
This is to certify that the

dissertation entitled

SENSITIVITY OF OAT TISSUES AND PROTOPLASTS TO HELMINTHOSPORIUM VICTORIAE TOXIN: ROLE OF TEMPERATURE AND OSMOTICA

presented by

Steven Paul Briggs

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Botany & Plant Pathology

Robert P. Scheffer
Major professor

Date Sept. 21, 1982



# SENSITIVITY OF OAT TISSUES AND PROTOPLASTS TO HELMINTHOSPORIUM VICTORIAE TOXIN: ROLE OF TEMPERATURE AND OSMOTICA

By

Steven Paul Briggs

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1982

ABSTRACT

SENSITIVITY OF OAT TISSUES AND PROTOPLASTS TO HELMINTHOSPORIUM VICTORIAE TOXIN: ROLE OF TEMPERATURE AND OSMOTICA

By

Steven Paul Briggs

A rapid, simple method for obtaining highly active preparations of Helminthosporium victoriae (HV) toxin was developed. Filtrates from 3 week-old cultures were precipitated with methanol, extracted with butanol, and chromatographed twice on an SP-Sephadex C25 column equilibrated and developed with water. Toxin was active at a concentration of 0.7 ng/ml.

HV-toxin caused the disruption of plasmalemma and tonoplast of sensitive but not resistant oat plants. Leakage of electrolytes from damaged cells was dependent on the fluidity of the membrane lipids. Electron spin resonance spectroscopy of a fatty acid spin label (5-doxylstearic acid) revealed that oat protoplast membranes undergo a phase change at 12°C. Leakage caused by toxin showed a similar temperature dependence which indicated that electrolytes may be transported across the membrane by a diffusible carrier; loss through pores is less likely. The rate of leakage caused by toxin was reduced more than 50% in the presence of plasmolyzing concentrations (0.2 M or greater) of sorbitol or mannitol. Levels of sorbitol which did not eliminate cell turgor were much less effective while higher concentrations were only slightly more effective. Neither stimulated uptake of electrolytes, changes in transverse pressure on the plasmalemma, nor osmotic shock appeared to be involved. Osmotica may act by preventing the flow of water into vacuoles of damaged cells.

Protoplasts were found to be as sensitive to toxin as were intact tissues. Mesophyll protoplasts were killed rapidly by toxin; collapse followed death at 35°C but not at 23°C. Isolated vacuoles were damaged by toxin but the presence of cytoplasmic contaminants on the vacuole surface may have been responsible for sensitivity to toxin. Vacuoles prepared by a method thought to preclude contaminants which adhere to the surface were not visibly affected by toxin.

The data show that toxin has a significant effect on the plasmalemma, and are compatible with the hypothesis that the initial biochemical lesion is in the plasmalemma or the cytoplasm. The data do not support the hypothesis that toxin has a primary effect on the cell wall. Further work is needed to evaluate the role of the tonoplast.

ACKNOWLEDGEMENTS

I wish to thank my major professor, Dr. R. P. Scheffer, and the members of my guidance committee, Drs. A. Haug, K. Poff and E. J. Klos, for the support they have given me throughout my graduate career. Thanks are also due Drs. A. H. Ellingboe and P. S. Carlson who served on my committee before leaving the university. Stimulating discussions with Dr. Ken Nadler lead to the quantitative model of leakage by mass flow presented in the General Discussion.

Thank you very much.

TABLE OF CONTENTS

Page

SECTION 4

RAPID KILLING OF OAT PROTOPLASTS BY
HELMINTHOSPORIUM VICTORIAE TOXIN

LIST OF TABLES

LIST OF FIGURES

Figure Page

SECTION 4

# GENERAL INTRODUCTION

Diseases involving host-selective toxins have long been used as model systems in plant pathology (9). A major advantage is the ability to study changes in diseased tissue without the confounding presence of the pathogen (9). One of the most studied cases is Victoria blight of oats, caused by Helminthosporium victoriae Meehan & Murphy. Work on this disease led to the long-standing hypothesis that plants are susceptible to the disease because they possess a receptor for the toxin (HV-toxin) (8). Resistant plants either lack the receptor or else possess a modified receptor which cannot bind toxin. HV-toxin causes the disruption of the plasmalemma of sensitive plants almost immediately (7); whether the effect is direct or indirect has been a subject of much controversy (4). However, all known changes in the host caused by the fungus or by toxin treatment can be attributed to disruption of the plasmalemma (8).

I have presented the work described in this thesis in four sections, to facilitate later publication elsewhere. A rapid, simple method for obtaining highly active toxin preparations is described in Section 1. The method does not provide an entirely homogenous preparation, but it is a good starting point for further purification; the preparation can be used for most studies of toxic effects. Older methods of toxin preparation made use of alumina, which can form a complex with toxin (Pringle,

personal communication); the complex could interfere with structural determinations and with toxic effects on cells.

Despite the extensive documentation of permeability changes caused by toxin (4), until now no attempts to measure direct effects on membrane structure have been made, other than some ultrastructural studies (4). The electron microscope studies failed to detect early effects of toxin, although disruption of membranes eventually become obvious. As a first step toward monitoring toxin-induced changes in membranes of living cells, I developed a method for using spin labels and electron spin resonance spectroscopy with isolated protoplasts (2,3). This study was the basis of my thesis for the M.S. degree (1). I used the spin label method to determine whether or not HV-toxin causes changes in membrane structure and to correlate aspects of structure such as phase changes (6) with properties of function such as permeability. The results of these experiments are reported in Section 2.

I used protoplasts to study toxin effects on membranes. Protoplasts were chosen because they form a relatively homogenous, living population which can be easily manipulated, and the plasmalemma is exposed. However, in the first stages of my research I was unable to detect significant damage to protoplasts even several hours after toxin treatment. General appearance and staining with Evan's blue or neutral red indicated the protoplasts were not sensitive to toxin. Attempts were made to determine what factor(s) caused protoplasts from sensitive tissue to become insensitive to toxin. An obvious candidate was the osmoticum in which protoplasts must be held to prevent lysis. I found that the sensitivity of tissues was much diminished in the presence of

plasmolyzing levels of osmoticum. This led to a more detailed hypothesis for toxin action which involves water flow into the vacuole of damaged cells. This idea and the data upon which it is based are presented in Section 3.

Fluorescein diacetate (FDA) was reported to be a better indicator of viability than are most vital stains (10). Hawes and Wheeler (5) used FDA to show that isolated root cap cells from oats were killed by HV-toxin. I found by staining with FDA that isolated protoplasts quickly lost their viability in the presence of toxin. Toxin-treated protoplasts collapsed within 3 hours after exposure if held at 35°C but remained normal in appearance if held at 23°C, even though viability was lost in both cases. These results are described in Section 4 which also describes work on the role of the cell wall, cytoskeleton, and vacuole in toxin response.

I have not prepared an extensive review on HV-toxin, or on host-selective toxins in general, because a number of such reviews have been published in recent years. Detailed background information may be found in a recent book edited by R.D. Durbin (4). In addition, the subject was reviewed thoroughly by Yoder (11) and by Scheffer (8).

LIST OF REFERENCES


1. Briggs, S.P. 1980. Location of spin labels in oat leaf protoplasts. M.S. Thesis, Michigan State University.

2. Briggs, S.P., A.R. Haug and R.P. Scheffer. 1982. Interaction of nitroxide spin labels with chloroplasts. Plant. Physiol. 70:668-670.

3. Briggs, S.P., A.R. Haug and R.P. Scheffer. 1982. Localization of spin labels in oat leaf protoplasts. Plant Physiol. 70:662-667.

4. Daly, J.M. 1981. Mechanisms of action. In "Toxins in Plant Disease", R.D. Durbin (ed.), Academic Press, NY. pp. 515.

5. Hawes, M.C. and H. Wheeler. 1982. Factors affecting victorin-induced root cap cell death: temperature and plasmolysis. Physiol. Plant Pathol. 20:137-144.

6. Quinn, P.J. 1981. The fluidity of cell membranes and its regulation. Prog. Biophys. Molec. Biol. 38:1-104.

7. Samaddar, K.R. and R.P. Scheffer. 1971. Early effects of Helminthosporium victoriae toxin on plasma membranes and counteraction by chemical treatments. Physiol. Plant Pathol. 1:319-328.

8. Scheffer, R.P. 1976. Host-specific toxins in relation to pathogenesis and disease resistance. In "Physiological Plant Pathology", R. Heitefuss and P.H. Williams (eds.), Springer-Verlag, NY. pp. 890.

9. Scheffer, R.P. and S.P. Briggs. 1981. A perspective of toxin studies in plant pathology. In "Toxins in Plant Disease", R.D. Durbin (ed.), Academic Press, NY. pp. 515.

10. Widholm, J. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technology 47:189-194.

11. Yoder, O.C. 1980. Toxins in pathogenesis. Ann. Rev. Phytopathology 18:103-129.

SECTION 1

# PREPARATION OF HELMINTHOSPORIUM VICTORIAE TOXIN

ABSTRACT

HV-toxin was isolated from 3 week-old cultures of Helminthosporium victoriae grown in Fries medium with yeast extract. Culture filtrate was precipitated with methanol and extracted with butanol. A thin-layer chromatography assay was developed for detecting contaminants in toxin preparations. Silica gel 60 plates were developed with ethanol, water, acetic acid (70:29:1); the major contaminant was detected by spraying the plates with vanillin-$H_2SO_4$. Cation-exchange chromatography separated toxin from the major contaminant. Toxin preparations were active at 0.7 ng/ml. High-performance liquid chromatography revealed that such preparations still contained impurities.

## INTRODUCTION

A satisfactory method for the purification of HV-toxin has not yet been elucidated. Highly active preparations have been obtained (1) but alumina columns were used. Alumina may form complexes with HV-toxin (Pringle, personal communication) and so should be avoided. We have established a simple, rapid method for preparation of HV-toxin which does not employ alumina. These preparations are of comparable activity to the best so far reported (2).

## EXPERIMENTAL

We retained the first steps in the established preparation procedure (2). These involve growing the fungus for 21 days in Fries medium with yeast extract, methanol precipitation of culture filtrate, and butanol extraction. The toxin preparation used for the experiments described in this section was then passed through a Sephadex LH20 column developed with water:methanol (1:1). This step was not effective in separating toxin from the major contaminant, as will be described later. The preparation at this stage had a dry weight of 500 mg/ml and diluted to $10^{-6}$ in the root growth inhibition assay. The next step was to establish a method for detecting contaminants; such a method is needed to evaluate the effectiveness of each attempted separation. Thin-layer chromatography was chosen because of its speed and simplicity. Several visualizing reagents were tested (Table 1). More spots were consistently detected on a fluorescent plate with ultra-violet light than by use of any other single method or reagent. Several compounds were separated in most solvent systems. However, use of vanillin-$H_2SO_4$ revealed that most of the contamination in crude toxin preparations migrated as a single spot or band; this substance is referred to as the major contaminant. The major contaminant was detected by reagents 2, 5 to 7, and 9 to 14 (see Table 1), suggesting that the contaminant is a complex molecule or mixture. The separation strategy was to first separate the

TABLE 1. Reagents used to visualize compounds on silica gel 60 thin layer chromatograms.[1]

| Reagent name | Method | Compounds detected |
|---|---|---|
| Iodine | Solution A: 5% $I_2$, 10% KI. Solution B: dilute 2 ml solution A with 3 ml $H_2O$ and 5 ml acetic acid. Spray plate with solution B. | alkaloids |
| Potassium iodoplatinate | Solution A: 10% KI, 45 ml. Solution B: 5% PtCl, 5 ml. Mix A and B and dilute to 100 ml with water; spray. | alkaloids |
| Dragendorff's | Solution A: 1.7 g $BiONO_3$ in 100 ml water, acetic acid (8:2). Solution B: 40 g KI in 100 ml water. Mix 5 ml A, 5 ml B, 20 ml acetic acid, 70 ml water; spray. | alkaloids, organic |
| Ninhydrin | Solution A: 0.2% ninhydrin in butanol, 95 ml. Solution B: 10% acetic acid, 5 ml. Mix A and B; spray; heat. | amino acids |
| Diphenylamine | Solution A: 2 g diphenylamine, 10 ml $H_3PO_4$, 2 ml aniline, 90 ml acetone; spray; heat. | carbohydrates |
| Anisaldehyde | Solution A: 1 ml anisaldehyde, 1 ml $H_2SO_4$, 18 ml ethanol; spray; mild heat. | carbohydrates |
| Bromcresol green | Solution A: 0.5% bromcresol green in alkaline ethanol; spray. | carboxylic acids |
| Potassium permanganate | Solution A: 0.25% $KMnO_4$ in water; spray. | diterpenoids |
| Hydroxamic acid-ferric acid | Solution A: 12.5% NaOH, 5% $NH_2OH$ mixed 1:1 in aqueous solution. Solution B: acetic acid. Solution C: 10% $FeCl_3$ (aq.). Spray with solution A; heat; spray with solution B; spray with solution C. | esters |

Table 1. (cont.)

| Reagent name | Method | Compounds detected |
|---|---|---|
| iodine | Bathe the TLC plate in vapor from heated iodine crystals. | general |
| ultraviolet (UV) light; long (366 nm) and short (254 nm) wavelengths | Examine fluorescent plates for dark spots or non-fluorescent plates for light spots. | general |
| ammonium bicarbonate | Bathe the non-fluorescent TLC plate in vapor from heated $NH_4HCO_3$ crystals; view under UV light. | general |
| vanillin | Solution A: 1% vanillin in $H_2SO_4$; spray; heat. | terpenes |
| antimony pentachloride | Solution A: 20% $SbCl_5$ in $CCl_4$; spray; heat. | terpenoids |

[1]Silica Gel 60, F-254 TLC plates were from Merck and had a thickness of 0.25 mm.

toxin from the major contaminant. To evaluate each attempted separation, TLC with vanillin-$H_2SO_4$ was used to assay for the contaminant and electrolyte leakage from leaf sections was used to assay for toxin.

