Selective Toxin of *Helminthosporium victoriae*: Thermal Relationships in Effects on Oat Tissues and Protoplasts

Steven P. Briggs, Robert P. Scheffer, and Alfred R. Haug

Graduate research assistant and professor, respectively, Department of Botany and Plant Pathology, and professor, Department of Microbiology, Pesticide Research Center, Michigan State University, East Lansing 48824. Present address of senior author: Department of Biotechnology Research, Pioneer Hi-Bred International, Inc., Box 38, Johnston, IA 50131.

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ABSTRACT

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Electrolyte leakage induced in oat leaves susceptible to toxin from *Helminthosporium victoriae* was minimal at temperatures below 12 C; above 12 C, the rate of leakage increased with increases in temperature. Protoplasts from leaves were held at temperatures from 0 to 40 C, and the fluidity of membranes was measured by electron spin resonance with 5-doxylstearic acid as a spin label. A membrane phase transformation was observed at 12 ± 1 C. The temperature correlation between toxin-induced leakage and membrane fluidity suggests that loss of electrolytes occurs through the membrane matrix, possibly via a shuttle-type carrier. Toxin rapidly killed oat mesophyll protoplasts as shown by the use of fluorescein diacetate, a vital stain. Collapse of protoplasts followed death at 35 C but not at 23 C. The vital stain showed that protoplasts are as sensitive to toxin as are leaf tissues and intact roots. Tissues treated with toxin at 0 C and held at that temperature had no toxin-induced leakage. Tissues exposed to toxin at 0 C, washed, and then warmed to 23 C lost as much as did tissues held at 23 C throughout. Presumably, the initial step in toxic activity can occur at 0 C, but processes leading to electrolyte leakage occur only at higher temperatures.

Additional key words: *Avena sativa*, *Cochliobolus victoriae*, host-selective toxin.

Helminthosporium victoriae Meehan & Murphy, the causal fungus of blight disease of oats (*Avena sativa* L.), produces HV toxin that selectively affects cultivars carrying the V_b allele (18); all cultivars with this allele also are susceptible to *H. victoriae*. The first effects of the toxin observed to date have been rapid losses of electrolytes from sensitive tissue (5,17,18,21) and changes in negative electropotential across the plasma membrane (9,12). Rates of leakage are proportional to the concentration of toxin to which tissues are exposed (7). Leakage continues after tissues are removed from toxin, until most of the electrolytes are lost from tissues (5). The mechanism by which toxin causes leakage of electrolytes is unknown; there is no evidence that the losses are a direct or primary effect of toxic action. However, there is no question that toxin affects the function of the plasma membrane, even though this may be a secondary effect.

Rapid killing of oat coleoptile protoplasts by the toxin produced by *H. victoriae* was reported by Samaddar and Scheffer (16). The toxin preparation used in that work killed 50% of susceptible protoplasts in 1 hr, at 0.16 ng/ml. Subsequently, Rancillac et al (15) observed that oat mesophyll protoplasts were killed by toxin at 32 C. Other workers have failed to confirm that toxin has a rapid effect on protoplasts. Hawes (10) reported that high concentrations of toxin killed susceptible protoplasts within 3–4 hr and that protoplasts from leaves were more sensitive than protoplasts from root cap cells. Hawes and Wheeler (11) and Briggs et al (3,4) reported that temperature and osmotica can influence the responses of tissues and protoplasts to toxin.

We have reexamined toxic reactions of tissues and protoplasts and have used spin labels with electron spin resonance (ESR) spectroscopy in attempts to correlate rates of leakage with molecular order in membranes. True lipid phase transitions are not known to occur in biological membranes, but transformations have been described that often are correlated with important physiological functions (14). The results of spin label studies were compared with the temperature-dependence of toxin-induced leakage of electrolytes. By analogies with other studies, the ESR and associated data suggest that leakage may be mediated by shuttle-type carriers. Other data reported here confirm that protoplasts are as sensitive to toxin as are intact cells in tissues.

