. Dose/response curves were always linear after 6 hr in the light, but ED_{50} values for susceptible leaves varied in several experiments between 5 and 50 ug/ml. ED_{50} for resistant leaves was about 5000 ug/ml (Fig 19).

The inhibition of chlorophyll synthesis was not expected because two other *in vivo* enzyme systems, nitrate reductase and the enzymes involved with CO_2 fixation, were stimulated by toxin (22,57). Thus, there is no basis for proposing a general disruptive effect on enzymatic pathways. An electrolyte leakage experiment with leaf tissues indicated no significant disruption of the plamalemma by a 2 hr exposure to toxin at 20 ug/ml (Table 3). Therefore, inhibition of chlorophyll synthesis is not associated with and can not be explained by leaky membranes or dead cells. Toxin-treated leaves showed no greater leakage of electrolytes than did control leaves exposed to water, even after 10 hr (Table 3). Based on these observations, a testable hypothesis is that toxin is blocking chlorophyll synthesis at some point. Blockage of ALA formation is a reasonable possibility because all other enzymes in the pathway are thought to be constitutive and nonlimiting in etiolated leaves (30,45).

A test of this hypothesis required prior determination of the ALA concentration required to eliminate the lag phase in chlorophyll synthesis. This is the concentration of ALA needed to support measurable chlorophyll synthesis

Table 3. Effect of HC toxin I (20 ug/ml) on electrolyte leakage from susceptible maize leaves.

Leaching Time	Conductivity[a] (umhos)	
(Hr)	Water	Toxin
0	2.4	2.6
2	8.0	6.7
4	10.2	9.6
6	12.7	12.0
8	15.2	15.4
10	22.2	16.9

[a]Solutions (10 ml per duplicate vial) were vacuum infiltrated into leaf tissue (200 mg per duplicate sample) for 15 min at the beginning of the exposure time. Leaf pieces were rinsed thouroughly after exposure to solutions, and 10 ml water was added as leaching solution.

in etiolated maize leaves. In early experiments, etiolated leaves were supplied with ALA and placed in light (33 uE/m^2/sec) to induce chlorophyll synthesis. This treatment caused necrosis of all leaves receiving ALA, presumable because they were synthesizing more chlorophyll than could be incorporated into membranes. When the experiment was conducted in dim light (1.7 uE/m^2/sec) control leaves in buffer made chlorophyll at a much slower rate than they did at the higher light intensity, and leaves receiving ALA made chlorophyll without necrosis. Thereafter, all experiments with ALA were performed in dim light. Similar data are available for barley (30), where use of low light intensities also eliminated photodestruction of etiolated leaves receiving ALA.

A minimum of 0.5 mg ALA per ml was required to eliminate the lag phase (Table 4). Also, the amount of chlorophyll made during the 2 hr exposure to light increased with ALA concentration up to at least 2.5 mg/ml (Table 4). The data are consistent with similar observations for barley (30). In my experiment, ALA at 1.0 mg/ml increased the amount of chlorophyll made during the first 2 hr in the light by 50% relative to control leaves in buffer (Table 4); therefore, the effect of ALA at 1.0 mg/ml was tested for its effects on toxin-induced inhibition of chlorophyll synthesis. This concentration of ALA prevented inhibition of chlorophyll synthesis by cycloheximide in barley (30).

Table 4. Effect of ALA on the lag phase of chlorophyll synthesis in etiolated maize leaves.

ALA [a] (mg/ml)	Chlorophyll (ug/gm FW)	% Increase
0	14.0	--
0.2	13.9	0
0.5	15.7	12
1.0	21.0	50
2.0	26.7	91
5.0	26.0	86

[a]The application of solutions through the transpiration stream began in the dark 2 hr prior to exposure (2 hr) to dim light (1.7 $uE/m^2/sec$).

The application of ALA (1.0 mg/ml) prevented the toxin-induced inhibition of chlorophyll synthesis (Fig 20). Toxin (175 ug/ml) in buffer caused approximately 50% inhibition in chlorophyll synthesis relative to control leaves without toxin after 6.5 hr exposure to dim light (1.7 uE/m^2/sec). Under the same conditions, leaves receiving toxin plus ALA made as much chlorophyll as did the control leaves without toxin (Fig 20). The results indicate that inhibition of chlorophyll synthesis by HC toxin may be attributed to a block in the ability of toxin-treated tissues to make ALA.

