COMPARATIVE EFFECTS OF HELMINTHOSPORIUM CARBONUM AND HELMINTHOSPORIUM VICTORIAE TOXINS ON SUSCEPTIBLE PLANT TISSUES

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
MONG-SHANG KUO
1968

This is to certify that the

thesis entitled

Comparative effects of Helminthosporium carbonum and Helminthosporium victoriae toxins on susceptible plant tissues

presented by

Mong-shang Kuo

has been accepted towards fulfillment of the requirements for

Ph. D. degree in Plant Pathology

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Major professor

Date July 2, 1968

O-169

ABSTRACT

COMPARATIVE EFFECTS OF HELMINTHOSPORIUM CARBONUM
AND HELMINTHOSPORIUM VICTORIAE TOXINS
ON SUSCEPTIBLE PLANT TISSUES

by Mong-shang Kuo

The pathogenicity of Helminthosporium carbonum to certain kinds of corn (Zea mays) depends on production of a substance (HC-toxin) which selectively affects susceptible corn. Thus H. carbonum and its toxin are similar to H. victoriae and its toxin (HV-toxin), which are specific to certain kinds of oats (Avena sativum). The objective of this work was to determine the physiological and biochemical effects of HC-toxin on plant tissues, and to compare such effects with those caused by HV-toxin. Many effects of HV-toxin are previously described in the literature. Experiments fall into the following comparative categories: (1) effects of toxin on growth of host and non-host plants; (2) factors affecting apparent uptake and activity of toxin; (3) effects on cellular permeability; and (4) effects on cellular metabolism, including respiration, protein and RNA synthesis, and carboxylation.

Corn inbred lines and hybrids which are susceptible, intermediate, or resistant to H. carbonum infection in the field were tested for sensitivity to HC-toxin. Susceptible corn was affected by low concentrations of toxin (0.5 μg/ml). Resistant corn required much higher concentrations (50 μg/ml) for comparable effects, while a corn line with

intermediate resistance to the fungus was intermediate in sensitivity to the toxin. This correlation is further evidence for the significant role of HC-toxin in disease development. Similar correlations are known for HV-toxin. The discovery of these intermediate types indicate that there are quantitative differences in toxin receptors or transporting system in cells.

It is known from previous work that HV-toxin uptake and activity is not closely coupled with expenditure of cellular energy. In contrast, HC-toxin was found to require energy from metabolism of susceptible tissue during the time of toxin exposure. Apparent uptake of HC-toxin by susceptible tissues was affected by temperature, lack of oxygen, uncouplers of oxidative phosphorylation, and respiratory inhibitors used during the limited time of toxin exposure. Activity of both toxins on susceptible tissues was affected by toxin concentrations and exposure times, suggesting that uptake may be governed by a rate limiting mechanism.

Growth of H. carbonum-susceptible seedlings was stimulated by HC-toxin at 0.028 μg/ml, and growth of resistant seedlings was stimulated by 0.69 μg/ml. Incorporation of amino acids and uridine into TCA-insoluble cellular components were also stimulated after brief exposures to toxin. These phenomena have never been observed with HV-toxin. Electrolyte leakage from toxin treated leaf tissues of susceptible corn developed very slowly with HC-toxin, in comparison with HV-toxin. There was no significant effect of toxin (25 μg/ml) on leakage of electrolytes after 4 hours exposure. A longer exposure time resulted in electrolyte

loss, decreased synthesis, and inhibition of growth. Previous work has shown that susceptible oat tissues had increased loss of electrolytes almost immediately after exposure to HV-toxin.

A well-known effect of H. victoriae-infection of HV-toxin treatment is a rapid and dramatic stimulation of gas exchange. In contrast, H. carbonum-infection and HC-toxin were found to cause a relatively slow and weak respiratory response in susceptible tissues.

The earliest effect of HC-toxin observed to date was an increase in CO_2 fixation by plant tissues in the dark. Data indicated that the stimulation of CO_2 fixation by HC-toxin was not a direct effect on carboxydismutase, but resulted from a stimulation in conversion of ribose-5-phosphate to ribulose-1, 5-diphosphate. However, the effect of HC-toxin on CO_2 dark fixation was shown to be a secondary event in action of the toxin.

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by

Mong-shang Kuo

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1968

TO MY PARENTS

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ACKNOWLEDGMENTS

The author wishes to express his heartiest gratitude to Dr. R. P. Scheffer, my major professor, for his guidance and constant encouragement during the study and help in preparation of this manuscript.

Appreciation is also due to Drs. N. E. Good, H. A. Lillevik, H. H. Murakishi and A. H. Ellingboe for their critical evaluation and help in preparation of this manuscript.

Finally, the author is indebted to his wife for the patience which she has shown.

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LIST OF ABBREVIATIONS

Most abbreviations follow those of the "Style Manual for Biological Journals." Abbreviations listed below follow Karlson, "Introduction to Modern Biochemistry", where possible.

ADP	:	Adenosine diphosphate
ATP	:	Adenosine triphosphate
CPM	:	Counts per minutes
CV	:	Cultivar
DNA	:	Deoxyribonucleic acid
DNP	:	2,4-Dinitrophenol
HC-toxin	:	The oat-specific toxin produced by Helminthosporium victoriae
HV-toxin	:	The corn-specific toxin produced by H. carbonum
OAA	:	Oxalacetate
OD	:	Optical density (absorbance)
PEP	:	Phosphoenol pyruvate
PGA	:	2,3-phosphoglycerate
P	:	Inorganic phosphate
RNA	:	Ribonucleic acid
Rul-1-P,5-P	:	Ribulose-1,5-diphosphate
Rib-5-P	:	Ribose-5-phosphate
TCA	:	Trichloroacetic acid
Tris	:	Tris(hydroxymethyl)amino methane

INTRODUCTION

Helminthosporium carbonum toxin (HC-toxin) was first isolated in 1965 by Scheffer and Ullstrup (45) from culture filtrates of *H. carbonum* Ullstrup race 1. Later, the host selective toxic compound was purified and crystallized (39). Several lines of evidence indicated that this substance is a primary determinant of pathogenicity of *H. carbonum* to corn. In addition to the host specific primary determinant, a second toxic metabolite was isolated and given the trivial name "carbtoxinine." The second compound was equally toxic to both susceptible and resistant plants. My studies have been confined to the effects of the host-specific compound on both susceptible and resistant plants. The role of carbtoxinine in disease development has not been evaluated.

There are indications that HC-toxin is chemically similar to a determinant of pathogenicity (HV-toxin) produced by *Helminthosporium victoriae* Meehan and Murphy. In each case the host-selective determinant is accompanied by a less toxic non-specific metabolite. The intact host-selective toxins do not react with ninhydrin, but after acid hydrolysis, amino acids are present. Therefore, at least part of each toxin molecule appears to be a peptide. The two pathogens, *H. carbonum* and *H. victoriae*, are sexually compatible, and approximately 1/4 of the progeny of a

cross produced both toxins (46). All information available to date suggests a similarity between these two host selective compounds in both structure and function. However, HC-toxin does not affect oats and HV-toxin has no effect on corn.

There are many studies of HV-toxin as a factor in disease development and as a model for the chemical basis of pathogenicity. The compound is extremely toxic to susceptible oat tissues and is highly selective in the plants that are affected. Plants resistant or immune to H. victoriae are not affected by HV-toxin. Several lines of evidence suggest that a primary or significant lesion of the toxin is in the plasma membrane of susceptible cells (41). The toxin causes increased O_2 uptake and loss of electrolytes from susceptible but not from resistant tissues. Among other biochemical effects, HV-toxin also inhibits incorporation of C^{14} amino acids and uridine into TCA precipitable cellular components of susceptible but not of resistant tissues. Uptake of HV-toxin does not seem to depend on cellular metabolism, but may be a simple physical process (43, 44).

Little is known about HC-toxin and its biochemical effects on host tissues, which are the subjects of my study. The plan of the work is a comparison of HC- and HV-toxins in their actions. Experiments were designed to fall into the following broad comparative categories: (1) effects of the

toxins on growth of host and non-host plants; (2) studies of toxin uptake; (3) effects on cellular permeability; and (4) effects of toxin on cellular metabolism, including respiration, protein and RNA synthesis, and carbon dioxide incorporation.

LITERATURE REVIEW

A leaf spot disease with symptoms similar to those caused by Cochliobolus heterostrophus Drechs. (Helminthosporium maydis Nishikado and Miyake) was first reported in 1941 on certain inbred lines of dent corn (50). The causal fungus was first identified as H. maydis because it was similar to that species in morphology and effects on corn plants. The fungus in question was designated as a new species in 1944. The binomial, Helminthosporium carbonum Ullstrup, was given because the fungus caused a characteristic charred appearance of infected corn ears (51). Two morphologically indistinguishable races of H. carbonum were recognized on the basis of host specificity, symptoms produced, and virulence (50).

H. carbonum race 1 proved to be much more virulent than race 2 to certain corn hybrid and inbred lines. Therefore, race 1 and its interactions with susceptible corn have received considerable attention. Resistance to race 1 is inherited in a simple dominant manner. The gene pair controlling resistance and susceptibility, Hm/hm, is located on chromosome 1 between the p and br loci (52). Another locus, Hm_2/hm_2 on chromosome

9, was found to function as a modifying gene, giving an intermediate reaction to the disease (35). Intermediate expressions of disease resistance are also determined by two additional alleles at the *hm* locus in chromosome 1.

Spore germination and host penetration by *H. carbonum* were similar on resistant and susceptible corn leaves (20). Penetration occurred about 12 hr after inoculation; no differences were noted in the rapidity with which resistant and susceptible leaves initially reacted to penetration by *H. carbonum*. The fungus then ramified rapidly through susceptible parenchyma and chlorenchyma tissues. In contrast, penetration pegs of the fungus were confined to the initially penetrated epidermal cells in resistant tissue. This can be taken as an indication that resistance is expressed within 12 hours of inoculation. The fungus was viable in resistant tissue for at least 60 days after inoculation, and was re-isolated at that time.

Several papers have been concerned with the nature of resistance to *H. carbonum*, but this problem has not been solved. A substance which inhibited growth of *H. carbonum* was found in infected tissues of resistant plants (23). The inhibitor was said to be localized near the sites of infection, and was extracted from the tissues 5 days after inoculation. Somewhat different results were reported by Hale and Roane (14), who indicated that growth of *H. carbonum in vitro* was inhibited by alcoholic extracts of diseased susceptible plants, but not by extracts of diseased resistant plants or by extracts of uninfected resistant and

susceptible plants. Therefore the role of the inhibitory substance in disease resistance should be re-examined.

Infection by obligate parasites has been reported to stimulate CO_2 fixation by plant tissues in the dark (48). Later, this effect was reported for infection by a non-obligate parasite (33). A considerable reduction of CO_2 fixation in the dark was observed in H. carbonum-infected susceptible corn tissues (28). Further studies using cell-free extracts from plants inoculated with a low concentration of inoculum (25×10^4 spores/ml) revealed that carboxylation was stimulated 4 hours after fungus penetration occurred (16 hours after inoculation), but before visible symptoms were evident. In contrast, inoculation with heavy spore suspensions (1×10^6 spores/ml) caused a reduction in carboxylations. The stimulation could have been caused by increased enzyme concentrations or by stimulatory factors, but this point was not clarified. Also, the extent to which either the host or parasite was responsible for increased CO_2 fixation was not evaluated. Following the initial stimulation, infection caused a rapid decrease in carboxylase activities by extracts from infected leaves. An inhibitor was implied as an explanation of reduced carboxylation (29).

Corn leaf spot caused by H. carbonum has been used as a model for still other studies of plant reactions to infection. Malic acid was decreased in infected susceptible seedlings, while isocitric acid was greatly increased. Malca (28) suggested a role for isocitric acid in development of disease caused by H.

carbonum, but just how this compound is involved is not clear. Changes in the levels of Krebs cycle intermediates following infections and chemical injuries have been reported by several workers (5, 7, 27, 58). It has been suggested that such changes could be traced to carboxylation of pyruvate or phosphoenol pyruvate. Three such carboxylases are known to be present in tissues of higher plants. They are the malic enzyme, phophoenol-pyruvate carboxykinase, and phosphoenolpyruvate carboxylase (58). The reduction of malic acid content in H. carbonum infected susceptible corn tissue was said to be correlated with a reduction in CO_2 fixation in the dark (28).

Carboxydismutase, a key enzyme of photosynthesis, is thought to be confined to chloroplasts. Activity of this enzyme is known to be altered in infected rice plants, resulting in a reduction in CO_2 dark fixation (1). Increased carboxylation of ribose-5-phosphate, forming 3-phosphoglycerate, was reported in cell-free extracts from H. carbonum infected corn leaf tissue (29). This preparation was identical to leaf protein fraction-I. For a long time leaf protein fraction-I was thought to be a multifunctional substance containing carboxydismutase, phosphoribose isomerase, and phosphoribulose kinase activities (1). Recently, however, carboxydismutase was separated by Trown (49) from the associated enzymes. Thus it is possible that the stimulation of carboxylation by H. carbonum infection could result from effects on intracellular sites other than on chloroplasts.

Malca et al (29) suggested that, in early stages of H. carbonum infection, stimulated activity in the pentose pathway might result in increased production of ribose-5-phosphate and stimulated photosynthetic activity. Infection has been reported to stimulate the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase in several plant tissues (22, 30, 47) (including H. carbonum-infected corn), and in tissues treated with growth regulators (17). This was correlated with increased participation of the pentose phosphate pathway. Increased dehydrogenase activity was observed in H. carbonum-infected susceptible and resistant corn leaves. The increase was much enhanced after the appearance of necrotic flecks and in tissues with more advanced symptoms. However, the possibility that the pathogen was contributing to the increased activity was not ruled out.