Toxin used in several experiments described here was active at 0.7 ng/ml but still contained some impurities. For this reason, resistant tissues or protoplasts were used as controls in every experiment, with no discernible effects of toxin. Also, there was no discernible effect on toxic action when the major contaminant in toxin preparations was added back to the highly active preparation. Results with toxins of other *Helminthosporium* spp. in highly active but not entirely pure form have been confirmed when more purified preparations became available (19).

MATERIALS AND METHODS

Electrolyte leakage assays were conducted as described previously (5), with variations in protocol as indicated in the various experiments. Leaf tissue samples (0.2 g of leaf pieces, each 5 mm long) were enclosed in cheesecloth and immersed in 10 ml of solution in a scintillation vial. Samples were infiltrated under reduced pressure with either water or toxin solution, incubated at room temperature for 1 hr, washed thoroughly in large volumes of water, and resuspended in 10 ml of water for leaching. The vials were held in water baths at the desired temperatures for the duration of the leaching period. The conductances of ambient solutions were measured with a conductivity meter equipped with a pipette-type, temperature-compensating electrode ($K = 1.0$). Readings were taken each hour unless indicated otherwise. Rates of leakage were determined by linear regression analysis; correlation coefficients were at least 0.95. Two oat cultivars were used; Park is susceptible and Garry is resistant to *H. victoriae* and to its toxin.

Plants used as a source of protoplasts were grown in vermiculite for 1 wk under fluorescent light at ~22 C (2). Protoplasts were isolated from primary leaves by peeling away the lower epidermis and floating the leaves (peeled surface down) on a solution containing 0.5% Cellulysin (Calbiochem-Behring Corp., San Diego, CA 92112) and 0.6 M sorbitol, adjusted to pH 5.6 with

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KOH. The preparation was incubated at 30 C for 2 hr in the dark, then was swirled gently to release protoplasts. The protoplasts were filtered through a layer of Miracloth and the suspension was centrifuged at 40 *g* for 10 min. The supernatant was removed and the pellet was washed by centrifugation in a suspension medium containing 0.6 M sorbitol and 10 mM $CaCl_2$, adjusted to pH 5.7 with KOH. Protoplasts were used at a concentration of approximately 10^6/ml.

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

Protoplasts (2 mg of chlorophyll per milliliter) in thick slurry suspensions were spin-labeled by adding 0.2 ml of suspension to a test tube containing a dry film of 5-doxylstearic acid (Syva Associates, Palo Alto, CA) and gently rotating for 5 min at 23 C. The film for labeling protoplasts was derived from either 10 or 20 μl of 30 mM spin label in ethanol, blown dry with air. Final spin label concentrations were 2 mM. Previous work has indicated that 5-doxylstearic acid partitions 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 that were placed in the Dewar vessel and scanned in the dark. ESR spectra were determined 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 within the cuvette. Relative ESR signal intensities were determined by dividing the peak-to-peak height of the midfield line by the amplifier gain. Line shape was analyzed in terms of the maximum hyperfine splitting parameter, $2T_{11}$. Phase transformation temperatures were determined by linear regression analysis.

Toxin was isolated from 3-wk-old cultures of *H. victoriae* grown in a modified Fries medium containing yeast extract (13). Culture filtrates were precipitated with methanol and extracted with butanol. Contaminants in toxin preparations were monitored by thin-layer chromatography on silica gel 60 plates developed with ethanol, water, and acetic acid (70:29:1); a major contaminant was detected by spraying the plates with vanillin-H_2SO_4. Partial separation of toxin from the major contaminant was achieved by cation exchange chromatography in an SP-Sephadex C25 column, which was equilibrated and developed with water. In a second pass through the column, toxin was strongly retained, allowing for complete separation from the major contaminant. The preparation at this stage of purification gave complete inhibition of seedling root growth at 0.7 ng/ml, which is approaching the highest activity so far reported (18). The preparation was chromatographed in an HPLC system by using a Whatman Partisil 10 column (0.5 × 25 cm) developed isocratically with 90% aqueous ethanol. Several peaks were detected by UV absorbance at 214 nm.