Recovery of Toxin from Homogenized Leaves

Host-selective toxin activity has not been recovered from susceptible or resistant maize leaves treated with HC toxin, despite intensive attempts (21). The procedures employed during these attempts to recover toxin from maize leaves were very similar to those used in the chlorophyll synthesis experiments. Cut leaves took up toxin solutions in the transpiration stream for a few hours. The leaves were then homogenized and the extract was assayed for toxic activity; none was found (21).

In my experiments, leaves were exposed to HC toxin I for 2 hr prior to homogenization in acetone, and the extract was analyzed for its chlorophyll content. When small aliquots (5 ul) of the acetone extract from toxin-treated leaves was subjected to TLC, an expoxide-containing compound was observed at R_f 0.35; no epoxide-containing

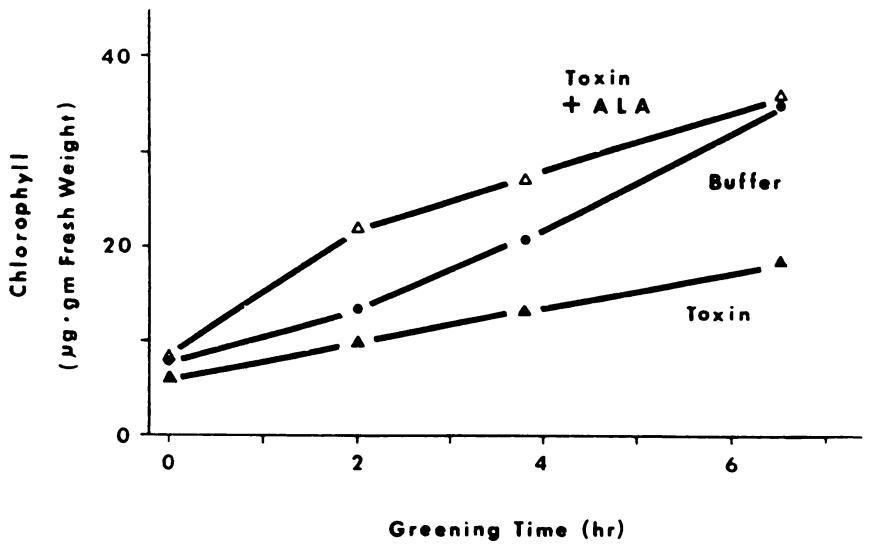


Figure 20. Effect of ALA (1.0 mg/ml) on toxin-induced inhibition of chlorophyll synthesis. Toxin concentration was 175 ug/ml. Leaves took up solutions in the transpiration stream in the dark for 2 hr prior to exposure to light (1.7 uE/m^2/sec).

spot was found at R_f 0.55 (HC toxin I) in plants exposed to toxin I. Plants receiving buffer but no toxin did not have the epoxide-containing spot at R_f 0.35. Comparable results were obtained with susceptible and resistant leaves. The TLC data suggest that susceptible and resistant leaves alter toxin to a more polar compound. The compound was purified from acetone extracts of treated leaves by flash chromatography on silica gel using acetone/methylene chloride (1/1, v/v). HPLC on partisil 5 column indicated the compound had a retention time identical to toxin IV standards. Toxin IV has much lower activity than does toxin I (Figs 5 and 13).

Discussion

The improved purification scheme for HC toxin is rapid, easy, and efficient, and makes possible the preparation of large quantities of crystalline HC toxin I. Limiting steps in the old procedures were the use of TLC plates as a preparative step, and use of analytical HPLC . These steps were eliminated by crystalization of toxin from eluate fractions from flash chromatography. The crystals, which were collected in high yields, gave a single peak on analytical HPLC (Fig 1); in bioassays, the preparation had specific activity equal to the highest reported for toxin (32). Many of the experiments in this research would not have been possible without large quantities of highly purified toxin I. The effect of toxin on chlorophyll synthesis in resistant seedlings (Fig 19), for example, required several hundred mg of toxin I. Other procedures that required large quantities of toxin were the $NaBH_4$ reduction of toxin I to confirm the structure of toxin IV; and hydrolysis of the epoxide to a diol for experiments with resistant tissues (Fig 6). Future chemical and biological studies of toxin I should be aided by the easy purification method.

