# EFFECTS OF A TOXIN FROM HELMINTHOSPORIUM MAYDIS RACE T ON RESPIRATION OF CORN TISSUES AND ON MITOCHONDRIAL REACTIONS

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ABSTRACT

EFFECTS OF A TOXIN FROM HELMINTHOSPORIUM MAYDIS RACE T ON RESPIRATION OF CORN TISSUES AND ON MITOCHONDRIAL REACTIONS

By

Mary Ann Bednarski

Helminthosporium maydis race T is the causal organism for the corn disease that reached epiphytotic proportions in 1970. The fungus is pathogenic to corn carrying Texas male sterile cytoplasm (Tmsc); corn with normal (N) or nonsterile cytoplasm is resistant. The greater virulence of race T over that of races known previously is correlated with the ability to produce a toxin (HM-T toxin) which selectively affects corn with T cytoplasm.

The aim of this work was to determine how the host-selective toxin acts at the cellular level. My data show that toxin-treated leaf and coleoptile tissues had 20 to 30 per cent higher respiration rates than did untreated control tissues. Respiration of resistant corn tissue was not affected by toxin. 2,4-Dinitrophenol (10 μM) caused increases in respiration of both resistant and susceptible tissues. Toxin also caused uncoupling of oxidative phosphorylation by mitochondria isolated from susceptible, but not from resistant tissues. Toxin-induced uncoupling was accompanied by decreased oxidation when malate-pyruvate was the substrate, but not when succinate or NADH was the substrate.

Maximum uncoupling of oxidative phosphorylation occurred approximately one minute after initial exposure of mitochondria to toxin. A lag in the response of nonphosphorylating mitochondria to toxin was also observed. In contrast, susceptible mitochondria responded immediately to 2,4-dinitrophenol, valinomycin, and phosphate. Toxin-induced uncoupling increased with increases in toxin concentration up to a saturating level; a plot of the response gave a hyperbolic curve characteristic of reversible inhibition.

Susceptible mitochondria were incubated for five minutes in a toxin concentration that gave approximately 50 per cent uncoupling; toxin-treated mitochondria were then washed in toxin-free media. Mitochondria treated in this way had $O_2$ and $P_i$ uptake values similar to those of control mitochondria (not exposed to toxin), when NADH, succinate or malate-pyruvate were the substrates. Assays showed that mitochondria did not remove measurable amounts of toxin from the ambient solution. These results show that toxin has a reversible effect on mitochondria, and that toxin is not bound firmly to a mitochondrial site.

Further studies on susceptible mitochondria showed that: 1) uncoupling induced by toxin did not require the presence of monovalent cations ($K^+$, $Na^+$, $Li^+$, or $NH_4^+$); 2) increased rates of mitochondrial swelling caused by toxin were independent of substrate oxidation, and the response was proportional to the amount of toxin applied; and 3) mitochondrial adenosine triphosphatase activity was stimulated by toxin. In addition to toxin sensitivity, two other differences in mitochondria from susceptible and resistant plants were evident. First, susceptible

mitochondria were more sensitive to low levels of nigericin than were resistant mitochondria. Second, the uptake rate of $K^+$ was slower in susceptible than in resistant mitochondria.

In vivo studies were concerned with glycolysis and levels of acid-labile phosphate in susceptible and resistant tissues. Toxin inhibited glycolysis in susceptible tissue, as measured by $CO_2$ release; the inhibition was not immediate and was not alleviated when intermediates of the glycolytic pathway were introduced along with toxin. Toxin did not affect glycolysis in resistant tissues and in cell-free preparations from susceptible or resistant plants. Levels of acid-labile organic phosphates decreased when susceptible tissues were exposed to toxin for one hour; toxin did not cause such changes in resistant tissues. In contrast, 2,4-dinitrophenol caused decreases in acid-labile phosphates in both resistant and susceptible tissues. These findings are compatible with the data on glycolysis, and support the hypothesis that toxin affects the mitochondrial site in vivo.

Toxin had no effect on two other physiological processes that were tested. Phosphorylation and electron transport in isolated chloroplast lamellae were not changed by toxin, although these systems are affected by other uncoupling agents. Adenosine triphosphatase activity associated with the microsomal fraction from roots was not affected by toxin in the presence or in the absence of $K^+$. My data indicate that the mitochondrion is the only important site for action of toxin in vivo.

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To my parents

Valentine John and Joan Irene

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| | |
|---|---|
| ADP | adenosine 5'-diphosphate |
| ATP | adenosine 5'-triphosphate |
| BSA | bovine serum albumin |
| c | Curie |
| C | degree Celsius |
| conc | concentration (in tables) |
| DNP | 2,4-dinitrophenol |
| g | gram(s) |
| HM-T toxin | *Helminthosporium maydis* race T toxin |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| HV-toxin | *Helminthosporium victoriae* toxin |
| MES | 2-(N-morpholino)ethanesulfonic acid |
| μ | micro (prefix $10^{-6}$) |
| N | Normal (conc) |
| N | Normal or nonsterile (cytoplasm) |
| NAD | β-nicotinamide adenine dinucleotide, oxidized form |
| NADH | β-nicotinamide adenine dinucleotide, reduced form (disodium salt) |
| $P_i$ | orthophosphate |
| P/O | ratio of ATP formed (moles) to atoms of oxygen consumed |
| RCR | respiratory control ratio |
| Tms | Texas male sterile (cytoplasm) |

| | |
|---|---|
| tris | N-tris(hydroxymethyl)aminomethane |
| Tricine | N-tris(hydroxymethyl)methylglycine |

# INTRODUCTION

Corn (Zea mays L.) is the major crop in the middle and south central United States. In 1970, severe damage to the corn crop was caused by a new race of Helminthosporium maydis. The disease reached epiphytotic dimensions and resulted in an estimated billion dollar loss (67,93). Consequently, the disease (Southern corn leaf blight) created much interest and stimulated many epidemiological, genetic, and physiological studies.

A common feature in many widespread epiphytotics is genetic uniformity in the crop. Uniformity usually results from demands for qualities of economic value; i.e., yield, insect and disease resistance, and suitability for mechanical harvesting (67,89). The genetic uniformity in relation to Southern corn leaf blight is in the cytoplasm of corn hybrids rather than in the nuclear genes (1,41). The cytoplasm with an extranuclear gene for male sterility was found by plant breeders in Texas, and is called Tms cytoplasm. Seed producers valued this characteristic because it made hybridization possible without the expense of detasseling.

More than 90 per cent of the corn grown in the Corn Belt prior to 1971 carried Tms cytoplasm (67); this was the determining factor for development of the epiphytotic of Southern corn leaf blight. Plants carrying Tms cytoplasm were susceptible, even when the necessary fertility-restoring genes were present. Corn with nonsterile or

normal (N) cytoplasm was resistant to H. maydis race T. Cytoplasmically inherited susceptibility to disease was previously unknown (20,40).

Susceptibility or resistance to the fungus was soon shown to be related to sensitivity or lack of sensitivity to a toxic product (HM-T toxin) from the fungus (17,41,99). The toxin is said to have a molecular weight of 500-600 (45), but its structure is not fully known.

Mitochondria isolated from Tms corn are affected by HM-T toxin (65); oxidation is uncoupled from phosphorylation (7). Resistant mitochondria are not affected by much higher concentrations of toxin. The specificity of toxin to a mitochondrial site was unexpected because other host-selective toxins do not affect isolated mitochondria (84). However, sensitivity to the other host-selective toxins is determined by nuclear genes rather than by cytoplasmic genes. To understand the relationship between cytoplasmically-controlled susceptibility and a toxin-sensitive site in the mitochondrion requires knowledge of cellular functions which are cytoplasmically inherited; certain properties of the mitochondrion are inherited in this way.

The direction of research for this thesis was guided by basic background questions such as the following: How does the toxin act? Does toxin affect tissue respiration in vivo? Can susceptibility be explained on the basis of a toxic effect on the mitochondrion in vivo? I have attempted to characterize the effects of toxin on mitochondria in some detail by comparing the toxin-induced effects with those of known uncoupling compounds. Several specific questions were asked in the experiments with mitochondria, including the following: Is the uncoupling effect reversible? Does toxin require monovalent cations

for uncoupling? Are there differences between resistant and susceptible mitochondria in addition to the difference in their response to the toxin? My work was not confined to mitochondria; other experiments were designed to determine the effects of toxin on tissue respiration, on glycolysis, and on photosynthetic phosphorylation by isolated chloroplasts. My tentative conclusion is that the primary site for action of HM-T toxin *in vivo* is in the mitochondrion, and that the other effects (e.g. on glycolysis) may result from the uncoupling that occurs in the mitochondrion.

# LITERATURE REVIEW

## Background and general considerations of the disease

Helminthosporium maydis (Nisikado and Miyake) is a pathogen of corn in areas of the world with mild winters and hot growing seasons. In the United States, H. maydis was formerly limited to the South, hence the disease it produces is commonly known as Southern corn leaf blight (67). The epiphytotic of Southern corn leaf blight throughout the Corn Belt in 1970 was caused by a new race of H. maydis, now known as race T (42). H. maydis race T causes severe blight only in corn containing Texas male sterile (Tms) cytoplasm, which constituted approximately 80 per cent of the total U. S. corn acreage in 1970 (93). The disease caused by H. maydis race T was unique because it was the first clear example of cytoplasmic inheritance of susceptibility. Philippine workers first noticed that Tms-cytoplasm corn was much more susceptible to H. maydis than was N-cytoplasm corn (61).

The origin of race T in the United States is still not fully understood (26,68). Based on studies of mating types, Leonard (53) has postulated that race T was either introduced into the Corn Belt or arose there by mutation several years prior to 1968, and that the fungus was transported from the Corn Belt into Southern states on infected seed. It is possible that endemic strains of H. maydis carried the genes for toxin production, and that this capacity became evident only when the

proper conditions occurred for spread of the fungus, and when a large acreage of susceptible corn was present.

Corn lines with several other types of cytoplasm were examined for reaction to H. maydis race T. Corn with normal (N), S-ms, or C-ms cytoplasms are resistant to the fungus. Plants with cytoplasm HA, P, and Q are susceptible and cannot be distinguished from those with Tms cytoplasm (58). Nuclear genes that restore full fertility to Tms plants do not provide resistance to H. maydis race T (31,37,99). Thus, cytoplasmically-controlled male sterility and susceptibility to disease probably depend on different mechanisms. Nuclear-gene/cytoplasm interactions can, however, modify both male sterility and disease susceptibility (30,40).

Morphologically, the two known races of H. maydis (races 0 and T) are indistinguishable (88). Race 0 is primarily a leaf pathogen, causing small lesions with parallel sides. Race T causes much larger, spindle-shaped lesions on susceptible Tms corn leaves; it also invades the ears and blackens the grain (41). Race T has a higher reproductive rate and a lower temperature optimum than does race 0; a new generation of spores is produced by race T within sixty hours after infection (42). Race T produces a toxin in culture and in infected plants (17,41,45,56); the toxin selectively affects corn with Tms cytoplasm. Race 0 is not known to produce a toxin that is involved in disease development. Thus, races 0 and T differ in symptoms induced, reproductive rates, temperature optima, cytoplasmic specificity to maize, and in ability to produce toxin. H. maydis race 0 from all parts of the world is pathogenic to some

genotypes of both N and Tms corn (88), and resistance to race O is controlled by nuclear genes (30,42).

## Toxin production in culture

Lim and Hooker (57) did the preliminary work showing that *H. maydis* race T produces substances that are more toxic to Tms than to N corn. These and other workers produced the toxin by growing the fungus in Fries' medium supplemented with 0.1 per cent yeast extract. Best yields were obtained when the fungus was grown in still culture for approximately fifteen days (17,99). Fungal isolates vary in ability to produce toxin in culture (17). The toxin was reported to be dialyzable, thermostable, and weakly positive to ninhydrin. Paper chromatography of a crude preparation from culture filtrates, using butanol:acetic acid:water (4:1:5, $^{v}$/v) as the solvent, showed a toxic substance with a $R_f$ value of 0.95. The toxin emitted weak yellow fluorescence under 366nm light. Its aqueous solution showed an absorption maximum at 270 nm (57). Similar absorption data were presented by Karr *et al.* (45).

## Genetic studies in relation to disease

*H. maydis* has a sexual stage known as *Cochliobolus heterostrophus* Drechsler (22); Lim and Hooker (55) have mated races O and T and analyzed the ascospore progeny for toxin production and pathogenicity. Ability to produce a host-specific toxin was correlated with selective virulence to Tms corn, and this ability was monogenic in inheritance. The amount of toxin produced and the degree of virulence probably are affected by many genes. However, this work was preliminary

and other reports on the genetics of the fungus must be considered. C. heterostrophus has a wide host range among species of the Graminaceae (69). Pathogenicity to each host species is inherited independently; at least thirteen genes for pathogenicity to different species have been identified (70). Race T appears to have the same degree of virulence on normal plants as does race 0, giving the same kinds of small necrotic flecks (88). Yoder and Gracen (103) reported that several gene pairs may be involved in the control of race T type pathogenicity.

## Bioassays

Several bioassay methods have been used to measure relative toxin activity. Inhibition of primary root elongation (17,99) is often used to assay HM-T toxin and other host-specific toxins (83). Relative inhibition of root growth caused by culture filtrates from isolates of H. maydis race T was correlated with relative severity of disease caused by the isolates in the field (99). The root growth assay showed that several hybrids and inbred corn lines were equally sensitive to the toxin (17). Arntzen et al. (5) modified the root growth bioassay to obtain readings within short time periods. Roots of control corn seedlings and of roots exposed to toxin were placed on a negative holder and enlarged photographs were taken at fifteen-minute intervals.

Leaf whorl and stem injection assays have been used in field screening to differentiate resistant from susceptible corn for plant breeding work. The assay response to toxin is the appearance of chlorotic or necrotic streaks in susceptible, but not in resistant, tissues (31,96). Leaf assays were also used in the laboratory to detect toxin

in crude preparations (45). Detached leaves were placed in a test tube with the basal end in water. Leaves were then punctured above the water line and a drop of toxin-containing solution was placed on the wound. Lesion formation was observed after incubation for twelve hours in a moist chamber.

Several other assays have been used to a limited extent. Toxin-induced inhibition of malate oxidation by mitochondria can be followed spectrophotometrically, using 2,6-dichlorophenolindophenol as an electron acceptor (72). Laughnan and Gabay (52) used toxin-induced inhibition of pollen germination as a bioassay; results can be obtained in two hours. Pollen from Tms (restored) plants was sensitive to toxin, and pollen from N plants was not sensitive. Leaf extracts from Tms corn infected with race T caused inhibition of pollen germination, whereas extracts from corn infected with race O did not.

