BIOASSAY AND PURIFICATION OF HELMINTHOSPORIUM VICTORIAE TOXIN AND ITS INTERACTION WITH OAT TISSUES AND WITH ISOLATED MEMBRANES

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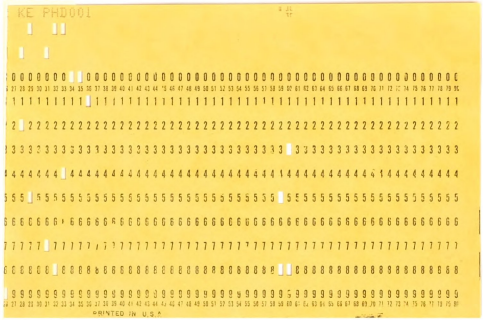
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ABSTRACT

BIOASSAY AND PURIFICATION OF HELMINTHOSPORIUM VICTORIAE TOXIN AND ITS INTERACTION WITH OAT TISSUES AND WITH ISOLATED MEMBRANES

By

Kenneth E. Damann, Jr.

Previous work makes it clear that Helminthosporium victoriae (HV) toxin selectively disrupts the plasmalemma of susceptible oat cells. The plasmalemma of resistant cells is not disrupted. However, the plasmalemma effect may be secondary; there is no direct evidence as to the location of the toxin-sensitive site in susceptible cells. It is not likely that a toxin receptor or sensitive site will be identified until a cell-free system from susceptible plants is found to be affected by toxin. Therefore, I have tested HV-toxin against several cell-free membrane systems in an attempt to discover an in vitro effect.

An assay based on toxin-induced leakage of electrolytes from susceptible but not from resistant tissues was developed as a rapid means of quantifying HV-toxin. The assay requires standardized procedures for growth of plants, and use of leaf samples of standard weight, section size, and age. The electrolyte leakage assay was used to guide the purification of HV-toxin by thin-layer chromatography, gel filtration, cation-exchange chromatography, and high-voltage electrophoresis. The quenching of ultraviolet-induced fluorescence on thin-layer chromatograms was correlated with toxin activity and appears to be a useful marker for locating HV-toxin.

There was no detectable change in toxin concentration in the residual solutions in which tissues had been incubated and removed. This indicated that large amounts of toxin are not bound by tissues. Similar amounts of toxin were recovered from resistant and susceptible cuttings when these had taken up toxin in the transpiration stream. Neither of these findings support the idea that resistance depends on degradation of the toxin by the resistant plant.

There was no evidence of the binding of toxin to a high molecular weight membrane component. Cell membranes were prepared from resistant and susceptible roots. The membranes were exposed to toxin, then solubilized and fractionated by gel filtration. The first fraction after the void volume contained host-specific activity. This activity probably was not carried through the column in a receptor-bound form, because gel filtration of toxin without membranes gave the same host-specific activity in the first fraction after the void volume.

A relatively crude toxin preparation inhibited ATPase activity from both resistant and susceptible roots; toxin purified by thin-layer chromatography was not inhibitory. Toxin-treated microsomes from susceptible and resistant roots bound 5 to 27 and 1 to 7 per cent more N-ethylmaleimide, respectively, than did the controls without toxin. These data do not confirm previous results indicating that toxin caused a decrease in N-ethylmaleimide binding by membrane preparations from susceptible but not from resistant plants.

No host-specific effect of HV-toxin on microsomal conformation was observed. A relatively crude toxin preparation caused an increase in absorbance of light by microsome suspensions prepared from both resistant and susceptible plants. This response is characteristic of either shrinkage or agglomeration of vesicles. A reduction in the flow

rate of a microsome suspension through a Millipore filter (0.45 micron pore size) was observed with microsomes from both resistant and susceptible plants. Presumably, the vesicles had agglomerated and plugged the filter pores. Microsomes treated with toxin purified by thin-layer chromatography caused no plugging.

The protection phenomenon previously ascribed to esterified toxin appears to be caused by some effect of methanol plus HCl, other than esterification. Diazomethane, which should esterify toxin, also inactivated it; the product failed to protect against active toxin.

Pretreatment with several compounds that block cytoplasmic protein synthesis caused tissues to lose sensitivity to toxin. Chloramphenicol, an inhibitor of organelle protein synthesis, gave no protection against toxin. These results suggest that a protein receptor may be synthesized in the cytoplasm. A variety of SH-binding reagents protected tissue from toxin. Non-ionic reagents which penetrate the membrane gave greater protection than ionic reagents which do not readily penetrate the plasma membrane. This suggests that the toxin sensitive site may not be located on the outside of the membrane.

None of the results indicate that HV-toxin is firmly bound to a receptor. On the basis of present information, the toxin-receptor association would appear to be more like that of H. maydis T toxin which has a readily reversible association with a mitochondrial site.

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Kenneth E. Damann, Jr.

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To fine parents:

Kenneth and Donelda Damann

Albert and Cora Koenig

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The understanding and aid of my wife, Catherine Ann, during the course of this work is appreciated. The enjoyment provided by my son, Kenneth III, also served as additional encouragement.

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LIST OF ABBREVIATIONS

Amo 1618	2' isopropyl 4'-(trimethylammonium chloride)-5-methyl-phenyl piperidine carboxylate
ATP	Adenosine 5' triphosphate
CCC	β-chloroethyl trimethylammonium chloride
cpm	Counts per minute
EDTA	Ethylene diamine tetraacetic acid
GA	Gibberellin
HV	*Helminthosporium victoriae*
MES	2-N-morpholino ethane sulfonic acid
NEM	N-ethylamaleimide
P_i	Orthophosphate
PPO	2,5-diphenyloxazole
POPOP	1,4-bis 2-(5-phenyloxazolyl) benzene
UV	Ultraviolet

INTRODUCTION

Host-specific toxins are required for pathogenicity and determine host-specificity of at least nine fungi which cause plant disease (4, 40, 52). The same visible, physiological, and biochemical symptoms caused by these fungi are also produced by their toxins alone. The toxins appear to be essential for colonization and establishment of the parasitic relationship (3, 39, 51).

The single gene control of sensitivity in oats to the toxin from Helminthosporium victoriae Meehan & Murphy (HV-toxin) (20) indicates that a specific site is affected. This initial specific interaction may then mediate the changes which are recognized as disease. Thus, it should be possible to study the chemical basis of resistance and susceptibility in plants by studying the initial interaction of toxins with susceptible and resistant tissues, cells, organelles, or organelle components.

Two general types of toxin-receptor interaction are indicated from work with other toxins: 1) Strobel concluded that H. sacchari toxin is firmly bound to proteins in the cell membrane (42); 2) Bednarski has shown that H. maydis T toxin is not firmly bound to the mitochondrial site, as indicated by the fact that mitochondria quickly recover when toxin is washed out (1). Some of my experiments were designed to determine whether HV-toxin follows one or the other of these patterns.

Previous work leaves no doubt that the plasmalemma of susceptible but not of resistant oats is affected by HV-toxin (36). However, there is still no direct evidence that the initial site of action is in the plasmalemma. I have attempted to obtain direct evidence via two approaches: 1) experiments designed to show whether or not toxin is bound to plasmalemma preparations from susceptible but not from resistant cells; 2) experiments designed to show whether or not toxin causes changes in some function or property of plasmalemma from susceptible but not from resistant cells. I have also used a third and less direct approach to understanding toxin sensitivity, which involved a study of the protective effects of certain compounds against toxin action.

The experiments outlined above were not possible without a rapid bioassay and highly purified toxin, neither of which was available at the time. Thus, my first concern was to develop an improved assay and a simple method of producing homogeneous toxin. Once a satisfactory assay and homogeneous toxin were available, I re-examined several hypotheses which attempt to explain the interaction of toxin with tissues. First, I considered the possibility that resistance and host-specificity are based on toxin inactivation, as first suggested by Romanko (33). Second, the possibility that toxin is bound by susceptible but not by resistant tissues was examined by assaying for toxin in ambient solutions after exposure to resistant and susceptible tissues. Third, attempts were made to detect possible binding of toxin to cell-free membrane fragments. The effects of HV-toxin on ATPase activity, light scattering, flow rate through Millipore filters, and N-ethylmaleimide binding by isolated membranes were examined to determine whether or not a toxin receptor or reactive site is located in the plasmalemma.

Finally, I have made further studies of the protective effects of certain compounds against the effects of HV-toxin *in vivo*. The purpose was to gain some understanding of toxin-sensitive sites in the cell by an indirect approach. HV-toxin was inactivated by treatment with methanol-HCl, which is known to esterify carboxyl groups in proteins (8). The products of this treatment protected tissues from the effects of active toxin. Is altered HV-toxin the protective molecule? If so, can the effects be traced to esterification of the molecule? Cycloheximide, a protein synthesis inhibitor, was previously shown to protect tissue against toxin-induced leakage (10). I have examined several protein synthesis inhibitors to determine whether or not they protect tissues in a similar manner. A variety of SH-binding reagents were also tested for their ability to protect tissues against toxin-induced loss of electrolytes.

An Appendix to the thesis is included, which describes attempts to introduce ^{14}C into the toxin molecule by biosynthesis. The feasability of the tracer approach for determining the site of action of HV-toxin is discussed.

Victoria blight of oats appeared after the Vb gene for crown rust resistance was introduced into the genome of commercial oat varieties. This pleiotropic gene conferred susceptibility to a previously unknown fungus. Meehan and Murphy first identified the causal fungus (Helminthosporium victoriae) in 1946 and showed that it produces a host-specific toxic principle (21, 22). This is an example of a fairly common situation in plant pathology, in which a disease problem is precipitated by a change in the genome of a crop plant.

H. victoriae produces high titers of host-specific toxin when grown in Fries' No. 3 medium. Luke and Wheeler (19) developed an assay based on inhibition of seedling root growth and showed that amount of toxin production in culture varied with different isolates. Maximum yields were obtained after 13-25 days in culture. Luke and Wheeler also showed that the ability of isolates to produce toxin was correlated with pathogenicity. Toxin was slowly inactivated in solutions above pH 4.0.

Pringle and Braun isolated a single host-specific toxin from culture filtrates in yields of approximately 1 mg per liter of filtrate (26). The toxin was inactivated by mild alkaline hydrolysis, and two ninhydrin positive components appeared (27). One of the products is a tricyclic secondary amine ($C_{17}H_{29}NO$) (25), known as victoxinine. Free victoxinine is also produced by H. victoriae and other Helminthosporium species in culture (28). The other ninhydrin positive component, a peptide, was hydrolyzed to aspartic acid, glutamic acid, glycine, valine, and one of the leucines. The molecular weight of HV-toxin is 867 daltons, if one set of amino acids is assumed (25). More recently, the structure

of victoxinine was determined by partial synthesis from prehelminthosporol. Several biosynthetic intermediates were found in culture filtrates of H. victoriae (6). The structure of the peptide portion of the toxin and the mode of attachment of the peptide to victoxinine are not known.

Toxin is rapidly inactivated when taken up by leaf cuttings, as shown by Romanko (33). Some toxin was recovered from susceptible cuttings, but none was recovered from resistant cuttings. Romanko hypothesized that resistance is based on ability to inactivate toxin. Scheffer and Pringle found no toxin activity in homogenates of resistant or susceptible cuttings which had taken up toxin in the transpiration stream (38). They proposed that toxin was bound to a receptor which then mediated toxic action in susceptible but not in resistant tissue. Wheeler (48) found that resistant and susceptible coleoptiles inactivated toxin at equal rates for the first 8 hours. After 8 hours the rate of toxin inactivation by resistant coleoptiles was greater than inactivation by susceptible coleoptiles.

Electrolyte losses are increased from toxin-treated susceptible but not from resistant tissue, as first shown by Wheeler and Black (49). Later work (35) showed that increased loss of electrolytes occurs almost at once following exposure of susceptible tissue to toxin. This suggested that the primary site of toxic action may be in the plasma membrane. Toxin has no effects on function or properties of isolated mitochondria (2, 37). Further work with protoplasts, apparent free space of tissues, and plasmolytic ability of root hairs leaves little doubt that the plasmalemma is affected at a very early time after exposure to toxin (36). Keck and Hodges, using a compartmental analysis technique, concluded that permeability of both plasmalemma and tonoplast are

affected by HV-toxin (14). These workers were not able to determine whether or not the primary effect was on one membrane; a direct effect on both membranes is possible.

Various chemical treatments affect the ability of susceptible tissue to respond to toxin (10, 35). Pre-treatment of leaf tissue with certain sulfhydryl binding compounds resulted in a 70 to 90% decrease in sensitivity to toxin, as determined from losses of electrolytes. These compounds gave no protection against leakage inducing agents other than toxin. N-Ethylmaleimide binding to membrane fragments from susceptible tissue was decreased by pretreating membranes with HV-toxin. This was the first report of an effect of a host-specific toxin on a cell-free system. It suggests that a toxin receptor is present in membranes and that toxin causes a decrease in accesible SH groups or NEM-binding sites. Pre-treatment with toxin did not affect binding of NEM by resistant membranes (9). The toxin-sensitive site in the susceptible cell may be a protein, as indicated by experiments with cycloheximide (10). Pre-treatment of tissues for 12 hr with cycloheximide resulted in a 70 to 90% decrease in toxin-induced loss of electrolytes. Tissues removed from cycloheximide regained sensitivity after 48 hr.

Treatment of toxin preparations with 0.2N HCl in methanol destroyed the ability of toxin to induce losses of electrolytes from tissues (9). This treatment is known to form methyl esters with carboxyl groups of proteins. The glutamic or aspartic acid carboxyl groups in the toxin molecule could be sensitive to this esterification. The presumed esterified toxin gave 40 to 60% protection against leakage induced by active toxin. This finding suggests that the carboxyl group is necessary

for toxin action, and that the presumed methyl ester derivative of toxin blocks the receptor site for active HV-toxin (9).

Another host-specific toxin, helminthosporoside, is involved in the eyespot disease of sugarcane caused by Helminthosporium sacchari (Van Breda de Haan) Butler (41). Steiner and Strobel developed an assay for helminthosporoside, based on the induction of runner lesions in susceptible leaves (41). They purified helminthosporoside by paper chromatography using the bioassay; yields were 7 to 9 mg per liter of culture medium. This toxin was characterized as 2-hydroxycyclopropyl D-galactopyranoside. Helminthosporoside was the major of two toxic compounds which were separated by paper chromatography or gel filtration (41).

Labelled helminthosporoside was produced in replacement cultures of H. sacchari when ^{14}C-sucrose was used as the substrate. Affected areas of leaves treated with ^{14}C-helminthosporoside were removed, homogenized, centrifuged, and the supernatant fractionated on Sephadex G-25. The elution pattern for susceptible tissue showed a small peak of radioactivity just after the void volume, followed by a large peak for the free ^{14}C-helminthosporoside. This indicated that the toxin was bound to a high molecular weight component which was excluded from the gel pores. The preparation from resistant tissue did not show a peak following the void volume; only the peak for free helminthosporoside was observed. Proteins from cell membranes of sugarcane were solubilized by incubation in Triton X-100 (1%); these proteins were mixed with ^{14}C-helminthosporoside and fractionated on a column of Bio-gel P-100. Preparations from susceptible plants had two major peaks of helminthosporoside binding. Preparations from resistant plants had no significant peaks other than the peak

for free helminthosporoside. The binding protein that eluted first had a molecular weight of 45,000 to 49,000 daltons, with 4 identical subunits of 11,700 daltons each (42). The amino acid sequence of the binding protein differed by 4 residues from a comparable protein isolated from resistant membranes which did not have toxin binding ability (43). This work should be confirmed, but it is the best evidence presented to date in support of the receptor hypothesis.