Root growth of resistant seedlings was inhibited by toxin concentrations that were 100-fold higher than was required to inhibit susceptible seedlings (Fig 6). This

confirms earlier observations based on inhibition of root growth with less pure toxin preparations (23). The epoxide is known to be essential for activity against susceptible seedlings (6,53). The data indicate that this observation can be extended to resistant seedlings as well (Fig 6); hydrolysis of the epoxide to a diol also destroyed toxic activity against resistant seedlings. Root growth by resistant seedlings was not affected by diol concentrations of 250 ug/ml. The data support but do not prove the hypothesis that resistant tissues have a toxin sensitive site similar to one in susceptible tissues. The other line of evidence for this hypothesis is that resistant plants gave the same response to toxin as did susceptible plants, provided the toxin concentrations are approximately 100-fold higher (Figs 6 and 19) (22,57,58).

Spectral data were collected on toxins II and III and reported elsewhere (47). Toxin II was shown to be the glycine-containing analog first reported by Kim et al. (19) Toxin III, a new analog, was found to contain trans-3-hydroxyproline rather than proline as its only chemical difference from toxin I (Fig 4). Thus, toxins I, II, and III all contain aoe. Toxin IV was characterized in this research. High resolution FAB MS and amino acid analyses indicated that toxin IV differed from toxin I only by the addition of 2 hydrogen atoms to the epxide-containing amino acid; toxin IV appeared to have an alteration in aoe. The data suggested that toxin IV contains a hydroxylated carbon

rather than a ketone adjacent to the epoxide (Fig 14). Such an alteration was detected in ^{13}C NMR spectra; the unique and easily detected carbonyl resonance at 203.7 ppm in toxin I was replaced by a resonance at 71.5 ppm (Table 2).

^{1}H NMR data for toxin IV (Fig 16) also support the proposed structure. Reduction of the ketone adjacent to the epoxide in toxin I with $NaBH_4$ produced a compound with the same spectral properties as toxin IV, and this is further proof that the carbon was hydroxylated. The structure is significant for two reasons: 1) the chemical alteration reduced the potency of the compound 100-fold (ED_{50} of toxin I and IV were 0.2 and 20 ug/ml, respectively); and 2) the amino acid aoe has been found in 3 biologically acitive metabolites from fungi other than HC toxins (9,16,49). Toxin IV is the first naturally occuring variation of the unusual amino acid to be reported.

The discovery of and the structural elucidation HC toxins II and III, each of which shows quantitative differences in levels of activity relative to each other and to toxin I, permits a more detailed examination of structure/activity relationships. Toxin I contains alanine and proline adjacent to aoe. Toxins II and III have slight variations in the residues adjacent to aoe; these differences do not alter the selectivity of toxin, but change the relative toxicities of the compounds. Toxin II

differs from toxin I by the substitution of a hydrogen for a methyl group, giving glycine rather than alanine in the molecule (Fig 4). Biological data indicate that on a dry weight or molar basis, toxin II is about one-half as potent as is toxin I, as determined by ED_{50} values in root growth assays (Fig 10). Toxin III is even less toxic. It differs from toxin I by the substitution of a hydroxyl for hydrogen at carbon 3 in the proline ring (Fig 4), giving a molecule that requires 10-fold higher concentrations for inhibition of root growth (Fig 11). Such differences in toxicity might be based on differences in affinity for receptors or on differences in permeation of plant cells, related to polarity of the compounds. These considerations may have a bearing on mode-of-action studies.

Kim et al. (19) reported an ED_{50} value for toxin II of 7.0 ug/ml in a similar but slightly different root growth inhibition assay using a toxin preparation purified by a different protocol. My preparation of the same toxin gave an ED_{50} of less than 0.4 ug/ml when assayed under their conditions (Table 1), perhaps indicating that my preparation is more pure.

The newly devised purification scheme for HC toxins is based on flash chromatography and HPLC with a silica gel. Perhaps it is not suprising that silica gel gave efficient chromatography of the toxins given their solubility and extractability in methylene chloride; silica is often chosen for chromatography of nonpolar compounds. Flash

chromatography eliminated the need for preparative TLC in purification of HC toxin; perhaps flash chromatography could simplify the purification of other relatively nonpolar selective toxins, such as T-toxin from H. maydis race T and several of the Alternaria toxins.