## Effects of toxin on host tissues, cells, and organelles

Susceptible tissue exposed to HM-T toxin for approximately four hours had greater losses of electrolytes into the ambient solution than did toxin-treated resistant tissues, or control tissues without toxin (5,14,32). Susceptible tissue infected with *H. maydis* race T also had a higher rate of electrolyte loss than did noninfected control tissue or tissue infected with race O (15,34). However, HM-T toxin-induced loss of electrolytes apparently was slow to develop; in short term studies, no increase in leakage was detected. Keck and Hodges (46) applied HM-T toxin to leaf sections that were pre-loaded with $^{86}Rb$. There was no toxin-induced efflux of $^{86}Rb$ for the first thirty

minutes, nor was there an immediate effect on tonoplast permeability (46). Uptake or release of $^{32}P$ by susceptible corn roots was not affected by toxin over a three-hour period (5). These reports suggest that HM-T toxin does not have an immediate effect on the plasmalemma and that it does not act by general disruption of the membrane, as is the case with the host-specific toxin from H. victoriae (84).

Mitochondria from Tms seedlings are severely affected by HM-T toxin, whereas mitochondria from resistant plants are not (65). Mitochondria isolated from susceptible corn swelled at an accelerated rate and lost respiratory control after exposure to the toxin, indicating an uncoupling effect. Toxin-treated and control mitochondria also had differences in the rates of substrate oxidation. Electron micrographs revealed that mitochondria in intact tissue are affected by the toxin (48). Control mitochondria in situ had the typical "contracted" configuration of mitochondria undergoing active phosphorylation, whereas mitochondria in tissue treated with toxin did not show this normal ultrastructural state. Also, mitochondria in leaf cells immediately adjacent to lesions produced by H. maydis race T were not in the "contracted" state, although mitochondria 3mm from the lesion were "contracted" and did not differ from the control mitochondria.

Texas male-sterility in corn has a cytoplasmic inheritance. Mitochondria contain mDNA; an association between cytoplasmic inheritance and mitochondrial DNA is implied. Susceptibility of the host plant to the fungus and to its toxin also has cytoplasmic inheritance. It is known that mitochondrial DNA determines some of the characteristics of the inner membrane in mitochondria (79). The task appears to

associate sensitivity to HM-T toxin with some mitochondrial gene product which can serve as a sensitive site for toxic action.

Chloroplasts also contain extra-nuclear DNA, but toxin does not appear to have a direct effect on this organelle (4). Electron transport by isolated chloroplast lamellae was not affected by HM-T toxin. HM-T toxin did inhibit $CO_2$ fixation in susceptible leaf tissues (4), but this was found to be an indirect effect correlated with a reduction in transpiration. Transpiration decreased, probably, because toxin caused stomates to close. The toxin was shown to inhibit light-induced $K^+$ uptake by guard cells (4).

Tipton et al. (94) reported that HM-T toxin inhibited the $K^+$-stimulated adenosine triphosphatase activity associated with the microsomes from susceptible roots. This suggests an interaction between toxin and an enzyme in the plasmalemma. There are no previous indications that characteristics of the plasmalemma are cytoplasmically determined.

The cell wall was also suggested as a possible initial site of action for the fungus. H. maydis race T produced in culture several cell wall degrading enzymes (6). The enzymes may aid in colonization of the host tissue by the pathogen; however, the genetic data (55) and the correlation of toxin sensitivity to disease susceptibility indicates that cell wall degrading enzymes are not key determining factors. Isolated cell wall preparations from N and Tms plants incubated with H. maydis race T were analyzed electrophoretically (87); preparations from susceptible (Tms) and resistant (N) plants had identical gel patterns of esterases, peroxidases, and acid phosphatases.

## Host-specific toxins and disease resistance

Toxins are known to determine host specificity for ten species of plant-infecting fungi (84). In all ten cases, susceptibility to the pathogen is related to sensitivity to a toxin produced by the fungus; resistance to the pathogen is associated with insensitivity to the toxin. In several of these cases, the reaction to the toxin is known to be controlled by one gene pair in the host. With *Phyllosticta maydis* and *H. maydis* race T, susceptibility is determined by extra-nuclear or maternal inheritance (16,102). The host-specific toxins can be used to study disease development and disease resistance at the molecular level, because the toxin duplicates changes in the host's metabolism which are also induced by the fungus. The results of my investigation of Southern corn leaf blight, described herein, suggest that mitochondria of susceptible maize may contain the initial site for toxin action.

## MATERIALS AND METHODS

### Corn plants

Inbred corn lines and hybrids were used as experimental materials. Corn susceptible to H. maydis race T, having Texas male sterile (Tms) cytoplasm, included inbred W64A and hybrids NK (P x 525), W64A x 5002, and G 4292-9901. Complementary corn inbred lines and hybrids with normal (N) cytoplasm were used; these are resistant to H. maydis race T.

Seeds, planted in vermiculite, were watered with a modified White's inorganic salt solution (100), containing in mg/l: $Ca(NO_3)_2$, 200; $Na_2SO_4$, 200; KCl, 80; $NaH_2PO_4$, 16.5; $MnSO_4$, 4.5; $ZnSO_4$, 1.5; $H_3BO_3$, 1.5; KI, 0.75; and $MgSO_4$, 360 (all values were corrected for the water of hydration). Seedlings were grown in the laboratory at room temperature (21-23 C) under Sylvania Gro-lux lamps, with a twelve-hour photoperiod. The second true leaf from fifteen-day-old plants was used in most experiments that required leaf tissue.

Roots were obtained from corn seedlings grown in the laboratory. In general, the growing conditions used by others (38,39) have been followed. Seeds were germinated for twenty-four hours at 30 C between layers of cheesecloth saturated with 0.2 mM $CaSO_4$ solution, in porcelain pans covered with plastic film (Saran wrap). At twenty-four hours, when the growing tips were visible, seeds were placed between layers of cheesecloth on a wire screen fixed on top of four-liter beakers. The

cheesecloth was saturated with the $CaSO_4$ solution and the solution in the beaker below the seeds was aerated with air that was warmed by bubbling through water at 40 C. The entire assembly was housed in a large cardboard box to exclude light. Subsequently, I will refer to this as "mist culture." Microsomes were obtained from roots grown in mist culture for 5-6 days (12-15 cm long).

Roots for respiration experiments were obtained from seedlings germinated and grown in the dark at 24 C for four days, or until roots were 4 to 6 cm long. Seeds (forty to fifty) were first placed embryo side down in 15 cm petri dishes, each containing 30 ml of 0.1 mM $CaSO_4$ solution.

Fungal cultures

Toxin-producing isolates of H. maydis race T were obtained from O. C. Yoder of Cornell University, and from infected corn in Michigan. H. maydis race O was obtained from A. J. Ullstrup of Purdue University. All stock cultures were grown on potato dextrose agar as test tube slants, and were stored under refrigeration. The titer of the toxin produced by these isolates decreased significantly after four transfers on agar.

Toxin production

Toxin was isolated from filtrates of cultures grown for 12-15 days in still culture at room temperature in Roux bottles, each containing 200 ml of medium. A modified Fries No. 3 medium (73) was used; yeast extract (0.1 per cent) was substituted for the minor elements and for $FeSO_4$. To harvest, the cultures were filtered through eight

layers of cheesecloth; the filtrate, adjusted to pH 3.5 with 6 N HCl, was concentrated under reduced pressure to one tenth of its original volume, at a temperature not exceeding 35 C. Two volumes of methanol were added, and the mixture was allowed to stand overnight at 4 C. The resulting precipitate was removed by filtration at 4 C, using Whatman No. 1 paper. Methanol was then withdrawn *in vacuo* at a temperature not exceeding 35 C. The aqueous concentrate was condensed to 0.2 of the original harvested volume and partitioned six times with equal volumes of chloroform. The chloroform fractions were pooled, concentrated *in vacuo*, and returned to an aqueous solution by the addition of 100 ml water. The aqueous solution was concentrated to 1 per cent of the harvested volume and immediately frozen in 1 ml aliquots. These procedures have been adapted from those used by other workers (74).

Inactive toxin was prepared by autoclaving 1 ml aliquots of the toxin stock preparation in sealed vials at 121 C, 15 lbs. pressure, for thirty minutes. Three repeated autoclavings at twenty-four hour intervals were required for complete inactivation.

## Toxin bioassay

Relative toxin activity was determined with a seedling bioassay (17). Five seeds were incubated embryo side down in 9 cm petri dish containing 10 ml of White's solution. After twenty-four hours incubation, the White's solutions were replaced with a series of toxin-containing solutions, and the seedlings were incubated for three to four days in the dark at room temperature. Serial dilutions of

toxin-containing preparations were made with White's inorganic salt solution. Root lengths were measured, and the dilution end-point was determined as the toxin dilution that gave 50 per cent inhibition of root growth. The partially purified HM-T toxin preparations varied in activity, with $ED_{50}$ ranging from 1.0 to 60 μg/ml. The activity of the toxin is indicated in each experiment. In all cases, resistant corn tolerated 100 times higher toxin concentration than did susceptible corn. Partially purified stock solutions of the toxin were assayed periodically against susceptible and resistant seedlings and no loss of activity was detected after one year in storage.

Manometry

Conventional techniques for Warburg manometry were followed (97). All experiments were performed at 25.5 C, with an initial twenty minute equilibration period before measurements of gas exchange were started. Oxygen uptake was measured with air as the gas phase. Carbon dioxide was removed with 0.1 ml of 20 per cent freshly prepared KOH solution absorbed to a piece of fluted filter paper in the center well.

Anaerobic conditions were obtained by using highly purified nitrogen as the gas phase. Air was flushed from the flasks, which were in series, with a constant flow of nitrogen for 20-30 minutes. Traces of oxygen were removed from the nitrogen by bubbling the gas through an alkaline pyrogallol mixture immediately before the gas entered the reaction vessels. Anaerobic conditions in the flasks were confirmed; there was no gas uptake when $Cu^{++}$ was added to ascorbic acid solutions in the Warburg flasks.

Preparation of tissues and acetone powder extracts for glycolysis experiments

Coleoptile tips (2 cm long) were cut from five-day-old etiolated corn seedlings. The tissue samples (0.5 g) were vacuum infiltrated for fifteen minutes in a solution containing 0.01 M glucose and 0.03 M phosphate buffer (pH 5.8), with or without toxin; infiltration was followed by incubation for fifteen minutes at room temperature on a reciprocal shaker. The coleoptile tips were removed from the incubating medium, blotted dry, and placed in Warburg flasks which contained filter paper discs wetted with 0.1 ml of the incubating medium. Roots were handled the same way, except that the vacuum infiltration step was omitted.

Acetone powder extracts were prepared from four-day-old etiolated coleoptiles (95). The tissue was cut in 1 cm sections and precooled; 50 g samples were homogenized for forty seconds in 500 ml of cold acetone with the temperature never above -10 C. The homogenate was quickly filtered with suction and the powdered precipitate was washed three times with 100 ml acetone (at -20 C) each. The fourth and final wash was with 100 ml of cold diethyl ether. The powder was then transferred to a watch glass and desiccated over $CaCl_2$ under reduced pressure for two hours. The $CaCl_2$ was then replaced with $P_2O_5$ and the preparation was held under vacuum for twenty-four hours. All manipulations except desiccation were at 4 C, using precooled (-5 C) glassware. Redistilled acetone was chilled to -20 C with dry ice.

For preparation of the enzyme extract, the powder was mixed in 0.03 M HEPES buffer (pH 7.6) using a powder to buffer ratio of

1:10 ($^{w}/v$). The mixture was centrifuged (13,000 x g for ten minutes at 4 C) and the supernatant solution was used as the enzyme source for experiments.

Isolation of mitochondria and methods for oxidative phosphorylation experiments

Standard procedures (64) were used to isolate mitochondria from leaves and coleoptiles. Corn plants were grown in the dark for one week in vermiculite watered with White's nutrient solution. The entire isolation procedure was at 4 C. Shoots (75 g) were cut into 1 cm pieces and chilled for one hour prior to grinding (without sand) for one minute with a cold mortar and pestle. The grinding medium (150 ml) contained 0.4 M sucrose, 0.03 M HEPES-NaOH (pH 7.5), 5 mM $Na_2EDTA$, 0.05 per cent cysteine, and 0.1 per cent bovine serum albumin. HEPES-Tris buffer replaced HEPES-NaOH in experiments with monovalent cations. The homogenate was filtered through eight layers of washed cheesecloth and immediately centrifuged for five minutes at 28,000 x g. A camel hair brush was used to resuspend the pellets in 30 ml of a modified grinding medium (minus cysteine). After a three-minute centrifugation at 2500 x g to remove cell debris, the mitochondria were spun down at 28,000 x g for five minutes. The final pellet was suspended in 2.0 ml of 0.4 M sucrose. Protein in the final stock suspension of mitochondria was adjusted to 5 mg/ml.

The reaction medium for experiments on oxidative phosphorylation contained 0.2 M KCl, 0.02 M HEPES-NaOH (pH 7.5), 2 mM $MgCl_2$, 0.1 per cent BSA, and 250 μg mitochondrial protein/ml. Substrates and

other reagents were added as indicated in tables and figures. In experiments with alkali metal cations, HEPES-Tris buffer was substituted for HEPES-NaOH buffer, and 0.4 M sucrose was the osmoticum.

The rate of electron transport (oxygen consumption) was monitored with a Clark-type oxygen electrode (Yellow Springs Instrument Co.) covered with a teflon membrane. The reaction vessels were held at 28 C. The signal from the electrode was amplified and fed into a Sargent recorder. The concentration of NADH added to the reaction mixture was determined optically, using the extinction coefficient of 6.2 at 340 nm for 1 mM NADH. The electrode holder was equipped with a narrow groove which accommodated a three-inch micropipette for all additions after equilibration was established. The contents of the reaction vessel were constantly stirred with a magnetic stirrer. These procedures were adopted from those used by others (64).