Study of the interaction of H. carbonum and corn tissue was simplified in 1964, when the host-selective toxin was isolated from culture filtrates by Scheffer and Ullstrup (45). Two toxic substances, HC-toxin and carbtoxinine, were later found (39). HC-toxin is host specific in that it caused 50% inhibition of root growth of susceptible hybrid corn (K61 x Pr) at 0.5 µg/ml, while a hundred-fold higher concentration was needed to affect the resistant hybrid (Prlx K61) to the same extent (39). Carbtoxinine caused 50% inhibition of root growth of both susceptible and resistant hybrids at 25 µg/ml. Crystalline

carbtoxinine reacts with ninhydrin and yields amino acids on acid hydrolysis. The crystallized host-specific toxin did not react with ninhydrin but yielded amino acids on acid hydrolysis. However, the exact structure of the substance is still under study.

A genetic analysis of interspecific hybrids between Cochliobolus carbonum (H. carbonum) and C. victoriae (H. victoriae) was reported by Scheffer et al. (46). One gene pair was shown to control toxin production in C. victoriae, whereas another gene pair controlled toxin in C. carbonum. Pathogenicity of each progeny isolate was correlated with ability to produce one or both host specific toxins. Progeny of C. carbonum race 1 x race 2 segregated 1:1 for ability or lack of ability to produce the corn specific toxin. Apparently the toxin-producing ability was qualitatively determined by one gene; however, the amount of toxin produced appeared to be controlled by multiple genes.

Hale et al. (15) in 1962, described effects of HC-culture filtrate that might have been caused by the host-specific toxin. They demonstrated that culture filtrates of H. carbonum inhibited growth of coleoptile sections from a susceptible inbred corn (K 44) but stimulated growth of a resistant inbred (K 41). Growth of non-host plants was also inhibited by the culture filtrates. Response of plants to biologically active substances is often dosage dependent. As indicated by French et al.,(11) in study of effect of growth regulators on plants, relatively

low concentrations of auxins increased growth and O_2 uptake, but high concentrations inhibited both. Hale's results might be expected if higher concentrations of a toxin are required to stimulate or inhibit growth of the resistant than are required for the susceptible plant.

Helminthosporium victoriae toxin from H. victoriae has received more study than any other model for host-parasite interactions. Several reviews of this subject have been published (38, 44). Cellular responses to toxin include increased O_2 uptake (44), decreased incorporation of amino acids and uridine into trichloroacetic acid precipitable cellular fractions (44), and rapid loss of electrolytes (3, 41). Apparent uptake of HV-toxin appears to be a simple process not affected by a wide range of temperatures or by metabolic inhibitors (43). The plasma membrane has been implicated as a primary site of action of the toxin (41), and a receptor in the susceptible cell is thought to account for toxin susceptibility (43). Resistance to the toxin is thought to be due to lack of receptors, or to decreased affinity of receptors in the plasma membrane of resistant plants.

Qualitatively, the toxic products of H. carbonum bear a striking similarity to the toxic products of H. victoriae (44). Both are low molecular weight peptides. Both host specific toxins are accompanied by a less toxic non-specific metabolite (44). The correlation of pathogenicity with ability to produce HC-toxin indicates that it is a primary determinant of pathogenicity (44),

as is HV-toxin. However, there are no previous comparative studies of the effects of these two toxins on plant tissue.

MATERIALS AND METHODS

Plant materials, inoculation methods, and bioassays: Corn hybrids Pr X K61 and Prl X K61 were used in most experiemnts. Pr X K61 is susceptible and Prl X K61 is resistant to H. carbonum race 1 and to H. carbonum toxin. Certain experiments required oat cultivars susceptible (cv. Park) and resistant (cv. Clinton) to H. victoriae infection and to H. victoriae toxin. Seeds were surface sterilized with 0.53% sodium hypochlorite (w/v) for 2 hours, then washed thoroughly with running water for 10 to 12 hours. Treated seeds were germinated on moist filter paper and were grown in vermiculite and watered daily with White's solution. In some experiments, plants were grown in the greenhouse in sand-soil-peat mixture (1:1:1) and watered with a soluble commercial fertilizer. Cuttings were taken from 10- to 14-day old plants with 3 or 4 fully expanded leaves. Unless otherwise stated, the first fully expanded leaf above the primary leaf was used as a source of leaf tissues.

For inoculation, spores from 10-day old cultures on mixed vegetable juice (V-8 juice) agar were used. The surfaces of cultures were flooded with distilled water and filtered through cheesecloth. The concentration of spores in suspensions was adjusted with distilled

water using a colorimeter (Spectronic 20, Bausch and Lomb Optical Co.) set at 600 mμ wave length. Plants were sprayed with the spore suspension, using a DeVilbis atomizer. They were kept in moist chambers for 12 to 24 hours after inoculation.

A standard seedling bioassay for toxin was used (46). Corn grains (3/plate) were placed embryo side down in 90-mm petri plates containing 10 ml White's solution. Twenty four hours later, White's solution was replaced by various dilutions of the toxin solution or with other test solutions. Water controls were used in all cases. After 3 to 4 days in test solutions, the longest root on each seedling was measured and the dilution end-point was determined. This was calculated from percent growth inhibition as the highest dilution which prevented root growth from exceeding 10 mm. The standard HV-toxin bioassay was used as required. Hulled oat seeds were germinated on moist filter paper for 24 hours, and placed in 5 ml toxin-containing solution in 60 mm petri plates. Serial dilutions of toxin-containing solutions were used. After incubation for 72 hours at 23 C, root growth was measured. The highest dilution which prevented roots from growing more than 10 mm was considered the dilution end point (38).

Isolation and purification of toxin: HC-toxin was isolated from culture filtrates of H. carbonum, using a slightly modified version of the method of Pringle and Scheffer (39).

High yielding isolates of H. carbonum race 1 were grown at 22 C for 3 weeks in Roux bottles, each containing 200 ml modified Fries no. 3 basal medium (39) supplemented with 0.1% Difco yeast extract. The culture liquid was filtered through several layers of cheesecloth, centrifuged (continuous flow) and filtered through paper on a Büchner funnel, with suction. The filtrates were then concentrated to 0.1 the original volume in vacuo below 45 C. After standing at 5 C for 24 hours, the concentrated solution was filtered by suction, an equal volume of methanol was added to the filtrate and the precipitate which formed was discarded. After filtration, methanol was removed by evaporation under reduced pressure at 40 C. The partially deproteinized concentrated culture filtrate was extracted with chloroform (7 parts culture filtrate: 1 part chloroform) several times, until no more color was removed. The chloroform extracts were combined, filtered, and evaporated in vacuo. The residue was dissolved in a small quantity of absolute ethanol, filtered, and evaporated in a flash evaporator to remove ethanol. HC-toxin in the residue was dissolved in distilled water and the volume brought to 100 ml. This preparation was further purified by gel filtration.

10 g dry spherical polyacrylamide (Bio Gel P-2, 200-400 mesh) was soaked overnight in water with constant stirring. The water and floating gels were decanted and the water replaced several times. The hydrated gels were then packed carefully in a K 15/30 Sephadex laboratory column. After packing, the water level in the column

was lowered to the top of the bed. A 5 ml sample of toxin preparation was added carefully to the top of the bed. The sample was drained to the bed, then was washed with 2 ml distilled water. Thereafter the column was developed with water at a constant flow rate (usually 10 ml/14-15 minutes). The effluent was collected in 10 ml fractions. Highest toxicity was in the third fraction (Fig. 1), as shown by bioassay. Fraction 3 also had the peak conductivity measurement, and developed a blue color with Folin-Dennis reagent (10). Toxicity peaks for both toxins correlated with peaks in conductivity and with blue color with phenol reagent (Fig. 1). Assays showed that 1.1 µg toxin/ml completely inhibited growth of susceptible corn. This activity is comparable to the highest reported for crystallin toxin.

Preparation of C^{14}-labelled toxin: H. carbonum race 1 was grown on modified Fries no. 3 basal medium supplemented with Difco yeast extract (39). To 500 ml of the medium, 0.2 mc sucrose-U-C^{14} (specific activity, 5.0 mc/µM) was added. HC-toxin was isolated and purified as described above. High toxicity and radioactivity were found in fractions 3 and 4 from the Bio Gel P-2 column.

Respiratory measurements: Standard manometric techniques were used to determine gas exchange (53). Cuttings of corn plants were exposed to toxin solutions for specified times. Following

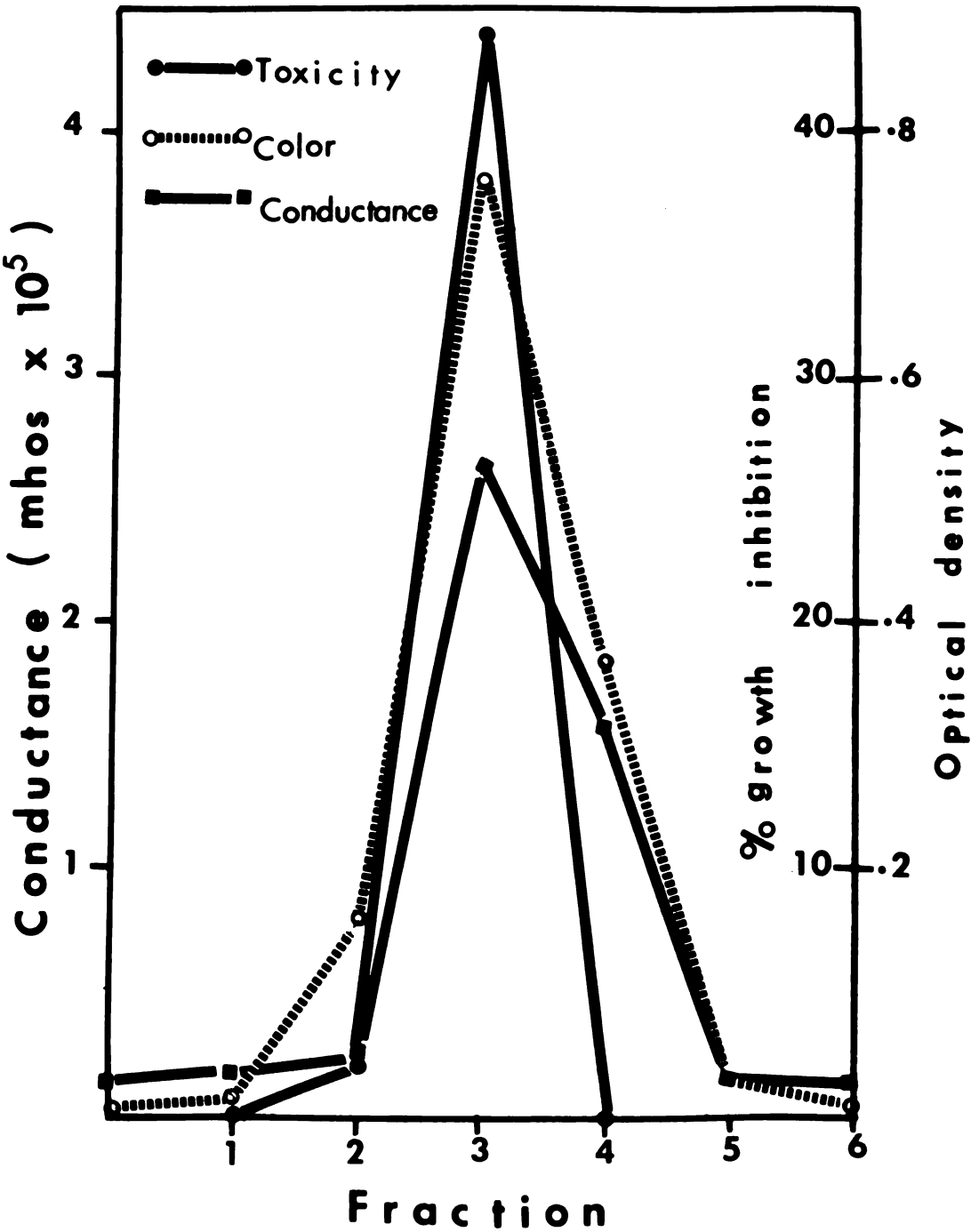


Fig. 1. Activity profile of HC-toxin from a polyacrylamide gel (Bio-Gel P-2).
Growth inhibition was determined in standard bioassays with susceptible corn seedlings. Optical density is absorbance at 660 mμ.

toxin treatment, 5 cm sections were cut from the centers of leaves. The leaf was cut longitudinally into two equal parts, the midrib was discarded, and one half of the leaf was used for respiratory measurements, while the other was used for fresh weight measurements. From 0.15 to 0.20 g leaf tissues (fresh weight) were placed on moistened filter paper in each Warburg flask at 25 C. H. carbonum infected leaves were required in some experiments. Leaves with uniform infection were selected from an equivalent position on each plant. Sections were cut from the middle of the leaf and placed on moistened filter paper in Warburg flasks. In experiments with roots, tissues were pre-treated with toxin and placed on moistened filter paper in each flask, or were placed in flasks containing 2 ml White's solution with or without toxin. Six roots were carefully selected and used in each flask. Experiments were at 25 C in all cases.

$C^{14}O_2$ uptake in the dark: Toxin pre-treated and non-treated control leaves of corn or oats were allowed to fix CO_2 in the dark for 3 to 4 hours. In come experiments, infected leaves were used along with non-inoculated controls. $C^{14}O_2$ was generated in a 1000 ml tightly sealed glass chamber from 25 μc C^{14} sodium bicarbonate by adding excess lactic acid to the container of radioactive carbonate. After dark fixation, leaves were removed from the chamber, washed first with 0.5 N HCl and then with distilled water to remove excess $C^{14}O_2$ adsorbed on the leaf surface. Washed leaves were blotted dry, cut into small pieces, and

dried for 24 hours at 80 C. Dried samples were degraded to CO_2 by wet combustion (2), using 5 ml liquid and 1.0 g solid van Slyke reagent per 14 mg dry samples (2). The labelled CO_2 released was trapped in an absorption flask containing 15 ml N/10 NAOH (2). Aliquots (0.1 or 0.2 ml) of the NAOH solution were placed on glass planchets for radiation counts with a gas flow G. M. counter (Nuclear-Chicago model 480) with a windowless detector.