Toxin-induced inhibition of chlorophyll synthesis 4 to 6 hr after exposure to light (Fig 17) is the most rapid inhibitory effect observed to date for HC toxin. Because the effect was so rapid, the procedure was tested as the basis for an improved assay. The only quantitative bioassay currently used for HC toxin is based on inhibition of root growth by susceptible seedlings; this assay requires 2 to 4 days to complete. In contrast, the chlorophyll synthesis experiments show substantial differences between the treatments by 6 hr after exposure to light (Fig 18), a time frame which would allow completion of an assay within one day. Doseage/response experiments were performed using the 6 hr exposure to light. Inhibition was evident in leaves of susceptible plants at toxin levels below 1 ug/ml, which is comparable to the minimal concentration that gives inhibition of root growth in assays requiring 72 hr toxin treatments (Fig 5). The dose/response effect on chlorophyll synthesis spans at least 5 orders of magnitude in toxin concentrations (Fig 19) whereas most inhibition in root growth inhibition occurs over only 2 orders of magnitude (Fig 5) (32). The

differences may be partly or entirely attributed to the differences in toxin exposure times.

ED_{50} for toxin in root growth inhibition assays is 0.2 ug/ml (Fig 5). ED_{50} concentrations in the experiments with chlorophyll varied from 5 to 50 ug/ml (Fig 19), using the same toxin preparations. The specific activity should not be considered a problem in the chlorophyll assay given the subtle effects of toxin and the short times of exposure to toxin and light; however variation in the ED_{50} must be reduced before the procedure can be considered reliable as a quantitative assay. A major factor in the variation probably is introduction of toxin to cut leaves through the transpiration stream. This in not desirable in a bioassay (56) because factors such as relative humidity, air temperature, and air circulation can markedly affect transpiration rates, and thus the amount of toxin taken up. Another problem may be fresh weights; researchers on chlorophyll synthesis in maize and barley have expressed chlorophyll levels on a fresh weight basis, as given herein. Perhaps a reference point other than fresh weight would improve the procedure.

There is a serious need for an improved bioassay for HC toxin, but I was more concerned with what the chlorophyll synthesis experiments could reveal about the biochemical effects of HC toxin. All other known rapid effects of toxin are stimulatory or enhanced; these include effects on respiration and CO_2 fixation in the dark (22), uptake of

certain amino acids and ions (58), in vivo enzymatic reduction of nitrate (57), and transitory increases in negative electropotential across the plasma membrane (13). The effect of toxin on chlorophyll synthesis was intriguing for a number of reasons. Control leaves in buffer make very little chlorophyll in the first 2 to 4 hr in the light (Fig 17) (15,30). The inhibition of chlorophyll synthesis by toxin was always evident in this time period. Therefore, assuming that the inhibition of chlorophyll synthesis is a secondary effect of toxin, the unknown primary effect may have been even more rapid than the chlorophyll data indicated. The pathway for chlorophyll synthesis is well known (5), and this gave an opportunity for further in-depth experiments on the effect of toxin on the pathway. The effect of toxin on chlorophyll synthesis was not expected because toxin had no rapid inhibitory effect on enzyme systems in maize involved in nitrate reduction and CO_2 fixation (22,57); therefore, the hypothesis emerged that toxin caused a block in the pathway for chlorophyll synthesis.

All enzymes in the chlorophyll synthesis pathway are thought to be constitutive and non-limiting in etiolated leaves, except for the enzyme complex that makes ALA, which is the first and limiting step of the pathway. De novo synthesis of the enzyme complex which makes ALA occurs during the 2 to 4 hr lag (30). It was hypothesized that

toxin may inhibit chlorophyll synthesis by preventing the formation of ALA. The application of ALA reversed the effect of toxin (Fig 20). This indicates that the toxin-induced inhibition of chlorophyll synthesis probably results from a block in the de novo formation of ALA, and may not be the result of a general disruptive effect of toxin on the pathway.

The experiment with ALA (Fig 20) was similar to experiments in which cycloheximide and other inhibitors of protein synthesis were used to prevent chlorophyll synthesis in barley (30). ALA restored the capacity of inhibitor-treated barley leaves to make chlorophyll (30). Other common features of the two experiments were the use of high levels of either HC toxin or cycloheximide (400 umolar) to reduce the amount of chlorophyll in leaves, continous feed of experimental solutions to replenish ALA as it was made into chlorophyll, and the use of low light intensities to prevent the tissues receiving ALA from making more chlorophyll than could be incorporated into membranes.