Phosphorylation rates and P/O ratios were measured as incorporation of $^{32}P$ into ATP. Upon complete utilization of the substrate (NADH), or after a reaction time of 3-4 minutes (for succinate or malate-pyruvate), a 1.0 ml aliquot of each 2.0 ml reaction medium was transferred to a Pyrex test tube. The sample was quickly frozen by immersing the tube in a bath of cold 50 per cent ethylene glycol. The unincorporated orthophosphate was extracted from the water-soluble ATP; this required less than fifteen minutes at room temperature following the standard procedures outlined by Saha and Good (80). Ten ml of a solution containing 10 per cent perchloric acid saturated with n-butanol-toluene(1:1, $^{v}/v$) was added to a 1.0 ml frozen sample. The following solutions were then added in order: 1.2 ml acetone; 1.0 ml

10 per cent ammonium molybdate; and 8.0 ml of butanol-toluene (1:1, v/v) saturated with 10 per cent perchloric acid. The ingredients were mixed for one minute with a glass plunger; the solvents were then allowed to separate. The phosphomolybdate-containing organic phase was removed by suction and the remaining aqueous solution was filtered through Whatman (9 cm) No. 4 paper wetted with 0.5 ml water. Additional ammonium molybdate (0.1 ml) was added to the filtered aqueous phase and extracted as before, using the above procedure but without acetone. After butanol-toluene was removed, radioactivity in each sample was determined.

A standard $^{32}P$ solution was used to determine the radioactivity expected from phosphorylation of 0.1 μmole ADP. A 0.1 ml sample of $^{32}P_i$ stock solution was added to 100 ml of cold 0.1 M $Na_2HPO_4$ and mixed thoroughly. A 1 ml fraction of this solution was added to 10 per cent perchloric acid and the total volume was brought to 13.8 ml. Radioactivity was determined by counting with a Geiger-Müller immersion tube. The counts represented the radioactivity expected from the formation of 0.1 μmole ATP.

The standard stock solution of $^{32}P$ was prepared as follows: Approximately 2 mc of $H_3{}^{32}PO_4$ were added to 1.0 ml of 0.2 N HCl and steamed overnight to insure breakdown of all pyrophosphates. The $^{32}P$ solution was then added to ten volumes of 0.11 M $Na_2HPO_4$, to make up a 0.1 M stock solution containing $^{32}P_i$. This stock preparation was stored in the refrigerator and was diluted with 0.1 M $Na_2HPO_4$ as needed for each experiment. The stock 0.11 M and 0.1 M $Na_2HPO_4$

solutions were purified with acid-washed charcoal (Norite) for use in all phosphate solutions.

Tris-$^{32}P$ was made by diluting carrier-free $^{32}P_i$ with phosphoric acid. The phosphoric acid solution (1.65 g/50 ml) was steamed for approximately four hours, allowed to cool, and further diluted to 100 ml. Tris-base crystals were added to bring the pH of the solution to 7.5. The volume was then brought to 153 ml, to obtain a 0.11 M $H_3PO_4$ solution. Acid-washed charcoal plus celite was added, and removed by centrifugation at 13,000 x g for five minutes at 4 C. The resulting supernatant solution was used to dilute the carrier-free $H_3{}^{32}PO_4$ (0.5 mc), following a procedure similar to the one given above.

Light scattering measurements of mitochondria

Changes in light scattering caused by changes in volume of mitochondria in suspension were measured as changes in absorbance (90), using a Bausch and Lomb Spectronic 505 spectrophotometer. Mitochondria (500 μg protein) were suspended in 2.0 ml of reaction medium containing 0.2 M KCl, 0.02 M HEPES-NaOH (pH 7.5), 2 mM $MgCl_2$, and 2 mg bovine serum albumin. Further additions are indicated in results. This reaction mixture was placed in cuvettes and changes in optical density at 520 nm were recorded. This gives an indication of the magnitude and rate of volume changes in the suspended mitochondria.

Determination of mitochondrial protein

The method of Lowry et al. (59) was used. A 0.5 ml aliquot of the mitochondrial preparation was precipitated with 10 per cent

trichloroacetic acid. After twenty-four hours at room temperature, the precipitate was collected by centrifugation and rinsed twice with 95 per cent ethanol. After air drying, the protein in the precipitate was dissolved in 1 N NaOH, and undissolved material was removed by centrifugation. Serial dilutions of the supernatant were made, Folin reagents were added, and absorbance was determined on a Klett-Summerson photoelectric colorimeter using a #66 filter. Protein concentration was determined by comparison with standard dilutions of bovine serum albumin dissolved in 1 N NaOH.

Isolation of plasma membranes

Plasma membranes were isolated by the methods established by Hodges and Leonard (38,39). Roots from six-day-old corn plants grown in mist culture were rinsed three times in cold deionized water, chilled, and cut into 1 cm sections. The sections (25-50 g) were placed in a cold mortar and ground for ninety seconds in four volumes of grinding medium, which contained 0.25 M sucrose, 3 mM $Na_2EDTA$, 25 mM Tris-MES (pH 7.2), and 2.5 mM dithiothreitol. The homogenate was strained through four layers of cheesecloth and sedimented by centrifugation at 13,000 x g for fifteen minutes. The pellet was then suspended in 5.0 ml of fresh grinding medium. After a thirty-minute centrifugation at 80,000 x g, the pellet was again washed in 10 ml of fresh washing medium. Pellets were then combined for a final thirty-minute centrifugation at 80,000 x g. This pellet contained the membrane vesicles (54) and was suspended in a solution containing sucrose (18 per cent, $^{w}/_{w}$), $MgSO_4$ (1 mM), and Tris-MES (33 mM, pH 7.2). The final suspension

contained 10 mg microsomal protein/ml as determined by the method of Lowry et al. (59) using bovine serum albumin as the standard. All steps in the procedure were carried out at 4 C. The Gilford 240 spectrophotometer was used for colorimetric analysis.

Assay for microsomal adenosine triphosphatase activity

The stock solution of ATP for assays was prepared as follows. Alkali cations were removed from disodium ATP solutions with $H^+$-Dowex resin. The resin was first washed several times with 95 per cent ethanol to remove a yellow pigment. $H^+$-Dowex 50-W-X-2 was added (in the cold, with mechanical stirring) to a solution of disodium ATP until the pH was 2.0; the solution was then filtered through a millipore disc (GSWP 025 00, 0.22 μm). The pH of the filtrate was adjusted to 6.5 with Tris crystals and the ATP concentration was adjusted by dilution to form a 6.66 mM solution; this stock solution was stored in aliquots suitable for each assay.

Assay for adenosine triphosphatase activity was based on the release of $P_i$ from ATP (54). The reaction mixture (5 ml) contained 3.33 mM Tris-ATP, 1.5 mM $MgSO_4$, 33 mM Tris-MES (pH 7.2), and 200 μg of freshly isolated membrane protein, with or without KCl (5 mM). Assays were performed at 38 C and the reaction mixture was agitated during the incubation time. At specific intervals, 1 ml aliquots were removed and added to a cold, acidified ammonium molybdate solution (1 per cent, w/v) and stored in the cold until determinations of $P_i$ were made. The $P_i$ released in each aliquot was estimated colorimetrically by the method of Fiske and Subbarow (24). In the assay, 7 ml of the reducing

agent was added, and color was allowed to develop at room temperature for thirty-five minutes. The reducing solution, freshly prepared for each assay, contained 0.1 g of 1-amino-2-naphthol-4-sulfonic acid, 5.8 g sodium bisulfite, and 0.2 g sodium sulfite in 700 ml water. Control solutions for the assay were terminated at once; the $P_i$ value for such controls was used as a correction factor. The spontaneous breakdown of ATP was monitored at intervals; little or no spontaneous breakdown occurred under assay conditions. A Gilford 240 spectrophotometer equipped with a digital readout and an automatic pipetting device was used to determine absorbance at 660 nm. The values were correlated with values for known concentrations of $P_i$, previously calibrated using the same procedure.

Assay for mitochondrial adenosine triphosphatase activity

The reaction medium contained 0.2 M KCl, 0.02 M HEPES-NaOH (pH 7.5), 0.1 per cent bovine serum albumin, 2 mM $MgCl_2$, and 6.66 mM ATP (disodium), in a total volume of 3.0 ml. The mitochondrial suspension (1 mg protein/ml) was added to the medium, and the mixture was incubated at 30 C. At timed intervals, 0.5 ml aliquots were removed and mixed with 3 ml cold 0.5 N trichloroacetic acid. The precipitated protein was removed by centrifugation for ten minutes at 13,000 x g. One ml of 4 per cent ammonium molybdate (in 2N $H_2SO_4$) and 0.2 ml of 10 per cent $FeSO_4$ (in 1N $H_2SO_4$) were added to the supernatant. After fifteen minutes incubation, the intensity of the resulting blue color was determined with a Gilford 240 spectrophotometer. Phosphate concentrations were determined by comparison with a standard curve for $P_i$

obtained by the same procedure. This is a modification of the procedure of Sumner (92). For assay of adenosine triphosphatase activity in a monovalent cation-free medium, sucrose (0.4 M) replaced KCl, and Tris-ATP was used as the substrate.

Isolation of chloroplast lamellae

Chloroplast lamellae were isolated by a method modified after that of Miflin and Hageman (62). The third leaves of fourteen-day-old corn plants were used. The plants were kept in the dark for twenty-four hours prior to use; this gives a decrease in starch content, which interferes with isolation of chloroplast lamellae (98). Chilled leaves (10 g) were cut in 1 cm segments, rinsed with cold distilled water, and ground in a Waring blender for fifteen seconds with eight volumes of homogenizing medium containing 0.01 M NaCl, 0.01 M Tricine-NaOH (pH 7.8), 2 mM $MgCl_2$, 0.4 M sucrose, 0.01 M glutathione, and 12 mg polyethylene glycol/ml. The homogenate was filtered through eight layers of washed cheesecloth and the chloroplasts were sedimented at 2500 x g for four minutes. The chloroplast pellet was dislodged with a camel hair brush and suspended in 40 ml of medium containing 0.01 M Tricine-NaOH (pH 7.5), 0.01 M NaCl, 2 mM $MgCl_2$, and 0.4 M sucrose. The suspension was centrifuged for forty-five seconds at 2,000 x g to remove cell debris. The chloroplasts were sedimented from the supernatant by centrifugation at 2500 x g for four minutes. Chloroplasts were then resuspended in 5 to 10 ml of the suspending medium, to make up a final concentration of about 0.4 mg chlorophyll/ml. All isolation procedures were at 4 C.

Chlorophyll content of the stock preparation was determined by an established procedure (3). A 0.05 ml aliquot of the plastid preparation was diluted to 10 ml with 80 per cent acetone. The acetone solution was centrifuged at 6500 x g for five minutes to remove particules. The absorbance of the resulting supernatant solution was determined at 663 and 645 nm using a Beckman DB spectrophotometer. The concentration of chlorophyll a plus b (μg/ml) in the acetone solution was calculated by the formula:

$$(A_{663} \times 8) + (A_{645} \times 20.3)$$

Reaction conditions for chloroplast lamellae

The basic reaction medium for experiments with chloroplast lamellae contained 0.1 M sucrose, 0.01 M KCl, 0.05 M Tricine-NaOH (pH 7.8), 1 mM $MgCl_2$, and 20 μg chlorophyll/ml. There were various additions, as indicated in the results section. Total volume of the reaction medium was 2 ml, and the temperature of the reaction vessel was 19 C.

Noncyclic electron transport was monitored with a modified spectrophotometer. The reduction rate of $K_3Fe(CN)_6$ (0.4 mM) was measured as loss of absorbance at 420 nm. The actinic light used was a broad band red light (620-700 nm). The spectrophotometer (Bausch and Lomb Spectronic 505) was fitted with filters to prevent the actinic illumination from interfering with the measurement of light at 420 nm. The amount of ferricyanide reduced was calculated using the millimolar extinction coefficient of 1.06.

Swelling and shrinking of chloroplasts were monitored as changes in light scattering, as determined by changes in absorbance at 540 nm. Details of the optical measurements are described by Izawa and Good (44).

# RESULTS

## I. Experiments with Tissue and Mitochondrial Respiration

### Effects of HM-T toxin on tissue respiration

Infection usually results in an increase in respiration of intact plant tissue; the host-specific toxins studied previously also have this effect (84). Therefore, HM-T toxin was tested for ability to cause respiratory changes in susceptible leaves, coleoptiles, and roots. The effects were compared to those of 2,4-dinitrophenol (DNP).

Leaf discs 1 cm in diameter, twenty discs per treatment, were vacuum infiltrated for fifteen minutes with toxin solutions at 10 or 50 μg/ml, or with DNP at 10 μM. Control discs were vacuum infiltrated with water. After treatment, excess solution was removed by blotting, and the discs were placed in Warburg flasks on wet filter paper to prevent desiccation. DNP-treated tissues consumed 32 μl $O_2$/hr·10 mg dry weight, whereas toxin-treated tissues took up an average 25 μl $O_2$/hr·10 mg. These oxygen uptake values were 30 per cent and 25 per cent greater than those for control tissues (Figure 1).