For most experiments, a toxin preparation that completely inhibited root growth of susceptible seedlings at 0.5 μg/ml was used at 50 μg/ml, unless stated otherwise. Experiments were repeated and confirmed with the preparation that completely inhibited root growth at 0.7 ng/ml, using 0.7 μg/ml unless stated otherwise. Reintroduction of the major contaminant into toxin preparations did not affect toxicity. Controls included toxin-treated resistant tissues or protoplasts and untreated controls. All experiments were repeated two or more times.

RESULTS

Effect of temperature on toxin-induced losses of electrolytes from tissues. The time course of toxin-induced loss of electrolytes from tissues was determined. Samples were infiltrated with water and washed thoroughly prior to exposure to toxin. Washing was necessary because each toxin-treated sample initially released electrolytes into the wash solution. Under these conditions, leakage caused by toxin did not become significant until ~50 min after addition of toxin, when the rate of loss became rapid and linear. Otherwise, the results are similar to those for experiments with different treatment protocols (5,7,16). The rate of electrolyte loss was only slightly greater at 35 C than at 23 C (Fig. 1). Above 40 C,

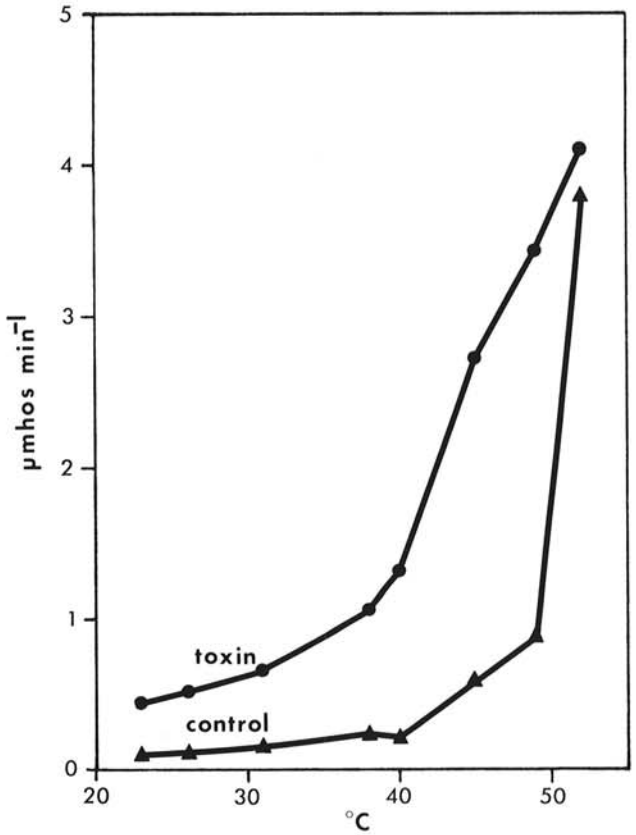

Fig. 1. Effect of temperature on the rate of electrolyte leakage from susceptible (cultivar Park) oat leaf tissues treated with selective toxin of *Helminthosporium victoriae*. Samples (0.2 g) were incubated in water or toxin solution for 1 hr at 23 C, washed, and held at the indicated temperatures for 5 hr. Conductivities of ambient solutions were measured at 1-hr intervals and rates were determined by linear regression analysis. Correlation coefficients were 0.95 or greater.