Otani, Nishimura, and Kohmoto (24) also have evidence that the host-specific toxin from Alternaria kikuchiana Tanaka binds firmly to a high molecular weight component from the susceptible pear. Leaves or fruit skins were infiltrated with toxin, homogenized in 1% Triton X-100 and centrifuged. The supernatant was fractionated on a column of G-50 Sephadex. When fractions immediately following the void volume were acidified with HCl, partitioned into ether, and placed on leaves, they gave the specific necrotic response characteristic of the toxin. This was interpreted as evidence for binding of toxin to a receptor. No toxin appeared to be bound by resistant tissue; only free toxin was eluted from the column. In contrast to these findings, Bednarski et al (1) reported that H. maydis T toxin is not firmly bound to the mitochondrial site.

Strobel (42) and Otani et al (24) have assumed that their preparations contained plasma membrane fragments. No evidence was presented that this is the case. However, a method was developed by Hodges et al (11), and evidence for the presence of plasma membrane fragments was presented. The fragments had K^+-stimulated ATPase activity characteristic of plasmalemma. They also had a high sterol to phospholipid ratio, high glucan synthetase activity, and took a stain which is specific for plant plasmalemma (12). More than 75% of the preparation was said to be derived from plasmalemma.

MATERIALS AND METHODS

Plant culture.--Oat cultivars 'Park' and 'Garry' were used as sources of tissue in all experiments. 'Park' contains the Vb gene and is susceptible to H. victoriae; 'Garry' contains the recessive allele and is resistant. Seeds for the root growth bioassay were hulled and germinated overnight at 27° on moist filter paper. Seedlings for electrolyte leakage assays were grown in vermiculite watered with a nutrient solution (50), at 21-23° under Gro-lux lamps with a 12 hr photoperiod. Unless otherwise stated, the first fully expanded true leaf was used.

Roots used for isolation of microsomes were grown in mist culture after the method of Hodges and Leonard (11). Oat seeds (20 g) were placed in water for 2 hr, then were incubated 24-48 hr at 27° on moist filter paper. Seedlings were placed between two layers of cheesecloth on an 8 gauge wire screen. The screen was placed on top of a 4 liter beaker that contained 2.5 liters of 1 mM $CaSO_4$. The $CaSO_4$ solution was stirred vigorously with a stream of compressed air warmed by pre-bubbling through water at 40°. Roots were 15 cm long after 5-6 days in the dark.

Production and purification of HV-toxin.--Stock cultures of H. victoriae were maintained on potato dextrose agar slants. To produce toxin, the fungus was grown for 3 weeks at 22° in 1 liter Roux bottles containing 200 ml of Fries' No. 3 medium (19) supplemented with yeast extract (0.1%), or in 125 ml Erlenmeyer flasks containing 25 ml medium. Toxin was isolated from culture filtrates by the method of Pringle and Braun (26). Filtrates were concentrated in vacuo to 0.1 volume, equal parts of methanol were added, and the precipitate was discarded. After

methanol was evaporated, the filtrate was partitioned 3 times against n-butanol. The butanol extracts were combined, concentrated in vacuo, and an equal volume of methanol was added to the concentrate. This solution was passed through an alumina column to which the toxin was adsorbed. The column was rinsed with methanol and aqueous methanol before toxin was eluted with 1% acetic acid. This partially purified toxin preparation (pH 3.5) was stable when stored at 4°; it was the stock preparation used for further purification.

The stock preparation from the alumina column was chromatographed by the descending method on paper (26) and on thin-layer plates. Thin-layer chromatography was on 20 x 20 cm plates of silica gel GF (Analtech Inc., Newark, Delaware 19711) or on silica gel F-254 (Brinkmann Instruments Inc., Westbury, N. Y. 11590). Three solvent systems were used: n-butanol, acetic acid, water, (3:1:1 v/v); n-propanol, acetic acid, water (200:3:100); and 2-butanone, propionic acid, water (15:5:6). Developed plates were viewed under long (366 nm) and short (254 nm) wavelength ultraviolet light. Ninhydrin and chlorine-tolidine sprays were used to indicate possible contamination of the toxin preparation by amino acids and peptides (31). To detect toxin, sequential portions of the chromatograms were removed and each portion was placed in water for bioassay by the electrolyte leakage method.

Toxin was also fractionated by gel filtration, using Sephadex G-15 columns (1.5 x 25 cm). Toxin was eluted with water; fractions (2 ml each) were collected and bioassayed by the electrolyte leakage method. Toxin was also separated by cation-exchange chromatography on SP-Sephadex C-25; toxin was eluted from the column with 0.05 or 0.1 M sodium chloride in 0.033 M sodium citrate-citric acid buffer at pH 3.6. Impurities in the

toxin sample were eluted with buffer; the ionic strength of the buffer was then increased with NaCl for further elution. Two ml fractions were collected and assayed by the electrolyte leakage method.

The ionic state and purity of the toxin molecule was examined by high-voltage paper electrophoresis at two pH levels: pH 1.9, in an 8.7% acetic acid, 2.5% formic acid buffer; and pH 6.5, in a 10% pyridine, 0.4% acetic acid buffer. Sequential areas of the Whatman 3 MM paper were assayed for the presence of toxin by the electrolyte leakage method.

Bioassays.--One bioassay was based on inhibition of seedling root growth (29), using a series of toxin dilutions. Five hulled, germinated seeds with roots less than 0.5 cm long were placed in each 60 x 15 mm petri dish with 5 ml of toxin solution or water. The root length was determined after incubation for 3 to 5 days. The toxin dilution which held susceptible root growth to less than 1 cm was termed the dilution end point. Growth of resistant roots was not affected by toxin.

An assay based on toxin-induced leakage of electrolytes was developed (5). The usual procedure was to cut 1 cm sections from the leaves of 1 to 3 week old plants; 0.5 g samples were then enclosed in cheesecloth bags. The samples were vacuum-infiltrated in water for 10 min at 2 cm Hg. Usually 3 samples were incubated for 60 min in 100 ml of toxin solution or water in a 600 ml beaker, on a shaker at 120 reciprocations per minute. Samples were then rinsed for 10 min in distilled water with a conductance of 1 umho; each sample was leached in 50 ml water in 125 ml Erlenmeyer flasks on a shaker. Conductance of ambient solutions was determined at intervals with a conductivity bridge (model RC 16 B2 Industrial Instruments) using a dip type electrode with a constant of 1.0. Conductance in umhos was expressed by the equation:

$$Ls = \frac{Kc}{Rm}$$

(Ls=specific conductance in mhos; Kc=cell constant (1.0); Rm=resistance in ohms at 22°). Data are given as the means of 3 samples. In some cases the values for single samples are given, and some data are expressed as a rate of leakage (umhos/hr). The inhibition of toxin-induced loss of electrolytes was calculated as per cent protection by the formula of Gardner (9):

$$\text{Per cent protection} = 1 - \frac{(\text{umhos I} - \text{T}) - (\text{umhos I})}{(\text{umhos T}) - (\text{umhos W})} \times 100$$

(umhos IT, I, T, or W = conductivity of leachates from inhibitor plus toxin, inhibitor, toxin, or water-treated tissues, respectively).

Membrane isolation.--Cell membranes were isolated by the procedure of Hodges and Leonard (11, 12). Oat roots (25-50 g) grown in mist culture were excised and held for 15 min in ice cold distilled water. Roots were then cut into 1 cm pieces and ground in a mortar for 60-90 seconds with 4 ml of homogenization medium per g fresh weight of roots. The homogenization medium contained 0.25 M sucrose, 3 mM EDTA, 2.5 mM dithiothreitol, and 25 mM tris-MES (2-N-morpholino ethane sulfonic acid) at pH 7.2. The homogenate was strained through cheesecloth and centrifuged at 13,000 g for 15 min (10,000 rpm in a Sorvall SS-34 head). The supernatant was then centrifuged at 80,000 g for 30 min, using the Spinco type 30 rotor at 30,000 rpm. The pellets were resuspended in fresh homogenization medium and combined into a single tube. This preparation was used as a source of membranes for NEM binding and ATPase experiments. To purify plasmalemma, the combined resuspended pellets were centrifuged again at 80,000 g for 30 min. The pellet was resuspended in a solution (2.5 ml) containing 30% sucrose (w/w) in 1 mM

tris-MES-$MgSO_4$ (pH 7.2). The suspension was layered on a sucrose gradient solution (8 ml 34% sucrose in buffer over 28 ml 45% sucrose in buffer) (16, 17) in a centrifuge tube. After centrifugation at 95,000 g for 2 hr (27,000 rpm in a SW-27 rotor) the band at the 34-45% sucrose interface was removed with a pipette. This band contained the purified plasmalemma fragments used in the light scattering and filter plugging experiments. The preparation was held at 2 - 5° throughout the procedure.

N-Ethylmaleimide binding assay.--The NEM binding assay was patterned after the procedure developed by Gardner (9). Toxin was an eluate from an alumina column; the preparation completely inhibited root growth at 0.035 ug/ml. Toxin (28 ug) in buffer was added to a suspension of membranes (13,000-80,000 g fraction, total volume 1 ml) and incubated for 10 min at room temperature. Controls contained buffer without toxin. The suspensions were then mixed with 0.5 ml of 0.3 mM NEM containing ^{3}H-NEM (5×10^5 cpm). After 15 min incubation, the reaction was stopped by adding 1.0 ml of ice cold 200 mM NEM. Homogenization medium (0.5 ml) was added to bring the total volume to 3.0 ml, and three aliquots (0.8 ml each) were removed. Ice cold 40% trichloroacetic acid (1 ml) was added to each aliquot, which was frozen and held overnight. The thawed, labeled preparation was filtered on a 2 cm Millipore prefilter (AP 2002000, Millipore Corp., Bedford, Mass. 01730) which retains proteins. The prefilter was moistened with 10% trichloroacetic acid prior to filtering, and the sample was washed 4 times on the filter with 15 ml portions of cold 10% trichloroacetic acid. Each sample on the prefilter was placed in a scintillation vial and dried at 50 to 60° for 1 hr. Ten ml of scintillation fluid (toluene containing 4 g PPO and 100 mg POPOP per liter was added to each vial. Vials were counted to 1% reliability in a Beckman LS-133 Liquid Scintillation Counter with 42% efficiency.

ATPase assays.--The method of Hodges and Leonard was followed with slight modifications (11). Tris-ATP was made from the Na salt in order to measure ion stimulated ATPase activity. Disodium ATP solution (6.66 mM) was adjusted to pH 2.0 with Dowex 50W-X-2 H^+ resin, which was removed by filtration on a Millipore filter. Tris crystals were added to bring the pH to 6.5, and the solution was frozen for storage. The buffer solution with KCl (111.0 mM) or without KCl contained 3.33 mM $MgSO_4$ and 73.4 mM tris adjusted to pH 6.5 with crystalline MES. The total substrate solution consisted of 2.3 ml buffer solution with or without KCl and 2.3 ml tris-ATP at 38°. Toxin, which completely inhibited seedling root growth at 0.035 ug/ml, was added to the total substrate solution to bring the final toxin concentration to 56 ug/ml. The membrane preparation (0.5 ml) was added to start the reaction, which was terminated at 5, 10, 15, or 20 minutes by pipetting 1 ml aliquots into 2 ml of ice cold 1% (w/v) ammonium molybdate in 2 N sulfuric acid.

Inorganic phosphate was determined by the method of Fiske and Subbarow (7). Seven ml of reducing agent solution (0.1 g 1-amino-2-napthol-4-sulfonic acid, 5.8 g sodium bisulfite, and 0.2 g sodium sulfite in 700 ml distilled water) was added to each aliquot. After 35 min at room temperature the absorbance at 660 nm was determined on a Gilford 240 Spectrophotometer fitted with a digital readout and a Gilson foot fed automatic pipetter. Results were calculated as umoles phosphate/hr/mg protein. Protein was determined by the method of Lowry et al (18).

Esterification procedures.--Two methods for methyl ester formation were used on the HV-toxin preparations. The first was the method of Fraenkel-Conrat (8), as modified by Gardner (9) for use with 0.2 N HCl in absolute methanol. Lyophilized toxin preparations were suspended in a

volume of methanol-HCl corresponding to the original volume of toxin solution eluted from an alumina column. After 48 hr at room temperature, the solutions were assayed for residual toxicity and for their ability to protect tissue from toxin-induced leakage of electrolytes.

Diazomethane was generated from 'Diazald' using the method given by the Aldrich Chemical Co. (Milwaukee, Wis. 53233), modified as suggested by J. A. D. Zeevaart. 'Diazald', ethanol, and KOH were used at 0.1 the amount suggested in the Aldrich method. Ethanol (95%, 2.5 ml) was added to a solution of KOH (0.5 g) in water (0.8 ml) in a 50 ml distilling flask fitted with a dropping funnel and an efficient condenser set downward for distillation. The condenser was connected to two receiving flasks in series, the second of which contained 20 to 30 ml of diethyl ether. The inlet tube of the second receiver dipped below the surface of the ether, and both receivers were cooled to 0°. The flask containing the alkali solution was heated in a water bath to 65° and a solution of 2.15 g (0.01 mole) of 'Diazald' (N-methyl-N-nitroso-p-toluenesulfonamide) in about 20 ml of ether was added through the dropping funnel in about 25 min. The rate of distillation equalled the rate of addition. When the dropping funnel was empty, another 4 ml of ether was added slowly and the distillation was continued until the distilling ether was colorless. The combined ethereal distillate contained about 0.3 g of diazomethane.

This diazomethane preparation was added to a methanol solution of toxin until a distinct yellow color was evident. The release of N_2 bubbles indicated that diazomethane was reacting with carboxyl groups in the preparation; esterification under these conditions is very rapid. Diazomethane and ether were evaporated under a stream of nitrogen. The preparation was then assayed and chromatographed on thin-layer plates.

Proper precautions were taken because diazomethane is explosive; all work was in a fume hood, and a safety shield was used. Ground glass fittings and scratched glassware were not used because they provide catalytic surfaces.

RESULTS

I. Development of an Assay Based on Toxin-Induced Leakage of Electrolytes

Effect of toxin concentration.--Gardner used toxin-induced leakage of electrolytes as the basis of a qualitative assay (9). His data were confirmed (Fig. 1) and the work was extended to develop a standardized quantitative assay for toxin. The toxin preparation was an eluate from an alumina column; its concentration at the dilution end-point in a seedling root growth assay was 0.16 ug/ml. Thus, the toxin preparation had relatively high activity, although it contained many impurities (25, 26). The danger inherent in use of impure preparations was overcome by use of resistant tissue controls in all cases. Leaf samples (0.6 g) were vacuum-infiltrated with water and incubated 60 min in various concentrations of HV-toxin (from 0.1 to 25 ug/ml) on the shaker at 120 reciprocations per minute. Triplicate samples were used for each treatment. Samples were rinsed for 10 min in distilled water and each sample was transferred to a flask of distilled water (50 ml; conductance, 1 umho) for leaching. Conductances of ambient solutions were taken at 30 min intervals. Results showed that the rate of toxin-induced leakage of electrolytes was constant for at least 3 hr, and that the leakage rate increased with increasing toxin concentration up to a saturating level (Fig. 2). The linearity allowed a rapid determination of HV-toxin concentration from 0.1 to 10.0 ug of this toxin preparation per ml of incubation solution. Toxin did not cause leakage from resistant tissue.

