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Effect of the Specific Toxin in Helminthosporium victoriae on Host Cell Membranes[1]

K. R. Samaddar and R. P. Scheffer
Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48823

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Abstract. ***Helminthosporium victoriae*** **toxin, which affects only hosts of the toxin-producing fungus, causes loss of electrolytes from roots, leaves, and coleoptiles of treated plants. Root hair cells lost the ability to plasmolyze after 20 minutes exposure to toxin in solution; comparable resistant cells retained plasmolytic ability during 3 hours exposure. Toxin stopped uptake of exogenous amino acids and Pi by susceptible but not by resistant tissue. Incorporation of ^{32}P into organic-P and ^{14}C-amino acids into protein was blocked in susceptible but not in resistant tissue. Apparent free space increased in susceptible but not in resistant roots. The increase was evident within 30 minutes, and reached 80 % free space after 2 hours exposure to toxin. When cell wall-free protoplasts were exposed to 0.16 μg toxin/ml, protoplasmic streaming stopped and all plasma membranes of susceptible protoplasts broke within 1 hour. Resistant protoplasts were not affected significantly. Data support the hypothesis of a primary lesion of toxin in the plasma membrane. Effects on synthesis could result from lack of transport of exogenous solutes to sites of synthesis. It is possible that all other observed effects of toxin are secondary to membrane damage.**

"Host-specific toxins" that are determinants of pathogenicity are now known from at least 5 different plant infecting fungi. These compounds reproduce all visible and all known biochemical symptoms of infection. In each case, loss of ability of a fungus isolate to produce its toxin has resulted in loss of pathogenicity. In each case, the substance is toxic only to the host of the toxin producing fungus (23). These are useful models for the study of disease, because many possible complexities of host-parasite interaction are bypassed.

One such substance, produced by *Helminthosporium victoriae* Meehan and Murphy (HV-toxin), is toxic to susceptible oat cultivars, but is harmless to resistant oat cultivars and to all other non-host plants tested (15). The toxin is composed of a cyclic secondary amine ($C_{17}H_{29}NO$) known as "victoxinine" and a peptide containing aspartic acid, glutamic acid, glycine, valine, and leucine. The complete toxin has a M.W. between 800 and 2000 but the exact structure is still unknown, largely because of problems with lability (15).

Among the many cellular responses that occur quickly after HV-toxin treatment are: increased O_2 uptake (21); decreased incorporation of ^{14}C amino acids and uridine into trichloroacetic acid insoluble cellular fractions (23); and rapid loss of electrolytes (24). Several lines of evidence suggest that these effects are secondary. Oxygen and Pi uptake by isolated mitochondria were not affected (4, 21, 23). Toxin uptake appears to be a simple process, not affected by wide ranges of temperature and by metabolic inhibitors. The peptide resulting from toxin breakdown inhibited toxin uptake, suggesting a competition for receptor sites present in susceptible cells (22).

Data to date suggest that the toxin causes a primary lesion in the plasma membrane of susceptible cells (23). The basis of resistance may be a lack of receptors or sensitive sites in the membrane. The possibility that the initial toxin lesion is in the plasma membrane was examined in this study by the use of intact tissues and cell free systems. The effects of toxin on ion leakage from tissues, cell plasmolysis, uptake of solutes, apparent free space, and behavior of cell wall free protoplasts were determined. The data indicate that toxin affects membrane physiology and transport systems of susceptible but not resistant cells. An abstract of part of the work was published (19).

Materials and Methods

Toxin susceptible (cv. Park) and resistant (cv. Clinton) oat seedlings were used in most experiments. Seeds were germinated on moist filter paper and seedlings were grown in the laboratory at 21 to 22° in White's nutrient solutions (14), or in vermiculite plus White's nutrients. In some cases larger plants grown in the greenhouse were used. The inhibition of seedling root growth in a dilution series of toxin was used as the standard bioassay (15).

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Toxin eluted from an alumina column, following the method of Pringle and Braun (14), was relatively stable at pH 3.5, and the concentrated solution was stored at 4°. This preparation gave complete inhibition of root growth of susceptible oat seedlings at a concentration of 0.0016 μg/ml but had no effect on growth of resistant seedling roots at 160 μg/ml. Further purification, achieved by passing this solution through a Bio-Gel P-2 column, gave a more toxic but less stable preparation.

Ion Leakage. The effect of toxin on ion leakage from coleoptile tissue was measured by changes in electrical conductivity of suspending solutions. One g tissue samples were vacuum infiltrated for 10 minutes with toxin, water, or inactivated toxin. The sample was rinsed in glass distilled water, enclosed in washed cheesecloth bags, and incubated on a shaker in 100 ml glass distilled water at 22°. Conductivity of the ambient solution was measured with a model RC 16B1 Industrial Instruments conductivity bridge, using a dip type electrode cell (k=1.0). Specific conductivity is given as reciprocal ohms (mhos).