A plant extract was used to determine dark fixation of CO_2. Three g (fresh weight) leaf tissue was cut into small pieces with scissors and placed in 15 ml 0.5 M tris buffer (pH 8.0) in a mortar at 2 C. An equal weight of washed sea sand was added, the mixture was ground to a fine pulp, and the resulting slurry was pressed through several layers of cheesecloth. The dark green homogenate was centrifuged to remove the sand and cellular debris. The turbid supernatant was centrifuged at 35,000 X g for 20 minutes and the dark green pellets, consisting mainly of broken chloroplasts and mitochondria, were discarded. The clarified extract was then centrifuged at 105,000 X g for 2 hours.The final supernatant of clear amber-colored fluid was used as an enzyme source (32).

Enzymatic assay methods were essentially similar to those of Malca et al. (29). Unless otherwise indicated, 0.3 ml of the enzyme preparation was added to 0.7 ml reaction mixture and incubated at the appropriate temperatures and times before the reaction was stopped by the addition of 0.1 ml of 1.0 N HCl.

Aliquots of the 1.1 ml reaction mixtures were placed on sand blasted glass planchets and dried for radiation counts. All reaction mixtures contained, in addition to 0.3 ml enzyme preparation, the following, in μ moles: $MgCl_2$,15; $NaHCO_3$, 5 (containing 2.5 μc); tris buffer (pH 8.0), 150; ATP, 2; and substrate, 1 or 2 (ribose-5-phosphate, 2; ribulose-1,5-diphosphate,1; phosphoenolpyruvate, 2; and phosphoglycerate, 2).

The following chemicals used in this experiment were from Sigma Chemical Co., St. Louis, Missouri: D-ribose-5-phosphate (disodium salt, 99% pure); D-ribulose-1,5,-diphosphate (tetrasodium salt, 1.64 μ moles/mg); adenosine-5,-triphosphate (disodium salt); phosphoenolpyruvate (trisodium salt); and D (-) 3-phosphoglycerate (tricyclohexlammonium salt).

The carboxylation systems studied were:

(1) PEP + CO_2 ---Mg++---> OAA + P
PEP carboxylase

(II) 3-PGA + CO_2 ---Mg++---> OAA + P
2,3-phosphoglycerate mutase, enolase, and PEP carboxylase

(III) Rul-1-P,5-P + CO_2 ---Mg++---> 2, 3-PGA
Carboxydismutase

(IV) Rib-5-P + ATP + CO_2 ---Mg++---> 2, 3-PGA + ADP
Phosphoriboisomerase, phosphoribulokinase, carboxydismutase

The following incubation temperatures and times were used for the 4 reactions: (I) 10 minutes at 22 C; (II) and (IV), 10 minutes

at 37 C; and (III), 8 minutes at 28 C. All assays were duplicated or triplicated and the experiments were repeated several times.

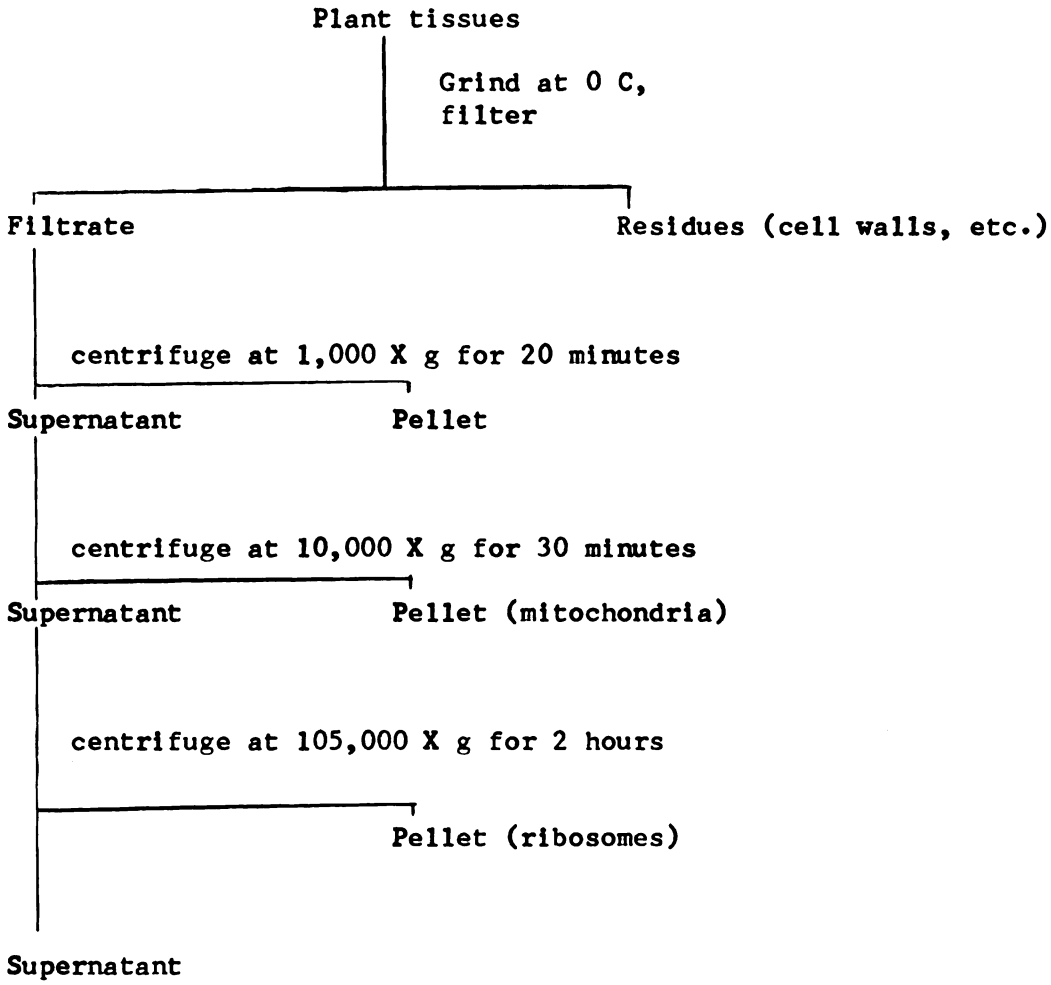
Incorporation of C^{14}-labelled amino acids and uridine: Fresh leaf tissues (0.5 g) were infiltrated for 15 minutes with DL-leucine-1-C^{14} or DL-valine-1-C^{14} (2.5 μc/5 ml water; specific activity 24.4 mc/mM) and then incubated for 4 hours at 22 C. After incubation, tissues were extracted 3 times with hot 80% ethanol. The homogenate was spun at 1,000 X g for 30 minutes. The pellet was washed 3 times with 80% ethanol ether (3:1, v/v). The washed pellet was then suspended in 3 ml 1.0 N NaOH and incubated at 100 C for 4.5 minutes. Aliquots (0.1 or 0.2 ml) of the supernatants were placed on sand blasted glass planchets and dried for determination of incorporated radioactivity (36).

For C^{14} uridine incorporation studies, 0.5 g leaf tissues were infiltrated with uridine-2-C^{14} (2.5 μc/5 ml; specific activity 20 mc/mM) for 15 minutes and incubated for 3 to 4 hours. The tissues were then extracted 3 times with hot 80% ethanol and ground in 10% TCA in a mortar. The homogenate was brought to 30 ml with 10% TCA and left overnight at 2 C. The TCA insoluble fraction was washed 3 times with 95% ethanol at room temperature. Each pellet was then suspended in 2.0 ml 0.3 N KOH and incubated for 18 hours at 37 C. After incubation, 60% perchloric acid was added to bring the final concentration of perchloric acid to

0.5 N. The sample was then centrifuged at 10,000 X g for 30 minutes. Aliquots (0.2 ml) of the supernatant were placed on glass planchets and dried for radiation counts.

Loss of electrolytes by tissues: A conductivity bridge (Industrial Instruments model RC 16 B1 with type BB1 conductivity cell, K = 1.00) was used for determination of conductivity of ambient solutions. One g fresh tissue was rinsed in glass distilled water and enclosed in washed cheesecloth. The bagged samples were further washed by shaking (100 strokes/ minute) in 100 ml glass distilled water for 2 hours. Tissues were placed in 50 ml fresh deionized glass distilled water and conductivity changes of the water were measured at specified intervals at 23 C. Specific conductance at 23 C was expressed in mohs. For this the following equation was used: $Ls = \frac{Kc}{Rm}$ (Ls = specific conductance, Kc = cell constant (K = 1.0), and Rm = measured resistance at 23 C) (6).

Cellular fractionation: Germinated corn seeds were grown in the dark for 3 days. Twenty five g samples of etiolated coleoptiles were vacuum infiltrated with HC-toxin (5.0 µg/ml). Tissues were ground in a mortar with 50 ml extraction medium (57) and 3 g fine sand for 3 to 5 minutes at 0 C. The homogenate was pressed through several layers of cheesecloth and the filtrate was subjected to differential centrifugation as indicated below:


Plant tissues
Grind at 0 C, filter
Filtrate
Residues (cell walls, etc.)
centrifuge at 1,000 X g for 20 minutes
Supernatant
Pellet
centrifuge at 10,000 X g for 30 minutes
Supernatant
Pellet (mitochondria)
centrifuge at 105,000 X g for 2 hours
Pellet (ribosomes)
Supernatant

RESULTS

Effects of Toxin on Growth of Host and Non-Host Plants

Response of resistant and susceptible corn seedlings to

HC-toxin: Highly purified HC-toxin was used to test effects on growth of corn plants. Both susceptible and resistant hybrids were used to assay the response to a range of toxin concentrations, using the seedling assay. The experiment was repeated 3 times with 10 to 19 plates per treatment and data were analyzed statistically. Toxin concentrations at 0.5 µg/ml gave >50% inhibition of root growth of susceptible corn (Table 1) whereas concentrations greater than 5.5 µg/ml were required to give significant inhibition of growth of resistant corn hybrids (Table 2). Low concentrations of toxin stimulated root growth of both resistant and susceptible plants. However, a higher concentration was required for a stimulatory effect on resistant plants (Table 2). H. carbonum resistant corn hybrids are not completely resistant to HC-toxin (39). It is known from the work of Pringle and Scheffer that 0.5 µg crystalline toxin/ml will give 50% inhibition of root growth in susceptible seedlings, whereas 50 µg toxin/ml will give comparable inhibition of resistant seedling roots.

Comparative toxin sensitivity of corn inbred lines and hybrids: Several corn inbreds are known to be intermediate in susceptibility to infection by H. carbonum race 1. Two gene loci and 2 additional alleles at the hm locus govern the phenotypes which range from resistance to susceptibility. The following experiments were designed to test whether or not the relative toxin sensitivity of corn is correlated with susceptibility and resistance to infection.

Two toxin preparations differing somewhat in activity were used to test sensitivity of corn inbred lines. Concentrated culture filtrate (75 ml) was precipitated with methanol. Precipitates were removed by filtration, the filtrate was evaporated to dryness (at 40 C), and residues were dissolved in 75 ml distilled water. The aqueous solution was filtered and extracted 3 times with 80% butanol. Butanol was removed from the extract and 20 ml methanol was added to bring the total volume to 40 ml. The methanol solution was used for chromatographic separation with an alumina column (2.2 x 27 cm). The column was prepared by washing first with distilled water and then with 50% methanol and allowed to stand for one night. Toxin was then eluted from the column with methanol. Forty-five ml fractions were collected immediately after absolute methanol was added. Most of the toxin appeared in fractions 1 and 2 and a little in fraction 3. For the second experiment, the first and second fractions were combined and the combined preparation was further purified by Bio-Gel separation.

Toxin preparations used in the first and second experiments caused complete inhibition of root growth of susceptible hybrid corn at concentrations of 5 µg/ml and 1.1 µg/ml, respectively. Crystallized toxin caused comparable effects at 1 µg/ml (39).

Seeds were treated with a range of HC-toxin concentrations and incubated at room temperature (22 C). The effects of toxin concentration on growth of roots were compared 4 days after treatment. Percent inhibition of root growth was taken as a measure of susceptibility. Each treatment was replicated 8 times and the experiment was repeated. Similar results were obtained with the two different toxin preparations. There was a correlation between sensitivity to toxin and susceptibility to the disease observed in the field (Table 3). The susceptible types (K 61, Pr, K 44, K 61 x Pr) fell into a group, the members of which probably did not differ significantly in toxin sensitivity. The resistant types (W 37-A, R 61, K 61 x Pr 1, Wf-9, PI 21740, TR, GE 440, K 61-1, and K 61-2) also appear to form a single group. The one inbred (P8) known to be intermediate in resistance and susceptibility in the field was clearly intermediate in toxin sensitivity (Table 3). Other intermediate types were not available, but on the basis of these data, it appears that inbred and hybrid corn types can be classified as resistant, intermediate, or susceptible to toxin, and that this correlates with reactions to H. carbonum infection observed in the field.

Table 1. Effects of HC-toxin on root growth of susceptible hybrid corn seedlings

Roxin concentration (μg/ml)	Experiment 1		Experiment 2		Experiment 3	
	Growth (mm)	% change	Growth (mm)	% change	Growth (mm)	% change
0	116	0	104	0	168	0
0.0068	119	+ 3	105	+ 1	-	-
0.014	110	- 5	118	+13	-	-
0.028	127	+ 9	110	+ 6	186[a/]	+11
0.055	122	+ 5	62	-41	-	-
0.55	44	-62	20	-80	-	-

[a/] Difference from control is significant at 5% level.