Effect of toxin exposure time.--Increased leakage from susceptible tissue was known to occur within 2 min after exposure to toxin (35). There were no data on the effect of length of incubation time on the rate

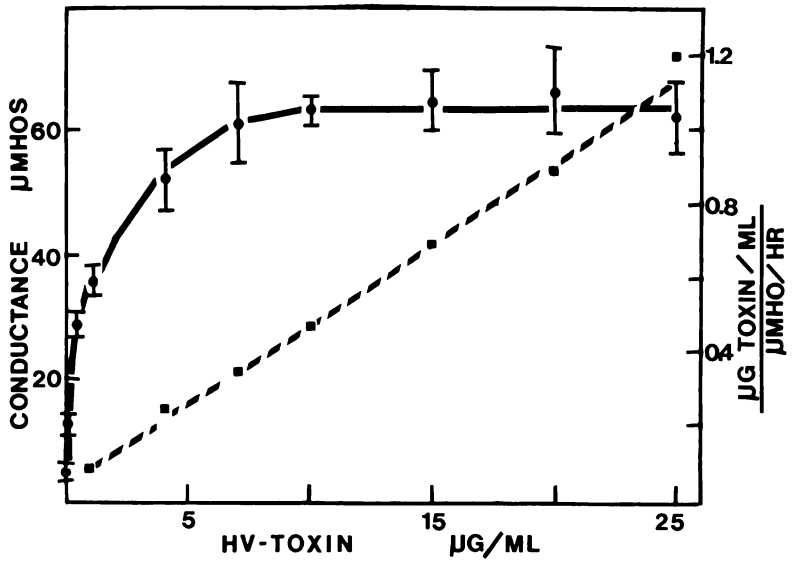


Fig. 1. Effects of HV-toxin at several concentrations on loss of electrolytes from oat leaves. Tissue samples (0.6 g each) were vacuum-infiltrated with water, incubated in toxin solution for 1 hr, rinsed, and leached for 3 hr in 50 ml distilled water for conductance measurements. Mean conductance and standard deviations are shown. Values for ambient solutions are plotted arithmatically (solid line, with scale to the left) and by the Woolf method (broken line, with scale to the right). The toxin solution completely inhibited susceptible seedling root growth at 0.16 ug/ml.

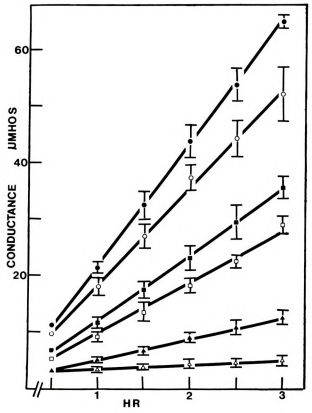


Fig. 2. Rates of electrolyte losses from oat leaves induced by HV-toxin at several concentrations. Data are from the experiment shown in Fig. 1. Toxin concentrations in ug/ml were: 0.1, ▲ ; 0.5, □ ; 1,0, ■ ; 4.0, ○ ; 10.0, ● ; and 0 (control), . Mean conductance and standard deviation are shown.

of leakage. Samples (0.6 g each) were vacuum-infiltrated with water and incubated in a subsaturating concentration of HV-toxin (1 ug/ml) for 1, 10, 30, 60, and 120 min. Samples were then rinsed and leached in the usual way. Leakage rates for the several exposure times were 2.5, 3.0, 6.5, 8.5, and 14.5 umhos/hr (Fig. 3). Thus, the rate of toxin-induced leakage increased with increases in toxin exposure time, indicating a time dependent diffusion of toxin to active sites throughout the tissue. Leakage in response to a saturating concentration of toxin (10 ug/ml) was 8.4, 14.7, 20.0, 25.0 and 21.7 umhos/hr for the same toxin exposure times, respectively. Maximum leakage in response to a saturating concentration of toxin was evident at 60 min, whereas longer times were required for maximum rate of leakage when subsaturating concentrations of toxin were used. The results for the 'uptake' of a saturating amount of toxin agree with the values reported for the accumulation of toxic amounts in the seedling root growth assay (38).

Effect of toxin-infiltration method on the rate of leakage.--Three procedures for infiltrating tissues with toxin were compared: a) Leaf samples were vacuum-infiltrated with toxin solutions for 10 min, incubated in the same solution for 50 min on the shaker, rinsed, and placed in water for leaching. b) Leaf samples were vacuum-infiltrated with water, transferred to toxin solutions for 60 min on the shaker, rinsed, and placed in water for leaching. c) Leaf samples were held in toxin solutions (without vacuum-infiltration) on the shaker for 60 min, rinsed, and placed in water for leaching. Three samples (0.6 g each) were used for each treatment. Leaching in distilled water (50 ml) was continued for 3 hr in all cases. Leaves infiltrated with toxin solution (10 ug/ml) (treatment a) changed conductance of ambient solutions at the

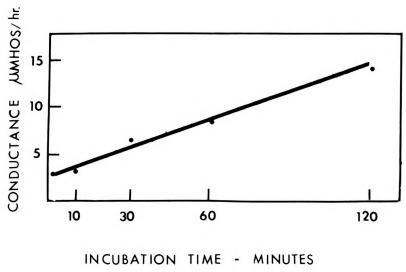


Fig. 3. Effect of time of incubation in toxin on subsequent rate of electrolyte loss from oat leaves. Samples (0.6 g each) were vacuum-infiltrated in water and incubated in a toxin preparation (1 ug/ml) for the times indicated. Samples were rinsed and leakage rates were determined from conductance of ambient solutions after a 3 hr leach. Each value is the mean of 3 samples. Each value was obtained by exposing a different set of samples to the toxin for the stated time.

rate of 18 umhos/hr, whereas leaves infiltrated with water and incubated in toxin solution (10 ug/ml) (treatment b) gave a value of 11 umhos/hr. Leaves treated with toxin but not with vacuum-infiltration changed conductance of the ambient solution at the rate of 7 umhos/hr. Control leaves infiltrated by vacuum with water did not have higher loss of electrolytes than did controls without vacuum-treatment (2-3 umhos/hr).

Methods a) and b) were compared in later experiments, using plants grown under slightly different conditions. Vacuum-infiltration with toxin at 1.0 and 10.0 ug/ml resulted in conductance changes at the rates of 15 and 24 umhos/hr, respectively. Vacuum-infiltration with water followed by a 60 min exposure to toxin (1.0 and 10.0 ug/ml) gave conductance rates of 8 and 16 umhos/hr.

Effect of size of leaf sections in the sample.--Another experiment which showed the complications of diffusion of toxin into leaves, or the efflux of electrolytes from leaves, was the effect of size of leaf sections in a sample. Leaves from 13 day old susceptible seedlings were cut into 2.0, 1.0, or 0.5 cm sections. Samples (0.6 g each) were vacuum-infiltrated for 10 min in water, incubated in toxin solution (10 ug/ml) or water for 60 min, rinsed, and monitored for electrolyte losses for 4 hr. Control tissues without toxin changed conductance of ambient solutions at the rate of 1 umho/hr regardless of the size of leaf sections. Toxin-treated leaf samples of 2.0, 1.0, and 0.5 cm sections changed conductance of ambient solutions at rates of 14, 26, and 32 umhos/hr respectively (Fig. 4). Thus, the rate of leakage is affected by the number of cut ends.

Effect of sample weight on toxin-induced leakage.--Leaf samples ranging from 0.1-2.0 g each were used to determine the effect of sample

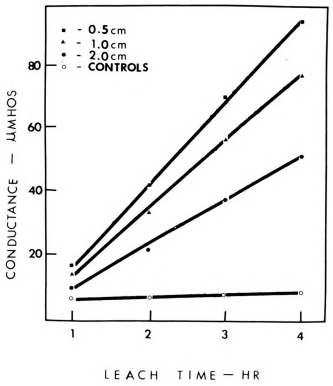


Fig. 4. Effect of size of leaf sections on toxin-induced loss of electrolytes. Samples (0.6 g each) of various leaf section lengths were vacuum-infiltrated with water, incubated in toxin solution (10 ug/ml) for 1 hr, rinsed, and leached. Conductance of ambient solutions was determined at the times indicated. The legend in the upper left indicates the length of leaf sections used in each sample. Controls in water leaked at the same rates regardless of section size.

weight on rate of toxin-induced loss of electrolytes. Tissues were vacuum-infiltrated with water, incubated for 60 min in a subsaturating toxin solution (1 ug/ml), or in water, rinsed, and monitored for electrolyte loss over a 3 hr period. Samples weighing 0.1, 0.5, 1.0, 1.5, and 2.0 g each changed conductance of ambient solutions by 0.4, 2.4, 5.0, 8.6, and 14.4 umhos/hr, respectively (Fig. 5). Calculations of conductance changes on a common weight basis gave values of 4.0, 4.8, 5.0, 5.7, and 7.2 umhos/hr/g (Fig. 5). The experiment was repeated with essentially the same results. The data indicate that a standard tissue sample weight is needed, and that large tissue samples may be better for detecting low concentrations of toxin.

Effect of leaf age on toxin-induced leakage.--Age of tissues is another variable to consider in toxin assays. Plants were grown in the laboratory for 6, 9, 12, 14, 18, or 21 days. Leaf samples from primary leaves were vacuum-infiltrated with water, incubated in toxin solutions (1.0 or 25 ug/ml) or in water for 1 hr on a shaker, and monitored for electrolyte loss. Age of plants had no effect on electrolyte loss from control leaves without toxin. Electrolyte loss from toxin-treated tissues varied with age of plants; primary leaves from 12-21 day old plants had greater losses than did leaves from younger plants (Fig. 6). The effect of age was more pronounced with a saturating (25 ug/ml) than with a subsaturating (1 ug/ml) concentration of toxin.

Effect of supplemental mineral nutrients on toxin-induced loss of electrolytes.---Oats were grown 12 days in vermiculite plus water or White's solution. Leaf samples (0.5 g each, 1 cm sections) were vacuum-infiltrated in water and incubated for 60 min in a saturating concentration (10 ug/ml) of HV-toxin. Three samples were used for each treatment.

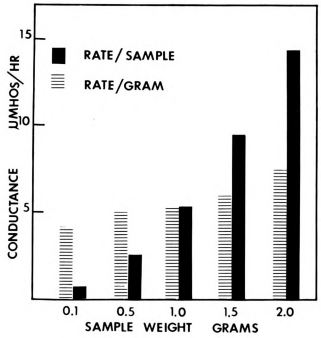


Fig. 5. Effect of sample weight on the rate of toxin-induced loss of electrolytes. Samples were vacuum-infiltrated in water, incubated in toxin solution (1 ug/ml) for 1 hr, rinsed, and leached for 3 hr.

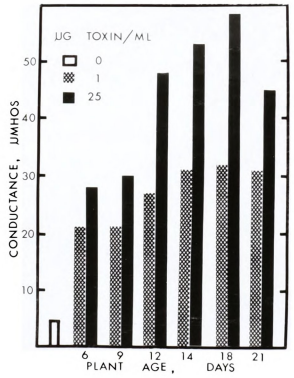


Fig. 6. Effect of leaf age on toxin-induced loss of electrolytes. Leaf samples (0.5 g each) from seedlings of ages indicated were vacuum-infiltrated in water, incubated in toxin solutions (1 or 25 ug/ml) or in water for 1 hr, rinsed, and leached for 3 hr. The legend in the upper left indicates the toxin concentrations used. Leakage from control samples of all ages was the same in the absence of toxin (left).

After rinsing, leakage rates were determined over a 3 hr period (Fig. 7). Toxin-induced leakage from plants grown in White's solution was twice the rate from plants grown without supplemental nutrients. This higher rate of leakage expands the scale within which differences in rate can be observed. This experiment also included a comparison of leakage from plants grown under continuous light, with plants grown under 12 hr light. These light treatments did not affect toxin-induced leakage of electrolytes.

Electrolyte loss vs. root growth assays.--HV-toxin from an alumina column was diluted 10^5 (0.42 ug/ml), 10^6, 10^7, 10^8, 10^9, and 10^{10} times for assay by the seedling root growth method. Susceptible control roots averaged 55 mm in length after 4 days. Roots in the toxin dilution series averaged 4, 7, 14, 23, 48, and 41 mm, respectively, after 4 days. A dilution end-point which gives 50% inhibition of growth is not easily established in repeated assays; therefore, I used the dilution (10^6) which limited growth to 10 mm as the end-point.

The same toxin dilutions were used to treat leaf tissue for the electrolyte leakage assay. Leaf samples (0.6 g each) from 16 day old plants were vacuum-infiltrated with water, incubated 4 hr in 100 ml toxin solution on the shaker, rinsed, and monitored for electrolyte loss. Tissues exposed to toxin at 10^5, 10^6, and 10^7 dilutions, or to water, changed the conductance of ambient solutions at rates of 14, 7, 3, and 2 umhos/hr, respectively, over a 3 hr period. Significant increases in leakage were not detected from tissues treated with more dilute toxin solutions. The dilution end-point for root growth inhibition bioassay was 10^6; this was also the highest dilution which caused detectable electrolyte leakage. Essentially the same results were obtained in two experiments.

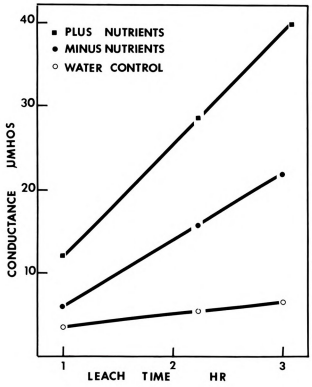


Fig. 7. Effect of supplemental nutrients given to growing seedlings on toxin-induced loss of electrolytes. Leaf samples (0.5 g each) were vacuum-infiltrated, incubated in toxin solution (10 ug/ml) for 1 hr, rinsed and leached 3 hr. Conductance of ambient solutions was determined at the intervals indicated. The "water control" samples were not exposed to toxin. Each value is the mean of 3 samples.

Effect of temperature during the leaching period.--Leaf samples (0.5 g each) were incubated in 100 ml toxin solution (10 ug/ml) for 1 hr on the shaker at 24°. Samples were then rinsed in distilled water and leached for 1.5 hr in 50 ml of water at either 11° or 24° (3 samples at each temperature). Since temperature affects the measurement of conductance, the data were converted to conductance at 25°. Results showed more electrolyte loss at 24° (19 umhos) than at 11° (7 umhos). The calculated Q_{10} value was 2.1, which confirms Gardner's results (9). Previous data (38) indicated that temperature over a wide range (5 to 37°) during the time of exposure to toxin had little effect on later growth of seedling roots. If growth and leakage experiments can be compared, it seems likely that the rate of leakage rather than the rate of a toxic reaction is affected by temperature.

II. Purification of HV-Toxin

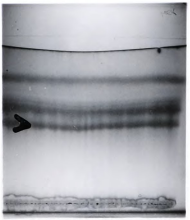
Toxin was purified by previously described methods (26) and by new methods. The leakage assay was much better than the root growth assay for guiding the isolation; the root assay could not be used to locate toxin on thin-layer chromatograms, or to assay other very small samples.