^{32}P Uptake and Incorporation. Tissue samples were placed in flasks containing 2 ml 1 mM acetate buffer (pH 5.5), 15 μg chloramphenicol and 200 μc ^{32}P. Flasks were incubated at 25° on a Dubnoff metabolic shaker (40 oscillations/min) for 2 hours. Samples were then rinsed thoroughly with cold 1 mM KH_2PO_4 and ground in boiling 80 % (v/v) ethanol. The homogenate was centrifuged at 10,000 × *g* for 10 minutes and the supernatant concentrated to 2 ml. A 50 μl aliquot was spotted on Whatman No. 1 paper. Marker ^{32}P was spotted on each paper for tracing the movement of Pi. Descending chromatographs using *n*-butyl alcohol: propionic acid:water (1246:620:874 v/v) were developed for 40 hours (2). Radioactive zones were detected with a Nuclear Chicago monitor. Radioautographs were made on Eastman Kodak X-ray films. Individual radioactive zones were removed, glued to planchets and counted in a Nuclear Chicago low background gas flow counter. Total uptake was calculated by adding the counts of all spots developed from a single origin. The amount of radioactivity in the inorganic and organic P spots was expressed as percent of total uptake. No attempt was made to identify the organic P compounds.

^{14}C-Amino Acid Uptake. Uptake was considered as the amount of labeled compound taken in from the medium and retained by tissues after repeated washings (18). Roots from 10 seedlings were incubated in 1.0 ml DL-leucine-1-^{14}C or DL-valine-U-^{14}C (pH 7.0). Concentrations of the amino acid solutions varied from 1 to 50 mM with specific activities from 0.5 to 1.0 μc per ml. Chloramphenicol (15 μg) was added to each flask and the mixture was incubated for 1 to 3 hours at 22° with gentle shaking. The roots were then washed for 1 hour in running tap water, blotted gently, placed into tightly stoppered tubes with 0.5 ml 95 % ethanol, and extracted with shaking for 12 hours. Aliquots of the ethanol extracts were counted on planchets in a Nuclear Chicago gas flow counter.

Apparent Free Space. Apparent free space of excised roots was determined by the India ink tagging method of Bernstein and Nieman (3). Roots were rinsed in distilled water, then equilibrated in 35 mM mannitol solution (50 ml/g root tissue) for 0.5 to 2 hours, with 4 or 5 changes of solution. During the last minute of equilibration, 1 ml India ink suspension (1 part/10 parts 35 mM mannitol solution) was added to each 20 ml equilibrating solution. The preparation was stirred for a few seconds before roots were removed, drained, and transferred to 100 ml glass distilled water for exodiffusion.

Exodiffusion solutions were stirred for 5 minutes, and samples were taken at intervals of 5, 10, 15, 30, and 60 minutes. Optical densities of 1:100 dilution of the equilibrating solutions and exodiffusion media were determined with a colorimeter at 500 mμ. A correction for the volume of equilibrating solution adhering to root surfaces was calculated from the optical density and volume of equilibrating and exodiffusion media (3). Mannitol concentrations in equilibration and exodiffusion media were determined by iodometric titration as described by Butler (6). Mannitol adhering to the root surface was subtracted from total mannitol quantity in the exodiffusion medium to obtain the amount of solute in free space. Toxin causes leakage of anthrone positive carbohydrates (4), which might give an error in mannitol determinations. Therefore, a correction for toxin treated samples kept in water was applied. Apparent free space was calculated from the diffusible solute (mg) in the exodiffusion medium and the concentration (mg/ml) of the equilibrating solution, expressed as percentage fresh weight of root tissue.

Preparation of Protoplasts. Protoplasts from coleoptiles of oats, corn, and sorghum were prepared with cellulase by the method of Ruesink and Thimann (17). Cellulase was prepared from culture filtrates of *Myrothecium verrucaria* isolate 460, obtained from Dr. Mary Mandels of the Quartermaster Laboratory at Natick, Massachusetts. The fungus was grown with continuous shaking for 14 days on a modified Whitaker's salt medium (17, 25). Culture filtrates were concentrated to 0.1 volume in a flash evaporator at 37°, and re-filtered. The filtrate was fractionated with solid $(NH_4)_2SO_4$ at 3° (7). The precipitate obtained at 35 to 70 % saturation was dissolved in a small volume of water and desalted in a Sephadex G-25 column. The column was prepared, washed, and developed with 0.1 % NaCl solutions. Maximum cellulase activity was in the second brown (1 ml) fraction eluted

from the column. Aliquots (200 μl) of this fraction were stored in separate vials at −20°, since activity was lost with repeated thawing and freezing.

