STUDIES ON THE SITES OF ACTION OF THE OST-SPECIFIC TOXINS FROM HELMINTHOSPORIUM VICTORIAE AND PERICONIA CIRCINATA

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Robert W. Scheffer

Major professor

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ABSTRACT

STUDIES ON THE SITES OF ACTION OF THE HOST-SPECIFIC TOXINS FROM HELMINTHOSPORIUM VICTORIAE AND PERICONIA CIRCINATA

By

John MacGregor Gardner

Pathogenicity of several plant-infecting fungi requires the production of certain compounds specifically toxic to host plants. This study is a further examination of the hypothesis that Helminthosporium victoriae (HV) and Periconia circinata (PC) toxins cause initial biochemical lesions in the plasma membranes of the susceptible plant cell. A related hypothesis is that resistant plants are not affected because they lack toxin receptor sites. Experiments described herein were focused on the biochemical factors which contribute to susceptibility to toxins. A convenient assay for toxins was employed, based on the toxins' ability to disrupt permeability barriers and to induce electrolyte loss from plant cells.

Twelve hr pretreatment of susceptible tissues with cycloheximide (CH) (2-5 μg/ml) gave 70 to 90% protection against both HV and PC-toxins. CH protection of oat tissues against HV-toxin was clearly reversible when CH was removed. CH did not protect oat tissues against electrolyte loss induced by nonspecific toxic compounds. Other inhibitors gave relatively little protection against either toxin in comparable experiments. It is possible that cycloheximide protects by inhibiting synthesis and turnover of receptor proteins.

When oat tissues were pretreated for 30 min with N-ethylmaleimide (NEM) (2 mM), 2,4-dinitrofluorobenzene (DNFB) (2 mM),

iodoacetate (2 mM), or arsenite (4 mM), all of which are sulfhydryl-binding compounds, there was 70 to 90% protection against toxin-induced loss of electrolytes. Significant protection was evident within one min after exposure to NEM or DNFB. Beta-mercaptoethanol reversed the protective effects of DNFB and arsenite. None of these reagents gave protection of susceptible sorghum tissue against PC-toxin, nor did they protect against loss of electrolytes induced by nonspecific toxic compounds. The protective compounds did not act via competitive effects with toxin. NEM did not react with the toxin molecule, as determined by a photometric assay.

HV-toxin breakdown products (TBP) partially countered NEM protection, as measured by a reduction in the protective effects of NEM. TBP also partially countered toxin activity, as determined by the electrolyte loss assay. The possibility that TBP was competing with NEM for the same sites was tested. Both TBP and toxin reduced ^{14}C-NEM labelling of cell-free, membrane enriched fractions from susceptible but not resistant oats. This indicates that HV-toxin and TBP may compete with NEM for sulfhydryl groups associated with toxin receptor sites. Other possible explanations have not been eliminated.

Certain other pretreatments with protein-binding reagents, pronase, and detergents, gave no protection against either toxin. Pretreatment of sorghum tissue with phospholipase D reduced subsequent losses of electrolytes caused by PC-toxin. Pretreatments with uranyl salts, which bind to membranes, gave partial protection against both toxins; however, uranyl salts also gave partial protection against loss of electrolytes induced by nonspecific toxic compounds. Carbonyl-binding reagents were confirmed as protectants against HV-toxin, but

not against PC-toxin.

HV and PC-toxins caused changes in single cell electropotentials, but under most conditions these came later than the rapid efflux of materials from cells into distilled water. No effects of toxins on isolated organelles have been demonstrated.

Both HV and PC-toxins were inactivated by treatment with dry methanol-HCl, a specific reagent for carboxyl groups. The esterified, inactivated toxins gave some protection against active toxin in electrolyte loss assays; it is possible that esterified toxin competes with active toxin for receptor sites. Carboxyl groups are therefore required for both HV and PC-toxin activity.

The data support the hypothesis of toxin receptors in plasma membranes of susceptible cells. Data with cycloheximide suggest that toxin receptors are proteins; data with certain sulfhydryl-binding reagents suggest that sulfhydryl groups are involved. More conclusive studies will require techniques for isolation of plant cell membranes.

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By

John MacGregor Gardner

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DEDICATION

TO FINE PARENTS

K.S.G.

R.F.L.

P.M.L.

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LIST OF ABBREVIATIONS

ATP - Adenosine 5'-triphosphate

CoA - Coenzyme A

CH - Cycloheximide

m-ClCCP - Carbonyl cyanide m-chlorophenylhydrazone

EDTA - Ethylenediamine tetraacetic acid

HC - *Helminthosporium carbonum*

HV - *Helminthosporium victoriae*

NAD - Nicotinamide adenosine diphosphate

NEM - N-ethylmaleimide

PC - *Periconia circinata*

POP - 2,5-diphenyloxazole

POPOP - 1,4-bis 2-(5-phenyloxazolyl) benzene

TCA - Trichloroacetic acid

TPP - Thiamine pyrophosphate

Tris - Tris (hydroxylmethyl) aminomethane

Part I

Experiments with *Helminthosporium Victoriae* Toxin

INTRODUCTION

Host-specific toxins are pathogen-produced determinants of disease which affect the same plants as does the pathogen. The toxins also reproduce the disease symptoms caused by the pathogen. At least eight host-specific toxins are now recognized (75,79,84). In addition, several non-specific toxins are known (53); the roles of non-specific toxins in disease development have seldom been evaluated. The mechanisms by which pathogens attack their hosts may differ in many respects, but parasitism and pathogenicity must have chemical bases. A primary biochemical lesion, or interaction, is therefore a logical prerequisite for parasite invasion. The initial or primary chemical lesion in the host is of interest in understanding both the action of toxin and the mechanism of pathogenicity.

HV-toxin is produced by the fungus Helminthosporium victoriae Meehan and Murphy, the causal agent of Victoria blight of oats (Avena sativa L.). Toxic activity was first described in 1947 (48) after H. victoriae had removed the popular cultivars of oats from production in the Midwest. The toxin principle was purified and partially characterized by Pringle and Braun (56,57). The toxin is produced by germinating spores and appears to be necessary for initial colonization of the fungus in susceptible host tissue (50). Toxin is highly specific for susceptible oats; resistant oats and other species will tolerate > 400,000 times higher concentrations than will susceptible

oats (39). Susceptibility or resistance to toxin is controlled by one dominant allele in the host. Therefore, this decrease may be defined both chemically and genetically.

The initial site of action for HV-toxin is believed to be in the plasma membrane (75). The rapid effects of toxin on electrolyte loss from tissues, and the destruction of cell wall-free protoplasts, were considered as evidence for an early lesion in the plasma membrane. Effects of toxin on apparent free space and plasmolytic ability further confirm the drastic effect on the membrane (75). Much evidence would indicate the toxin-induced symptoms such as increased respiration (72,62), decreased uptake and incorporation of various solutes (68), and damage to cellular organelles (45), are all secondary and result from initial effects of toxin on the plasma membrane. If the hypothesis of initial action on the membrane is correct, this toxin is the only known substance which can selectively destroy the plasma membrane of a specific organism.

Previous workers (73) postulated that the plasma membrane of the susceptible cell has a distinct receptor site for toxin. They also postulated that the resistant cell lacks a receptor site, or that the receptor site has lost its affinity for toxin. There is no direct evidence for these hypotheses, but several observations seem to offer support (68,73).

The standard toxin bioassay based on root growth inhibition proved to be impractical for many of my experiments. An assay based on toxin-induced efflux of electrolytes from susceptible cells, originally suggested by Wheeler and Black (91), was therefore developed. This assay proved to have many advantages over the seedling root growth assay.

The aim of my work was to examine the receptor site hypothesis. First, I have used the counteracting effects of various substances to develop some information on the general nature of hypothetical receptor sites. I have then attempted to devise a technique to isolate the receptor sites, based on an indirect chemical assay for the receptor components. Such an in vitro assay should help to locate the receptor in a subcellular fraction. This approach, if successful, would be a major step in understanding toxin action. A covalent labelling of the receptor site was a major concern in my approach to identification of toxin receptor sites.

Many substances were tested for ability to alter the sensitivity of susceptible cells to toxin, in the hope of finding a clue to the nature of the receptor site. Cycloheximide, which primarily inhibits protein synthesis, was found to decrease toxin susceptibility. N-ethylmaleimide (NEM), which reacts covalently with sulfhydryl groups in protein, was found to have strong protective properties against toxin. The role of protein and sulfhydryl groups in membranes is well documented (22,49,64,77,83,85). The idea that receptor sites are proteins gradually evolved. Further experiments were designed to show the specificity of the NEM effects, and possible applications to isolation and characterization of receptor sites for toxin.

The data in this thesis are consistent with the original receptor site hypothesis outlined by Scheffer and Pringle (73). The data presented should aid and stimulate more definitive investigations on the toxin-receptor site interactions.

LITERATURE REVIEW

Much of the literature on host-specific toxins is discussed in several recent reviews (53,74,75). HV-toxin was first characterized by Pringle and Braun (57) as a low molecular weight substance (< 2000) which breaks down under mild alkaline conditions to give two ninhydrin-positive components. One, named victoxinine, was described as a tricyclic secondary amine ($C_{17}H_{29}NO$) with a molecular weight of 264. The other moiety, a peptide, had an empirical ratio of 1 leucine:1 valine:1 glycine:1 glutamic acid:1 aspartic acid. The peptide was thought to account for specificity, although it has not been characterized further. Victoxinine has a non-specific activity about 0.0001 as much as the complete toxin. The linkage between peptide and victoxinine apparently involves the single nitrogen of victoxinine and the terminal amino nitrogen of the peptide. The complete toxin molecule does not react with ninhydrin. Recently, victoxinine was characterized as a sesquiterpene having properties of both secondary and tertiary amines (unpublished data from R. B. Pringle).

The first physiological work with HV toxin was concerned with the increase in respiration occurring in diseased and toxin-treated susceptible oats (93). More definitive data on this phenomenon came from Scheffer and Pringle (72), who demonstrated that toxin has no effect on oxygen uptake by isolated mitochondria. Also, respiratory

responses are readily abolished by rinsing tissue in distilled water (1). It is therefore safe to conclude that increased respiration in tissues is an indirect consequence of toxin damage.

Wheeler and Black (91) reported that efflux of electrolytes from susceptible tissues increased as early as 15 min after toxin treatment. Respiration increases were detected 10 to 20 min after toxin treatment (72). Wheeler and Black found that toxin-treated tissues took up less electrolytes than control tissues 10 min after toxin treatment, and thereafter toxin-treated tissues lost more electrolytes than controls. These data were clarified by Scheffer and Samaddar (75) who detected toxin-induced electrolyte efflux as early as 2 min after exposure to toxin.

Wheeler and Luke prophetically stated in a 1963 review (93) that the primary effect of toxin was most likely on cellular permeability and that all other changes were probably indirect effects. Five years later, Sammadar and Scheffer (68) showed by several criteria that toxin had a very rapid effect on the plasma membrane. Extrapolation of the electrolyte efflux curve to zero time would suggest that the plasma membrane effects are almost immediate (75). However, it is still possible that the initial effect may occur elsewhere, and that changes in the plasma membrane are secondary. Final conclusions should be withheld until effects on isolated plasma membranes are demonstrated.

Toxin action does not appear to require metabolic energy and apparently involves a physical interaction with receptors not present or nonfunctional in resistant tissues (73). The hypothesis is that receptors are present at the cell surface, but so far there is no

conclusive supporting evidence. There are some observations that seem to fit the hypothesis. First, breakdown products of toxin, but not victoxinine, reduced toxin activity in a seedling bioassay. Second, bisulfite and other carbonyl-binding compounds also reduced toxicity, perhaps by affecting the hypothetical receptor sites (70, 73).

An alternative was suggested by Romanko (62), who postulated that resistance is based on ability to inactivate toxin. Romanko's hypothesis was based on apparent recovery of toxin from treated susceptible cuttings, and lack of recovery from resistant cuttings. This implied that resistant tissues had toxin sensitive sites, but that toxin was inactivated too rapidly for the sites to be affected. Romanko's results could not be repeated (73,90). Theoretically, toxin would have to be inactivated immediately to account for resistance. All existing data would argue against this possibility.

Recently, still another hypothesis was proposed by Wheeler and Pirone (94) to account for resistance to toxin. They observed that when bean cuttings were given moderate doses of toxin for 36 hr, the leaves of this toxin-insensitive plant were resistant to tobacco mosaic and alfalfa mosaic viruses. The authors suggested that toxin stimulates a repair mechanism in the plant membrane. Even if this hypothesis were true, we would still need to know why the susceptible cell membrane is affected, while the resistant membrane is stimulated to repair.

Direct proof for the toxin receptor site requires experiments to show that specific sites are affected in susceptible cells, and that these sites in resistant cells are missing, nonfunctional, or have low

affinity for toxin. Attempts to detect adsorption of toxin to tissues by assaying removal of toxin from solution were unsuccessful (73). This leads to two possibilities: that toxin is not bound or inactivated on the cell surface or within the cell; or, that the assays for toxin are not sensitive enough to detect removal of small quantities of toxin. Very few molecules of toxin may be required to disrupt the membrane.

Several attempts have been made to alter susceptibility to toxin by chemical treatments. One report (16) indicates that calcium at a high concentration (0.1 M) will protect oat cuttings from toxin, but only when toxin is mixed with the calcium solution. This was interpreted as a membrane stabilizing effect. However, calcium is known to inhibit transpiration, and the protective effect could very well be an artifact. Similarly, reports of synergistic effects of cytokinins on toxin action (46) were later shown to be artifacts (43). Cytokinins increase transpiration and therefore more toxin was taken up by intact cuttings.

Uranyl ions were reported to protect cells against toxin when added to toxin solutions (27,70). However, binding of uranyl ion by toxin in solution was demonstrated (70). To circumvent mixing of toxin and uranyl ion, cuttings were pretreated for 12 hr with uranyl nitrate (27). The pretreatment also protected against toxin, but there is still a possibility of uranyl ions accumulating in tissues and accounting for toxin inactivation. Hanchey nevertheless interpreted her data to indicate that toxin acts on the cell surface, since it is known that uranyl ion does not penetrate the plasma membrane of yeast cells (65). Uranyl ion has a strong affinity for phosphoryl and

carboxyl groups (66), and has some affinity for sulfhydryl groups (60).

The damage to the plasma membrane by toxin appears to be general and massive, since leakage of potassium, phosphate, sugars, and amino acids increased after exposure of cells to toxin (8). The rate of electrolyte efflux was said to be unaffected by oxygen tension and was characterized by a low temperature coefficient (Q_{10} 1.2).

Electron microscope studies of toxic effects have given ambiguous results. Derangement of nuclear membranes, endoplasmic reticula, chloroplasts and mitochondria have been observed (45), but these observations were made after long exposures to toxin. The plasma membrane appeared to be intact, but smoother than in untreated cells; the bi-layer appearance was retained for 24 hr after toxin exposure. Other studies have shown that definite changes in membrane characteristics are not apparent in electron microscope pictures (28). The long toxin treatment periods used in these studies make results difficult to interpret.

The genetics of susceptibility and resistance to toxin is especially interesting since susceptibility to HV-toxin is linked to resistance to several strains of crown rust (Puccinia coronata) (58). Susceptibility to toxin in oats is dominant over resistance, and the reaction is controlled by the Vb locus. Other genes have been found in certain oat selections which may modify the reaction (44). It is possible that resistance to H. victoriae and susceptibility to P. coronata are not controlled by the same gene pair, but are only linked very closely. The qualitative ability of H. victoriae to produce HV-toxin also is controlled by a single gene, as shown by mating

experiments and genetic analysis (71). A major question is whether or not the primary gene product of the Vb allele in susceptible plants is the toxin receptor.

If the toxin receptor is established as being located in the plasma membrane, then the final understanding of the mechanism of toxin action will result on a basic knowledge of the structure and function of the plasma membrane. Therefore, certain information related to the plasma membrane is included here. Progress in this general field is summarized in several recent reviews (10,38,47).

Danielli and Davson's original hypothesis that the membrane is basically a protein-lipid-protein sandwich has survived 30 yr of criticism; only recently have solid objections been raised, based on data from work with electron microscopy, infrared spectroscopy, circular dichroism, and other techniques. Recent data have emphasized that protein-protein interactions are major determinants of membrane structure (38). Even though the role of lipid in membrane structure has been de-emphasized, there is good evidence to show that hydrophobic lipid-protein interactions determine some of the structural characteristics of proteins in membranes (88).

The most striking demonstration of the importance of structural proteins in membranes was provided by Fleisher et al (21). In their experiments, 95% of the lipid was extracted from mitochondrial membranes without altering their bilayer appearance. Nevertheless, phospholipids do play an important role in electron transport and succinate oxidation (26). Furthermore, membranes of the gas vacuoles from Microcystis aeruginosa are composed entirely of protein, and apparently only a single protein moiety is involved (33).

Structural proteins from many membrane types bind hydrophobically to lipid (35,38). Such proteins are heterogeneous, resistant to pronase, and soluble only in detergent or non-aqueous solvent systems. Alkaline butanol is very effective in extracting protein from membranes (3,38). Spectroscopic studies on plasma membranes from human erythrocytes (88) have shown that membrane proteins are, to a great extent, globular with a high helical content. These studies suggest that the architecture of membrane proteins is dependent on lipid-protein and/or lipid sensitive protein-protein interactions.

Opposing the bilayer lipid-protein hypothesis is the view that membranes are composed basically of repeating subunits, each unit composed of independent sets of catalytic and structural proteins (26). Electron microscopy in part has supported this hypothesis, but there may be artifacts in the techniques (10).

Many so-called transport proteins for various sugars and amino acids have been described (54). The techniques involved in identifying and isolating transport proteins have potential application to studies on toxin receptors. Therefore, some of the more important experimental procedures, especially those concerned with sulfhydryl functions, will be described in some detail.

Stein (83) was the first to develop a technique for isolating a membrane transport protein. Previous work (15) had shown that fluorodinitrobenzene (FDNB) inhibited the glucose transport system in red blood cells. Therefore, Stein labelled cells induced for glucose transport with ^{14}C-FDNB, and cells not induced for glucose transport with ^{3}H-FDNB. The cell cultures were then mixed, homogenized, and the protein fraction chromatographed on DEAE cellulose. The protein

fractions with the highest $^{14}C/^{3}H$ ratio were presumed to be specific for, or at least associated with, the induction of glucose transport.

Kolber and Stein (37) tried another double labelling technique with the galactoside transport system, also inducible by substrate. In this technique, induced cultures of E. coli were labelled with ^{3}H-phenylalanine and non-induced cultures with ^{14}C-phenylalanine. The same type of fractionation method used with the glucose transport system was successful in showing 3 proteins with high $^{3}H/^{14}C$ ratios. Two of these proteins were galactosidase and transacetylase; the other was presumably a soluble protein associated with galactoside transport, although more definitive work by Fox and Kennedy (22) has cast some doubt on the significance of this protein. Fox and Kennedy developed a labelling approach using N-ethylmaleimide (NEM), a covalent sulfhydryl reagent. With thiodigalactoside, a galactose analogue, they protected a specific binding protein against inactivation by NEM. Using induced and non-induced cultures of E. coli, they were able to show that induced cultures had a binding protein. They were able to block 23% of the reactive sulfhydryl groups in the membrane fraction from NEM labelling by adding thiodigalactoside to the NEM solution. The binding protein, called M protein, was extracted with detergent from membrane fractions and therefore appeared to be a lipoprotein.

ATPase is another well-studied membrane protein. NEM completely inhibited this enzyme (86). ATP can partially protect against NEM inactivation, and against ^{14}C-NEM labelling of red blood cell ghosts (86). However, ATP blocked many sulfhydryl groups not related to

ATPase activity. The author suggested that ATP could have induced conformational changes in the membrane and therefore was affecting nonspecific sulfhydryl groups. Membrane ATPase is probably a lipoprotein, since attempts to solubilize it have not been successful (47).

Sulfhydryl groups are important for other membrane functions. Glucagon and epinephrine binding to the rat liver plasma membrane is inhibited by parachloromercuribenzoate (PCMB) and 5',5' dithiobisnitrobenzoic acid (DTNB) (85). These studies demonstrated that a hormone binding protein was different from adenyl cyclase, which mediates the hormonal action by producing cyclic 3',5' adenosine monophosphate. Similarly, the acetylcholine receptor was shown to involve a distinct acetylcholine receptor protein in the membrane of the eel electroplax (13). Experiments with equilibrium dialysis demonstrate that the receptor protein is blocked by PCMB (51). Reversible disulfide linkages are involved in the regulation of both the enzyme and the receptor protein (49).

The evidence is strong that sulfhydryl groups are important in membrane function. However, Benson (6) pointed out that many membrane types have very low content of sulfhydryl groups (e.g., the red blood cell membrane has 1.4% cysteine). This implies the near absence of disulfide cross-linkages in membrane protein and a freedom of configurational alteration.

The environment of the sulfhydryl group usually determines what reagent will react with it (2,12). For example, iodoacetate and arsenite do not inhibit the glucose transport system, whereas NEM, dinitrofluorobenzene (DNFB), and several mercurials are effective inhibitors (15). NEM (9 mM) gave maximal inhibition of glucose transport

in red blood cells after 30 min of exposure. The red blood cell membrane was not penetrated to any extent by three mercurials: PCMB, parachloromercuriphenylsulfonate (PCMPS), and chlormerodrin. This made it possible to locate the glucose transport system on the outer surface of the membrane (87). The same approach was used by Pardee and Watanabe (55) to locate the sulfate binding protein on the membrane of Salmonella typhimurium; in this case, diazonium salts were used to react with histidine and tyrosine groups in protein.

Two inherently different types of membrane proteins, soluble and insoluble, have been described. Insoluble proteins are firmly bound to lipid and their isolation requires organic solvents and detergents. Soluble membrane proteins have been found mostly in bacteria. They are released by an osmotic shock technique described by Heppel (29).

Purification of membranes by density gradient centrifugation is done routinely for animal and bacteria cells, but apparently difficulties with plant cells have been encountered. It is evident that biochemical markers will be necessary for plant cell membrane work.

MATERIALS AND METHODS

Plants and Fungus Cultures: Oat cvs. Park, Rodney and Clinton were used in most experiments. Park oat has the dominant Vb allele for susceptibility to H. victoriae and to its toxin; Rodney and Clinton are resistant. Seedlings were grown in the laboratory at 22 C in vermiculite plus White's nutrient solution (95). Light (100-200 ft. candles) was furnished by Gro-lux fluorescent tubes (Sylvania). Unless otherwise stated, the first true leaf above the primary leaf was used as a source of leaf tissue for experiments.

For toxin production, highly virulent strains of H. victoriae were grown in one liter Roux bottles, each containing 200 ml modified Fries no. 3 basal medium. Liquid medium was seeded with small pieces of mycelium from potato dextrose agar slant cultures. The still cultures were incubated for 3 weeks at 22 C.

Preparation of Toxin and Toxin Breakdown Products (TBP): Toxin was isolated from culture filtrates by the method of Pringle and Braun (56). Filtrates were concentrated in vacuo, equal parts of methanol were added, and the precipitate was discarded. After methanol was removed by evaporation in vacuo, the filtrate was extracted 3 times with n-butanol. The butanol extracts were combined and concentrated in vacuo, and an equal volume of methanol was added to the concentrate. This solution was then passed through an alumina column.

Methanol and aqueous methanol, respectively, were run through the column. The toxin, which was adsorbed to the alumina, was eluted with 1% acetic acid. Unless otherwise mentioned, the eluate from alumina was used for experiments. This preparation completely inhibited root growth of susceptible plants at 0.001 μg/ml. For some experiments, toxin was further purified by gel filtration (39) using Biogel P-2 (200-400 mesh) or Sephadex G-10 or G-15. Unless otherwise mentioned, columns were 1.5 cm x 25 cm. The toxin at this stage of purity could not be stored for very long without gradual loss of activity.

Toxin was broken down by the following method. The active fractions from gel filtration were pooled, and NaOH was added to bring the pH to 11.5. The solution was held at room temperature for one week, when the solution was readjusted to pH 7.0 to 8.0 with HCl. The preparation was evaporated to dryness *in vacuo*, and repeatedly dried with acetone to yield a white friable powder. This preparation was called toxin breakdown product (TBP). A small amount of residual activity remained, but this was reduced still further after storage at room temperature. Descending chromatography of TBP on Whatman no. 1 paper using propanol : acetic acid : water (PAW, 200 : 3 : 100 v/v) resulted in a major ninhydrin positive spot at R_f 0.65 - 0.70. This was the R_f reported for peptide in breakdown products of toxin (57). A ninhydrin positive spot at R_f 0.80 - 0.90 was seen occasionally; this was presumed to be victoxinine or a rearrangement thereof. The latter interpretation was favored since the spot was not reactive with iodoplatinate reagent (59), whereas authentic victoxinine gave an R_f of 0.84 - 0.89 and was strongly reactive with iodoplatinate. On

silica gel thin layer plates (Brinkman MN silica gel N-HR/UV_{254}), using the same solvent system, authentic victoxinine gave an R_f of 0.70 - 0.75, whereas TBP preparations gave a ninhydrin positive spot of R_f 0.65 - 0.70. A much better separation was obtained when butanol : acetic acid : water (BAW, 8 : 2 : 2 v/v) was used. In this system, authentic victoxinine had an R_f of 0.67 - 0.72, whereas the TBP preparation gave a major ninhydrin positive spot with R_f of 0.52 - 0.56. Victoxinine was not detected in TBP with the iodoplatinate reagent.

Toxin Assays: A seedling root growth bioassay (73) for toxin was used. Hulled oat seeds were germinated for 24 hr between moist filter paper. Serial dilutions of toxin in White's solution or in water were prepared, and 5 ml of each dilution was placed in each of two 60 x 15 mm Petri dishes. Five seedlings were placed in each dish, and root growth was measured after 48 to 72 hr incubation time. The highest dilution which restricted root growth to 1 cm or less was considered the dilution end-point. Roots of control plants were approximately 6 cm long.

A toxin conductance assay was developed, modified from methods of Wheeler and Black (91). Toxin-treated or control tissue samples (0.5g - 1.0g) were enclosed in cheesecloth, rinsed thoroughly in distilled water, and suspended in 50 ml toxin solution in 125 ml flasks which were placed on a reciprocal shaker (70 - 100 strokes/min). After toxin treatment, tissue samples were washed 4 to 5 times in 100 ml of distilled water over a 10 min period, then suspended in 50 ml distilled water (conductance approximately 1 μmho) and shaken

(70 - 100 strokes/min) for up to 6 hr. Conductivity of the ambient solution was measured at intervals with a model RC 16B1 Industrial Instruments conductivity bridge, using a dip type electrode with a constant of 1.0 for solutions below 200,000 ohms and a constant of 0.1 for solutions above 200,000 ohms. Specific conductivity was expressed as reciprocal ohms by the following equation:

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

(Ls = specific conductance, Kc = cell constant, and Rm = resistance at 22 C). Oat leaf tissue, from plants 5 to 15 days old, was used. In all experiments, conductance values obtained from water control samples were subtracted from the values of toxin-treated samples to give toxin-induced electrolyte efflux. In experiments with inhibition of toxin action being estimated, the term "percent protection" was used to show the reduction in toxin-induced electrolyte loss. The following equation was used to calculate percent protection:

$$\% \text{ protection} = 1 - \frac{(\mu\text{mhos I-T}) - (\mu\text{mhos I})}{(\mu\text{mhos T}) - (\mu\text{mhos W})} \times 100$$

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

Measurement of Sodium and Potassium Contents in Extracts and Leachates from Tissues: HCl was added to leachates from toxin-treated and control tissues to bring HCl concentration to 0.2N. All solutions were held at 3 C prior to analysis. To obtain tissue extracts, 100 mg coleoptile tissue was extracted in boiling water for 10 to 15 min and the final volume was adjusted to 10 ml. This solution was filtered through Whatman ash free filter paper. A Jarrel-Ash "Dial

Atom" Atomic absorption photometer or a Coleman flame photometer was used to measure the ion content of both leachates and tissue extracts. Standard solutions of sodium and potassium were used to obtain estimates of ion concentration.

Electropotentials of Single Plant Cells: These experiments were conducted in the laboratory of Dr. Noe Higinbotham of the Department of Botany, Washington State University. Methods of measuring electropotentials in coleoptile cells of oat, corn and sorghum were essentially those used by Etherton and Higinbotham (19). The changes in potential between the cytoplasm and bathing solutions were measured with microelectrodes prepared with an automatic electrode puller. Cell microelectrodes were filled with electrolyte by boiling in 3M KCl for 15 min; tip diameters were approximately 0.5μ. Reference electrodes were filled with 3M KCl in agar; tip diameters were approximately 10 - 20μ. The electrodes were connected to a measuring circuit with Ag-AgCl wires. The measuring circuit consisted of a Keithly electrometer with preamplifier connected to a Heath variable time recorder.

Coleoptile sections were cut into 1 cm sections 3 to 6 hr before use and floated on a 1X nutrient solution (a salt solution 1mM with respect to sodium, potassium, and calcium) (30) before they were mounted in a perfusion chamber. The perfusion chamber was continually flushed with the 1X nutrient solution. Coleoptile sections were mounted so that a cut surface could be observed microscopically. The cell microelectrode was then inserted into a cell and the reference microelectrode was placed in the bathing solution. This made it

possible to determine the resting potential across the plasma membrane.