Paper chromatography.--A toxin-containing eluate from an alumina column was chromatographed on Whatman No. 1 paper by the descending method described by Pringle and Braun (26). The solvent was n-propanol, acetic acid, water, (200:3:100 v/v). Most activity was recovered from the R_f 0.7 area, as determined by direct electrolyte leakage assay of sequential segments of the chromatogram. This confirms previous findings (26). Some activity was spread over the R_f 0.5-0.8 area; this area also

contained several ninhydrin positive and ultraviolet fluorescing and absorbing bands. HV-toxin is reported to have no such ninhydrin positive, fluorescing, or absorbing properties. Thus, paper chromatography did not give good separation of toxin from contaminating materials.

Thin-layer chromatography of HV-toxin.--An attempt was made to separate toxin from contaminants by use of thin-layer chromatography. The toxin from an alumina column was spotted on silica gel GF or on silica gel F-254 plates which contained a fluorochrome that fluoresced on exposure to ultraviolet light at 254 nm. Chromatograms were developed with n-butanol, acetic acid, water (3:1:1 v/v); with n-propanol, acetic acid, water (200:3:100 v/v); or with 2-butanone, propionic acid, water (15:5:6 v/v). The chromatograms showed characteristic quenching of fluorescence at 254 nm (Fig. 8). Areas of the chromatograms (at R_f 0.45 for the butanol, at R_f 0.60 for the propanol, and at R_f 0.55 for the butanone solvents) contained toxin which induced electrolyte leakage. This was determined by scraping areas from the plates into 50 ml of water for assay. There was a correlation between the area of the chromatogram that gave quenching, and the area that induced leakage from susceptible but not from resistant tissues (Tables 1 and 2). These experiments were repeated many times with essentially the same results.

This procedure made possible a rapid (4 hr) separation of toxin from contaminants in preparations from an alumina column. In addition, the amount of toxin in a preparation was correlated with the size and intensity of the quenching area on the chromatogram, as demonstrated by ability to induce leakage. Toxin preparations with low activity did not give discernable quenching in the appropriate R_f area unless a large volume of solution was used on the chromatograms. Apparently, lower



a b c

Fig. 8. Quenching of UV-induced fluorescence on thin-layer chromatograms characteristic of HV-toxin. Three solvent systems were used: a) n-butanol, acetic acid, water (3:1:1), (R_f of toxin, 0.45); b) n-propanol, acetic acid, water (200:3:100), (R_f 0.60); c) 2-butanone, propionic acid, water (15:5:6), (R_f 0.55). The arrows indicate the position of the quenching area which caused leakage from susceptible but not from resistant tissue.

Table 1. Correlation of Toxin Activity with Ultraviolet Quenching on Thin-Layer Chromatograms

A one cm^2 area of the quenching band (Q) and 3 sequential areas (each, 1 cm^2) above (+) and below (-) the quenching zone were removed and placed in 50 ml distilled water. Each zone was assayed with susceptible tissue by the electrolyte leakage method. Three solvent systems were used: BAW = n-butanol, acetic acid, water (3:1:1); PAW = n-propanol, acetic acid water (200:3:100); BPW = 2-butanone, pripionic acid, water (15:5:6).

Location	Conductance in umhos		
	BAW	PAW	BPW
+3cm	9	7	8
+2	9	8	10
+1	12	15	12
*Q	61	48	50
-1	22	19	18
-2	13	13	12
-3	13	12	10

Table 2. Recovery of Host-Specific Toxin from an Ultraviolet Quenching Area of a Thin-Layer Chromatogram

The chromatogram was developed with n-butanol, acetic acid, water (3:1:1). A cm^2 area from the quenching zone was placed in 50 ml water and assayed for ability to induce leakage of electrolytes from resistant and susceptible tissue. Tissues were leached for 3 hr prior to conductivity determinations of ambient solutions. The control was a toxin-free area from the chromatogram.

Tissue Type	Quenching zone umhos	Control umhos
Susceptible	50	5
Resistant	6	7

concentrations of toxin can be detected by bioassay than by quenching; thus, a relatively high threshold concentration may be needed to observe quenching. However, as little as 0.01 ml of the toxin preparation from alumina caused quenching; this toxin preparation had a dilution end point of $1:10^6$ (0.04 ug/ml) in the root assay. There was no other proof that HV-toxin was responsible for the quenching, but the technique was useful in detecting and purifying HV-toxin. Toxin was eluted from silica gel with distilled water and adjusted to pH 3.6 with HCl. Silica gel was removed from the suspension by filtration through a 0.22 u Millipore filter. At least 50% of toxin applied to a chromatogram was recovered by this method, as determined by electrolyte leakage assay.

Gel filtration.--Previously, (9) HV-toxin was separated on 1.5 X 25 cm columns of Sephadex G-10, G-15, or Bio-gel P-2. Ten ml fractions were collected in each case, and toxin was present in the third fraction. Thin-layer chromatography of each toxin-containing fraction revealed a variety of fluorescing, absorbing, and ninhydrin-positive contaminants. Toxin separation was improved when two ml fractions were collected and assayed; in this case, toxin was present in fractions 10 through 12. However, these fractions also contained other materials, as revealed by thin-layer chromatography. Again, there was a correlation between toxin activity in fractions from the column and the appearance of quenching on thin-layer plates, at the R_f characteristic of toxin.

Electrophoresis of HV-toxin.--The possibility of separating toxin from contaminants by use of high-voltage paper electrophoresis was tested. One ml of a toxin preparation (28 mg/ml) from an alumina column was spotted in a 20 cm strip on Whatman 3MM paper (27 cm wide). Two buffers were used: a) 8.7% acetic acid, 2.5% formic acid (pH 1.9); and

b) 10% pyridine, 0.4% acetic acid (pH 6.5). Electrophoresis in buffer a was conducted for 2 hr at 120 mA and 5 kV, whereas electrophoresis in buffer b was conducted for 2 or 3 hr at 80 mA and 2 kV. Papers were dried and cut into sequential 1 in^2 sections. Each section was placed in 50 ml of water with 0.5 g of susceptible tissue, for electrolyte leakage bioassay. Results of the bioassays showed that toxin migrated as a cation at pH 1.9, and as an anion at pH 6.5 (Table 3). The experiment was not repeated and neutral standard was not used.

Cation-exchange chromatography of HV-toxin.--Cation-exchange chromatography is a logical method of purifying toxin, since the molecule behaves as a cation at pH 1.9. SP-Sephadex, a strong cation exchanger, was used with 0.033 M sodium citrate-citric acid buffer at pH 3.6. Toxin was not eluted when a 1.5 x 25 cm column was developed with 50 or 100 ml of buffer. When columns were developed with buffer solutions containing NaCl (0.05 or 0.1 M) and 2 ml fractions were collected, toxin was present in the 10^{th} to 20^{th} fractions after NaCl was added (Fig. 9). When toxin-containing fractions from the column were chromatographed on thin-layer plates, the ultraviolet quenching zone always appeared at the R_f for toxin. Fractions without toxin never gave the ultraviolet quenching zone on thin-layer chromatograms. Thin-layer chromatograms of toxin containing fractions from the column had no spots that were ninhydrin positive, and there was no evidence of materials that fluoresced in ultraviolet light. At least 75% of toxic activity was recovered from the cation column as estimated by the leakage bioassay.

Methanol precipitation of contaminants in a toxin-containing eluate from an alumina column.--These toxin preparations from alumina columns contained a variety of contaminating compounds, as shown by previous

Table 3. Electrophoretic Migration of HV-Toxin at pH 1.9 and pH 6.5, as Shown by Electrolyte Leakage Assays

Electrophoresis in experiments 1, 2, and 3 was respectively for 2 hr at 120 mA and 5 kV, 2 hr at 80 mA and 2 kV, and for 3 hr at 80 mA and 2 kV. Position numbers indicate the position on the paper (3MM), in sequential square inch pieces from the origin (0, ± ½ inch), toward the anode (+) or cathode (-). Samples of susceptible tissue were incubated for 1 hr with solutions eluted from each position, rinsed, and leached for 3 hr; conductance values are for ambient solutions.

pH 1.9 Buffer		pH 6.5 Buffer		
Position	Conductance umhos Exp 1	Position	Conductance umhos Exp 2	Exp 3
-1	4	+4	4	7
-2	4	+3	4	6
-3	4	+2	5	16
-4	4	+1	22	10
-5	4	0	19	6
-6	4	-1	7	4
-7	4	-2	5	4
-8	7	-3	4	5
-9	18	-4	6	4
-10	18			
-11	6			
-12	4			

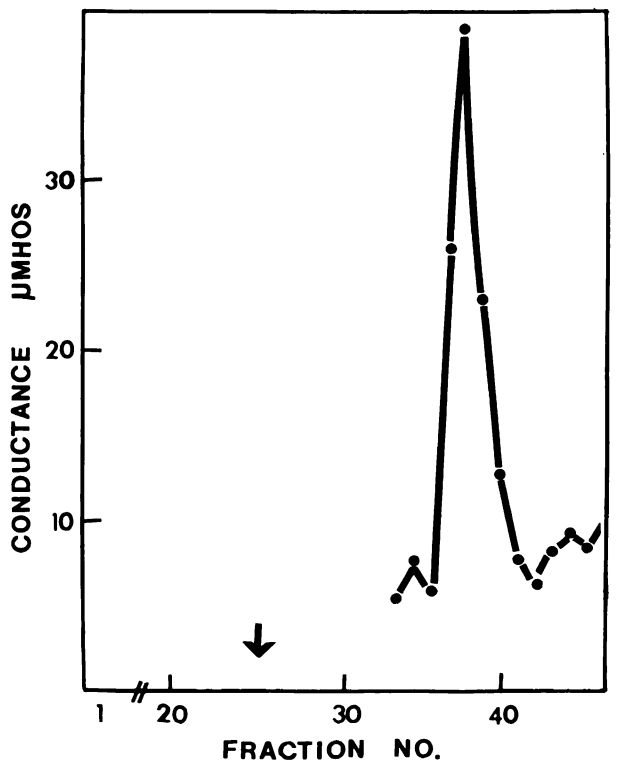


Fig. 9. Toxin content of fractions from the cation-exchange column. Toxin from an alumina column (0.5 ml containing 21 mg dry weight) was chromatographed on a 1.5 x 25 cm column of SP-Sephadex. The column was developed first with 50 ml of 0.033 M sodium citrate-citric acid buffer (25 fractions). Buffer containing 0.1 M NaCl was then added (arrow) and the column was developed further. Aliquots (0.01 ml) of the fractions were incubated in 50 ml water with susceptible tissue samples (0.5 g each) for 2 hr. Samples were then rinsed and leached for 3 hr; conductance of ambient solutions was determined.

experiments. One ml of toxin solution (16 mg) from the alumina column was evaporated to dryness, and 16 ml absolute methanol was added. A precipitate appeared and was removed by centrifugation for 20 min at 13,000 g. The supernatant was assayed by the electrolyte leakage method; no activity was lost and much inert material was removed. Previously, it was reported that drying results in loss of activity (26); my preparation appears to be more stable.

III. Toxin Inactivation and Possible Binding by Tissues and Isolated Membranes

Recovery of HV-toxin from resistant and susceptible oat leaves.-- Romanko (33) concluded that specificity of HV-toxin is based on ability of resistant tissue to completely inactivate toxin. Others (38) were not able to confirm this. Therefore, I have re-examined the question of toxin inactivation by tissues, using the assay based on electrolyte leakage rather than the assay based on inhibition of root growth.

First, is toxin inactivated or adsorbed when added to homogenates of resistant or susceptible leaves? Leaf samples (1 g each) were homogenized with an Omni-mixer in 100 ml water. HV-toxin from an alumina column was added to each homogenate so that the final toxin concentration was 7 ug/ml; this was a near-saturating concentration of toxin. Water controls and toxin-containing controls were used. After 30 min, the homogenates were assayed for toxin content by the electrolyte leakage method. Results (Table 4) show that equal activity was recovered from homogenates of resistant or susceptible leaves or from control toxin solutions. Thus, activity was not lost when toxin was added to tissue homogenates. The experiment was not repeated.

Table 4. Recovery of Toxin Added to Homogenates of Resistant and Susceptible Leaves, as Indicated by the Electrolyte Leakage Assay

Resistant and susceptible cuttings (1 g each) were homogenized in 100 ml water. Toxin (7 ug/ml) was added to the homogenates and to water as a toxin control. After 30 min the homogenates and toxin control were assayed for toxin content. Tissue samples (0.6 g each) were incubated for 1 hr with each treatment, rinsed, and leached for 3 hr; conductance of ambient solutions was determined. Each value is the mean for 3 samples, with a standard deviation.

Treatment	Conductance umhos
Water control	4.3 ± 0.3
Susceptible homogenate + toxin	37.7 ± 3.0
Resistant homogenate + toxin	39.0 ± 5.0
Toxin control	40.7 ± 3.0

Is toxin inactivated during the process of homogenization? One g of susceptible tissue was homogenized in 100 ml water containing the same HV-toxin preparation (7 ug/ml). The homogenate was centrifuged for 10 min at 13,000 g and the supernatant was separated into 50 ml portions. Each portion was assayed for toxin activity using resistant and susceptible tissues (0.6 g each). Toxin (7 ug/ml) and water control solutions were included. Tissues were incubated for 1 hr, rinsed, and leached as previously described. Results (Table 5) show that HV-toxin was not inactivated by homogenization for 60 seconds. Thus, toxin is not inactivated during homogenization. The experiment was not repeated.

Recovery of HV-toxin from resistant and susceptible tissue was then compared. One g of susceptible or resistant leaf cuttings was placed in 5 ml of toxin solution (500 ug/ml). After 80 min under light the resistant cuttings took up 0.8 ml of toxin solution and the susceptible cuttings took up 0.6 ml. The cuttings were removed from solution, rinsed, homogenized in 100 ml water, and centrifuged as previously described. The supernatant was assayed for toxin content. Standards for comparison were prepared by assaying control toxin solutions at 0.1, 1.0, and 10 ug/ml. Results (Table 6) show 33% recovery of toxin from susceptible and 25% from resistant homogenates. This experiment was repeated with similar results. These data do not support the hypothesis that resistance is based on inactivation of toxin.

The toxin recovery experiment was repeated with cuttings that took up toxin solution for 5 hours rather than 80 minutes. Control cuttings took up 2.3 ml water, whereas resistant and susceptible cuttings took up, respectively, 1.8 and 0.8 ml of toxin solution (500 ug/ml). Lower uptake by susceptible cuttings probably results from stomatal closure (45) and a

Table 5. Recovery of Toxin Present During Homogenization of Tissues, as Indicated by the Electrolyte Leakage Assay

One g of susceptible oat leaves was homogenized in 100 ml toxin solution (7 ug/ml) and centrifuged. The supernatant was divided into two equal portions and assayed for toxin content. One assay sample (0.6 g) of resistant or susceptible tissue was placed in each 50 ml portion, incubated 1 hr, rinsed, and leached 3 hr before conductance was determined for the ambient solution.

Treatment	Conductance of solutions from:	
	Resistant	Susceptible
	umhos	
Water control	3.5	4.6
Homogenate	6.0	42.0
Toxin control	7.5	39.0

Table 6. Recovery of HV-Toxin Activity after 80 min Uptake by Resistant and Susceptible Oat Leaf Cuttings

Cuttings (1 g, approximately 10 leaves) of resistant and susceptible oats took up the volumes of toxin solution (500 ug/ml) as indicated. Cuttings were then homogenized in 100 ml water and centrifuged. The supernatants for each treatment were assayed for toxin by the electrolyte leakage method, using susceptible leaf samples (0.6 g each). The assay samples were incubated for 1 hr, rinsed, and leached for 3 hr. Conductance of ambient solutions was determined; each value is the mean for 3 samples.