Several solvent systems were tested (Table 2), in part to help develop a large scale separation process. Separation of the toxin from the major contaminant in the butanol extract was achieved with systems 1 to 3 and 11 to 13. In systems 1 to 3 the toxin remained at the origin making these systems unsatisfactory. Systems 11 to 13 were not satisfactory because the toxin smeared over a large area at the base of the plate rather than running as a tight band. The major contaminant ran as a very tight band in systems 9 and 10, but chromatograms developed faster in system 9 than in 10. In further work, system 9 (ethanol:water:acetic acid, 70:29:1) was routinely used to detect the major contaminant in column chromatographic eluants.

An attempt was made to separate toxin from the major contaminants in butanol extracts by use of molecular exclusion chromatography on BioGel P-2 columns (Table 3). Toxin eluted from the column over a very large volume (40 ml) indicating either adsorption to the column bed or interaction with a contaminant in solution. Similar results were obtained with Sephadex G-15 in water or in 30% aqueous methanol, and with LH20 in 30% or 50% aqueous methanol; in each case the toxin eluted over very large volumes. Thus, toxin was not separated from the major contaminants by molecular exclusion chromatography.

Adsorption chromatography on straight-phase silica flash columns was unsuccessful because HV-toxin is insoluble in non-polar solvents such as dichloromethane. More polar solvents such as ethanol or

TABLE 2. Solvent systems tested to separate toxin from major contaminants on silica gel 60 thin layer chromatograms.[1]

| Solvent system | $R_f$ toxin | $R_f$ major contaminant[2] |
|---|---|---|
| 1. acetone, water (93:7) | origin | 0.6-0.25 |
| 2. acetone, water (85:15) | origin-0.22 | 0.31-0.50 |
| 3. acetone, acetic acid, water (85:5:10) | origin | 0.16-0.45 |
| 4. butanol, acetic acid, water (5:1:4) | 0.11-0.43 | 0.33-0.43 |
| 5. butanol, acetic acid water (3:1:1) | 0.22-0.37 | 0.20-0.30 |
| 6. butanol, methylethyl ketone, acetic acid (5:4:1) | | |
| 7. chloroform, methanol (4:1) | not assayed | origin-0.22 (smear) |
| 8. ethanol, water, acetic acid (95:4:1) | origin | 0.01-0.09 |
| 9. ethanol, water, acetic acid (70:29:1) | not assayed | 0.38 (center of smear) |
| 10. ethanol, water, acetic acid (50:49:1) | 0.50-0.70 | 0.56-0.70 |
| 11. methanol | 0.65-0.78 | 0.65-0.78 |
| | origin-0.33 | 0.43-0.57 (much tailing) |
| 12. methanol, water (95:5) | 0.03-0.30 | 0.38-0.59 |
| 13. methanol, water (93:7) | 0.03:0.43 | 0.43-0.64 |
| 14. methanol, acetic acid, water (7:1:2) | 0.50-0.66 | 0.50-0.66 |
| 15. methylethyl ketone, methanol, acetic acid (7.5:2.4:1) | origin-0.24 | origin-0.24 (much tailing) |
| 16. n-propanol, ammonium hydroxide (58%), water (7:1:2) | origin-0.29 | 0.12-0.26 |
| 17. 2-propanol, ethyl acetate, water (7:1:2) | 0.17-0.23 | 0.17-0.22 |

[1]TLC plates were prepared by applying 20 µl of the toxin preparation from an LH20 column (diluted 1:10 in methanol) in a line 8 cm long, 1 cm above the base of a 10 x 10 cm plate. The starting preparation had a dry weight of 500 mg/ml and diluted to $10^{-6}$ in the root growth bioassay. For bioassay of the TLC plates, the center 4 cm were scraped off at the indicated $R_f$. Toxin was eluted from silica with water and the eluant was used in the standard electrolyte leakage assay (1). The silica remaining on the plate was sprayed with reagent to visualize contaminants. Solvent systems were prepared 1 hour before use and allowed to equilibrate in the development chamber.

[2]Contaminant was detected with vanillin-$H_2SO_4$.

TABLE 3. Molecular exclusion chromatography of HV-toxin on Biogel P-2 columns.

| Column fraction No.[2] | Leakage rate[3] | Presence of contaminants at indicated $R_f$[1] | | | | |
|---|---|---|---|---|---|---|
| | | 0.22 | 0.33 | 0.41 | 0.52 | 0.53 |
| 21-25 | 0.04 | | | | | |
| 26-30 | 0.06 | | | | | |
| 31-35 | 0.07 | | | | | |
| 36-40 | 0.05 | | | | | |
| 41-45 | 0.06 | | | | | |
| 46 | 0.44 | | | | | |
| 47 | 0.44 | | | | | |
| 48 | 0.44 | | | | | + |
| 49 | 0.44 | | | | | + |
| 50 | 0.44 | + | + | | | ++ |
| 51 | 0.56 | ++ | + | + | | ++ |
| 52 | 0.56 | +++ | | ++ | | |
| 53 | 0.56 | ++ | | +++ | | |
| 54 | 0.56 | + | | +++ | | |
| 55 | 0.56 | | | +++ | | |
| 56 | 0.44 | | | +++ | | |
| 57 | 0.44 | | | +++ | + | |
| 58 | 0.44 | | | + | ++ | |
| 59 | 0.44 | | | | ++ | |
| 60 | 0.44 | | | | ++ | |
| 61 | 0.15 | | | | + | |
| 62 | 0.15 | | | | | |
| 63 | 0.15 | | | | | |
| 64 | 0.15 | | | | | |
| 65 | 0.15 | | | | | |
| 66-70 | 0.06 | | | | | |
| $H_2O$ | 0.02 | | | | | |
| $5 \times 10^{-4}$ dilution of LH20 prep. | 0.48 | | | | | |

[1]Contaminants were detected on thin layer chromatograms developed in solvent system 17 (Table 2). Contaminants are visualized with vanillin-$H_2SO_4$. Color of contaminants at several $R_f$ zones: 0.22, blue; 0.33, gray; 0.41, purple; 0.52, yellow; 0.53, purple.

[2]A 1.3 x 110 cm column was loaded with 1 ml of the toxin preparation from an LH20 column (see footnote 1 in Table 2) and eluted with water (7 ml per hour). Fraction volume was 2 ml; void volume was approximately 45 ml.

[3]Fractions were assayed by removing 10 µl, dilution to 10 ml in water and assaying by the electrolyte leakage method. The leakage rate is in units of µmhos/minute.

methanol caused toxin to elute with the major contaminant without adsorption occurring.

Both the toxin and the contaminant passed through a QAE-Sephadex A25 column, equilibrated and developed with water, without being retarded. A brown pigment in the toxin preparation was adsorbed to the column and was not desorbed by 0.1 M NaCl. However, anion-exchange chromatography failed to separate toxin from the major contaminant (Table 4).

Several attempts were made to separate toxin from the major contaminants in butanol extracts by use of cation-exchange chromatography. A partial separation was achieved on the first pass through an SP-Sephadex C25 column equilibrated and developed with water. Separation was not enhanced by a linear NaCl gradient (from 0 to 0.1 M). The gradient accelerated the elution of toxin but did not affect the major contaminant, which eluted in the void volume. A second pass through the column caused the toxin to be strongly retained. Toxin did not begin to elute until fraction 43 and continued at least until fraction 77. The major contaminant was also retained more than on the first pass; it eluted in fractions 16-19 rather than 8-11. The toxin-containing fractions were pooled and the dry weight and toxicity were determined. This preparation of toxin completely inhibited root growth of susceptible seedlings at 0.7 ng/ml.

The large ratio of contaminants to toxin present in the first pass through the SP-Sephadex C25 column inhibited interaction with the ion-exchange resin, possibly by competing for binding sites. On the second pass, toxin bound strongly, indicating that it behaves as a cation at neutral pH. Elution with a NaCl gradient on the second pass should

TABLE 4. Anion-exchange chromatography of HV-toxin on QAE-Sephadex A25 column.

| Fraction No.[1] | Major contaminant[2] | Conductivity[2] |
|---|---|---|
| 2 | | 8 |
| 3 | | 7 |
| 4 | | 10 |
| 5 | ++ | 57 |
| 6 | +++ | 71 |
| 7 | +++ | 70 |
| 8 | ++ | 79 |
| 9 | + | 64 |
| 10 | | 43 |
| 11 | | 35 |
| 12 | | 19 |
| 13 | | 13 |
| water | | 7 |

[1]Fraction volume was 1 ml. Toxin preparation was the same as for Table 4; sample sizes were 0.5 ml each. The column bed was 0.9 x 7 cm (4.5 ml bed volume). The column was equilibrated and developed with water until fraction 17 was collected; at that point a linear NaCl gradient (to 0.1 M) was started. The NaCl solution did not elute any more toxin or major contaminant.

[2]Assay is given in Figure 4.

TABLE 5. Cation-exchange chromatography of HV-toxin on SP-Sephadex C25 columns.

| | Column development conditions[1] | | | | | |
|---|---|---|---|---|---|---|
| | Zero to 0.1 M NaCl | | Water; first pass | | Water; second pass | |
| Fraction No. | Major contaminant[2] | leakage rate[3] | Major contaminant | Leakage rate | Major contaminant | Leakage rate |
| 1-5 | | 0.04 | | | | |
| 5-6 | | 0.26 | | 0.06 | | |
| 7-8 | ++ | 0.26 | + | 0.05 | | |
| 9-10 | +++ | 0.26 | +++ | 0.12 | | |
| 11-12 | + | 0.29 | + | 0.32 | | |
| 13-14 | | 0.33 | | 0.32 | | |
| 15-16 | | 0.26 | | 0.40 | ++ | |
| 17-18 | | 0.09 | | 0.37 | +++ | |
| 19-20 | | 0.06 | | 0.19 | + | |
| 21-22 | | 0.03 | | 0.07 | | |
| 23-42 | | | | | | avg. = 0.06 |
| 43 | | | | | | 0.12 |
| 44 | | | | | | 0.17 |
| 45 | | | | | | 0.20 |
| 46 | | | | | | 2.25 |
| 47 | | | | | | 0.37 |
| 48 | | | | | | 0.47 |
| 49 | | | | | | 0.54 |
| 50 | | | | | | 0.52 |
| 51 | | | | | | 0.58 |
| 52 | | | | | | 0.48 |
| 53 | | | | | | 0.68 |
| 54 | | | | | | 0.58 |
| 55 | | | | | | 0.81 |

TABLE 5. (cont.)

| Fraction No. | Column development conditions[1] | | | | | |
|---|---|---|---|---|---|---|
| | Zero to 0.1 M NaCl | | Water; first pass | | Water; second pass[4] | |
| | Major contaminant[2] | leakage rate[3] | Major contaminant | Leakage rate | Major contaminant | Leakage rate |
| 56 | | | | | | 0.74 |
| 57 | | | | | | 0.55 |
| 58 | | | | | | 0.44 |
| 59 | | | | | | 0.50 |
| 60 | | | | | | 0.51 |
| 61 | | | | | | 0.45 |
| 62 | | | | | | 0.52 |
| 63 | | | | | | 0.51 |
| 64 | | | | | | 0.46 |
| 65 | | | | | | 0.45 |
| 66 | | | | | | 0.60 |
| 67 | | | | | | 0.54 |
| 68 | | | | | | 0.59 |
| 69 | | | | | | 0.50 |
| 70 | | | | | | 0.52 |
| 71 | | | | | | 0.51 |
| 72 | | | | | | 0.48 |
| 73 | | | | | | 0.48 |
| 74 | | | | | | 0.55 |
| 75 | | | | | | 0.59 |
| 76 | | | | | | 0.50 |
| 77 | | | | | | 0.35 |

TABLE 5. (cont.)