Isolation of Nuclei and Determination of Protein Synthesis: Nuclei were isolated using standard methods developed for plants (7,20). Oat seedlings were grown in the dark for 5 to 6 days, then leaf tissue was cut into 2 - 4 cm sections, washed with distilled water and floated on White's solution at 3 C for 0.5 to 1 hr. The tissue was chopped rather than ground to eliminate shear forces which injure nuclei. The leaf sections were placed in a 250 ml beaker with a polyethylene-covered bottom. Two ml/g tissue of the following buffer was used: sucrose, 0.5M; $MgCl_2$, 5mM; $CaCl_2$, 5mM; EDTA, 5mM; BSA, 0.2%; mercaptoethanol, 10 mM. Tissue was chopped to a fine mince with single edge razor blades held firmly with long-nose pliers. Total chopping time was no longer than 10 min. The mince was filtered through 4 layers of cheesecloth followed by filtration through one layer Miracloth. The filtrate was centrifuged at 350g for 10 min and the pellet was resuspended in approximately 1 ml chopping buffer. The suspension was layered on a 1.2 M sucrose solution buffered with Tris-HCl (0.05M, pH 7.4). After centrifugation at 450g for 15 min, the pellet was resuspended in 1 ml chopping buffer and once more centrifuged through a 1.2 M sucrose solution. The final pellet was resuspended in a buffer containing Tris-HCl (0.08M, pH 7.4), sucrose (0.6M) and chloramphenicol (300 μg/ml). Many nuclei were evident by microscopic examination.

Toxin in 0.05M Tris-HCl (pH 7.4) or Tris buffer alone was added to aliquots of the nuclear suspension and held on ice 10 to 15 min

before addition of other materials. At 0 time, after the tubes had equilibrated for 5 min at 32 C in a water bath, radioactive substrate was added. This was either lysine ^{14}C (uniformly labelled, 269 mc/mM) or leucine ^{14}C (carboxyl labelled, 55.3 mc/mM). The final activity was from 0.5 to 1.0 μc/ml. Further additions were required for experiments with an ATP regenerating system as follows: Tris-ATP, 0.1mM; phosphocreatine (Na salt), 2mM; and creatine kinase, 100 μg/ml. Total volume of the reaction mixture was 0.9 ml. Duplicate tubes were incubated at 32 C, and the reaction was terminated by the addition of 40% TCA (1 ml). The 40% TCA step was followed by the addition of 5 ml cold 20% TCA containing, in some cases, approximately 10 mg/ml D,L lysine-HCl. Samples were chilled, centrifuged at 10,000g for 30 min, and pellets were redissolved in 0.2N HCl. The tubes were incubated at 90 C for 30 min to degrade amino acyl RNA. The tubes were then cooled, and an equal volume of 40% TCA was added.

After centrifugation, the pellets were washed once in 20% TCA followed by two washings in ethanol:ether (1;1). Precipitates were redispersed in 20% TCA, filtered on Millipore filters (type PHWP, 0.3μ, 47mm) and washed with 50 ml cold 10% TCA. The filter pad was dried with 20 ml ethyl ether and placed in scintillation vials at 50 C for several hours. Scintillation solution (10 ml, containing 4g PPO and 0.1g POPOP per liter of toluene) was added to each vial and vials were counted in a Packard Tri-Carb scintillation counter with approximately 40% efficiency. In each experiment a sample of the nuclear suspension was frozen and later assayed for protein by the modified Folin-Ciocalteau method (42) using BSA as the standard. The calculation of the number of μMoles amino acid incorporated into

protein was as follows:

cpm incorporated/eff. x $(dpm/\mu c)^{-1}$ x $S.A.^{-1}$ x mg $protein^{-1}$ = μmoles incorporated per mg protein

(eff. = counter efficiency; dpm/μc = 2.2×10^6; S.A. = specific activity in μc/μM)

Preparation and Labelling of (Presumed) Membrane Fragments: Oat seedlings were grown in the dark for 5 to 6 days and the leaves were cut, sectioned into pieces 2-3 cm long, rinsed with distilled water, and placed in White's solution at 3 C for 30 min. Tissue was then placed in a buffer solution in a 250 ml beaker with a polyethylene-covered bottom. Approximately 2 ml buffer was used per g tissue. The buffer contained sucrose, 0.5M; Tris-HCl, 0.05M (pH 7.4); KCl, 10mM; $CaCl_2$, 2mM; $MgCl_2$, 2mM; EDTA·Na_2, 2mM; BSA, 0.1%; and beta-mercapto-ethanol, 20mM. Tissue was chopped rapidly with single edge razor blades held with long-nose pliers, and the brei was filtered through 2 layers of cheesecloth and 1 layer Miracloth. The filtrate was centrifuged for 10 min at 12,000g and the pellet discarded. The supernatant was centrifuged for 1 hr at 50,000g, or (in some cases) for 40 min at 30,000g. The two methods gave comparable results. The pellets were resuspended in 1M sucrose buffered with 0.1M Tris-HCl, pH 7.2; or in 0.1M K-phosphate buffer, pH 6.7. This preparation was clarified at 2000g for 5 min. The supernatant was called the particulate fraction and is believed to contain plasma membrane fragments (41).

The particulate fraction was incubated with NEM-^{14}C, with and without toxin or its breakdown products (TBP). A 0.3 ml aliquot of the particulate preparation was mixed with 0.3 ml buffer (Tris-HCl,

pH 7.4 or K-phosphate, pH 6.7; 0.05M), TBP (final conc. of TBP, 500 μg/ml) in buffer, or toxin (final conc. 16 μg/ml) in buffer. NEM-^{14}C (New England Nuclear Corp., 2.9 mc/mM) was diluted with NEM to give a final concentration of 0.1 - 0.03mM (activity 0.1 - 0.5 μc/ml) in the reaction mixture. After 10 min, 0.3 ml of the NEM solution was added. After incubation for 15 min, 1 ml 200mM NEM was added, and the reaction was terminated by adding an equal volume of 40% TCA. Triplicate tubes were used for each sample. The solution was diluted by adding 5 ml 10% TCA containing excess NEM, and the mixtures were centrifuged at 10,000g for 30 min. The pellet was washed again with 20% TCA, followed by 1 or 2 washes each with ethanol and ethanol:ether (1:1 v/v). The pellets were resuspended in 10% TCA, filtered on Millipore filters (pore size 0.3μ or 0.45μ) and washed with 60 ml 10% TCA. The filter pads were washed with 20 ml ethyl ether, placed in scintillation vials, dried for several hr at 50 C, and then 10 ml scintillation solution (toluene, 1 liter; PPO, 4g; POPOP, 100mg) was added. Preparations were counted with a Packard Tri-Carb scintillation counter with approximately 40% efficiency; samples were counted long enough to be within 1% reliability. In many experiments, control tubes terminated at 0 time were used to determine adsorption of radioactivity; these values, plus the background counts, were subtracted from the other sample counts. In a few experiments, protein in the particulate fraction was estimated by the modified Folin-Ciocalteau reaction (42).

All experiments described were repeated one or more times with essentially the same results.

RESULTS

Effects of Toxin on Ion Fluxes and Electropotentials of Cells:

Toxin-induced electrolyte efflux from cells: HV-toxin-treated tissues lose more organic and inorganic substances than do untreated control tissues (8). Within 2 min after toxin exposure, an increase in loss of total electrolytes over the control can be detected (75). Electrolyte efflux has been suggested as a possible toxin assay system (91), but there are not enough data to evaluate such an assay. Therefore, the relationship between toxin concentration and electrolyte efflux was examined and characteristics of toxin-induced efflux were determined.

When toxin concentrations were plotted against conductance of leaching solutions (in μmho), hyperbolic curves resulted (Figure 1, top). These resemble a plot of substrate concentration versus enzyme activity. Plots based on log toxin concentration were also similar to enzyme plots (Figure 1, top). This relationship suggested that data could be plotted reciprocally; therefore, a modified Lineweaver-Burk (Wolff) plot is shown, which yields a straight line and an estimation of the Km, or an estimation of the affinity of the toxin for its receptor (Figure 1, bottom). Km values were approximately 1 μM, assuming a molecular weight for toxin of about 1000. This use of reciprocal plots was helpful for checking on the competitive or noncompetitive nature of a toxin inhibitor, as will be examined in a

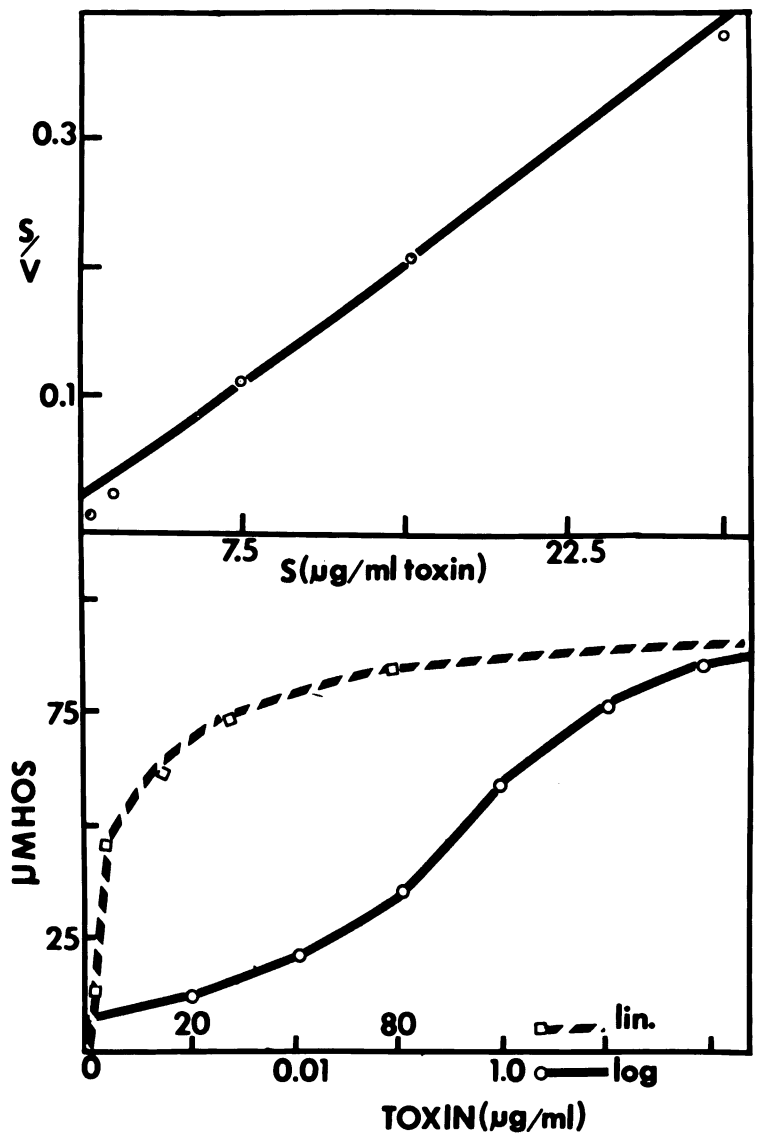

Fig. 1. The effect of HV-toxin concentration on efflux of electrolytes from leaf tissue. Leaf samples (900 mg) from susceptible oat plants (6-10 days old) were sectioned into 1 cm long pieces and enclosed in cheesecloth. Samples were suspended in a toxin solution (0.001-100 µg/ml), rinsed, and placed in 50 ml water for leaching. Toxin-induced electrolyte efflux was plotted against log and linear toxin concentrations (below, B). The same data were used in a Wolff Plot of S (toxin concentration) versus S/V (µmhos) (above, A).

later section. The Hill equation (76) has been used for enzymes which give sigmoidal rather than hyperbolic substrate versus activity curves. A value (n) is obtained which is often called an "interaction coefficient," which is a measure of the number of different sites on an enzyme interacting with substrate. A Hill plot for the toxin data from very low to high concentrations of toxin gave a value of less than one, suggesting that one kind of site is interacting with toxin (Figure 2). This approach, of course, assumes an analogy between toxin and substrate, and between toxin receptor and enzyme. The suggestion that one type of receptor site is interacting with toxin is consistent with genetic data on toxin susceptibility, since one gene determines susceptibility.

Leachates from toxin-treated and control tissue were analyzed by flame photometry. Most of the electrolytes lost on initial exposure to toxin was potassium (Figure 3). This is reasonable since potassium is known to exist in very high concentrations in plant cells (30). Analyses also show that sodium efflux was not increased at the time of rapid potassium efflux. These data agree with results reported by Black and Wheeler (8). The lack of sodium efflux appears to be due to lack of sodium available for leaking out of the tissue, because destruction of the membrane with toluene and methanol gave losses of potassium but not sodium. These results, plus previous observations (8) suggest that the permeability changes induced by toxin are non-specific and are not limited to specific transport systems.

The temperature coefficient (Q_{10}) of toxin-induced electrolyte efflux (efflux beyond that of efflux from control tissue) was of interest in considering a possible role of metabolism in toxin action.

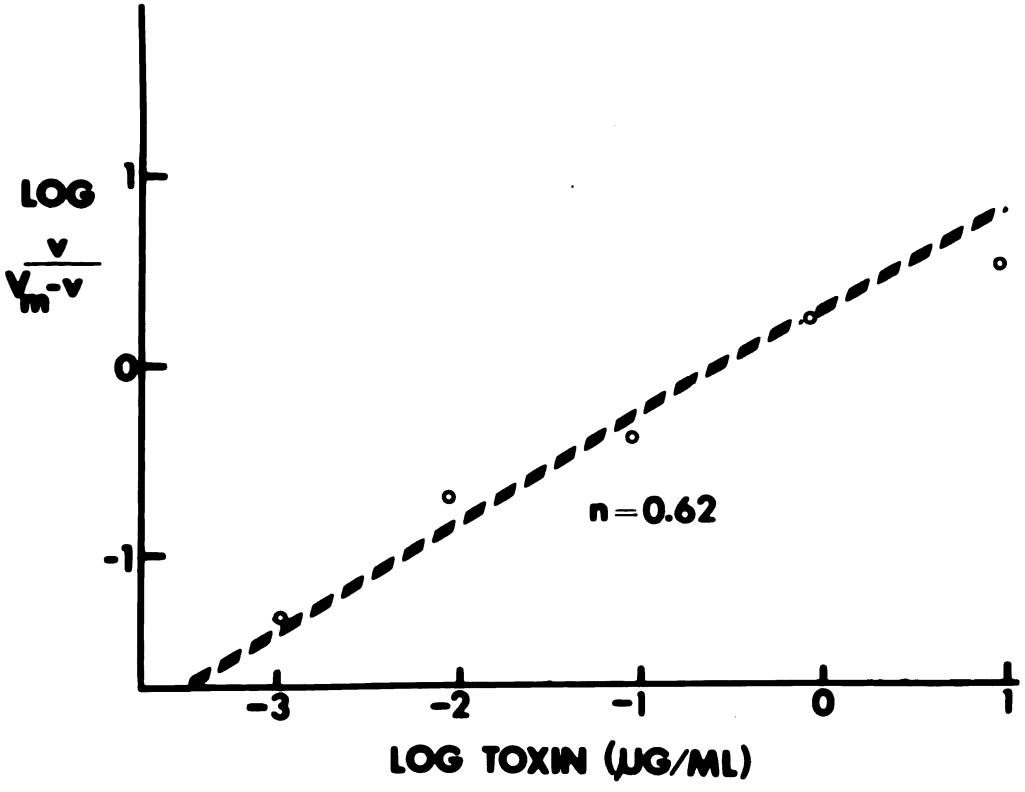

Fig. 2. Hill plot of HV-toxin-induced efflux of electrolytes. Data are from Fig. 2. v = µmhos toxin-induced efflux of electrolytes; Vm = maximum µmhos toxin-induced efflux of electrolytes at saturating toxin concentrations; n = slope (y/x), which indicates the interaction coefficient. The slope indicates the number of types of sites interacting with toxin.

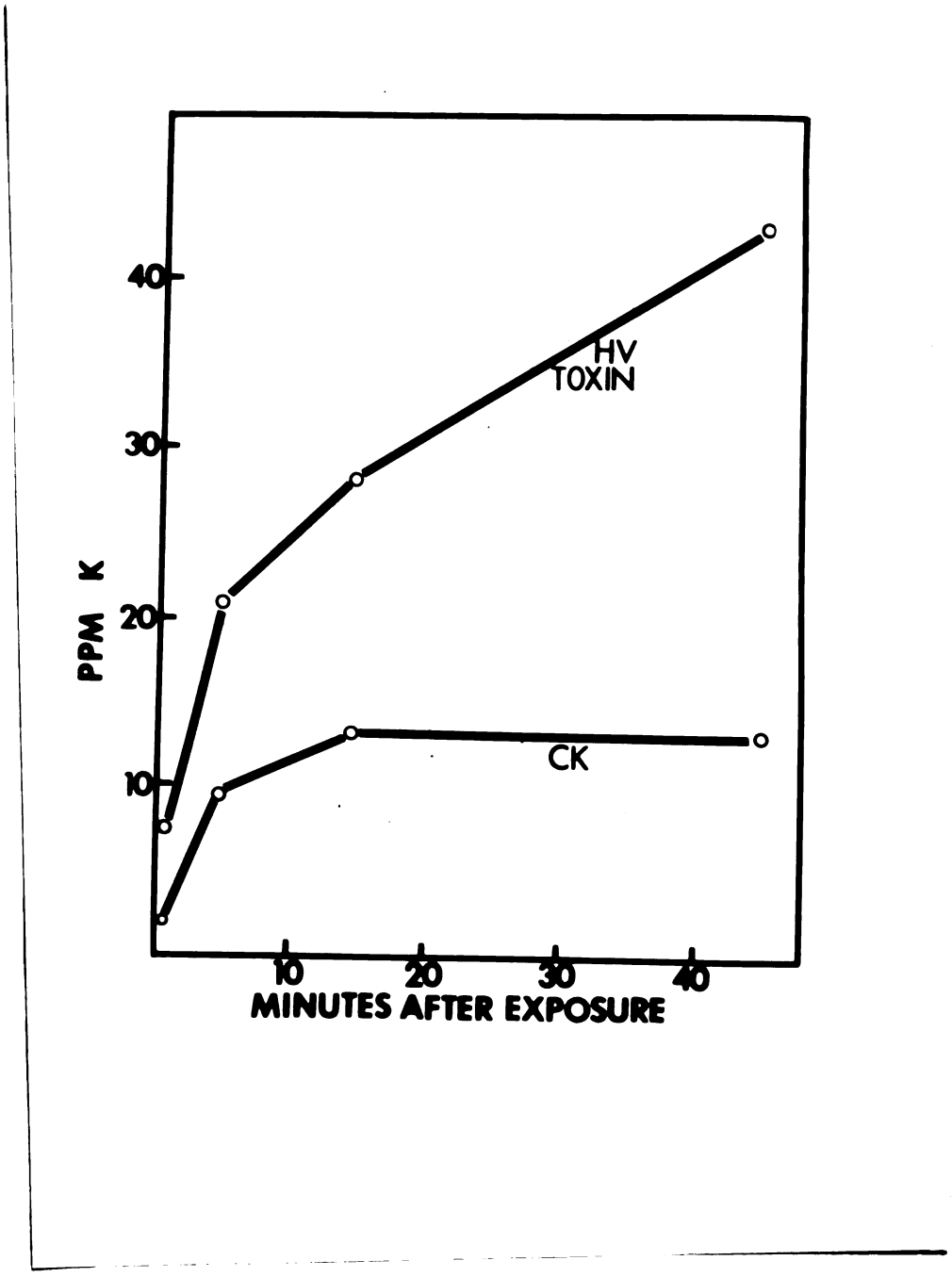

Fig. 3. Initial effect of HV-toxin on potassium efflux from oat coleoptile tissue. Coleoptile tissue from 5 day old plants (2.0g/ sample) was enclosed in cheesecloth and incubated for 2 hr in White's solution. Tissue samples were then rinsed and shaken in 100 ml toxin (0.5 μg/ml) or distilled water. Samples were removed, concentrated to 1/3 volume, and potassium was measured with a flame photometer.

This aspect had been examined by Wheeler and Black (91), who reported that toxin-induced electrolyte efflux had a Q_{10} of 1.2. Simple diffusion processes usually have Q_{10} values near one. In my Q_{10} experiments, all tissue samples were exposed to toxin at 22 C, and leached at temperatures ranging from 5 to 22 C. These conditions always gave Q_{10} values that were always higher than 2 (Table 1). High Q_{10} values, however, do not necessarily indicate a metabolic involvement in toxin action. When the temperature was varied during the toxin uptake period rather than during the leaching period, little or no effect of temperature on toxin-induced electrolyte efflux was noted. This suggests that the effect of temperature is on membrane structure, with membrane damage reduced at the lower temperature. Many effects of temperature on plant cell membranes are known (17), but previously reported results may have little bearing on my data. When leaf sections were floated on toxin solutions, symptoms appeared in 16 hr at 22 C, but 4 days were required at 5 C. The delay in appearance of symptoms may be caused by effects of temperature on toxin-induced electrolyte efflux.

Effect of toxin on electropotentials in single cells of oat coleoptiles: It is logical that electrolyte efflux should cause a charge imbalance across the plasma membrane. One possible way to determine whether or not the initial effect of toxin is on the plasma membrane is to demonstrate whether or not there is an immediate effect on this structure. Data on electrolyte losses indicated an effect of toxin within 2 min (75). Therefore, experiments with single cell electropotentials were designed to detect the earliest possible effect on

Table 1. Temperature Coefficients (Q_{10}) of HV-Toxin-Induced Electrolyte Efflux.

Leaf tissue was treated with toxin solution (16 µg/ml) for 2 hr, rinsed, and then leached in 50 ml distilled water for 6 hr. Only the temperature of the leaching solution was varied.

Temperature[1/] (C)	Conductivity[2/] (µmhos)	Q_{10}
5	19	2.6
22	114	
12	39	2.9
22	114	

1/ Temperature during the leaching period.

2/ This is the electrolyte loss, induced by toxin treatment. It is in addition to the value obtained in control tissue. Controls ranged from 3.5 to 7.2 µmhos.

the plasma membrane. The results were not entirely conclusive or striking. In some of the experiments toxin had a fairly rapid but mild effect on the electropotential. In general, it was clear that the drastic effects of toxin on electrolyte efflux were not paralleled by similar changes in electropotential. However, these results are of interest in regard to postulated electrogenic ion pumps associated with the membrane and their role in the generation of the electropotential (19,31).

Coleoptile sections 1 to 2 cm long (from 4 to 5 day old plants) were floated on a 1X nutrient solution for 3 to 6 hr. A 1X solution is a solution containing 1 mM amounts of potassium, sodium, and calcium; a 0.01X solution is a 1 to 100 dilution of the standard solution. The tissue was then mounted in a plexiglas perfusion chamber where it could be observed microscopically. Nutrient solution or toxin dissolved in nutrient solution was flushed through the chamber at a constant rate. Penetration of the cell with the electrode was considered successful if the potential stabilized at one value for 5 min. Toxin or test solution was then added and changes in potential were recorded, as described in Materials and Methods.

Experimental results using the standard 1X nutrient solution showed that toxin, even at high concentrations (16 μg/ml) had no consistent effect on the potential of susceptible cells within 5 min after exposure to toxin. Although small changes were observed initially in some cases, the more typical results indicated a slow drop in potential after 5 min (Figure 4). When a 0.01X nutrient solution was used, the potential of susceptible cells responded to toxin within one min. The potential dropped about 30 mv for several min, then recovered to

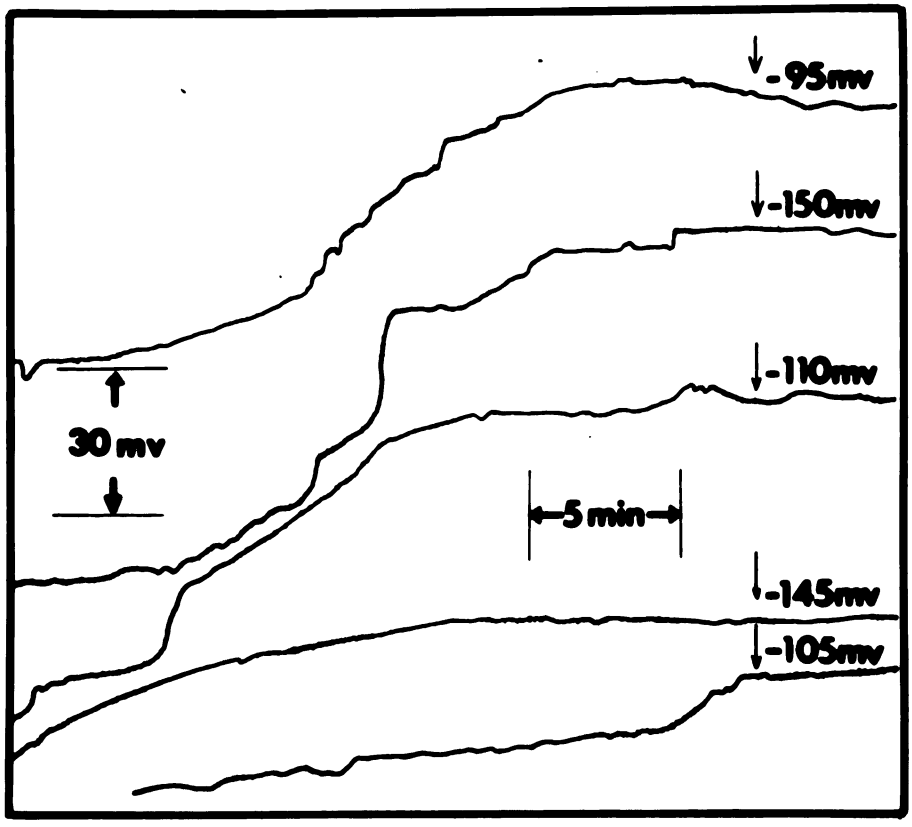

Fig. 4. The effects of HV-toxin (16 μg/ml) on membrane electropotentials in 5 single cells of susceptible oat coleoptiles. Potentials were recorded from right to left. After potentials had stabilized for 5 min, toxin was added at the times indicated by the arrows (right). Bathing solution was the standard 1X solution with or without toxin. Solutions without toxin gave a relatively straight line.

near the original value (Figure 5). There was no change in the potential of resistant cells after toxin treatment.

Carbonyl cyanide m-chlorophenylhydrazone (m-ClCCP), an uncoupler, had striking and consistent effects on the electropotential of oat coleoptile cells. Within 3 min of exposure, the potentials dropped precipitously from above -100 mv to about -50 mv, where the potentials levelled off (Figure 6). These results indicate that the membrane potential is dependent on metabolic energy. The 50 mv which were not affected by m-ClCCP may be related to a "diffusion potential" or a "Donnan potential." Toxin should theoretically abolish the diffusion potential if the potential is caused by free ions in the cell. Since toxin plus CCP did not reduce the potential much below the values given by CCP alone, a Donnan potential seems probable.

The host-specific toxins from Periconia circinata (PC) and Helminthosporium carbonum (HC) were also examined for the possibility of rapid effects on potentials of susceptible oat and corn cells. The results with PC-toxin showed no initial effects on potentials. HC-toxin, also thought to be a membrane active toxin, gave more interesting results. Five μg/ml HC-toxin caused an increase in the potential (10-40 mv) of corn cells within a min or two after toxin application (Figure 7). The effect on resistant plants was not tested. These results suggest a membrane effect, which seems to be in accord with previous data showing that HC-toxin can stimulate growth and uptake of amino acids and nitrate (39,40,97,98).

The results with HV-toxin indicate that some other system is affected before the system supporting the membrane potential is affected. Electrogenic pumps, however, may not be generated in the

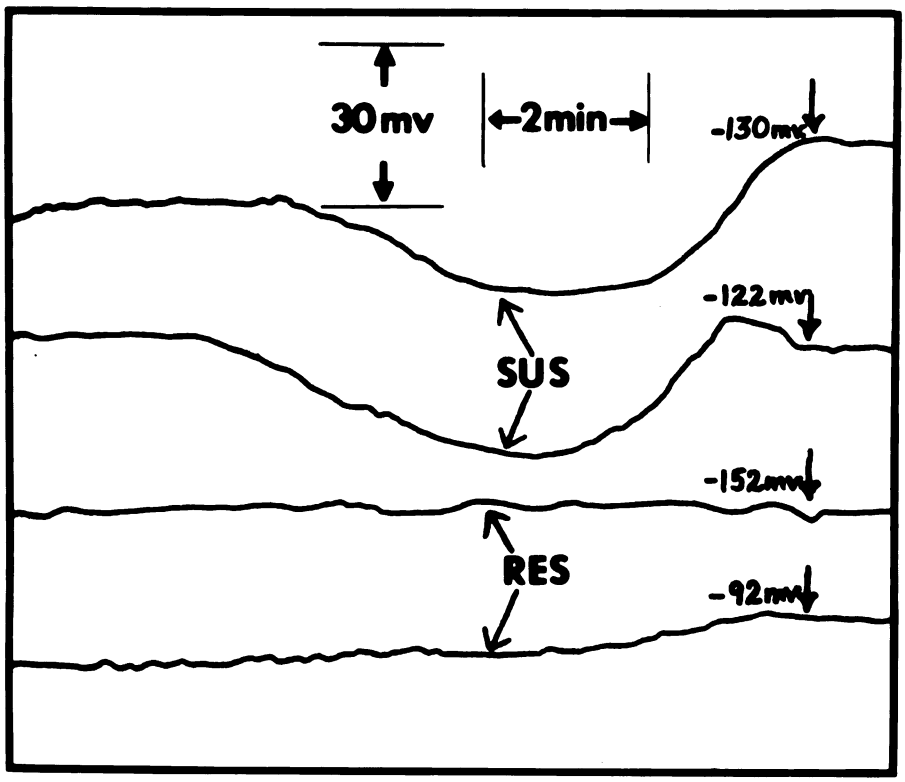

Fig. 5. The effect of HV-toxin (16 μg/ml) on membrane electropotentials in 4 single cells of susceptible or resistant oat coleoptiles. Potentials were recorded from right to left. After potentials had stabilized for 5 min, toxin was added at the times indicated by arrows (right). Bathing solution was 0.01X solution with or without toxin. Solutions without toxin gave a relatively straight line.