Coleoptiles from seedlings grown in dim red light were harvested 72 hours after germination. Sections 1 mm long were treated with cellulase solution diluted 1:1 with 1.0 M mannitol buffered with 25 mM sodium phosphate buffer (pH 6.5). After 2 hours incubation in the dark at 22°, 10 volumes of 0.5 M mannitol buffered at pH 6.5 were added. Protoplasts settled to the bottom of the tube in 10 minutes, and the supernatant was removed with a pipet. Mannitol solution was again added and the procedure was repeated to remove cellulase.

Results

Effect of Toxin on Coleoptile Tissue. Since coleoptiles were to be used in some experiments, it was necessary to see if they have the usual differential response of resistant and susceptible plants to toxin. Toxin increases respiration (21) and ion leakage (24) in leaf and root tissues; these responses were chosen as possible indicators of toxic effects on coleoptiles.

Seedlings were grown in the dark, in diffuse light, or in red light. Subapical sections (1 cm long) of coleoptiles were removed and split 3 days after germination. Samples (0.5 g) were vacuum infiltrated with toxin solution (0.16 μg/ml) or water at pH 6.5 for 10 minutes. Coleoptile tissue of susceptible toxin-treated oats released electrolytes at a much faster rate than did the controls and the treated resistant tissue (fig 1). Eight hours after toxin treatment, the specific conductance of the ambient solution of treated susceptible tissue was 5 times greater than that of any other treatment solution.

Oxygen uptake by toxin treated and control coleoptile tissue was determined manometrically. Toxin caused little or no increase in O_2 uptake by susceptible coleoptiles in 4 experiments. Increased O_2 uptake caused by toxin apparently depends on the type of tissue, while an increase in ion leakage is evident in all susceptible tissues so far tested (15).

Effect of Toxin on Plasmolytic Ability of Cells. Cells with damaged membranes will not plasmolyze when placed in hypertonic solutions. Therefore, we can test whether or not toxin destroys or damages the plasma membrane *in situ*. The selective effects of toxin were compared with the effect of 2,4-dinitrophenol (DNP) on cell membranes, using concentrations of DNP known to uncouple oxidation from phosphorylation. Roots of 4 day old plants were placed in either toxin solutions (0.16 μg/ml), DNP (10 and 100 μM), or water for varying times. After treatment roots were quickly rinsed in water, placed in 0.5 M mannitol solutions, and observed under a microscope. Within 20 minutes after toxin exposure, susceptible root hair cells lost the ability to plasmolyze in hypertonic solutions (table I). Toxin treated resistant cells showed plasmolytic shrinkage even after 180 minutes of exposure to toxin. Resistant and susceptible untreated controls plasmolyzed normally. DNP at 100 μM acted much more slowly than toxin and destroyed the plasmolytic ability of both susceptible and resistant cells (table I).

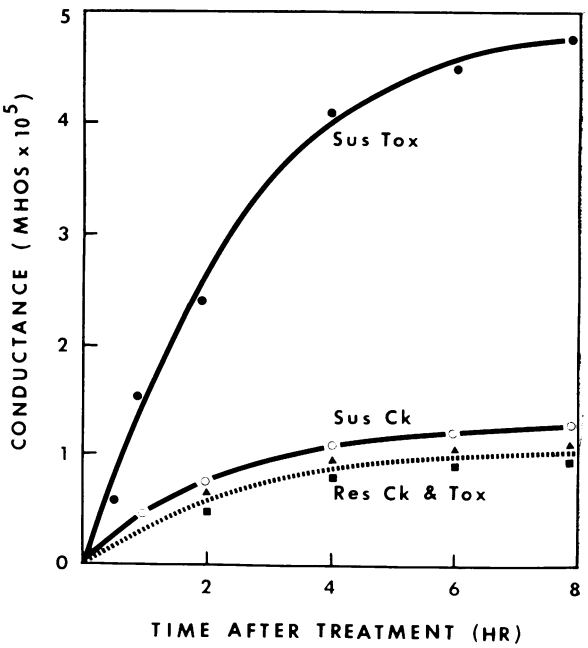

FIG. 1. Effect of toxin on loss of electrolytes from *Helminthosporium victoriae* resistant (Res) and susceptible (Sus) coleoptiles. Tissue samples (0.5 g) were infiltrated with toxin solution (0.16 μg/ml) for 10 minutes, and suspended in glass distilled water. Electrolyte loss was determined from conductivity of the water. ● = Susceptible toxin treated tissue; ○ = susceptible control; ▲ = resistant toxin treated; and ■ = resistant control tissue.