[1]The same column was used in each case. Column size was 1.5 x 23 cm (40 ml bed volume). The column was developed with either a linear NaCl gradient (from 0 to 0.1 M NaCl) which was begun immediately after the sample entered the column bed or else distilled water alone was used for development. When water alone was used, the sample was passed through the column twice; the results of both the first and second passes are shown. The toxin sample had a dry weight of 142 mg/ml and a dilution endpoint of $10^{-4}$; the sample was the supernatant (in water) of a methanol precipitation of toxin from an LH20 column. One ml of sample was applied to the column. Fraction volumes were 2 ml.

[2]The major contaminant ($R_f$ = 0.41) was visualized with vanillin-$H_2SO_4$.

[3]Leakage rate was determined by the electrolyte leakage assay. Toxin samples (10 µl per fraction) were pooled and diluted to 10 ml.

[4]Fractions eluted after elution of the major contaminant in the first pass were pooled and concentrated; the results of 7 different runs were concentrated to 1 ml. This sample was applied to the column for the second pass.

enhance the chromatography but would introduce the problem of desalting the toxin. We were unable to de-salt by gel exclusion chromatography (data not shown).

The sample, prepared by two passes through SP-Sephadex, was chromatographed on a Waters high performance liquid chromatography (HPLC) system using a Whatman Partisil 10, 0.5 x 25 cm column developed isocratically with 90% aqueous ethanol. At least five peaks were detected by UV-absorbance at 214 nm. We did not determine whether any of the peaks observed were caused by toxin. Reverse-phase chromatography on C-18 or C-8 columns did not result in detectable separation.

Re-introduction of the major contaminant into toxin preparations from which it had been removed did not decrease the toxicity of the solutions (data not shown).

## LIST OF REFERENCES


1. Samaddar, K.R. and R.P. Scheffer. 1968. Effect of the specific toxin in Helminthosporium victoriae on host cell membranes. Plant Physiol. 43:21-28.

2. Scheffer, R.P. and R.B. Pringle. 1963. Respiratory effects of the selective toxin of Helminthosporium victoriae. Phytopathology 53:465-468.

SECTION 2

# FLUIDITY OF OAT CELL MEMBRANES AFFECTS ELECTROLYTE LEAKAGE INDUCED BY HV-TOXIN

## ABSTRACT

Protoplasts from oat leaves were held at temperatures from 0°C to 40°C, and the fluidity of the membranes was monitored with the spin label 5-doxylstearic acid. A membrane phase change was observed at approximately 12°C. There was relatively little toxin-induced loss of electrolytes from susceptible leaves at temperatures below 12°C; losses increased rapidly with increases in temperature above 12°C. The correlation between leakage rate and membrane fluidity suggests that loss of electrolytes occurs through the matrix of the membrane, possibly via a shuttle-type carrier. There was no toxin-induced leakage from leaf tissues that were treated with toxin at 0°C, and subsequently incubated at that temperature. Tissues which were treated with toxin at 0°C, washed, and then warmed to 23°C leaked as if they had been held at 23°C throughout. Therefore, toxin may be taken up or bound at 0°C but does not initiate leakage until the tissues are warmed.

# INTRODUCTION

Helminthosporium victoriae, the causal agent of Victoria blight of oats, produces a toxin which selectively affects cultivars carrying the $V_b$ allele. To date, the first detectable effect of the toxin is rapid loss of electrolytes from sensitive tissue (16). The rate of leakage is proportional to the concentration of toxin to which tissues are exposed (9). Leakage continues, even after removal of toxin, until most of the free electrolytes are lost from tissues.

The mechanism by which HV-toxin causes electrolytes to be lost is unknown. The longest-standing hypothesis is that toxin binds to a receptor in the plasmalemma, leading to a breakdown of semi-permeability. Resistant plants are thought to either lack such a receptor or else possess a modified receptor which does not bind toxin (16). This hypothesis has been applied to several host-selective toxins, but to date there is no conclusive proof that it is correct (12).

Experiments described here were designed to clarify several questions. Does HV-toxin cause a large change in the structural matrix of protoplast membranes? Do the electrolytes which are lost because of toxin treatment pass through holes created by membrane rupture or must they diffuse through the matrix of the membrane? Finally, if electrolytes do move through the membrane, what type of transport mechanism may be involved?

There are two well-characterized models of membrane transport, both elucidated by studies with ionophores. Gramicidin A forms a bimolecular tunnel which spans the bilayer and mediates the passage of monovalent cations (15). Valinomycin is circular and sequesters $K^+$ inside. These two ionophores are representative of the pore-forming and cage-forming classes of ionophores. Valinomycin can diffuse freely across membranes because of its hydrophobic surface. Studies on phospholipid bilayers reveal that the lipids must be in a fluid state for valinomycin to function as an ionophore (6). When the temperature of the sample drops below the lipid phase transition temperature, valinomycin-mediated conductance across the bilayer stops. Presumably, this is because valinomycin cannot diffuse through gel phase lipid and is, essentially, "frozen-out" of the membrane. In contrast, gramicidin A can function normally even when the lipids are in the gel phase (6). Once the bimolecular tunnel is formed, the structure is stable and functionally independent of the lipid phase outside the tunnel.

We have used electron spin resonance (ESR[1]) spectroscopy and a fatty acid spin label to characterize the temperature-dependence of molecular ordering in membranes of oat leaf protoplasts. While true lipid phase transitions do not occur in biological membranes, transformations have been described which are often correlated with important physiological functions (13). Low temperature inhibition of membrane activities often occurs below phase transformation temperatures. Transformations are characterized by a cooperative increase in molecular ordering but are not simple liquid-crystal to gel lipid phase

---

[1]ESR, electron spin resonance; I(12,3), 5-doxylstearic acid

transitions. We have compared the results of our spin label studies with experiments on the temperature-dependence of toxin-induced electrolyte leakage. The data indicate that leakage may be mediated by a shuttle-type carrier.

## MATERIALS AND METHODS

Plant materials were prepared as previously described (3). The effect of toxin on electrolyte leakage was determined by use of leaf segments (0.5 cm long). The tissue samples (0.2 g) were wrapped in cheesecloth and immersed in 10 ml of solution in a scintillation vial. The samples were infiltrated under reduced pressure with either water or toxin solution, incubated at room temperature for 1 hour, washed thoroughly, and resuspended in 10 ml of $H_2O$. They were then placed in water baths at the treatment temperature and held at that temperature for the duration of the experiment. Electrolytes in ambient solutions were determined with a Markson conductivity meter equipped with a pipet-type temperature-compensating electrode (K=1.0).

Protoplasts were isolated by peeling away the lower epidermis of the leaves with forceps and floating the leaves (peeled surface down) on a solution containing 0.5% Cellulysin (Calbiochem-Behring Corp.) and 0.6 M sorbitol, adjusted to pH 5.6 with KOH. The preparation was incubated at 28°C for 3 hours in the light, then was swirled gently to release protoplasts. The protoplasts were filtered through a layer of Miracloth and the suspension was centrifuged at 40xg for 10 minutes. The supernatant was removed and the pellet was washed by centrifugation in a suspension medium containing 0.6M sorbitol and 10mM $CaCl_2$ (pH 5.7).

Protoplasts (approximately 2 mg chlorophyll/ml) in thick slurry suspensions were spin-labeled by adding 0.2 ml to a test tube containing a dry film of 5-doxylstearic acid (Syva Associates, Palo Alto, CA) and gently rotating for 5 minutes at 23°C. The film for labelling protoplasts was derived from 10 or 20 μl, respectively, of 30 mM spin label in ethanol, blown dry with air. Final spin label concentrations were approximately 2 mM. Previous work has indicated that fatty acid spin labels partition throughout all membranes of the cell (2), and that the observed fluidity is a composite value. The samples were immediately pipetted into Varian low temperature quartz cuvettes, placed in the dewar and scanned in the dark. ESR spectra were recorded with a Varian model E-112 X-band spectrometer. The sample temperature was controlled by a Varian variable temperature controller and was monitored by an Omega model 250 thermocouple positioned within the cuvette.

Relative ESR signal intensities were determined by dividing the peak-to-peak height of the mid-field line by the amplifier gain. Lineshape was analyzed in terms of the maximum hyperfine splitting parameter, $2T_{11}$. Phase transformation temperatures were determined by linear regression analysis.

## RESULTS

Many membrane functions are inhibited at low temperature (13). Therefore, oat leaf tissues were exposed to toxin at 0°C to determine whether or not toxin-sensitivity was affected. Tissues which were treated and held at 0°C did not lose electrolytes faster than did untreated tissues (Table 1); the tissues at 0°C appeared to be insensitive to toxin. This inability to respond to toxin could be caused either by interference with toxin action at its initial site or by inhibition of membrane transport in general.

To determine the effect of low temperature on membrane transport, tissues were incubated in toxin solutions at 23°C for 1 hour, washed, and then cooled to 0°C for leakage monitoring. Electrolyte loss was only 22% of the rate for the sample held at 23°C (Table 1). Nevertheless, the treated-and-cooled samples still leaked considerably more than did the controls (7.4 times more), indicating that once toxin-induced leakage has begun, it is not totally stopped by lowering the temperature to 0°C. Therefore, low temperature inhibits membrane transport and may also inhibit toxin action at its initial site. This tentative conclusion is based on the data showing that leakage is not induced at 0°C, even though leakage can continue at that temperature once it has been induced at 23°C.

The possibility that low temperature inhibits toxin action at its

TABLE 1. Effect of temperature on HV toxin-induced leakage of electrolytes.

| Temperature | | Leakage rate | | |
|---|---|---|---|---|
| Treatment[a] | Measurement[b] | Toxin-treated | Control | Toxin-treated /control |
| °C | °C | μmhos/min | μmhos/min | |
| 23 | 23 | 0.864 ± 0.057 | 0.026 ± 0.011 | 33.2 |
| 23 | 0 | 0.193 ± 0.012 | 0.026 ± 0.008 | 7.4 |
| 0 | 0 | 0.028 ± 0.004 | 0.029 ± 0.004 | 1.0 |
| 0 | 23 | 0.770 ± 0.157 | 0.023 ± 0.007 | 33.5 |

[a]Oat leaf samples were held at the indicated temperatures during infiltration with toxin (25 μg/ml), incubated for 60 minutes, and washed.

[b]Treated samples were held at the indicated temperatures for 5 hours. Conductivity was measured at 60 minute intervals. The rate of leakage was determined by linear regression analysis.

initial site was examined further by treating tissues with toxin at 0°C, washing extensively (three to four hours) in water or alkaline water, followed by warming to 23°C. Loss of electrolytes from these samples was equal to that from samples which had been treated and held at 23°C throughout (Table 1). This suggests that toxin was taken up or bound tightly by the tissue at 0°C (it was not washed away) but was unable to trigger the next step leading to electrolyte leakage. The next step, which may simply be a conformational change of the receptor, appears crucial to toxin action because cell death (11) as well as electrolyte leakage is inhibited by low temperature. Washing the tissues with alkaline water did not affect the results.

Spin labels and electron spin resonance spectroscopy were used to determine whether or not a membrane phase transformation occurs above 0°C in oat leaf protoplasts. Results show that membrane microviscosity ($2T_{11}$) decreased linearly as the temperature of the sample increased from 0°C to approximately 12°C (Figure 1). An abrupt change in slope, indicating a change in the dependence of molecular ordering on temperature, occurred at 12.02 ± 79°C. Membrane microviscosity decreased faster with increasing temperature > 12°C than it did at temperatures < 12°C. The possibility of another transformation occurring at a temperature above 38°C was not ascertained because at 40 to 45°C the ESR spectrum of the spin label I(12,3) became too weak and narrow to determine $2T_{11}$ with accuracy.