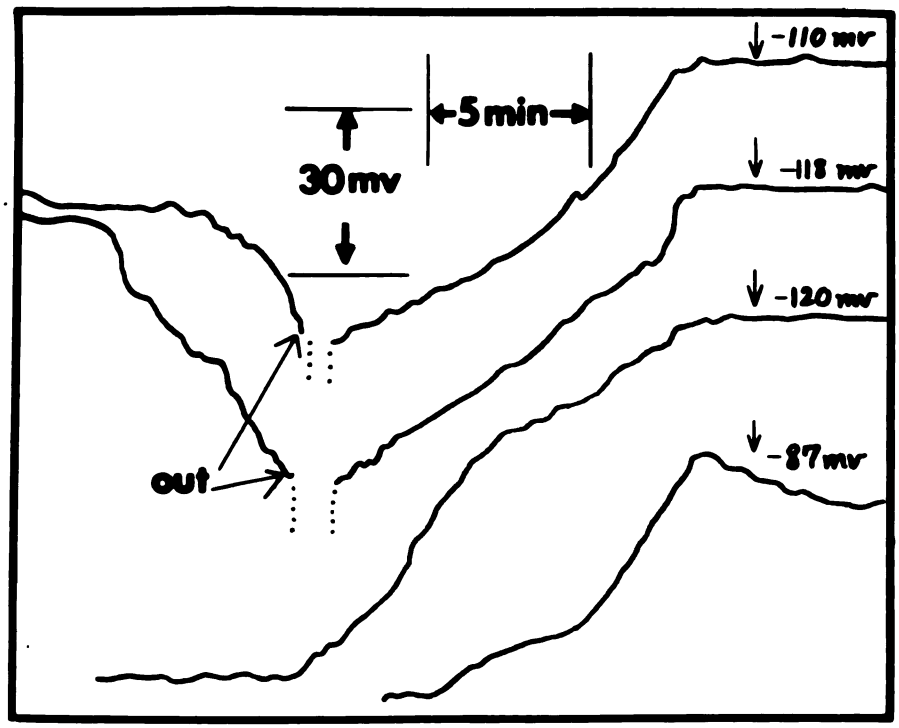

Fig. 6. Effect of m-ClCCP on membrane electropotentials in 4 single cells of oat coleoptiles. Potentials were recorded from right to left. After potentials had stabilized for 5 min, CCP (20 μM) was added at the times indicated by the arrows (right). CCP was removed at the times indicated by the dotted lines. A neighboring cell was penetrated to show recovery of potential. Solutions without CCP gave a relatively straight line. Nutrient solution (1X) was used in all cases.

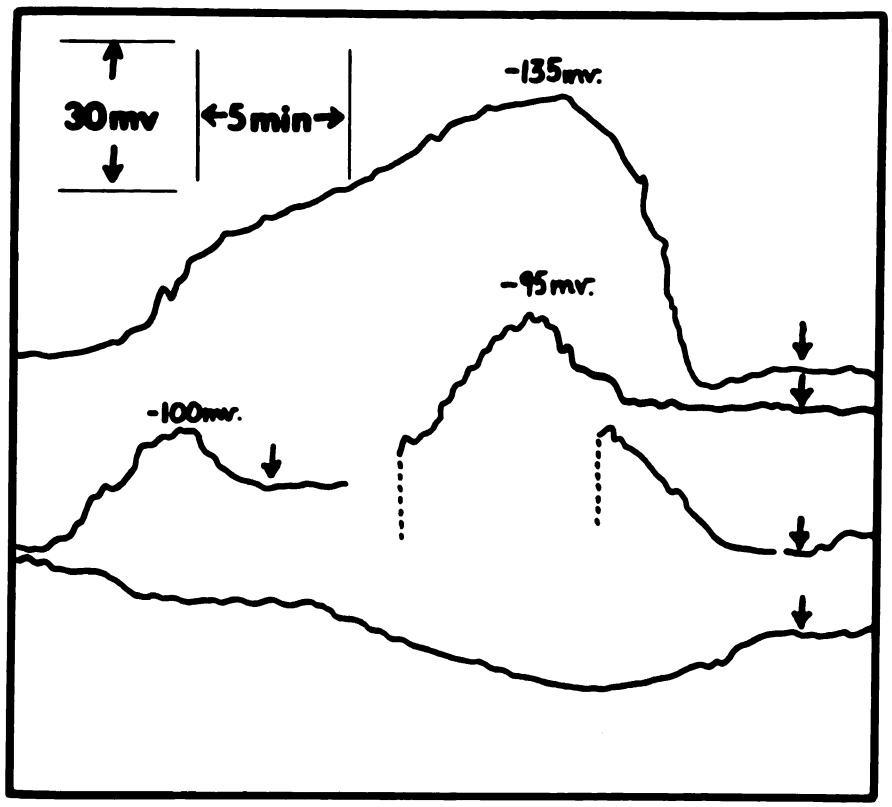

Fig. 7. Effect of HC-toxin (5 μg/ml) on membrane electropotentials in 5 single cells of susceptible corn coleoptiles. Potentials were recorded from right to left. After potentials had stabilized for 5 min, toxin was added at the times indicated by arrows (right). Bathing solution was the standard 1X solution with or without toxin. Controls without toxin gave a relatively straight line. Dotted line indicates that the seal with the electrode was broken.

membrane but could originate in the cytoplasm. The leakage of potassium, which would be expected to raise the potential, may be neutralized by flow of counter-ions. Potassium depletion from coleoptile sections was estimated by measuring the tissue content after treatment with toxin or control solutions (Table 2). These coleoptile sections were comparable to those used for electropotential measurements. Toxin-induced potassium depletion from coleoptile cells could only account for a fraction of the toxin-induced drop in potential, based on predictions of the Nernst equation and assuming a potassium diffusion potential. Therefore, indirect or secondary effects on electrogenic potential probably occur. A recent discussion on electrogenic pumps in higher plants cells by Higinbotham et al (31) confirms observations made here with oat coleoptiles.

Counteracting Effects of Various Substances on Toxin Action:

Carbonyl reagents: Previous data show that carbonyl reagents can counteract or delay the effects of toxin on susceptible tissue, as determined by an electrolyte leakage assay. There appears to be no reaction between toxin and these reagents.

First, I attempted to determine whether or not semicarbazide was competitive with toxin for receptor sites. Semicarbazide (1 mM in 10 mM acetate buffer, pH 4.5) was mixed with a wide range of toxin concentrations. Results confirmed the previous report, and show that semicarbazide protects susceptible tissue against HV-toxin. A reciprocal plot of data indicated that semicarbazide inhibition is noncompetitive (Figure 8). Protection was still evident when toxin concentrations were raised until saturating levels of toxin were reached

Table 2. Effect of HV-Toxin on Potassium and Sodium Contents of Avena Coleoptile Cells.

Coleoptile sections (1 cm long) from susceptible oats were floated on toxin (9 μg/ml) in nutrient solution or in nutrient solution alone. After designated times, samples (100 mg) were removed, rinsed with water, extracted in boiling water, and K and Na in the extracts assayed with an absorption spectrophotometer.

Treatment Time in		Ion content (μeq/g fresh wt)		
1X[1] (hr)	1X+toxin[1] (hr)	K (μeq)	Na (μeq)	Toxin-induced K loss[2] (%)
0	0	55	3.2	
4	0	69	5.5	
3	1	54	7.2	18
7	0	58	8.2	
4	3	29	8.7	50
4	6	10	6.2	83

1/ 1X is the standard nutrient solution (30), 1 mM with respect to K and Na.

2/ According to the Nerst equation, a 50% K loss should cause an electropotential drop of 18 mv, assuming a potential of 116 mv. However, the potential drop with toxin in 3 hr was 70 to 80 mv (see Figure 5).

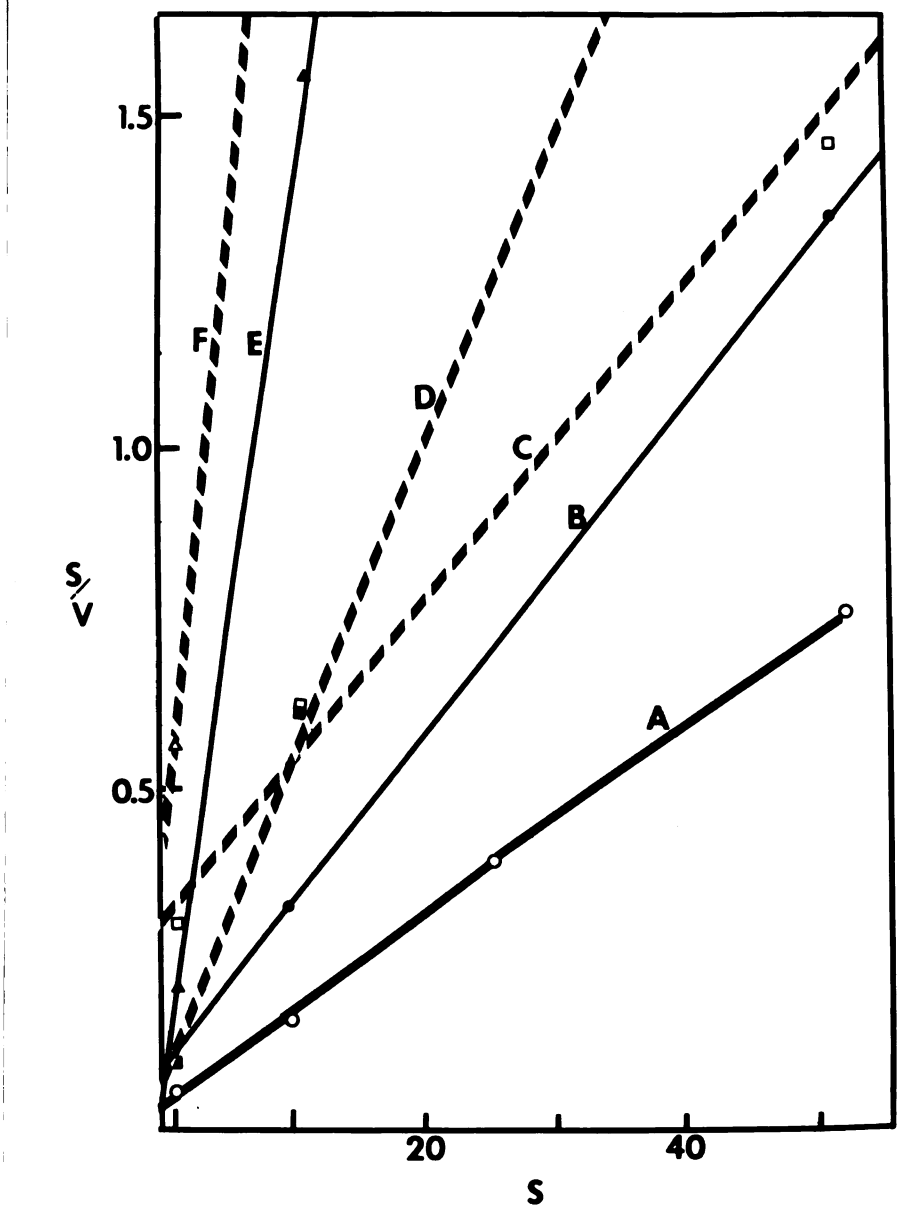

Fig. 8. Wolff plots showing effects of inhibitors on HV-toxin-induced loss of electrolytes. After pretreatment, leaf tissue (1g/sample) from susceptible oats was rinsed, treated with toxin (16 μg/ml) for 3 hr, rinsed again, and suspended in 50 ml distilled water for 3 hr. Conductance of the ambient solution was measured. Treatments: A, control, without inhibitors; B, semicarbazide-HCl (2 mM, pH 4.8) in toxin solution; C, uranyl nitrate (5 mM, pH 4.8) pretreated for 40 min; D, fluorodinitrobenzene (5 mM, pH 5.2) pretreated for 15 min; E, N-ethylmaleimide (5 mM, pH 7.4) pretreated for 15 min; F, cycloheximide (5 μg/ml) pretreated for 10 hr.

(Figure 9).

Simultaneous treatments of toxin and semicarbazine are effective in protection, but pretreatments have previously failed to show the same effect (70). However, some protection was evident in my experiments when high concentrations of bisulfite, semicarbazide, and cyanide (or combinations thereof) were used in 20 to 50 min pretreatments. It is known that carbonyl complexes are dissociable (11).

Mercaptoethanol gave some protection when tissues were pretreated with high concentrations of this sulfhydryl donor. Other experiments showed that mercaptoethanol gave some protection against toxin when the two were applied simultaneously (Table 3). Protection in pretreatments apparently depends on long periods of exposure to mercaptoethanol. This is consistent with the data on carbonyl reagents, since mercaptoethanol is known to be an effective carbonyl-binding reagent (11).

Uranyl salts: Interest in uranyl salts as membrane reactive compounds came from the work of Rothstein and colleagues (63-66). Hanchey (27) claimed that uranyl nitrate pretreatments for 12 hr gave 50 to 60% protection of oat tissues against toxin-induced electrolyte loss. Samaddar and Scheffer (70), on the other hand, showed that toxin solutions were apparently binding uranyl ion, which could explain the high protection which developed when uranyl salts and toxin were mixed, or when tissues had accumulated uranyl salts for 12 hr periods. An attempt was made to clarify this situation and to examine the significance of the toxin counteraction by uranyl salts.

Oat leaf sections from 6 to 10 day old plants were infiltrated

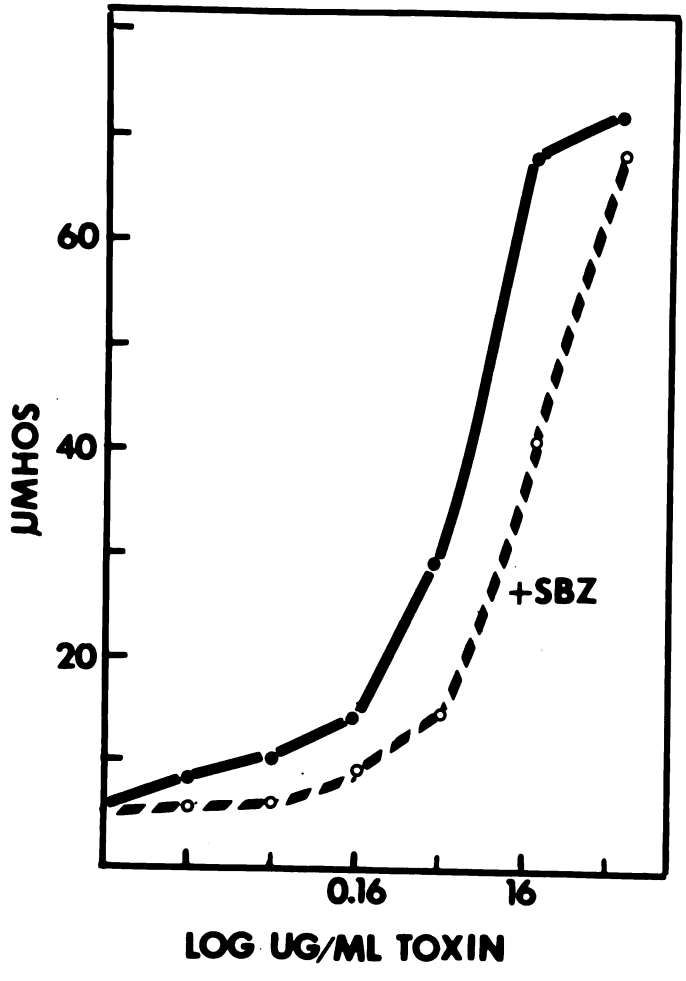

Fig. 9. Effect of semicarbazide-HCl (SBZ) on HV-toxin-induced efflux of electrolytes as a function of toxin concentration. Susceptible oat leaf tissue (1.5g/sample) was treated with toxin (0.0016-160 μg/ml), toxin plus semicarbazide (1 mM, pH 4.5), or with control solutions for 3 hr. Samples were rinsed and leached in 50 ml distilled water for 2 hr.

Table 3. Effect of 2-Mercaptoethanol (ME) Treatments on HV-Toxin-Induced Efflux of Electrolytes.

Susceptible oat leaf tissue (500 mg/sample) was infiltrated for 1 min in test or control solutions and then incubated in the same solutions for indicated times. Tissues were then rinsed, treated with toxin (16 μg/ml) for 1 hr, rinsed again, and leached for 1 hr in 50 ml distilled water. Conductance of the leaching solution was measured. ME concentration, 80 mM.

Expt	Pretreatment	Treatment	Conductance (Δ μmho)[1/]	Protection (%)
1	none	Toxin	30.4	
	none	ME + Toxin	26.2	14
2	Water	Toxin	24.3	
	ME, 60 min	Toxin	18.5	24
	ME, 120 min	Toxin	16.5	32

1/ ME and water controls had about 7 μmhos conductance. This was subtracted to obtain the values given.

with 4 mM uranyl nitrate (pH 3.5) for 5 min, rinsed with 100 ml glass distilled water, treated with excess toxin (32 μg/ml), and assayed for toxin-induced electrolyte efflux. Results (Table 4) show that uranyl nitrate gave striking protection against toxin in a 5 min pretreatment. These results are consistent with the data of Rothstein and Larabee (65) who reported that uranyl ion coats the plasma membrane of yeast cells within one or 2 min at pH 3.5. The uranyl protective effect for toxin was decreased by distilled water, tap water, and phosphate buffer solution (with increasing removal of protection in that order), when these solutions were used to rinse tissue samples before toxin treatment (Table 5). In these experiments, control and uranyl-treated tissue samples were treated with toxin in the same flask, so that inactivation of toxin by uranyl ions dissociating from tissues would not be a factor. It is not clear whether inhibition of toxin-induced electrolyte loss by uranyl pretreatment is competitive or noncompetitive, or mixed competitive-noncompetitive (Figure 8). Possibly higher toxin concentrations are able to wash off some of the uranyl ions coating the cell, thereby reducing protection.

These experiments indicate that uranyl protects against toxin-induced electrolyte efflux. However, is the protection related to the interaction of the toxin with the receptor site? To examine this possibility, uranyl pretreated tissues were exposed and leached in methanol (20%) or in m-ClCCP (100 μM). These substances are known to damage membranes. The results indicated that pretreatment of tissue with uranyl nitrate gave 40 to 60% protection against electrolyte efflux induced by methanol or m-ClCCP (Table 6). This result, along with the observation that uranyl ion can also protect sorghum against

Table 4. Effect of Uranyl Nitrate Pretreatments on HV-Toxin-Induced Efflux of Electrolytes.

Susceptible oat leaf tissue (1.0 g/sample) was treated with uranyl nitrate solutions (4 mM, pH 3.5) or buffer (pH 3.5) for 5 to 30 min, including a 1 min infiltration. Samples were rinsed in glass distilled water and treated with toxin (32 µg/ml) for 30 min. After rinsing with distilled water, samples were leached in 50 ml distilled water for 2 hr. Conductance values are for leaching solutions.

Pretreatment	Treatment	Conductance (µmhos)	Protection (%)
Water	Water	8.3	
Uranyl nitrate (30 min)	Water	5.7	31 1/
Water	Toxin	82.0	
Uranyl nitrate (5 min)	Toxin	18.2	77
Uranyl nitrate (30 min)	Toxin	12.3	85

1/ This value represents protection against normal electrolyte efflux, rather than toxin-induced efflux.

Table 5. Effect of Various Rinse Treatments on Uranyl (UO_2) Nitrate Protection against HV-Toxin.

Leaf tissue samples (1.0g) were infiltrated for 10 min with 4 mM uranyl nitrate (pH 3.5) and then washed 2 times with 100 ml potassium phosphate (0.05 M, pH 6.7), tap water, or distilled water for 10 min. After thorough rinsing, samples were treated with toxin (16 µg/ml) or water for 1 hr and then leached in 50 ml distilled water for 2 hr. Conductance values are for leaching solutions.

Pretreatment	Rinse	Treatment	Conductance[1/] (µmhos)	Protection (%)
Water	Water	Toxin	54.1	
UO_2	Water	Toxin	20.1	63
UO_2	Tap Water	Toxin	28.7	47
UO_2	PO_4	Toxin	40.0	26

1/ Control values were subtracted.

Table 6. Effect of Uranyl Nitrate on Leakage of Electrolytes Induced by mCl-CCP and Methanol.

Leaf tissue samples (1.0g) were infiltrated for 5 min with 4 mM uranyl nitrate (pH 3.5) and then washed thoroughly with glass distilled water. Samples are then suspended in 50 ml mCl-CCP (100 μM), methanol (20%), or water and the conductivity of the solution measured after 2 hr.

Pretreatment	Treatment	Conductance of ambient solution (μmhos)	Protection by uranyl nitrate (%)
Water	Water	13.7	
Uranyl nitrate	Water	10.2	25
Water	CCP	37.7	
Uranyl nitrate	CCP	19.0	68
Water	Methanol	33.2	
Uranyl nitrate	Methanol	12.9	88

PC-toxin, suggests that uranyl reduces efflux of electrolytes by coating and stabilizing the membrane. When oat cuttings were placed in 2 mM uranyl nitrate, transpiration was reduced 44 to 58%. This also suggests general effects of uranyl ions on cell membranes. Hence, experiments with uranyl salts and toxin could be misleading, particularly since they give questionable information about the site or mechanism of toxin action. It is possible that uranyl ions interfere directly with the action of toxin. It is also possible that uranyl ions simply change the membrane so that electrolytes are not lost, even though toxin has acted.

Pretreatment with cycloheximide and other inhibitors: Several other biologically active compounds were tested for possible protective effects against the action of HV-toxin on susceptible oat tissues. Among the materials tested, cycloheximide gave the most striking results. Cycloheximide is known to be a potent inhibitor of protein synthesis in plant cells; however, other effects on respiration and ion uptake have raised questions about its specificity (18).

Cuttings from susceptible oat seedlings (6-12 days old), or uniform size, were weighed (0.5 - 1.0g) and the freshly cut ends were immersed in 2 ml cycloheximide (CH) solution (5 μg/ml), or in water as a control. Cuttings were allowed to transpire under fluorescent lamps (100-200 ft-candles) for 2 to 12 hr. The basal 1 cm from each cutting was removed and discarded, and cuttings were sectioned into 1 cm pieces and enclosed in cheesecloth. Duplicate or triplicate samples were rinsed, placed in toxin solution (16 μg/ml) for 1 to 2 hr, rinsed 4 to 5 times with 100 ml distilled water to remove free space

electrolytes, and finally placed in 50 ml distilled water for 1 to 4 hr for leaching. Flasks (125 ml) on a reciprocal shaker (80-100 strokes/min) were used for toxin and leaching treatments. Resistance of the leaching solution was measured in the usual way.

A 12 hr pretreatment with CH reduced toxin-induced electrolyte efflux by 80 to 90% (Table 7). The 12 hr pretreatment period was necessary for maximum protection; data show that CH protection developed rapidly between 8 and 12 hr after initial exposure (Figure 10). The initial delay, plus the rapid development of protection suggest that the phenomenon could be linked with protein synthesis and/or turnover. CH alone did not cause a significant loss of electrolytes after exposure for 12 hr. This may indicate that nonspecific damage, such as the damage caused by uncouplers, was not an important factor in CH protection of oat tissues against the effects of HV-toxin. CH did not affect resistance to toxin in resistant plants.

The available electrolytes in CH-treated and control tissues were measured by extracting tissue with boiling water. In no case were the available electrolytes decreased by CH. Pretreatments with uncouplers often depleted the available electrolytes because they caused leakiness of membranes. On later treatment with toxin, there simply was less electrolytes to be lost. To determine whether or not the CH effect was specific for toxin-induced electrolyte efflux, CH-treated and control tissue were exposed to and leached in methanol (20%) or CCP (100μM). The results show that CH did not protect against electrolyte efflux induced by these substances.

CH prevents elongation of the peptide chain on the ribosome; apparently CH can be removed from its site of action without irreparable

Table 7. Effect of Cycloheximide (CH) Pretreatments on HV-Toxin-Induced Electrolyte Efflux from Susceptible Oat Tissue.

Susceptible oat shoots (1.0g/sample) took up CH (5 μg/ml) or water for 12 hr. Shoots were cut into 1 cm long pieces, enclosed in cheesecloth, and treated with toxin (9 μg/ml) or water for 1 hr, rinsed, and leached in 50 ml distilled water for 1.5 hr. Samples 5 through 8 were placed on White's solution (WS) for 48 hr before toxin or water treatment and leaching. Note the reversal of protection after 48 hr on White's solution.

Sample	Pretreatment (12 hr)	Treatment	Conductance (μmhos)	Protection (%)
1	Water	Water	8	
2	Water	Toxin	39	
3	CH	Water	10	
4	CH	Toxin	14	87
5	Water + WS(48 hr)	Water	11	
6	Water + WS(48 hr)	Toxin	47	
7	CH + WS(48 hr)	Water	12	
8	CH + WS(48 hr)	Toxin	44	11

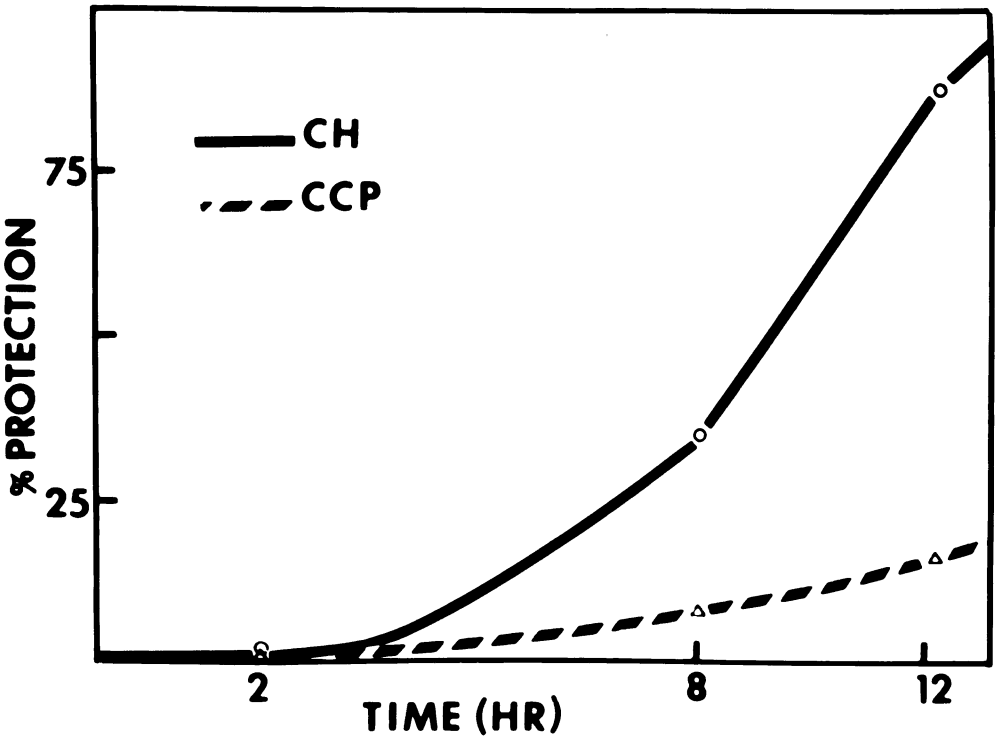

Fig. 10. Time required for development of cycloheximide (CH) protection against HV-toxin-induced efflux of electrolytes. Susceptible oat cuttings (1.0g/sample) took up 5 μg/ml CH, 50 μM m-ClCCP, or water for 12 hr. One to 2 ml of solution was taken up in 12 hr. Cuttings were removed at intervals, sectioned, enclosed in cheesecloth, and suspended in toxin (16 μg/ml) for 1 hr. After toxin treatment, samples were rinsed and suspended in 50 ml distilled water for 2 hr. Conductance of the ambient solution was measured.

damage to protein synthesis (78). An attempt was made to determine whether or not the CH-protective effects can be reversed in time. CH-treated and control cuttings were cut into 1 cm sections (each sample was 0.5 - 1.0g) and placed on White's solution (15 ml) in 9 cm covered Petri dishes under fluorescent lights. Duplicate samples were removed at different time intervals and tested for toxin susceptibility. White's solution was renewed every 12 hr; CH was added back to samples at appropriate times to regain the protective effect. The results indicate that the CH effect is readily reversible (Table 7, Figure 11). Recovery of insensitivity to toxin was even more rapid in samples which had been previously exposed to CH. The conductance values in Table 7 are typical values for all protection and reversal cycles shown in Figure 11. The control samples treated with cycloheximide were always slightly more leaky than controls treated with water.