Table 2. Effect of HC-toxin on root growth of resistant hybrid corn seedlings

Toxin concentration (µg/ml)	Growth (mm)	% change
0	93	0
0.69	115[a/]	+19
1.40	100	+ 7
2.75	95	+ 3
5.5	88	- 6

[a/] Difference from control is significant at 1.0% level.

Table 3. The relationship between toxin susceptibility and disease resistance[a/]

Corn lines	Effect of toxin on growth[c/]	Reaction to infection[b/]	Genotype[d/]
K61	-75	Susceptible	$hm/hm;hm_2/hm_2$
K44	-72	"	?
Pr	-70	"	$hm/hm;hm_2/hm_2$
K61 x Pr	-64	"	"
P 8	-37	Intermediate	Hm^A/Hm^A; hm_2/hm_2
K61-2	-11	Resistant	?
W37-A	- 8	"	?
R61	- 5	"	?
K61-1	0	"	?
GE 440	0	"	?
K61 x Pr 1	+ 8	"	$Hm/hm;Hm_2/hm_2$
Wf-9	+12	"	$Hm/Hm;Hm_2/Hm_2$
PI 21740	+18	"	?
TR	+14	"	?

a/ Seeds were pre-treated in White's solution for 24 hours, then placed in petri plates containing 10 ml toxin (0.6 µg/ml) solution for 4 days.

b/ Ullstrup, A. J. (Personal communication).

c/ % inhibition (-) or stimulation (+) of root growth 4 days after toxin treatment, as compared to non-treated controls.

d/ Reference 35.

Effect of HC-toxin on several non-host plants: Several species, known to be immune to H. carbonum, were used to test susceptibility to HC-toxin. Seeds were placed on filter papers moistened with White's nutrient solution and incubated in the dark at room temperature or at 30 C for 1 to 3 days. Germinated seeds were exposed to HC-toxin solutions for 4 days and length of root growth was measured. Root growth of all host and non-host plants was increasingly inhibited by increasing concentrations of HC-toxin. All the monocots tested (barley, wheat, Sudan grass, rye grass, sorghum and oats) were relatively insensitive to the toxin. There was little or no effect of HC-toxin at 5 µg/ml on root growth of these plants. Growth of susceptible hybrid corn was completely inhibited at this toxin concentration. At higher concentrations, growth of resistant corns was inhibited by HC-toxin, indicating that resistance to HC-toxin is relative rather than absolute. Tomato and cucumber seedling roots were inhibited at all concentrations of HC-toxin used. Tomato and cucumber appeared to be more sensitive than resistant corn, but less sensitive than susceptible corn (Table 4).

HC-toxin used in this experiment was desalted twice with a polyacrylamide gel (Bio-Gel P-2, Particle size 200-400) column. The preparation completely inhibited root growth of susceptible corn at concentration of 2.5 µg/ml. Crystalline toxin at 1 µg/ml is known to inhibit all root growth in susceptible seedlings.

Table 4. Effect of HC-toxin on root growth of host and non-host plants[a/]

Plants	Growth of controls[b/]	Effect of toxin on growth[c/] Toxin concentration (µg/ml) 6.25	12.5	25.0	50.0
	mm	%	%	%	%
Corn (resistant)	103	+5.8	-12.6	-32.0	-46.6
Corn (susceptible)	111	-100	-100	-100	-100
Tomato	39	-58.9	-74.4	-87.2	-87.2
Cucumber	66	-12.1	-34.8	-54.5	-72.7

a/ Seeds were germinated on moist filter papers in the dark for 1 or 3 days before toxin was added.

b/ Controls in water without toxin, average values for 10 seedlings.

c/ Per cent inhibition (-) or stimulation (+) of growth, as compared with growth of controls. This toxin preparation completely inhibited root growth of susceptible corn seedlings at 2.5 ug/ml.

Effect of HV-toxin on growth of susceptible and resistant plants: HV-toxin is known to be highly selective and to have high activity. A toxin preparation that did not affect resistant oats in a solution of 1 mg/ml was diluted 10 million times with water and still inhibited root growth in susceptible seedlings (44). HC-toxin has a lower order of toxicity and selectivity. Growth of susceptible hybrid seedlings was inhibited 50% by 0.5 µg/ml of crystallized HC-toxin, while a hundred fold higher concentration (50 µg/ml) of the same preparation inhibited root growth of resistant corn seedlings (39).

Host selectivity and toxicity of HV-toxin was re-examined by using desalted HV-toxin. Ten ml of an HV-toxin preparation from an alumina column was adjusted to pH 6.5, and the precipitates which formed were removed by filtration (Whatman no. 1 filter paper). The filtrate was further purified by Bio-Gel filtration. A five ml sample was applied on the top of the column and drained to the bed. Two ml distilled water was added and drained to the bed. The column was developed with distilled water (flow rate: 2 ml/3.5 minutes), and the 21st to 26th fractions (2 ml each) were combined and used for bioassay against several species of seedling plants.

HV-toxin proved much more selective than HC-toxin, with only the susceptible Park oats being affected by 36 µg toxin/ml (Table 5). All species tested, including both monocots and dicots, were affected somewhat by 3.6 mg/ml; the resistant oat was among the

least sensitive of all plants tested. This particular toxin preparation gave complete inhibition of susceptible oats at 0.009 µg/ml. Therefore, resistant plants tolerate at least 400,000 times higher toxin concentrations than do susceptible plants, in contrast to 100 times higher with resistant corns to HC-toxin.

Table 5. Effect of HV-toxin on seedling root growth of host and non-host plants

Plants	Growth in controls	% inhibition at toxin concentration (µg/ml) of 36.0	360	3600
Oats	mm	%	%	%
cv. Park	50	100[a/]	100	100
cv. Clinton	88	0	0	55
Tomato	49	0	0	100
Barley	76	0	0	55
Wheat	81	0	0	80
Sorghum	80	0	0	73
Corn	100	0	0	80

a/ Root growth of susceptible oats (cv. Park) was completely inhibited by toxin at 0.009 µg/ml.

Toxin Uptake and Recovery Studies

Temperature and concentration effects: Germinated susceptible corn seeds (K61 x Pr) were first equilibrated at 5, 23, or 37 C for 30 minutes, and then exposed to HC-toxin (7.0 µg/ml) for 1 to 10 hours. Seedlings were then rinsed thoroughly with 10 changes of distilled water and incubated at room temperature (22 C) for 3 days. Per cent inhibition of root growth after 4 days was taken as a measure of toxin uptake. Since the treatment conditions were varied only for the time of toxin exposure, the effects are believed to be on toxin uptake. However, toxic action may also be involved, and the effects are stated to be on "apparent toxin uptake." The experiment was repeated 4 times with similar results.

The results indicated that toxin uptake is temperature and time dependent (Fig. 2). Four hours toxin treatment resulted in inhibition of 3, 5, and 70% for 5, 23, and 37 C, respectively. Ten hours toxin treatment gave 13, 50, and 93% inhibition of growth at 5, 23, and 37 C, respectively. Generally, toxic effects were more pronounced after exposure at the higher temperatures.

Some conflicting results were obtained with short exposure times, when growth stimulatory effects were obtained. The reason for this stimulatory effect is not known, but it could be due to sub-lethal amounts adsorbed, which are known to stimulate

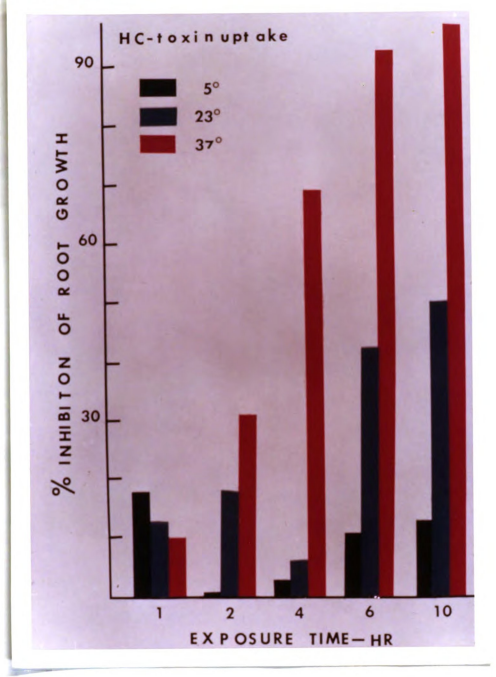


Fig. 2. Effect of temperature on apparent uptake and activity of HC-toxin.

root growth as indicated in the previous experiment. Results of this experiment could indicate that temperature affects toxin uptake, but they could also be interpreted as temperature effects on toxin sensitivity of tissues, or on speed of toxic reactions.

Three concentrations (4.5, 9.0, and 18 µg/ml, plus a water control) and 4 exposure times were used to test the effects on HC-toxin uptake. Seeds were germinated in White's solution for 2 days. Germinated seeds were exposed in test solutions for 6, 12, 16, or 18 hours, then were washed thoroughly with 10 changes of distilled water. Five seedlings were placed in each Petri plate containing 10 ml distilled water and incubated at room temperature for 5 days. Five plates were used for each concentration and exposure time.

Results showed that toxin uptake by H. carbonum susceptible corn seedlings was affected by toxin concentration and exposure time (Table 6). Twelve hours exposure was needed for seedlings to take up enough toxin from 9 or 18 µg toxin/ml to significantly inhibit later growth. Uptake of HC-toxin did not increase indefinitely with increasing toxin concentrations. Instead, it tended to become saturated at each temperature, indicating that uptake may be governed by a rate limiting mechanism (Table 6).

Table 6. Effect of toxin concentration and exposure time on apparent uptake and activity of HC-toxin, as determined by root growth bioassay

Concentration[a] (µg/ml)	Growth of roots after exposure for hr					
	12		16		18	
	growth	% inhibition	growth	% inhibition	growth	% inhibition
	mm		mm		mm	
0	80	0	84	0	82	0
4.5	69	14	57	32	35	57
9.0	61	24	41	51	28	66
18.0	59	26	46	45	31	62

[a] Concentration of 2.3 µg/ml completely inhibited root growth of susceptible seedlings in the standard bioassay, in which seedlings were continuously exposed to toxin.

Effect of metabolic inhibitors on HC-toxin uptake: If HC-toxin uptake is an energy requiring process, then inhibition of respiration or uncoupling of phosphorylation from oxidation should cause reduction of toxin uptake. The following experiments were designed to see if uncouplers and respiration inhibitors will prevent toxin uptake. Seeds were germinated in White's dolution for 2 days, and the seedlings were treated in a test solution containing inhibitor and HC-toxin for 12 to 20 hours. Preliminary experiments showed that exposure of seedlings to the inhibitors prior to the toxin treatment did not affect the action of the inhibitor; therefore, prior exposure to inhibitors was omitted and inhibitors and HC-toxin were added at the same time. After seedlings were treated in test solutions for desired times, they were washed thoroughly with 10 changes of distilled water, placed in 10 ml distilled water per petri dish, and incubated at 23 C for another 2 days. Both water and inhibitor controls (without toxin) were used. Root growth was measured and inhibition of root growth was taken as a measure of toxin uptake. The experiment contained 10 replicate plates for each treatment, and each inhibitor was used in at least 2 experiments.

Sodium azide (10^{-4}M), 2, 4-dinitrophenol (DNP) (10^{-4}M), and Potassium cyanide (5×10^{-3}M) reduced toxicity of HC-toxin to susceptible plants (Table 7). The possibility that these compounds might react directly with toxin molecules rather than with plant cells was ruled out by the use of appropriate controls;

the preparation containing HC-toxin and DNP was left overnight in a refrigerator before diluting and assaying. There was no decrease in toxicity as a result of exposure of toxin to the test compound. The results suggest that HC-toxin uptake requires metabolic energy.

Sodium bisulfite and sodiem arsenate were also tested for effects on toxin uptake or action. Concentrations of bisulfite from 6×10^{-3} to 4×10^{-4}M and sodium arsenate from 6×10^{-3} to 4×10^{-4}M did not reduce the toxicity of HC-toxin. Higher concentrations were not used because they inhibit growth too drastically.

Anaerobic effects: The previous experiment suggested that energy metabolism of host tissues might be an important factor in HC-toxin uptake. Therefore, more experiments were designed to test this possibility. Germinated susceptible seeds were exposed to HC-toxin under anaerobic conditions to inhibit respiration; seeds exposed to toxin under aerobic conditions served as controls. In the first experiment, seedlings were immersed in a 100 ml beaker containing 40 ml toxin solution (40 µg/ml) for 20 hours. Seeds were at the bottom of beakers, about 2.5 cm below the water surface, during the time of exposure to toxin. Seedlings were then washed and incubated at room temperature for 2 days. The controls were placed in 40 ml water in beakers. The other treatment and its control were seedlings placed in 10 ml

Table 7. Effect of some metabolic inhibitors on apparent uptake and activity of HC-toxin

Inhibitor	Root growth in			
	Inhibitor control	Inhibitor + toxin	Water control	Toxin control
DNP, 10^{-4}M	76[a/]	78	93	51
KCN, 5×10^{-3}M	39	40	85	24
NaN_3, 10^{-4}M	64	58	89	44

a/ Average root growth of 10 seedlings, in mm.