Table I. *Effects of Toxin and 2,4-Dinitrophenol on Plasmolysis of Root-hair Cells in Hypertonic Solutions*

Treatment	Exposure time[1] required to destroy plasmolysis ability	
	Susceptible	*Resistant*
	Min	*Min*
Control[2]	>180	>180
Toxin, 0.16 μg/ml	20	>180
DNP, 100 μM	90	120
DNP, 10 μM	180	>180

[1] Exposure times were 10, 20, 30, 40, 50, 90, 120, 150, and 180 minutes prior to placing cells in hypertonic solution.

[2] Control roots were placed in water or deactivated toxin.

Table II. *Effect of Toxin on ^{32}P Uptake and Incorporation by Oat Leaf Tissue*

Toxin concentration was 0.16 μg/ml. Each tissue sample was 0.5 g, pre-treated with toxin or water for 4 hours and incubated with ^{32}P for 2 hours. Treatments were duplicated.

Oat type and treatment	Radioactivity		
	Total uptake	Inorganic P	Organic P
	cpm	*cpm*	*cpm*
Susceptible control	12,895	9555	3245
Susceptible plus toxin	1560	1215	120
Resistant control	14,400	10,160	3550
Resistant plus toxin	15,800	11,990	3780

Table III. *Effect of Toxin on Uptake of ^{14}C Amino Acids by Susceptible and Resistant Oat Roots*

Reaction mixture (1.0 ml) contained amino acid as indicated; 1 drop of chloramphenicol solution (0.5 mg/ml); 30 mM phosphate buffer (pH 7.0); 250 mg fresh root tissue. After incubation for 2 hours at 22° on a shaker, tissue was washed 1 hour with water and extracted with 0.5 ml ethanol. Aliquots (0.1 ml) on planchets were counted. Toxin concentration was 0.16 μg/ml. The valine-U-^{14}C was 1.0 μc/ml. The leucine-1-^{14}C was 0.5 μc/ml.

Exposure time	Treatment	Uptake of ^{14}C			
		Valine (50 mM)		Leucine (1 mM)	
min		*SUS*	*RES*	*SUS*	*RES*
		cpm	*cpm*	*cpm*	*cpm*
30	Control	9280	10,950	1540	...
	Toxin	585	11,240	255	...
60	Control	9570	9525	1700	...
	Toxin	580	10,040	270	...
120	Control	9985	10,320	1960	1815
	Toxin	460	10,895	190	1795

Effect of Toxin on ^{32}P Uptake and Incorporation. Transpiring cuttings of resistant and susceptible 10 day old plants were allowed to take up toxin solution (0.16 μg/ml) or water for 4 hours. Following treatment, 0.5 g leaf samples were incubated with buffered ^{32}P as described previously. Radioactivity (cpm) of the ethanol extract was used to estimate total uptake. Counts of the several spots on paper chromatograms gave the distribution of P in organic and inorganic fractions. Toxin treated susceptible tissue had less ^{32}P uptake than did susceptible control tissue (table II). Uptake was not inhibited in resistant treated tissues. Controls and treated resistant tissues incorporated 20 to 30 % of the total ^{32}P into organic P-compounds, while incorporation was completely blocked in treated susceptible tissues. The experiment was done 3 times with essentially the same results. Similar results were obtained with root and coleoptile tissues.

In another experiment, the ethanol extracts were added to paper until all spots had approximately equal counts. An autoradiograph was made of the developed chromatogram. Labeled organic P-compounds occurred in extracts of control and treated resistant tissues but little or none were found in extracts of toxin treated susceptible tissue.

Effect of Toxin on Active Uptake of Amino Acids. Amino acid uptake and retention in roots treated with toxin were compared with uptake and retention in untreated controls. Roots were treated with toxin solution (0.16 μg/ml), incubated in labeled amino acid solutions, washed and extracted with ethanol. The radioactivity (cpm) in ethanol extracts was used as a measure of intracellular free pool amino acids. Susceptible roots exposed to toxin for only 30 minutes showed a 90 % decrease in the active uptake of labeled amino acids (table III). Similar results were obtained with labeled valine and leucine at concentrations from 1 to 50 mM.

Effect of Toxin on Apparent Free Space of Tissue. Apparent free space in roots is thought to consist of cell wall and intercellular spaces (5). The possibility of toxin-induced changes in apparent free space in roots was examined as a further measure of membrane damage. The experiment was based on the hypothesis that if toxin disrupts the plasma membrane, the barrier for free permeation will disappear and apparent free space should increase.