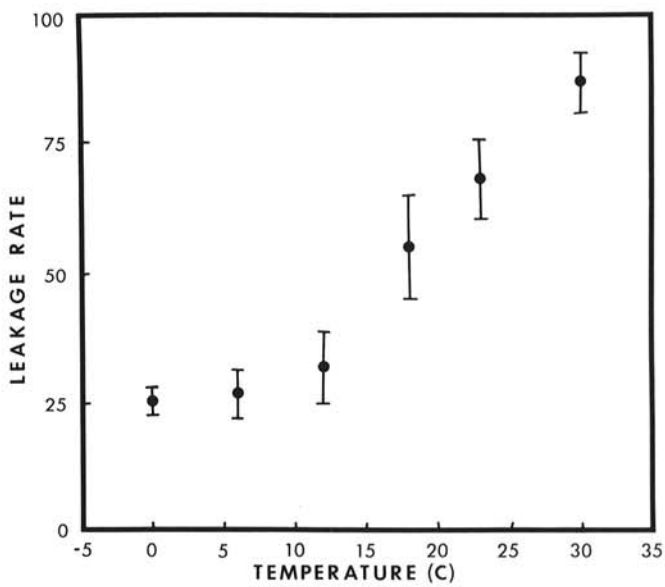

Fig. 2. Effect of temperature during the leaching period on the rate of electrolyte loss (μmhos·min^{-1} × 100) induced by selective toxin of *Helminthosporium victoriae*. Tissues were treated with toxin, washed at 23 C, and incubated at the indicated temperatures. Variability is shown.

the rate of electrolyte leakage was much greater than at lower temperatures. Toxin-treated tissues were more responsive to increases in temperature than were control tissues. Next, tissue samples were treated with toxin and washed at 23 C; a 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 (Fig. 2).

Sensitivities of tissues to several toxins are known to be affected by temperature prior to exposure (5). Pretreatment of oat tissues at temperatures up to 40 C had no effect on the subsequent response to toxin at 23 C. However, pretreatment at 44 C or higher eliminated sensitivity to toxin, as determined by electrolyte leakage (Fig. 3). Loss of sensitivity was not caused by depletion of electrolytes during pretreatment of tissues at 44–48 C; when such tissues were heated to 52 C, they lost electrolytes at the same rate as did controls that were pretreated at 23 C rather than at 44–48 C.

Many membrane functions are inhibited at low temperature (14). We exposed oat leaf tissues to toxin at 0 C to determine whether toxin-sensitivity was affected (Table 1). Tissues that were treated and held at 0 C appeared to be insensitive to toxin and did not lose electrolytes faster than did control tissues. 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 efflux of electrolytes, tissues were incubated in toxin solutions at 23 C for 1 hr, washed, and then cooled to 0 C to monitor leakage. Electrolyte loss was only 22% of the rate for the sample held at 23 C (Table 1). Nevertheless, samples treated at 23 C and cooled at 0 C still lost more electrolytes than did the controls without toxin, indicating that once toxin-induced leakage has begun, it cannot be completely stopped by lowering the temperature. Therefore, low temperature inhibits efflux of electrolytes across the membrane and may also inhibit the initial action of the toxin. 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.

Further indication that low temperature may affect toxin action at its initial site was obtained by treating tissues with toxin at 0 C, washing thoroughly (3–4 hr) in large volumes of water or alkaline water near 0 C followed by incubation at 23 C. Loss of electrolytes from these samples was equal to that from samples that had been treated and held at 23 C throughout (Table 1). This suggests that toxin was taken up or bound by the tissue at 0 C but was unable to trigger the next step leading to electrolyte leakage. The next step, which could be only a conformational change in a receptor, appears crucial to toxin action, because cell death (11) as well as electrolyte leakage is inhibited by low temperature. The location of a receptor or sensitive site, whether in plasma membrane or elsewhere, is not indicated by these data.

Effect of temperature on spin label motion in protoplasts. Membrane activities are often inhibited at temperatures below phase transformation temperatures. Transformations are characterized by a cooperative increase in molecular ordering but are not simple liquid-crystal to gel lipid-phase transitions. We used spin labels and ESR spectroscopy to determine whether a membrane phase transformation occurred above 0 C in oat leaf protoplasts. Results showed that membrane microviscosity, as detected by $2T_{II}$, decreased linearly as the temperature of the sample increased from 0 to 12 C (Fig. 4). An abrupt change in slope, indicating a change in the dependence of molecular ordering on temperature, occurred at 12 ± 1 C. Membrane microviscosity decreased faster with increasing temperature above 12 C than below. The possibility of a second lipid phase transformation at a higher temperature could not be ascertained, because at temperatures above 38 C the ESR signal became too weak and narrow to determine $2T_{II}$ with accuracy. The abrupt change in molecular ordering at 12 C is correlated with the change in rate of toxin-induced leakage from tissues, which also occurs at 12 C (Fig. 3).