The presence of an abrupt change in molecular ordering at 12°C suggested that toxin-induced electrolyte leakage might also decrease abruptly at 12°C. Samples were treated with toxin and washed at 23°C; a

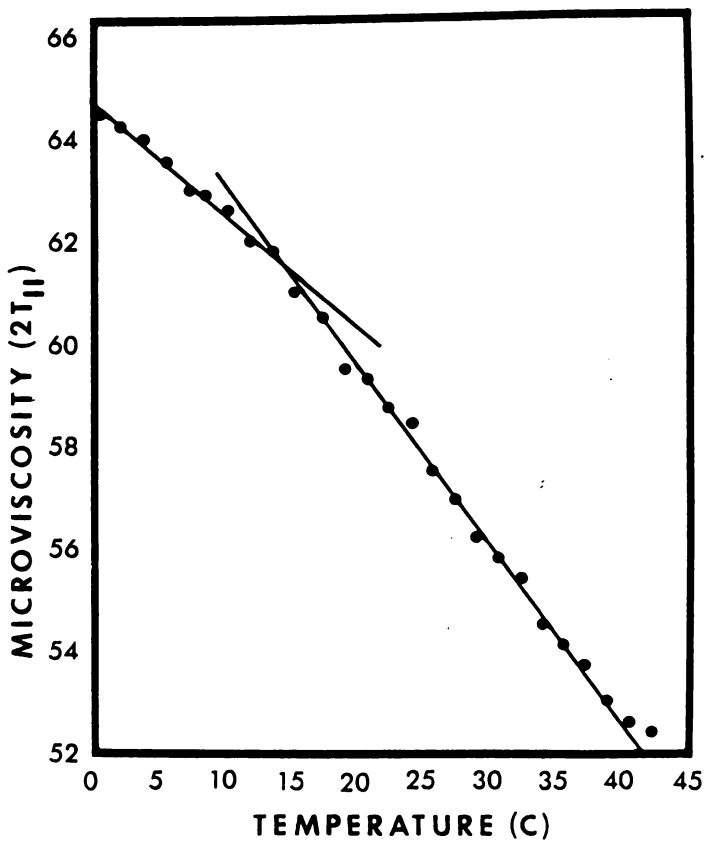


Figure 1. The effect of temperature on spin label motion. Oat leaf protoplasts were spin labeled with 5-doxylstearic acid; relative membrane microviscosity values were measured as $2T_{11}$.

uniform rate of electrolyte leakage was induced. Samples were then held at various temperatures during the leakage period. The rate of leakage was much suppressed at 12°C and below but increased rapidly with increasing temperature above 12°C (Figure 2). Apparently, the membrane must be in a particular state of organization for toxin-induced leakage of electrolytes to occur; the transformation which occurs upon cooling below 12°C inhibits transport of electrolytes.

Oat leaf protoplasts were exposed to toxin and ESR spectra of 5-doxylstearic acid in their membranes were recorded (data not shown). Toxin caused no significant changes in membrane fluidity within 3 hours after exposure. Apparently, toxin does not cause a rapid, drastic disruption of the lipid matrix in the membrane.

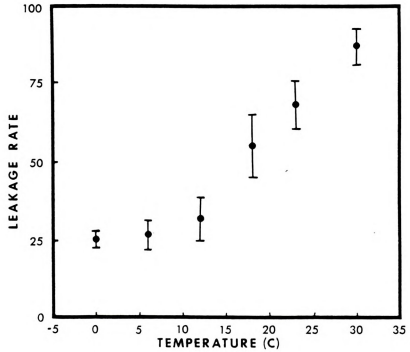


Figure 2. Effect of incubation temperature on the rate of toxin-induced electrolyte leakage (μmhos $min^{-1}$ x 100) from susceptible tissues. Leaf samples (pieces 5 mm long; 0.2 g) were infiltrated with water or toxin solution (25 μg/ml) at 23°C and incubated at the indicated temperature.

## DISCUSSION

The results shown in Table 1 indicate that toxin binds or is taken up by tissues at 0°C but that higher temperatures are required for electrolyte leakage to be initiated. There are at least 3 possible explanations of the failure to induce leakage at 0°C. One possibility is that the toxin binds to its receptor at 0°C but the toxin-receptor complex is unable to catalyze a subsequent step or undergo a conformational change leading to leakage. Many enzymes display similar behavior in that they can bind to their substrate at 0°C but are unable to catalyze the reaction leading to products. The second possibility is that lateral diffusion within the membrane bilayer is required for the toxin-receptor complex to induce leakage; such diffusion would be inhibited if the membranes were in a highly ordered state. This possibility is similar to the mobile receptor hypothesis put forward by Cuatrecasas (8) to account for hormone action. The hypothesis assumes that the hormone- or toxin-receptor complex is membrane-bound. The third possibility is that the toxin is taken into a cell compartment at 0°C but is unable to bind or activate the toxin receptor until the sample is warmed. Which, if any, of these mechanisms is correct will probably not be established until a toxin receptor is isolated.

A change in molecular ordering of oat leaf protoplast membranes was observed at 12°C. A precise understanding of what such observations

indicate at the molecular level is not presently available. However, it is widely thought (13) that lateral phase separations could be responsible. Such separations occur in a bilayer when a heterogenous mixture of lipids separates into domains of pure lipid below the melting temperature of one lipid species. Thus, patches of gel lipid may exist in a liquid-crystal matrix of fluid lipid and protein. Proteins which normally associate with the now "frozen" lipid are excluded and must associate with other fluid membrane lipids. This may inactivate catalytic proteins (1,13). Alternatively, a change in molecular ordering of the membrane as a whole could occur. Such a phase transformation could also cause catalytic proteins to become inactive by extruding them from the bilayer (1). Whatever the details of the molecular change, it is clear that toxin-induced passage of electrolytes through the membrane is strongly inhibited below the transformation temperature (12°C) (Figure 2).

Since fatty acid spin labels detect changes in the bulk lipid, electrolyte leakage appears to be a function of the fluidity of the bulk lipid. Extrapolation from work with ionophores (5) suggests that electrolytes from toxin-damaged cells are carried through the membrane by facilitated diffusion through the lipid phase. Passage through pores seems unlikely since pore-forming ionophores such as gramicidin A do not require a fluid membrane for activity (6). On the other hand, the transport of water through the membranes of phosphatidylcholine liposomes is also much reduced below the lipid phase transition temperature (2). Therefore, the possibility that the electrolytes move through a pore with water cannot be ruled out. Whether it is the

toxin-receptor complex or some other entity which mediates the passage of electrolytes is not known.

No changes in fluidity of oat protoplast membranes were detected following HV-toxin treatment. This is in contrast to the effects of cercosporin on tobacco protoplast membranes (10). Cercosporin, a photosensitizing agent, causes the oxidation of polyunsaturated fatty acids which leads to a rigid, leaky membrane and cell death. Spin labels detect such damage because it occurs in the bulk lipid. More subtle changes, such as modification of only a receptor protein, would almost certainly not perturb bulk lipid fluidity and would go undetected by spin labels. We suggest that damage by HV-toxin is of this latter type.

In conclusion, our data suggest and are compatible with the hypothesis that HV-toxin binds to a receptor in susceptible plants. If the membrane is fluid, the toxin-receptor complex is irreversibly transformed to a quasi-ionophore or else activates an ionophore. The ionophore requires at least a partially fluid membrane to function and, therefore, may be a diffusible carrier rather than a pore.

## LIST OF REFERENCES


1. Armond, P.A. and L.A. Staehelin. 1979. Lateral and vertical displacement of integral membrane proteins during lipid phase transition in Anacystis nidulans. Proc. Natl. Acad. Sci. USA 76:1901-1905.

2. Blok, M.C., L.L.M. van Deenen and J. DeGier. 1976. Effect of the gel to liquid crystalline phase transition on the osmotic behavior of phosphatidylcholine liposomes. Biochim. Biophys. Acta 433:1-12.

3. Briggs, S.P., A.R. Haug and R.P. Scheffer. 1981. Localization of spin labels in oat leaf protoplasts. Plant Physiol. 70:662-667.

4. Briggs, S.P., R.P. Scheffer and A.R. Haug. 1981. Loss of turgor suppresses sensitivity of oats to Helminthosporium victoriae toxin. Phytopathology 71:863.

5. Briggs, S.P., R.P. Scheffer, and A.R. Haug. 1981. Membrane fluidity modulates sensitivity of oats to Helminthosporium victoriae toxin. Phytopathology 71:863.

6. Boheim, G., W. Hanke and H. Eibl. 1980. Lipid phase transition in planar bilayer membrane and its effect on carrier- and pore-mediated ion transport. Proc. Natl. Acad. Sci. (USA) 77:3403-3407.

7. Bronson, C.R. and R.P. Scheffer. 1977. Heat- and aging-induced tolerance of sorghum and oat tissues to host-selective toxins. Phytopathology 67:1232-1238.

8. Cuatrecasas, P. and M.D. Hollenberg. 1976. Membrane receptors and hormone action. In "Advances in Protein Chemistry", Vol. 30. Academic Press, New York.

9. Damann, K.E., J.M. Gardner and R.P. Scheffer. 1974. An assay for Helminthosporium victoriae toxin based on inducated leakage of electrolytes from oat tissue. Phytopathology 64:652-654.

10. Daub, M.E. and S.P. Briggs. 1982. Changes in tobacco cell membrane composition and structure caused by cercosporin. Plant Physiol. (submitted).

11. Hawes, M.C. and H. Wheeler. 1982. Factors affecting victorin-induced root cap cell death: temperature and plasmolysis. Physiol. Plant Pathol. 20:137-144.

12. Lesney, M.S., R.S. Livingston and R.P. Scheffer. 1982. Effects of toxin from Helminthosporium sacchari on nongreen tissues and a reexamination of toxin binding. Phytopathology 72:844-849.

13. Quinn, P.J. 1981. The fluidity of cell membranes and its regulation. Prog. Biophys. Molec. Biol. 38:1-104.

14. Quinn, P.J. and P.W. Williams. 1978. Plant lipids and their role in membrane function. Prog. Biophys. Molec. Biol. 34:109-173.

15. Weinstein, S., Wallace, B.A., E.R. Blout, J.S. Morrow and W. Veatch. 1979. Conformation of gramicidin A channel in phospholipid vesicles: a $^{13}C$ and $^{19}F$ nuclear magnetic resonance study. Proc. Natl. Acad. Sci. (USA) 76:4230-4234.

16. Yoder, O.C. 1980. Toxins in pathogenesis. Ann. Rev. Phytopathol. 18:103-129.

# SECTION 3

# OSMOTIC CONDITIONS AFFECT SENSITIVITY OF OAT TISSUES TO TOXIN FROM HELMINTHOSPORIUM VICTORIAE

ABSTRACT

Osmotica (sorbitol and mannitol) at concentrations of approximately 0.2 M or greater suppress the rate of electrolyte leakage from oat tissues caused by Helminthosporium victoriae toxin. The effect plateaus at approximately 0.2 M osmoticum which coincides with incipient plasmolysis. Neither stimulated uptake of electrolytes, changes in transverse pressure on the plasmalemma, nor osmotic shock appear to account for the protection. Inhibition of water flow into the vacuole is suggested as an explanation.

# INTRODUCTION

Samaddar and Scheffer (8) first reported that HV-toxin rapidly kills isolated protoplasts from oat coleoptiles. This report has become controversial; there are claims that protoplasts do not respond rapidly to toxin (9). We have investigated the response of tissues to HV-toxin, using the same conditions in which protoplasts were held, i.e. in an external osmoticum. Electrolyte losses from diseased tissues can be either enhanced, inhibited or unaffected by the presence of an external osmoticum, depending upon the disease in question (4). Therefore, the response of any given diseased tissue to an osmoticum cannot be predicted. The basis for an osmotic effect on electrolyte loss is unknown. Hawes and Wheeler (5) reported that oat root cap cells can be partially protected from HV-toxin by mannitol solutions. Their data indicated that protection increases with increases in mannitol concentration, with no sudden loss of protection at the point of incipient plasmolysis. In contrast, we find a sudden loss of protection when the osmotic potential of the external solution is insufficient to eliminate turgor. These results will be discussed in terms of a new hypothesis to account for the effect of cellular water state on action of toxin. Part of this work has been presented as an abstract (2).

## MATERIALS AND METHODS

Plants were grown in vermiculite under fluorescent lights as described previously (1). Avena sativa cv. Park and cv. Korwood were used as toxin sensitive and resistant types, respectively. The electrolyte leakage assay for toxin (3) was conducted with primary leaves of 1 week-old oat seedlings. The leaves were cut into 0.5 cm segments and 0.2 g batches were placed in cheesecloth bags. Each bag was submerged in 10 ml of water or treatment solution which was infiltrated into the tissue under reduced pressure. After 1 hour, the samples were washed repeatedly with water or sorbitol solution and then immersed in 10 ml of the indicated solution. The conductivity of the solution was measured with a conductivity meter equipped with a pipet-type electrode (K=1). Leakage rates were determined by linear regression analysis; the correlation coefficients were 0.95 or greater.