Resistant and susceptible oat plants were grown in staining dishes with removable trays (22). Toxin or other substances were added to the solutions of some dishes, while other dishes were used as untreated controls. Apparent free space was similar with different equilibration and exodiffusion times up to 2 hours and with excised roots and roots of intact plants. In most cases roots with the several different toxin treatment times were excised and equilibrated for 1 hour in 35 mM mannitol, followed by 1 hour exodiffusion in glass distilled water.

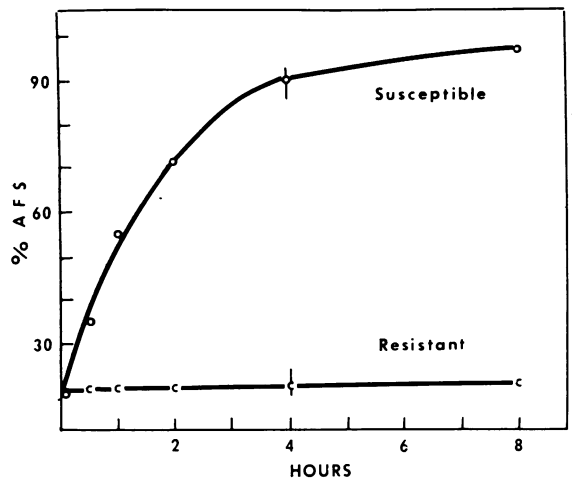

FIG. 2. Effect of toxin (0.16 μg/ml) on apparent free space (AFS) of susceptible and resistant oat roots. Roots were treated with toxin, excised, and equilibrated in 35 mM mannitol for 1 hour, then placed in glass distilled water for exodiffusion. Mannitol in the exodiffusion medium was determined after 1 hour, for apparent free space calculations. Maximum and minimum values are indicated for hour 4.

Control and treated resistant roots had apparent free space values ranging from 13 to 20 % and are therefore in good agreement with published values for *Gramineae* (3, 6). Within 30 minutes, free space in toxin treated susceptible roots increased to 40 %. As the time of treatment increased, values also increased until after 8 hours 90 to 100 % of the total root volume became free space (fig 2). Membranes of more cells appear to be destroyed as toxin treatment time increases, with corresponding increases in free space. There was no change in apparent free space in treated resistant roots, even after 8 hours. Apparent free space appears to change more slowly after toxin treatment than does the response as measured by amino acid uptake. However, the apparent free space measurements are for whole tissues, while the amino acid uptake experiments may include only the outer cells.

The effects of toxin (0.16 μg/ml) were compared with the effects of 0.1 mM DNP, 1 mM NaF, and 1 mM NaN_3 (table IV). After 2 hours exposure to toxin about 80 % of the root volume in susceptible tissue was freely permeable, while apparent free space of resistant roots remained unchanged. NaF and NaN_3 had no effect on apparent free space, although the concentrations used are known to inhibit metabolism. DNP increased apparent free space in both susceptible and resistant roots, and therefore appears to affect the plasma membranes non-specifically.

Effect of Toxin on Free Protoplasts. Cell wall-free protoplasts (17) were prepared from coleoptiles of corn, sorghum, and susceptible and resistant oats. The spherical protoplasts were enveloped by plasma membranes and showed active protoplasmic streaming. The protoplasts were transferred to concavity microscope slides and treated with various concentrations of toxin or inhibitors. The basic reaction mixture was 10 μl protoplast suspension plus 10 μl treatment solution, buffered at pH 6.5. Coverslips were placed over the cell suspensions, and slides were incubated at 22° in a moist chamber. A microscope was used to take zero time counts of intact protoplasts, followed by counts at varying intervals. Survival percentages were based on zero time counts.

Table IV. *Effects of Toxin and Various Metabolic Inhibitors on Apparent Free Space of Oat Roots*
The data were calculated as % of total root volume.

Treatment[1]	% Apparent free space	
	Susceptible	Resistant
Control	16 ± 4	22 ± 4
Toxin, 0.16 μg/ml	80 ± 5	22 ± 4
DNP, 0.1 mM	45 ± 5	45 ± 5
NaF, 1 mM	16 ± 4	22 ± 4
NaN_3, 1 mM	20 ± 2	20 ± 2

[1] Treatment time, 2 hours; equilibrium time in 35 mM mannitol, 2 hour; exodiffusion time in glass distilled water, 1 hour.