Oat leaf protoplasts were exposed to toxin, and ESR spectra of

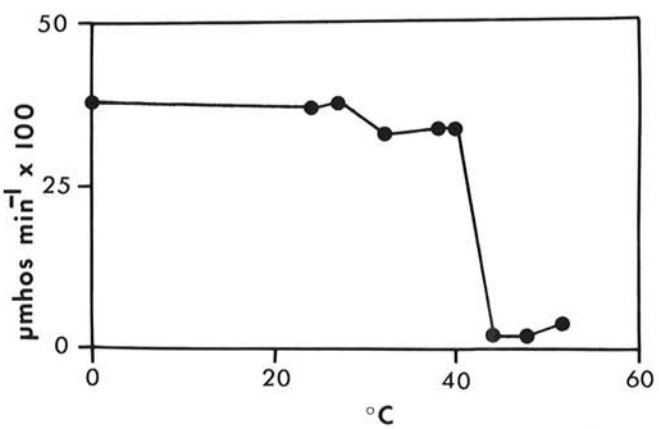

Fig. 3. Effect of pretreatments at several temperatures on electrolyte leakage induced by selective toxin of *Helminthosporium victoriae* in oat (cultivar Park) leaf tissues. Samples (0.2 g) were held at the indicated temperatures for 1 hr, washed, treated with toxin for 1 hr at 23 C, washed, and incubated at 23 C. Rates were determined as indicated in Fig. 1. Losses from susceptible and resistant controls (no toxin) and from toxin-treated resistant tissues were no greater than the lowest values shown.

TABLE 1. Effect of temperature during exposure to toxin and during the leaching period on HV toxin-induced leakage of electrolyte from oat tissues

Temperature (C)		Leakage rate		Ratio of
Treatment[a]	Measurement[b]	Toxin-treated (μmhos/min)	Control (μmhos/min)	toxin-treated to control
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), incubation for 60 min, and washing.

[b] Treated samples were leached at the indicated temperatures for 5 hr after toxin treatment and washing. Conductivity was measured at 60-min intervals. The rate of leakage was determined by linear regression analysis.

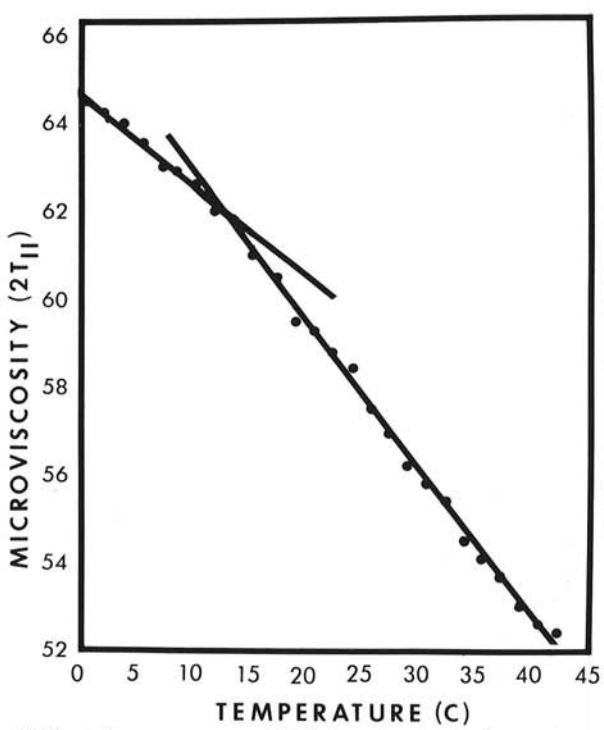

Fig. 4. Effect of temperature on spin label motion. Oat leaf protoplasts were spin-labeled with 5-doxylstearic acid; relative membrane microviscosity values were measured as $2T_{II}$.