In other experiments, transpiring leaf cuttings were allowed to take up toxin solutions for four to six hours under light and with constant air movement; leaf discs were then cut and placed in Warburg flasks on wet paper. Rates of oxygen uptake were 20-30 per cent higher

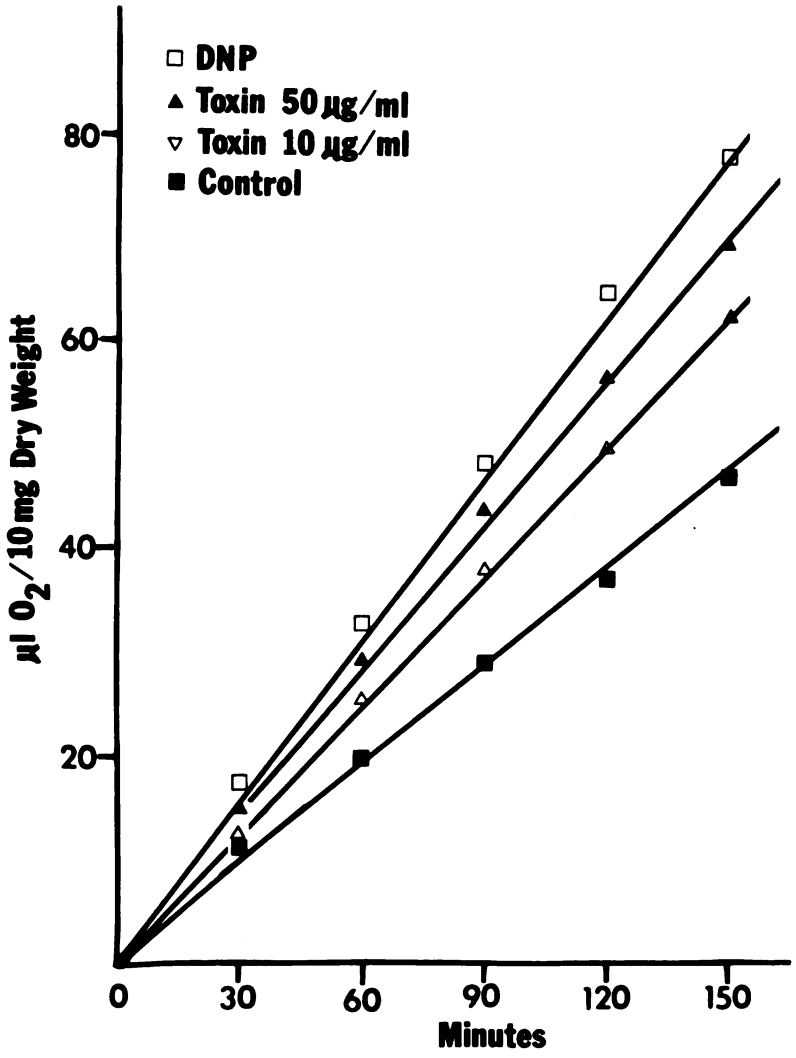



Figure 1. Effect of HM-T toxin on oxygen uptake by leaves from susceptible corn plants. Leaf discs (1 cm diameter) from the second true leaves of fourteen-day-old plants were infiltrated with water, with toxin solutions (50 µg or 10 µg/ml), or with 2,4-dinitrophenol (DNP) (10 µM). Standard manometric techniques were used; reaction vessels were held in the dark at 25.5 C. The equilibration time was fifteen minutes. $ED_{50}$ for the toxin preparation was 20 µg/ml in root growth assay.

for toxin-treated susceptible tissue than for control tissue. The maximum effect was about the same as with infiltration.

Similar, striking effects on respiration of coleoptile tips treated with toxin were observed. In these experiments, coleoptile tips were exposed to toxin (20 μg/ml), using several vacuum infiltration times (15, 30, 45, or 60 minutes) as a variable. Vacuum infiltration caused more than 50 per cent decrease in the rate of respiration by coleoptiles (Figure 2). However, the toxin-infiltrated tissue again had greater oxygen uptake than did the respective control tissue. Maximum stimulation was observed after fifteen minutes infiltration time; longer times of infiltration gave no further increases in oxygen uptake, as determined in several experiments. Therefore, the fifteen-minute vacuum infiltration time was used in all later experiments.

Respiration by susceptible coleoptiles was increased approximately 20 per cent after treatment with HM-T toxin. The experiment was repeated a number of times with similar results; data from representative experiments are given in Figure 3. Thus, the response of coleoptiles was comparable to that of leaves, except that DNP (10 μM) gave about the same degree of stimulation as did toxin. Respiration of resistant tissue was not affected by toxin but the expected response to DNP was observed.

Other experiments showed that oxygen uptake by corn root tips was not affected by toxin. There were no differences in rates of respiration by toxin-treated susceptible tissue, toxin-treated resistant tissue, and control tissues without toxin. The apparent lack of effect

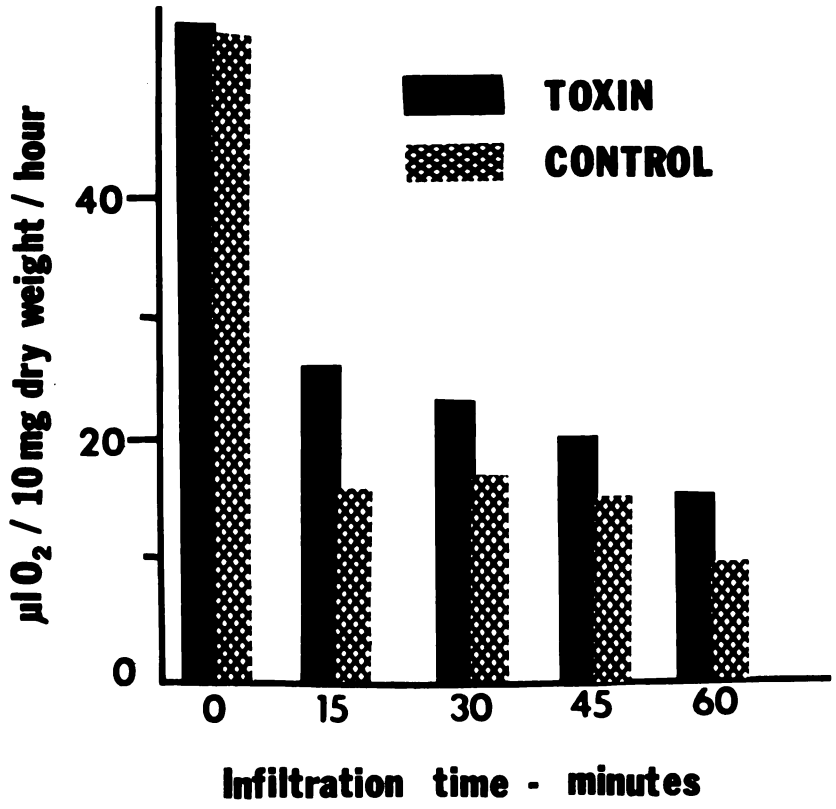



Figure 2. The effect of infiltration on respiration of toxin-treated and control coleoptiles. Three-day-old coleoptile tips (1 to 1.5 cm long) were vacuum infiltrated with water or with toxin (20 µg/ml) for the indicated times. Coleoptile tips were then blotted dry, placed in Warburg flasks (0.5 g tissue/flask) on wet paper, and allowed to equilibrate for fifteen minutes at 25.5 C. $ED_{50}$ for the toxin preparation was 20 µg/ml in root growth assay.

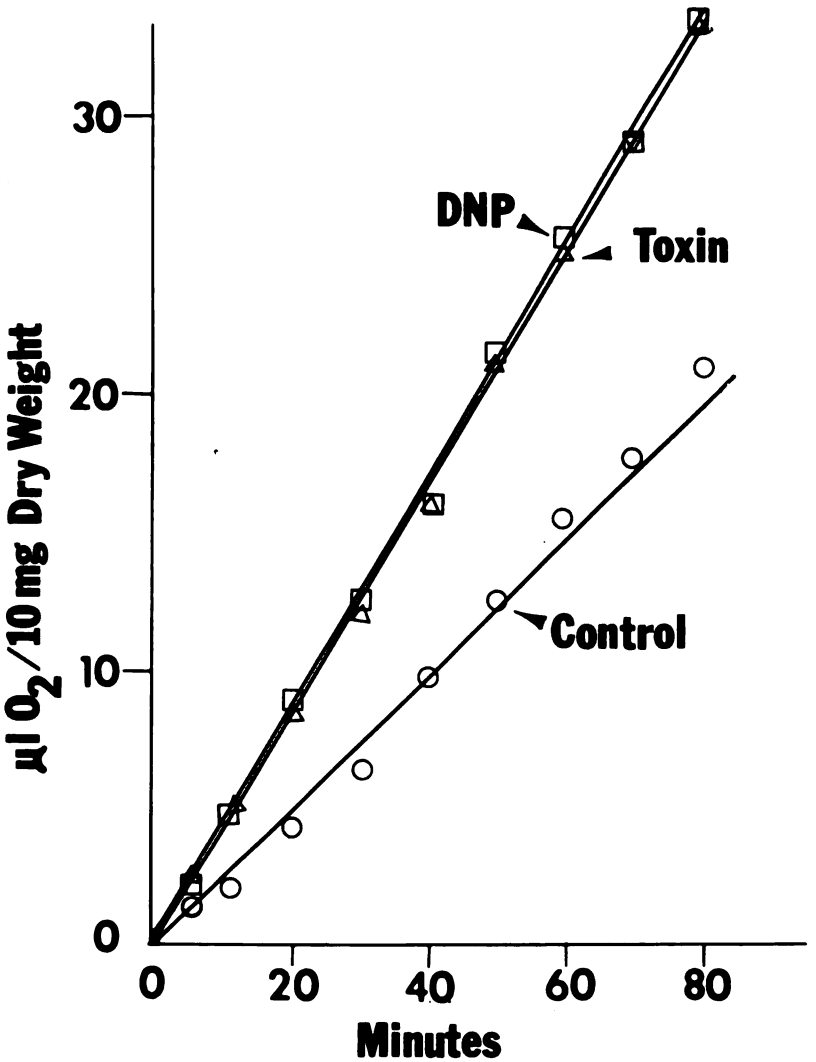



Figure 3. Effect of HM-T toxin and 2,4-dinitrophenol (DNP) on respiration by coleoptiles from susceptible corn seedlings. Approximately twenty coleoptile tips (each, 2 cm long) weighing 0.5 g were placed in a cheesecloth bag and vacuum infiltrated for fifteen minutes with 20 ml of a solution containing toxin (12.5 µg/ml) or DNP (10 µM); control treatments lacked these. Other ingredients of the reaction medium were glucose (10 mM) and potassium phosphate buffer (33 mM, pH 5.0). The tissues were incubated for thirty minutes on a reciprocal shaker at 120 strokes per minute, then were blotted dry and placed in Warburg flasks on wet paper. Equilibration time was twenty minutes and temperature was 25.5 C. $ED_{50}$ for the toxin preparation was 20 µg/ml in root growth assay.

on susceptible tissues does not necessarily indicate absence of uncoupling; other factors may be limiting.

## The effect of HM-T toxin on NADH oxidation and phosphorylation by mitochondria

One possible explanation for the increased respiration in intact tissue is that toxin causes uncoupling of oxidative phosphorylation. This possibility was tested directly on isolated mitochondria from susceptible and resistant plants. In the absence of toxin, oxidation rates of susceptible mitochondria in the presence or absence of added $P_i$ were comparable to those reported by other workers (63). The P/O ratio was 1.22. HM-T toxin added along with $^{32}P_i$ resulted in a 25 per cent decrease in the P/O ratio. On the other hand, addition of HM-T toxin three minutes prior to the addition of $^{32}P_i$ resulted in a 60 per cent decrease in the P/O ratio (Table 1). Thus, there appears to be a lag time in the development of a maximum effect of toxin on coupling in susceptible mitochondria.

These phenomena were examined further by comparing the changes in rates of oxygen uptake by mitochondria after addition of inorganic phosphate, DNP, valinomycin (an ionophorous uncoupler), or HM-T toxin. Data were taken as polarigraph tracings showing changes in oxygen concentration in mitochondrial suspensions. A different mitochondrial preparation was used to test each substance, but in each case the mitochondria were oxidizing exogenous NADH at a comparable rate. Each of these substances caused a stimulation of oxygen uptake. The stimulation of respiration by DNP, $P_i$, and valinomycin was immediate, whereas there

Table 1. Effect of HM-T toxin on oxidative phosphorylation by susceptible mitochondria. In treatment A, mitochondria were preincubated for three minutes in the medium (2 ml) containing buffer, ADP, and $Mg^{++}$; NADH was then added and the mixture was incubated one minute before $P_i$ was added. In treatment B, toxin (1.2 µg) was added immediately before the addition of $P_i$. In treatment C, toxin was present from the beginning of the preincubation period. In all cases, the reaction time after the addition of $P_i$ was three minutes. For the basic components of the reaction medium, see Figure 4. $ED_{50}$ for the toxin preparation was 2 µg/ml in root growth assay.

| Treatment | P/O |
|---|---|
| A - Control | 1.22 |
| B - Toxin added with $P_i$ | 0.91 |
| C - Toxin added three minutes prior to $P_i$ addition | 0.44 |

was a lag of approximately sixty seconds before the maximum rate of electron transport induced by toxin was established. Representative data from three different experiments are shown in Figure 4. The delayed effect of toxin suggests that there may be a barrier in the mitochondrion which must be penetrated by toxin molecules. Data on the effect of toxin on P/O ratios (Table 1) could also be interpreted in this way. Based on these experiments, I have routinely added toxin to mitochondria two to three minutes before the substrate was added. Control mitochondria prepared from resistant (N) seedlings were not affected even by very high concentrations of HM-T toxin (500 μg/ml). The resistant mitochondria had the expected response to all substrates tested and to DNP.

Several different toxin concentrations were tested for effects on susceptible mitochondria. Freshly prepared toxin dilutions were used with mitochondria oxidizing exogenous NADH in the absence of phosphate. There was a sharp increase in the rate of electron transport with increases in toxin concentration up to about 1.0 μg/ml; the rate increased gradually thereafter until it was near the rate for mitochondria under phosphorylating conditions. No inhibitory effect of toxin on electron transport by nonphosphorylating mitochondria was observed, even with high concentrations of toxin. The rate of electron transport by phosphorylating mitochondria was not affected by toxin (Figure 5).