Treatment	Amount taken up:		Conductance	Recovery:	
	ml	ug[a]	umhos	ug	%
Water control	-	-	6.2	-	-
Toxin (0.1 ug/ml)[a]	-	-	10.1	-	-
Toxin (1.0 ug/ml)[a]	-	-	24.8	-	-
Toxin 10 ug/ml)[a]	-	-	41.5	-	-
Homogenates from:					
Resistant cuttings	0.8	400	23.5	100	25
Susceptible cuttings	0.6	300	21.8	100	33

[a]Dry weight of a toxin-containing preparation (from an alumina column) which gave maximum loss of electrolytes from leaves at 10 ug/ml.

decrease in transpiration. Results, expressed as per cent recovery, show about one half as much toxin recovered from resistant (11%) as from susceptible tissue (25%), even though the absolute amount recovered was similar (100 ug) (Table 7). Toxin was used at 50 ug/ml in a similar experiment. Again, the total amounts recovered from resistant and susceptible tissues were similar (10 ug), but the amount taken up by resistant tissue (100 ug) was twice that taken up by susceptible tissue (45 ug). This resulted in a lower per cent recovery from resistant tissue (10%) than from susceptible tissue (22%).

The data from the 5 hr uptake experiment, in contrast to the 80 min experiment, suggest that toxin is inactivated more rapidly by resistant than by susceptible tissue. However, others have found that resistant and susceptible tissues inactivate toxin at equal rates until the susceptible cells are damaged and leaky (48). Resistant cells are not damaged and continue to inactivate toxin. The results of experiments with 5 hr uptake times probably reflect the damage to susceptible cells. These considerations, plus the fact that resistance is evident within 2 min of exposure to toxin (35), support the conclusion that resistance does not depend on ability to inactivate toxin.

Attempts to show removal of HV-toxin from solution by resistant and susceptible leaves.--If a significant amount of toxin is bound by receptors in plant tissue, then it should be possible to detect removal of toxin from ambient solutions. A differential affinity of toxin for susceptible tissues would be indicated if the residual solution from susceptible tissues contained less toxic activity than did the residual solution from resistant tissues.

Table 7. Recovery of HV-Toxin Activity after 5 hr Uptake by Resistant and Susceptible Oat Leaf Cuttings

Cuttings (1 g, approximately 10 leaves) of resistant and susceptible oats took up the volumes of toxin solution (500 ug/ml) as indicated. Cuttings were then homogenized in 100 ml water and centrifuged. The supernatants for each treatment were assayed for toxin by the electrolyte leakage method, using susceptible leaf samples (0.6 g each). The samples were incubated for 1 hr, rinsed, and leached for 1.5 hr. Conductance of ambient solutions was determined; each value is the mean and standard deviation for 3 samples.

Treatment	Amount taken up:		Conductance	Recovery:	
	ml	ug [a]	umhos	ug	%
Water control	-	-	4.9 ± 0.8	-	-
Toxin (0.11 ug/ml)[a]	-	-	12.6 ± 2.6	-	-
Toxin (0.33 ug/ml)	-	-	19.0 ± 2.4	-	-
Toxin (1.00 ug/ml)	-	-	30.3 ± 5.0	-	-
Homogenates from:					
Control cuttings without toxin	2.3	0	6.3 ± 1.7	-	-
Resistant cuttings with toxin	1.8	900	32.3 ± 1.9	100	11
Susceptible cuttings with toxin	0.8	400	27.0 ± 1.8	100	25

[a]Dry weight of a toxin preparation (from an alumina column) which gave maximum loss of electrolytes from leaves at 10 ug/ml.

Previous results, using the root growth assay, showed no loss of toxin from ambient solutions when large amounts of root tissues were exposed (38). I have repeated this experiment using leaf tissue and the electrolyte leakage assay. Leaf samples from resistant or susceptible plants were incubated in toxin solutions long enough to attain a toxin dose. In many such experiments 5-9 g of leaf tissues were incubated for 1-2 hr on the shaker in 100 ml of toxin solution (0.1-9.1 ug/ml). This toxin preparation gave maximum leakage at 10 ug/ml. Control solutions were held without exposure to plant tissues. Assay of the residual solutions, brought to the initial volume, showed no loss of activity from ambient solutions of susceptible or resistant tissues. In each case, the activity in ambient solution was comparable to the activity in a toxin control solution without tissue. These data suggest that toxin is not firmly bound in detectable amounts by either resistant or susceptible tissues. Limitations of the assay may preclude detection of a small amount of toxin which might be firmly bound.

Attempts to show binding of HV-toxin by proteins from cell membranes.--Possible binding of toxin to proteins from membranes of oat roots was tested in experiments similar to those of Otani et al (24). Membrane fragments sedimenting between 13,000-80,000 g were prepared as described in Materials and Methods and resuspended in 1 mM tris-MES buffer (pH 7.2) containing 1 mM $MgSO_4$. The toxin preparation from an alumina column gave complete inhibition of seedling root growth at a minimum concentration of 0.035 ug/ml. Toxin (final concentration 7 mg/ml) was added to a 2.0 ml suspension of membranes. To solubilize proteins, the suspension was treated with Triton X-100 (0.03 ml) and ground in a Ten Broeck homogenizer. The homogenate was placed on a gel filtration column

(Sephadex G-50, 1.5 x 25 cm) and eluted with water. A one ml aliquot of each 2 ml fraction from the column was assayed for free toxin by the electrolyte leakage method. The remaining 1 ml of each fraction was diluted with 10 ml water and acidified with 5 drops of 6N HCl. The acidified solution was partitioned 3 times against 10 ml of n-butanol. The butanol extracts were combined, evaporated to dryness, taken up in 50 ml water, and assayed with resistant and susceptible tissue (0.5 g samples) to detect any HV-toxin which may have been released from proteins solubilized from the membrane.

Results showed that free toxin came through the column after fraction 17. Thus, toxin bound to solubilized membrane components should have appeared between the void volume and fraction 17. The fraction immediately following the void volume (fraction 9) caused some leakage from susceptible but not from resistant tissue (Fig. 10). This fraction contained very little toxin, because leakage from assay tissues was detected only after 15 hr of leaching; it was not apparent after 3 hr, as was the case for leakage induced by fractions 18 and 19. Acid treatment and partitioning of fraction 9 did not increase its ability to induce leakage from tissues. The experiment was repeated 3 times with similar results.

These data do not prove that toxic activity came through the column is a toxin-protein complex. Therefore, two additional experiments included a toxin control with triton X-100, but without the membrane preparation. Again, the fraction immediately following the void volume caused leakage from susceptible but not resistant assay tissues (Fig. 10, insert). The host-specific toxic activity in fraction 9 does not appear to be the result of binding and release of toxin by a membrane component.

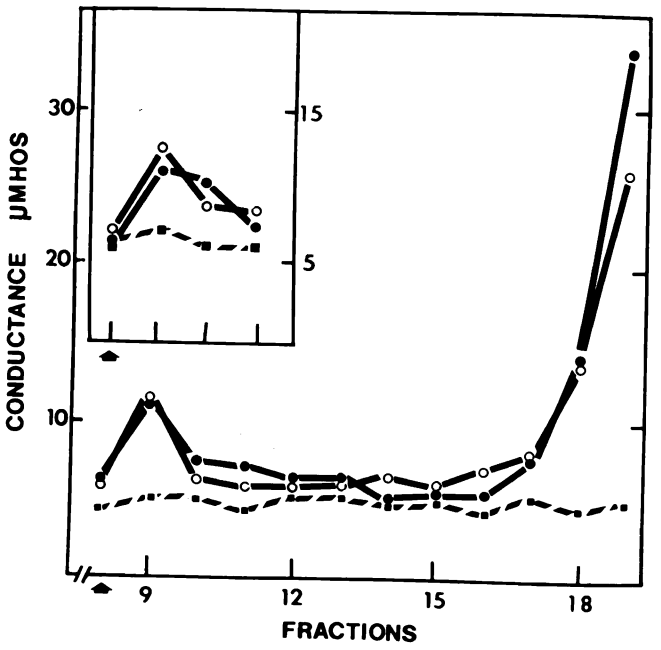


Fig. 10. Toxin content of fractions from a column used to separate components of solubilized membranes. Membrane vesicles were mixed with toxin plus Triton X-100 and homogenized. The homogenate was placed on a Sephadex G-50 column and eluted with water; two ml fractions were collected. One ml was assayed for toxin without further treatment (—o—). The other ml was diluted, acidified, partitioned into butanol, evaporated and assayed for toxin using resistant (▪▪), and susceptible (—●—) tissue samples. Conductance was determined after leaching for 15 hr. The insert shows toxicity of fractions from a column separation of an homogenate of toxin, Triton X-100 , and buffer without membranes. The arrows indicate the void volume of the columns.

IV. Possible Effects of HV-Toxin on Function or Properties of Isolated Membranes

Effect of HV-toxin on N-ethylmaleimide binding by oat microsomes.--NEM and other sulfhydryl-binding compounds are known to protect susceptible tissues against toxin-induced loss of electrolytes (10). This was confirmed; NEM gave 87% protection (Table 8). Gardner (9) also showed that toxin reduced ^{14}C-NEM binding to microsomes isolated by differential centrifugation (12,000-50,000 g) from etiolated susceptible leaves. The ability of membrane fragments from resistant plants to bind NEM was not affected by toxin. I have repeated Gardner's experiments using the 13,000-80,000 g fraction from resistant and susceptible oat roots. Isolation procedures were those of Hodges and Leonard (11); the binding assay was similar to that of Gardner (9), as described in Materials and Methods. Results showed little or no effect of toxin on NEM-binding by membranes from resistant plants. There was a possible toxin-induced increase in binding by preparations from susceptible plants (Table 9). These data do not confirm the results obtained by Gardner. However, the oat tissues, membrane isolation conditions, and binding assays differed from those of Gardner.

Effect of HV-toxin on ATPase activity of oat microsomes.--*H. maydis* T toxin is said to inhibit ATPase in microsomes (44). Possibility of a similar effect by HV-toxin on oat membrane fragments was tested. The 13,000-80,000 g pellet was resuspended in 1 mM tris-MES buffer (pH 7.2) containing 1 mM $MgSO_4$, or in homogenization medium. ATPase activity was assayed by the method of Hodges and Leonard (11), modified as described in Materials and Methods. The toxin preparation was an alumina column eluate, which gave maximum inhibition at a minimum concentration of

Table 8. N-Ethylmaleimide Protection against Toxin-Induced Loss of Electrolytes from Oat Tissues

Toxin-sensitive leaf tissue (0.6 g) was exposed to 2 mM NEM or to water for 0.5 hr. After rinsing, tissues were incubated in toxin solution (16 ug/ml) or in water for 1.5 hr, rinsed, and leached. Data are expressed as the conductance (umhos) of ambient solutions after leaching for 1.5 hr. Each value is the mean for 3 samples; standard deviations are shown.

Treatment	Conductance umhos	% Protection
Water + water	2.7 ± 0.3	-
NEM + water	5.2 ± 0.2	-
NEM + toxin	7.5 ± 1.0	87
Water + toxin	20.3 ± 1.2	-

Table 9. Effect of Toxin on N-Ethylmaleimide Binding by Membrane Preparations from Resistant and Susceptible Oat Roots

Membrane preparations (0.9 ml) were incubated 10 min in toxin (28 ug) or in buffer solutions before ^{3}N-NEM was added. The reaction was stopped after 15 min by adding 200 mM NEM. Membranes were precipitated by freezing overnight in trichloroacetic acid: membranes were then collected and washed on Millipore filters. Radioactivity on the filters was determined in a Beckman scintillation counter. Values are the mean counts and standard deviation of triplicate samples. % change in binding after toxin treatment is indicated.

Treatment	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	cpm	%Change	cpm	%Change	cpm	%Change	cpm	%Change
Susceptible + buffer	4466 ± 257	-	5565 ± 107	-	6622 ± 115	-	1726 ± 109	-
Susceptible + toxin	5664 ± 937	+27	5833 ± 137	+5	8220 ± 756	+23	1939 ± 48	+12
Resistant + buffer	4370 ± 272	-	4887 ± 315	-	7298 ± 90	-	1572 ± 141	-
Resistant + toxin	4535 ± 343	+ 4	4954 ± 405	+1	7823 ± 489	+ 7	1627 ± 94	+ 4

0.035 ug/ml in a seedling growth assay. Results showed that ATPase activity in membranes from both resistant or susceptible roots was inhibited slightly by the preparation, suggesting that some factor other than HV-toxin was involved. Therefore, toxin purified by thin-layer chromatography was used in another experiment. The more highly purified preparation had no effect on ATPase activity (Table 10). The experiment was repeated with similar results.

Effect of HV-toxin on light scattering and filterability of oat microsomes.--The microsome preparation was the 13,000-80,000 g fraction from homogenates of resistant or susceptible tissues. In some cases, the membrane preparation was purified further by use of a sucrose gradient. Toxin was an eluate from alumina, which gave complete inhibition of seedling root growth at 0.042 ug/ml. Effect of toxin on light scattering by the microsomes was tested at 340 nm, using a Beckman DU spectrophotometer adapted for such purposes. One ml of microsome suspension was added to the cuvette, toxin was added to bring the concentration to 420 ug/ml, and the solution was stirred. Stirring and addition of water or toxin solutions did not alter the absorbance of buffer solutions without membrane fragments. An increase in absorbance occurred when toxin was added to microsomes from both resistant and susceptible tissues.

The increase in absorbance could be caused by shrinkage or by agglomeration of microsomes. To determine which, filterability of microsomes through Millipore filters was tested. A calibrated Millipore filter apparatus with a volume of 15 ml was layered with 5 ml of 45% sucrose, 0.4 ml of a microsome suspension with or without toxin, and 9.6 ml of 34% sucrose. The toxin preparation, an eluate from an alumina

Table 10. Effect of Toxin on K^+ Stimulated ATPase Activity of Membranes Isolated from Resistant and Susceptible Oat Roots

Toxin preparations were evaporated to dryness and taken up in buffer. Buffer solution (0.01 ml) with toxin (280 ug) or without toxin was added to 5.0 ml of reaction mixture. ATPase activity was assayed by the method of Hodges and Leonard (11), modified as stated in Materials and Methods.

Treatment	Enzyme activity umoles Pi/hr/mg protein			
	-KCl	% Change	+KCl	% Change
Toxin from alumina column:				
Susceptible control	18.8	-	26.6	-
Susceptible + toxin	12.8	-32	20.6	-24
Resistant control	19.1	-	26.0	-
Resistant + toxin	14.8	-23	7.6	-24
Toxin from thin-layer chromatogram:				
Susceptible control	4.5	-	6.6	-
Susceptible + toxin	4.2	- 7	7.6	+15
Resistant control	5.9	-	8.5	-
Resistant + toxin	5.2	-12	8.3	- 2

column, gave complete inhibition of root growth at 0.090 ug/ml. The filter was placed on a vacuum flask, and the time required for each ml to flow from the filter funnel was recorded. Data were plotted as rates of flow. Toxin from the alumina column caused the flow rate of microsome suspensions to decrease from 6.0 to 0.6 ml/minute. The decrease was similar for preparations from resistant and susceptible plants. Toxin purified by thin-layer chromatography caused no decreases in rates of flow of microsome preparations. Apparently, some factor other than HV-toxin in the preparation from alumina caused an agglomeration of microsomes that plugged the filters and caused an increase in absorbance.