5-doxylstearic acid in their membranes were recorded (*unpublished*). Toxin caused no significant changes in membrane fluidity within 3 hr after exposure.

Effect of temperature on viability of toxin-treated protoplasts. Protoplasts were exposed to toxin and both fluorescein-stained and unstained protoplasts were examined with the microscope. Viability of toxin-sensitive protoplasts declined rapidly, as determined by the ability to retain fluorescein (22) (Table 2). Viability of nontreated control protoplasts and of treated and control resistant protoplasts was not significantly reduced during the experiment (see Fig. 5). In contrast, microscopic appearance of sensitive protoplasts gave no indication of a rapid lethal effect of toxin (Table 2). The unstained protoplasts appeared normal 3 hr after exposure, even though 73% of them were killed. Protoplast surfaces were consistently distorted at 90 min after exposure to toxin, but the change was subtle. The distortion was no longer evident by 180 min after exposure, when the unstained protoplasts had returned to a normal appearance. The results were confirmed with a more active toxin preparation, which completely inhibited seedling root growth at 0.7 ng/ml. Data were not taken at all the exposure times indicated in Table 2, but this toxin preparation at 1.4 μg/ml killed all protoplasts within 60 min at 23 C. The effect was almost as rapid as that reported previously (17).

Temperature had a significant effect on viability and collapse of toxin-treated protoplasts. Collapse of significant numbers of protoplasts was not observed until 4.5 hr after exposure at 23 C. Collapsed protoplasts had a nonspherical outline, a fuzzy surface, and aggregated chloroplasts; collapse was usually associated with agglutination of several cells. Toxin-treated protoplasts at 35 C lost viability in about half the time required at 23 C (Fig. 5). Furthermore, toxin-treated protoplasts at 35 C collapsed with loss of viability. All toxin-treated protoplasts at 35 C disintegrated by 3 hr after exposure, with only cellular debris remaining. Susceptible control protoplasts and resistant protoplasts (toxin-treated and control) remained in good condition.

Several significant details were discovered during the course of these experiments. Both Evans blue and neutral red dyes proved to be poor indicators of protoplast viability, no better than was detected microscopically without dyes. Control protoplasts without toxin maintained viability for 2 days or more in the dark but not in the light, where protoplast senescence appeared to be accelerated.

Toxin sensitivities of protoplasts, roots, and leaves were compared; all three were about equal in sensitivity to toxin, with detectable effects of the best toxin preparation at 0.7 to 1.4 ng/ml (Table 3).

TABLE 2. Effects of selective toxin from *Helminthosporium victoriae* on oat leaf protoplasts, as determined by protoplast appearance and by staining at 22 C with fluorescein diacetate (FDA). Temperature during the experiment was 22 C

Toxin exposure (hr)	Viable protoplasts[a] (%)	Protoplast appearance[b]
0	95 ± 2	Normal
1.5	78 ± 14	Distorted surface
3.0	27 ± 13	Normal
4.5	11 ± 5	Collapsed[c]
6.3	3 ± 1	Collapsed[c]

[a] Viable = 100 × (number observed fluorescing with FDA)/(number observed in dark-field). A minimum of 100 protoplasts was counted at each time. The results are the means for four experiments. A toxin preparation that completely inhibited root growth at 0.5 μg/ml was used at 50 μg/ml. Toxin (1.4 μg/ml) that was active at 0.7 ng/ml gave comparable results but with more rapid killing; all protoplasts were dead in 1 hr.

[b] Normal = spherical protoplasts, with an even distribution of chloroplasts around the periphery of the cell, and no agglutination; distorted surface = rough or wrinkled outline of protoplast in contrast to the normally smooth surface; collapsed = shrunken, agglutinated protoplasts with a fuzzy surface, a nonspherical outline, and indistinct chloroplasts. Dark-field microscopy was used to examine protoplasts.