Unless stated otherwise, a crude toxin preparation which completely inhibited root growth of oat seedlings at 0.5 µg/ml was used. Toxin was prepared by methanol precipitation of culture filtrate followed by butanol extraction and passage through an LH20 column. A much more active preparation was obtained by further chromatography on an SP-Sephadex column. Key experiments were confirmed with the more purified preparation which completely inhibited root growth at 0.7 ng/ml.

The water potential of primary leaves of 1 week-old oat seedlings was determined as described by Nelsen et al. (6). A section 13 mm long was removed from the center of each leaf with a sharp razor blade and immediately placed in a 9 x 5 mm sample well of a Wescor dewpoint hygrometer. Leaf water potential was determined 3 hours later, after the samples had equilibrated with the chamber. Each sample was then wrapped in foil, frozen in liquid nitrogen, thawed, and returned to the sample well of the hygrometer. After a 15 minute equilibration period, another reading was taken to determine the solute potential of the tissue. The hygrometer was calibrated with sorbitol solutions of known osmotic potential.

The effects of pressure on leakage were determined on leaf sections treated with toxin and washed as described for the standard electrolyte leakage assay. Immediately after washing, the samples were placed in a portable pressure bomb (PMS Instrument Company, Corvallis, OR). The pressure was brought to 10 bars at a rate of 1 bar per minute using compressed air. The samples were decompressed at a rate of 2 bars per minute; conductivity measurements were then made, and the samples were again pressurized. Controls were kept in the dark at atmospheric pressure.

The potassium concentration of the ambient solution was measured with an Orion solid-state potassium-selective electrode (model 93-19) coupled to a double-junction reference electrode. The electrode was calibrated with KCl solutions of known concentration. The electrode response was linear from 0.01 to 100.00 mM KCl.

## RESULTS

Protoplasts were normally held in 0.6 M sorbitol (1). Therefore, we first determined the effect of 0.6 M sorbitol on the toxin dose-response curve for leaf tissues, using the electrolyte leakage assay (Figure 1). The sorbitol solution suppressed the rate of electrolyte leakage from sensitive tissue. When diluted to $10^{-3}$, the toxin preparation caused nearly the same rate of leakage as it did at a $10^{-6}$ dilution in water. At saturating toxin levels, the tissue in sorbitol leaked only one-third as fast as did the tissue in water. However, the toxin dilution endpoint did not appear to be changed by sorbitol. Toxin-treated tissue from resistant plants leaked slightly more in the presence of sorbitol than in water. Similarly, susceptible tissue which was not exposed to toxin had a higher rate of leakage in sorbitol than in water (data not shown). Sorbitol was as effective when it was present only during the time that leakage was monitored as when it was present during the time of treatment, washing, and monitoring.

Sorbitol was tested at various concentrations to determine effects on rate of leakage from leaf tissues (Figure 2). At concentrations of 0.18 M or greater, sorbitol suppressed the leakage rate to approximately one-third the value for controls in water. Increasing the sorbitol concentration to 0.73 M caused little decrease in the leakage rate, compared with that at 0.18 M. However, at sorbitol concentrations lower

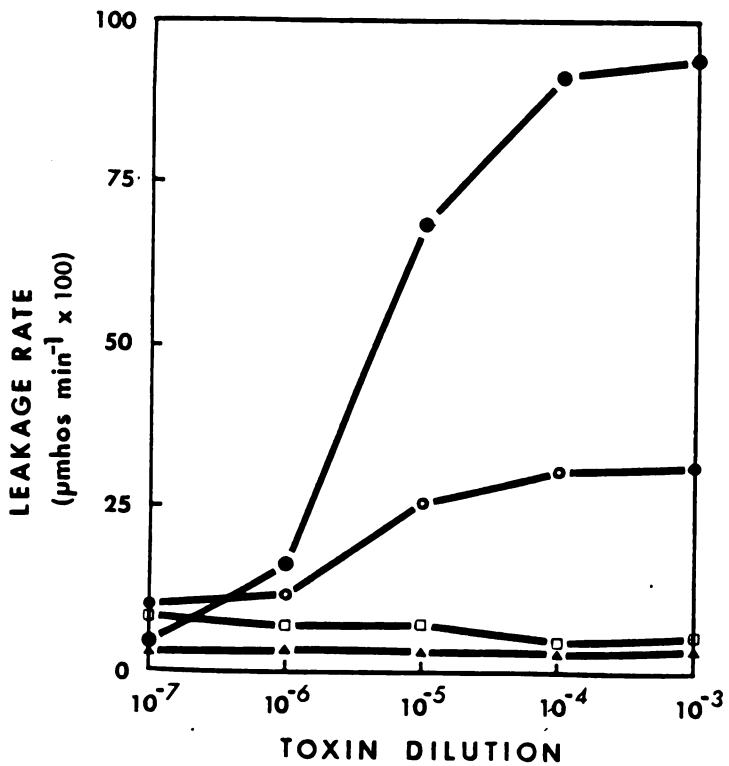


Figure 1. Effect of sorbitol on the toxin dose-response curve. Oat leaf segments (5 mm long; 0.2 g) were treated with toxin at the indicated dilution in the presence or absence of 0.6 M sorbitol. A $10^{-4}$ dilution had 50 μg toxin per ml. Samples were washed and resuspended in either water or sorbitol. Electrolyte leakage was monitored with a conductivity meter; rates of leakage were determined by linear regression analysis. (●) Park (sensitive) in water; (o) Park in 0.6 M sorbitol; (□) Korwood (resistant) in 0.6 M sorbitol; (▲) Korwood in water. Sorbitol (0.6 M) also suppressed leakage caused by a more purified toxin preparation (dilution endpoint = 0.7 ng/ml; used at a concentration of 0.14 μg/ml).

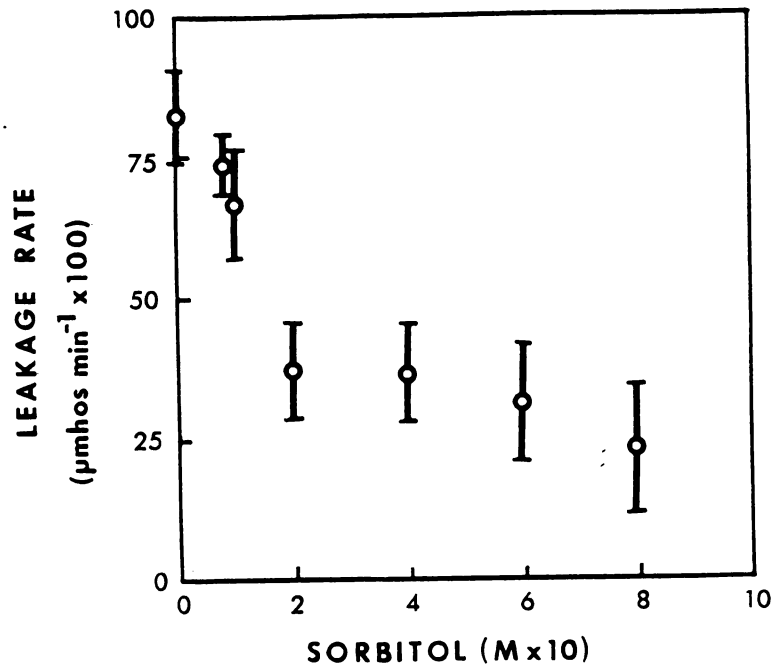


Figure 2. Effect of sorbitol concentration on the rate of electrolyte leakage from toxin-treated tissue. Oat leaf segments (5 mm long; 0.2 g) were treated with toxin (50 μg/ml) plus the indicated concentration of sorbitol. Subsequent washing and monitoring was in a sorbitol solution of the same osmolarity as the treatment solution. Rates were determined as in Figure 1. Bars indicate standard deviations.

than 0.18 M, the protective effect rapidly dropped off. The rate of leakage in 0.07 M sorbitol was 89% of the rate in water. The same results were obtained when mannitol rather than sorbitol was used.

The turgor pressure of the sample tissue was determined to ascertain whether or not the rapid loss of protection at low sorbitol concentrations was correlated with incipient plasmolysis (Table 1). Both the water and solute potentials of the leaves were measured directly and, from them, the turgor pressure was calculated. The turgor of tissues of the resistant cv. Korwood was 7.77 bars, which was slightly greater than that of tissues of susceptible cv. Park (6.70 bars). The difference was caused mostly by a greater solute potential in Korwood (-10.17 bars vs -9.33 bars for Park). The overall water potentials of the two cultivars were approximately equal (-2.63 bars for Park, -2.40 bars for Korwood). The concentration of sorbitol which gave a solution water potential equal to the turgor of the tissues was calculated (Table 1). Park tissue was calculated to have zero turgor in a bathing solution of 0.21 M sorbitol whereas Korwood required 0.24 M sorbitol; these are the values at which incipient plasmolysis should occur. These concentrations are only slightly greater than the sorbitol concentration (0.18 M) below which the osmo-protection is lost.

There was a series of experiments to determine how sorbitol and mannitol exert their protective effect. The possibility that osmotica reduce net leakage by stimulating uptake of electrolytes (10) was investigated first. Simultaneous measurements of solution conductivity and KCl concentration (using a $K^+$-specific electrode) during leakage of electrolytes from toxin-treated tissue indicated that essentially all

TABLE 1. Water ($\psi$), solute ($\psi_\pi$), and pressure ($\psi_p$) potentials of oat leaves.

| Cultivar | Bars | | | Equivalent sorbitol molarity[2] |
|---|---|---|---|---|
| | $\psi$ | $\psi_\pi$ | $\psi p$[1] | |
| Susceptible | -2.63 ± 0.79 | -9.33 ± 0.93 | 6.70 | 0.21 |
| Resistant | -2.40 ± 0.67 | -10.17 ± 0.61 | 7.77 | 0.24 |

[1] $\psi - \psi_\pi = \psi_p$

[2]The measured water potential of a 0.91 M sorbitol solution was -29.37 (± 0.59) bars. The equivalent sorbitol molarity (esm) is the concentration of sorbitol in the bathing solution which causes the turgor of the cells to be zero, i.e., esm = $\psi_p$ (0.91)/29.37.

of the increased conductivity was due to KCl (unpublished). The KCl concentration in the sample chambers reached 1 to 2 mM at 5 hours after washing. Therefore, tissue samples were suspended in 1 mM KCl and in 0.1, 0.2, 0.4, 0.6, or 0.8 M sorbitol. The conductivity of the ambient solution was measured at hourly intervals. Sorbitol did not stimulate uptake of KCl. The conductivity of the solution slowly increased at all sorbitol concentrations at a rate similar to that of the water control (data not shown). The suppression of leakage by sorbitol probably is not an artifact caused by stimulated uptake.

A second possibility is that the reduced transverse pressure on the plasmalemma (caused by the loss of turgor) changes the permeability of the membrane (10). Tissues were treated with toxin and then held at a pressure of 10 bars for the leakage period (Table 2). The rate of electrolyte loss at 10 bars (0.297 ± 0.086 μmhos $min^{-1}$) was the same as at ambient pressure (0.280 ± 0.033 μmhos $min^{-1}$). Compression of the membrane does not appear to affect permeability.

Osmotic shock is known to modify the plasmalemma of oats (7). We investigated the possibility that osmotic shock might account for the protective effect of sorbitol. Tissues were immersed in 0.3 M sorbitol for 15, 30 or 60 minutes prior to toxin treatment and monitoring of electrolyte leakage in water. The sorbitol pretreatment had little effect on the rate of leakage (Table 3). Pretreatment for 30 and 60 minutes slightly increased the rate of toxin-induced leakage, as compared to leakage induced following the 15 minute pretreatment and the untreated control. Thus, tissues were not protected by prior osmotic shock.

TABLE 2. Effect of ambient pressure on electrolyte leakage rate.

| | Electrolyte leakage rate (μmhos $min^{-1}$) | | | |
|---|---|---|---|---|
| | at Ambient pressure | | at 10 bars | |
| Cultivar | control | toxin[1] | control | toxin |
| Sensitive | 0.025 ± 0.007 | 0.280 ± 0.033 | 0.025 ± 0.007 | 0.297 ± 0.086 |
| Resistant | 0.031 ± 0.005 | 0.025 ± 0.002 | 0.023 ± 0.001 | 0.019 ± 0.000 |

[1]Leaf pieces (0.2 g) were exposed to HV-toxin at 0.5 μg/ml.