V. Protection of Toxin-Sensitive Tissues by Altered Toxin, Protein Synthesis Inhibitors, and SH-Binding Compounds

Effect of "esterified" toxin on toxin-induced loss of electrolytes.--Gardner used the Fraenkel-Conrat procedure for esterification of toxin. Toxin from an alumina column was lyophilized and dissolved in methanol plus HCl (0.2N), and left for 48 hr at room temperature (9). This procedure was assumed to esterify the toxin. The preparation did not cause leakage of electrolytes. When the esterified preparation (48 to 80 ug/ml) was added to an active toxin preparation (0.8 to 8.0 ug/ml) there was a 40-60% decrease in toxic effects (9). Gardner interpreted these data to mean that esterification destroys toxicity, and that esterified toxin competes for or blocks toxin receptor sites. I have tested several predictions based on the hypothesis. The protection phenomenon should be concentration dependent and should exhibit saturation kinetics. Pretreatment with inactivated or esterified toxin should protect the site against later exposure to active toxin. It

should be possible to isolate the esterified toxin or the protective principle. Finally, other methods of esterification should produce inactive toxin which can protect tissue from leakage induced by active toxin.

First, I repeated and confirmed Gardner's experiment (9) which showed that the esterified preparation protected tissues against active toxin. The esterified preparations lost most of their activity and gave 70% protection against active toxin, as shown by electrolyte leakage data (Table 11). Next, I attempted to determine the concentration of an esterified preparation required for maximum protection against active toxin. Two different toxin preparations were used: preparation a gave complete inhibition of seedling root growth at 0.009 ug/ml; and preparation b gave comparable inhibition at 0.016 ug/ml. Both preparations were treated by the Fraenkel-Conrat procedure (8), and each was tested at concentrations of 1, 10, 40, and 100 ug/ml against saturating concentrations of the active toxin preparation from which it was derived. Electrolyte loss assays showed that the 4 concentrations of inactivated preparation a gave 8, 27, 68, and 77% protection, respectively, against active toxin (10 ug/ml); inactivated preparation b gave 4, 18, 62, and 69% protection (Fig. 11). Thus, 40 ug of esterified toxin/ml gave maximum protection against active toxin at 10 ug/ml.

Does the inactivated toxin protect tissues from active toxin by firm binding to a receptor, making it unavailable to the active molecule, or does inactive toxin affect the toxin molecule *per se*? Tissue samples (0.5 g each) were exposed to toxin plus esterified toxin for 60 min, or to esterified toxin alone for 60 min, and then were exposed to active toxin for 60 min. Both cotreatment and pretreatment with inactivated

Table 11. Protective Effect of Inactivated Toxin against Toxin-Induced Loss of Electrolytes

Susceptible leaf tissue (0.6 g samples) were vacuum-infiltrated with water and incubated in the treatment solutions for 1 hr. Tissues were rinsed for 10 min and placed in 50 ml water; conductance of ambient solutions was determined after 2 hr.

Treatment	Conductance umhos	Protection %
Water control	3.8	-
Inactivated toxin (45 ug/ml)	5.6	-
Toxin control (9.1 ug/ml)	32.3	-
Inactivated toxin (45 ug/ml) + toxin (9.1 ug/ml)	14.1	70

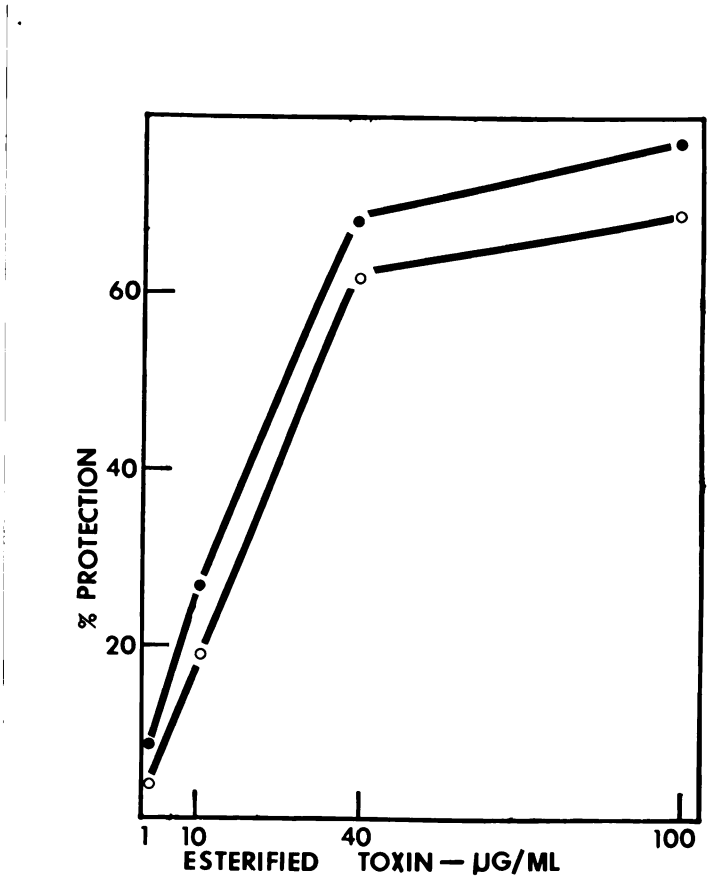


Fig. 11. Protective effects of several concentrations of inactivated toxin against active toxin. Two different toxin preparations were used (—●—, —○—). Susceptible leaf samples (0.6 g each) were vacuum-infiltrated in water and incubated for 30 min in inactivated toxin at the concentrations shown. Samples were then exposed to active toxin (10 ug/ml) for 1 hr. Samples were rinsed and leached 3 hr before conductances of ambient solutions were determined. Data were plotted as per cent protection; each value is the mean of 3 samples.

toxin gave protection against active toxin (Table 12), indicating that inactivated toxin affects the tissues and not the active toxin molecule. This experiment was repeated with essentially the same results. Rinsing the tissues for 60 min with water did not remove the protective effects of the inactivated preparation (Table 13). Thus, it appears that the inactivated preparation is bound firmly to a site, or that it has altered an active site.

Gardner did not include a control to test possible protective effects of methanol-HCl alone. Therefore, a control of methanol plus 0.1 N HCl (0.5 ml) was used. Toxin (45 ug/ml) was treated with methanol plus 0.1N HCl (0.5 ml). Leaf samples were incubated in 100 ml of water containing either methanol-HCl or inactivated toxin, with or without active toxin (10 ug/ml). Samples (3 per treatment) were then rinsed for 10 min and leached 3 hr before conductance of ambient solutions were determined. Methanol plus HCl control gave 29% protection, whereas the inactivated toxin preparation containing methanol plus HCl gave 70% protection (Table 14). The experiment was repeated with two other inactivated toxin preparations; in each case, the HCl-methanol control gave some protection, but the protection was always much less than that given by the inactivated toxin preparations.

A preparation of inactivated toxin in methanol plus HCl was evaporated to dryness and dissolved in water; a control preparation was not evaporated. The preparation after evaporation and resuspension had a pH above 6.0. The evaporated and non-evaporated preparations gave approximately equal protection against active toxin (Table 15). The experiment was repeated with essentially the same results. If methanol plus HCl contributes to protection by inactivated toxin, then evaporation

Table 12. Comparative Protective Effects of Inactivated Toxin Applied as a Pretreatment and a Co-treatment with Active Toxin

Leaf samples (0.5 g each) were vacuum-infiltrated in water and incubated in water or in inactivated toxin (40 ug/ml) for 1 hr. Samples were then rinsed and incubated for 1 hr with the second treatment as indicated. Active toxin was at a saturating concentration (10 ug/ml). Samples were then rinsed and leached 3 hr before conductances of ambient solutions were determined. Each value is the mean conductance for 3 samples.

First treatment	Second treatment	Conductance umhos	Protection %
Water	Water	5.3	-
Water	Toxin	45.7	-
Inactivated toxin	Toxin	19.4	65
Water	Inactivated toxin + toxin	20.3	63

Table 13. Attempts to Remove the Protection Given by Inactivated Toxin

Leaf samples (0.5 g each) were vacuum-infiltrated in water, incubated in water or in inactivated toxin (methanol-HCl treatment) (40 ug/ml) for 1 hr, and rinsed with water for the times indicated. Samples were then incubated for 1 hr with the second treatments as indicated, rinsed, and leached 3 hr before conductances of ambient solutions were determined. Active toxin was used at 10 ug/ml. Data are the means for 3 samples.

Treatments:			Conductance	Protection
First	Rinse	Second		
	min		umhos	%
Water	60	water	3.9	-
Water	60	active toxin	47.3	-
Inactivated toxin	10	water	5.0	-
Inactivated toxin	10	active toxin	20.3	65
Inactivated toxin	60	water	5.8	-
Inactivated toxin	60	active toxin	20.2	67

Table 14. Protective Effect of HCl in Methanol Against Toxin-Induced Loss of Electrolytes from Oat Leaves

Tissue samples (0.5 g each) were vacuum-infiltrated in water and incubated in: a) water containing 0.5 ml of 0.1 N HCl-methanol, with or without active toxin; b) water containing 0.5 ml of inactivated toxin solution, with or without active toxin. After 1 hr, samples were rinsed, and leached for 3 hr. Conductances were determined for ambient solutions. Each value is the mean of 3 samples.

Treatment	Conductance umhos	Protection %
Water control	3.8	-
Active toxin[a] control	32.3	-
Methanol-HCl	4.7	-
Inactivated toxin[b]	5.6	-
Methanol-HCl + active toxin[a]	24.8	29
Inactivated toxin[b] + active toxin[a]	14.1	70

[a]The final concentration of active toxin (10 ug/ml) gave maximum leakage.
[b]Final concentration of inactivated toxin was 45 ug/ml.

Table 15. Effect of Methanol-HCl on Protective Effects of Inactivated Toxin against Toxin-Induced Loss of Electrolytes

Leaf samples (0.5 g each) were vacuum-infiltrated in water and incubated in an active toxin preparation (10 ug/ml) containing either esterified toxin (40 ug/ml), inactivated toxin from which methanol-HCl was evaporated, or a methanol-HCl preparation without inactivated toxin. Samples were rinsed and leached 3 hr; conductance of ambient solutions was determined. Data are the means of 3 samples.

Treatment	Conductance umhos	Protection %
Water control	5.0	-
Active toxin control	22.5	-
Inactivated toxin + active toxin	13.3	52
Evaporated inactivated[a] toxin + active toxin	11.6	62
Methanol-HCl + active toxin	17.8	27

[a]Toxin inactivated by methanol plus HCl was evaporated and resuspended in water.

should have decreased the protective effect. Thus, the protective effect of methanol plus HCl (Table 14) appears to differ from the protective effect of inactivated toxin.

Toxin from a thin-layer chromatogram was compared with a preparation from the alumina column to determine whether or not a highly purified toxin can be converted to a protective product. Inactivated preparations were evaporated to dryness to remove methanol and HCl. Inactivated toxin from the alumina column gave 58% protection against active toxin; inactivated toxin from thin-layer chromatograms gave 30% protection (Table 16). The experiment was repeated with similar results. Only 50% of the toxin applied to thin-layer plates was recovered; thus, the inactivated toxin from thin-layer chromatograms had only half the concentration of the inactivated toxin from alumina. These data indicate that the altered toxin in the preparation is the protective factor.

Many attempts were made to purify inactivated toxin by thin-layer chromatography. A protective product was not recovered when inactivated preparations were chromatographed and assayed against active toxin.

Toxin from alumina and toxin from a thin-layer chromatogram were esterified by the diazomethane method; inactivation and protective effects were tested. Inactivated ("esterified") preparations from the alumina column and from thin-layer chromatograms failed to induce electrolyte leakage (Table 17). The experiment was repeated with the same results.

The inactivated toxin preparation from the alumina column was analyzed by thin-layer chromatography to determine whether or not inactivated toxin could be detected. The chromatogram of active toxin had the zone which quenched fluorescence induced by ultraviolet light. The chromatogram of the diazomethane-inactivated toxin did not have the

Table 16. Protective Effects of Inactivated Toxin from Two Sources against Toxin-Induced Loss of Electrolytes

Toxin preparations from an alumina column and from thin-layer chromatograms were treated with methanol plus HCl. Leaf samples (0.5 g each) were vacuum-infiltrated in water and incubated in inactivated toxin (40 ug/ml) with or without active toxin (10 ug/ml) for 1 hr. Samples were then rinsed and leached for 3 hr; conductance of ambient solutions was determined. Each value is the mean of 3 samples.

Source[a]	Treatment	Conductance umhos	Protection %
-	Water control	6.8	-
Alumina column	Inactivated toxin	5.0	-
Thin-layer chromatogram	Inactivated toxin	5.1	-
Alumina column	Inactivated + active toxin	16.0	58
Thin-layer chromatogram	Inactivated + active toxin	23.5	30
-	Active toxin[b]	33.2	-

[a] Source of toxin used for inactivation.
[b] Active toxin gave complete inhibition of root growth at a minimum concentration of .042 ug/ml.

Table 17. Inactivation of Two HV-toxin Preparations by Diazomethane Treatment

Toxin from a thin-layer chromatogram and toxin from an alumina column were used. Leaf samples (0.5 g each) were vacuum-infiltrated in water, incubated for 1 hr in the toxin preparations (4.2 ug/ml), rinsed, and leached 3 hr; conductance of ambient solutions was determined. Each value is the mean of 3 samples.

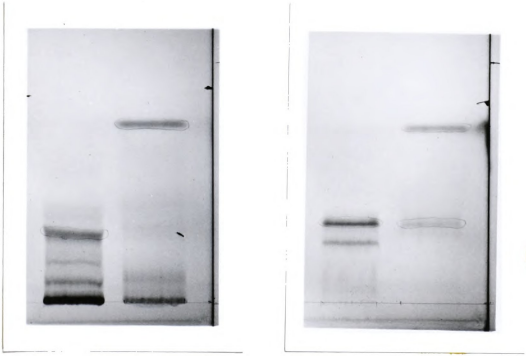
Toxin source	Treatment	Conductance umhos	Inactivation %
-	Water control	5.7	-
Thin-layer chromatogram	Toxin control	25.7	-
Thin-layer chromatogram	Diazomethane	11.4	72
Alumina column	Toxin control	34.0	-
Alumina column	Diazomethane	7.0	95

quenching zone characteristic of toxin. However, it had a quenching area near the solvent front which might be either esterified toxin or a by-product of the reaction. The toxin that was isolated by thin-layer chromatography and treated with diazomethane lost 72% of its activity; when this partly inactivated preparation was chromatographed, there was a small quenching zone at the R_f of toxin (Fig. 12). When both esterified preparations were chromatographed in benzene, a single quenching spot (R_f 0.33) appeared. Again this spot may be esterified toxin or a by-product of the reaction.

The inactive diazomethane treated preparations gave no protection against toxin-induced loss of electrolytes. This raises doubts that the esterified toxin molecule is the product that protects against active toxin. A molecular rearrangement other than esterification may be the basis of protection. Esterification with diazomethane-treatment inactivates toxin, but does not give a protective compound.