[c] 20% of protoplasts were collapsed, 80% appeared normal.

DISCUSSION

Selective toxin produced by *H. victoriae* causes rapid losses of electrolytes from susceptible tissues (17,21). Toxin also causes a rapid change in electropotential across the plasma membrane (9,12,18), indicating that early losses of electrolytes are more than simple releases from cell walls or intercellular spaces. However, such rapid responses are detected only under certain conditions (17). With our protocol and experimental conditions, toxin-induced leakage from tissues was not clearly evident until 50 min after exposure. Thus, both tissues and protoplasts may require some time for membrane damage to be detected. The time of detection also depends on activity of the toxin preparation; for example, our highly active toxin preparation at 1.4 μg/ml killed all

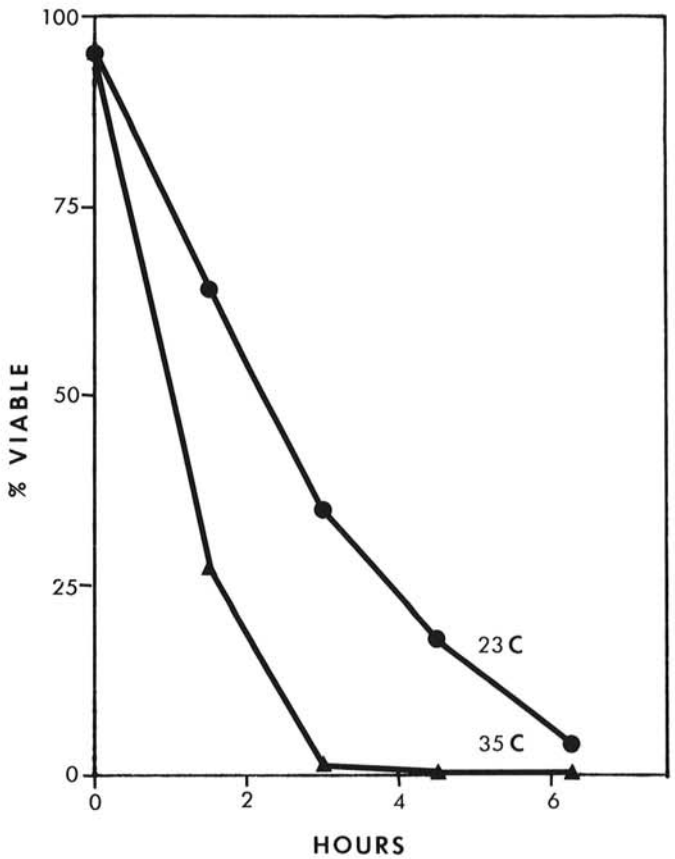

Fig. 5. Effect of temperature on viability of HV toxin-treated protoplasts from susceptible (cultivar Park) oat leaves. Viability was determined by the fluorescein diacetate assay. At the end of the experiment, the viabilities of control protoplasts with no toxin were: resistant (cultivar Garry) at 23 C, 91 ± 4%; resistant at 35 C = 92 ± 3%; susceptible at 23 C = 95 ± 2%; susceptible at 35 C = 93 ± 1%. Viability of resistant, toxin-treated protoplasts at 23 C was 84 ± 8%; at 35 C it was 77 ± 10%.

TABLE 3. Comparative sensitivities of susceptible oat roots, leaves, and protoplasts to the selective toxin of *Helminthosporium victoriae* [a]

Assay method	Criterion of activity	Assay time	Dilution end point[b] (ng/ml)
Root growth inhibition	Complete inhibition	3 days	0.7
Protoplast viability[c]	>80% kill	19 hr	1.4
Electrolyte leakage from leaves	Toxin-induced increase	1 hr	1.4

[a] There were no obvious effects on protoplasts and tissues of resistant oats (cultivar Garry).