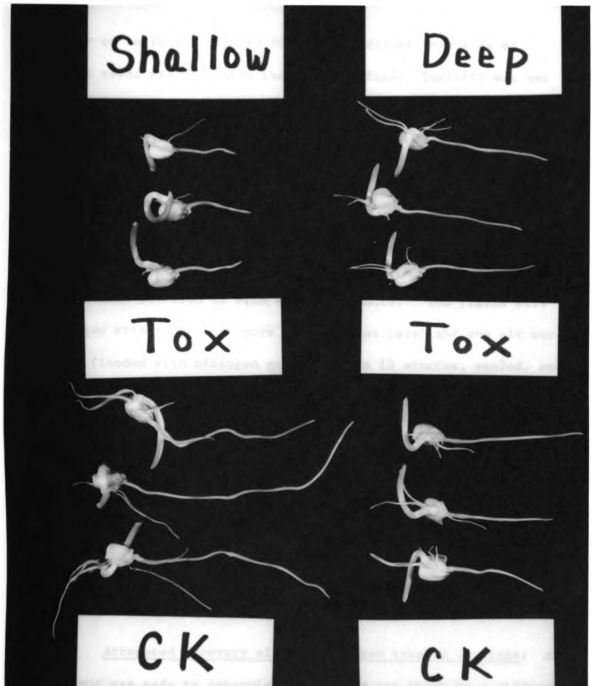


Fig. 3. Effect of anaerobic conditions on apparent uptake and activity of HC-toxin.

water or toxin solution in 90 mm petri dishes; one side of these seeds was not below the water surface. Toxicity was not expressed in seedlings which were thus exposed anaerobically to toxin in solution for 20 hours, as shown by equal growth of control and treated plants 2 days after toxin exposure (Fig. 3).

In a second experiment, seeds in toxin solutions were held under nitrogen or in air during the exposure time. Five germinated seeds were placed in a 125-ml Erlenmeyer flask with 5 ml toxin solution or with water as control. The flasks were plugged with rubber stoppers with one gas inlet and one air outlet, flooded with nitrogen gas for 10 to 15 minutes, sealed, and kept in the dark for 20 hours at room temperature. After 20 hours exposure to toxin or to water, the seedlings were washed and placed in 90 mm dishes containing 10 ml water and incubated for 2 days before root growth was measured. Again, toxicity of HC-toxin was reduced when seedlings were exposed under anaerobic conditions, as indicated by comparable growth of treated and non-treated seedling roots (Table 8).

Attempted recovery of HC-toxin from treated cuttings: An attempt was made to determine whether or not there is a difference in HC-toxin uptake or inactivation by susceptible and resistant corn tissues. A preliminary experiment (Table 9) indicated that corn cuttings took in enough HC-toxin with the transpiration stream in 2 hours to give visible symptoms. Therefore, cuttings

Table 8. Effect of anaerobic conditions during the time of exposure to HC-toxin on subsequent growth of susceptible seedling roots.

Treatments[a/]	Experiment no. 1	Experiment no. 2
N_2 control	62[b/]	55
N_2 toxin	56	46
Air control	96	77
Air toxin	22	15

a/ HC-toxin (40 µg/ml) was used. The preparation gave complete inhibition of root growth of susceptible seedlings at 2.0 µg toxin/ml in the standard assay.

b/ Average root growth in mm for 18 seedlings.

were exposed to a high concentration (500 µg/ml) of HC-toxin for 20 hours. This treatment caused typical toxin symptoms to both susceptible and resistant plants in 24 hours. Cuttings were harvested after 20 hours exposure and were ground in a mortar with fine sea sand. The homogenates were extracted with water and the water extract was assayed for selective toxicity. Host-specific toxic activity was not recovered from either resistant or susceptible cuttings. This experiment was repeated 2 times with the same negative results.

Susceptible and resistant corn seedlings were grown in 90 x 73 x 60 mm duplex staining dishes, with removable trays, each containing 100 ml White's solution. Cheesecloth was fitted with rubber bands over the bottom of the tray; the tray was then supported just above the solution level and germinated seeds were placed on the cheesecloth. The trays thus prepared were incubated at room temperature for 2 weeks, until plants had made appreciable growth. The solution was then replaced with a fresh solution containing radioactive HC-toxin (activity, 25.3 CPM/ml). After exposure for 12 hours, roots were washed with water and the washes were combined with the radioactive solution, to which a second tray of plants was exposed for another 12 hours. This procedure was repeated for a third tray of plants. After exposing 3 trays of plants, 4 ml aliquots of residual radioactive solution were placed in aluminum dish planchets and dried for radioactivity counts. Differences in radioactivity

between control solutions which had not contained plants and the solutions which had contained plants were used as the measure of toxin uptake. Approximately half the radioactivity disappeared from the solution in which plant roots were exposed. However, nonspecific adsorption of the toxin to glassware and to the plant roots might partially account for the loss of radioactivity in residual solutions.

In a second experiment, no loss of toxin was detected in residual solutions by bioassays. Susceptible and resistant seedlings were grown in White's solution for 2 weeks and then exposed to HC-toxin for 24 hours. After the exposure period, plant roots were washed with distilled water and the washes were combined with the residual solution to bring it back to 100 ml. Toxicity of residual solution was tested by the standard bioassay. Residual solution in which susceptible plants had been exposed was as toxic as was the original solution (Table 10).

Table 9. Effect of HC-toxin concentration and exposure time on symptom expression by cuttings[a/]

Exposure time (hr)	Concentration (µg/ml)		
	12.5	25.0	50.0
0	0[b/]	0	0
2	1	2	2
4	2	3	3
8	3	4	4

a/ Susceptible cuttings were exposed in toxin solution for intervals indicated. Cuttings were then washed and placed in distilled water and held for 48 hours before results were taken.

b/ 0, no symptoms; 1, leaf margin slightly damaged and few necrotic spots on leaf tips; 2, 1/3 of leaf blades collapsed; 3, 2/3 of leaf blades collapsed; 4 all leaves collapsed.

Table 10. Root growth bioassay of residual solutions to determine removal of toxin by intact resistent and susceptible plants in 24 hours[a/]

Treatments	Dilution of test solution			Amount of solution uptake
	1:200	1:800	1:3200	
	mm	mm	mm	ml
Fresh toxin control	<10	20	48	-
Aged toxin control[b/]	<10	18	43	27
Solution exposed to susceptible plants	<10	20	45	23
Solution exposed to resistant plants	<10	23	48	25

a/ Results were taken after 4 days growth of seedlings. Roots of resistant and susceptible seedlings in water averaged 107 and 105 mm, respectively. Values are average root length of 10 seedlings, in mm.

b/ This control was not exposed to plants, but was kept under the conditions used for plants. All solutions were brought back to original volume (100 ml), which was a 1:200 dilution of a toxin preparation.

Distribution of HC-toxin in sub-cellular fractions:

Etiolated susceptible corn coleoptiles (25 g) were infiltrated with radioactive HC-toxin. The infiltrated tissues were washed with cold toxin solution and homogenized in 0.45 M sucrose containing 0.05 M Tris-buffer and 0.005 M $MgCl_2$. After filtration through two layers of cheesecloth, subcellular particles in the filtrate were separated by differential centrifugation. The resulting pellets were suspended in water or in 1 N KOH solution and 0.1 or 0.2 ml aliquots from each fraction were placed on glass planchets for radioactivity counts.

Very little radioactivity was associated with the particulate fractions (Table 11); most was found in the soluble fraction. Several explanations are possible. Much of the labelled toxin might be bound loosely to subcellular organelles and leaked into the supernatant during grinding, centrifuging, and washing. The radioactivity might be from labelled toxin molecules in inter-cellular spaces but not in the protoplasm. With little or no incubation of treated tissues after toxin infiltration, there might be insufficient time for toxin to enter the plant cells. The last possibility is suggested from the fact that HC-toxin treated leaves do not leak electrolytes unless they are infiltrated thoroughly and incubated for at least 1 hour. At any rate, the results were inconclusive as to the site of toxin action. Furthermore, there is an inherent difficulty with this kind of experiment. Very small amounts of HC-toxin are needed to

affect susceptible tissues; therefore, a high radioactive toxin preparation is needed for meaningful results. It seems difficult at this time to prepare toxin with sufficient radioactivity by biosynthesis. The same problem was encountered in studies of the action of gibberellic acid on plant tissues (31, 59).

Comparison with HV-toxin studies: Apparent uptake and activity of HV-toxin with susceptible oat tissues appeared to differ from HC-toxin uptake by susceptible corn. HV-toxin uptake was not affected by temperature over a wide range and by various metabolic inhibitors. The results suggest that HV-toxin uptake did not depend on expenditure of cellular energy(43). In contrast, HC-toxin uptake by HC-susceptible tissues was affected by temperature, lack of oxygen, uncouplers of oxidative phosphorylation, and respiratory inhibitors. All data indicated that HC-toxin uptake required energy of metabolism. The uptake of both toxins by their susceptible tissues is affected by toxin concentrations and exposure times, suggesting that uptake may be governed by a rate limiting mechanism. It has been suggested but not proved that HV-toxin has receptor sites in susceptible tissues that are lacking in resistant tissues (43). HC-toxin would appear to have receptor sites in both resistant and susceptible tissue; the resistant tissue is affected if toxin concentrations are high enough. HC-toxin resistant and susceptible tissue could differ in either the number of receptor sites or the affinity of each site for toxin. No conclusions are possible, since all

Table 11. Distribution of radioactivity in subcellular fractions of C^{14}-toxin treated corn tissues[a/]

Fractions	Total radioactivity (CPM)
Nuclei & chloroplasts	5
Mitochondria	250
Ribosomes, endoplasmic reticula, and other membranes	322
Supernatant	2250

a/ Etiolated HC-susceptible coleoptiles (25 g) were vacuum infiltrated with 100 ml C^{14}-labelled HC-toxin (specific activity: 4,414 cpm/260 µg/100 ml). Coleoptiles were homogenized in 0.45 M sucrose containing 0.05 M Tris-buffer and 0.005 M $MgCl_2$. Fractions were separated by differential centrifugation.

attempts to recover HV- or HC-toxins from toxin treated tissues have failed.

Effect of Toxins on Electrolyte Leakage from Tissues

Electrolyte leakage from HC-toxin treated tissues: Ion leakage of HC-toxin treated leaf tissues was compared with non-treated controls, using conductivity of the ambient solution as a measure of leakage. Cuttings were allowed to take up HC-toxin (25 μg/ml) with the transpiration stream for either 4 or 24 hours. One g tissue was cut from the leaves and used for each replicate of each treatment. The tissue was washed with 100 ml deionized glass distilled water for 2 hours by shaking (100 strokes/minute). Tissues were then placed in 50 ml deionized glass distilled water and leached for specified times. Electrolyte loss from susceptible leaf tissues was not affected by 4 hours exposure to HC-toxin (Fig. 4-A). The ambient solutions of toxin treated and control leaf tissues had equal conductivity. When leaf tissues were exposed to HC-toxin for 24 hours, electrolyte loss to the ambient medium was very high (Fig. 4-B). Ten hours after toxin treatment, (2 hours washing and 8 hours leaching times), conductivity of ambient solutions of treated susceptible leaf tissues was 2.64-fold higher than that of controls.

The experiment was repeated, using vacuum infiltration rather than toxin uptake by transpiring cuttings. Electrolyte

loss was measured at 2, 4, 6, 8, and 10 hours after toxin exposure. One g leaf tissue was infiltrated with toxin (50 μg/ml) for 15 minutes *in vacuo*. Infiltrated tissues were incubated for 2 hours at room temperature, followed immediately by 2 hours washing with deionized glass distilled water in flasks on a reciprocal shaker. Results of a representative experiment showed that conductivity of ambient solutions of treated susceptible tissues was above the controls within 6 hours, and was 74% higher than the controls by 12 hours (Table 12). These times include both the incubation and leaching times.

Comparison with HV-toxin: Electrolyte leakage from leaf tissues is a very early response of susceptible oat tissues to *H. victoriae* infection or HV-toxin treatment. The increase in conductivity of ambient solutions is correlated with relative susceptibility of plants to the disease (44). HV-toxin caused loss of electrolytes from roots, leaves, coleoptiles and aleurone cells of treated plants, which in each case reflects permeability changes in the cell membranes (3, 41). Wheeler and Black found that dilute culture filtrates caused a permeability change which was said to be expressed earlier than other measurable responses to toxin (55). Careful experiments have shown a measurable response by 2 minutes after exposure to toxin (40). The rate of electrolyte loss has a low temperature coefficient and is not dependent on oxygen, which supports the idea of HV-toxin uptake being independent

of energy metabolism. Comparable results were obtained by infiltrating susceptible coleoptiles with HV-toxin for 10 minutes. Eight hours after toxin treatment, the specific conductance of the ambient solution of the treated susceptible tissues was 5 times greater than that of controls.

In contrast, HC-toxin caused ion leakage to develop much more slowly in susceptible corn tissues. No increase in leakage was detected in 4 hours, as compared to non-treated controls (Fig. 4-A). A considerably longer exposure time is required for HC-toxin to show increased loss of electrolytes, and the magnitude of the loss is not as great as with HV-toxin on susceptible oats.