Protoplasmic streaming (cyclosis) stopped in many protoplasts from susceptible oat plants within 10 minutes after exposure to toxin. Toxin at 0.16 μg/ml caused 100 % bursting in 1 hour (table V). Most broken protoplasts and the remains of their plasma membranes soon lysed and disappeared, leaving mitochondria apparently unharmed. Protoplasts from corn, sorghum, and resistant oats were not affected. Cyclosis in the resistant protoplasts did not stop and there was no more bursting or lysis than in controls. In several experiments done with slight variations in procedure, free protoplasts from susceptible and resistant plants clearly retained their specific differential response to HV-toxin. Since free protoplasts lack cell walls, we can eliminate this structure as a necessary site of action of the toxin.

Toxin concentrations from 1.6 to 1.6×10^{-7} μg/ml were used in another experiment. Again toxin had a dramatic effect on susceptible but no effect on resistant protoplasts (fig 3). Toxin at 1.6×10^{-4} μg/ml caused 50 % bursting of susceptible protoplasts in 1 hour, while 1.6 μg/ml caused 100 % bursting. Cyclosis was not affected in the toxin treated resistant and untreated control protoplasts.

DNP was used at concentrations (10 and 100 μM) known from preliminary experiments to damage oat cuttings. Again the highly specific effect of toxin was evident (table V). Thirty minutes after exposure to toxin (0.16 μg/ml), 84 %

Table V. *Comparative Effects of Toxin and 2,4-Dinitrophenol on Oat Protoplast Survival*
Toxin concentration was 0.16 μg/ml.

Protoplast type	Treatment[1]	Protoplast survival after[2] 30 min	60 min	120 min
		%	%	%
Susceptible (cv. Park)	Control	94	90	90
	Toxin	16	0	0
	DNP 100 μM	82	60	38
	DNP 10 μM	88	76	45
Resistant (cv. Clinton)	Control	96	92	92
	Toxin	91	90	90
	DNP 100 μM	80	60	40
	DNP 10 μM	87	72	52

[1] Solutions were made with 25 mM phosphate buffer (pH 6.5).
[2] Calculated as % of intact protoplasts at zero time.

of susceptible protoplasts were destroyed and the remainder showed no cyclosis. Within 1 hour all susceptible protoplasts were broken. DNP acted more slowly, and affected susceptible and resistant protoplasts equally. Cyclosis stopped 15 minutes after exposure to DNP, and after 2 hours 48 to 62 % of the protoplasts had lysed or burst (table V).

Filipin and ribonuclease are known to cause bursting of free protoplasts (9, 17). Therefore, these substances were tested for possible differential effects on HV-toxin resistant and susceptible cells. Filipin at 50 μg/ml caused 20 to 30 % of protoplasts to break in 2 hours, while 0.03 % solutions of ribonuclease caused about 50 % bursting in 1 hour. Resistant and susceptible protoplasts were affected equally by both filipin and ribonuclease.

Bisulfite is known from previous work to reduce the effect of HV-toxin on susceptible oat seedlings (22). Therefore, the effect of sodium bisulfite on toxic action against protoplasts was tested. When toxin was diluted with freshly prepared $NaHSO_3$ solution at pH 6.5, toxic effects were delayed. Toxin at 0.16 μg/ml caused 100 % bursting of protoplasts in 1 hour. In the presence of 0.8 mM $NaHSO_3$, only 37 % of the protoplasts lysed in 1 hour, and only 56 % in 2 hours. This concentration of bisulfite alone did not affect protoplast survival. Bisulfite did not completely counteract the effects of HV-toxin, since most of the surviving protoplasts later collapsed. Cyclosis was vigorous in both water and $NaHSO_3$ controls. The bisulfite effect on toxicity to protoplasts parallels the effect on seedlings (22).

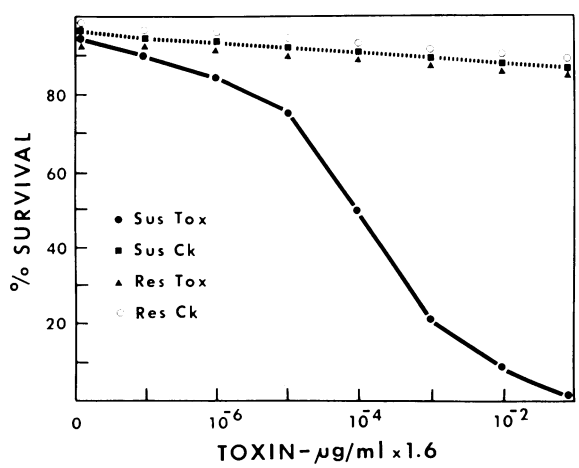

FIG. 3. Effect of toxin concentration on survival of resistant and susceptible protoplasts. Intact protoplasts were counted at zero time and after 1 hour exposure to toxin or water (controls). ● = Susceptible toxin treated; ■ = susceptible control; ▲ = resistant toxin treated; and ○ = resistant control tissue.