TABLE 3. Effect of osmotic shock on sensitivity of oat tissue to HV-toxin.

| Osmotic shock period (min)[1] | Electrolyte leakage rate (μmhos $min^{-1}$) | | | |
|---|---|---|---|---|
| | susceptible | | resistant | |
| | control | toxin | control | toxin |
| 0 | 0.007 | 0.157 | 0.012 | 0.013 |
| 15 | 0.017 | 0.152 | 0.016 | 0.010 |
| 30 | 0.017 | 0.175 | 0.013 | 0.011 |
| 60 | 0.019 | 0.171 | 0.013 | 0.011 |

[1]Tissues (0.2 g) were immersed in water (0) or 0.3 M sorbitol for 15, 30, or 60 minutes followed by a wash with water, exposure to toxin (0.5 μg/ml) for 1 hour, another wash, and resuspension in water for conductivity measurements.

# DISCUSSION

The rate of electrolyte loss from toxin-treated oat tissue was substantially less in sorbitol or mannitol solutions at concentrations greater than 0.2 M than in water. This supports the findings of Hawes and Wheeler (5) that oat root cap cells are partially protected from HV-toxin by mannitol. Osmotica probably do not prevent the initial damage caused by toxin because the dilution endpoint in the presence of sorbitol is unchanged. Osmotica appear to affect expression of primary damage. Protection by osmotica may be part of the reason why rapid effects of toxin on protoplasts are not always observed. Other possibilities such as a change in the transverse pressure on the plasmalemma, osmotic shock, or stimulated uptake of solutes do not appear to be involved. The results of direct experiments on protoplasts and further explanation of this problem will be presented elsewhere.

There is some discrepancy between our results and those of Hawes and Wheeler (5). We find a sharp loss of protection at osmoticum concentrations less than approximately 0.2 M whereas they report that the osmoticum dose-response curve is gradual, without a sharp loss at lower concentrations. The fact that entirely different assays and different tissues were used by the two groups may explain the difference. However, the data in their Figure 2 (5) show that of four mannitol concentrations tested (0.2, 0.4, 0.6, and 0.8 M) only the 0.2 M solution was significantly different from the others. This resembles the pattern

we report in Figure 2.

Our data indicate that the protective effect of sorbitol plateaus when the turgor of the cells drops to zero; higher sorbitol levels give little increase in protection. This suggests that sorbitol protects by inhibiting the flow of water down its chemical potential gradient into damaged cells. One hypothesis for toxin action which takes these findings into account is that toxin acts by first disrupting the plasmalemma. In the case where no osmoticum is present, as solutes are lost from the cytoplasm, water will flow into the vacuole because the osmotic potential of the cytoplasm will be less negative than that of the vacuole. Of course, the water potentials of both the cytoplasm and vacuole remain much less than that of the apoplast. The swelling of the vacuole which results could lead to a disruption of the tonoplast and release of the vacuolar contents. This relationship is expressed in the following equations. Before toxin exposure: $\psi^c = \psi^v$, $\psi_\pi{}^c = \psi\pi^v$, and $\psi_p{}^c = \psi_p{}^v$. After toxin exposure: $\psi_\pi{}^c > \psi_\pi{}^v$, $\psi_p{}^c = \psi^v \psi_p$ and, therefore, $\psi^c > \psi^v$, where c = cytoplasm and v = vacuole. If $\psi^c > \psi^v$ then water flows from the cytoplasm to the vacuole.

In the second case, where plasmolyzing levels of osmoticum are present, most of these relationships are unchanged. However, the low osmotic potential of the apoplast must now be taken into account. Under these conditions, we propose that the solutes lost from the cytoplasm following toxin damage cause water to flow more to the apoplast than to the vacuole. This change reflects the fact that the apoplast is, essentially, an infinite pool of constant, low water potential whereas the water potential of the vacuole increases as it loses solutes and takes up water. Thus, swelling of the vacuole and disruption of the tonoplast are inhibited.

## LIST OF REFERENCES


1. Briggs, S.P., A.R. Haug and R.P. Scheffer. 1982. Localization of spin labels in oat leaf protoplasts. Plant Physiol. 70:662-667.

2. Briggs, S.P., R.P. Scheffer and A.R. Haug. 1981. Loss of turgor suppressess sensitivity of oats to Helminthosporium victoriae toxin. Phytopathology 71:863.

3. Damann, K.E., J.M. Gardner and R.P. Scheffer. 1974. An assay for Helminthosporium victoriae toxin based on induced leakage of electrolytes from oat tissue. Phytopathology 64:652-654.

4. Hancock, J.G. 1981. Osmotic conditions influence estimation of passive permeability changes in diseased tissues. Physiol. Plant Pathol. 18:117-122.

5. Hawes, M.C. and H. Wheeler. 1982. Factors affecting victorin-induced root cap cell death: temperature and plasmolysis. Physiol. Plant Pathol. 20:137-144.

6. Nelsen, C.E., G.R. Safir and A.D. Hanson. 1978. Water potential in excised leaf tissue. Comparison of a commercial dew point hygrometer and a thermocouple psychrometer on soybean, wheat and barley. Plant Physiol. 61:131-133.

7. Rubinstein, B. 1977. Osmotic shock inhibits auxin-stimulated acidification and growth. Plant Physiol. 59:369-371

8. Samaddar, K.R. and R.P. Scheffer. 1968. Effect of the specific toxin in Helminthosporium victoriae on host cell membranes. Plant Physiol. 43:21-28.

9. Wheeler, H. 1981. The role of toxins in pathogenesis. In "Toxins in Plant Disease", R.D. Durbin (ed), pp. 477-494. Academic Press, New York.

10. Zimmermann, U . 1978. Physics of turgor- and osmoregulation. Ann. Rev. Plant Physiol. 29:121-148.

SECTION 4

# RAPID KILLING OF OAT PROTOPLASTS BY HELMINTHOSPORIUM VICTORIAE TOXIN

ABSTRACT

HV-toxin rapidly killed oat mesophyll protoplasts, as shown by use of the vital stain fluorescein diacetate. Collapse of protoplasts followed death at 35°C but not at 23°C. Tissues lost sensitivity to toxin above 40-44°C. The vital stain showed that protoplasts were as sensitive as were leaf sections and intact roots to toxin. Toxin caused protoplasts to leak $K^+$. Overall, the results indicate that neither the cytoskeleton nor the cell wall is required for the action of toxin. Vacuoles prepared by a method which leaves cytoplasmic contamination on the surface were shown to respond to toxin. Vacuoles prepared by another method did not respond to toxin; thus, sensitivity of the tonoplast is uncertain.

## INTRODUCTION

Rapid killing of oat coleoptile protoplasts by HV-toxin was first reported by Samaddar and Scheffer (14). Subsequently, Rancillac et al. (13) observed that oat mesophyll protoplasts were lysed by toxin at 32°C; lysis at 23°C was not reported. Other workers have failed to confirm that HV-toxin has a rapid effect on protoplasts (18). Recent reports by Hawes and Wheeler (7) and Briggs et al. (3,4) describe how temperature and osmotica may influence the response of tissues and protoplasts to toxin. We have investigated directly the response of protoplasts to toxin and the possibility that cell structures which are lost when protoplasts are prepared (e.g., the cytoskeleton and cell wall) may modify toxin sensitivity. The speed with which toxin affects protoplasts and tissues was compared. Possible effects of toxin on isolated vacuoles were determined.

## MATERIALS AND METHODS

One week-old oat plants were grown in vermiculite under fluorescent lights as previously described (2). Protoplasts were prepared from primary leaves by peeling away the lower epidermis and floating the peeled leaf surface on a solution of 0.5% Cellulysin and 0.6 M sorbitol, pH 5.7. After 2 hours at 30°C in the dark, the protoplasts were filtered through Miracloth and washed by repeated centrifugation at 40xg in 0.6 M sorbitol. Protoplasts were used at a concentration of approximately $10^6$ per ml.

Protoplasts and vacuoles were observed with a Zeiss Universal microscope in both dark-field and in dark-field with UV light (for observations of fluorescence); barrier filter No. 50 and exciter filter No. 1 were used. Samples were stained with fluorescein by adding 5 µl of 0.5% fluorescein diacetate in acetone to 0.5 ml of the sample and incubating for 5 minutes. The samples were viewed first in dark-field and then with UV light so that the same protoplasts or vacuoles could be scored for viability. At least 100 protoplasts were scored in each sample.

Cell walls were isolated from both etiolated coleoptiles and green, primary leaves of oats according to the method of Kivilaan et al. (9). Tissues (25 g) were homogenized on ice in 180 ml glycerol with 37 g of glass beads (200 µm diameter). The supernatant was decanted onto a bed of glass beads and filtered. The filtrate was discarded and the glass

bead filter bed was suspended in 50 ml glycerol. The supernatant was filtered two more times on clean glass beads after which the beads were removed by centrifugation. The cell walls were pelleted by centrifugation at 25,000xg for 5 minutes and were resuspended in water. The walls were washed three times by repeating this procedure.

An Orion solid-state, potassium-selective electrode (model 93-19) coupled to a double-junction reference electrode was used to measure the potassium concentration of the ambient solution. Potassium chloride solutions of known concentration were used to calibrate the electrode. The electrode response was linear over the 0.01 to 100.00 mM KCl concentration range.

Electrolyte leakage experiments were conducted as previously described (3). Leaf tissue samples were infiltrated under reduced pressure with the treatment solution, incubated for 1 hour, washed, and resuspended in water for the leakage period. The conductivity of the ambient solutions was measured with a conductivity meter equipped with a pipet type electrode (K=1.0). Readings were taken each hour for 5 hours unless indicated otherwise. Rates of leakage were determined by linear regression analysis; correlation coefficients were at least 0.95.

Vacuoles were prepared by the method of Lorz et al. (11) and also by the method of Martinoia et al. (12). In the first method (11), protoplasts in 0.6 M mannitol were diluted 1:1 with 0.254 M $CaCl_2$. This solution (4 ml) was pipetted onto a density gradient consisting of 0.6 mannitol (12 ml) above 0.54 M sucrose (10 ml). The sample was centrifuged at 30,000xg for 3 hours and the vacuoles were removed from the top layer. The second method (12) requires that the protoplasts be

lysed by passage through a syringe. The lysate in 0.4 M sucrose, 2.5% Ficoll, 15 mM sodium phosphate, 2 mM sodium EDTA, pH 7.6 (5 ml total volume) was overlaid by a density gradient consisting of 0.2 M sorbitol, 0.2 M sucrose, 15 mM sodium phosphate, 2 mM sodium EDTA, pH 7.5 (5 ml total volume) which was overlaid by a top layer of 0.4 M sorbitol, 15 mM sodium phosphate, 2 mM sodium EDTA, pH 7.6 (2 ml total volume). The sample was centrifuged for 2 minutes at 200xg and then for 3 minutes at 1000xg. The vacuoles floated to the top layer.

For most experiments a toxin preparation which completely inhibited root growth of oat seedlings at 0.5 µg/ml was used at a concentration of 50 µg/ml. A second preparation with only one-tenth the toxicity of the first was used for experiments on $K^+$-loss from protoplasts. Experiments on protoplasts (except $K^+$-leakage) and vacuoles were repeated and confirmed with a third toxin preparation which completely inhibited root growth at 0.7 ng/ml; a concentration of 0.7 µg/ml was used unless stated otherwise. Resistant and untreated controls were used in all experiments.

## RESULTS

Protoplasts were exposed to toxin and observed with the microscope (Table 1). Viability rapidly declined, as determined by the ability to retain fluorescein (21). Resistant and untreated controls had only a slight loss of viability (data not shown). A toxin preparation which completely inhibited root growth at 0.7 ng/ml was used at a concentration of 1.4 µg/ml. This treatment caused all protoplasts to lose viability within 60 minutes at 23°C. In contrast, microscopic appearance of the protoplasts in both dark- and light-fields did not indicate a rapid, lethal effect of toxin (Table 1). Most protoplasts appeared normal 3 hours after exposure even though 73% had lost viability, as indicated by the fluorescein diacetate treatment. Distortion of protoplast outline was consistently observed approximately 90 minutes after exposure to toxin; the change was subtle but nearly all protoplasts were affected. By 180 minutes after exposure, the distortion was no longer noticeable; the protoplasts appeared normal again. Significant levels of protoplast collapse were not observed until 4.5 hours after exposure. Collapse was characterized by a non-spherical, fuzzy surface around a shrunken core of indistinguishable chloroplasts, and was generally associated with agglutination. Evan's blue and neutral red were found to be poor indicators of viability. Both stains failed to detect loss of viability beyond that which could be determined without stain.