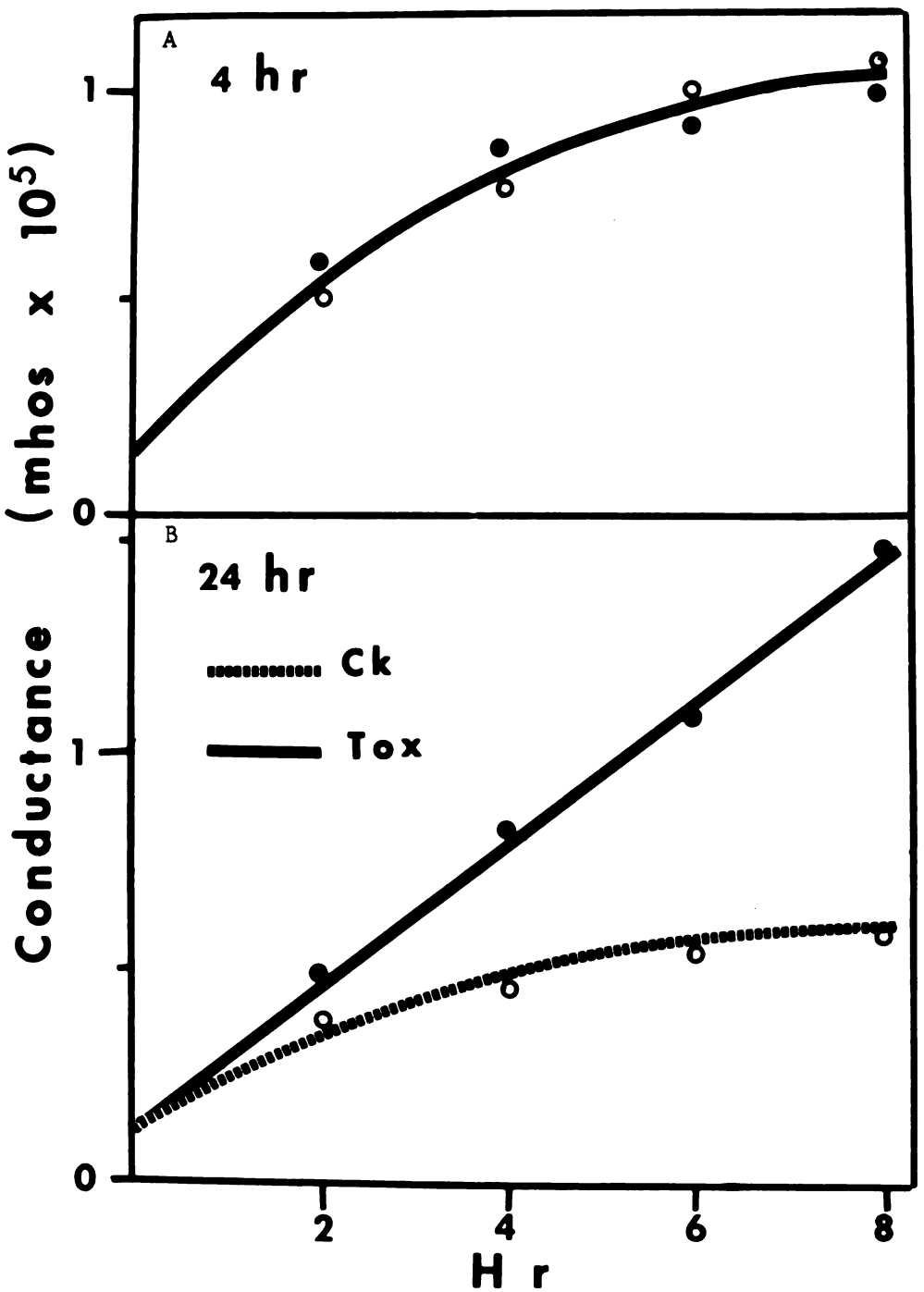


Fig. 4. Effect of HC-toxin on electrolyte loss from leaves of toxin-treated susceptible corn cuttings.
CK = control; Tox = toxin treated; hr = hours pre-treatment with toxin before determinations were started.

Table 12. Effect of infiltration with HC-toxin on electrolyte loss from susceptible corn leaves[a/]

Leaching time	Conductance x 10^{-5} Mohs		% increase in conductance
	Control	Toxin treated	
0	0.125	0.125	-
2	0.455	0.526	16
4	0.781	0.893	14
6	0.926	1.220	32
8	0.961	1.670	74
10	1.060	2.380	125

a/ One g susceptible leaf tissues was infiltrated with HC-toxin (50 µg/ml) for 15 minutes followed by 2 hours incubation at room temperature. After washing, the tissues were enclosed in a cheesecloth bag and bathed in 50 ml deionized glass distilled water for leaching.

Effects of Toxins on Metabolism of Suscepts

Effect of HC-infection and HC-toxin on tissue respiration: Plants with 4 to 5 fully expanded leaves were inoculated with H. carbonum spore suspension (7 to 8 spores/microscopic field, 100 magnification). Inoculated and control plants were held in a fog chamber for 24 hours. Effects of infection on gas exchange were then determined by standard Warburg manometry, using leaf sections. Oxygen uptake by inoculated leaf tissues of susceptible plants was somewhat higher than in control tissues, but the differences developed slowly and were not striking. There were little or no differences in respiration of inoculated and control leaves in 24 hours. The peak in infection-stimulated respiration came 48 to 72 hours after inoculation. Thereafter, respiration in diseased tissue gradually decreased (Fig. 5), possibly because of tissue disintegration. It is likely that the infecting mycelium contributed to gas exchange; thus it is impossible to attribute the stimulation entirely to host tissue.

The respiratory response of both root and leaf tissues to toxin was measured, since different tissues might differ in toxin sensitivity. Cuttings of corn plants were allowed to take up HC-toxin (5 μg/ml) for varying periods of time. Sections of leaves were cut from treated and control plants, and oxygen uptake was measured as described above. Two to 4 hours

treatment with toxin (5 µg/ml) caused no effect on respiration of leaf tissues (Table 13). These cuttings had taken up enough toxin to cause visible symptoms to appear later, as was shown by holding some of the treated cuttings at room temperature for 2 days. Eight hour exposure to toxin caused a 30% increase in oxygen uptake, which was still evident after 30 hours exposure. There was no visible damage to treated leaf tissues at the time of O_2 uptake measurements. However, in some other cases, visible toxic effects were evident by 30 hours. Variations in environmental conditions during the experiments and different conditions of test plants might cause such differences. Oxygen uptake by susceptible leaf tissues varied with concentration of HC-toxin. Low concentrations had little or no effect on O_2 uptake (Table 14).

Effects of toxin on gas exchange by root tissue was determined with roots of 5 day old seedlings, carefully selected for uniformity. Roots of 6 plants were placed in each Warburg flask containing 0.2 ml HC-toxin (500 µg/ml) and 1.8 ml White's solution. Readings were taken at 20 minute intervals, after 10 minutes equbration at 25 C. Respiration of roots was not affected by HC-toxin over a 2 hour period.

Susceptible oat tissue responds to HV-infection with an increase in respiration. Respiratory rates of susceptible infected and control plants are similar for 2 days following inoculation, but on the third day the rate in infected plants rises sharply.

It reaches a maximum of nearly twice the control rate on the fourth day and then falls until on the eighth day it is well below the control value (13). Comparable respiratory increases are obtained with crude culture filtrates (38) and purified toxin (44). The intensity of the response is modified greatly by varying the conditions of treatment. Both roots and leaves can be stimulated almost immediately after exposure to HV-toxin (44).

Generally, respiratory response of susceptible corn tissue to HC-infection followed the same pattern, but with smaller increases than with HV-infection. It reached a maximum 2 or 3 days after inoculation, with about 30% increase over controls. A comparable increase in respiration can be achieved by treating susceptible corn leaves with HC-toxin. However, the response is much slower than with HV-toxin on oats. Exposure of corn tissues for about 8 hours is required for a 30% increase in respiration to develop.

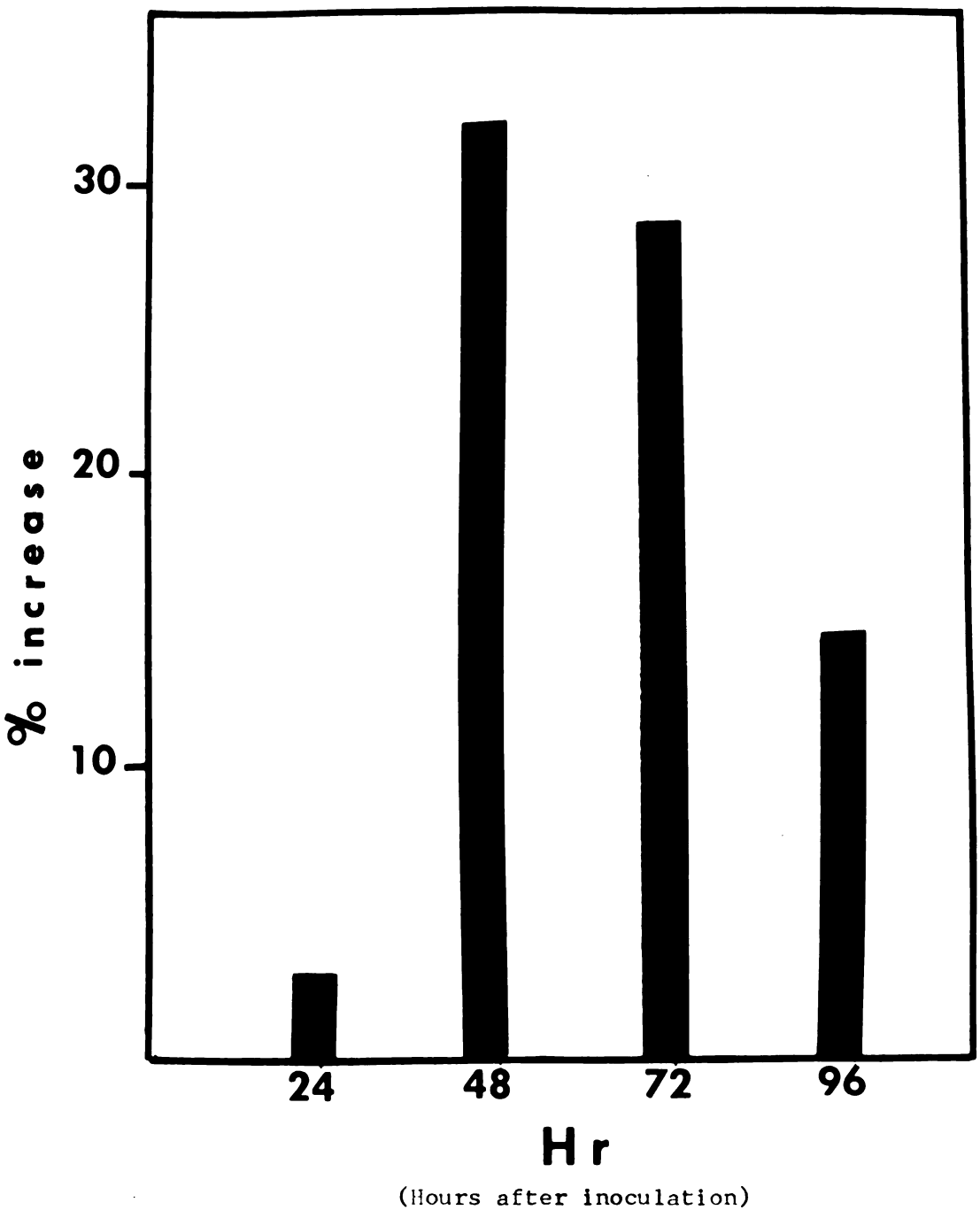


Fig. 5. Effect of H. carbonum race 1 infection on oxygen uptake of susceptible corn leaves.

Table 13. Effect of HC-toxin on respiration of susceptible corn leaves

Experiment	Exposure to toxin (hr)	O_2 uptake[a]	% change
1.	0	35	-
	2	33	-6
	4	34	-3
2.	0	545	-
	8	709	+30
3.	0	43	-
	30	60	+38

a/ µl O_2 uptake/10 mg tissue (dry weight)/hr in experiments 1 and 3. Values for experiment 2 are per g fresh weight/hr.

Table 14. Effect of HC-toxin concentration on respiratory response of susceptible corn leaves[a/]

Concentration[a/] (µg/ml)	O_2 uptake/10 mg dry tissue/hr			
	Experiment 1		Experiment 2	
	µl	% change	µl	% change
0	41	-	46	-
0.25	44	+7	43	-7
1.0	45	+10	46	0
4.5	47	+15	55	+20

a/ Cuttings were exposed to HC-toxin for 4 hr before these determinations were made. This preparation gave complete inhibition of root growth in the standard bioassay at 4.5 µg/ml.

Effect of HC-toxin on incorporation of amino acids and uridine: The effect of toxin on incorporation of C^{14}-amino acids or C^{14}-uridine by tissues was determined by measuring the amount of radioactivity retained in TCA insoluble cellular fractions. The protocol for these experiments is described in the methods section. Incorporation of amino acids into TCA insoluble cellular components was not affected by HC-toxin in exposures up to 6 hours. Longer exposures to toxin caused stimulation of amino acid incorporation. Eight hours toxin (5 µg/ml) treatment followed by a 4 hours incubation with C^{14}-labelled leucine or valine at room temperature resulted in 23% increase in incorporation by susceptible tissues and 43% by resistant tissues (Table 15). The stimulation of amino acid incorporation might be related in some way to increased growth of susceptible and resistant plant roots caused by relatively low concentration of HC-toxin.

Similar results were obtained with uridine incorporation. There was little or no effect of toxin on uridine incorporation into TCA insoluble cellular components with toxin exposures of 4 hours. Eight hours exposure to toxin (5 µg/ml) followed by 4 hours incubation with C^{14}-uridine (2.5 µc/ml) gave stimulation in incorporation (30% by susceptible and 20% by resistant tissues). Still longer exposures (22 hours) resulted in a 45% decreased incorporation in susceptible tissues but a 20% increase in incorporation in the resistant tissues (Table 16). After 22 hours toxin treatment, there was mild flaccidity of leaves of both resistant and susceptible plants.

There is a fundamental difference in effect of HV- and HC-toxin on protein and RNA synthesis of susceptible tissues. Incorporation of the C^{14}-amino acids by resistant oat tissue was not affected by HV-toxin, whereas incorporation by susceptible oat tissue was practically stopped in a very short time by very dilute toxin solutions (44). Synthesis of RNA *in vivo* also appears to be stopped very quickly and by very dilute HV-toxin solutions (44).

Effect of HC-infection on dark fixation of CO_2 by leaves: Malca et al (28) have described the effects of HC-infection on $C^{14}O_2$ uptake in the dark. Susceptible seedlings inoculated with *H. carbonum* race 1 fixed less CO_2 in the dark, while CO_2 uptake by resistant tissue was not affected. However, this effect was evident at 3 days after inoculation; earlier responses by intact tissue were not detected. An earlier response might be expected, since these workers reported increased CO_2 uptake by homogenates prepared from the tissue shortly after inoculation (29). I have repeated and tried to expand these experiments.