Discussion

H. victoriae causes increased respiration in host tissue, as do many other plant pathogens. HV-toxin can reproduce this effect. The primary lesion was once thought to be a toxin-induced uncoupling of oxidation from phosphorylation, but the only evidence was a lack of response by toxin treated tissues to known uncoupling agents (16). Primary effects of toxin clearly are not here, since O_2 and Pi uptake by isolated mitochondria are not affected by toxin (4, 20, 21, 23). Slightly decreased P/O ratios by mitochondria from plants previously treated with toxin (4, 23) may be a secondary effect of cell breakdown products on the mitochondria. Furthermore, coleoptile tissue and aleurone cells (20) are toxin-sensitive, but do not respond by increased gas exchange.

Another effect of HV-toxin is to stop the incorporation of ^{14}C-labeled amino acids into trichloroacetic acid insoluble cellular components (23), suggesting an effect on protein synthesis. However, ribosomes from oats, when prepared carefully to prevent bacterial growth, had such low synthetic activity that no conclusions were possible (23). Ribosomes from reticulocyte cells, which are af-

fected by all known inhibitors of protein synthesis, were not affected by HV-toxin (20). We tentatively conclude that the apparent effect on protein synthesis in tissues is another secondary effect. A breakdown in transport to synthetic sites could explain the effect of toxin on incorporation of amino acids. Similarly, the lack of P- incorporation into organic compounds could result from disruption of transport to the active site of synthesis. Drastic damage to the plasma membrane can result in leakage of intracellular ions as well as inhibition of active uptake of exogenous solutes. However, there is no direct evidence ruling out interference with energy metabolism *in vivo* as part of the explanation of inhibited synthesis.

There are several indications that the site of a primary lesion of toxin is in the plasma membrane. Previous data were suggestive but not conclusive (23). Data presented here are more conclusive, and may be summarized as follows. A) Root hair cells exposed briefly to traces of toxin cannot be plasmolyzed. This is a known effect of membrane damage. B) Toxin inhibits or stops membrane regulated, active uptake of exogenous solutes such as amino acids (8) and P, after brief exposures. C) Apparent free space in tissues increases after toxin treatment. The plasma membrane is considered as the permeability barrier in tissues; disruptions are expected to lead to increased apparent free space. D) The plasma membranes of isolated susceptible protoplasts break after brief exposure to toxin. Furthermore, there is evidence of membrane damage from the electron microscopic work of Luke *et al.* (12); however, these workers used only tissues that had been exposed to toxin for 24 hours.

The experiments with isolated protoplasts are of special interest. Since the toxin acts selectively on protoplasts without cell walls, we can eliminate the wall as a necessary lesion site. The stability of isolated protoplasts depends on intact membranes, and agents affecting this structure can cause bursting. Filipin, a polyene antibiotic, breaks *Neurospora* protoplasts, presumably by binding with membrane sterols (9). Proteases and lipases break *Bacillus megaterium* protoplasts (10), but have no effect on *Avena* protoplasts. Basic proteins such as ribonuclease, cytochrome C, and protamine caused bursting of *Avena* protoplasts, presumably after binding with the membrane (17). We found that filipin, ribonuclease, and DNP affect HV-toxin susceptible and resistant protoplasts, and that the toxin effects are more drastic than those of any other substance tested. The toxin seems to have a strong affinity for membranes of susceptible cells.

Our working hypothesis is that toxin combines with or affects an unknown component in the susceptible cell, resulting in disorganization of the surface. Such disruptions could account for all the effects of toxin described to date. The resistant cell membrane appears to lack the receptor or sensitive site, since such cells do not respond in any observable way. Membrane damage by HV-toxin could lead to the "biochemical symptoms" observed in susceptible cells. This postulation is based in part on published data for several biological systems. Studies on the action of colicines indicate that membrane damage can lead to temporarily increased respiration and collapse of synthetic systems (13). Membrane damage may affect many other cellular components because of physical and metabolic interconnections (1, 11).

HV-toxin and 2 other host-specific determinants of pathogenicity, 1 from *Helminthosporium carbonum* and 1 from *Periconia circinata,* are being used as models for the study of disease development and disease resistance in plants (23). Susceptibility or resistance to all these diseases is based on reaction with or lack of effect by specific toxins. Thus resistance and susceptibility appear to be based on constitutive factors. The extreme specificity of these low molecular weight substances is of interest for other reasons as well. For example, the fact that reactions of isolated mitochondria are not affected indicates selective effects on the different membranes within the susceptible cell.