The cycles of protection and reversal suggest that an effect on protein turnover, involving a protein or proteins with a short half-life (6 to 12 hr), could be responsible for the CH effect. If this is true, the number of receptors should be limiting. To examine whether the effect on toxin susceptibility was qualitative (all receptor sites affected to some extent) or quantitative (some receptor sites affected, i.e. limiting the number of receptor sites), CH (5 μg/ml)-treated (9.5 hr) and control tissues were treated with a with range of toxin concentrations. The results (Figure 12) show little protection by CH against toxin at 1.6 μg/ml. When toxin concentration was 16 μg/ml or higher, the protection by CH was striking. One possible explanation of the results is that receptor sites are

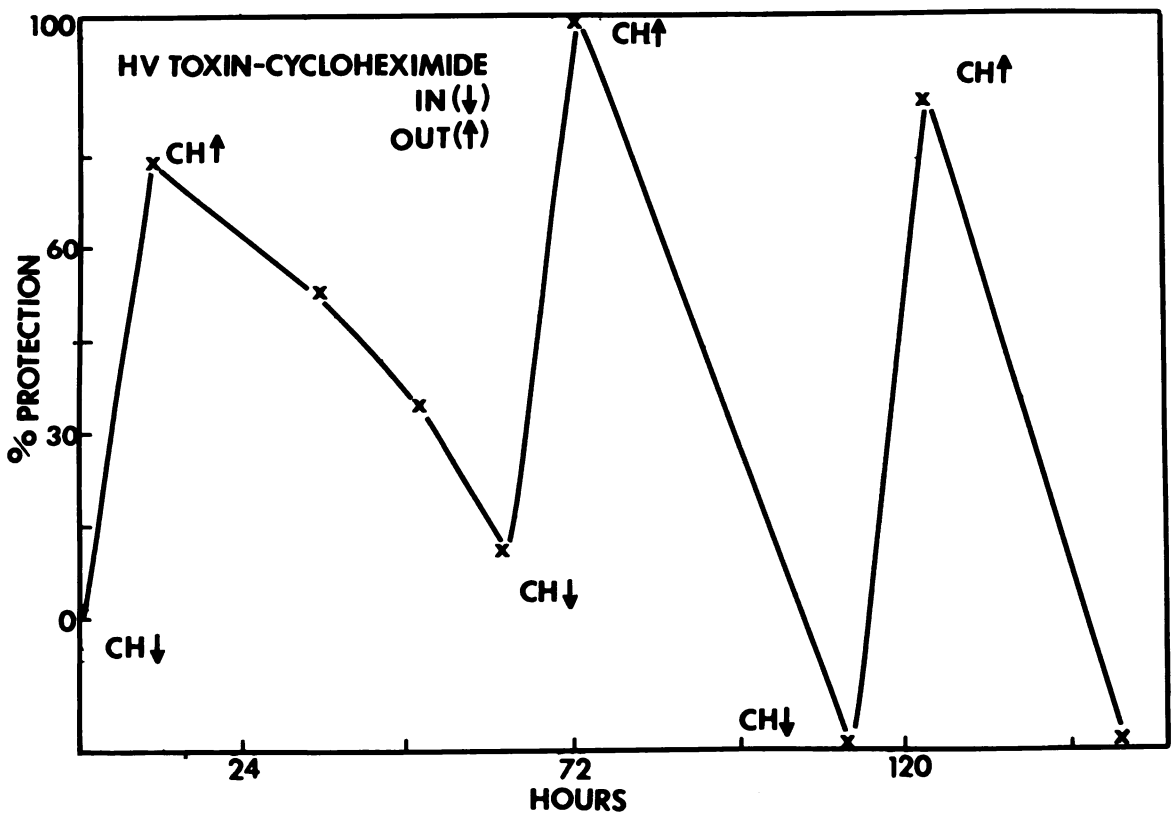

Fig. 11. Cycloheximide (CH) protection against HV-toxin-induced efflux of electrolytes and reversal of the protective effect. Susceptible oat cuttings (1.0g/sample), which took up 1 to 2 ml CH (5 μg/ml) or water for 12 hr, were sectioned into 1 cm long pieces and floated on White's solution. White's solution was replaced every 12 hr. At appropriate times, sections were enclosed in cheesecloth, treated with toxin (9 μg/ml) for 1 hr, rinsed, and leached in 50 ml distilled water. Conductance of the ambient solution was measured. Samples were discarded after toxin treatment and assay, and separate samples were tested for each protection and reversal cycle. White's solution was replaced with CH in White's solution to regain protection. Conductance values for control samples were comparable to those given in Table 7.

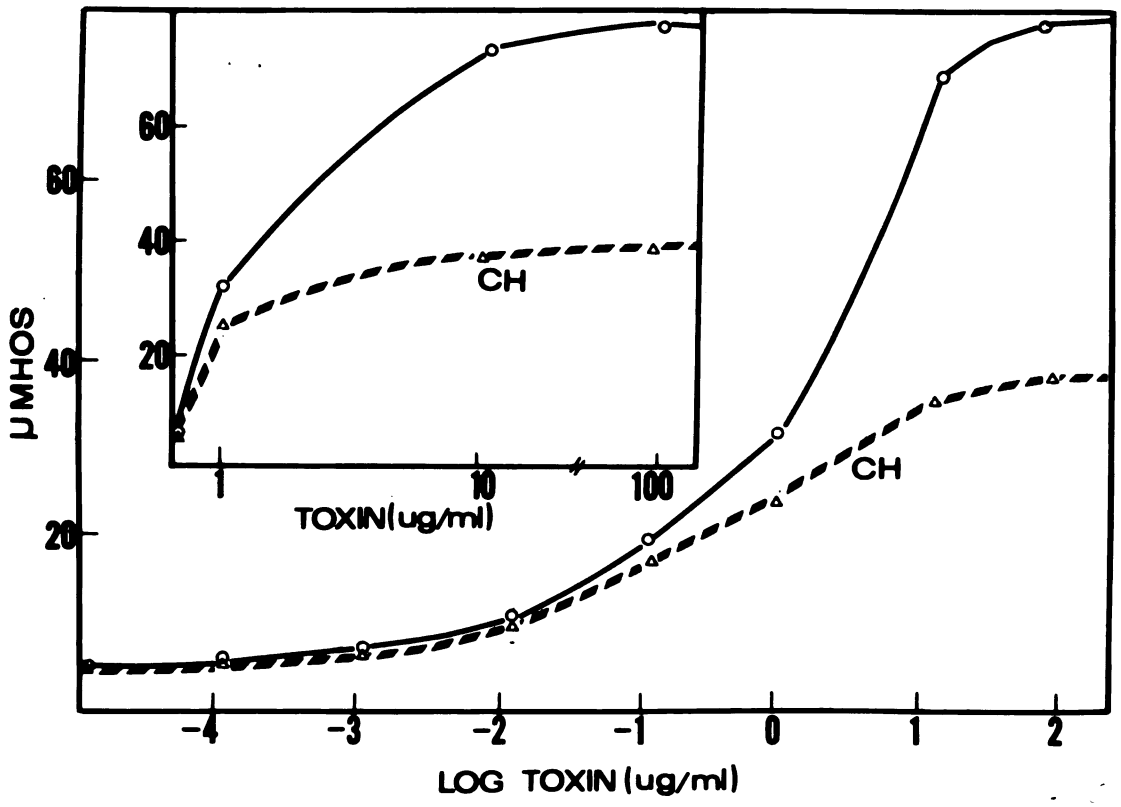

Fig. 12. Effect of HV-toxin concentration on cycloheximide (CH) protection against HV-toxin-induced efflux of electrolytes. Susceptible oat cuttings (1.0g/sample) took up approximately one ml CH (5 μg/ml) or water for 9.5 hr. Samples were then treated with toxin in White's solution or White's solution alone for 4 hr. After toxin treatment, samples were rinsed and suspended in 50 ml distilled water for 4 hr. Conductance of the leaching solution was measured.

depleted by protein turnover. At low toxin concentrations the limiting factor would not be the number of receptor sites.

Several other metabolic inhibitors were tested for protective effects against HV-toxin. Tissues were exposed to sodium azide, sodium fluoride, sodium arsenate, 2,4-dinitrophenol, and CCP for 2 to 12 hr, then treated with toxin and assayed for toxin-induced electrolyte efflux. Even at high concentrations, 12 hr pretreatments with these substances gave little or no protection against toxin (Table 8). However, these inhibitors often damaged membranes, causing increased electrolyte efflux prior to toxin treatment. Thus the results with inhibitors may not be reliable, because available electrolyte reserves in the tissue were depleted. Leakiness was much greater with tissues floated on inhibitor solutions than with cuttings which took up inhibitors. The results suggest that membrane damage of many kinds does not necessarily give protection against toxin.

SKF-7997-A_3 (Tris-(2-diethylaminethyl)-phosphate trihydrochloride) inhibits the conversion of lanosterol to sterols, a process necessary for the maintenance of membrane sterols. This inhibitor is known to affect sterol synthesis in plants (9). Solutions of the SKF-7997-A_3 were prepared fresh before each experiment, and the pH was adjusted to 7.2 with phosphate buffer (10 mM). Susceptible cuttings were allowed to take up inhibitor (250 μg/ml), or tissue sections were floated on the inhibitor solution. The results of 24 hr pretreatments indicated that SKF-7997-A_3 gave no protection against HV-toxin, even when damaging concentrations of the inhibitor were used.

Actinomycin D, which inhibits DNA-dependent RNA synthesis, was

Table 8. Effect of Metabolic Inhibitors on HV-Toxin-Induced Loss of Electrolytes from Susceptible Oat Tissue.

Cuttings (1.0g; from 6 to 10 day old plants) took up approximately 1 ml inhibitor with 13 or 24 hr exposure. Cuttings were cut into pieces (1 cm long pieces) and enclosed in cheesecloth for toxin (9-16 μg/ml) treatment (2 hr). Tissue was then rinsed and leached in distilled water for 4 hr prior to conductivity determinations.

Pretreatment with	Pretreatment time (hr)	Conc. (mM)	Protection[1/] (%)
Cycloheximide	13	0.05	81
Sodium Fluoride	13	4.0	30
Sodium Azide	13	1.0	21
DNP	13	1.0	26
m-ClCCP	13	0.08	15
Actinomycin D	24	0.03	61
SKF-7997-A_3	24	0.8	0

1/ Conductance values for control leaching solutions: Toxin = 57.5 μmho; H_2O = 2.6 μmho.

also tested as a possible protective agent against toxin. In 24 hr pretreatments, protective effects with this inhibitor (30 - 100 μg/ml) ranged from 20 to 80%. These results, as do those with CH, suggest some role of protein synthesis in maintaining receptor sites.

Short exposures to the inhibitors mentioned above, including CH, gave no protection against subsequent toxin-induced electrolyte efflux.

Pretreatment with sulfhydryl-binding reagents: N-ethylmaleimide (NEM), iodoacetic acid (IA), 2,4-dinitrofluorobenzene (DNFB), sodium arsenite, parachloromercuribenzoate (PCMB), parachloromercuriphenylsulfonate, and chlormerodrin were used because they are sulfhydryl-binding reagents. N-ethylmaleimide is the most specific of these for sulfhydryl groups although it can react very slowly with amino groups (81). Iodoacetate and dinitrofluorobenzene (at low pH) and arsenite react rapidly, under gentle conditions, with sulfhydryl groups (89).

The first true leaves from 5 to 20 day old seedlings were cut into 1 cm sections, randomized, weighed, and enclosed in cheesecloth. Tissue samples were infiltrated under vacuum for about 1 min and then incubated in the sulfhydryl reagent for 30 min. Samples were then rinsed 4 to 5 times with distilled water (10 min total time). A rinse with 5 mM mercaptoethanol was used in experiments with NEM. Following these treatments, samples were exposed to toxin (16 μg/ml) for 0.5 to 2 hr, then were rinsed several times in 100 ml distilled water (10 min total time) and placed in 50 ml distilled water for a leach period of 1 to 4 hr. Flasks (125 ml) on a reciprocal shaker (80 - 100 strokes/min) were used for treatments and leaching.

Resistance of the ambient solution was measured with a conductivity bridge.

NEM, DNFB, arsenite, and iodoacetate gave 60 to 90% protection against toxin-induced electrolyte efflux (Figure 13). PCMB and other mercurials gave little protection against toxin. PCMB, PCMPS, and chlormerodrin do not penetrate the membrane readily, and for this reason they have been used to locate proteins on the membrane of the red blood cell (87). Lack of protection against toxin would not appear to be correlated with the ability to permeate the membrane, since mercuric ion, which should penetrate the membrane, gave no protection. Mercuric ion caused considerable membrane damage and leakiness.

NEM was chosen as the most specific of the sulfhydryl reagents. It usually gave about 80% protection. Tissue samples were treated with NEM for 10 to 60 min, rinsed, treated with toxin or control solutions, and leached in distilled water. The results indicate that NEM induces some leakiness, but the electrolyte loss was insignificant in comparison to toxin-induced leakiness (Figure 14). The results also show that reliable protection is evident for at least 4 hr.

Do sulfhydryl-binding reagents actually protect tissues against toxin? The apparent protective effect could result from damage to the membrane, or prior depletion of electrolytes from tissue. There are several reasons for believing that protective effects are involved. First, the reagents which protected against HV-toxin did not protect sorghum tissue against PC-toxin, for which the bioassay was also toxin-induced electrolyte efflux. Second, if NEM plus toxin

Fig. 13. Effect of brief pretreatments with sulfhydryl binding reagents on HV-toxin-induced loss of electrolytes. Susceptible leaf tissue (0.5-1.0g/sample) was cut into 1 cm pieces, enclosed in cheesecloth, and suspended in reagent or water, infiltrated under vacuum, and held for 30 min. Tissues were rinsed, treated with toxin (16 μg/ml), and assayed for toxin-induced electrolyte loss. The following reagents were used with concentrations as indicated: N-ethylmaleimide (NEM), 2mM (pH 6.7-7.4); dinitrofluorobenzene (DNFB), 2mM (pH 5.2); iodoacetate (IA), 2mM (pH 4.0-6.0); sodium arsenite (AsO_2), 5mM (pH 7.5); parachloromercuribenzoate (PCMB), 2mM (pH 7.5-9.0). The mean of at least 3 experiments and the range from the mean is indicated.

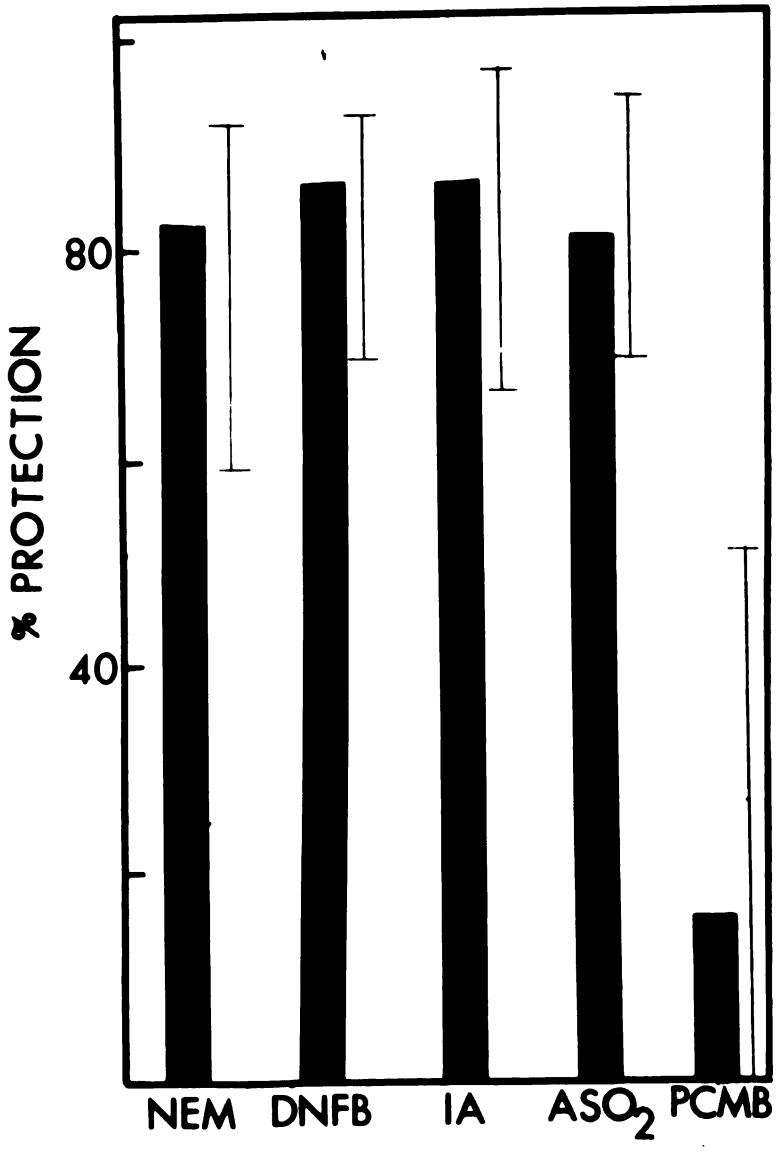

Fig. 13

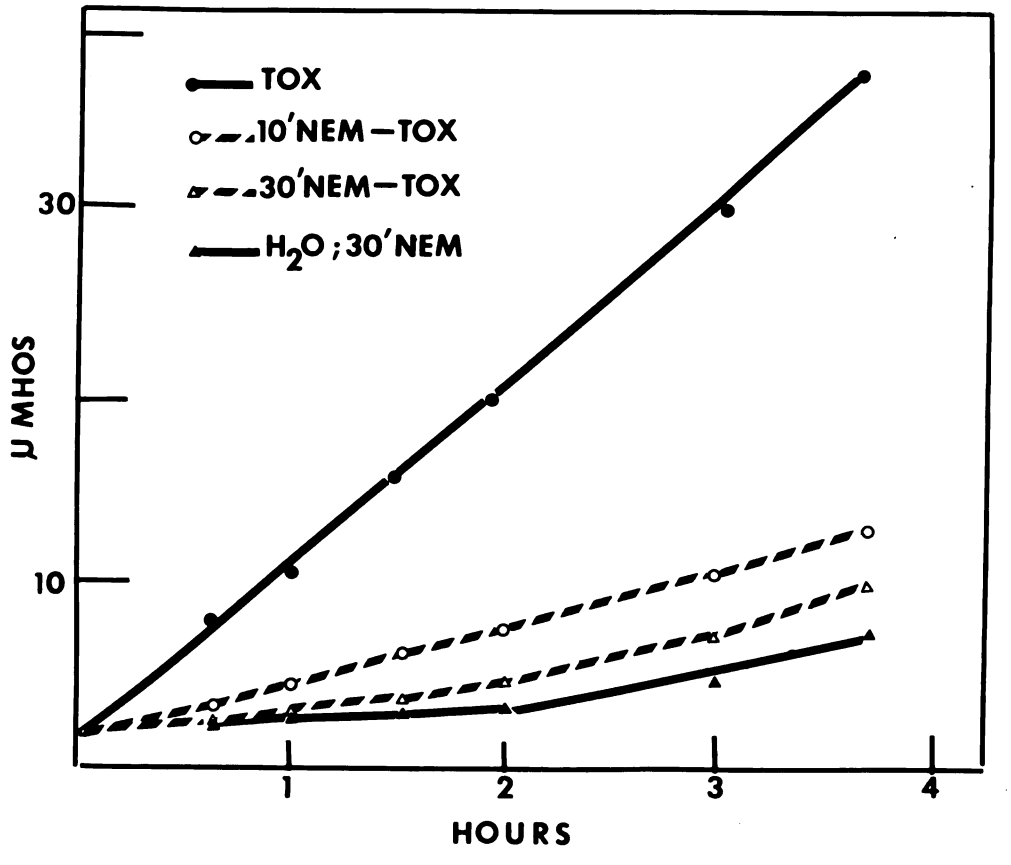

Fig. 14. Effect of n-ethylmaleimide (NEM) pretreatment on HV-toxin-induced electrolyte efflux from susceptible oat leaf tissue. Sections of leaves were treated with 5 mM NEM (pH 7.3) for 10 to 60 min. After rinsing with 5 mM mercaptoethanol and distilled water, tissue was treated with toxin solution (16 μg/ml) for 30 min, rinsed, and leached in 50 ml distilled water. Conductance of the ambient solution was measured. 10' denotes 10 min.

treatment was depleting the tissue of electrolytes and giving reduced electrolyte efflux in the leaching treatment, then a post-treatment (toxin, then NEM) should also give an apparent protective effect. Post-treatment, however, gave no protection. Thirdly, NEM had no protective effect on electrolyte loss induced by methanol and m-ClCCP. Rinses between treatments were checked for undetected depletion of electrolytes from tissue, and results were negative. The observations indicate that the protective effect is specific for HV-toxin-induced electrolyte efflux.

Effects of DNFB, arsenite, and PCMB on enzyme activity are often reversed with a sulfhydryl donor (14,89). Beta-mercaptoethanol was chosen as a suitable sulfhydryl donor in an attempt to reverse the protective effects against toxin-induced electrolyte loss. After pretreatment of the susceptible leaf tissue with sulfhydryl reagent, tissue was rinsed, incubated with mercaptoethanol (90 mM) for 40 min, then treated with toxin and assayed for toxin-induced electrolyte efflux. Controls were treated similarly but without mercaptoethanol. The results indicated that the effects of arsenite and dinitrofluorobenzene could be reversed with a sulfhydryl donor (Tables 9 and 10).

Red blood cells and yeast lose much of their potassium when exposed to mercurials (5,63,77). Partially reactive sulfhydryl groups do not react with NEM in red blood cells, but do react with organic mercurials and mercuric chloride, resulting in potassium leakage. Mercuric chloride has an all-or-nothing effect on the red blood cell membrane so that when a threshold concentration of reagent is reached, the membrane becomes disorganized and potassium loss occurs

Table 9. Dinitrofluorobenzene (DNFB) Protection against HV-Toxin-Induced Loss of Electrolytes, and Reversal of the Protective Effect by Beta-mercaptoethanol (ME).

Susceptible oat leaf tissue (800 mg/sample) was infiltrated with 2 mM DNFB (pH 5.2) for 30 min. Tissue was then treated with 80 mM ME or water for 40 min, rinsed, and treated with toxin (16 μg/ml) for 1 hr. After toxin treatment, tissue was rinsed and suspended in 50 ml distilled water for 4 hr. Conductance of the ambient solution was measured with a conductivity bridge.

Treatments			Conductance	Protection
1	2	3	(μmhos)	(%)
Water	Water	Toxin	153	
Water	Water	Water	5	
DNFB	Water	Toxin	56	81
DNFB	Water	Water	29	
DNFB	ME	Toxin	134	14
DNFB	ME	Water	7	

Table 10. Arsenite Protection against HV-Toxin-Induced Loss of Electrolytes, and Reversal of the Protective Effect by Beta-mercaptoethanol (ME).

Susceptible leaf tissue (750 mg/sample) was infiltrated with 5 mM sodium arsenite (pH 7.0) and incubated for 30 min. Tissue was then suspended in 80 mM mercaptoethanol or in water for 40 min, rinsed, and treated with toxin (16 µg/ml) for 1 hr. After rinsing, tissue was suspended in 50 ml distilled water for 2 hr. Conductance of the ambient solution was measured.

Treatments			Conductivity	Protection
1	2	3	(µmhos)	(%)
Water	Water	Toxin	45.5	
Water	Water	Water	5.0	
Arsenite	Water	Toxin	20.0	73
Arsenite	Water	Water	9.0	
Arsenite	ME	Toxin	47.5	0
Arsenite	ME	Water	9.0	

(63). To test this possibility for NEM protective effects against toxin, concentrations of NEM from 0.1 mM to 10 mM NEM were examined in protection experiments. The results indicate that there is no all-or-nothing effect on membrane integrity, since protection was directly related to log toxin concentration (Figure 15). An all-or-nothing effect on the membrane would result in a sigmoid curve.

Are the protective effects of sulfhydryl reagents, as well as the other protective reagents, competitive or non-competitive? In other words, can excess toxin overcome the protective effects? Tissue samples were treated with a protective reagent and then assayed for toxin-induced electrolyte loss. The results, plotted on a reciprocal basis, indicated that the inhibition by sulfhydryl reagents, as well as inhibition by cycloheximide and semicarbazide, was non-competitive and could not be overcome by raising toxin concentration (Figure 8).

An attempt was made to determine the time required for NEM and DNFB to react with tissues and effect resistance to toxin. Tissue samples were exposed to NEM (5 mM) or DNFB (5 mM) for 0.45 to 30 min, rinsed with distilled water plus a mercaptoethanol (5 mM) rinse for NEM samples, treated with toxin (16 μg/ml) and assayed for toxin-induced electrolyte efflux. The results indicated that within one min of exposure to either reagent, significant protection developed against toxin (Figure 16). Maximum protection was obtained by a 10 min pretreatment. This may indicate that affected sites are on the outer surface of the cell, but it is possible that both reagents are permeating rapidly. The rapidity of the effect might also indicate that the protective effects are directly associated with the

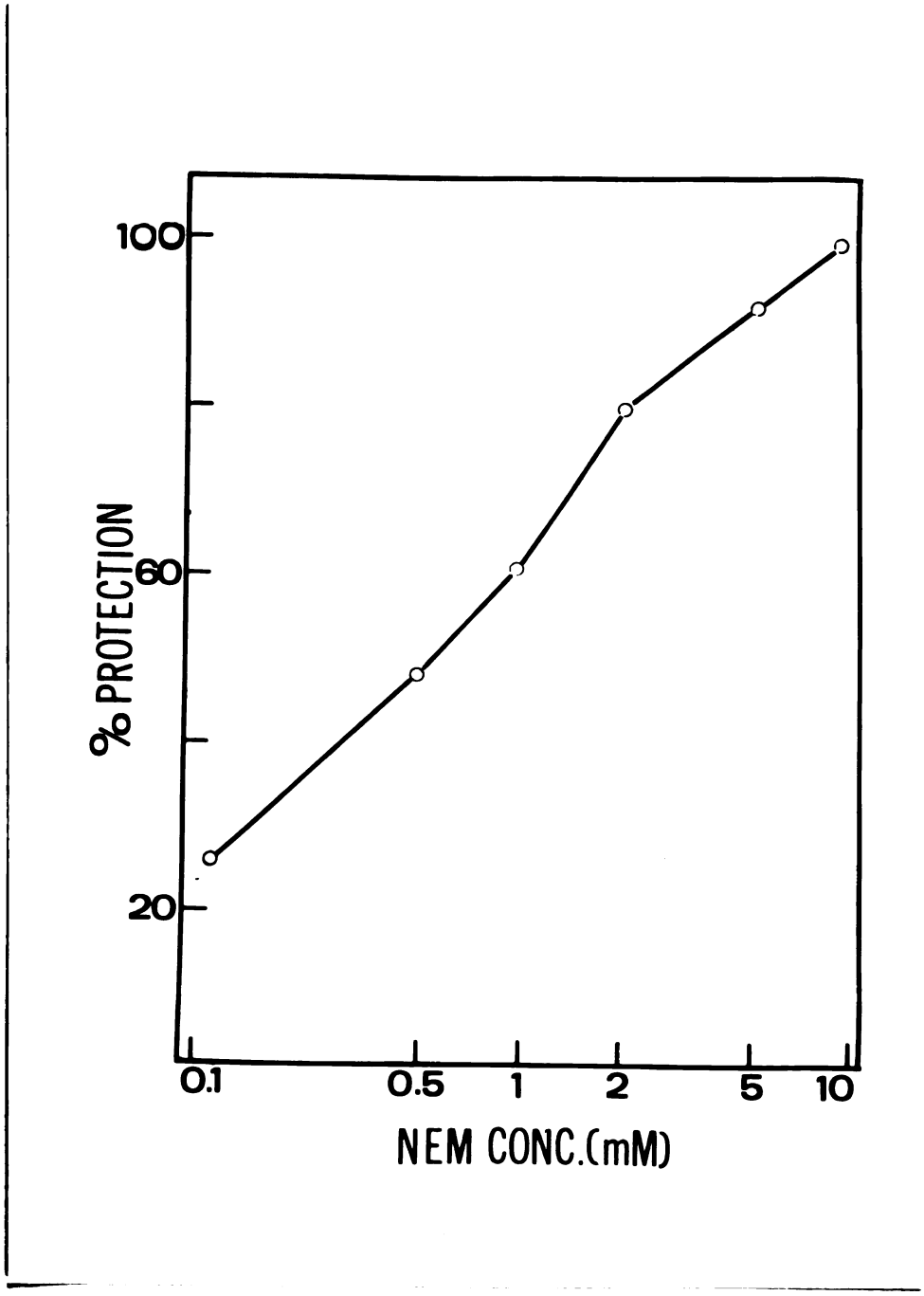

Fig. 15. Protection against HV-toxin-induced loss of electrolytes by n-ethylmaleimide (NEM) at several concentrations. Leaf tissue sections (600 mg/sample) from susceptible oat were treated with NEM (0.1 to 10 mM; pH 7.4), rinsed with 5 mM mercaptoethanol, then with distilled water, and suspended in toxin (16 μg/ml) for 1 hr. After toxin treatment, tissues were rinsed and suspended in 50 ml distilled water for 1 to 2 hr prior to conductivity determinations on the leaching solutions. Conductivity values for toxin-treated and water control samples were 56 and 9 μmhos, respectively.