Toxin-induced uncoupling of oxidative phosphorylation was examined further, by determining effects on the rate of ATP formation. ATP formation decreased with increases in toxin concentrations, as measured by assaying an aliquot of each reaction mixture for the amount

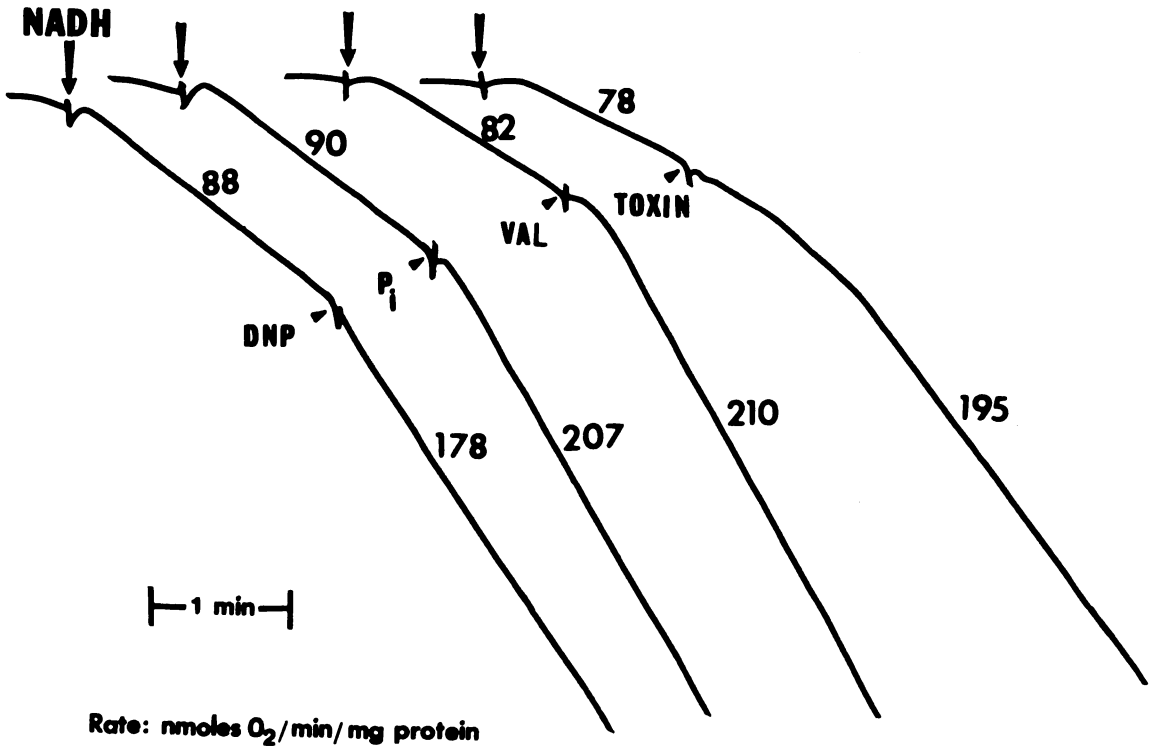



Figure 4. The delayed effect of HM-T toxin on mitochondrial oxidation of NADH, as compared to the response elicited by other agents. The reaction mixture (2 ml) contained 0.2 M KCl, 20 mM HEPES-NaOH (pH 7.5), 2 mM $MgCl_2$, 2 mg bovine serum albumin, 1 mM ADP, and 500 µg mitochondrial protein. The arrow ( ⬇ ) indicates the time that NADH (0.5 mM) was added. Additions of 2,4-dinitrophenol (DNP) ($2.4 \times 10^{-5}$M), $P_i$ (2.5 mM), valinomycin (VAL) ($5 \times 10^{-7}$M), or toxin (0.5 µg) are indicated by pointers ( ▲ ). $ED_{50}$ for toxin preparation was 4.5 µg/ml in root growth assay.

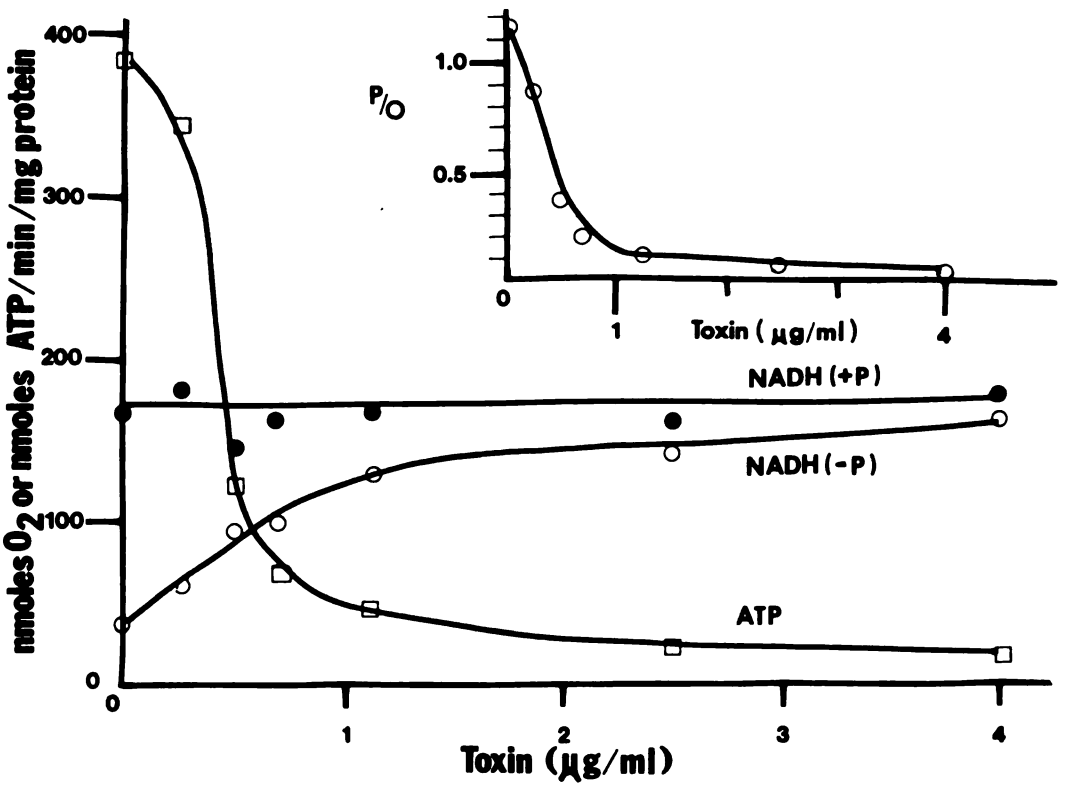



Figure 5. Effect of concentrations of HM-T toxin on ATP formation ( □ ) and on the oxidation of NADH by susceptible corn mitochondria. Phosphorylating ( ● ) and nonphosphorylating ( ○ ) conditions were used for oxidation of NADH. The reaction mixture (2 ml) contained 0.2 M KCl, 20 mM HEPES-NaOH (pH 7.5), 2 mM $MgCl_2$, 2 mg bovine serum albumin, 1 mM ADP, and 500 µg mitochondrial protein. NADH was 0.5 mM and $P_i$ ($Na_2H^{32}PO_4$) was 2.5 mM. Insert shows the effect of toxin on P/O ratios. The toxin preparation at 60 µg/ml gave 50 per cent inhibition of root growth by susceptible seedlings.

of $^{32}P_i$ incorporated into organic phosphate. A plot of the data on the inhibition of ATP formation (Figure 5) gave a curve that is roughly hyperbolic. When the reciprocals of the reaction rate were plotted against toxin concentrations, a straight line was obtained; this suggests a reversible interaction of the toxin with its mitochondrial site. These data show that the toxin acts as an uncoupler.

Effects of toxin on oxidative phosphorylation by mitochondria, with succinate, malate-pyruvate, and NADH as substrates

Toxin-induced changes in the ADP/O ratio for susceptible mitochondria were determined using a toxin concentration that gave a 50 per cent decrease in the P/O ratio (see Figure 5). In determining the ADP/O ratios, the assumption was made that all the ADP added was phosphorylated to ATP; the calculations were based on the amount of ADP added and the atoms of oxygen consumed. Respiratory control ratios were calculated, based on the oxidation rates obtained after the addition and after the complete utilization of ADP. Toxin appears to act as a conventional uncoupler, as shown by decreases in mitochondrial respiratory control, and by decreases in the ADP/O ratios (Table 2). These are the same criteria used to show that DNP is an uncoupling agent.

A more precise measurement is the incorporation of $^{32}P_i$ into organic phosphate (ATP synthesis); this was determined in two experiments (Table 2). Susceptible and resistant mitochondria were incubated in a medium containing ADP and one of three substrates, to which $P_i$ was added. Rates of nonphosphorylating and phosphorylating electron transport were measured. Results showed that toxin caused a 60 per cent

Table 2. Oxidation and phosphorylation by toxin-treated and control susceptible and resistant mitochondria, using NADH (1 mM), succinate (10 mM) and malate-pyruvate (10 mM each) as substrates. The basic reaction mixture (2 ml) is given in Figure 5. Toxin was present throughout the reaction, including the preincubation period of three minutes. The toxin preparation at 60 µg/ml gave 50 per cent inhibition of root growth by susceptible seedlings.

| Expt. No. | Mitochondrial Type | Substrate | Toxin (µg/ml) | Respiration Rate (nmoles $O_2$/min/mg protein) | | | Respiratory Control Ratio | Phosphorylating Ratio |
|---|---|---|---|---|---|---|---|---|
| | | | | +ADP,-$P_i$ | -ADP,+$P_i$ | +ADP,+$P_i$ | | |
| | | | | | | | | ADP/O |
| I | Susceptible | NADH | 0 | - | 91 | 182 | 2.0 | 1.14 |
| | | | 0.2 | - | 102 | 188 | 1.84 | 0.45 |
| | | | 0.5 | - | 112 | 188 | 1.66 | 0.33 |
| | | | | | | | | P/O |
| II | Susceptible | NADH | 0 | 80 | - | 164 | - | 1.18 |
| | | | 0.5 | 129 | - | 139 | - | 0.35 |
| | | Succinate | 0 | 68 | - | 80 | - | 1.43 |
| | | | 0.5 | 75 | - | 108 | - | 0.84 |
| | | Malate-Pyruvate | 0 | 39 | - | 97 | - | 2.18 |
| | | | 0.5 | 17 | - | 21 | - | 0.97 |
| III | Resistant | NADH | 0 | 78 | - | 166 | - | 1.07 |
| | | | 50 | 87 | - | 183 | - | 1.09 |
| | | Succinate | 0 | 60 | - | 91 | - | 1.32 |
| | | | 50 | 58 | - | 88 | - | 1.37 |
| | | Malate-Pyruvate | 0 | 26 | - | 94 | - | 1.87 |
| | | | 50 | 31 | - | 91 | - | 1.70 |

increase in nonphosphorylating electron transport, when mitochondria from susceptible plants were oxidizing NADH. There was much less stimulation (10 per cent) when sodium succinate was the substrate. However, the rate of oxygen uptake was approximately 35 per cent above the rate for control mitochondria when succinate was the substrate and phosphorylating conditions were used. With malate-pyruvate as the substrate, no stimulation of electron transport was observed, either in the presence or absence of inorganic phosphate; instead, there was a marked inhibition. In other experiments, electron transport was completely inhibited by higher concentrations of toxin, when susceptible mitochondria were oxidizing malate-pyruvate.

Toxin prevented incorporation of inorganic phosphate into ATP by susceptible mitochondria, regardless of the substrate. This was shown by decreases in P/O ratios. When NADH was the substrate, the P/O ratio was 1.18 for control, and 0.35 for toxin-treated mitochondria. Likewise, there was a 50 per cent decrease in the amount of phosphate incorporated, when succinate or malate-pyruvate was the substrate. This suggests that probably all three sites of phosphorylation were affected by toxin.

Mitochondria from resistant corn were used in some of the experiments. Toxin did not cause uncoupling or changes in the respiration rate (Table 2), even when the toxin concentration was 100-fold greater than the concentration used on susceptible mitochondria.

Effect of several preparations from Helminthosporium species on NADH oxidation and phosphorylation by isolated corn mitochondria

Resistant and susceptible mitochondria were compared in their response to three other "toxin" preparations. The first of these was a preparation from culture filtrates of H. maydis race 0. Cultures of H. maydis race 0 were grown in the same medium and the culture filtrate was processed by the same methods that were used to obtain HM-T toxin from race T. The second toxin tested against corn mitochondria was HV-toxin prepared by the procedure of Pringle and Braun (73). HV-toxin is known to have a rapid effect on susceptible oat tissues, causing large increases in respiration; it has no effect on mitochondria from oats (10,81). The third "toxin" preparation used was HM-T toxin inactivated by heat, as described by Comstock (17). All of these preparations were tested in two experiments against mitochondria from N and Tms cytoplasm corn; the effects were compared with those of active HM-T toxin.

Susceptible mitochondria were sensitive to HM-T toxin, as indicated by an increase in oxygen uptake in the absence of $P_i$ and by a decrease in the P/O ratio (Table 3). Resistant mitochondria were not affected. The heat inactivated HM-T toxin was used on susceptible mitochondria at 10 μg/ml; it caused 40 per cent stimulation in non-phosphorylating electron transport and 10 per cent decrease in P/O ratio. The experiment was repeated with similar results. The results with inactivated toxin might be caused by high concentrations of extraneous materials in the preparation, or by incomplete inactivation

Table 3. Effect of culture extracts from Helminthosporium spp. on NADH oxidation by corn mitochondria. See Figure 5 for the reaction conditions. Inactivated HM-T toxin at 200 µg/ml and HM-0 "toxin" at 400 µg/ml did not affect growth of susceptible (Tms) or resistant (N) corn roots. HV-toxin inhibited growth of susceptible oat roots at 0.1 µg/ml, and HM-T toxin inhibited growth of susceptible (Tms) corn roots at 50 µg/ml.

| Expt. No. | Toxin | Concentration (µg/ml) | Susceptible | | | Resistant | | |
|---|---|---|---|---|---|---|---|---|
| | | | $-P_i$ | $+P_i$ | P/O | $-P_i$ | $+P_i$ | P/O |
| | | | (nmoles $O_2$/min/mg protein) | | | (nmoles $O_2$/min/mg protein) | | |
| I | None | 0 | 80 | 191 | 0.90 | 84 | 142 | 0.91 |
| | HM-T | 1.0 | 180 | 199 | 0.22 | 73 | 124 | 1.00 |
| | Inactive HM-T | 10 | 129 | 199 | 0.80 | - | - | - |
| II | None | 0 | 127 | 250 | 1.04 | 84 | 142 | 0.91 |
| | HM-0 | 10 | 116 | 232 | 0.82 | 90 | 132 | 0.83 |
| | HV | 10 | 138 | 229 | 0.80 | 86 | 127 | 0.92 |

of toxin. No active toxin was detected in a seedling root growth bioassay. However, mitochondria were shown by other experiments to be more sensitive to HM-T toxin than are seedling roots.

HM-O "toxin" and HV-toxin gave comparable results with mitochondria from both Tms and N corn (Table 3). Coupling was not significantly affected by high concentrations of either preparation. There was a 10 per cent decrease in P/O ratio after exposure to the HM-O preparation (Table 3); this may be experimental error, or it may reflect the high concentration of the relatively crude preparation added to the mitochondrial suspension. Thus, no host-specific activity against corn mitochondria was evident with either of these fungal culture extracts. The amount of HV-toxin added to the mitochondrial suspension was approximately 2000 times greater than is required to completely inhibit root growth in susceptible oats. This experiment was not repeated.

### Comparative effects of HM-T toxin, ionophores, and 2,4-dinitrophenol on electron transport by mitochondria

The uncoupling action of HM-T toxin was compared with that of known uncouplers, including several ionophores. The antibiotic ionophores used were valinomycin, nigericin, and gramicidin D; each was dissolved in a minimal amount of 95 per cent ethanol. Comparable amounts of ethanol, without the antibiotics, caused no apparent change in mitochondria. Valinomycin is known to increase $K^+$ permeability in mitochondria, and uncouples only in the presence of $K^+$. $K^+$ uptake is energy-dependent, and the uptake leads to increased electron transport.