Effect of several inhibitors of protein synthesis on sensitivity of oat tissue to HV-toxin.--Previous work (10) has shown that cycloheximide causes susceptible leaves to lose sensitivity to toxin; the effect is reversible. The decrease in sensitivity was thought to result from inhibition of synthesis of a protein receptor with a short half-life. I have repeated and extended these experiments with cycloheximide, and have used other inhibitors of protein synthesis.

Resistant and susceptible oat cuttings (0.6 g) were allowed to take up actinomycin D, chloramphenicol, cycloheximide, or gougerotin solutions (10^{-4}M) for 12 hr under fluorescent lights. Leaves were then cut into 1 cm sections, enclosed in cheesecloth, vacuum-infiltrated in water for 10 min, and incubated in toxin solution (10 ug/ml) or in water for 60 min.



A. B.

Fig. 12. Thin-layer chromatograms of toxin and diazomethane-treated toxin. Plates were developed with n-butanol, acetic acid, water (3:1:1).

A. Effect of diazomethane treatment of toxin from an alumina column on UV-induced fluorescence. Left, untreated toxin, with quenching of fluorescence induced by UV, characteristic of active toxin; right, diazomethane-treated toxin (95% inactivated) with no quenching of fluorescence at R_f 0.45.

B. Effect of diazomethane-treatment of toxin from a thin-layer chromatogram on quenching of fluorescence induced by UV. Left, untreated toxin, with quenching of fluorescence characteristic of active toxin. Right, diazomethane-treated toxin (72% inactivated), with some quenching of fluorescence characteristic of active toxin. This preparation retained 28% of its toxic activity.

The quenching area at high R_f in the diazomethane-treated toxin preparations may be inactivated ("esterified") toxin or a by-product of the reaction.

Tissues were rinsed, leached, and monitored for electrolyte loss. Results (Table 18) showed that actinomycin D, cycloheximide, and gougerotin gave protection against toxin-induced loss of electrolytes. These are inhibitors of cytoplasmic protein synthesis. Chloramphenicol, an inhibitor of organelle protein synthesis, gave no significant protection against toxin. This experiment was repeated with essentially the same results. Cycloheximide at 10^{-4}M gave the best protection of the inhibitors tested; therefore, concentrations from 1.8×10^{-8}M to 1.8×10^{-5}M were tested for protective effects. Concentrations of 1.8×10^{-5}M and 1.8×10^{-6}M gave 73 and 70% protection, which compares well with the 79% protection given by cycloheximide at 10^{-4}M (Table 19). Cycloheximide at 1.8×10^{-7}M gave slight protection (11%) and 1.8×10^{-8}M gave no protection.

Effects of sulfhydryl-binding compounds on toxin-induced loss of electrolytes.--Gardner has shown that N-ethylmaleimide (NEM) sodium arsenite, iodoacetic acid, and dinitrofluorobenzene cause approximately 80% decrease in sensitivity of susceptible tissue to toxin (10). Parachloromercuribenzoate gave 20% protection against toxin; this compound does not penetrate the mitochondrial membrane (23). However, there was not a good correlation between protective ability and ability to penetrate membranes; mercuric ion should penetrate readily but it did not protect tissues against the effects of toxin. However, the effects of mercuric ion were uncertain because it caused considerable leakage from oat tissues (9, 10).

I have reinvestigated the protective effects of several sulfhydryl reagents against toxin-induced loss of electrolytes. NEM, iodoacetamide, dinitrofluorobenzene, mersalyl, p-chloromercuribenzoate, and mercuric

Table 18. Effect of Protein Synthesis Inhibitors on Toxin-Induced Loss of Electrolytes from Oat Cuttings

Leaf cuttings (0.6 g) took up the inhibitor solutions (10^{-4}M) in the transpiration stream during a 12 hr light period. Leaf sections from the cuttings were then incubated in water or in toxin solutions (10 ug/ml). After 1 hr, the leaf samples were rinsed and placed in water for leaching. Conductance of ambient solutions was determined after 4 hr leaching. Each value is the conductance for one sample.

Treatment	Conductance umhos	Protection %
All controls without toxin	10	-
Toxin control without inhibitors	82	-
Actinomycin D + toxin	33	66
Chloramphenicol + toxin	80	3
Cycloheximide + toxin	26	79
Gougerotin + toxin	29	73

Table 19. Protective Effect of Cycloheximide against Toxin-Induced Loss of Electrolytes

Susceptible oat leaf cuttings (0.6 g samples) took up cycloheximide (CH) for 12 hr under lights. Leaf sections (1 cm long) from cuttings were enclosed in cheesecloth and vacuum-infiltrated with water. Samples were then incubated in active toxin solutions (10 ug/ml) for 1 hr, rinsed, and leached for 4 hr before conductances of ambient solutions were determined. Each value is the mean of 3 samples.

Treatment	Conductance umhos	Protection %
Water control	9	-
Toxin control	82	-
CH 1.8×10^{-5}M + toxin	29	73
CH 1.8×10^{-6}M + toxin	31	70
CH 1.8×10^{-7}M + toxin	74	11
CH 1.8×10^{-8}M + toxin	82	0

chloride were tested, each at a concentration of 2 mM. Results (Table 20) show 86-91% protection by NEM, dinitrofluorobenzene and iodoacetamide. These reagents readily penetrate mitochondrial membranes (24). Parachloromercuribenzoate and mersalyl, which gave 51-53% protection, do not penetrate mitochondrial membranes (23). Mercuric chloride caused nonspecific leakage. Thus, sulfhydryl-binding reagents capable of penetrating cell membranes were much more effective than were the non-penetrating reagents in protecting tissues against toxin.

Table 20. Protective Effects of Sulfhydryl-Binding Reagents against Toxin-Induced Loss of Electrolytes

Leaf samples (2 susceptible and 1 resistant, 0.5 g each) were vacuum-infiltrated in water and incubated 20 min in the SH-binding reagent (2 mM). Samples were then incubated for 1 hr in toxin (10 ug/ml). Samples were rinsed for 10 min and leached 3 hr before conductance of ambient solutions was determined.

$$\text{Per cent protection} = 1 - \frac{(\text{umhos ITS}) - (\text{umhos ITR})}{(\text{umhos TS}) - (\text{umhos TR})} \times 100$$

umhos ITS, ITR, TS, or TR = conductance of ambient solutions from inhibitor plus toxin-treated susceptible, inhibitor plus toxin-treated resistant, toxin-treated susceptible, or toxin-treated resistant tissues, respectively. Each value is the mean of three samples.

Treatment	Leakage from tissues: Susceptible umhos	Leakage from tissues: Resistant umhos	Protection %
Water control	6.3	5.7	-
Toxin control	22.3	5.0	-
$HgCl_2$ + toxin	52.0	37.0	13
PCMB[a] + toxin	24.0	15.5	51
Mersalyl + toxin	14.3	6.2	53
DNFB[b] + toxin	8.1	5.7	86
Iodoacetamide + toxin	6.1	4.8	87
NEM[c] + toxin	6.8	5.3	91

a PCMB = parachloromercuribenzoate
b DNFB = dinitrofluorobenzene
c NEM = N-ethylmaleimide

DISCUSSION

The conventional assay for host-specific toxin, based on inhibition of root growth of susceptible seedlings (30), requires 4-5 days for completion. This is too long for accurate assays of unstable, highly purified toxin. Long-term assays may be complicated by secondary effects involving growth and metabolism by test seedlings. The assay based on toxin-induced loss of electrolytes is an improvement; it can be completed 4-7 hr after initial exposure of tissues. Still more rapid assays are desirable, but none are available at this time.

The electrolyte leakage data show several characteristics not clearly evident in previously published data. One is the saturation limit for leakage induced by HV-toxin; a rational explanation is that the number of receptor sites per cell becomes a limiting factor. A second important characteristic of toxin-induced leakage is linearity; the rate of leakage induced by a given concentration is constant for 3 hr or more, and increases with increases in toxin concentration over 3 orders of magnitude in the sub-saturating range. Rates of electrolyte leakage approximately double with 10 fold increases in toxin concentration. Thus, it appears that the number of available receptor sites affected is a function of toxin concentration, and that affected sites continue to leak electrolytes for some time after tissues are removed from toxin solutions. The dosage-response data, including saturation kinetics, appear to be consistent with the hypothesis of toxin receptor sites or substances.

Several factors were tested individually to determine how each might affect the rate of electrolyte losses induced by toxin. Electrolyte

losses increased in a direct relationship with increases in exposure time (from 1 min to 2 hr). Leakage also became more rapid with increases in temperature over a range from 11° to 24°. Within limits, rates of electrolyte loss increased with increasing age of tissues and weight of leaf samples, and decreased with increasing size of leaf sections comprising the sample. These data indicate the conditions to be used for the most sensitive assays. Further experiments have shown that the amount of toxin in unknown solutions can be estimated reliably by the ability to induce electrolyte loss from susceptible oat leaves. Several dilutions of an unknown solution usually are required, plus comparison with a known standard; these are relatively simple operations.

Assays based on electrolyte leakage do not require use of all conditions that are optimal; it is more important to use the same procedures in each assay, for the sake of reproducibility. A satisfactory assay procedure which I have used is started with leaf samples (0.5 g, cut into 1 cm sections) from 12 to 21 days old seedlings. Samples are enclosed in cheesecloth, vacuum-infiltrated with water, incubated 1-4 hr in toxin solution, rinsed, and leached to determine electrolyte loss. The assay has many uses for which root growth assay is unsatisfactory. As examples, the electrolyte loss assay has been used to determine the protective effects of various compounds which presumably affect toxin receptor sites (10), to locate toxin on chromatograms, and to identify toxin in fractionation procedures.

Purification of toxin as described herein was directly dependent on the use of the leakage assay. A useful finding was the correlation between toxin activity and the quenching of ultraviolet-induced fluorescence which occured on thin-layer plates. This made it possible

to locate toxin on these chromatograms without an assay. I have not been able to separate toxicity from quenching at the R_f characteristic of toxin. The two were correlated after thin-layer chromatography with three solvents, after cation-exchange chromatography, and after electrophoresis.

Highly purified toxin was obtained from a cation-exchange column. The criteria of purity were a lack of ninhydrin positive or ultraviolet-detectable material on thin-layer chromatograms. High voltage paper electrophoresis indicated that toxin migrated rapidly as a cation at pH 1.9 and slowly as an anion at pH 6.5, and that the preparation was homogenous. This would fit with the report that the toxin contains glutamate and aspartate residues which would contribute ionizable carboxyl groups, giving the molecule a net negative charge at high pH (27).

HV-toxin as described by Pringle and Braun (26, 27) was unstable during evaporation or purification. In my experience, toxin was stable in a saturated solution of sodium bicarbonate. According to Pringle and Braun, this treatment inactivated toxin and split the toxin molecule into victoxinine and a peptide. Although rigorous attempts were not made, I failed to detect any iodoplatinate-positive victoxinine from toxin preparations after treatment with sodium bicarbonate, or from toxin preparations inactivated by autoclaving at pH 10. Authentic victoxinine was readily detected on thin-layer plates with iodoplatinate (31). Others have failed to detect victoxinine in toxin breakdown products (9, and personal communication from R. B. Pringle); however, victoxinine may have been present in amounts to low to be detected by iodoplatinate. It is possible that I am dealing with a different toxic entity than was

studied by Pringle and Braun (28). This consideration awaits the determination of the chemical structure of HV-toxin. Other fungi are known to produce several related forms of a host-specific toxin (13, 26, 30).

The first hypothesis attempting to explain specificity of HV-toxin was that resistant oat tissues inactivate toxin more efficiently than do susceptible tissues. Romanko (33) recovered toxin from susceptible cuttings but not from resistant cuttings which had taken up toxin. Scheffer and Pringle, using the root growth assay, were unable to demonstrate toxin in homogenates of either resistant or susceptible cuttings which had taken up toxin. My data, based on the electrolyte leakage assay, showed that toxin was recovered in equal amounts from toxin-treated cuttings of both resistant and susceptible plants. If resistance is based on toxin inactivation, overloading the tissue with toxin should destroy resistance. Toxin was recovered from resistant tissues in all cases, indicating an excess of active toxin, these results make untenable the hypothesis that resistance depends on ability to inactivate toxin.

There are indications that some host-specific toxins are firmly bound to membranes, and that others are not firmly bound. Strobel (42) presented evidence that helminthosporoside from H. sacchari binds to a protein in the cell membrane. Otani et al (24) presented comparable evidence for Alternaria kikuchiana toxin. On the other hand, Bednarski et al (1) have found that toxin from H. maydis race T uncoupled electron transport in mitochondria only when toxin was present. When toxin-treated mitochondria were resuspended in a toxin-free medium, their normal functions were fully restored. Any possible binding of toxin appears to be readily reversible.

Several types of experiments designed to show binding of HV-toxin by resistant or susceptible oats gave negative results. I attempted without success to show binding of toxin to tissues by assaying residual solutions which had previously been incubated with resistant or susceptible leaves. An experiment patterned after that of Otani et al (24) failed to show binding of HV-toxin to membranes isolated from susceptible oats. With present data, it appears that HV-toxin is more like H. maydis T toxin in its association with a receptor. On the other hand, the toxin effects of HV-toxin do not appear to be reversible.

One objective of this research was to determine whether or not cell-free membranes from susceptible oats are affected by HV-toxin. Several properties of the membranes were chosen for analysis, including effects of toxin on N-ethylmaleimide binding, ATPase activity, light scattering, and flow rate of membrane suspensions through filters. My results indicate that toxin did not decrease the binding of NEM by membranes from susceptible or resistant plants. In contrast, Gardner's data indicated that toxin decreased the binding of NEM by membranes from susceptible but not from resistant plants (9). The reason for the discrepency in these results is not known. However, we did not use the same plant tissues or membrane isolation procedures, and there were differences in our binding assays.

An effect of toxin on K^+-stimulated ATPase activity should indicate that there is a toxin receptor in the membrane (12). This seems logical, because toxin causes isolated susceptible protoplasts to burst. My results show that K^+-stimulated ATPase activities in membranes from resistant and susceptible roots were not affected by toxin from a thin-layer chromatogram. Tipton et al (44) presented evidence that H. maydis

race T toxin inhibited K^+-stimulated ATPase of microsomes from susceptible but not from resistant roots. Bednarski (unpublished results) could not repeat the work of Tipton; her results showed no inhibition of microsomal ATPase by HM-T toxin preparations which gave striking inhibition of root growth and mitochondrial phosphorylation.

The previously described protection of oat tissues against active toxin by inactivated preparations of toxin (9) was studied further. First, is HV-toxin esterified to give a protective compound as reported (9), or is some other constitutent of an impure preparation altered to give a protective product? If it is the toxin molecule, do we have any reason to believe that esterification is the process leading to protection? Toxin purified by thin-layer chromatography was inactive after treatment with methanol-HCl, and the inactive preparation gave protection against active toxin. Toxin preparations treated with diazomethane also were inactivated, but the inactive product did not protect against leakage induced by active toxin. There may be problems with solubility of the products of these treatments. However, the results suggest that the changes caused by methanol-HCl are something other than esterification. The best proof of methanol-HCl esterification of toxin would be the rigorous demonstration of recovery of toxin activity when the inactive (presumably esterified) toxin was placed under saponifying conditions.

Gardner interpreted the protection data as indicating that esterified toxin is bound to a site, thus blocking active toxin (9). I have attempted without success to reverse protection by washing out the protective substance, which suggests that altered toxin may be firmly bound. Rinses with low salt solutions to remove protection were not tried because of complicating effects on monitoring the leakage.