Corn seedlings were inoculated with several different concentrations of HC-spores ranging from O. D. 0.7 to 1.5 at 600 mμ, as determined with a Spectronic 20 Colorimeter (Bausch and Lomb). This amounted to more than 50 spores/microscopic field (100X magnification). After inoculation, plants were held in a moist chamber for 12 to 24 hours. Heavy infection was evident on

Table 15. Effect of HC-toxin on incorporation of C^{14}-amino acids into TCA insoluble cellular fraction by leaf tissues of corn[a/]

Plants	Treatments	Exposure time			
		6 hours		8 hours	
		CPM[b/]	% change	CPM	% change
Susceptible	Control	309	-	372	-
	Toxin	307	-1	457	+23
Resistant	Control	-	-	320	-
	Toxin	-	-	458	+43

a/ Cuttings were exposed in HC-toxin (5 µg/ml) for times indicated. Incubation time with C^{14}-amino acids was 4 hours.

b/ CPM/0.1 ml/planchet, above background.

Table 16. Effect of HC-toxin on uridine-C^{14} incorporation into TCA insoluble cellular component by corn leaf tissues[a/]

Plants	Treatments	Exposure time					
		4 hours		8 hours		22 hours	
		CPM[b/]	% change	CPM	% change	CPM	% change
Susceptible	Control	79	-	218	-	218	-
	Toxin	87	+10	282	+29	119	-45
Resistant	Control	105	-	173	-	169	-
	Toxin	116	+11	206	+19	202	+20

a/ Cuttings were exposed to HC-toxin (5 µg/ml) for times indicated, followed by 4 hours incubation with C^{14}-uridine.

b/ CPM, above background/0.2 ml/planchet.

on inoculated leaves after 24 hours incubation in moist chambers at room temperature. The second leaf above the primary leaves was cut and used for CO_2 fixation experiments. CO_2 fixation in the dark was decreased by infection in all cases, with all incubation times and inoculum concentrations used. The reduction ranged from 65 to 83% over non-inoculated controls.

Malca et al did not include non-pathogenic control inoculations in their experiments. Accordingly, susceptible corn seedlings (K61 x Pr) were inoculated with a spore suspension of H. victoriae (OD 0.9 at 600 mμ) and kept in the moist chamber for 24 hours. The inoculated leaves were then used to determine CO_2 fixation in the dark. This fungus penetrates corn tissues, but infection fails to develop. Corn leaves inoculated with H. victoriae had 55% lower CO_2 fixation in the dark than did comparable non-inoculated control leaves.

Effect of HC-toxin on dark fixation of CO_2 by leaves: Transpiring susceptible cuttings were allowed to take up HC-toxin for 4 hours and then were exposed in $C^{14}O_2$ for another 4 hours. The leaves thus treated were combusted to CO_2 and the $C^{14}O_2$ fixed by tissue was determined (see "Materials and Methods"). CO_2 fixation in the dark by both resistant and susceptible tissues was stimulated after 4 hours exposure to toxin (Table 17). Longer exposures to toxin tended to decrease the dark fixation of CO_2. The results indicate that stimulation of CO_2 dark fixation is correlated with toxin concentrations, since 50 μg toxin/ml caused much more

stimulation than did 5 μg toxin/ml (Table 17). Both susceptible and resistant tissues showed the same tendency, although the resistant tissue was stimulated significantly less than was susceptible tissue under the conditions of the experiment. This difference appears to reflect the difference in response of resistant and susceptible tissue as measured by other parameters. These data support the idea that resistant and susceptible plants differ quantitatively rather than qualitatively.

HC-toxin treated cuttings were placed in the dark for 2 hours, followed by a 4 hour exposure to $C^{14}O_2$ in the dark. The purpose of this treatment was to see if pre-treatment in the dark affects later dark fixations, as was shown by Daly and Livne (8) in a study of rust infections of wheat and bean. CO_2 fixation in the dark was increased only 16% by toxin treatment (50 μg/ml) when a dark period preceded the $C^{14}O_2$ incubation period. In contrast, without the pre-dark treatment, HC-toxin (50 μg/ml) caused 90% increase in fixation.

Chemical or other non-specific injuries might also cause increased CO_2 fixation in the dark, as is the case with soybeans fumigated with hydrogen fluoride (58). Corn cuttings were allowed to take up 5×10^{-4}M copper sulfate solution for 4 hours, followed by 4 hours exposure to $C^{14}O_2$ in the dark. The amount of radioactivity fixed by leaf tissues was compared to that fixed by untreated control tissues. Copper sulfate solution caused a mild flaccidity in the treated leaves after 4 hours, and a decrease in CO_2 fixation in the dark (Table 18).

Table 17. Effect of HC-toxin on $C^{14}O_2$ dark fixation by corn tissues

Experiment No.	Tissue type	Treatments[a]	Toxin Concentration (µg/ml)	CO_2 Fixation (CPM)[b]	% change
1.	Susceptible	Control	0	464	-
		Toxin	50	1,379	+198
2.	Susceptible	Control	0	50	-
		Toxin	50	95	+90
3.	Resistant	Control	0	361	-
		Toxin	50	485	+34
4.	Susceptible	Control	0	490	-
		Toxin	5	706	+44
5.	Susceptible	Control	0	355	-
		Toxin	5	461	+30
6.	Resistant	Control	0	105	-
		Toxin	5	128	+22

a/ Cuttings were exposed to toxin solution for 4 hours before exposure to $C^{14}O_2$ in the dark.

b/ Above background.

Table 18. Effect of copper sulfate on $C^{14}O_2$ fixation in the dark by corn leaves

Experiment No.	Treatment[a/]	$C^{14}O_2$ fixed[b/]	% change
1.	Control	425	-
	Copper sulfate	291	-32
2.	Control	442	-
	Copper sulfate	277	-37

a/ Cuttings took up copper sulfate solution (5×10^{-4}M) for 4 hours before exposure to $C^{14}O_2$ for an additional 4 hours.

b/ CPM/0.2 ml/planchet, above background.

Effect of HC-toxin on CO_2 incorporation by cell free systems: The previous experiment indicated that carboxylations by corn tissue were stimulated by HC-toxin. There are several known mechanisms for carboxylations by plant tissues. Malca et al (29), in their study of HC-infected tissues indicated that the increase in conversion of ribose-5-phosphate to ribulose-1,5-diphosphate following HC-infection provided high substrate levels for carboxydismutase activity, thus stimulating CO_2 fixation. The effect of HC-toxin on this and other carboxylation mechanisms was examined.

Susceptible corn cuttings were allowed to take up HC-toxin (5 µg/ml) for 4 hours, followed by 4 hours incubation in the dark at room temperature. Leaves were then cut and ground in a mortar with washed sea sand. The homogenate was filtered, centrifuged, and the supernatant was used as an enzyme source. Several carboxylation systems were tested (see "Materials and Methods") with appropriate substrates and cofactors.

The phosphoenol pyruvate reaction system was affected slightly by HC-toxin treatment, with a 10 to 40% decrease in CO_2 fixation. The 3-phosphoglyceric acid reaction system was not affected significantly by HC-toxin. Extracts of HC-toxin treated cuttings showed a marked increase in the activity of the ribose-5-phosphate recation system. Four separate experiments showed the same tendency (Tables 19, 20), with a 47 to 99% increase in activity of the Rib-5-P reaction system. Similar

results were obtained by Malca et al (29) with extracts made shortly after HC-inoculation with a low concentration of spores. However, when susceptible plants were inoculated with a heavy concentration of spores, there was a reduction in activity of Rib-5-P reaction system. The data are not conclusive for the ribulose-1,5-diphosphate reaction system, since HC-toxin caused a 22% increase in one experiment and a 23% decrease in another (Table 19).

The conversion rate of Rib-5-P to Rul-1-P,5-P was calculated by the method described by Malca (29), with slight modification. Briefly, the conversion rate is calculated as follows:

$$\frac{\text{CPM in Rib-5-P-toxin / CPM in Rul-1-P,5-P-toxin}}{\text{CPM in Rib-5-P-Ck / CPM in Rul-1-P,5-P-Ck}}$$

This ratio provides an index of the formation of Rul-1-P,5-P from Rib-5-P by extracts of toxin treated cuttings in relation to that of control cuttings. A ratio of 1 indicates that the conversion is proceeding at an equal rate in toxin treated and control extracts; in other words, no effect of toxin. A ratio of more than 1 indicates a higher rate of conversion in extracts of toxin treated leaves. A ratio of less than 1 indicates less activity in toxin treated than in the control leaf extracts. The calculated conversion rate of Rib-5-P to Rul-1-P,5-P from experiments 1 and 2 (Table 19), was respectively 1.32 and 1.93. The data indicate that stimulation of the Rib-5-P reaction system by HC-treatment was caused by stimulation of either phosphoriboisomerase or phosphoribulokinase or both, but not of carboxydismutase. These results are comparable

to those of Malca et al, using susceptible corn tissues and a low inoculum level (29).

The effect of HC-toxin was tested further by use of cell-free carboxylation system from corn. A supernatant enzyme was extracted from healthy tissue and toxin was added after extraction. The following substances (in μ moles) were added to 0.3 ml of the supernatant enzyme: $MgCl_2$, 15; $NaHCO_3$, 5 (containing 25 μc); Tris buffer pH 8.0, 150; ATP, 2; and Rib-5-P, 2. Water was added to bring the total volume to 1 ml. Proper controls were included for comparisons. Before addition of $HC^{14}O_3^-$, HC-toxin at 0.5, 2,5, or 5.0 μg/ml was added and the reaction mixture was incubated at room temperature for 30 minutes. Immediately after addition of $HC^{14}O_3^-$, the mixture was placed at 37 C for 10 minutes. After 37 C incubation, 0.1 ml 1N HCl was added to stop the reaction, and 0.2 ml aliquots were taken for radioactivity counts. The results indicated that HC-toxin caused a reduction in carboxylation by carboxydismutase activity, and indicated that the Rib-5-P reaction system is not a primary site of toxin action. However, the experiment should be repeated, since incubation of enzyme preparation in the reaction mixture at room temperature for 30 minutes before addition of radioactive sodium bicarbonate might use up Rib-5-P in the reaction mixture and affect further reactions.

Table 19. Effect of HC-toxin, added before extraction, on the activities of cell-free carboxylation systems

Reaction systems[a]	Radioactivity fixed (CPM)					
	Experiment 1			Experiment 2		
	Control	Treated	Change	Control	Treated	Change
	CPM	CPM	%	CPM	CPM	%
PEP+CO_2+Mg^{++}	714	637	-11	90	57	-37
3PGA+CO_2+Mg^{++}	765	641	-16	36	33	- 8
Rul-1-P,5-P+CO_2+Mg^{++}	263	321	+22	86	66	-23
Rib-5-P+ATP+CO_2+Mg^{++}	400	645	+61	36	53	+47
Mg^{++} + CO_2	7	16		-2	-0.5	
Mg^{++}+CO_2 + ATP	14	23		0.2	-2.5	
Mg^{++}+ CO_2+Rib-5-P	23	26		2.5	0.9	

a/ All reaction mixtures had a total volume of 1.0 ml, including 0.3 ml supernatant enzymes. Other conponents, as needed (in μ moles) were $MgCl_2$, 15; $NaHCO_3$, 5 (with 25 μc $HC^{14}O_3^-$); Tris buffer, pH 8.0, 150; ATP, 2; Rib-5-P, 2; Rul-1-P,5-P, 1; PEP, 2; and PGA, 2.

Table 20. Cell-free carboxylation activities from HC-toxin treated tissues

Reaction systems[a]	Radioactivity fixed (CPM)					
	Experiment 1			Experiment 2		
	Control	Treated	Change	Control	Treated	Change
	CPM	CPM	%	CPM	CPM	%
Rib-5-P+ATP+CO_2+Mg^{++}	89	177	+99	448	848	+89
Mg^{++} + CO_2 + ATP	1	5		30	30	
Mg^{++} + CO_2 + Rib-5-P	2	6		66	44	
Rib-5-P+ATP+CO_2+Mg^{++}+ boiled enzyme preparation[b]	-2	-2		-	-	

a/ All reaction mixtures had a total volume of 1.0 ml, including 0.3 ml supernatant enzymes. Other components, as needed (in μ moles) were $MgCl_2$, 15; $NaHCO_3$, 5 (with 25 μc $HC^{14}O_3^-$); Tris buffer, pH 8.0, 150; ATP, 2; Rib-5-P, 2; Rul-1-P,5-P, 1; PEP, 2; and PGA, 2.

b/ 0.3 ml boiled supernatant enzymes was used with other specified components.

Effect of HV-toxin on CO_2 dark fixation by oat tissues: Leaves of oats (cvs. Clinton and Park) were treated with HV-toxin before being exposed to $C^{14}O_2$, and thereafter the radioactivity fixed by treated leaves was compared to that fixed by untreated control leaves. CO_2 fixation by susceptible leaf tissues in the dark was stimulated by HV-toxin (Table 21). Highest stimulation was obtained after 4 hours treatment with 0.32 µg HV-toxin/ml. CO_2 fixation by resistant oat leaves was not affected by the same treatment. In some cases, there may have been a decrease in CO_2 fixation by treated resistant leaves (Table 22), but this is probably due to experimental error.

Table 21. Effect of HV-toxin on $C^{14}O_2$ fixation in the dark by susceptible oat leaves (cv. Park)

Experiment No.	Treatment	Exposure time	$C^{14}O_2$ fixed (CPM[a])	% change
1.	Control	0	74	-
	Toxin (0.8 µg/ml)[b]	30 min.	80	+8
2.	Control	0	147	-
	Toxin (0.8 µg/ml)	30 min.	157	+7
3.	Control	0	123	-
	Toxin (0.32 µg/ml)	4 hr	222	+81
4.	Control	0	117	-
	Toxin (0.16 µg/ml)	4 hr	158	+35

a/ Counts for experiment 1 are the av. CPM (above background)/ 0.1 ml/ planchet (2 planchets); Counts for experiments 2, 3 and 4 are av. CPM/0.2 ml/planchet.

b/ Toxin preparation completely inhibited root growth of susceptible seedlings at 0.016 µg/ml.