Synthesis of ALA is the limiting and controlling point of the pathway (1,2,5), and an exogenous supply of ALA makes possible the formation of excessive amounts of chlorophyll. The limiting role of ALA in the chlorophyll synthesis pathway was confirmed (Table 4); ALA eliminated the lag phase in chlorophyll synthesis in maize leaves. Also, when leaves were supplied ALA, more chlorophyll was

made during the first 4 hr in the light than was made by control leaves (Fig 20). The control leaves in buffer eventually made as much chlorophyll as did the leaves with exogenous ALA, but only after the lag phase of 2 to 4 hr (Fig 20).

The data provide no clues as to how toxin might prevent the formation of ALA in etiolated leaves exposed to light. At least two hypotheses are tenable: toxin inhibits the formation of the enzyme which makes ALA, or toxin inhibits the activity of the enzyme which makes ALA. Events leading to the synthesis of the enzyme include the recognition of the light signal, transduction of the signal, and synthesis of the enzyme complex. Synthesis of the enzyme which makes ALA obviously involves translation (30), but there is controversy as to whether transcription is required (20,30,45). If toxin interfered with enzyme formation, it could theoretically be at any of these levels.

Present status of work on the enzyme which makes ALA does not allow for measurement of enzyme levels in terms of how much protein is present; nor is activity of the enzyme complex easily determined in maize. This limited the scope of experiments that could be performed to determine why exogenous application of ALA prevented the inhibition of chlorophyll synthesis by toxin.

There is an earlier report that the toxic effect of HC toxin can be alleviated. Inhibition of susceptible seedling root growth by the toxin was reduced but not totally prevented when seedlings were exposed to toxin under anaerobic conditions or in the presence of respiratory inhibitors (21). The data were interpretted to mean that the uptake or activity of HC toxin may require metabolic energy (21). Those data (21) have no bearing on the interpretation of the experiment with ALA (Fig 20).

Toxin at 20 ug/ml was used in the experiments on synthesis of chlorophyll (Fig 17). It must be asked if this is a reasonable concentration of toxin to use in such experiments, or is the inhibitory effect of toxin simply an artifact of high toxin concentrations? I argue that the toxin concentrations are reasonable based on three observations. First, one of the most critical experiments that established HC toxin as a pathogenicity factor demonstrated that exogenous application of toxin allowed fungal spores that did not produce toxin to colonize susceptible maize (7). Published data indicate that the minimum toxin concentration for this kind of result is 20 ug/ml (7); in other words, this concentration was used to define the biological significance of toxin.

Second, the results of my experiments can be explained by proposing that toxin may in some fashion inhibit the synthesis of protein or RNA. Cycloheximide, actinomycin-D, and acetocycloheximide are common metabolic inhibitors used

in biological studies. These compounds have been used to study synthesis of chlorophyll; effective concentrations are on the order of a few hundred uMolar (30,45). HC toxin I at 20 ug/ml is approximately 46 uMolar; therefore, the concentration is very reasonable for metabolic inhibition.

Third, there is no basis for proposing a general disruptive effect of toxin at 20 ug/ml. In my experiments, this concentration had no effect on membrane integrity as determined by electrolyte leakage (Table 4). In other research, this concentration enhanced rather than disrupted processes of solute uptake and nitrate reductase activity (57). Still higher concentrations (50 ug/ml) were reported to stimulate CO_2 fixation in the dark (22).

Various lines of evidence suggest that resistant maize cells may have a toxin-sensitive site that is similar to a site in susceptible cells. This implies that toxin may affect processes critical to all living cells. Thus, we cannot rule our metabollic processes such as RNA synthesis or protein synthesis as possible target sites for toxin.

Appendix

An understanding of how toxin prevents the formation of ALA may provide valuable insight to the mode of HC toxin action. One possible explanation is that toxin prevents the formation of the enzyme complex which makes ALA. This hypothesis is not easily tested, so another question was addressed: does HC toxin prevent the synthesis of other inducible proteins in maize? This question was asked to get indirectly at the question: does HC toxin prevent the formation of the enzyme which makes ALA?