Results with protein synthesis inhibitors support the hypothesis that continual protein synthesis is necessary for tissue to remain sensitive to toxin. The lack of a protective effect by chloramphenicol may indicate that organelle protein synthesis is not necessary, but that protein synthesis in cytoplasm is necessary for sensitivity to toxin. Cycloheximide protection develops 8-12 hr after treatment, and is reversed within 48 hr after removal of cuttings from cycloheximide (9, 10). This suggested that the protective effect is based on inhibition of protein synthesis and depletion by turnover of the receptor. The protective effect does not appear to be an immediate effect such as occurs in cycloheximide inhibition of indole acetic acid-induced proton release from elongating coleoptiles (32). Protective effects of protein synthesis inhibitors supports the concept that sensitivity to toxin is based on the final product of the Vb gene, a protein which confers susceptibility.

SH-binding reagents which penetrate the plasmalemma gave greater protection against toxin than did those which did not penetrate. This indicates that the toxin receptor may not be on the outer surface of the plasmalemma. Instead, the toxin receptor may be in hydrophobic areas within the membrane, in the cytoplasm, or in the tonoplast.

The initial objectives of this research were to develop a rapid and sensitive assay for toxin, and convenient methods for obtaining relatively pure toxin. These objectives were met; the procedures developed were basic to further work concerned with understanding the interaction of toxin with susceptible oats. The possibility that inactivation or firm binding plays a role in host-specificity was also investigated. There were no differences in inactivation by resistant and

susceptible tissues, and there were no indications of firm binding of toxin to cellular receptors. Thus, these processes were ruled out as contributing to toxin specificity. There are contradictory indications regarding a possible toxin-sensitive site in the plasmalemma. My data indicate that toxin does not affect isolated membrane vesicles. Negative evidence rules out effects on the parameters tested, but the crucial parameter may not have been monitored. The indirect approach dealing with protective effects of certain chemicals against toxin supports the hypothesis that toxin sensitive sites exist.

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APPENDIX

Attempts to introduce radioactive label into Helminthosporium victoriae toxin.--An obvious way to study the interaction of HV-toxin with resistant and susceptible oat tissue is to use radioactive toxin as a tracer. Several attempts were made to introduce ^{14}C by biosynthesis into the HV-toxin molecule. These experiments are described for the record and the practicability of the approach is discussed.

No methods to incorporate radioactive label into HV-toxin have been published. Wheeler grew H. victoriae on Fries' medium containing ^{14}C-sucrose (30 uC/ml). Spores from such cultures, harvested and germinated over a 60 day period, retained their ability to infect susceptible plants and to produce toxin (46). Speculations for future work to detect microbial toxins by the use of radioisotopes were published in 1953 (47), but no further work has been reported. Samaddar (34) grew H. victoriae with ^{14}C-sucrose and partially purified the toxin by use of alumina and Bio-Gel P-2 columns. He reported a correlation between toxicity and radioactivity in fractions from such columns. However, the fractions from Bio-Gel columns contain 50% or more of non-toxic materials, on a dry weight basis.

The presence of a sesquiterpene in the HV-toxin molecule (25) indicated the possibility of using acetate or mevalonate to incorporate label into HV-toxin. Zweig and DeVay (53) studied the incorporation of various ^{14}C substrates into diterpenoid gibberellins (GA) of Fusarium moniliformae. Per cent ^{14}C incorporation was calculated as the ratio of counts in isolated GA to counts in substrate added. The most efficient precursors (<2%) were methyl crotonic acid, and sodium acetate. Mevalonic

acid or sucrose, glucose and fructose were not incorporated into GA in detectable levels.

In my work, radioactive culture filtrates were produced by growing H. victoriae in Fries' No. 3 medium supplemented with several different ^{14}C-substrates. The following radioactive solutions were sterilized by filtration (Millipore, with 0.22u pores) and added to the medium to bring it to 1 uC/ml: UL-^{14}C-sucrose, specific activity 300 mC/mmole (Volk Radiochemical Co., Burbank, Calif. 91502); UL-^{14}C-leucine, 270 mC/mmole; sodium acetate-2-^{14}C, 2 mC/mmole; DL-mevalonic-2-^{14}C acid, 14.32 mC/mmole (New England Nuclear, Boston, Mass. 02118); and DL-mevalonic acid-2-^{14}C lactone, 7.1 mC/mmole (Amersham Searle, Arlington Heights, Ill. 60005). The fungus was shown to produce toxin on Fries' No. 3 salts plus 0.01 M l-glutamic acid. UL-^{14}C-l-glutamic acid, 180 mC/mmole (New England Nuclear) was added to the Fries' No. 3 salts at 1 uC/ml and supplemented with non-labelled glutamic acid to 0.01 M.

H. victoriae was grown on the radioactive media for 21 days and harvested by filtration. The filtrates were evaporated to 0.1 the original volume and fractionated by thin-layer chromatography, gel filtration, or cation-exchange chromatography as previously described. Radioactivity on thin-layer chromatograms was determined with a Packard model 7200 radiochromatogram scanner fitted with a recording ratemeter. Radioactivities in aliquots of fractions from the column were determined with a GM tube (Nuclear Chicago model 1010). Corresponding areas from a chromatogram or aliquots from column fractions were bioassayed by the electrolyte leakage method.

Results of analysis of the filtrates from the ^{14}C-sucrose-amended cultures are presented, since they were characteristic of the results for

other labelled substrates. Scans of thin-layer chromatograms revealed broad peaks of ^{14}C activity which overlapped areas occupied by toxin. Fractionation of the radioactive culture filtrate on Sephadex G-15 showed an overlap of the toxin activity and radioactivity peaks (Fig. 13). When the filtrate was fractionated on a SP-Sephadex cation exchange column, there was a distinct separation of radioactivity from toxin activity (Fig. 14). This was evidence that the toxin did not contain enough label for detection.

A calculation of the amount of label that might be incorporated into toxin can be based on dry weight. The fungus grown in 200 ml of medium had a dry weight of approximately 0.7 g (20) when the filtrate was harvested. The filtrate had a dilution end point for toxin of approximately $1:10^5$ in a root growth assay. The highest activity of purified toxin so far obtained gave complete inhibition of root growth at 0.2 ng/ml. From these facts it was calculated that 200 ml of filtrate should contain about 4 mg of toxin. If the filtrate was amended with ^{14}C-sucrose at 1 uC/ml and the assumption is made that all the ^{14}C was metabolized into non-volatile products, then the amount of label in toxin should be roughly equal to the ratio of toxin dry weight to fungus dry weight times the total radioactivity of the filtrate. This ratio is:

$$\frac{4 \text{ mg}}{700 \text{ mg}} \times 200 \text{ uC} = 1.14 \text{ uC}.$$

The cpm/ml at the dilution end point (10^5 dilution) would be

$$\frac{1.14 \times 10^6 \text{ cpm}}{200 \times 10^5 \text{ ml}} = 0.05 \text{ cpm/ml}$$

These are the conditions under which my labelling experiments were done. One ml of the culture filtrate could be expected to contain approximately 5000 cpm in toxin if the assumptions are valid. Clearly no such levels of

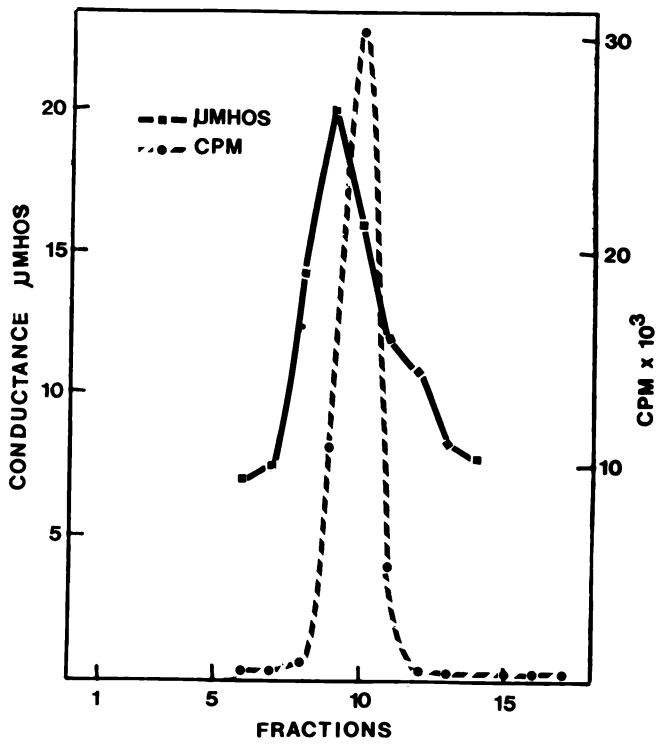

Fig. 13. Toxic activity and radioactivity in fractions from a gel column used to separate toxin from ^{14}C-labelled culture filtrate. An aliquot (1.0 ml) from each 2 ml fraction was added to 50 ml water and assayed for toxin by the electrolyte leakage method. Radioactivity in an aliquot (0.1 ml) from each fraction was counted with a Geiger-Mueller tube.

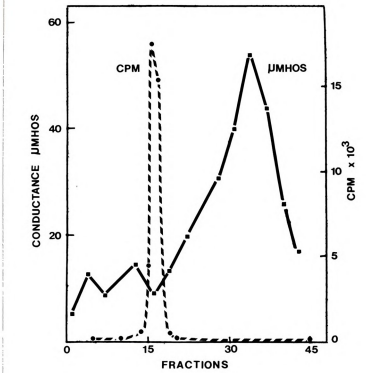

Fig. 14. Toxic activity and radioactivity in fractions from a cation-exchange column used to separate toxin from a ^{14}C-labelled culture filtrate. An aliquot (1.0 ml) from each 2 ml fraction was added to 50 ml water and assayed for toxin by the electrolyte leakage method. Radioactivity in an aliquot (0.1 ml) from each fraction was counted with a Geiger-Mueller tube.

radioactivity were detected when toxin was purified by cation-exchange chromatography (Fig. 14).

The failure to attain labelling using ^{14}C-sucrose was thought to be due to the great dilution involved in the metabolism of sucrose. Compounds which could be toxin precursors or components were incorporated into the Fries' No. 3 medium in a radioactive form. Bioassay of radioactive glutamate, leucine, acetate and mevalonate-amended culture filtrates showed that they contained assayable levels of HV-toxin. Thin-layer chromatography, gel filtration and cation-exchange chromatography of these preparations failed to establish a correlation between radioactivity and HV-toxin activity.

Attempts were made to increase toxin synthesis by blocking terpenoid synthesis with anti-gibberellins. The assumption was that by preventing diterpenoid cyclization (personal communication J. A. D. Zeevaart) more sesquiterpene-containing toxin precursor (victoxinine) and thus more HV-toxin would be synthesized. Antigibberellins CCC or Amo 1618 at concentrations which effectively prevent Gibberella fujikora from synthesizing GA (15) did not inhibit or stimulate the production of HV-toxin. Victoxinine production was not monitored.

Very little is known of the biosynthetic production of HV-toxin in culture. This remains an open area of research for determining kinetics, effects of various carbon sources, growth conditions, and effects of protein synthesis inhibitors on toxin production.

Calculations of maximum theoretical labelling and their implications for detecting toxin binding.--The failure to label toxin biosynthetically prompted some calculations of the maximum theoretical biosynthetic labelling. Many assumptions were made. The first was that toxin is a

sesquiterpene bound to a peptide with 5 amino acids. The nine acetate precursors in the sesquiterpene should make acetate a good avenue of introducing label into the toxin. Carbon-14 at 100% isotopic enrichment contains 62 mC/matom. If one atom of ^{14}C was present at the 2 position in each acetate molecule, 1 mmole of acetate-2-^{14}C would contain 62 mC. One mmole of sesquiterpene would contain 3 x 186 mC/mmole of mevalonic acid, or 558 mC. One mmole of HV-toxin containing the one mmole of labelled sesquiterpene would also contain 558 mC of ^{14}C. This result does not take into account the dilution which would occur by internal biosynthesis and utilization of unlabelled acetate.

Nevertheless, further calculations comparing the detection of HV-toxin by bioassay vs radioactivity can be made, even though it is impossible to attain the maximum theoretical labelling. Assuming root growth bioassay can detect HV-toxin at 0.2 ng/ml, 1 mmole of HV-toxin (MW=867) could be diluted in 4.3×10^9 ml of water and give a dilution end point which inhibits root growth to less than 1 cm. HV-toxin at 558 mC/mmole contains 222×10^7 dpm/ml. Assuming 50% counting efficiency, this becomes 111×10^7 cpm/ml. This is equal to 62×10^{10} cpm/mmole of toxin. When diluted in 4.3×10^9 ml water, which is the dilution end point, there are approximately 150 cpm/ml water. The possibility of detecting any binding of toxin would be near impossible when one considers the large amount of toxin known to be unbound in the solution which gives the dilution end point.

Since tritium has a much higher specific activity than does ^{14}C (29 C/mA vs 62 mC/mA) the same calculations result in a higher specific activity for a mmole of toxin labelled in the same way with tritium. Going through the same steps as before, HV-toxin would contain 260 curies per

mmole. Again this is an unattainable maximum theoretical value under the conditions imposed. This amount of tritium would contain 29 x 10^{13} cpm/mmole in toxin. Dilution to 4.3 x 10^{9} ml, which is the dilution end point, would give 70,000 cpm/ml. This high activity rapidly diminishes when it is remembered that toxin inhibits root growth of oats even at 100 times less than the dilution end point concentration. At that concentration there would be 700 cpm/ml and most of that activity would not be bound. The outlook for detecting binding of toxin even at these high specific activities seems very improbable. The realization that these calculations are based on the presence of 9 labelled atoms per molecule of toxin and that every molecule is labelled suggests that even chemical methods of labelling, including organic synthesis of labelled toxin, would not be useful in determining sites of binding.

There are two other factors to consider regarding the possibility of detecting toxin binding; one is the apparent K_m or affinity of toxin for a site, and the other is the number of such sites per cell. Gardner (9) has calculated a K_m of 1 uM from the electrolyte leakage data, assuming a MW of 1000 for toxin and using the dry weights of impure preparations of toxin as if they were pure toxin. I have recalculated the K_m value making the following adjustments. The 1/2 maximal rate of leakage was obtained with 1 ug of an impure toxin preparation per ml. A concentration of 0.1 ug/ml of the same toxin preparation was barely detectable by the leakage assay; 0.1 ug/ml was also the dilution end point concentration in the root growth bioassay. Scheffer and Pringle stated that the most active toxin preparation obtained had a dilution end point concentration of 0.2 ng/ml (37). Therefore, the actual concentration of toxin in the 0.1 ug/ml preparation was 0.2 ng toxin per ml; 99.8% of the dry weight of this

preparation would be substances other than active toxin. Thus, the preparation would contain 2 ng toxin per ml or 2000 ng per liter. Assuming a toxin molecular weight of 867, this would mean that a concentration of 2.3 nM will give 1/2 the maximal rate of leakage. Thus, the K_m is very low, indicating a high affinity interaction. A low K_m should favor detection of binding, if there are a large number of binding sites. All indications are that there are less than 100 such sites per cell (29).

My conclusion is that labelled toxin probably will not be useful for locating or identifying toxin binding sites in plant cells, because of the extremely high biological activity of HV-toxin.