DNP is not dependent on a cation; it probably changes the electrical potential of the mitochondrial membrane by inducing a leakage of protons.

Changes in the rates of oxidation of exogenous NADH by treated and control susceptible and resistant mitochondria were monitored. NADH was used in preference to other substrates because it does not produce an oxidizable product, nor is it a permeant anion as is succinate, malate and pyruvate. There were other experiments to determine whether or not toxin action requires monovalent cations, such as $K^+$. The isolation and reaction media were free of alkali metals; pH was adjusted with Tris crystals, as described in Materials and Methods.

Rates of oxidation of NADH by untreated control mitochondria, with and without $K^+$ (Figure 6), were comparable to rates reported elsewhere (35,63). HM-T toxin caused an increase in NADH oxidation; a further increase in the rate was seen when $K^+$ was added. However, the toxin appears to have much less dependence on $K^+$ for action than does valinomycin. DNP caused uncoupling of mitochondrial electron transport in the absence of $K^+$, although $K^+$ caused a slight increase in oxygen uptake by both DNP-treated and control mitochondria (Figure 6). Toxin had no effect on mitochondria from resistant plants, either in the presence or absence of $K^+$. Valinomycin and DNP had equal effects on resistant and susceptible mitochondria.

Phosphorylating mitochondria were treated with several different concentrations of toxin, with and without $K^+$, in order to determine the influence of $K^+$ on the uncoupling effect of toxin. The data

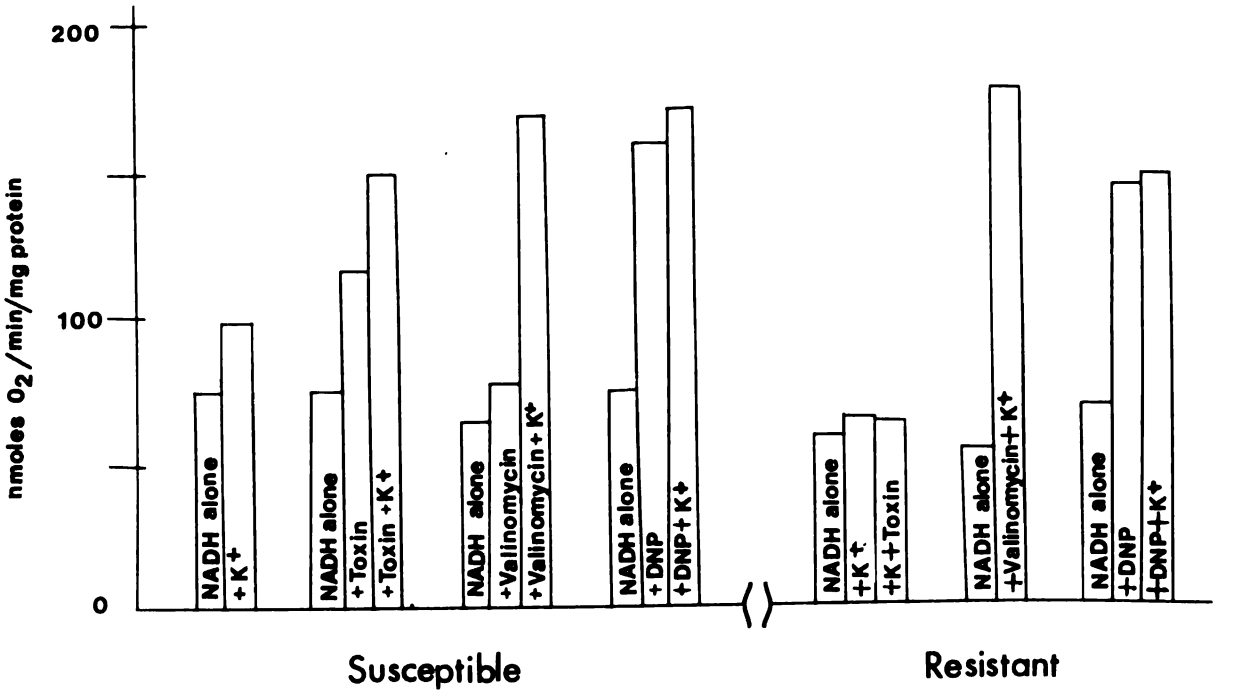



Figure 6. Comparative effects of HM-T toxin and uncouplers on NADH oxidation (under nonphosphorylating conditions) by mitochondria from susceptible and resistant plants. Each value is the average of three replications. The basic reaction mixture was $K^+$-free. Further additions were: 0.5 mM NADH; 50 mM $K^+$ as KCl; 2.0 µg toxin; 20 µM DNP; and 0.01 µM valinomycin. The toxin preparation inhibited 50 per cent of susceptible root growth at 60 µg/ml.

(Table 4) show that toxin caused equal uncoupling, with and without $K^+$ (50 mM). Similar negative results were obtained with $Li^+$, $Na^+$, and $NH_4^+$.

Potassium flux of mitochondria determined with a $K^+$ electrode

An Orion-type $K^+$ sensitive electrode was used to obtain more direct and sensitive determinations of initial influx of $K^+$ into mitochondria and to test further the possibility that toxin does not require $K^+$ for uncoupling. The reaction medium contained sucrose (400 mM), HEPES-Tris (20 mM; pH 7.5), $MgCl_2$ (2 mM), and bovine serum albumin (4 mg), in a total volume of 4 ml. The sucrose osmoticum minimizes passive swelling and maximizes active accumulation of $K^+$ during the oxidation of NADH. The potassium chloride concentration (1 mM) was within the sensitivity range of the $K^+$ electrode.

Only a slight uptake of $K^+$ was seen when susceptible mitochondria were given exogenous NADH (0.125 mM) (Figure 7). A correlation between $K^+$ influx, substrate oxidation, and hydrogen ions is known (47, 78); an active accumulation of $K^+$ was expected. Toxin did not cause a change in $K^+$ influx in susceptible mitochondria. Gramicidin D caused a small efflux of $K^+$ from the mitochondria. Susceptible mitochondria had no further changes in $K^+$ flux. The rate of uptake did not change when substrate was added; further uptake may not occur because the mitochondria were already swollen.

In contrast to susceptible mitochondria, the resistant mitochondria did take up $K^+$ during NADH oxidation. Gramicidin D added after NADH was exhausted caused a release of the accumulated $K^+$, but toxin had no effect on $K^+$ movement. Thus, there was no evidence that

Table 4. Effect of HM-T toxin on oxidation and phosphorylation by susceptible mitochondria with and without $K^+$, using NADH as the substrate. Mitochondria were suspended in a reaction medium free of alkali metal ions except for a small amount of $Na^+$ (1 mM) from the sodium salt of NADH. HEPES-Tris (20 mM, pH 7.5), Tris-ADP (1 mM), and Tris-$P_i$ (2.5 mM) were substituted for HEPES-NaOH, $Na_2ADP$ and $Na_2HPO_4$. $K^+$ was added as KCl (50 mM). The $ED_{50}$ for susceptible roots was 60 µg/ml.

| Toxin concentration (µg/ml) | P/O ratio | |
|---|---|---|
| | $-K^+$ | $+K^+$ |
| 0 | 1.11 | 1.27 |
| 0.5 | 0.70 | 0.99 |
| 1.0 | 0.32 | 0.44 |
| 2.20 | 0.15 | 0.21 |

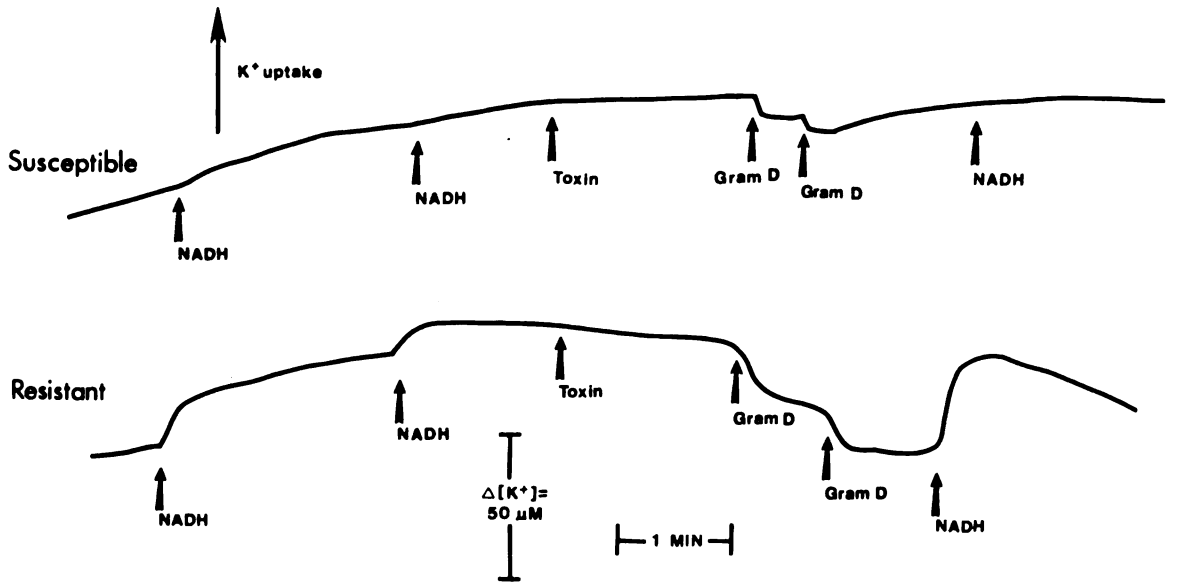



Figure 7. Effects of toxin and gramicidin D on $K^+$ flux in susceptible and resistant mitochondria. Reaction medium contained 0.4 M sucrose, 20 mM HEPES-Tris (pH 7.5), 2 mM $MgCl_2$, 4 mg BSA, and 1 mM KCl, in a total volume of 4 ml. NADH (final concentration, 0.125 mM), gramicidin D (2 μg) and toxin (5 μg) were added as indicated. Changes in concentration of $K^+$ were determined at 28 C with an Orion-type $K^+$-sensitive electrode, with a cation exchanger. $ED_{50}$ for this toxin preparation in the root seedling assay was 20 μg/ml.

toxin affects active uptake of $K^+$ by either type of mitochondrion. However, the $K^+$ experiments raised the interesting possibility that toxin sensitivity of susceptible mitochondria may in some way be related to a deficiency in the $K^+$ uptake mechanism. This line of experimentation was not pursued further.

## Comparative effects of nigericin and toxin on mitochondria from susceptible and resistant corn plants

Nigericin, an antibiotic and an ionophore, is a weak carboxylic acid which complexes $K^+$ and therefore can carry either $K^+$ or $H^+$ but not both. Nigericin at low concentrations does not affect respiratory control in the presence of $K^+$, nor does it accelerate adenosine triphosphatase activity in intact mitochondria (23,51,76).

Nigericin (0.45 μg/ml) was added to susceptible and resistant mitochondria in the presence of NADH. Resistant mitochondria did not respond to nigericin, but oxygen uptake by susceptible mitochondria was almost doubled. The elevated rate of oxygen uptake by susceptible mitochondria was further increased when HM-T toxin was added (Table 5). No such rate change was observed when toxin was added to resistant mitochondria. Resistant mitochondria were also insensitive to nigericin under phosphorylating conditions.

Nigericin at several different concentrations was then tested for effects on susceptible and resistant mitochondria. Nigericin at 0.2 μM caused a 17 per cent decrease in phosphate incorporation in resistant mitochondria and a 27 per cent decrease in susceptible mitochondria. Nigericin at 0.5 μM caused a 20 per cent decrease in

Table 5. Effect of nigericin and HM-T toxin on NADH oxidation by mitochondria from susceptible and resistant corn, under nonphosphorylating conditions. Mitochondria (500 μg protein) were suspended in a medium (2 ml) containing 0.4 M sucrose, 20 mM HEPES-Tris (pH 7.5), 2 mM $MgCl_2$, 50 mM KCl, and 2 mg BSA. Final concentrations of NADH, nigericin, and toxin were 0.5 μM, 0.45 and 0.5 μg/ml, respectively. $ED_{50}$ for the toxin preparation in a root seedling assay was 60 μg/ml.

| Treatment | Oxygen uptake | |
|---|---|---|
| | Resistant | Susceptible |
| | (nmoles/min/mg protein) | |
| NADH | 66 | 62 |
| NADH + nigericin | 73 | 116 |
| NADH + nigericin + toxin | 75 | 168 |

phosphate incorporation by resistant and a 60 per cent decrease in incorporation by susceptible mitochondria (Table 6). The experiment was repeated with essentially the same results. These responses suggest that Tms and N mitochondria differ in characteristics other than toxin sensitivity. The possible significance of these differences will be discussed later.

## Comparative effects of calcium and HM-T toxin on mitochondrial phosphorylation

Data presented above show that $K^+$ and other monovalent cations have little or no effect on the action of HM-T toxin. Studies were made with $Ca^{++}$, to test further any possibility that toxin might be interacting with a cation pump in mitochondria.

Calcium accumulation by maize mitochondria is an energy dependent process supported either by substrate oxidation or by ATP (64). An attempt was made, using phosphorylation as the criterion, to detect interference by toxin with the supply of energy required for the transport of $Ca^{++}$, or to detect any interference of $Ca^{++}$ with toxin. Results showed that mitochondria were affected by $Ca^{++}$ ($10^{-3}$M), as indicated by the drop in P/O value from 0.96 to 0.31 after $Ca^{++}$ was added. Toxin (3 µg/ml) alone caused the P/O value to drop to 0.20. Susceptible mitochondria incubated with $Ca^{++}$ ($10^{-3}$M) plus toxin (3 µg/ml) had a P/O ratio of 0.15. Thus, $Ca^{++}$ did not interfere with uncoupling induced by toxin.