Many proteins associated with chloroplasts in maize are made de novo in response to light, and quantitative analyses are possible (11,44,48). However, another inducible system, the synthesis of heat shock proteins, was chosen for study. This system was attractive for a number of reasons. Heat shock proteins are formed rapidly by tissues, requiring only about 2 hr of incubation at 40 to 45 C for formation (8). The experiment is relatively simple (8), the root tips of only a few seedlings are required (8) rather than gram quantities of leaf tissue necessary for the light induced proteins. Heat shock proteins are good models because they are made in response to heat rather than to light which initiates chlorophyll synthesis. Many of the light induced proteins, along with

chlorophyll, are associated with developing plastids. The synthesis of some induced proteins is closely tied with de novo syntheis of other constituents of the plastid (44). For example, the synthesis of the light-induced chlorophyll a/b binding protein is closely associated with the synthesis of chlorophyll. How, or even whether, synthesis of one induced constituent is influenced by or influences the synthesis of other constituents of the plastid is an active area of research, and a possible confounding factor in the question about the influence of HC toxin on synthesis of induced proteins. The heat shock protein system appears to be relatively simple biochemically; therefore, some of the potential problems may be avoided.

Three hr incubations of root tips in ^{35}S-methionine resulted in substantial uptake of label (Table 5). Buffer extracts of root tips were precipitated with 5% and 10% trichloroacetic acid, and counted for radioactiviy. Depending upon the treatment, 1 to 10% of the cpm was incorporated into protein (Table 5). Heat shock (45 C) for 3 hr reduced the uptake of ^{35}S-methionine relative to control root tips at 25 C (Table 5); the reduced efficiency of incorporation of radioactivity into protein by heat shocked tissues was even more striking (Table 5). For example, a typical root tip of susceptible seedlings in water took up 50,570 cpm at 25 C with nearly 5% (2,411 cpm) incorporated into protein. Heat shock reduced uptake to

Table 5. Uptake and incorporation of ^{35}S-methionine by root tips of susceptible and resistant maize at 25 and 45 C.

Treatment	(cpm/root tip/ul buffer)[a] Uptake[b]	Incorporation[c]	% Incorporation
25 C			
sus water	50,570	2,411	4.8
res water	40,780	2,625	6.4
sus toxin	39,320	1,638	4.2
res toxin	56,465	5,074	9.0
45 C			
sus water	27,138	454	1.6
res water	32,652	539	1.6
sus toxin	22,536	360	1.6
res toxin	45,885	954	2.0

[a]Root tips (4 per treatment) incubated in 100 ul of appropriate solution for 1 hr at 25 C before addition of ^{35}S-methionine (50 uCi) and two hr incubation at indicated temperatures. Root tips were then homogenized in an extraction buffer (100 ul).

[b]Radioactivity in buffer extract prior to precipitation with 5% and 10% trichloroacetic acid (TCA) in water.

[c]Radioactivity in buffer extract after precipitation with 5% and 10% TCA in water. This value is taken to be cpm incorporated into protein.

27,138 cpm per root tip, but only 1.6% of the cpm were incorporated into protein (Table 5).

Polyacrylamide gel electrophoresis indicated that heat shock (45 C) for 3 hr resulted in the formation of proteins not found in control roots (25 C). These proteins had apparent MW of 83, 81, and 68. The data are consistent with those from other studies on heat shock proteins in maize (8). Exposure to HC toxin I at 20 ug/ml for one hr prior to heat treatment reduced subsequent formation of heat shock proteins in susceptible maize. This response was evident by a specific decrease in the intensity of the heat shock protein bands. The intensity of the other protein bands (proteins not induced by heat shock) were not obviously affected by toxin.

The experiment indicates that toxin inhibits the formation of at least some induced proteins as one of its earliest known effect. The experiment on heat shock proteins supports the hypothesis that the toxin-induced inhibition of chlorophyll synthesis resulted from tissues not making the enzyme that forms ALA. The data may suggest a rapid inhibitory effect of toxin on the synthesis of at least some inducible proteins. There is no indication in the data as to which steps leading to synthesis may be inhibited. The inhibition of heat shock protein formation could have resulted from an inhibition of RNA synthesis (transcription) or of protein synthesis (translation); these are perhaps the most obvious possibilities. However,

we can not rule out other possibilities. Perhaps toxin interferes with the ability of tissues to sense environmental stimuli, or to transduct the sensing of the stimuli.

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