Fig. 16. Time required for development of protective effects of N-ethylmaleimide (NEM) and dinitrofluorobenzene (DNFB) against HV-toxin-induced loss of electrolytes. Leaf tissue (800 mg/sample) from susceptible oats was infiltrated with NEM (5 mM, pH 7.4) and DNFB (5 mM, pH 5.2) for 30 sec and removed from treatment solution at the designated time for immediate rinsing (5 mM mercaptoethanol rinse for NEM; distilled water rinse for DNFB). Tissues were then treated with toxin (9 μg/ml), rinsed, and leached in 50 ml of distilled water for 3 hr prior to conductivity determinations on the leaching solutions. Conductivity values for toxin-treated water control samples were 77 and 6 μmhos, respectively.

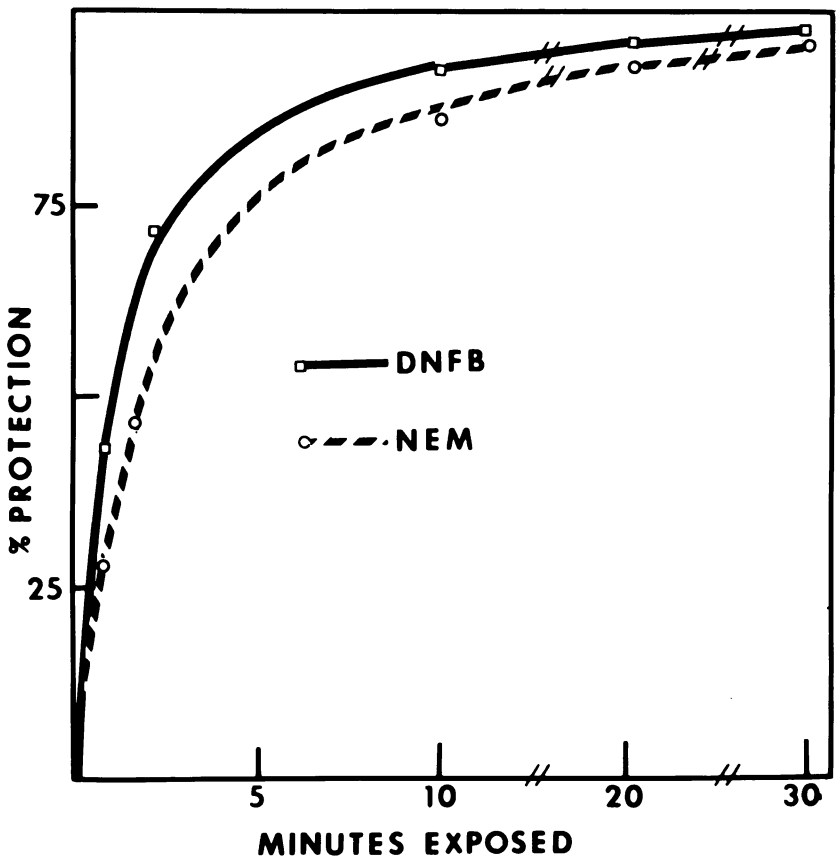

Fig. 16

sensitive sites for HV-toxin.

To test whether or not NEM was reacting with the toxin molecule, a spectrophotometric assay based on the maximum adsorption of NEM at 305 mμ was used (61). One ml toxin (32 μg/ml) in Tris-HCl (50 mM, pH 7.4) or potassium phosphate (50 mM, pH 6.7), or one ml buffer (control), was added to 1 ml 3 mM NEM plus buffer in a cuvette. Results were recorded as the change in absorbance at 305 mμ. Neither toxin nor toxin breakdown products (TBP) (1 to 20 mg/ml) caused a change in absorbance within one hr (Table 11). Glycine at high concentrations (56 mg/ml) reacted with NEM, indicating that NEM is not completely specific. However, paper or silica gel thin layer chromatograms of toxin or its breakdown products showed no significant amounts of free amino groups, with the exception of the peptide associated with toxin. The effects of NEM on host tissue susceptibility to toxin are rapid, and it is unlikely that this protective effect involves amino groups.

The sulfhydryl reagents which protect against toxin could be reacting with a sulfhydryl group which is involved in general conformation of the membrane, or they could be reacting with a sulfhydryl group which is closely associated with the hypothetical toxin receptor. To examine these possibilities, TBP was used as a substance which could possibly adsorb to the toxin receptor site yet be relatively inactive. This toxin analog was tested for its ability to reduce the protective effect of NEM, possibly by blocking the toxin-specific sites. It had been previously noted that TBP, which contains victoxinine (or a rearrangement thereof) and a peptide, partially counteracted toxin in a seedling bioassay (73). If TBP did compete, this

Table 11. Photometric Assay for a Possible Interaction Between HV-Toxin Breakdown Products (TBP) and NEM.

One ml NEM (3 mM, pH 6.7 phosphate buffer) was mixed with 1 ml test solution in a cuvette, and the change in absorbance of NEM at 305 mμ was measured with a Beckman D3 recording spectrophotometer. Glycine, Cysteine, and Bovine Serum Albumin (BSA) were used for comparison. The product had an R_f value similar to that reported for the peptide from HV-toxin.

Addition	Final Conc. (mg/ml)	Reaction Time (min)	OD_{305}	NEM Remaining (%)
Toxin	1	35	0.00	100
TBP	20	60	0.00	100
Glycine	1	20	0.00	100
Glycine	56	35	0.53	73
BSA	66	1	0.18	80
BSA	66	10	0.24	73
Cysteine	0.18	1	0.75	16
Cysteine	0.18	10	0.90	0

would be evidence for a close association between the sensitive sulfhydryl groups and the receptor site for toxin. TBP (from alkali-treated Biogel purified toxin) was weighed as an acetone powder and dissolved in NEM solutions buffered with 50 mM potassium phosphate, pH 6.7, or with 50 mM Tris-HCl, pH 7.4. pH of solutions was checked to eliminate any possibility of a pH effect on the NEM reaction. TBP (200 to 800 μg/ml) usually had slight residual toxicity; assay values for samples containing TBP were adjusted accordingly. That is, NEM and NEM-TBP treated control values in toxicity assays were subtracted from the assay values of their toxin-treated counterparts. The results of many experiments indicated that TBP, when present in the NEM treatment solution, reduces NEM protection against toxin-induced electrolyte efflux (Tables 12 and 13). In a typical experiment, TBP gave 20 to 40% reversal of NEM protection against toxin. High concentrations of either PC-toxin breakdown products or glycine, serving as controls for HV-toxin TBP, failed to give reversal of protection.

The results suggest that NEM protects oat tissue against HV-toxin by affecting a receptor site protein. However, the actual relationship between the sensitive sulfhydryl groups and the hypothetical toxin receptor is not known. Experiments with TBP as described above suggest a direct relationship.

Reagents which react with amino, histidine, and tyrosine groups did not protect oat tissue against HV-toxin. The following reagents were tested: diazosulfanilic acid (DSA), diazo-7-amino-1,3-napthalene-disulfonate (NDS), 4-acetamido 4' isothiocyano stilbene 2,2' disulfonic acid, fluorescein isothiocyanate, and iodine-potassium-iodate. These compounds used as pretreatments (30 min) with

Table 12. N-ethylmaleimide (NEM) Protection against HV-Toxin-Induced Loss of Electrolytes, and Countereffects of Toxin Breakdown Products (TBP).

Susceptible oat leaf tissue (800 mg/sample) was infiltrated with TBP (500 µg/ml) or water for 5 min, then exposed to NEM (2 mM, pH 7.4), NEM plus TBP, or control solutions for 35 min. Samples were rinsed, treated with toxin (16 µg/ml) for 30 min, and leached in 50 ml distilled water. Conductance of the leaching solution was measured.

Pretreatment	Treatment	Conductance Expt 1[1/] (µmhos)	Conductance Expt 2[2/] (µmhos)	Protection Expt 1 (%)	Protection Expt 2 (%)
Water	Water	5.0	5.6		
Water	Toxin	51.3	17.8		
NEM	Water	10.0	6.6		
NEM	Toxin	18.4	7.7	82	91
NEM + TBP	Water	10.3	8.3		
NEM + TBP	Toxin	25.9	12.4	66	58
NEM + PC-TBP[3/]	Water	10.0	7.7		
NEM + PC-TBP	Toxin	19.7	8.0	79	97

1/ Leaching time was 90 min.

2/ Leaching time was 45 min.

3/ PC-TBP breakdown products from the host-specific toxin of *Periconia circinata*, used as a control.

Table 13. N-ethylmaleimide (NEM) Protection against HV-Toxin-Induced Loss of Electrolytes, and Countereffects of Toxin Breakdown Products (TBP).

Susceptible oat leaf tissue (500 mg/sample) was infiltrated with TBP (500 μg/ml) or water for 5 min, then exposed to NEM (2 mM, pH 7.1), NEM + TBP, NEM + glycine, or control solutions for 30 min. Samples were rinsed with mercaptoethanol (5 mM) and distilled water, treated with toxin (9 μg/ml) for 1.5 hr, and then leached in 50 ml distilled water for 4 hr. Glycine (2 mg/ml) was used as a control to test possible reactions of NEM with free amino groups.

Pretreatment	Treatment	Conductance (μmhos)	Protection (%)	Reversal of Protection (%)
Water	Water	12.5		
Water	Toxin	98.5		
NEM	Water	29.5		
NEM	Toxin	62.7	62	
NEM + TBP	Water	28.0		
NEM + TBP	Toxin	77.5	43	31
NEM + glycine	Water	35.6		
NEM + glycine	Toxin	65.2	66	0

millimolar concentrations were not effective. NDS and DSA were synthesized prior to use according to Pardee and Watanabe (53). Such results strengthen the suggestion that the effects of sulfhydryl-binding compounds are highly specific.

Sulfhydryl groups are known to be associated with active sites in enzymes. In many cases, the active site sulfhydryl is the most, if not the only, reactive sulfhydryl group in the enzyme molecule (89).

Competition between toxin breakdown products (TBP) and toxin:

TBP preparations were shown previously to counteract in part the effects of toxin on seedling root growth (73). An attempt was made to confirm this observation by assaying the effects of toxin on efflux of electrolytes from susceptible tissue. Toxin was used in concentrations from 0.15 to 9.0 μg/ml and TBP, prepared from Biogel purified HV-toxin, was added in amounts from 100 to 500 μg/ml. Results showed that TBP partially counteracted the effects of toxin. TBP did not counteract toxin effects at high concentrations of toxin (Figure 17). It was also clear that TBP never gave more than 30% protection against toxin-induced electrolyte efflux, even at high concentrations of TBP. This is consistent with the observation that TBP in seedling bioassays only reduced the dilution endpoint of toxin from $1/10^6$ to $1/10^5$. The experiment was repeated a number of times with essentially the same results.

A procedure was developed to further purify breakdown products. The toxin preparation (16 mg/ml) from the alumina column was diluted 1 to 10 with water and adjusted to pH 7 to 8 with NaOH. The cloudy flocculation which developed was collected on a Millipore filter

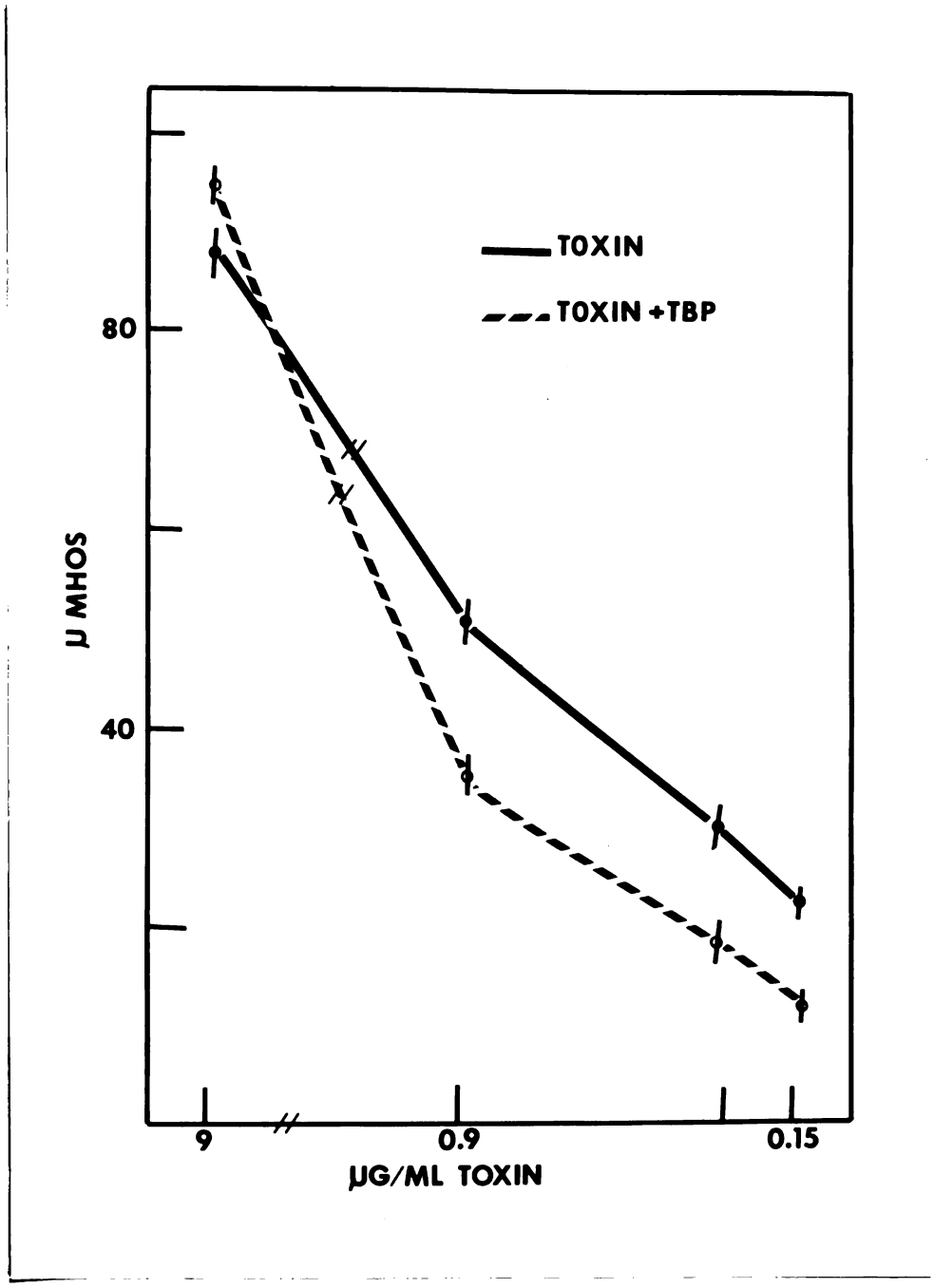

Fig. 17. Countereffects of toxin breakdown products (TBP) on HV-toxin-induced loss of electrolytes for oat leaves. Susceptible leaf tissue (500 mg/sample) was treated with toxin (0.15 to 9.0 μg/ml), toxin plus TBP (500 μg/ml), TBP, or water for 2 hr. Samples were leached in 25 ml distilled water for 4 hr and conductance of ambient solution was measured. Conductance (μmhos) of controls: TBP, 26.9; water, 17.7. This conductance of 9.2 μmhos can be attributed to residual toxicity in TBP. A correction was made for this value. Variability between replications is indicated by vertical lines through each point.

(type GSWP, 0.22 μ), rinsed with a small volume of water, and discarded. The filtrate was evaporated in vacuo to 1/10 volume, adjusted to pH 11.5 with NaOH and left for one week at 22 C. pH was readjusted to 8.0 and the preparation was dried in vacuo. Butanol was added (a volume equal to that of the pH 7.0 filtrate); insoluble material was collected on a Millipore filter and discarded. The filtrate was evaporated to dryness and the residue was washed with 75 ml cold ethyl ether on a Millipore filter. The residue on the filter was redissolved in water and lyophilized. The discarded precipitates from the previous steps contained little ninhydrin positive material whereas the ether insoluble residue was strongly ninhydrin positive and gave an R_f value with paper chromatography corresponding to the peptide reported to be a toxin breakdown product (57). The ether soluble material gave a negative iodoplatinate test and little or no color in the ninhydrin test, indicating that it did not contain victoxinine.

The ether insoluble material was chromatographed on a Sephadex G-15 column (1.5 x 88 cm). Two ml fractions were collected and tested with methyl cellulose ninhydrin reagent (4). One ninhydrin positive peak was obtained well after the void volume of the gel, indicating a molecular weight of less than 1500 (Figure 18). Peak fractions from two columns were lyophilized to a white powder. The material had R_f 0.55 on silica gel thin layer plates (Butanol:acetic acid:water. 8:2:2). The product was not victoxinine, which has R_f 0.70 in this system. The material was hydrolyzed in 6N HCl in evacuated ampules and subjected to two dimensional chromatography. There were at least 4 ninhydrin positive spots, indicating that the

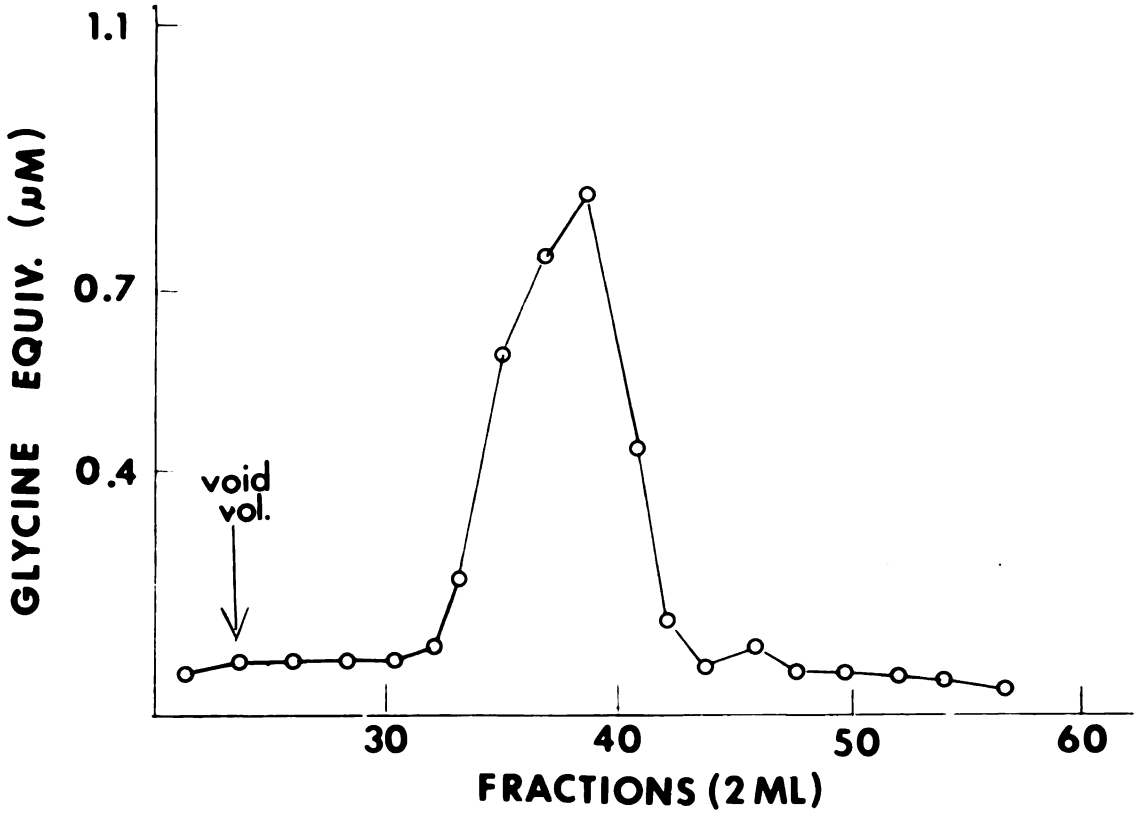

Fig. 18. Chromatography of HV-toxin breakdown products on a Sephadex G-15 column (1.5 x 88 cm). Approximately 50 mg material was placed on the column and eluted with distilled water. Two ml fractions were collected and assayed with methyl cellulose ninhydrin reagent, with glycine as the standard. The flow rate was 6 ml/hr.

substance was a peptide.

Competition between esterified, inactive toxin and the active toxin: Since HV-toxin was 2 dicarboxylic acids (aspartic and glutamic) in the peptide, free carboxyl groups may be available for reaction. Methanol-HCl is a fairly specific reagent for esterifying the carboxyl group (23,96). Toxin is soluble in acidified methanol.

HV-toxin was lyophilized and dissolved in anhydrous methanol-0.2N HCl. The methanol was made acidic by bubbling gaseous HCl through a stock solution of methanol, the acid was titrated with Tris (1N), and the solution was diluted to 0.2N HCl before use. After 48 hr, toxin was assayed for its ability to induce electrolyte loss from oat leaves. Results showed that toxin lost most of its activity. There was little or no loss in activity of toxin in methanol or HCl alone. The inactive toxin was reactivated in part by diluting the preparation 1 to 50 with water and adjusting to pH 7 to 8. Assay showed a 38% increase in toxin activity after one week at 5 C.

Esterified toxin was then tested for its ability to reduce the effects of an active toxin preparation. Solutions containing from 0.08 to 8.0 μg toxin/ml were added to solutions containing esterified toxin (48 to 80 μg/ml). Toxin and control solutions were buffered at pH 4.5 with 10 mM sodium acetate. The test and appropriate control solutions were then assayed for ability to induce efflux of electrolytes from oat leaves. The results indicated that esterified toxin reduced the activity of toxin, even at high toxin concentrations (Table 14). In some cases there was slight residual activity in

Table 14. Countereffects of Esterified (Inactive) Toxin (ET) on HV-Toxin-Induced Loss of Electrolytes.

Susceptible oat leaf tissue (800 mg/sample) was exposed to toxin plus ET in acetate buffer (pH 4.5), or to toxin in buffer, for 2 hr. Tissue was then rinsed and suspended in 50 ml water for 2 hr. Conductance values are for ambient solutions.

Treatment[1/]	Conductance (μmhos)	Toxicity Decrease (%)
Water	8.3	
ET (80 μg/ml)	10.8	
Toxin (0.8 μg/ml)	24.4	
Toxin (0.8 μg/ml) + ET (80 μg/ml)	17.6	58
Toxin (8.0 μg/ml)	44.2	
Toxin (8.0 μg/ml) + ET (80 μg/ml)	34.3	36

1/ ET was prepared from the toxin preparation used in this experiment.

esterified toxin, so that conductance values were corrected slightly for this factor. The experiment was repeated twice with essentially the same results.

Methanol-HCl treatment may be inactivating toxin by esterifying carboxyl groups. It appears, however, that esterification does not change the toxin molecule enough to prevent it from counteracting the effects of fully active toxin, possibly by competing with it for toxin receptor sites.

Experiments with Cell Particles and Nuclei:

Interactions of a particulate fraction from cells with ^{14}C-NEM, toxin, and toxin breakdown products (TBP): Results described earlier indicate that the protective effect of NEM was reduced by TBP. Therefore, an attempt was made to label oat cells with ^{14}C-NEM in the presence of TBP. The results indicated that TBP reduced the amount of NEM bound to the cell. However, the experiment was not satisfactory because of possible residual toxicity in TBP and problems in labelling tissue. Therefore, an *in vitro* labelling procedure was attempted. Previous work (68,75) indicates a role of plasma membrane in toxin action. On the basis of some preliminary information on the isolation of plasma membranes (personal communication, D. J. Morre), a particulate fraction obtained by centrifugation between 12,000g and 50,000g, was used as a possible enriched preparation of plasma membrane fragments.

Etiolated oat seedlings (100-150g) were chopped quickly with a razor blade in a buffer and the 12,000-50,000g fraction was isolated as described in Materials and Methods. Toxin (0.3 ml, containing 15

μg) or TBP (0.3 ml, containing 0.45 mg) in Tris-HCl buffer was added to 0.3 ml of the resuspended particulate fraction (3 to 6 mg protein/ml). Both toxin and TBP were pre-filtered through Millipore filters to eliminate material insoluble at pH 7.4. After 5 to 10 min, 0.3 ml ^{14}C-NEM (3 to 300 μM; 0.1 to 1 μc/ml) was added. The final reaction mixture was buffered at pH 6.7 with 0.05 M potassium phosphate or at pH 7.4 with 0.05 M Tris-HCl. The mixture was incubated at 22 C for 15 min, with occasional stirring; the reaction was then terminated by the addition of 2 ml NEM (200 mM) plus 2 ml 40% TCA, followed by 10 ml 20% cold TCA. The precipitate was washed with 10% TCA, followed by 95% ethanol, and then with ethanol:ether (1:1). The precipitate was collected on Millipore filters and washed with 50 ml TCA (10%). The filter disks were dried and suspended in scintillation mixture for counting.

Results showed that both toxin and TBP caused a reduction in labelling of the particulate fraction from susceptible oats (Tables 15 and 16). In no case was there a significant reduction in labelling (at the 5% level) with particulate fractions from resistant oats. Bovine serum albumin was labelled with ^{14}C-NEM in the presence of toxin or TBP; no reduction in labelling from untreated (minus toxin or TBP) controls occurred (Table 17). The results therefore suggest that toxin or TBP is having a specific effect in a cell-free system from susceptible tissue. Whether or not the membrane fraction *per se* is involved has not been determined.

Table 15. Effect of HV-Toxin Breakdown Products (TBP) on Labelling of Particulate Fractions[1/] with ^{14}C-NEM.

Expt #	Tissue from	Treatment[2/]	CPM in protein[3/] (CPM)	Percent reduction[4/] (%)
1	Susceptible oats	Buffer	11,597	
		TBP (500 µg/ml)	9,179	21 (0.01)
2	Susceptible oats	Buffer	14,475	
		TBP	11,433	20 (0.005)
3	Resistant oats	Buffer	3,532	
		TBP	3,305	6 (n.s.)
4	Resistant oats	Buffer	3,836	
		TBP	3,517	8 (n.s.)
5	Susceptible oats	Buffer	5,897	
		TBP	4,901	17 (0.005)
	Susceptible-Resistant (mixed)	Buffer	6,498	
		TBP	5,715	12 (0.05)
	Resistant oats	Buffer	5,612	
		TBP	5,256	6 (n.s.)
6	Susceptible oats	Buffer	3,719	
		TBP	3,095	17 (0.05)
	Resistant oats	Buffer	4,589	
		TBP	4,676	-2 (n.s.)

1/ Particulate fraction was obtained by centrifugation at 12,000g - 50,000g.

2/ TBP was obtained by inactivation of toxin prepared by gel filtration.

3/ CPM in background was subtracted. Protein in the particulate fraction was 1 to 2 mg/sample.

4/ Figures in parentheses indicate the minimum level of probability; n.s. indicates that the difference is not significant from the control at the 5% level (0.05).

Table 16. Effect of HV-Toxin and HV-Toxin Breakdown Products (TBP) on the Labelling of a Particulate Fraction[1/] with ^{14}C-NEM.

Expt #	Tissue from	Treatment[2/]	CPM in protein[3/] (CPM)	Reduction in CPM[4/] (%)
1	Susceptible oats	Buffer	2,335	
		TBP	1,738	26 (0.05)
		Toxin	1,804	23 (0.1)
	Resistant oats	Buffer	3,024	
		TBP	2,762	9 (n.s.)
		Toxin	3,216	-7 (n.s.)
2	Susceptible oats	Buffer	1,561	
		Toxin	876	44 (0.05)
	Resistant oats	Buffer	2,304	
		Toxin	2,066	10 (n.s.)
3	Susceptible oats	Buffer	834	
		Toxin	659	21 (0.05)
4	Susceptible oats	Buffer	550	
		Toxin	499	9 (0.05)
	Resistant oats	Buffer	418	
		Toxin	474	-14 (n.s.)

1/ Fraction sedimenting between 12,000g (10 min) and 30,000g (40 min).

2/ Toxin concentration was 16 μg/ml in all experiments.

3/ The CPM in background and 0 time samples were subtracted from CPM values.

4/ Figures in parentheses indicate the minimum level of probability for the difference from control. n.s. = not significant at 5% level (0.05) in a paired t test.

Table 17. Labelling of Bovine Serum Albumin (BSA) with ^{14}C-NEM in the Presence of HV-Toxin Breakdown Products (TBP) and HV-Toxin.

^{14}C-NEM (0.3 ml; 30 μM; 1 μc/ml) was added to an equal volume of TBP (1.5 mg/ml) or toxin (48 μg/ml) at 22° C. After 5 min, BSA (0.3 ml; 6 mg/ml) was added and the mixture was incubated for 15 min. All solutions were buffered with 0.05 M Tris-HCl, pH 7.2. Reactions were terminated with 10 mM mercaptoethanol and 20% TCA.