TABLE 1. Comparative effects of toxin on protoplasts, as determined by protoplast appearance and staining with fluorescein diacetate (FDA).

| Minutes exposure to toxin | % viable[1] | Protoplast appearance[2] |
|---|---|---|
| 0 | 95 ± 2 | normal |
| 90 | 78 ± 14 | distorted surface |
| 180 | 27 ± 13 | normal |
| 270 | 11 ± 5 | 20% collapsed, others OK |
| 375 | 3 ± 1 | 20% collapsed, others OK |

[1]% viable = number observed fluorescing with FDA ÷ number observed in dark-field. A minimum of 100 protoplasts were counted at each time. The results are the means for 4 experiments.

[2]normal = spherical with an even distribution of chloroplasts around the cell periphery, no agglutination; distorted surface = rough or wrinkled outline of protoplast in contrast to the normally smooth surface; collapsed = non-spherical outline, fuzzy surface, shrunken, much agglutinated with indistinct chloroplasts. Protoplasts were viewed in dark-field.

Control protoplasts without toxin maintained viability in the dark but not in the light. Conducting the experiment in the light accelerated the senescence of the controls such that death caused by toxin was sometimes difficult to detect.

The sensitivity of protoplasts to toxin was compared with that of seedling roots and leaves (Table 2). The sensitivity of the three materials was similar, being affected by toxin at approximately 1 ng/ml.

A more rapid effect of toxin on protoplasts was observed at elevated temperature (Figure 1). Toxin-treated protoplasts held at 35°C lost viability about twice as fast as did those at 23°C. Collapse of the treated protoplasts at 35°C paralleled their loss of viability. All protoplasts had disintegrated so that only cellular debris was left 3 hours after exposure to toxin at 35°C. Resistant or untreated protoplasts remained healthy.

The effect of elevated temperature on loss of electrolytes from toxin-treated leaves was determined (Figure 2). At temperatures above 40°C, the rate of electrolyte leakage was much greater than at lower temperatures. Controls and toxin-treated susceptible tissues were both affected. The rate of electrolyte loss at 35°C was only slightly greater than at 23°C. The sensitivity of tissues to HV-toxin was reduced or eliminated by pretreatment at elevated temperature (Figure 3). Holding the tissues at temperatures up to 40°C had no effect on their subsequent response to toxin at 23°C. However, pretreatment at 44°C or higher eliminated sensitivity to toxin, as determined by electrolyte leakage. There was evidence that loss of sensitivity was not caused by depletion of electrolytes during the pretreatment period.

TABLE 2. Comparative sensitivity of HV-toxin assays.

| Assay[1] | Dilution endpoint of toxin (ng/ml) |
|---|---|
| Root growth inhibition | 0.7 |
| Protoplast viability | 1.4 |
| Electrolyte leakage from tissues | 1.4 |

[1]The dilution endpoints are defined as follows: root growth inhibition, the amount needed to totally inhibit root growth of germinated seeds; protoplast viability, the amount needed to cause death of significant numbers of protoplasts as determined by the fluorescein diacetate assay (in this case, 84% of the protoplasts were dead 19 hours after exposure to toxin); electrolyte leakage, the amount needed to cause significant leakage from leaves in 1 hour.

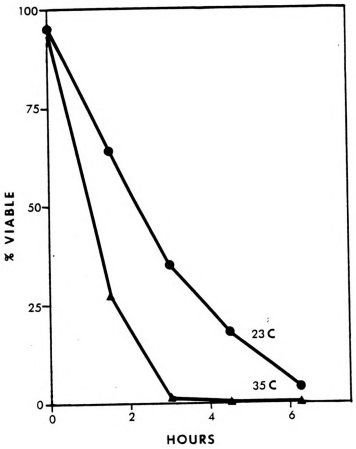


Figure 1. Effect of temperature on the rate of toxin-induced death of susceptible protoplasts. Viability was determined using the fluorescein diacetate assay. At the end of the experiment, the viabilities of the controls were: resistant, untreated, 23°C, 91 ± 4%; resistant, untreated, 35°C, 92 ± 3%; resistant, toxin-treated, 23°C, 84 ± 8%; resistant, toxin-treated, 35°C, 77 ± 10%; susceptible, untreated, 23°C, 95 ± 2%; susceptible, untreated, 35°C, 93 ± 1%.

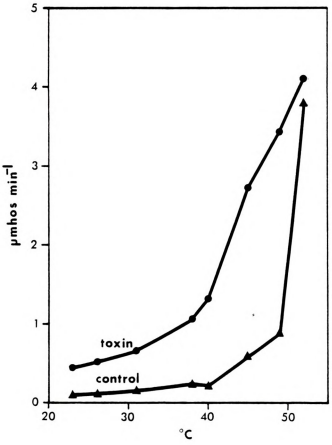


Figure 2. Effect of temperature on the rate of electrolyte leakage from susceptible leaf tissues treated with toxin. Samples (0.2 g) were incubated in water or toxin solution for 1 hour at 23°C, washed, and then held at the indicated temperature for 5 hours. Conductivity of the ambient solution was measured at 1 hour intervals and rates were determined by linear regression analysis. Correlation coefficients were 0.95 or greater.

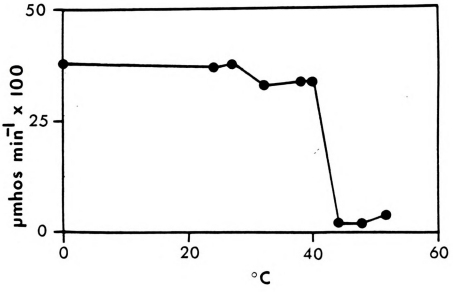


Figure 3. Effect of temperature pretreatments on toxin sensitivity of leaf tissues. Samples (0.2 g) were held at the indicated temperature for 1 hour, washed, treated with toxin for 1 hour at 23°C, washed, and allowed to leak at 23°C. Rates were determined as in Figure 2.

The cytoskeleton appears to control membrane receptor mobility and function (17). Release of protoplasts can disrupt the cytoskeleton (10). We exposed tissues to inhibitors which disrupt the cytoskeleton by causing the loss of either microtubules (colchicine, vinblastine, or podophyllotoxin) or microfilaments (cytochalasin B). The inhibitors alone caused little or no leakage and did not suppress or enhance the rate of leakage caused by toxin (Table 3).

Cell walls were isolated and tested to determine whether or not they are needed for toxin action, as proposed by others (6,20). Cell walls were mixed with toxin solution which was then removed by centrifugation. The residual toxicity of the recovered solution was unchanged, suggesting that the walls did not bind toxin. The conductivity of solution containing cell walls and toxin was monitored, but no release of electrolytes was observed. No interaction between cell walls and HV-toxin was evident.

The effect of toxin on the permeability of protoplast membranes was determined by monitoring loss of $K^+$ with an ion-selective electrode (Figure 4). $K^+$ was quickly removed from solution by freshly suspended toxin-treated and control protoplasts. $K^+$ losses began in every case at approximately 3 hours after exposure to toxin. Toxin- treated protoplasts from susceptible leaves lost $K^+$ faster than did the controls. Twenty hours after exposure, the concentration of $K^+$ in the ambient solutions of treated, susceptible protoplasts was nearly 1 mM whereas the solutions with control protoplasts were approximately 0.5 mM. The toxin-induced leakage from protoplasts was clearly evident only when the experiment was performed in the dark.

TABLE 3. Effect of microtubule and microfilament inhibitors on toxin-induced electrolyte leakage from susceptible leaf tissue (0.2 g samples).[1]

| Inhibitor | Rate of leakage (μmhos $min^{-1}$) | |
|---|---|---|
| | toxin-treated | control |
| Colchicine | 0.65 | 0.11 |
| Vinblastine | 0.70 | 0.02 |
| Podophyllotoxin | 0.66 | - |
| Cytochalasin B | 0.69 | 0.03 |
| Water | 0.75 | 0.02 |

[1]Concentrations of the inhibitors were as follows: colchicine, 0.25 mM; vinblastine sulfate, 100 μM; podophyllotoxin, 50 μM; cytochalasin B, 20 μM. Rates are from a representative experiment.

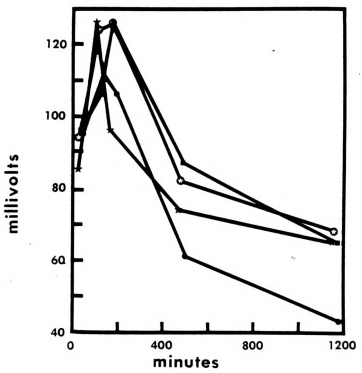


Figure 4. Toxin-induced loss of $K^+$ from protoplasts. Decreasing millivolts indicates an increasing concentration of $K^+$ in the ambient solution. A value of 40 mV corresponds to 1 mM KCl. A decrease of 60 mV is equivalent to a 10-fold increase in KCl concentration. (■) untreated, susceptible; (★) untreated, resistant; (●) toxin-treated, susceptible; (✪) toxin-treated, resistant.

The time-course of toxin-induced loss of electrolytes from tissues was determined (Figure 5). Samples were infiltrated with water and washed prior to exposure to toxin. Washing was necessary because each sample released substantial quantities of electrolytes into the wash solution. The conductivity of the ambient solution increased at once after addition of toxin, because of the electrolytes present in the toxin preparation. Toxin-treated tissues removed electrolytes from solution for the first several minutes. Leakage caused by toxin did not become significant until approximately 50 minutes after addition of toxin. The rate of loss became rapid and linear.

Vacuoles were prepared from leaf protoplasts by the method of Lorz et al. (11) and tested for sensitivity to toxin. Fluorescein diacetate was used as an indicator of membrane integrity; approximately one-half to three-fourths of the vacuoles in each sample were stained with fluorescein diacetate (Figure 6). Exposure to toxin caused the proportion of stained vacuoles from susceptible plants to drop significantly whereas the controls changed only slightly. Vacuoles were also prepared by the method of Martinoia et al. (12). None of the vacuoles prepared by this method stained with fluorescein diacetate, possibly indicating no contamination of tonoplasts with debris from other cell constituents (1). No obvious effect of toxin on the vacuoles was observed in dark- or light-field.

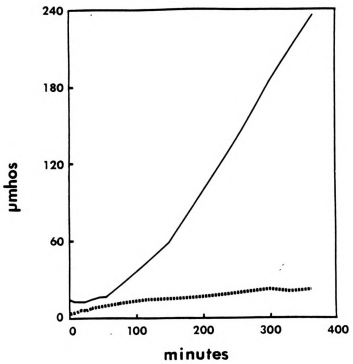


Figure 5. Time course of electrolyte loss from oat leaf tissues. Samples (0.2 g) were infiltrated with water under reduced pressure, washed, and then resuspended in water or toxin solution at time zero. Conductivity measurements were made at once and every 5 minutes for the first hour; after that, determinations were made every 30 minutes. The solid line indicates leakage from the sample in toxin, the dashed line is for the untreated control.

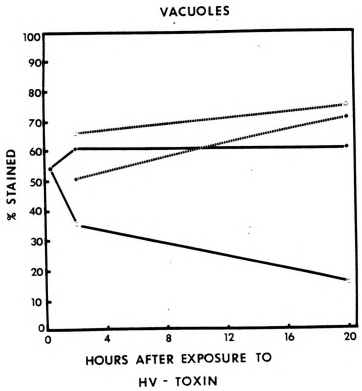


Figure 6. Effect of HV-toxin on retention of fluorescein by vacuoles. Vacuoles were prepared by the method of Lorz et al. (11), exposed to toxin, and treated with fluorescein diacetate at the indicated times after toxin exposure. (o, dashed line) resistant, toxin-treated; (●, dashed line) resistant, untreated; (o, solid line) susceptible, toxin-treated; (●, solid line) susceptible, untreated.

## DISCUSSION

There have been reports of very rapid effects of HV-toxin on loss of electrolytes from susceptible plants (15,19). Pretreatment with inactivated toxin is required to demonstrate a very rapid response to toxin (15). Under our experimental conditions, the tissues briefly took up electrolytes when first exposed to HV-toxin; toxin-induced leakage was not evident until 50 minutes after exposure. Thus, protoplasts may require 50 minutes for membrane damage or killing to develop in the presence of similar toxin concentrations; higher concentrations decrease the lag time.

The effect of toxin on $K^+$ uptake and exit from isolated protoplasts was determined. Protoplasts removed $K^+$ from the ambient solution upon transfer to fresh solutions. Loss of $K^+$ from the protoplasts started about 3 hours after transfer and was fastest from susceptible, toxin-treated protoplasts. Toxin concentration in the protoplast experiment was only 0.1 of that used in the experiment with tissues, which may explain the greater lag time for response to toxin. Alternatively, the responses of protoplasts in tissues may differ from the responses of protoplasts that are freed from tissue.