$Ca^{++}$ at $10^{-4}$M was used in a second experiment; results were similar to those reported for $10^{-3}$M $Ca^{++}$. Thus, both $Ca^{++}$ and toxin

Table 6. Effect of nigericin on oxidative phosphorylation by susceptible and resistant mitochondria, using NADH as the substrate. The reaction medium given in Table 5 was used, except that 1 mM ADP and 2.5 mM $Na_2H^{32}PO_4$ were added.

| Experiment No. | Nigericin concentration (μM) | P/O ratios | |
|---|---|---|---|
| | | Resistant | Susceptible |
| I | 0 | 0.99 | 0.99 |
| | 0.2 | 0.81 | 0.72 |
| | 0.5 | 0.76 | 0.51 |
| II | 0 | 0.96 | 0.94 |
| | 0.5 | 0.90 | 0.43 |
| III* | 0 | 1.03 | 1.02 |
| | 0.01 | 0.96 | 0.91 |
| | 0.1 | 0.83 | 0.75 |
| | 1.0 | 0.80 | 0.62 |

*The nigericin used in this experiment had been in solution for three weeks and had lost activity.

lowered the P/O ratio of susceptible mitochondria; in combination, there may have been a slight additive effect (Table 7). Resistant mitochondria responded to $Ca^{++}$ the same as did susceptible mitochondria; toxin did not affect the P/O ratio, and toxin did not affect uncoupling induced by $Ca^{++}$ (Table 7).

## The effect of HM-T toxin on mitochondrial swelling

Volume changes by mitochondria in suspension cause changes in light scattering, and this can be measured spectrophotometrically. Volume changes are caused by movement of ions and water into and out of the organelle; for example, corn mitochondria swell passively when suspended in a KCl medium (90). Contractile changes resulting from ion flux require ATP or substrate oxidation (90). Thus, volume changes also reflect the integrity of the mitochondrial membrane.

Changes in light scattering by mitochondria suspended in a buffered-KCl medium were measured with a Bausch and Lomb spectrophotometer. Initial passive swelling, seen as a decrease in absorbance at 520 nm, was observed in all experiments (Figure 8). The addition of NADH stopped swelling and caused a slight contraction in the mitochondria. Toxin stimulated passive swelling; addition of NADH had no stabilizing effect on toxin-treated mitochondria (Figure 9). Gramicidin D also induced swelling but its effect was reversed by addition of NADH. Toxin-induced swelling of isolated mitochondria appeared to be independent of respiration, since susceptible mitochondria responded to toxin similarly in the presence or absence of substrate.

Table 7. Comparative effects of $Ca^{++}$ and HM-T toxin on oxidative phosphorylation by mitochondria, using NADH as the substrate. The reaction medium (2 ml) contained 0.4 M sucrose, 20 mM HEPES-Tris (pH 7.5), 2 mM $MgCl_2$, 2 mg BSA, 1 mM Tris-ADP, 0.5 mM NADH, 2.5 mM Tris-$P_i$, 500 µg mitochondrial protein, and $Ca^{++}$ as indicated. The toxin preparation gave 50 per cent inhibition of susceptible root growth at 20 µg/ml.

| Expt. No. | Mitochondrial type | Toxin concentration (µg/ml) | P/O ratios | | |
|---|---|---|---|---|---|
| | | | $-Ca^{++}$ | $+Ca^{++}$ ($10^{-3}$M) | $+Ca^{++}$ ($10^{-4}$M) |
| I | Susceptible | 0 | 0.96 | 0.31 | - |
| | | 3 | 0.20 | 0.15 | - |
| II | Susceptible | 0 | 1.06 | 0.37 | 0.90 |
| | | 3 | 0.21 | 0.12 | 0.15 |
| | Resistant | 0 | 1.19 | 0.41 | - |
| | | 3 | 1.10 | 0.35 | - |

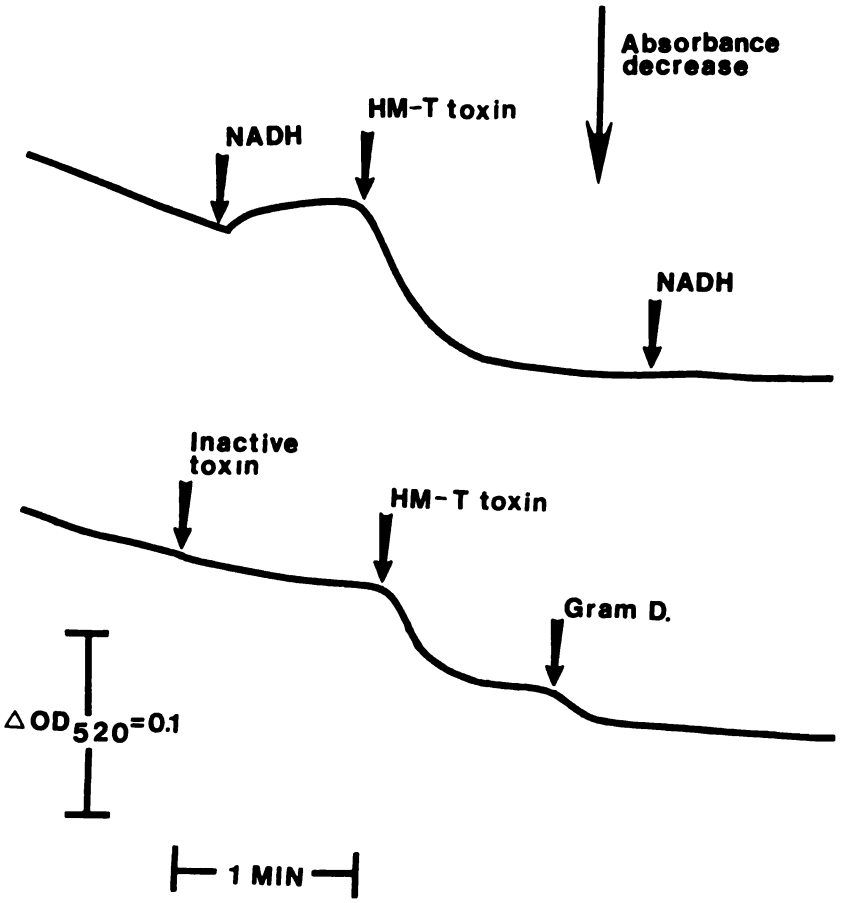



Figure 8. Effect of HM-T toxin on swelling of mitochondria in suspension. Light scattering at 520 nm was monitored with a Bausch and Lomb spectrophotometer 505. The reaction mixture is given in Figure 9. Controls included heat-inactivated HM-T toxin and gramicidin D (Gram D). The toxin preparation inhibited root growth 50 per cent when used at 2 μg/ml.

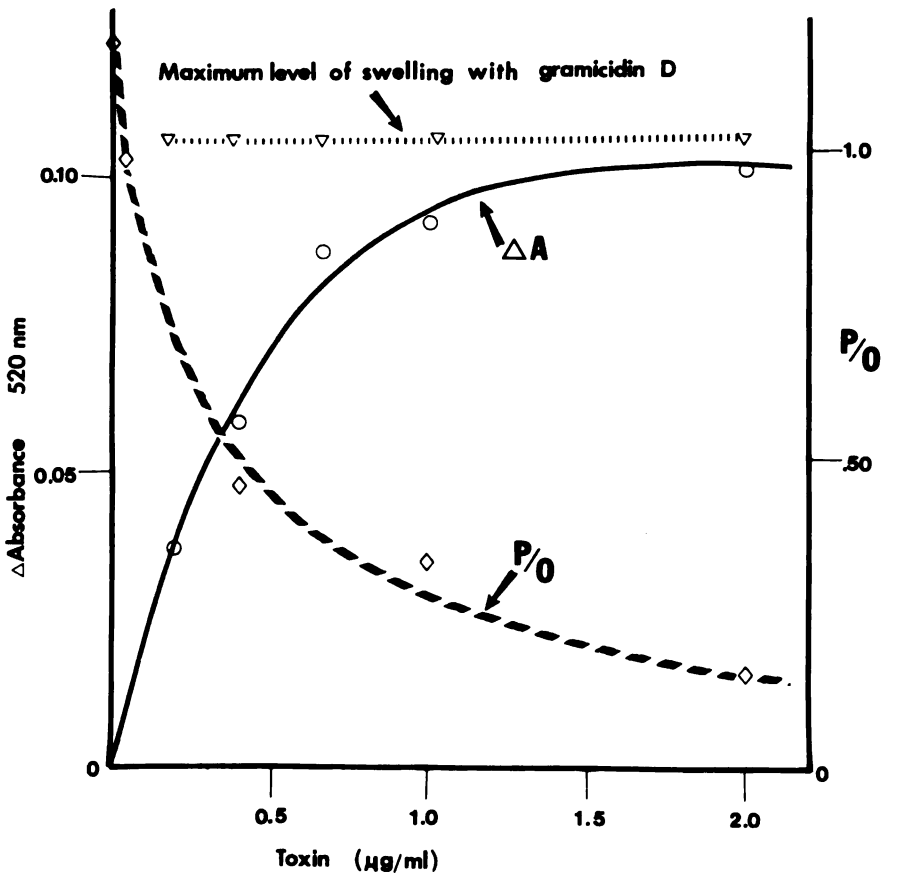



Figure 9. Effect of HM-T toxin at several concentrations on swelling of mitochondria and on concomitant oxidative phosphorylation. The △ A curve shows toxin-induced changes in absorbance at 520 nm for mitochondria suspended in KCl reaction medium. The reaction mixture (2 ml) contained 0.2 M KCl, 20 mM HEPES-NaOH (pH 7.5), 2 mM $MgCl_2$, 2 mg BSA, 0.5 mM NADH, and 500 μg mitochondrial protein. After absorbance values for toxin-induced changes were determined, gramicidin D was added to determine maximum swelling capacity (top line). $ED_{50}$ for the toxin preparation in a root bioassay was 2 μg/ml.

Susceptible mitochondria did not respond to inactivated HM-T toxin; when active toxin was added to the same mitochondria, there was an immediate increase in swelling (Figure 8). Gramicidin D caused only a small further increase in swelling. In other experiments, gramicidin D was added prior to addition of toxin, and there was no change in the gramicidin-induced swelling rate. Swelling in resistant mitochondria was not affected by toxin. Each of these experiments was repeated at least three times.

Swelling induced by HM-T toxin was a quantitative response which increased with increases in toxin concentration up to about 1.5 μg/ml (Figure 9). The degree of swelling caused by toxin was correlated with the degree of uncoupling when mitochondria were oxidizing NADH in the presence of ADP and $P_i$ (Figure 9). Gengenbach et al. (29) reported that toxin-induced swelling is correlated with per cent stimulation of oxygen uptake. However, they did not look for changes in efficiency of phosphorylation.

## Reversibility of the effect of HM-T toxin on susceptible mitochondria

A plot of toxin concentration vs. ATP formation (see Figures 5 and 9) gave a hyperbolic curve; this is consistent with the hypothesis of a reversible interaction between toxin and a sensitive site.

The possibility of firm binding of toxin to a mitochondrial site was tested directly; if there is firm binding, toxin should be removed from solution by mitochondria. A concentration of toxin which caused 50 per cent inhibition of $P_i$ incorporation by mitochondria was used. Toxin was incubated in a mitochondrial suspension that had

twice the protein concentration than that used to determine the 50 per cent inhibition point. The mitochondria were then removed by centrifugation, and fresh mitochondria were added to the supernatant solution. The effect of the supernatant solution on fresh mitochondria should give an indication of how much the toxin concentration was reduced. Results showed that essentially all toxin remained in solution (Figure 10). This implies that little or no toxin was bound firmly to sites in the mitochondria, and that reversible binding is a reasonable possibility.

In another experiment, susceptible mitochondria were exposed to a toxin concentration that was known to give 50 per cent inhibition of phosphorylation. Control mitochondria were placed in a reaction mixture without toxin. After five minutes incubation, the mitochondrial suspension was diluted with ten volumes of washing medium minus BSA and centrifuged for five minutes at 13,000 x g. The supernatant solution was removed carefully and the sides of the test tubes were wiped to remove traces of toxin solution. The pelleted mitochondria were then resuspended in a fresh reaction medium without toxin; most (75-80 per cent) of the mitochondrial protein was recovered by centrifugation. Samples of the mitochondria previously exposed to toxin were then tested for oxidative phosphorylation, using NADH, succinate and malate-pyruvate as substrates. The results showed that the mitochondria (which were initially uncoupled by toxin) recovered, after washing, to almost the original phosphorylating capacity (Table 8). The washing procedure caused some lowering of electron transport rates in control mitochondria, particularly in the malate-pyruvate system. However,

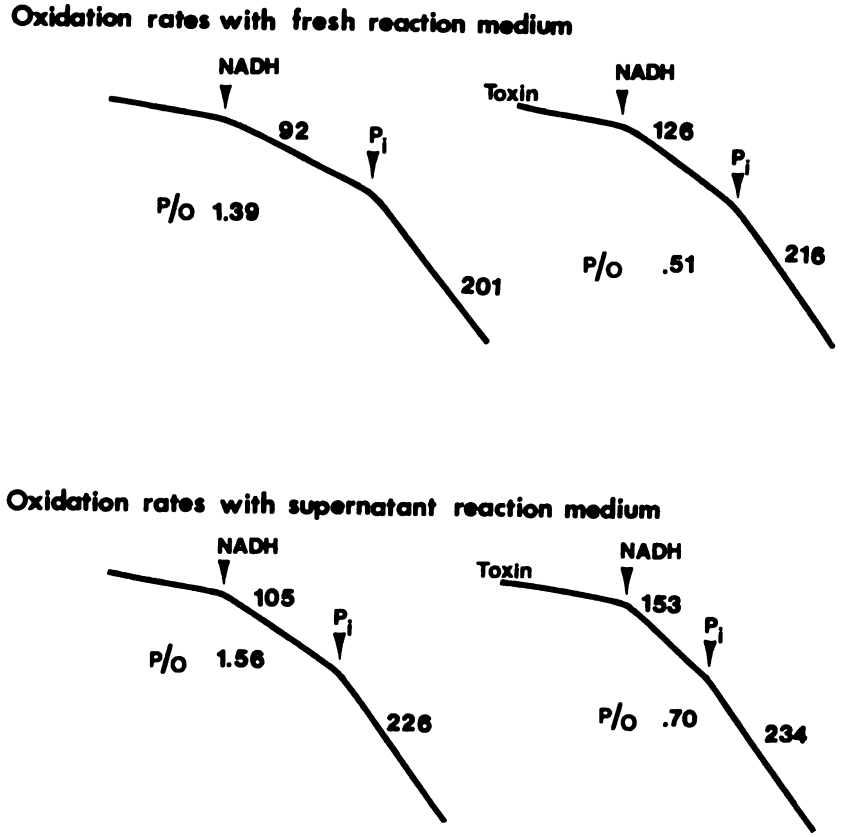



Figure 10. Tests for the presence of toxin left in solution after removal of susceptible mitochondria, as determined by uncoupling effects. The top curves represent the experimental controls. Oxygen uptake and P/O ratios of susceptible mitochondria (0.5 mg protein) incubated in the presence (upper right) and absence (upper left) of HM-T toxin indicate the uncoupling activity of the toxin preparation. This same amount of toxin was preincubated for five minutes with 1.0 mg mitochondrial protein; the mitochondria were then removed by centrifugation and the supernatant was assayed against fresh mitochondria (0.5 mg), (lower tracings). The basic reaction mixture is given in Figure 9. Oxygen uptake rates (μmoles/min·mg protein) are shown. The toxin preparation gave 50 per cent inhibition of susceptible seedlings at 4.5 μg/ml.