Addition	CPM[a] in BSA (cpm)	Reduction in labelling (%)
Buffer	22,201	
TBP	24,093	-8[b]
Toxin	22,633	-2[b]

a. CPM values are the mean of triplicate samples, minus CPM for background and 0 time samples.

b. Differences from control (buffer) not significant at 5% level of probability.

Effect of toxin on uptake and incorporation of amino acids by isolated nuclei: Nuclei are capable of actively taking up amino acids (36) and incorporating them into protein (7). The nuclear membrane is continuous with the endoplasmic membrane (47). Therefore, the incorporation of amino acids into protein of isolated nuclei was selected as a test for the integrity of the nuclear membrane in experiments with toxin.

Nuclei were isolated by centrifugation through a 1.2 M sucrose solution (32). They were incubated for 15 min in the cold in Tris buffer (0.05 M, pH 7.4) with or without toxin. Chloramphenicol (100 μg/ml) was added to inhibit incorporation by bacteria. Lysine-^{14}C was added and incorporation into protein at 32 C was measured at time intervals up to 60 min. The results of two experiments indicated that toxin had no effect on incorporation of lysine into protein (Table 18). Previous work (75) has shown that toxin has no effect on isolated mitochondria and chloroplasts.

Table 18. Effect of HV-Toxin on Incorporation of ^{14}C-Lysine into Protein of Nuclei Isolated from Susceptible Oat Leaves.

Toxin (16 μg/ml) in Tris-HCl (0.05 M, pH 7.4) or Tris-HCl control was added to a suspension of nuclei in 0.6 M sucrose plus 0.08 M Tris-HCl. Preparation was incubated on ice for 15 min. After 5 min equilibration at 32°C, D,L-Lysine (1 μc/ml; 3 to 30 μM/ml) was added and the preparations were incubated for 45 and 60 min, respectively, for experiments 1 and 2. The reaction was stopped by adding 40% TCA. Radioactivity (cpm) in samples with TCA added at 0 time was subtracted from cpm in samples with TCA added at 45 or 60 min.

Expt. #	Treatment	CPM incorporation (cpm)	Lysine incorporation (mμmoles/mg protein
1	Water	173	0.302
	Toxin (16 μg/ml)	185	0.323 1/
2	Water	1,423	4.86
	Toxin (16 μg/ml)	1,281	4.38 1/

1/ Differences from control values are not significantly different at 5% level of probability.

DISCUSSION

Eight different host-specific toxins are not known, one from each of the following fungi: Helminthosporium victoriae, H. carbonum, H. maydis, H. sacchari, Alternaria citri, A. mali, A. kikuchiana, and Periconia circinata (75,79,84). These substances are known to be involved in disease development (75), and they can account for the visible and biochemical symptoms of the respective infections. Therefore, these toxins are useful models for studies of the biochemical basis of disease. They are required for pathogenicity of the producing fungi (75) and they are useful models for studying this phenomenon. Host-specific toxins can differentiate between resistant and susceptible plants; thus, they should be useful tools for the study of disease resistance.

HV-toxin can reproduce all the symptoms of Victoria blight of oats. The drastic effects of HV-toxin may constitute an unusual case, whereas more subtle toxins may go undetected. However, it has been shown histologically that the invasion of susceptible oat tissue by H. victoriae is typical of most plant pathogens (98).

The very rapid effects of HV-toxin on electrolyte efflux from cells and the bursting of cell wall-free protoplasts is convincing evidence for an early toxin-induced lesion in the plasma membrane. Other evidence for membrane damage includes an increase in apparent free space, inhibition of plasmolysis, and a decrease in uptake and

incorporation of several organic and inorganic solutes (68). Increase in electrolyte loss appears to be a general phenomenon following infection (92). The alteration in permeability by HV-toxin is apparently non-specific, since leachates contained increased amounts of the organic and inorganic solutes usually present in plant cells (8). Thus, HV-toxin seems to create a hole in the plasma membrane whereby available solutes can escape to a medium of lower osmotic potential. However, a very early effect of toxin on the membrane does not prove that the initial lesion is in the membrane. Alteration of membrane characteristics could result from an initial lesion elsewhere.

Resistance to HV-toxin appears to be a passive or constitutive rather than an induced or dynamic characteristic (69). The simplest hypothesis is that resistant cells lack a functional receptor site (73). Others have suggested that resistance is based on toxin inactivation (62,90), or on some vague repair mechanism (94). There is much evidence against the idea that resistance is based on toxin inactivation. Also, the hypothesis of membrane self-repair, based on toxin-induced viral resistance in a non-host plant, does not seem to be consistent with data showing no correlation between level of metabolism and resistance (69,73).

Susceptibility to HV-toxin and to the toxins of H. carbonum and P. circinata, is controlled by one gene locus in each case. It seems reasonable that the gene product for susceptibility in each case is the toxin receptor. A receptor site for HV-toxin should be a molecule which interacts with toxin. However, we must have conclusive chemical proof that the receptor is located in the plasma

membrane before final conclusions about the site of action are made. The rapidity of the toxin responses and the absence of effects on isolated organelles (75) suggest that the site of action is in the plasma membrane, and all data are consistent with this hypothesis.

Little is known about the plant cell membrane, because of difficulties in isolation. Therefore, indirect approaches to determine the site of toxin action have been used. Uranyl salts, known to be membrane active (65), will counteract the effects of toxin on cells (27). However, toxin solutions bind uranyl ions (70), which complicates any interpretation of protective effects of uranyl against toxin. Other evidence indicates that uranyl ions bind to the membrane, stabilizing it and inhibiting the loss of electrolytes, regardless of the type of damage inducing the loss. Uranyl protection against toxin might indicate only that an early effect of toxin action, electrolyte efflux from host cells, is blocked by uranyl ion. The experiments with uranyl may tell us nothing about the site of toxin action.

Bisulfite, a carbonyl reactive compound, was reported by Scheffer and Pringle (73) to counteract partially the effects of toxin in seedling bioassay. This was later confirmed and certain other carbonyl reagents were shown to act in a similar manner (70). The fact that carbonyl reagents protect best when the reagent is added to the toxin solution is consistent with the known reversible nature of carbonyl complexes (11). Also, the data suggest that the inhibition of toxin action by carbonyl reagents is noncompetitive, which is consistent with evidence that a toxin receptor, rather than the toxin molecule, is affected (70). However, the protective effect of

carbonyl reagents is difficult to reconcile with our limited knowledge of the chemical composition and structure of the plasma membrane. Phospholipids and proteins do not contain highly reactive carbonyl groups. Furthermore, carbonyl reagents do not offer a way to covalently label affected sites, nor have they given unequivocal clues about the chemical nature of toxin receptor sites.

Experiments to locate conclusively the hypothetical receptor site in vivo are difficult for several reasons. First, toxin quickly disorganizes the cell, which could introduce artifacts into methods, such as autoradiography for detecting a labelled form of toxin in the cell. Second, toxin disappears in tissues, presumably because of instability. Third, adsorption of toxin to the cell has been difficult to detect by bioassay (73), suggesting that toxin does not bind tenaciously to receptor sites. Adsorption to few sites per cell may be sufficient for toxicity, and this may be beyond the limits of the bioassay. Indirect approaches in locating the receptor were used because of these difficulties. Certain sulfhydryl reagents [parachloromercuribenzoate (PCMB), parachloromercuriphenylsulfonate (PCMPS), and chlormerodrin] which do not readily permeate the plasma membrane, have been used to locate glucose transport proteins on the membrane of red blood cells (87). Unfortunately, the technique cannot be applied to the toxin receptor problem, since these reagents gave little or no protection against toxin. The failure of PCMB, PCMPS, and other mercurials to protect effectively may be due to a lack of permeation. Several other sulfhydryl reagents (NEM, DNFB, Iodoacetate) protect tissues against HV-toxin.

Experiments with cycloheximide effect gave some indication that

proteins may play a role in the toxin receptor site. Protection against toxin by cycloheximide pretreatments could be interpreted in terms of toxin receptor site turnover and depletion in the plasma membrane. The rapid development of protection 8 to 12 hr after exposure to cycloheximide is consistent with this interpretation, as is the reversal of protection. It is possible, however, that cycloheximide does not need to inhibit the synthesis of toxin receptor sites to protect against toxin; conceivably, cycloheximide could change membrane properties enough to affect the receptor sites. Evidence for turnover of membrane components is often difficult to interpret, since some components may be transiently located in the membrane yet perform an integral role in membrane function. The half-life of the protein in the plasma membrane of Mycoplasma laidlawii, for example, has been estimated to be 3 hr (35). In Amoeba proteus, turnover half-life of an antibody-specific membrane protein was 5 hr (47). On the other hand, turnover half-life of total protein in the rat liver endoplasmic reticulum was from 72 to 120 hr (52). Cycloheximide has been shown to inhibit other cellular processes (18); interpretation of the protective effect must be made with caution.

Protective effects of certain sulfhydryl-binding reagents against toxin also suggest that proteins are involved as toxin receptor sites. These protective reagents (14,23) do not react with or affect the toxin molecule. This is in agreement with our knowledge of the chemical composition of toxin. Noncompetitive inhibition of toxicity by sulfhydryl-binding reagents suggests that the receptor site is being affected. Reversibility of their effects suggests that

membrane damage is not a factor in the protection against toxin action. However, as mentioned in the case of cycloheximide, indirect alteration of receptor sites by membrane conformational changes could account for these results. The fact that membrane damage caused by many other treatments will not protect against toxin indicates that the membrane must be altered in a particular way, if indeed membrane conformation is responsible for protective effects.

The plot of toxin concentration versus toxin-induced electrolyte efflux is hyperbolic, which suggests an analogy between toxin-receptor interactions and substrate-enzyme interactions. If these data are plotted reciprocally, inhibitor effects can be examined for competitiveness with toxin. This is consistent with the enzyme analogy.

If a reagent inhibits an enzyme, two main possibilities are usually considered. First, the active site and the reagent may have reacted directly. Second, the conformation of the enzyme may have been altered, indirectly protecting the active site on the enzyme. The consideration with the toxin-receptor interaction is similar, except that the only concern is whether or not the receptor site or the conformation of the membrane as a whole (assuming a membrane receptor) is involved in the protective effect.

N-ethylmaleimide (NEM) reacts rapidly with sulfhydryl groups (61) and protects tissues against toxin. NEM protection can be countered to some extent by toxin breakdown products. This suggests a possible direct inactivation of the toxin receptor by NEM. Experiments with ^{14}C-NEM were designed to test this idea further. ^{14}C-NEM was previously used by Fox and Kennedy (22) to label membrane proteins

specifically involved on galactoside transport. Since this transport system is inducible, noninduced E. coli cells were used as controls. Two of Fox and Kennedy's experiments are of interest with respect to experiments with toxin. First, an in vivo double labelling procedure was developed to label only the specific sites for galactoside. Second, a membrane fraction from cells was used to label all except the sites specific for galactoside. Logically, one would expect that the experiment wherein only the specific sites were labelled would give higher differences between induced and control preparations than in experiments where all except specific sites were being labelled. However, Fox and Kennedy actually obtained a 23% reduction in NEM labelling of membrane preparations. This could be explained if non-specific sites were being protected by the galactoside. It does not necessarily mean that 23% of the sulfhydryl groups in the membrane are associated with the galactoside transport system.

Selective NEM labelling of toxin receptor sites required the use of an inactive but specific derivative of toxin, or a toxin breakdown product (TBP). Toxin breakdown products (TBP) are relatively inactive but appear to counter the effects of both toxin action and NEM protection against toxin. However, residual toxicity in TBP complicated the experiment with oat coleoptile cells. Therefore, membrane preparations from oat cells were considered as the best possible material for labelling experiments. Preliminary information from another laboratory (D. J. Morre, Purdue Univ.) (41) suggested that a particulate fraction sedimenting between 12,000g and 50,000g is enriched in plasma membrane fragments. I have used such preparations from susceptible and resistant leaf tissue in ^{14}C-NEM labelling

experiments. Theoretically, if NEM reacts with the toxin receptor site, ^{14}C-NEM labelling of membrane fragments from susceptible but not resistant cells would be reduced in the presence of toxin or TBP.

The results of many experiments indicated that differential effects occurred in ^{14}C-NEM labelling of membrane-enriched preparations from susceptible and resistant plants. Reduction of labelling by toxin or TBP in susceptible membrane preparations was 10 to 25%, whereas little or no reduction occurred with resistant preparations. The unexpectedly high labelling reduction in susceptible preparations suggests that toxin or TBP affect more than just the toxin-specific sites, as was suggested for effects of galactoside on NEM labelling in Fox and Kennedy's experiment. If this finding can be confirmed by other methods, it will be the first in vitro effect discovered for toxin. However, identification of plant cell membranes with biochemical markers will be necessary to determine whether or not the effect is actually associated with sites on the plasma membrane.

A more difficult problem is to label toxin-specific sites with NEM, rather than protecting them, as described above. Direct labelling would be more desirable than the NEM procedure for identifying or isolating a receptor substance. This aspect of the Fox and Kennedy experiment was not completed successfully. An alternative labelling procedure described by Kolber and Stein (37) for the galactoside transport system might be applicable to the toxin system. This would require isogenic lines of oats differing only in the gene locus for toxin susceptibility. Susceptible cells could be labelled with ^{3}H-amino acid, and resistant cells with ^{14}C-amino acid. The cells would then be homogenized, the homogenates from resistant and

susceptible cells would be mixed and fractionated for proteins. A high $^{3}H/^{14}C$ ratio in a protein would indicate that it is related to the gene for susceptibility.

The finding that esterification causes toxin to lose activity and counteracts the toxin molecule is of considerable interest. The reduction of toxicity may be related to competition between esterified toxin and unaltered toxin for receptor sites. More data are needed to establish this. The results suggest that toxin activity depends on carboxyl groups whereas adsorption to the toxin receptor site does not require carboxyl groups. Toxin analogs could be useful in future studies with toxin.

The evidence presented here suggests that the toxin receptor consists of a protein or that proteins are associated with the receptor site. This does not exclude the possibility that lipid molecules may be involved. In fact, an hypothesis based on lipoprotein receptors would be consistent with our knowledge of membrane proteins, including membrane ATPase (47,80), acetylcholine receptor protein (49), and the M (galactoside transport) protein (22). Conclusive evidence for or against a lipoprotein receptor could come from a knowledge of the solubility characteristics of the toxin receptor, once it is located and at least partially characterized. The <u>in vitro</u> NEM experiment described above may provide a way of identifying receptor sites. However, these studies must await further knowledge of plant cell membranes and their isolation.

SUMMARY

HV-toxin disrupts the permeability barrier in susceptible but not resistant cells. A bioassay based on loss of electrolytes was used to determine the nature of the interaction of toxin with hypothetical receptor sites. Cycloheximide and sulfhydryl-binding compounds (for example, N-ethylmaleimide) decrease the sensitivity of tissue to HV-toxin, indicating that the receptor may be a protein or closely associated with a protein. ^{14}C-NEM binding studies indicated that toxin and its breakdown products can reduce NEM labelling in particulate fractions which are thought to contain plasma membrane fragments. Such preparations from resistant cells did not bind with toxin. Esterified toxin is inactive, which suggests that free carboxyl groups on the toxin peptide are required for toxicity. The carboxyl groups do not appear to be required for toxin adsorption to receptors, since esterified toxin counteracts the effects of active toxin. The results suggest that toxin interacts with a specific proteinaceous receptor in the plasma membrane.

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LITERATURE CITED

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Part II

Experiments with *Periconia Circinata* Toxin

INTRODUCTION

Periconia circinata (Mangin) Sacc., a pathogen of grain sorghum, produces several related toxic substances (referred to herein as PC-toxin) with properties not unlike those of Helminthosporium victoriae toxin (25). Thus comparative studies of both substances could be meaningful. There is a distinct possibility that both PC-toxin and HV-toxin have their primary site of action in the plasma membrane, and therefore the same basic questions can be asked about their sites and mechanisms of action.

PC-toxin and HV-toxin are very specific for certain cultivars of sorghum and oat, respectively. Both cause loss of materials from sensitive cells and inhibit various metabolic processes. Both appear to act by simple physical interaction with cellular receptors. These effects can be compared to those described by Thatcher, who first proposed that alteration of cell permeability is important for successful host-parasite interactions (28). Data with host-specific toxins would seem to support Thatcher's hypothesis, although his hypothesis was originally formulated for obligate parasites.

Helminthosporium carbonum toxin (HC-toxin) differs significantly from HV and PC-toxins in its effects on plant tissues. HC-toxin is host selective, but susceptible and resistant corn do not differ in tolerance to the degree expressed by HV and PC-toxins. HC-toxin increases growth and uptake of solutes at low concentrations, and

apparent uptake of toxin is inhibited under anaerobic conditions (12, 33). However, evidence again suggests that the primary site of action may be the plasma membrane (33). There is little or no information on the mechanism of action of the 5 other known host-specific toxins.

Previous data have shown that PC-toxin causes increased respiration, inhibition of solute uptake and incorporation, and electrolyte leakage from susceptible tissues within 5 hr of toxin application (13). However, effects similar to those of HV-toxin on cell wall-free protoplasts (25) were not observed (13). It was also evident that PC-toxin has less drastic effects on susceptible tissues and is much less toxic per unit weight than is HV-toxin.

It is logical to believe that there are distinct receptor sites for both toxins. The specificity of both toxins is based on one gene differences between resistant and susceptible hosts (25,26). Presumably, the hypothetical receptor is in each case a product of the gene for susceptibility. However, toxin receptor sites must be located and identified chemically before we can fully understand the mechanism of toxin action. In the cell, the receptor may be able to transmit and amplify the initial interaction with toxin, thereby destroying the integrity of the plasma membrane. In vitro, the receptor should interact with toxin; with the correct techniques, we should be able to detect this interaction.

My investigation was based on the premises stated above. The questions asked were: (1) what is the location and chemical nature of the toxin receptors? ; (2) what is the primary effect of toxin on the cell? ; (3) what is the mechanism of toxin action? In effect,

these questions put to test the receptor hypothesis originally stated by Scheffer and Pringle (23) for HV-toxin.

My data do not give complete and unequivocal answers to these difficult questions, but do provide indications which may be a foundation for further work. Much preliminary work was necessary in order to develop the tools and to gain perspective for the problem. The effects of toxin on membrane functions, on cellular metabolism, and on organelles were studied to narrow the possibilities. The nature of susceptibility and resistance to toxin was studied in various ways, and the toxin itself was altered in the hope of gaining some clue to its interaction with sensitive tissue.

In pathogenesis, one event leads to another until confusion exists. Too often the secondary effects lead to wrong interpretations. The host-specific toxins are valuable not only in eliminating the second party, the pathogen, but they also strengthen the hypothesis that pathogenesis has a chemical basis, and that a single compound may be a key to understanding pathogenesis. Host-specific toxins are herein considered as models for the study of disease development and disease resistance.

LITERATURE REVIEW

Much of the information on PC-toxin is discussed in recent reviews (24,25). Some of the literature pertaining to HV-toxin is also pertinent to the study of PC-toxin and will not be repeated here. Several publications on the chemistry of PC-toxin (15-18) and a thesis on the physiological effects of PC-toxin (13) will form the basis of this review.

P. circinata causes the Milo disease of grain sorghum, first recognized in the United States in 1924. However, until 1948 the disease was thought to be caused by Pythium arrhenamanes (11), even though this fungus infected both resistant and susceptible varieties of sorghum. Leukel (11) clarified this and observed that a hypothetical selectively toxic principle was left in sand by the fungus. Scheffer and Pringle (22) extracted the toxin from culture filtrates and characterized it as a low molecular weight peptide (15). Like HV-toxin, PC-toxin had no effect on many non-host plants, even at high concentrations. Resistant sorghum plants tolerated at least 26,000 times higher concentration of toxin than were required to affect susceptible plants (13).

PC-toxin was absorbed on Norite-Celite columns and was eluted by aqueous pyridine. The toxin was further purified by crystallization, ion exchange chromatography, and countercurrent distribution (17,18). Some batches of culture filtrates gave two distinct host-specific

toxins on countercurrent distribution. The first, PC-toxin A, was found to have an empirical ratio of 2 serine: 2 glutamic acid: 4 aspartic acid; 6 alanine. The molecular weight is less than 2000 as judged by molecular seiving, which implies that the toxin contains fourteen amino acids (17). The crystalline toxin affects susceptible roots at 0.1 μg/ml. More recent evidence suggests several variants of PC-toxin, apparently all host specific (18). PC-toxins A and B are known to contain free amino groups (unpublished information from R. B. Pringle).

PC and HV-toxins have several effects in common. Both induce increases in respiration (13,25). Mansour (13) did not report the exact time required for PC-toxin to cause an increase in respiration, but apparently the increase occurred earlier than one hr after toxin exposure. However, there was no effect of PC-toxin on succinoxidase activity by isolated mitochondria. The time required for PC-toxin to induce electrolyte efflux was not determined. Uptake and incorporation of amino acids and uridine by susceptible tissue was decreased after toxin treatment. Thus it was apparent that PC-toxin has effects similar to HV-toxin, but less drastic. However, several differences were noted. In contrast to HV-toxin, PC-toxin could be recovered from susceptible and resistant plants. Also, PC-toxin had negligible effects on susceptible protoplasts (13). Apparent uptake of PC-toxin but not HV-toxin by susceptible cells was not affected by temperature (13,23).

A single gene pair controls resistance and susceptibility to PC-toxin (26). This is similar to the case with HV-toxin, for which the dominant host Vb allele is in control of toxin resistance and

susceptibility. In both cases, susceptibility to the disease and to the toxin have the same genetic basis.

The multiple toxins of P. circinata may be traced to variability in the fungus. The fungus isolates may not be genetically uniform, because it has not been possible to germinate the conidia. Isolates were derived from single conidiophores, and the nuclear condition of the heterocaryotic fungus is unknown. Each isolate may be producing a slightly different toxin. Another possibility may be that toxins are produced under different conditions or times in the growth cycle. In any case, the several forms of PC-toxin probably have the same site of action since susceptibility to them is controlled by a single gene locus. For convenience, I will refer to these toxic compounds by the collective name PC-toxin.

MATERIALS AND METHODS

Host Plants and Fungus Cultures: Sorghum cultivars resistant (cv. RS-610, and a resistant Colby selection) and susceptible (cv. Colby) to P. circinata and to its toxin were used. Seeds were germinated at 30 C between moist filter paper in petri dishes, or in vermiculite in trays at 22-24 C. Seedlings were grown for 20 days or less in White's nutrient solution (31) under fluorescent lights (100 to 200 foot-candles) in the laboratory.

Toxin was obtained from a highly virulent strain of P. circinata grown for 3 weeks on a modified Fries No. 3 basal medium supplemented with 0.1% Difco yeast extract (22). The fungus was grown in stationary cultures in Roux bottles containing 200 ml medium at 22 C.

Purification and Isolation of PC-toxin: Cultures were harvested by filtration after 3 weeks growth. The filtrate was concentrated in vacuo, deproteinized with methanol, and absorbed on a Norite-Celite column (15). Toxin was eluted from the column with a 10% pyridine solution. After removal of the pyridine in vacuo, the aqueous toxin solution was stored at 5 C. Unless otherwise mentioned, the Norite-Celite toxin eluate was used for experiments. This preparation completely inhibited root growth of susceptible plants at 0.1 μg/ml. For some experiments, toxin was further purified by gel filtration with Biogel P-2 (200-400 mesh), Sephadex G-10, or Sephadex G-15.

Two ml of the toxin-containing Norite-Celite eluate was placed on a 1.5 x 25 cm column, and eluted with distilled water. Five ml fractions were collected: fractions 5 through 7 had maximum toxin activity. Fractions were diluted and assayed by the standard seedling bioassay (22), or by a conductance assay based on the ability of the toxin to induce electrolyte leakage from susceptible leaf tissue. Dry weights were determined and the toxin was stored at -20 C without significant loss of activity. Elution of toxin from Sephadex G-15 with phosphate buffer (0.05 M, pH 6.4) rather than water gave two toxic peaks.

Toxin Assays: The standard assay was based on the ability of toxin to inhibit seedling root growth (22). Seeds were germinated between moist filter paper for 24 hr at 30 C. Serial dilutions of toxin solutions were made with distilled water or White's solution. Five ml of each dilution were placed in 60 x 15 mm Petri dishes with 5 germinated seeds. The dilution endpoint, determined after 2 to 4 days at 22 C, is defined as the highest toxin dilution which restricts the growth of susceptible roots to 1.0 cm or less. Controls were resistant seedlings in toxin solutions as well as susceptible seedlings in water. Control roots grew 4 to 8 cm long during this time.

An assay was developed to measure the ability of toxin to induce electrolyte loss from susceptible leaf tissue. Leaf tissue (0.5-1.0g) from 6 to 20 day old sorghum seedlings was sectioned into 1 cm pieces, enclosed in cheesecloth, and suspended in 50 ml toxin solution in 125 ml flasks. Flasks were placed on a reciprocal shaker at 70-100 strokes/min. After toxin treatment, tissue samples were

washed 4 or 5 times, using 100 ml distilled water each time, for a total wash time of 10 min. Tissues were then suspended in 50 ml distilled water (conductance approximately 1 μmho) for up to 8 hr. Conductivity of the ambient solution was measured at intervals with a model 16B1 Industrial Instruments conductivity bridge, using a dip type cell with a constant of 0.1 for solutions below 200,000 ohms and a constant of 0.1 for solutions above 200,000 ohms. Specific conductivity was expressed as reciprocal ohms. In all experiments, conductance values obtained from water control samples were subtracted from values for toxin-treated samples to give the toxin-induced electrolyte efflux, which is proportional to toxin concentration within a given range. Percent protection was calculated, for experiments with toxin inhibitors, as protection against toxin-induced loss of electrolyte. The term is defined in the section on HV-toxin.

Amylase Production by Aleurone Cells: Gibberellic acid-induced amylase production in sorghum aleurone cells was determined, as outlined by Jones and Varner (7) for barley aleurone layers. Embryos and 80 to 90% of the endosperm were removed from sorghum seeds with a sharp corner of a single edge razor blade. The embryoless seeds were sterilized in 1% hypochlorite for 20 min, rinsed thoroughly in sterile distilled water, and transferred to sterile moist sand in Petri dishes covered with aluminum foil. Aseptic conditions were used throughout. After 48 hr imbibition on sand at 22 C, the embryoless seeds were transferred to 50 ml Erlenmeyer flasks containing gibberellic acid (1 μM), chloramphenicol (30 μg/ml), $CaCl_2$ (10 mM), Na acetate (pH 4.8, 1.0 mM), and PC-toxin (1.0-100 μg/ml of a preparation

that had a dilution endpoint 0.1 μg/ml). Ten embryoless seeds per flask were incubated on a reciprocal shaker (70-100 strokes/min) for 8, 12, 16, 24, and 30 hr at 22 to 26 C. The medium was then decanted into test tubes; in all experiments, this medium had negligible amylase activity. Embryoless seeds were then ground in a mortar with a pinch of sand and 1.0 ml 0.2 M NaCl. The thick paste was mixed with 4.0 ml additional NaCl solution, and the resulting solution was centrifuged at 5000g for 10 min. The extract or supernatant was assayed for amylase activity.

The α-amylase activity was measured, using a conversion factor of 2.7 μg α-amylase per unit O.D. change based on purified malt α-amylase (7). From 0.05 to 0.5 ml of the extract or medium was diluted to 1.0 ml with water. The assay involved adding 1.0 ml starch solution to the test solution and terminating the reaction after 5 to 10 min with 1.0 ml IKI reagent. The mixture was diluted with 5 ml water and the blue color which developed was measured with a Klett photometer, using a no. 62 filter. The following equation was used to convert O.D. units into μg α-amylase:

$$\mu g\ \alpha\text{-amylase} = \frac{\text{O.D. x (ml total extract) x 2.7}}{\text{time of reaction (min) x ml extract added}}$$

Preparation of Mitochondria: Grinding and washing buffers for the isolation of mitochondria were those used by Bonner (1). The grinding buffer contained 0.3 M mannitol, 1.0 mM EDTA·Na_2, 0.1% Bovine Serum Albumin (BSA), 0.05% glutathione (GSH), and 0.05 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes-NaOH, pH 7.2). The washing buffer was the same except for the omission of GSH. Approximately 100 g etiolated sorghum leaf tissue, from 5 to 7 day old

plants, was ground in a porcelain mortar with grinding buffer (2 ml/g tissue) and a trace of washed sea sand. Total grinding time was no more than 10 min. After filtration of the homogenate through 4 layers of cheesecloth, the homogenate was centrifuged at 1000g for 15 min and the resulting supernatant was centrifuged at 10,000g for 15 min. The resulting pellet was resuspended in an equal volume of wash buffer and this was centrifuged at 6000g for 15 min. The pellet was resuspended in 0.25 M sucrose (enough for approximately 10 flasks, 0.4 ml/flask). All manipulations were at 3 C.

Respiratory Measurements: Standard manometric techniques were followed in measuring gas exchange (29). To measure respiration of seeds imbibing water or toxin, duplicate batches of 50 seeds each were allowed to imbibe in 2 ml solution in Warburg flasks. At intervals, stopcocks were closed and oxygen uptake was measured for 30 min at 30 C. Equilibration time was 10 min.