Death of protoplasts was determined with the vital stain fluorescein diacetate (21). Protoplasts with intact membranes retain fluorescein, which is released from the diacetate by esterases. In

contrast, the membranes of dead protoplasts cannot retain fluorescein. Therefore, viability in this case is defined as having an intact plasmalemma. Thus, viable protoplasts will fluoresce pale green in UV light whereas dead protoplasts emit a dim, blood-red fluorescence. All protoplasts were killed within 60 minutes after exposure to a highly active toxin preparation at a concentration of 1.4 µg/ml. This confirms the report of Samaddar and Scheffer (14) who observed total lysis of coleoptile protoplasts 60 minutes after exposure to toxin. In contrast to their observations, we found that mesophyll protoplasts do not lyse or collapse in large numbers until long after death, when the cells are held at 23°C. The difference in results may be related to differences in ambient temperatures. At 35°C, mesophyll protoplasts quickly die and collapse when treated with toxin. Similar observations were made by Rancillac et al. (13). Inadvertent warming of the coleoptile protoplasts (e.g., by illumination) could have brought about the collapse reported in the earlier work (14).

Temperature can inhibit as well as enhance toxicity. Both low temperature (3) and high temperature (5) can block the action of toxin. Temperatures above 40 to 44°C made the tissue insensitive to toxin, whereas tissues at all temperatures below this threshold were about equally sensitive. Protection by heating may be the result of a direct effect on proteins or a physical alteration in the lipid phase (16). Further work is required.

Protoplasts were examined to determine their level of sensitivity to toxin. If the site of action is not in the cell wall or cytoskeleton, then protoplasts should be as sensitive as are intact tissues.

HV-toxin killed protoplasts at the lowest concentration that inhibited root growth and caused leakage of electrolytes. This indicates that sensitivities of tissues and protoplasts to toxin are approximately equal. Therefore, it is unlikely that toxin acts on a site external to the plasmalemma.

We have tested isolated vacuoles for toxin sensitivity. Earlier studies indicated that the tonoplast and plasmalemma may be disrupted simultaneously (8). Vacuoles isolated from mesophyll protoplasts by the method of Lorz et al. (11) were found to stain with fluorescein diacetate. Using this stain as an assay for membrane integrity, we observed that vacuoles were disrupted by toxin. Vacuoles prepared by the method of Martinoia et al. (12) did not stain, and no effect of toxin was seen in the microscope. Other workers have suggested that vacuoles which are enveloped by plasmalemma from the parent cell will stain with fluorescein diacetate and that vacuoles not contaminated with plasmalemma will not stain (1). If true, this could explain the difference in behavior of the vacuoles prepared by the two different methods; those prepared by the method of Lorz et al. (11) may be enveloped by plasmalemma. Thus, the plasmalemma or some other organelle trapped on the vacuole could be the site of toxin action and the vacuole itself could be immune. A different assay will be required to test vacuoles prepared by the method of Martinoia et al. (12), to confirm their lack of sensitivity to toxin.

The cytoskeleton of cells is disrupted when protoplasts are released from tissues (10). The cytoskeleton plays an intricate role in membrane function; this includes the control of membrane receptors

(17). Therefore, membrane permeability changes such as those caused by toxins could be modified by conversion of cells to protoplasts. We found, however, that tissues treated with either microtubule (colchicine, vinblastine, podophyllotoxin) or microfilament (cytochalasin B) inhibitors were unchanged in response to toxin. The inhibitors did not cause permeability changes when used alone. Thus, the cytoskeleton does not appear to be a target of HV-toxin and does not appear to mediate toxin action.

Cell walls were also considered as potential sites of toxin action (6,20). Isolated cell walls failed to remove toxin from solution, suggesting the absence of binding sites. Cell walls which were treated with toxin did not release electrolytes into the ambient solution. We tentatively conclude that HV-toxin does not act on cell walls, and that cell walls are not required for toxic action.

## LIST OF REFERENCES


1. Admon, A., B. Jacoby and E.E. Goldschmidt. 1980. Assessment of cytoplasmic contaminations in isolated vacuole preparations. Plant Physiol. 65:85-87.

2. Briggs, S.P., A.R. Haug and R.P. Scheffer. 1982. Localization of spin labels in oat leaf protoplasts. Plant Physiol. 70:662-667.

3. Briggs, S.P., R.P. Scheffer and A.R. Haug. 1982. Fluidity of oat cell membranes affects electrolyte leakage induced by HV-toxin. Physiol. Plant Pathol. (submitted).

4. Briggs, S.P., R.P. Scheffer and A.R. Haug. 1982. Osmotic conditions affect sensitivity of oat tissues to toxin from Helminthosporium victoriae. Physiol. Plant Pathol. (submitted)

5. Bronson, C.R. and R.P. Scheffer. 1977. Heat- and aging-induced tolerance of sorghum and oat tissues to host-selective toxins. Phytopathology 67:1232-1238.

6. Hanchey, P. 1980. Histochemical changes in oat cell walls after victorin treatment. Phytopathology 70:377-381.

7. Hawes, M.C. and H. Wheeler. 1982. Factors affecting victorin-induced root cap cell death: temperature and plasmolysis. Physiol. Plant Pathol. 20:137-144.

8. Keck, R.W. and T.K Hodges. 1973. Membrane permeability in plants: changes induced by host-specific pathotoxins. Phytopathology 63:226-230.

9. Kivilaan, A., T.C. Beaman and R.S. Bandurski. 1959. A partial chemical characterization of maize coleoptile cell walls prepared with the acid of a continually renewable filter. Nature 184:81-82.

10. Lloyd, C.W., A.R. Slabas, A.J. Powell and S.B. Lowe. 1980. Microtubules, protoplasts, and plant cell shape. Planta 147:500-506.

11. Lorz, H., C.T. Harms and I. Potrykus. 1976. Isolation of "vacuoplasts" from protoplasts of higher plants. Biochem. Physiol. Pflanzen. 169:617-620.

12. Martinoia, E., U. Heck and A. Wiemken. 1981. Vacuoles as storage compartments for nitrate in barley leaves. Nature 289:292-294.

13. Rancillac, M., R. Kaur-Sawhney, B. Staskawicz and A.W. Galston. 1976. Effects of cycloheximide and kinetin pretreatments on responses of susceptible and resistant Avena leaf protoplasts to the phytotoxin victorin. Plant and Cell Physiol. 17:987-995.

14. Samaddar K.R. and R.P. Scheffer. 1968. Effect of the specific toxin in Helminthosporium victoriae on host cell membranes. Plant Physiol. 43:21-28.

15. Sammaddar, K.R. and R.P. Scheffer. 1971. Early effects of Helminthosproium victoriae toxin on plasma membranes and counteraction by chemical treatments. Physiol. Plant Pathol. 1:319-328.

16. Volger, H. and K.A. Santarius. 1981. Release of membrane proteins in relation to heat injury of spinach chloroplasts. Physiol. Plant 51:195-200.

17. Weihing, R.R. 1979. The cytoskeleton and plasma membrane. Meth. Achiev. exp. Pathol. 8:42-109.

18. Wheeler, H. 1981. The role of toxins in pathogenesis. In "Toxins in Plant Disease", R.D Durbin (ed.), pp. 477-494. Academic Press, NY.

19. Wheeler, H. and H.S. Black. 1963. Effects of Helminthosporium victoriae and victorin upon permebility. Amer. J. Bot. 50:686-693.

20. Wheeler, H. and E. Elbel. 1979. Time-course and anti-oxidant inhibition of ethylene production by victorin-treated oat leaves. Phytopathology 69:32-34.

21. Widholm, J. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technology 47:189-194.

# GENERAL DISCUSSION

## GENERAL DISCUSSION

The data presented in this thesis are compatible with the hypothesis that HV-toxin has an initial site of action in the plasma membrane or the cytoplasm. The initial step, perhaps binding to a receptor, is soon followed by leakage of electrolytes from the cell. Electrolytes may be moved across the plasma membrane by a diffusible carrier, or they may move through pores created by toxin. Movement by a diffusible carrier seems more likely, for reasons discussed in thesis sections 2 and 3.

There are alternative explanations of toxic action. It is possible that toxin acts by activating a lipase, but this seems unlikely. While degradation of membrane lipids could conceivably account for known toxin effects, the kinetics of electrolyte loss caused by toxin do not support the hypothesis. The rate of leakage caused by a given concentration of toxin is linear, as shown in thesis section 4, figure 5. Therefore, the number of lesions in the membrane through which electrolytes pass is constant. Degradation of membranes by lipase should result in a constant increase in the number of membrane lesions, which would give an exponential rate of electrolyte loss.

There are several possible explanations for the effects of external osmotica on the rates of toxin-induced leakage from tissues. The osmoticum affects cell turgor; at 0.2 M or greater, the rate of toxin-induced leakage is suppressed. A favored hypothesis is that cell turgor

affects water flow into the vacuole; if this is extensive, because of toxic effects on the plasmalemma, then the tonoplast may break, with further leakage from the cell. Another possible explanation, not ruled out by the data, is that cell turgor affects mass flow from the cell. The hydraulic pressure in a turgid cell could cause water and solutes to flow out rapidly if there is an adequate hole in the membrane. Leakage by mass flow would be through a continuous water channel spanning the membrane. Many different solutes would be lost.

The kinetics of solute loss from a toxin-damaged cell can be described mathematically according to the mass flow hypothesis.

$$\text{Rate of solute loss} = -\frac{dm}{dt} = C_t \times R \times N$$

$$\frac{1}{V}\frac{dm}{dt} = \frac{dCt}{dt} \qquad -\frac{dCt}{dt} = \frac{Ct \times R \times N}{V}$$

$$R = \alpha P = \alpha\beta C_t \qquad -\frac{dC_t}{dt} = \frac{\alpha\beta}{V} C^2{}_t N$$

$$\int_{C_o}^{C_t} C^{-2}\, dc = -\int_{o}^{t} \frac{\alpha\beta N}{V} dt$$

$$-\frac{1}{C} + \frac{1}{C_o} = -\frac{\alpha\beta N t}{V}$$

$$C_t = \frac{C_o}{\frac{\alpha\beta C_o N t}{V} + 1}$$

where $C_t$ = concentration of solute inside the cell at time t, m = number of moles of solute inside the cell, R = rate of water flow through a pore in the membrane, N = number of pores in the membrane (proportional to the toxin concentration), V = volume of the cell, P = turgor of the cell, $\alpha$ and $\beta$ are proportionality constants. The assumptions of the model are that the rate of solute loss from the cell is proportional to the concentration of solute in the cell, the number of

pores in the membrane, and the rate of water flow through each pore.

The rate of water flow through each pore is assumed to be proportional to the turgor pressure of the cell, which is proportional to the concentration of solute in the cell. The cell is assumed to be a single compartment surrounded by an infinitely dilute solution. The hypothesis predicts that loss of solute will be hyperbolic with time. Unfortunately, the model is too simple to be used in analyzing electrolyte leakage experiments from tissues. Loss of radioactive solute from isolated root cap cells may allow the model to be tested. A better test may be to examine the solute specificity of leakage caused by toxin. If many different solutes are lost soon after exposure to toxin, then the activation of a shuttle-type carrier which must bind the solute would seem unlikely; a conduit across the membrane through which any molecule of the appropriate size could pass would be more likely. The mass flow hypothesis and the hypothesis presented in Section 3 for water flow into the vacuole may be compatible with each other; they differ primarily in the explanation of how osmotica affect the rate of toxin-induced leakage. We feel that the mass flow hypothesis is less likely because water and solutes are believed to move through separate channels in the membrane. The results given in Section 2, which suggest that loss of electrolytes occurs via a shuttle-type carrier rather than a continuous channel spanning the membrane, reinforces this view but still do not allow for a firm conclusion.

Future work on the mode of action of HV-toxin will be aided by the use of cell-free preparations which respond to toxin. The sensitivity of vacuoles described in Section 4 is an important step in this direction.

Even though cytoplamsic contaminants may be responsible for the response of these vacuoles to toxin, this is the first observation of an effect of HV-toxin on a cell-free preparation. Analysis of the protein in these preparations by two-dimensional gel electrophoresis may lead to the identification of a toxin receptor; several oat cultivars of varying sensitivity to toxin should be compared and the effects on the gel pattern caused by factors which destroy toxin sensitivity, such as cycloheximide and heat, should be examined. Conclusive identification of the putative toxin receptor will require binding analysis with radioactive toxin. Therefore, a method for obtaining labeled, homogenous toxin is a high priority.