[b] Minimum concentration needed to give stated result.

[c] As determined by fluorescein diacetate as a vital stain.

susceptible protoplasts in 1 hr, whereas at 1.4 ng/ml only 84% were killed in 19 hr (Table 3).

Death of protoplasts was determined with fluorescein diacetate, a vital stain (22). Protoplasts with intact membranes retain fluorescein, which is released from the diacetate by cellular esterases. The membranes of dead protoplasts do not retain fluorescein. Therefore, viability in this case is defined as possession of an intact and functional plasmalemma. Viable protoplasts fluoresce pale green in UV light, whereas dead protoplasts emit a dim, blood-red fluorescence. All protoplasts were killed within 60 min after exposure to a highly active toxin preparation at a concentration of 1.4 μg/ml. This time scale is comparable to that reported in previous work (16) with protoplasts from coleoptiles. In contrast to the earlier observations (16), the protoplasts used in our experiments at 23 C did not lyse or collapse in large numbers for 2 hr or more following death. However, the toxin-treated protoplasts died and collapsed relatively quickly when held at 35 C. Similar observations were made by Rancillac et al (15).

Temperature can inhibit as well as enhance toxicity. Both low temperature (4) and pretreatment at high temperature (5) can partially block the action of toxin. Pretreatment at temperatures above 40–44 C made tissue insensitive to toxin, whereas tissues pretreated at all temperatures from 0 to 40 C 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 in which the proteins are embedded (20).

The sensitivity of oat protoplasts to toxin was compared to that of cells in tissues. If the site of action is not in the cell wall (10) or cytoskeleton, then protoplasts should be as sensitive as intact tissues. Protoplast viability, root growth, and electrolyte leakage were affected by toxin at minimal concentrations from 0.7 to 1.4 ng/ml (Table 3), which is remarkably close considering the limitations of the assays. This indicates that sensitivities of tissues and protoplasts to toxin are approximately equal. Therefore, a site external to the plasmalemma is not likely to be required for toxic action.

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 occur. There are at least three possible explanations of the failure to induce leakage at 0 C. One possibility is that the toxin binds to a 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 explanation fits the mobile receptor hypothesis developed by Cuatrecasas and Hollenberg (6) 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 temperature is increased. Which, if any, of these mechanisms is correct will probably not be established until a toxin receptor or sensitive site is isolated.

A change in molecular ordering of oat leaf protoplast membranes was observed at 12 C. A precise understanding of this phenomenon at the molecular level is not presently available. However, it is widely thought that lateral phase separations could be responsible (14). Whatever the details of the molecular change, it is clear that below the transformation temperature (12 C) toxin-induced passage of electrolytes through the membrane is inhibited (Fig. 3). 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 (1) 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 (1). Whether it is the toxin-receptor complex or some other entity that mediates the passage of electrolytes is not known. Presumably, the membrane must be in a particular state of organization for toxin-induced leakage of electrolytes to occur; the transformation that occurs with cooling below 12 C inhibits efflux of electrolytes.

No changes in fluidity of oat protoplast membranes were detected following exposure to HV-toxin, in contrast to the effects of cercosporin on tobacco protoplast membranes (8). Cercopsorin, a photosensitizing agent, causes the oxidation of polyunsaturated fatty acids that 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 a protein probably would not perturb bulk lipid fluidity and would go undetected by spin labels and ESR. We suggest that damage by the toxin of *H. victoriae* is of this latter type.

In conclusion, the data confirm that isolated protoplasts are sensitive to the selective toxin of *H. victoriae* and that an early effect on protoplasts can be demonstrated. Also, the data indicate that HV-toxin causes changes in the properties of plasma membranes, perhaps after binding to a receptor site that is not necessarily in the plasma membrane. If the membrane is fluid, the toxin-receptor complex may be irreversibly transformed to an ionophore or else may activate an ionophore. The ionophore requires a fluid membrane to function and, therefore, appears to be a diffusible carrier rather than a pore.

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