Table 22. Effect of HV-toxin on $C^{14}O_2$ fixation by resistant oat leaves (cv. Clinton)

Experiment No.	Treatment	Exposure time (hr)	$C^{14}O_2$ fixed (CPM)[a]	% change
1.	Control	0	148	-
	Toxin[b] (0.32 µg/ml)	4	120	-19
2.	Control	0	83	-
	Toxin (0.32 µg/ml)	4	81	-2

a/ Av. CPM (above background)/0.2 ml/planchet (2 planchets).

b/ Toxin preparation completely inhibited root growth of susceptible seedlings at 0.016 µg/ml.

DISCUSSION

The discovery in 1965 of a host specific disease determinant from Helminthosporium carbonum strengthened the concept of chemical determinants of pathogenicity (45). This concept is not widely accepted simply because so few examples are known. Therefore, the objections to the concept are based on negative evidence which is never conclusive. Technical difficulties might account for the few successes in finding host-specific or other toxins involved in pathogenicity. More successful examples should be expected from careful studies of other host-pathogen combinations.

The correlation between HC-toxin sensitivity and susceptibility to H. carbonum-infection, with different types of corn, strengthened the idea of HC-toxin as a primary determinant of pathogenicity. H. carbonum susceptible corn varieties were highly sensitive to HC-toxin, while H. carbonum resistant corn was much less sensitive to the toxin. An inbred corn line that was intermediate in the resistant to susceptible scale in field evaluations was also intermediate in sensitivity to HC-toxin. Similar correlations are known for H. victoriae-infection and HV-toxin. The discovery of these intermediate types indicate that there are quantitative differences in toxin receptors or transporting system in cells.

Several cases are described in the literature which offer good promise for finding still more host specific toxins. A good example is leaf scorch disease of sugarcane, caused by Stagonospora sacchari Lo et Lin. Several aspects of the disease are analogous to Victoria blight of oats caused by Cochliobolus victoriae (38, 44). Leaf scorch appeared suddenly in Taiwan in 1949 on sugarcane varieties newly introduced from India. The disease spread quickly over all sugarcane plantations on the island. Many highly desirable commercial varieties were abandoned before the disease was finally controlled by development and adoption of resistant sugarcane varieties. The origin of the causal fungus, S. sacchari, received considerable attention. Evidences for the endemic occurrence of the fungus came from isolation of the fungus from grasses other than sugarcane. Furthermore, the fungus was observed on herbarium specimens collected by Sawada in 1909. The fungus was found as a weak parasite on a red-skinned sugarcane (cv. Badila) and on a number of wild grasses, as shown by isolation from Miscanthus japonica and Miscanthus sinensis (24). The disease is now under control, and is ignored by contemporary sugarcane breeders and plant pathologists. Possible production of a host-specific toxin by the pathogen was never tested.

Hale et al in 1962 described effects of H. carbonum culture filtrates that might have been caused by the host-specific toxin (15). The growth of excised stems of pea, a non-host plant, was inhibited by a relatively low dilution of culture

filtrate. Growth of coleoptiles from susceptible corn plants was also inhibited, while growth of comparable resistant coleoptiles was stimulated. My data show that low concentrations of highly purified HC-toxin stimulate growth of both susceptible and resistant seedlings. The explanation of this stimulation is unknown. It could possibly depend on stimulated protein and RNA synthesis resulting from release of bound enzymes, on effects at the translation level of protein synthesis, or on changes in cell permeability. The use of an active ribosomal preparation might help to answer such questions.

HC-toxin had an inhibitory effect on growth of several non-host plants, including tomato, cabbage and cucumber. Tomato plants, for example, were more sensitive than HC-resistant corn, but less sensitive than HC-susceptible corn. These effects might be caused by impurities in the toxin preparation used. However, this seemed unlikely, since the toxin was carefully purified and the concentration which caused inhibition of non-host plants was relatively low (5 μg/ml). Growth of seedling roots varies with species in sensitivity to many deleterious factors. Some are extremely sensitive to many substances. This possible explanation of the results was not examined further.

Very high concentration of HV-toxin (3.6 mg/ml) also caused inhibition of root growth of a resistant oat cultivar (Clinton), sorghum, and other non-host plants. Incomplete separation of victoxinine from toxin might explain the effect on

resistant plants at high concentrations of toxin. Separation of victoxinine from HV-toxin by gel filtration (Bio Gel P-2, 100-200) was somewhat unsatisfactory (unpublished data). A better separation method should be developed in order to rule out the possibility. However, the fact that wheat and sorghum were more sensitive to high concentrations of the HV-toxin preparation than were the resistant oats (cv. Clinton) argues against victoxinine contamination as a factor. Sorghum and wheat are more resistant to victoxinine-HCl than are oats (42). High concentrations of some amino acids are known to be toxic to growth of microorganisms (25, 54). Similar non-specific mechanisms could inhibit the growth of higher plants because increase of toxin concentration means an increase in peptide concentration (38).

High concentrations of HC-toxin inhibited root growth of corn plants, while low concentrations were stimulatory. This dual effect of HC-toxin on growth appears to be a basic difference between HC- and HV-toxins. HC-toxin behaves like some growth regulators. Low concentrations of IAA and several other plant hormones caused increased growth and respiration of coleoptiles, but concentrations greater than 10^{-4} or 10^{-3}M were inhobitory to both growth and respiration (11).

The plasma membrane is the main barrier in plant tissues to diffusable substances. Membranes of cells are known to be poorly permeable to hydrophilic substances (21). HV- and HC-toxins have this property; therefore it seems unlikely that they

cross plasma membrane simply by diffusion. This fact, plus the high host-selectivity of the toxins indicate the need of specialized mechanisms for toxin uptake if they do not act on the outer surface of the plasma membrane (21). A receptor or binding site in the susceptible plasma membrane is thought to account for susceptibility to HV-toxin (43).

All attempts to obtain direct evidence for HC-toxin uptake by corn plants were inconclusive, as was the case with HV-toxin (43). The use of C^{14} labelled toxin and cell fractionation, as well as attempted recovery of the toxin from treated cuttings, gave no conclusive data. However, indirect evidence indicated that HC-toxin was taken up by corn plants. The rate of toxin uptake, as measured by later toxin effects, varied directly with toxin concentration and exposure time. Inhibition of root growth following earlier toxin exposure (used as a measure of toxin uptake) did not increase linearly with increasing concentration of HC-toxin. Instead, the response leveled off at higher concentrations, suggesting the existance of binding sites which governed a rate limiting step of uptake. HC-toxin activity toward susceptible corn plants was temperature-dependent during the time of exposure to toxin. Toxic activity was not expressed if plants were held under anaerobic conditions during the limited time of toxin exposure. 2,4-dinitrophenol, sodium azide, and potassium cyanide treatment durint the time of exposure to toxin likewise decreased later evidences of toxin action.

All evidence indicated that toxin uptake required energy of metabolism. The data with DNP fit this conclusion, because it would make less ATP available for energy-requiring reactions, although it probably has effects other than uncoupling of phosphorylation from oxidation. Data from sodium azide and potassium cyanide treatment also fit a concept of an energy requiring step in toxin uptake, since these substances are known to interfere with energy metabolism.

In contrast to the case with HC-toxin, all data indicate that HV-toxin uptake is a simple process, possibly a 1-step adsorption to a receptor (43), which does not require metabolic energy.

An active transport system requiring metabolic energy is postulated for uptake of HC-toxin. Such a system may be comparable to known systems, such as that for sulfate and thiosulfate uptake by Salmonella typhimurium (9, 37). The sulfate transport system is energy and temperature dependent. Results of genetic studies indicated that three discrete cistrons were responsible for forming the elements of the transport system (9). Binding and transport of sulfate ions require separate factors. The binding component was isolated by Pardee et al (37), and shown to be a protein with a molecular weight of 32,000. Binding of sulfate ion to binding sites was complete in 20 seconds and was energy independent. However, the transport phase required expended cellular energy (9). This fact might be useful in understanding

the difference between HV- and HC-toxin uptake. For HV-toxin, binding of toxin molecules to binding sites could result in immediate disruption of plasma membranes. HC-toxin may be moved by a transporting system.

It seems illogical that corn cells would have an active transport specific to a toxic substance. A more reasonable speculation is that toxin is carried by a transport system which carries similar substances normally involved in metabolism. A parallel is known for lysine, whose transport system will carry arginine as well (12). Resistance of corn plants to HC-toxin may not be based on lack of a toxin transporting system; growth of resistant corn can be stimulated or inhibited by toxin, although higher concentrations are needed for resistant than for comparable effects on susceptible tissue. Resistance and susceptibility might be based on differences in affinity for binding sites.

Several observations indicate that HV- and HC-toxins have quantitative and/or qualitative differences in their effects on host tissue, the most significant of these being the difference in uptake. Otherwise, HC-toxin caused stimulation of protein and RNA synthesis after brief exposure of tissues. Low concentrations of HC-toxin also stimulated growth of both resistant and susceptible seedlings. These phenomena have never been observed with HV-toxin. Electrolyte leakage from toxin treated leaf tissues of susceptible corn developed very slowly with HC-toxin, although

long treatments and high toxin concentrations resulted in electrolyte loss, decreased synthesis, and inhibition of growth. In contrast, susceptible oat tissues had increased electrolyte loss almost immediately after exposure to HV-toxin.

HV- and HC-toxins also differ in the speed and magnitude of the respiratory responses elicited in susceptible tissue. H. victoriae-infection or HV-toxin treatment results in a rapid and dramatic stimulation in gas exchange. This could result from a quick disruption of membranes by the toxin, which leads to ion leakage and activation of anion respiration (4, 41). H. carbonum-infection and HC-toxin cause a relatively slow and weak respiratory response in susceptible tissue. HC-toxin appears to use an active transport system, and all the secondary effects of toxicity may be limited by this system. There is of course no available evidence to show that anion respiration is responsible for stimulated respiration in either corn or oats after toxin treatment.

The earliest effect of HC-toxin observed to date was an increase in CO_2 fixation by tissues in the dark. A series of experiments indicated that the stimulated CO_2 fixation was specific to toxin action. A tissue-invading non-pathogenic fungus and chemical injuries did not stimulate CO_2 fixation in the dark. With oats and HV-toxin, susceptible but not resistant oats had a stimulation in CO_2 dark fixation. Very high concentrations of HV-toxin were not tested on resistant oats, but it is clear that the difference between resistant and susceptible oats in this category

is much greater than with HC-resistant and susceptible corn.

The significance of infection-stimulated CO_2 fixation in the dark is not known. Its function as a nutritional factor in parasitism was suggested by Micocha et al (34), but this has been questioned by Daly (8). It could affect pH or permeability of infected cells. All previous studies have not determined whether increased CO_2 dark fixation reflected a change in host tissue or was simply reflection of fungus metabolism. The use of HC-toxin rather than fungus infection shows clearly that this change occurs in host tissue.

H. carbonum infection causes a decrease rather than an increase in dark fixation of CO_2 (28). My results confirm this negative finding. On the surface, this appears to conflict with the finding that toxin causes increased fixation. However, Malca et al. (29), using cell-free extracts from infected tissue, found that increased potential for CO_2 fixation in the dark existed in tissues shortly after inoculation. The increase was soon followed by a sharp decrease in CO_2 fixing potential. Data were presented to show that the decrease was caused by accumulation of metabolic inhibitors in infected tissue. Decreased CO_2 fixation occurred with injuries such as those caused by copper sulfate and invasion by non-pathogens. It seems likely that any injury to tissue will soon lead to decreased CO_2 fixing potential. Overall, the data on effects of infection and toxin treatment do not contradict the hypothesis that toxin causes the biochemical

changes of disease development.

Several enzymes can be responsible for dark CO_2 fixation (56), but factors other than enzymes may be rate limiting and can lead to increased rate of CO_2 fixation in the dark. My data indicate that the activities of phosphoriboisomerase (19), or of phosphoribulokinase (18), or both, which are involved in conversion of ribose-5-phosphate to ribulose-1,5-diphosphate were stimulated by HC-toxin treatment. These results parallel the findings of Malca et al. (29) with H. carbonum infection. These activities should provide higher substrate concentrations for carboxydismutase activity.

Carboxydismutase is a key enzyme in photosynthesis and is located mainly in chloroplasts (16, 26). It would be easy to conclude from these data that toxin has a direct effect on chloroplasts. Carboxydismutase, phosphoriboisomerase, and phosphoribulokinase activities were once thought to be an enzyme complex in fraction-I of leaf protein (32). Recently, carboxydismutase was separated from the other two enzymes (49). My data indicate that the stimulation of CO_2 fixation by HC-toxin is not a direct effect on carboxydismutase, but results from a stimulation in conversion of Rib-5-P to Rul-1-P,5-P, which provides more substrate for carboxydismutase. Therefore, HC-toxin could act directly on intracellular sites other than chloroplasts. Increased glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities in H. carbonum infected tissues were reported (30),

and a stimulation in the pentose pathway is possible. Parallel effects of HC-toxin would be expected. Even though stimulation of CO_2 fixation in the dark is an early result of HC-toxin action, there is some reason to believe that the whole effect is a secondary event in action of the toxin. No increase in CO_2 fixation resulted when HC-toxin was added to plant extracts in a medium capable of supporting the reactions leading to CO_2 fixation. Toxin appears to affect other systems in intact tissues, which in turn leads to stimulation in the CO_2 fixing reactions.

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