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Literature Cited

1. Bain, J. M. and F. V. Mercer. 1966. Subcellular organization of the developing cotyledons of *Pisum sativum* L. Australian J. Biol. Sci. 19: 49–67.
2. Benson, A. A., J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Hass, and W. Stepka. 1950. The path of carbon in photosynthesis. V. Paper chromatography and radioautography of the products. J. Am. Chem. Soc. 72: 1710–19.
3. Bernstein, L. and R. H. Nieman. 1960. Apparent free space of plant roots. Plant Physiol. 35: 589–98.
4. Black, H. S. and H. Wheeler. 1966. Biochemical effects of victorin on oat tissues and mitochondria. Am. J. Botany 53: 1108–12.
5. Briggs, G. E. and R. N. Robertson. 1958. Apparent free space. Ann. Rev. Plant Physiol. 8: 11–30.
6. Butler, G. W. 1953. Ion uptake by young wheat plants. II. The apparent free space of wheat roots. Physiol. Plantarum 6: 617–35.
7. Green, A. A. and W. L. Hughes. 1955. Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. In: Methods in Enzymology. I. S. P. Colowick and N. O. Kaplan, eds. Academic Press, New York. p 76–77.

8. HOKIN, L. E. AND M. R. HOKIN. 1963. Biological transport. Ann. Rev. Biochem. 32: 533–78.
9. KINSKY, S. C. 1962. Effect of polyene antibiotics on protoplasts of *Neurospora crassa*. J. Bacteriol. 83: 351–58.
10. LANDMAN, O. E. AND S. SPIEGELMAN. 1955. Enzyme formation in protoplasts of *Bacillus megaterium*. Proc. Natl. Acad. Sci. 41: 698–704.
11. LIPS, S. H. AND H. BEEVERS. 1966. Compartmentation of organic acids in corn roots. II. The cytoplasmic pool of malic acid. Plant Physiol. 41: 713–17.
12. LUKE, H. H., H. E. WARMKE, AND P. HANCHEY. 1966. Effects of the pathotoxin victorin on ultrastructure of root and leaf tissue of *Avena* species. Phytopathology 56: 1178–83.
13. NOMURA, M. 1964. Mechanism of action of colicines. Proc. Natl. Acad. Sci. 52: 1514–21.
14. PRINGLE, R. B. AND A. C. BRAUN. 1957. The isolation of the toxin of *Helminthosporium victoriae*. Phytopathology 47: 369–71.
15. PRINGLE, R. B. AND R. P. SCHEFFER. 1964. Host-specific plant toxins. Ann. Rev. Phytopathology 2: 133–56.
16. ROMANKO, R. R. 1959. A physiological basis for resistance of oats to Victoria blight. Phytopathology 49: 32–36.
17. RUESINK, A. W. AND K. V. THIMANN. 1965. Protoplasts from the *Avena* coleoptile. Proc. Natl. Acad. Sci. 54: 56–64.
18. SACHER, J. A. 1966. Permeability characteristics and amino acid incorporation during senescence (ripening) of banana tissue. Plant Physiol. 41: 701–08.
19. SAMADDAR, K. R. AND R. P. SCHEFFER. 1966. Effect of *Helminthosporium victoriae* toxin on protoplasts from *Avena* coleoptiles. Phytopathology (Abstract) 56: 898.
20. SAMADDAR, K. R. 1968. Mechanism of action of the primary determinant of pathogenicity from *Helminthosporium victoriae*. Ph.D. thesis, Michigan State University.
21. SCHEFFER, R. P. AND R. B. PRINGLE. 1963. Respiratory effects of the selective toxin of *Helminthosporium victoriae*. Phytopathology 53: 465–68.
22. SCHEFFER, R. P. AND R. B. PRINGLE. 1964. Uptake of *Helminthosporium victoriae* toxin by oat tissue. Phytopathology 54: 832–35.
23. SCHEFFER, R. P. AND R. B. PRINGLE. 1967. Pathogen produced determinants of disease and their effects on host plants. In: The Dynamic Role of Molecular Constituents in Plant-Parasite Interaction. C. J. Mirocha and I. Uritani, eds. Bruce Publishing Company, St. Paul, Minnesota. p 217–36.
24. WHEELER, H. AND H. S. BLACK. 1963. Effects of *Helminthosporium victoriae* and victorin upon permeability. Am. J. Botany 50: 686–93.
25. WHITAKER, D. R. 1953. Purification of *Myrothecium verrucaria* cellulase. Arch. Biochem. Biophys. 43: 253–68.