In experiments with isolated mitochondria, each Warburg flask contained 2.0 ml total volume reaction mixture with and without additions, including 0.4 ml mitochondrial suspension. Flasks were chilled in the coldroom and the following substances were added, with the final concentrations as indicated: substrates (potassium salts of succinic acid or alpha-ketoglutaric acid), 0.25 M; sucrose, 0.2 M; KH_2PO_4, 0.07 M; $MgSo_4$, 0.0075 M; $ATP{\cdot}Na_2$, 1 mM; cytochrome C, 0.1 mM; NAD, 0.13 mM; TPP, 0.13 mM; CoA, 0.013 mM; malonate, 0.025 M; and PC-toxin, 1.0 to 200 μg/ml. The toxin preparation had a dilution endpoint at 0.1 μg/ml in a seedling root growth assay. For P:O ratio determinations, 0.2 ml hexokinase solution (2 mg/ml in 0.5 M glucose)

was added to the side arm and tipped into the reaction mixture after 5 min equilibration time. Ten min equilibration was used for succinoxidase measurements. Readings were taken every 10 min and the temperature was 30 C.

Phosphorylation efficiency (P/O ratio) was measured by the uptake of orthophosphate from the reaction mixture. After 45 min of oxygen uptake, 2.0 ml cold 10% TCA was added to the reaction mixture in each flask and a 2.0 ml sample was removed and centrifuged to remove precipitates. A sample (1.0 ml) was diluted with 9.0 ml water. From the resulting solution, 0.2 ml samples were diluted with 8.4 ml water, to which was added 1.0 ml 2.5% ammonium molybdate and 0.4 ml 0.25% aminonaptholsulfonic acid. After 15 min, the color which developed was measured with a Klett photometer using a no. 66 filter (4). Duplicate samples were used for each Warburg flask. Separate flasks were used to determine phosphate at the beginning of each experiment; after equilibration, TCA was added before hexokinase was tipped in. Pi uptake was expressed as the difference in μMoles of Pi per flask at the beginning and end of the experiment. A standard KH_2PO_4 solution was used to calibrate the Pi uptake (μMoles/flask). This value was divided by the number of μ atoms of oxygen respired to give a P/O ratio.

Measurement of Electropotentials in Single Cells: See Materials and Methods, HV-toxin section.

Incorporation of Amino Acids by Nuclei: See Materials and Methods, HV-toxin section.

RESULTS

Effect of PC-toxin on Membrane Function: Efflux of electrolytes from susceptible tissue into distilled water is the most rapid and direct test found to date for membrane damage by HV-toxin. Therefore, similar studies were conducted with PC-toxin. Mansour (13) showed that tissues treated with PC-toxin lost more electrolytes than controls within 4 to 5 hr. She did not test for earlier effects.

Resistant and susceptible sorghum leaves were cut into 1 cm pieces, enclosed in cheesecloth, leached in distilled water for 3 hr, and then treated with a high concentration of toxin for 7 min. After a short rinse, samples were leached in 50 ml distilled water. Conductivity of the leaching solution was measured at 2 to 4 min intervals with a conductivity bridge and electrode. The first toxin-induced increase in electrolyte loss was detected 15 min after first exposure to toxin, or 6 min after being placed in the final leaching solution (Figure 19). Resistant tissues were not affected.

Experiments with a wide range of toxin concentrations showed that the ability to induce loss of electrolytes can be used to estimate toxicity on a quantitative basis. When conductance (μmhos) of ambient solutions was plotted against log or linear toxin concentrations (Figure 20, bottom), curves similar to those observed with HV-toxin were obtained. A reciprocal plot (Wolff plot) of the data gave a straight line (Figure 20, top). Estimations of Km, or apparent

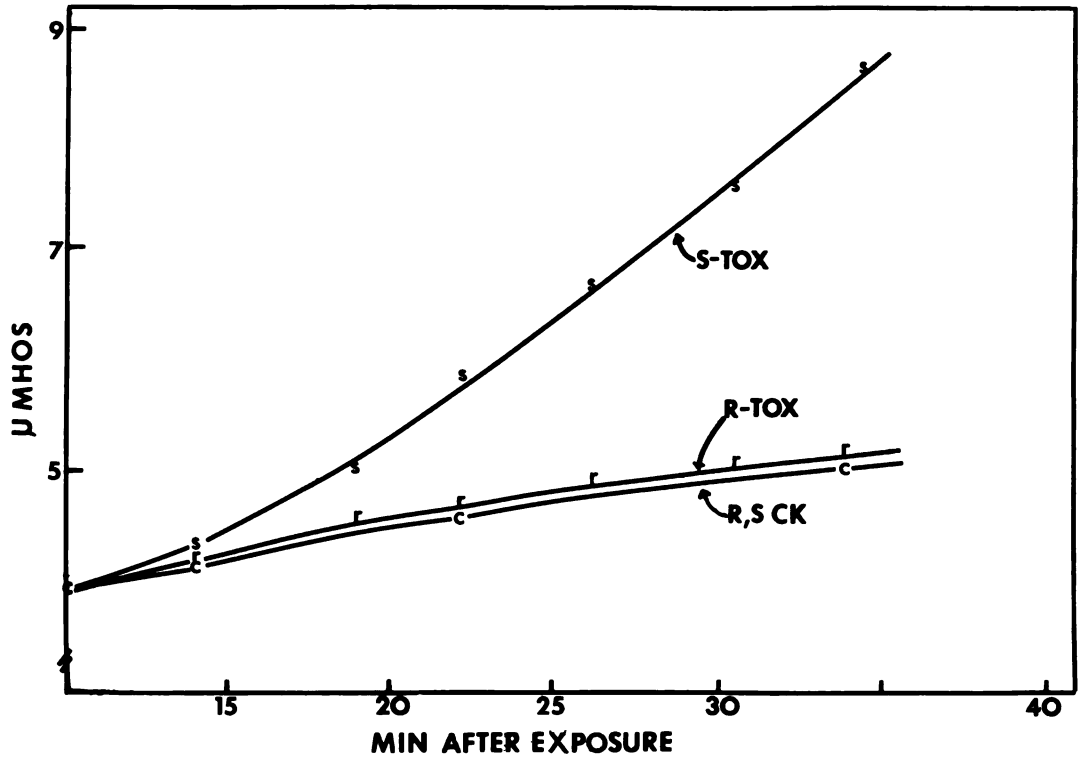

Fig. 19. Time course of electrolyte efflux induced by PC-toxin. Two g each of susceptible (S, cv. Colby) and resistant (R, cv. RS-610) leaf tissue were leached in distilled water for 3 hr, infiltrated in vacuo with toxin (400 μg/ml), rinsed in several changes of distilled water, and placed in 50 ml distilled water 9 min after first exposure to toxin. CK indicates controls without toxin.

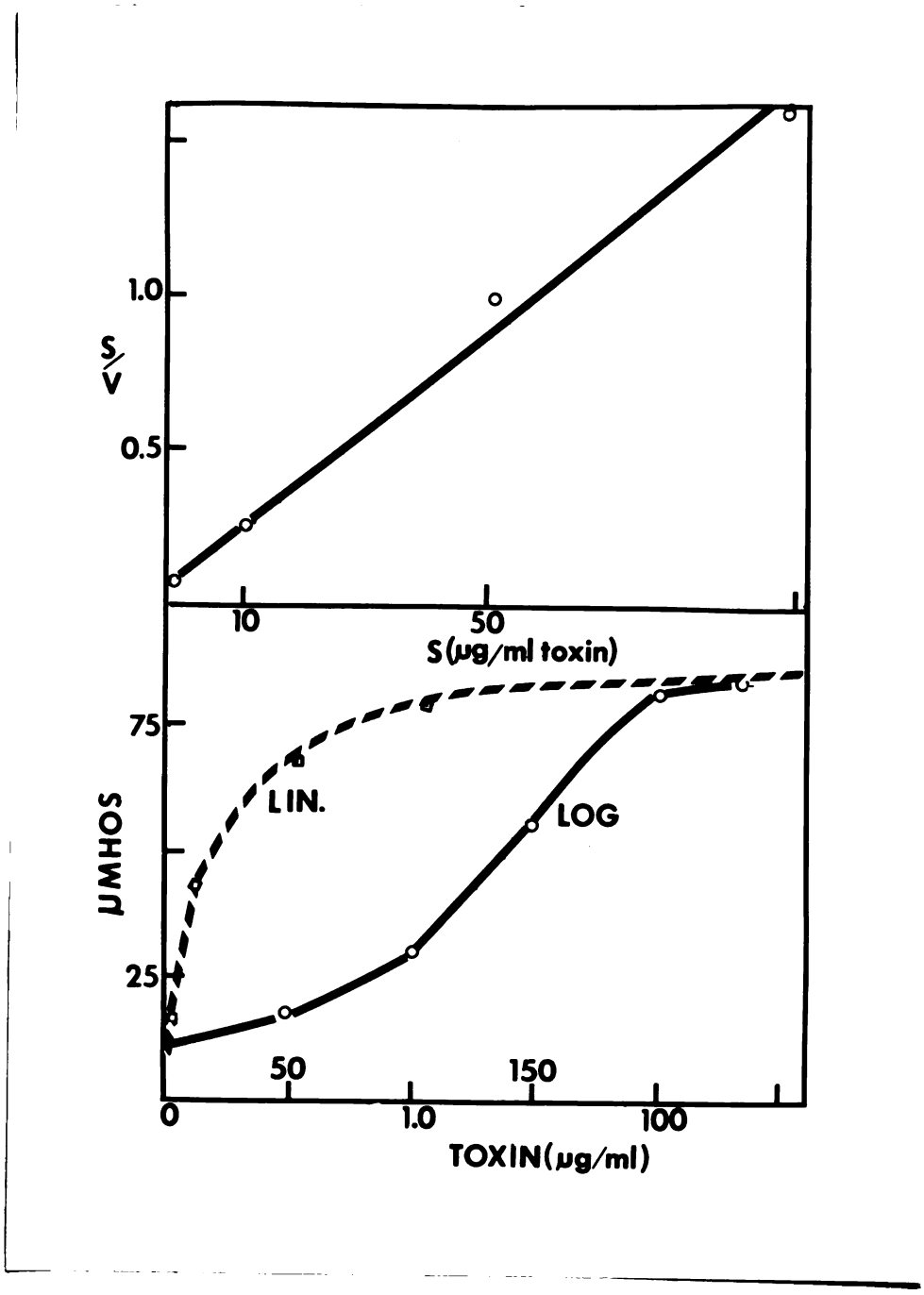

Fig. 20. The effects of PC-toxin at several concentrations on loss of electrolytes from sorghum leaves. Young leaf tissue (900 mg) was treated with PC-toxin for 3.5 hr, rinsed several times in distilled water, then leached for 6 to 8 hr in 50 ml distilled water for conductance measurements. Upper diagram is a Wolff plot of S (toxin conc.) versus S/V (V=µmhos conductance). In the lower diagram, conductance of ambient solutions is plotted against log or linear toxin concentrations. The toxin preparation completely inhibited susceptible seedling root growth at 0.1 µg/ml.

affinity of toxin for its receptor, gave values from 0.1 to 1.0 μM. Data were plotted by the Hill method, resulting in a straight line with a slope of less than one (Figure 21). This might suggest that there is only one type of site for toxin (27).

An assay for toxin based on the ability to induce loss of electrolytes has several advantages over the seedling bioassay. It is at least as sensitive as the seedling bioassay, and the results are not complicated by possible toxin inactivation in long-term assays, and by long-term effects on growth and metabolism. The relationship between toxin concentration and electrolyte efflux is direct, suggesting that the primary toxin lesion, if not in the plasma membrane per se, must be very closely associated with membrane integrity. In all these respects, PC-toxin appears to be similar to HV-toxin.

As with HV-toxin, much of the PC-toxin-induced efflux of electrolytes consists of potassium. A toxin-induced change in sodium efflux was not detected (Figure 22). The rapid efflux of potassium, the most available cation in the cell, suggests that electrolyte efflux is nonspecific. This should be confirmed, however, by a complete ion analysis of leachates.

Electropotentials across the plasma membrane were measured in single cells of toxin-treated sorghum coleoptiles as a possible indication of toxin damage to the membrane. Sorghum coleoptile cells which gave a stable membrane potential (-100 to -150 mv) were placed in a perfusion chamber and exposed to a steady flow of toxin solution (80 μg/ml). In no case was there an indication of a rapid or drastic effect of toxin on the potential (Figure 23). In one case, the potential did not increase in the presence of toxin for more than 45 min.

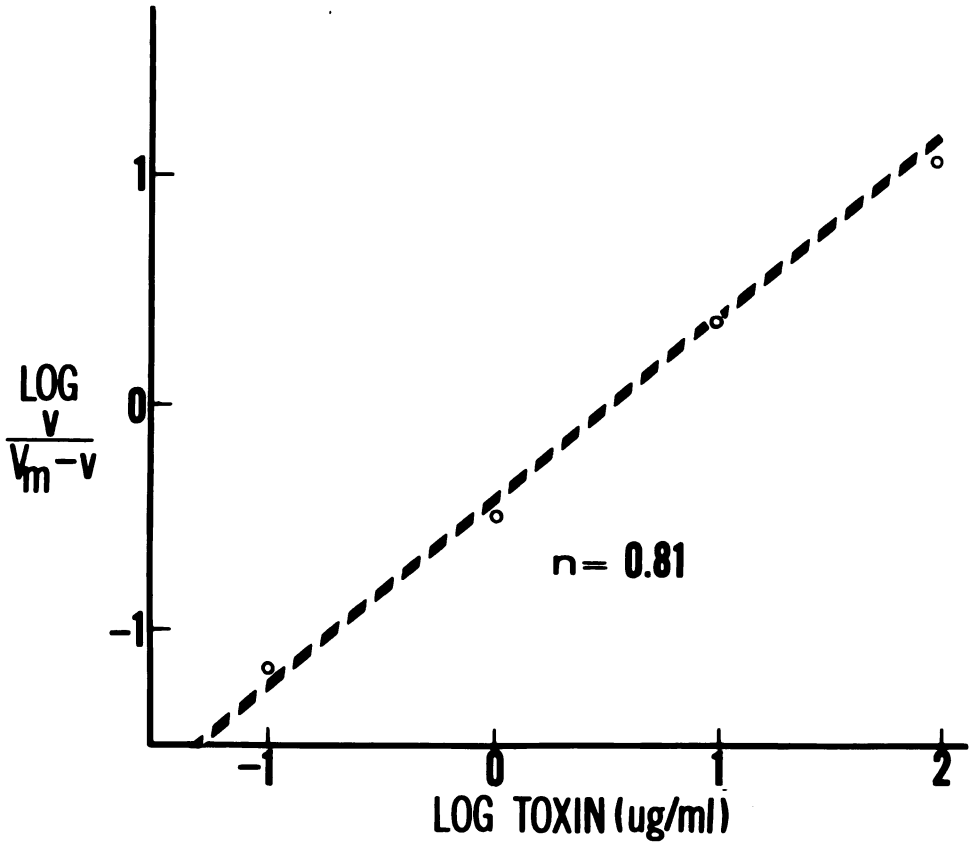

Fig. 21. Hill plot of data from Fig. 20. v = conductivity values (μmhos) for preparations treated with toxin. V_m = max. conductivity values (μmhos) for preparations treated with saturating levels of toxin. n = slope (y/x), or interaction coefficient. The slope gives an indication of the number of types of receptor sites.

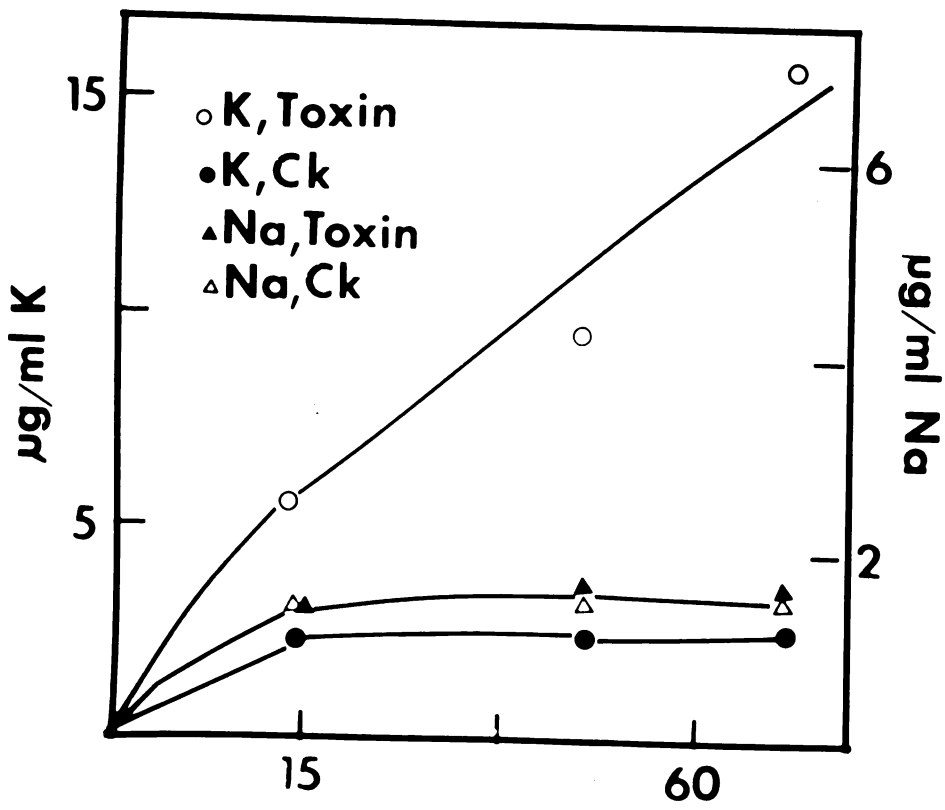

Fig. 22. Loss of Na and K from PC-toxin-treated sorghum coleoptiles. Two g coleoptile tissue (from 5 day old seedlings) for each sample was enclosed in cheesecloth and floated on White's solution for 2 hr. Samples were then rinsed with distilled water and suspended in 50 ml PC-toxin solution (80 µg/ml) for 45 min. Samples were then rinsed and leached in 50 ml distilled water. Leachates were examined for Na and K content by flame photometry.

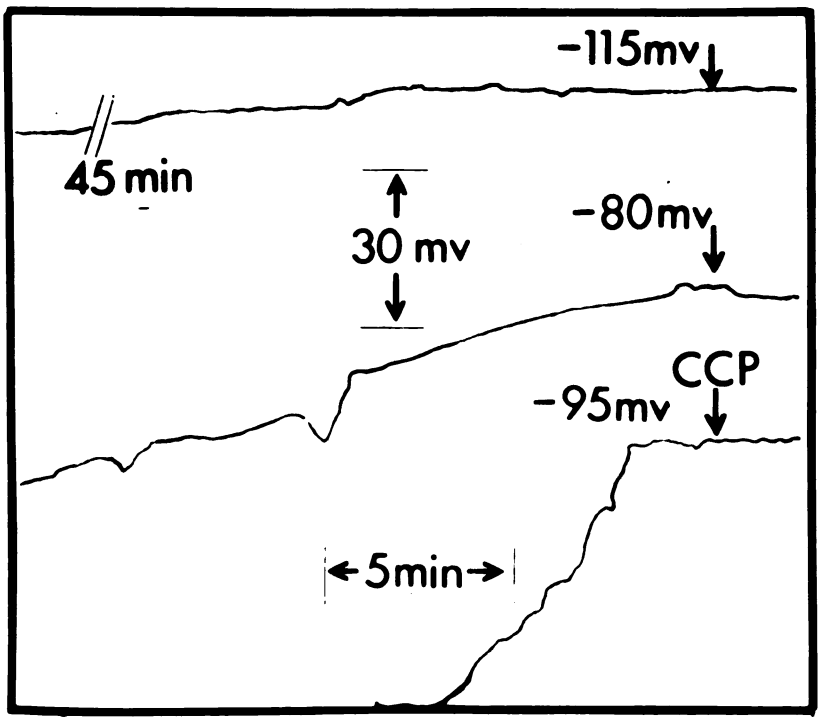

Fig. 23. The effect of PC-toxin (80 μg/ml) and m-ClCCP (20 μM) on membrane electropotentials in 3 single cells of susceptible sorghum coleoptiles. Potentials were recorded from right to left. After potentials had stabilized for 5 min, toxin or m-ClCCP was added at the times indicated by arrows (right). Bathing solution was standard 1X solution. Control potentials showed a relatively straight line.

There were not enough successful penetrations of these cells to eliminate the possibility of more subtle effects of toxin. The results are similar to those obtained with HV-toxin. Cells exposed to the toxins appeared to have more breaks than the control cells in the seal of the membrane around the electrode, although no data were collected on this. If either toxin had affected electrogenic pumps associated with the membrane, potentials should have dropped precipitously. In contrast to toxin, an uncoupler of oxidative phosphorylation, m-ClCCP, caused a rapid drop in potential within 3 min (Figure 23).

The most sensitive test for toxin-induced changes in the membrane integrity still appears to be a change in the efflux of electrolytes from treated cells. This is the basis for a useful assay. Changes in membrane properties which come later are questionable for evaluating the primary site of toxin action. Conclusive evidence for a primary effect of toxin in the membrane might be obtained with cell-free membrane preparations from sorghum.

Some Effects of PC-Toxin on Metabolism and on Subcellular Organelles: Effects of PC-toxin on various metabolic systems have been noted previously (13). I have examined further the possibility of direct effects of PC-toxin on protein synthesis, mitochondrial oxidation, phosphorylation, and uptake and incorporation of amino acids by nuclei.

An inducible enzyme, α-amylase, was used to determine effects of PC-toxin on synthesis of a protein. With this system, synthesis can be measured without the complication of amino acid uptake systems. Cycloheximide, a known protein synthesis inhibitor, was used as a

control treatment. Embryoless seeds of susceptible sorghum were sterilized with hypochlorite solution and allowed to imbibe water on moist sterile sand. Gibberellic acid (1 μM) was added to the incubation medium to induce synthesis of α-amylase, and the preparation was incubated for 8 to 30 hr. Toxin was added at various times and samples were thereafter withdrawn for enzyme assay. Results showed that amylase activity remained in the seed during the first 30 hr. High concentrations of toxin (30 μg/ml) did not affect amylase synthesis with less than 6 hr treatment. Cycloheximide, however, halted synthesis within 30 min (Figure 24). The results indicate that an effect of PC-toxin on protein synthesis (13) may not be a direct effect such as that caused by cycloheximide.

Earlier data (13) showed no effect of PC-toxin on activities of isolated mitochondria. An attempt was made to confirm this finding, and to extend it by using higher concentrations of toxin. Mitochondria were isolated as described previously (1) and incubated in the usual reaction mixtures with and without toxin. Succinic acid and α-ketoglutaric acid were used as substrates. PC-toxin at high concentrations (230 μg/ml) did not affect oxidation of these substrates by mitochondria, whereas DNP (10 μM) caused a slight increase in oxidation (Figure 25). Phosphorus uptake by mitochondria was measured, using α-ketoglutarate as the substrate. The cyclic reaction beyond succinate were inhibited by adding malonate to the reaction mixture, and hexokinase in glucose was used to couple the system so that ATP was not accumulated. Results showed much variability from flask to flask, but there was no indication that toxin affected phosphate uptake. Again, this confirms the results of

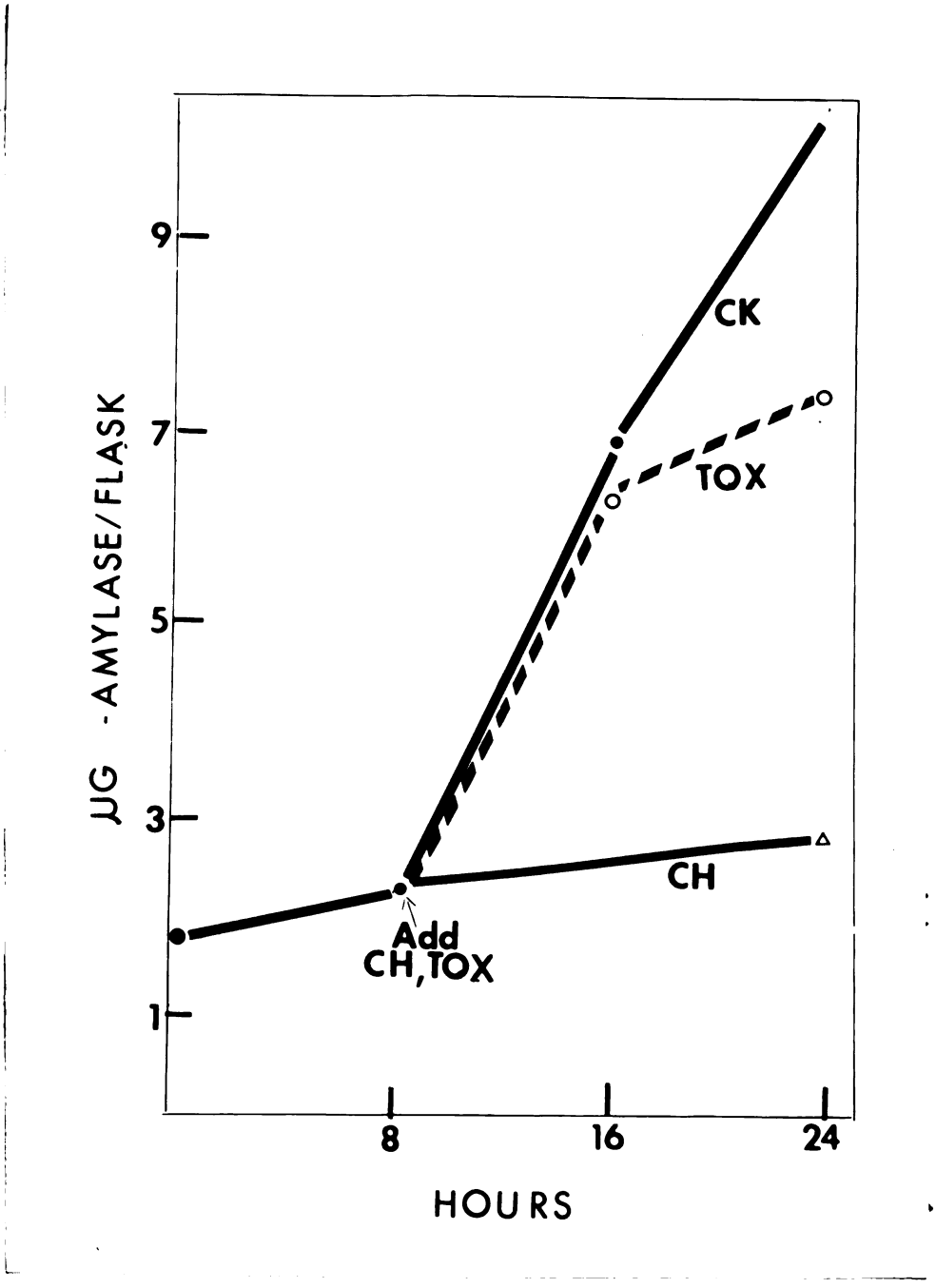

Fig. 24. Effects of PC-toxin (Tox) and cycloheximide (CH) on gibberellic acid (GA) induction of α-amylase in sorghum aleurone cells. Ten embryoless seeds were allowed to imbibe on moist sterile sand for 48 hr, then were transferred at 0 time to sterile media containing GA (1 μM), $CaCl_2$, (10 mM), Na acetate, (1 mM), and chloramphenicol, (30 μg/ml). Toxin (30 μg/ml) or CH (5 μg/ml) was added as indicated. Toxin had no effect on aleurones from resistant seeds. There was little or no amylase activity in the medium. CK indicates control without toxin or CH treatment.

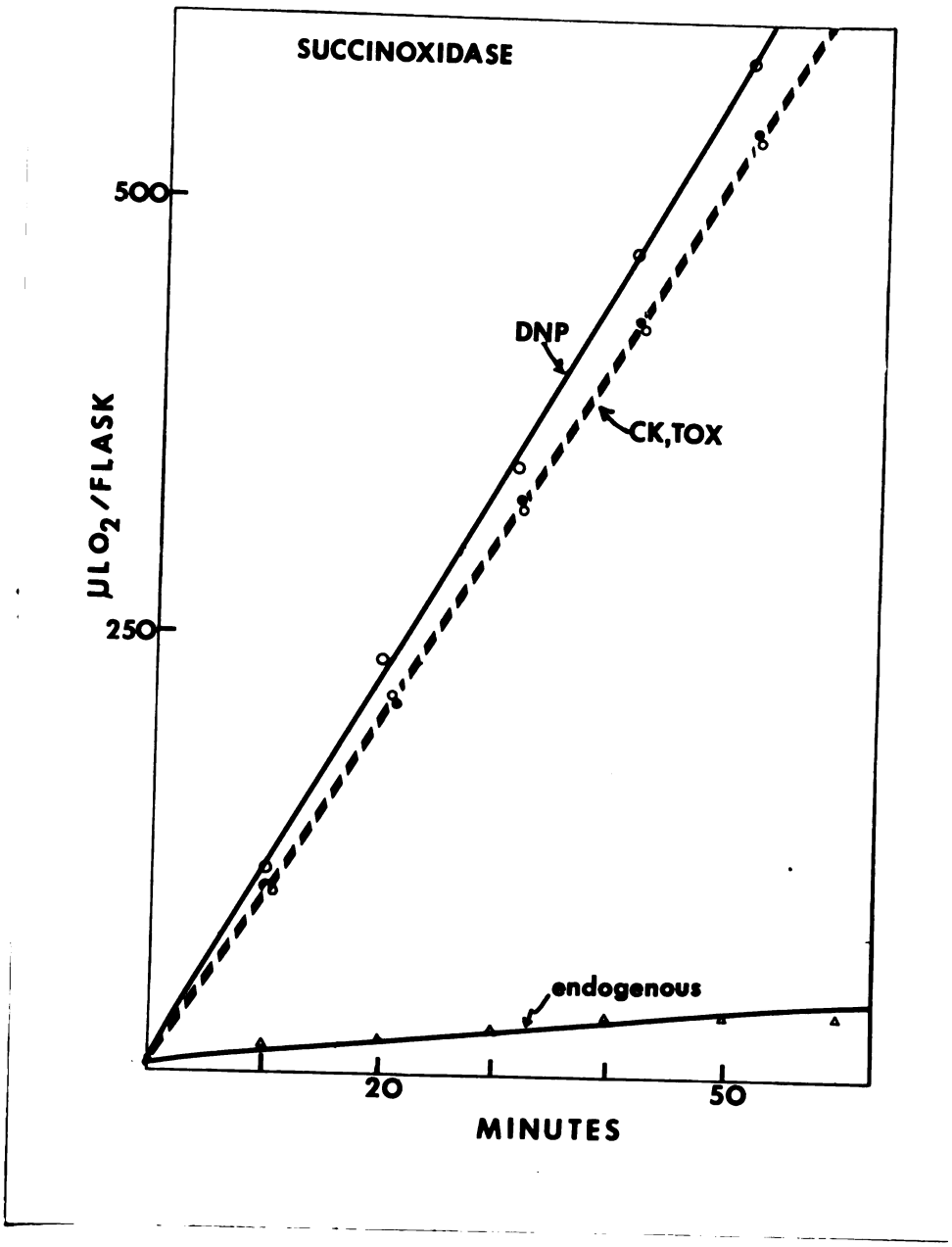

Fig. 25. Succinoxidase activity of isolated mitochondria, with and without PC-toxin. Concentration of toxin (tox) was 230 μg/ml, and 2,4-dinitrophenol was 10 μM. CK indicates control without toxin or DNP. The reaction mixture contained the following, in μmoles/flask: sucrose, 400; potassium succinate, 50; KH_2PO_4, 15; ATP·Na_2, 2; cytochrome c, 0.2; DPN, 0.26; TPP, 0.26; CoA, 0.026; mitochondrial suspension in 0.25 M sucrose, 0.4 ml. Total volume per flask was 2.0 ml. Temperature was 30 C.

Mansour (13). The respiratory increase induced by PC-toxin in intact tissue (13) appears to be some indirect or secondary effect of toxin.

Nuclei have uptake systems for amino acids (8), and such systems should be sensitive to the disruption of the nuclear membrane. A possible effect of PC-toxin on uptake and incorporation of amino acids by nuclei was measured. Nuclei were isolated from etiolated leaf tissue and incubated with a reaction mixture containing Tris-HCl buffer (0.05 M), sucrose (0.2 M), and chloramphenicol (100 μg/ml), and PC-toxin (5.0 to 230 μg/ml) or water. Nuclei were incubated in toxin 15 min before the addition of radioactive amino acid (0.5 to 1.0 μc/ml; 4 to 20 μM). Replicate tubes, incubated for 0 to 60 min, were removed and processed as described in "Materials and Methods". The results of these experiments were variable but they showed no significant effect of PC-toxin on uptake and incorporation of amino acids by nuclei.

Effects of Inhibitors on Susceptibility of Sorghum to PC-Toxin: Previous experiments with HV-toxin suggested that cycloheximide protected tissues against toxin by inhibiting protein synthesis or turnover. PC-toxin was used in the same kind of experiment. Cuttings were allowed to take up cycloheximide (2 to 5 μg/ml) for various times, then removed and tested for the ability of toxin to induce electrolyte loss. Many separate experiments showed that cycloheximide reduced the susceptibility of sorghum tissues to PC-toxin by 80 to 90%. Results of a representative experiment are shown in Table 19. Protection against toxin developed rapidly 8 to 12 hr after

Table 19. The Effect of Cycloheximide (CH) on PC-Toxin Sensitivity of Susceptible Sorghum Tissue.

Susceptible cuttings (1.0g/sample) took up approximately one ml of CH (5 μg/ml) or water. Cuttings were cut into 1 cm pieces, enclosed in cheesecloth, treated with toxin (82 μg/ml) and assayed for toxin-induced loss of electrolytes.

12 hour 1/ Pretreatment	2/ Treatment	3/ Conductivity (μmhos)	Protection (%)
Water	Water	10.0	
Water	Toxin	34.5	
CH	Water	11.4	
CH	Toxin	15.0	86

1/ Cuttings took up approximately 1 ml water or CH (5 μg/ml) per gram.

2/ Cuttings were cut into sections (1 cm long), wrapped in cheesecloth, treated with toxin (80 μg/ml) for 2 hr, and assayed for toxin-induced electrolyte loss.

3/ Conductivity of ambient solution was taken after 2 hr leaching time.

initial exposure to cycloheximide (Figure 26), which is similar to the case with HV-toxin. Cycloheximide may reduce the available number of receptor sites, but other kinds of evidence are needed for a firm conclusion. Cycloheximide may also affect reactions other than protein synthesis (3). Little is known about the turnover and depletion of specific membrane proteins.

The effects of cycloheximide were compared with the effects of m-ClCCP, DNP, sodium azide, magnesium fluoride, Actinomycin D, and SKF-7997-A_3. Results showed that these inhibitors have some protection against toxin; for example, DNP gave protection, but it was much less than that obtained with cycloheximide (Figure 26). Actinomycin D gave very high protection against PC-toxin when used as a 24 hr pretreatment. SKF-7997-A_3, a steroid synthesis inhibitor, gave 40% protection in a 24 hr pretreatment. Of all the materials tested, cycloheximide, in contrast to some of the other inhibitors, did not cause significant leakiness of cells in 12 hr pretreatment.

Sulfhydryl reagents were tested for a protective effect against PC-toxin similar to that obtained for HV-toxin. Results gave no indication of an effect by NEM, PCMB, or DNFB. A possible role of histidine and tyrosine groups was examined by the use of two diazonium salts, diazosulfanilic acid and diazonapthalenedisulfonic acid. Solutions of reagents were prepared immediately before each experiment by diazotizing according to the methods of Pardee and Watanabe (14). The results were inconclusive. One problem was that PC-toxin appears to react with these reagents, possibly by reaction with carboxyl groups, since bioassays indicated that toxin activity was reduced.

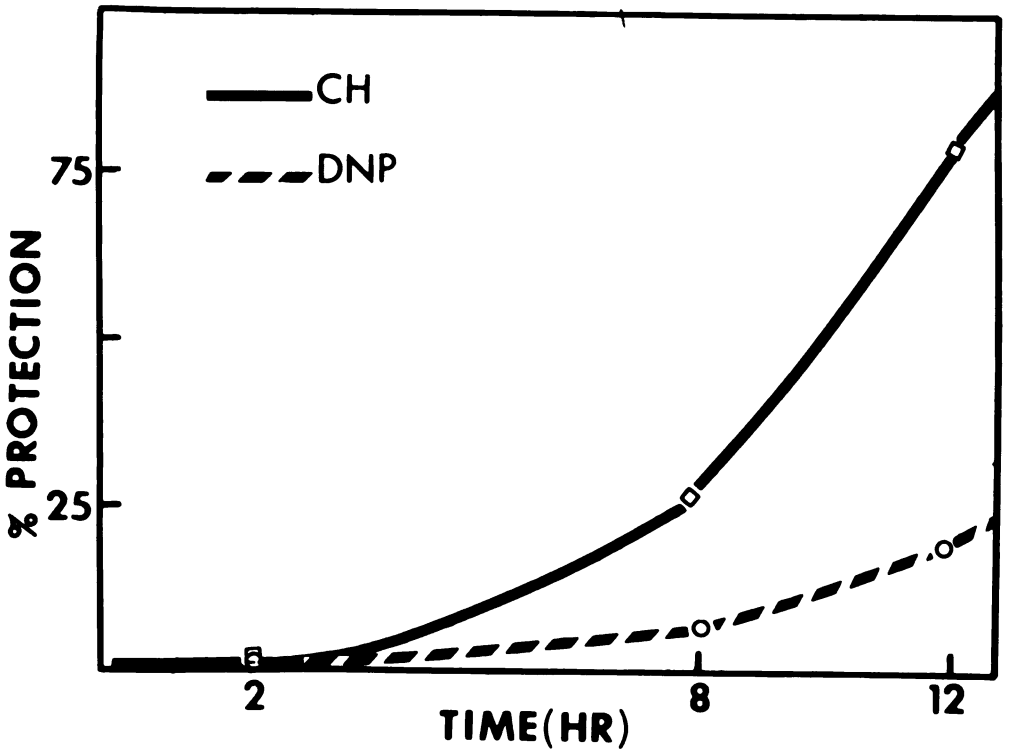

Fig. 26. Development of cycloheximide (CH) insensitivity to PC-toxin by sorghum tissue. Cuttings from 8 to 12 day old plants took up approximately 1 ml cycloheximide (5 μg/ml) or 0.1 mM dinitrophenol (DNP), or water, for 12 hr. Cuttings were removed at times indicated, cut into pieces, enclosed in cheesecloth, treated with toxin (80 μg/ml) for 2.5 hr, and leached in distilled water for 2 hr before readings were taken.

Phospholipases have been used to investigate membrane properties (30). Phospholipase D hydrolyzes phosphoryl choline and ethanolamine residues from phospholipids and is found in plant tissues (2). Many membrane proteins in higher organisms may occur as phospholipoprotein (10). Phospholipase D was therefore tested for its effect on susceptibility of cells to PC-toxin. Coleoptile sections were incubated in White's solution containing 5 mg/ml phospholipase D. Control sections were incubated in White's solution. Samples (200 mg) were then treated with toxin or water and assayed for toxin-induced electrolyte loss. The results of several experiments indicated that phospholipase treatment reduced susceptibility to toxin (Table 20). Although this experiment can not be considered conclusive, it may suggest a role of phospholipids, directly or indirectly, in the toxin receptor site.

Uranyl salts have been shown to act on the membrane surface of yeast cells by Rothstein and colleagues (19,20). However, experiments with HV-toxin and uranyl salts were not conclusive, because the toxin molecule was affected. Results of a ferrocyanide colorimetric assay for uranyl ion (20) and a bioassay showed that PC-toxin did not complex with uranyl ions. Further experiments were designed to determine the possible protective effects of uranyl salts against PC-toxin. The results showed that uranyl nitrate pretreatments for 12 hr gave up to 80% protection against PC-toxin-induced loss of electrolytes. Simultaneous application of uranyl salts and PC-toxin gave no significant protective effect. Nonspecific effects of uranyl ion are suspected, since uranyl ion can reduce electrolyte loss induced by methanol or m-ClCCP in oat tissue. Uranyl ion may bind with

Table 20. Effect of Phospholipase D (PL-D) Pretreatment on Susceptibility to PC-Toxin.

Susceptible coleoptile sections (300 mg/sample) were floated on 5 mg/ml PL-D (pH 5.6) for indicated times. Samples were then treated with toxin (82 µg/ml) for 1 hr and leached in 25 ml distilled water.

Pretreatment	Treatment	Conductance		Protection	
		Exp. 1[1/] (µmhos)	Exp. 2[2/] (µmhos)	Exp. 1 (%)	Exp. 2 (%)
Water	Water	7.6	4.0		
Water	Toxin	40.0	12.1		
PL-D, 2 hr	Water	7.6	6.0		
PL-D, 2 hr	Toxin	22.7	6.0	53	100
PL-D, 3 hr	Toxin	25.6		44	

1,2/ Leaching time was 2 hr in experiment 1 and 1 hr in experiment 2.

the membrane and reduce the loss of electrolytes following membrane damage. Therefore, we can not be certain that uranyl blocks toxin receptors.

Uptake and Recovery of PC-toxin, and its Action on Resting Seeds: Previous data (13) indicated that toxin uptake might be linked to metabolism. To test this possibility, seedlings were exposed to toxin under partially anaerobic conditions and in the presence of dinitrophenol. Seedlings which had germinated for 24 hr were exposed to toxin in serial dilutions plus dinitrophenol (10 μM), for 2 to 6 hr, then were removed from the inhibitor and toxin and placed under the usual assay conditions. In another experiment, partially anaerobic conditions were obtained by submerging seedlings in water or toxin solution to a depth of 1 cm. Again, seedlings were removed after 2 to 6 hr and placed under standard conditions for growth. Root growth was measured 3 days later. Results of both experiments indicated no effect of these treatments on apparent uptake of toxin.

HV-toxin is inactivated rapidly *in vivo*, but previous experiments have shown that PC-toxin can be recovered from resistant and susceptible tissue (13). The possibility of detecting adsorption to hypothetical receptors by recovery of toxin from tissue was considered. In most experiments designed to test recovery of toxin, cuttings were allowed to take up a measured amount of toxin, then were rinsed and homogenized in distilled water. Up to 80% of the original toxin was accounted for in extracts from susceptible and resistant plants (Table 21). However, estimations varied because of limitations of the bioassay. There were no significant differences in % recovery from

Table 21. Recovery of PC-Toxin from Cuttings and Seeds as Determined by Seedling Bioassay.

Susceptible (Sus) and resistant (Res) cuttings took up toxin for indicated time. Tissue was homogenized and centrifuged at 5000g for 10 min. The supernatant was diluted and tested for its effect on growth of resistant and susceptible seedling roots. The dilution endpoint (DEP) was the concentration (μg/ml) of toxin that gave inhibition of seedling root growth.

Expt #	Hours uptake	Type of extract	Input[1/] toxin (units)	Extracted[2/] toxin (units)	Mean[3/] recovery (%)
1	20	Sus	4.2	2.9	69
		Res	4.2	1.7	40
	30	Sus	4.2	1.3	31
		Res	4.2	2.6	62
	60	Sus	4.2	1.5	36
		Res	4.2	1.5	36
2	32	Sus	8.3	6.7	81
		Res	8.3	5.9	71
3	14	Sus	6.2	2.5	40
		Res	6.2	2.5	40
	30	Sus	6.2	2.5	40
		Res	6.2	2.5	40
4	24	Sus (seeds)[4/]	12.5	3.9	31
		Res (seeds)	12.5	4.8	38

1/ DEP of toxin solution = units of toxin input.

2/ $\frac{\text{(dilution in ml to give DEP)}}{\text{(μg/ml of toxin) x (ml of toxin input)}}$ = units of toxin extracted

3/ $\frac{\text{units of toxin extracted}}{\text{units of toxin input}}$ x 100 = percent recovery

4/ 100 seeds imbibed toxin; extracts were assayed, as described for cuttings.

susceptible and resistant tissues. Results indicate that resistance to toxin is not caused by greater ability of resistant tissue to inactivate toxin.

Intact resistant and susceptible seedlings which had been grown in nutrient solutions for 12 days were exposed by submerging the root tips (2 to 3 cm) in toxin solutions (80 μg/ml). The seedlings were removed 20 hr later, and the leaf tissue was assayed for the presence of toxin. No toxin was detected in leaves of resistant plants. Toxin disorganizes the membranes of susceptible roots, and toxin was recovered from leaves of these plants (Table 22). The results suggest that toxin does not pass through intact cell membranes until the membranes are disrupted by toxin, and that toxin acts on the outer surface of the cell.

HV-toxin acts on resting seeds before germination is irreversibly activated (21), suggesting that susceptibility is constitutive. Susceptible and resistant Colby sorghum seeds were treated with toxin for 2 to 12 hr, washed for 2 hr, dried over $CaCl_2$ for several days, then stored for 30 months. At that time, susceptible seeds exposed to toxin for 2 hr (30 months previously) did not germinate (Table 23). Results were similar for seeds exposed to toxin for 12 hr. Toxin kills susceptible resting seeds at a time when germination is reversible and when respiration is azide insensitive, as shown by respiratory determinations similar to those reported for oat seeds and HV-toxin (21). Results suggest that susceptibility and resistance to toxin are constitutive characteristics and not correlated with the rate of metabolism. These results indicate that seed treatment with toxin could be used to screen for disease resistance in

Table 22. Recovery of PC-Toxin from Leaf Tissues of Resistant and Susceptible Seedlings Following Exposure of Intact Roots to Toxin.

Intact roots of 12 day old plants were exposed to toxin (80 μg/ml) for 20 hr. Primary leaves were excised and 1 g was homogenized in 40 ml phosphate buffer (pH 6.7). The equivalent extracts from resistant (Sus. extract) and susceptible leaves (Res. extract) were assayed against susceptible seeds. Inhibition of root growth was a measure of the toxin recovered.

Dilution of extract	Root growth in seedling assay	
	Sus. extract (cm)	Res. extract (cm)
1:1	0	6-8
1:20	0	6-8
1:100	2	6-8
1:1000	2-6	6-8
control	6-8	6-8

Table 23. Germination of Resistant and Susceptible Sorghum Seeds Exposed to PC-Toxin 3 Weeks to 30 Months Previously.

Susceptible and resistant seeds imbibed in toxin solution (80 µg/ml) or water for 2 hr. Seeds were then washed in running tap water for 2 hr, dried over $CaCl_2$ under vacuum, stored for 30 months, and germinated between moist filter paper at 30 C. Each value was determined from germination of 150 to 200 seeds.

Seed type	Treatment (2 hr)	% Germination at		
		3 weeks	3 months	30 months
Susceptible	None		68	68
Susceptible	Water	66	64	68
Susceptible	Toxin	28	42	0.7
Resistant	None		73	73
Resistant	Water	67	65	62
Resistant	Toxin	60	66	63

plant breeding programs.

Effects of Chemically Altering the Toxin Molecule: PC-toxin contains dicarboxylic acids (glutamic and aspartic), and free carboxyl groups might be expected. At least one free amino group in the toxin molecule is indicated by the ninhydrin positive test given by toxin. Therefore, the possibility of altering these groups was examined.

Methanol-HCl esterification is specific for carboxyl groups, if conditions are mild (5,32). The only side reaction which is known to occur is the N to O acyl shift. Since toxin has 2 serine groups it is possible that toxin molecules could be altered in this way. Esterification of carboxyl groups can be reversed by neutralizing the HCl in the reaction mixture.

PC-toxin (80 μg/ml) was lyophilized and dissolved in dry methanol-HCl (0.2 N). Methanol was acidified by HCl gas and titrated with Tris buffer (1 N); this stock solution was diluted with dry methanol. The reaction mixture was incubated for 48 hr. The toxin preparation was not completely soluble in methanol, but part of the insoluble material was dissolved in the acidified methanol during the incubation period. Toxin treated by this procedure was assayed and found to be almost completely inactivated (Table 24). No significant reduction in toxin activity occurred in methanol or 0.2 N HCl alone. When the pH of the toxin-methanol-HCl mixture was raised to pH 7.0 to 8.0 with NaOH, toxin activity was restored to 80% of its original level in 48 hr. This is consistent with known reversibility of esterification (5,32). Results suggest that toxin activity requires free carboxyl groups.

The ability of esterified, inactive toxin to counteract the

Table 24. Inactivation of PC-Toxin by Esterification, and Reversal of the Inactivation at pH 7.0.

PC-toxin was lyophilized and treated with dry methanol + 0.2 N HCl for 48 hr. Controls were toxin treated with HCl alone and methanol alone. After 48 hr in methanol-HCl, which gave almost complete inactivation, the preparation was adjusted to pH 7.0-8.0 and left for 48 hr. All preparations were diluted and assayed for toxin-induced loss of electrolytes.

Treatment	Conductance (μmhos)	Toxin Activity (%)
Toxin in water	17.6	100
Toxin in HCl (0.2 N)	17.2	96
Toxin in MeOH (anhyd.)	20.4	123
Toxin in MeOH + HCl (inactive)	8.9	5
Toxin in MeOH + HCl followed by 48 hr at pH 7.0	16.0	83
Water control	8.4	

effects of active toxin was tested. Toxin was mixed with either esterified toxin in buffer (0.05 M sodium acetate, pH 4.5) or with buffer alone, and then assayed for its ability to induce electrolyte loss from susceptible tissue. The results indicated that inactive, esterified toxin reduced the activity of fully active toxin (Figure 27). Results suggest that toxin interaction with a receptor does not depend on carboxyl groups, and that esterified toxin is competing with active toxin for sites.

Toxin was treated with fluorescent dyes (fluorescein isothiocyanate and Rhodamine B-200)known to label free amino groups, using the labelling procedure of Rinderknect. The separation of the labelled molecules in the toxin preparation from the free dye molecules was observed. When the labelled toxin preparations were diluted and assayed for ability to induce loss of electrolytes, there was no significant reduction in toxicity compared to unlabelled toxin. However, before final conclusions are possible, the possibility of having labelled impurities instead of toxin must be eliminated.

The results of esterification experiments with HV- and PC-toxins are similar and suggest a role of carboxyl groups in toxin action. Also, counteracting effects of inactive toxin on active toxin was observed in experiments with both toxins. More study is needed on the nature of this apparent competitive effect.

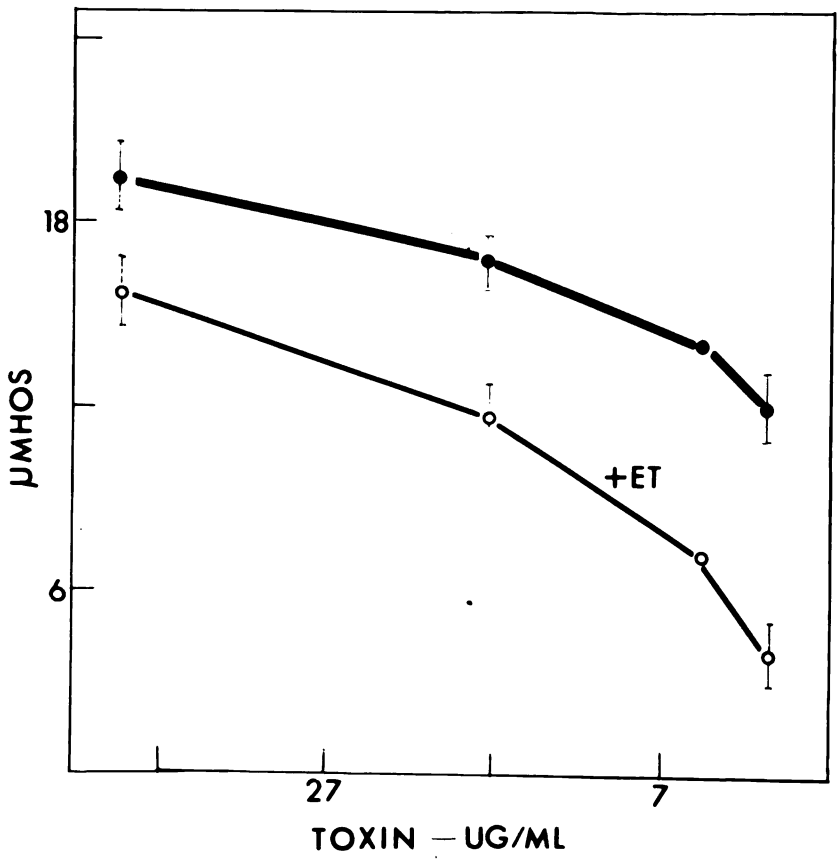

Fig. 27. Counteracting effects of esterified toxin (ET) on toxin induced loss of electrolytes from sorghum leaves. Toxin at the appropriate concentration was mixed with ET (400 μg/ml) in sodium acetate buffer (pH 4.5) or mixed with buffer alone. Susceptible sorghum leaf tissues (800 mg/samples) were treated with these solutions for 2 hr. After thorough rinsing, samples were leached in 50 ml distilled water for 2 hr before conductivity readings were taken. A value of 2.2 μmho, representing residual activity in ET alone over water control, was subtracted from values for samples containing ET.

DISCUSSION

PC and HV-toxins cause many similar physiological effects in their respective susceptible plants. Several aspects of their chemistry and the nature of toxin susceptibility and resistance are also similar (Table 25). However, PC-toxin is less potent on a weight basis than is HV-toxin. Also, the effects of PC-toxin on susceptible host cells are much less drastic than those observed with HV-toxin. Therefore, PC-toxin may be a more difficult model than HV-toxin for location of the initial lesion.

Increased electrolyte efflux, the most direct criterion for toxin-induced membrane damage, was observed within 15 min of exposure of susceptible cells to high concentrations of PC-toxin. Damage to the plasma membrane might account for all the symptoms caused by PC-toxin. However, more conclusive evidence is needed before the hypothesis of a site of action in the plasma membrane is accepted without reservation. Respiratory responses of susceptible tissues to toxin are indirect and secondary, because toxin had no effect on oxidation and phosphorylation by isolated mitochondria.

Apparent uptake of PC-toxin, as measured by toxic effects, does not appear to be an active process but may consist of a simple, one-step adsorption to receptor sites. Thus the receptor site hypothesis, as originally proposed for HV-toxin (23), seems equally appropriate to PC-toxin. Susceptibility to both toxins is controlled by a single

Table 25. Comparative Characteristics of HV and PC-Toxins.

Characteristics of toxin molecule	HV-toxin	PC-toxin
Conc. of toxin that affects susceptible tissue	0.0002 μg/ml	0.1 μg/ml
Conc. of toxin that affects resistant tissue	3.6 mg/ml	> 2.6 mg/ml
Number of molecular variants	one known	two or more
Chemical nature	peptide	peptide
Ninhydrin reaction	negative	positive
Effect of esterification on toxicity	inactivates	inactivates
Physiological effects of toxins on susceptible plants		
Effects on:		
Respiration	increase	increase
Uptake and incorporation of solutes	decrease	decrease
Reactions of isolated organelles	none	none
Free space	increase	not tested
Electrolyte efflux	increase	increase
Susceptible protoplasts	broken	no effect
Behavior of toxin in host cells		
Effects of cycloheximide on sensitivity to toxin	decrease	decrease
Effects of sulfhydryl reagents on sensitivity	decrease	no effect
Effects of carbonyl reagents on sensitivity	decrease	no effect
Effects of toxin breakdown products on sensitivity	decrease	no effect
Effects of esterified toxin on sensitivity	decrease	decrease
Effects of uranyl ions on sensitivity	decrease	decrease
Recovery of toxin from tissues	none	40 to 80%

gene locus; therefore, one gene product, presumably a receptor site, has been proposed.

The data from experiments with cycloheximide can be interpreted according to the toxin receptor hypothesis. Cycloheximide protection against toxin could mean that the receptor is a protein with a 6 to 12 hr turnover time. However, other possible explanations have not been ruled out. Even the data with phospholipase D pretreatments are not counter to the receptor hypothesis. Phospholipase D protection against toxin-induced electrolyte loss could mean that the sensitive component is a lipoprotein, or is closely associated with a lipoprotein.

The apparent competition of esterified PC-toxin with active toxin is also consistent with the hypothesis of a receptor site. Each molecule of PC-toxin contains two glutamic acid residues and four aspartic acid residues; some or all of these may be esterified by methanol-HCl. The latter treatment is quite specific for carboxyl groups (5,32). The reduction of toxicity by esterified toxin could be explained if carboxyl groups are necessary for toxin activity, but not required for stereospecific adsorption of toxin to receptor sites. A toxin analog might be useful in future studies of toxin.

The identification of a receptor site for PC-toxin need not utilize a reagent that binds with a specific group. Another approach, based on the incorporation of amino acids into the protein in question, would appear to be a more direct way to identify receptor proteins. This double labelling procedure was used by Kolber and Stein (9) to isolate membrane proteins associated with galactoside transport. This type of experiment would require that sorghum cultivars

be isogenic, except for the single gene controlling susceptibility. The susceptible cultivar would be labelled with ^{14}C-amino acid and the resistant cultivar with ^{3}H-amino acid. The tissues would be homogenized, the homogenates mixed, and then proteins would be fractionated. A protein with a high $^{14}C/^{3}H$ ratio should be the specific gene product for susceptibility.

PC-toxin is similar to HV-toxin in many respects. Resistance and susceptibility to each is based on single gene differences, and probably is based on lack of receptor sites. The mechanism of toxin action, in terms of interaction with receptors, could differ in each case. However, the available data are in accord with the hypothesis of receptor sites in plasma membranes.

SUMMARY

The earliest observed response of susceptible tissues to PC-toxin was increased loss of electrolytes. A toxin assay based on the ability to induce loss of electrolytes was developed; the assay was shown to be practical, and was similar in nature to the assay used for HV-toxin. PC and HV-toxins are similar in many other respects. The data suggest that susceptibility and resistance to PC-toxin are constitutive characteristics which do not depend on active metabolism. Increased respiration and decreased amino acid incorporation are apparently secondary effects in toxic action. Pretreatment of tissues for 12 hr with cycloheximide caused them to become insensitive to PC-toxin. Esterification inactivated PC-toxin; the inactive form counteracted in part the effects of active toxin. The results show there are receptor sites in the plasma membrane of susceptible cells. Resistant cells may lack such receptor sites.

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