Table 8. Reversible effects of HM-T toxin on susceptible mitochondria. In all treatments, mitochondria were preincubated with the reaction mixture (minus $P_i$ and substrate) in the presence (+) or absence (-) of toxin. In "Standard Treatment," mitochondrial activity was then measured with the addition of a substrate and $P_i$. In "After Incubation" treatments, the toxin-treated (+) and control (-) mitochondria were washed, resuspended in fresh reaction medium and tested for oxidative phosphorylation. Reaction media and conditions are given in Figure 9.

| Expt. No. | Substrate | Toxin | Standard Treatment | | | After Incubation and Washing | | |
|---|---|---|---|---|---|---|---|---|
| | | | Oxygen uptake (nmoles/min·mg protein) | P/O | Change in P/O (%) | Oxygen uptake (nmoles/min·mg protein) | P/O | Change in P/O (%) |
| I | NADH | - | 182 | 1.40 | | 173 | 1.11 | |
| | | + | 205 | 0.65 | -54 | 148 | 1.16 | 0 |
| II | NADH | - | 208 | 1.13 | | 228 | 1.10 | |
| | | + | 194 | 0.54 | -52 | 126 | 0.91 | -17 |
| III | NADH | - | 229 | 1.28 | | 183 | 1.30 | |
| | | + | 196 | 0.30 | -77 | 162 | 1.10 | -15 |
| | Succinate | - | 113 | 1.80 | | 91 | 1.60 | |
| | | + | 83 | 0.55 | -69 | 65 | 1.51 | - 6 |
| | Malate-Pyruvate | - | 102 | 1.97 | | 64 | 1.85 | |
| | | + | 38 | 0.72 | -63 | 57 | 1.69 | - 9 |

malate dehydrogenase is known to be a soluble matrix enzyme which is released by swollen mitochondria (21). HM-T toxin induces swelling of mitochondria; hence, some malate dehydrogenase may be lost, and this could account for the slightly lower oxidation rates in treated mitochondria.

These results support the concept of a toxin-mitochondrial interaction which is essentially reversible in short term experiments. The toxin removal data, presented earlier, and the hyperbolic nature of the inhibition curve all support the tentative conclusion that toxin does not form a firm or irreversible bond with the mitochondrion.

## II. Experiments Involving Glycolytic Respiration

### Glycolysis by cell-free preparations in the presence of HM-T toxin

The extreme sensitivity of susceptible Tms mitochondria to HM-T toxin suggests that the mitochondrion may be the primary site of toxin action. The stimulatory effect of HM-T toxin on tissue respiration may also be explained by the uncoupling effect of the toxin on mitochondria *in vivo*. If the only direct effect of toxin is on mitochondria, then preparations of soluble glycolytic enzymes should not be affected.

Glycolytic enzymes were extracted from the acetone powder of coleoptile tissues with a buffer solution (0.03 M HEPES, pH 7.6) as described earlier. This enzyme preparation was placed in Warburg flasks (1.5 ml/flask) and the following were added, to a final volume of 2.3 ml: $KH_2PO_4$, 24 mM; fructose, 10 mM; glucose-1-P, 50 mM; $MgCl_2$,

20 mM; $NAD^+$, 0.3 mM; and ATP, 0.13 mM. When a linear rate of $CO_2$ release was established, toxin was introduced from the side arm of the flask. Glycolysis by this complete system released 16.4 µl $CO_2$/hour·flask. There was no significant change in the rate after addition of toxin; the rates for toxin-treated (4 and 12 µg/ml) preparations were 15.5 µl and 17.1 µl $CO_2$ released/hour·flask. This experiment was repeated four times with negative effects of toxin. Similar negative results were obtained with enzyme preparations from resistant tissues.

In another experiment, coleoptile tips from five-day-old plants were frozen quickly and then treated with toxin. Control sections were treated with water. Although the rate of glycolytic $CO_2$ evolution was low, there was no difference between toxin-treated and control preparations. The experiment was repeated twice with similar results. Cell-free tissue homogenates were then prepared and supplemented with $P_i$ (0.4 M), fructose (1.0 M), glucose-1-P (0.5 M), $NAD^+$ (10 mM), and ATP (6.6 mM). Again, there was no difference in rates of $CO_2$ release by toxin-treated and control preparations.

Effect of HM-T toxin on tissue glycolysis

Glycolysis by cell-free enzyme preparations from susceptible and resistant tissues was not affected by toxin. Toxin was then tested for an effect on glycolysis in intact tissues. Coleoptile sections from susceptible plants were treated with toxin and placed under anaerobic conditions; these tissues had lower rates of $CO_2$ evolution than did control tissues without toxin (Figure 11). In contrast,

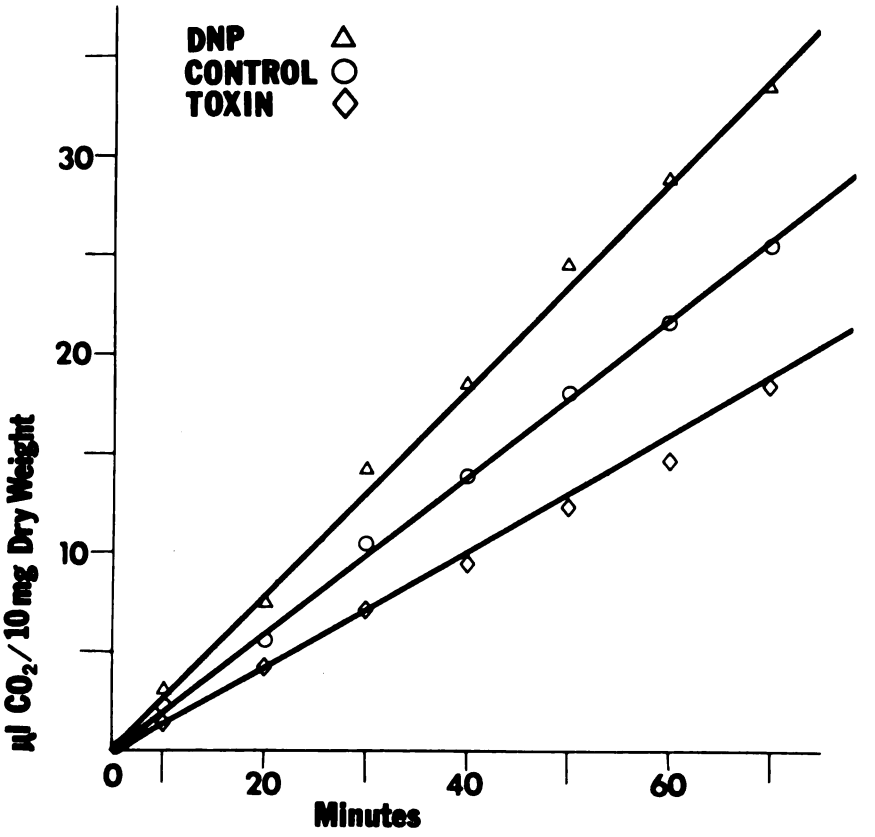



Figure 11. Effect of HM-T toxin on glycolytic respiration by coleoptile tips from susceptible plants. Tissue sections (2 cm long) were vacuum infiltrated in a solution containing glucose (0.01 M) and phosphate buffer (pH 5.8), with or without toxin (12.5 µg/ml) or 2,4-dinitrophenol (DNP) (50 µM). Sections were blotted dry and placed in Warburg flasks (0.5 g tissue/flask) which were then flushed with nitrogen. After equilibration for fifteen minutes at 25.5 C, $CO_2$ evolution was determined manometrically. The $ED_{50}$ for the toxin preparation in a root growth assay was 20 µg/ml.

tissues pre-treated with 2,4-dinitrophenol (50 μM) released more $CO_2$ than did control tissues. In a second experiment, several concentrations of HM-T toxin and DNP were used. Results showed that low concentrations of DNP stimulated $CO_2$ evolution, but high concentrations (3 mM or more) completely inhibited glycolysis. HM-T toxin did not stimulate $CO_2$ evolution at any concentration; low concentrations gave rates comparable to those of controls. Toxin at 10 μg/ml generally caused a 30 per cent decrease in $CO_2$ output, whereas 100 μg/ml gave approximately 50 per cent inhibition of glycolysis. The maximum inhibition by toxin never exceeded 60 per cent.

The effects of varying the toxin exposure time were determined in another set of experiments. Coleoptile tips were incubated in a toxin solution for 0, 15, 30, 45 or 60 minutes. With no preincubation and no vacuum inflitration, tissues in toxin solution for fifteen minutes had less $CO_2$ evolution than did control tissues. Tissues exposed for thirty minutes had even less $CO_2$ output, but no further decrease was evident with exposures > thirty minutes (Figure 12). Thus, thirty minutes exposure is needed to insure a maximum effect. Release of $CO_2$ by resistant tissue was not affected by toxin.

Susceptible tissues were then treated with various intermediates and cofactors of the glycolytic cycle, prior to or along with the application of toxin. ATP, ADP, $Na^+$, $K^+$, fructose, glucose, glucose-1-P, $NAD^+$, and $PO_4^{-3}$ were applied alone and in many combinations; none of these affected toxin-induced inhibition of glycolysis.

The possibility that the inhibition of glycolysis caused by toxin was a secondary response and an indirect consequence of the

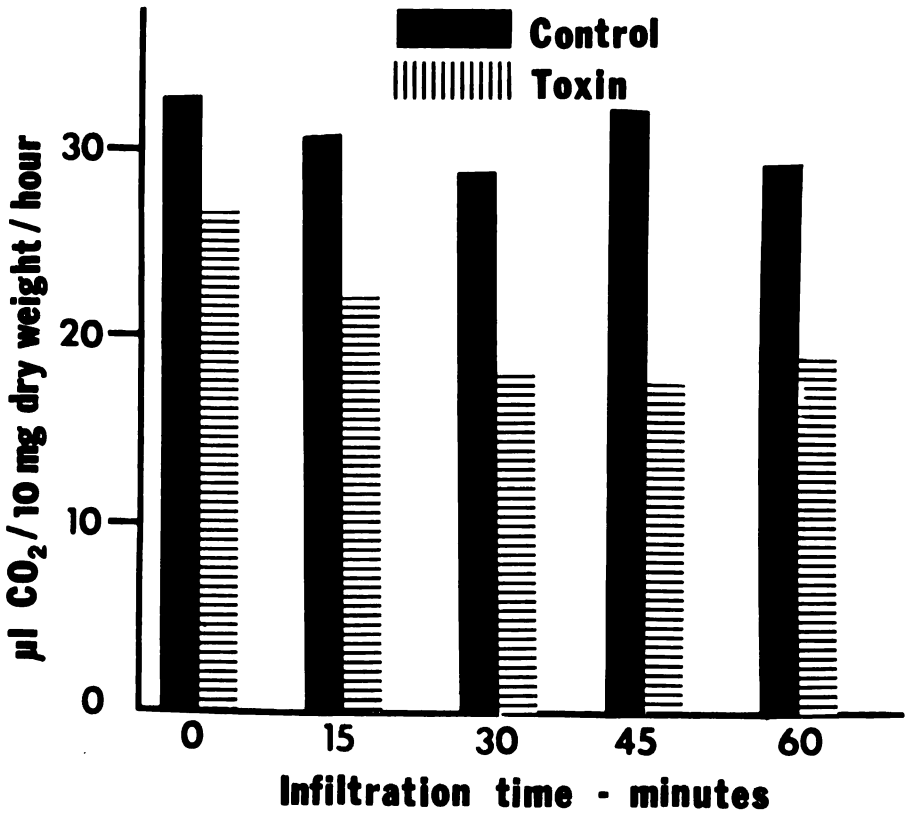



Figure 12. Effect of toxin exposure time on glycolysis by coleoptile tissue. Three-day-old coleoptile tips (2 cm long) were vacuum infiltrated in water or in toxin solution (20 µg/ml) for 0, 15, 30, 45 and 60 minutes. Tissues (0.5 g/flask) were blotted dry and placed in Warburg flasks, which were flushed with nitrogen for twenty-five minutes. Equilibration time was fifteen minutes, and temperature was 25.5 C. $CO_2$ evolution was determined manometrically. The $ED_{50}$ for the toxin preparation in a root growth assay was 20 µg/ml.

mitochondrial effect was examined. Root tissue was first placed under anaerobic conditions and a linear rate of glycolysis was established prior to the introduction of toxin. No immediate change in the rate of glycolysis was seen in tissue exposed to toxin in this manner. DNP caused a decrease, within thirty minutes, in the amount of $CO_2$ released (Figure 13).

The effects on glycolysis discussed thus far were obtained in short term experiments. Next, the effect of toxin over a longer time was examined. Coleoptile sections were treated with toxin (20 µg/ml), DNP (0.5 mM), oligomycin (50 µg/ml), or toxin plus oligomycin mixture for twenty minutes and monitored for $CO_2$ evolution for four hours. Controls were incubated in the glucose/phosphate buffer. Results were plotted as per cent change in $CO_2$ release at sixty-minute intervals. The control, DNP, and oligomycin-treated tissue had a constant rate of $CO_2$ release over the four-hour period. $CO_2$ evolution was inhibited in the toxin-treated and in the toxin plus oligomycin-treated tissues. However, the tissues appeared to recover from the initial inhibitory effects of toxin; almost half of the inhibitory effect on glycolysis had disappeared within four hours after treatment (Figure 14). The rates of $CO_2$ released do not extrapolate to identical starting values, indicating differences in early responses of the tissues to treatment, as seen previously (Figure 12).

Oligomycin, an inhibitor of mitochondrial adenosine triphosphatase (71),was applied along with toxin, using appropriate controls. Oligomycin alone caused only a 10 per cent decrease in $CO_2$ evolution, whereas toxin plus oligomycin caused a 50 per cent decrease and toxin

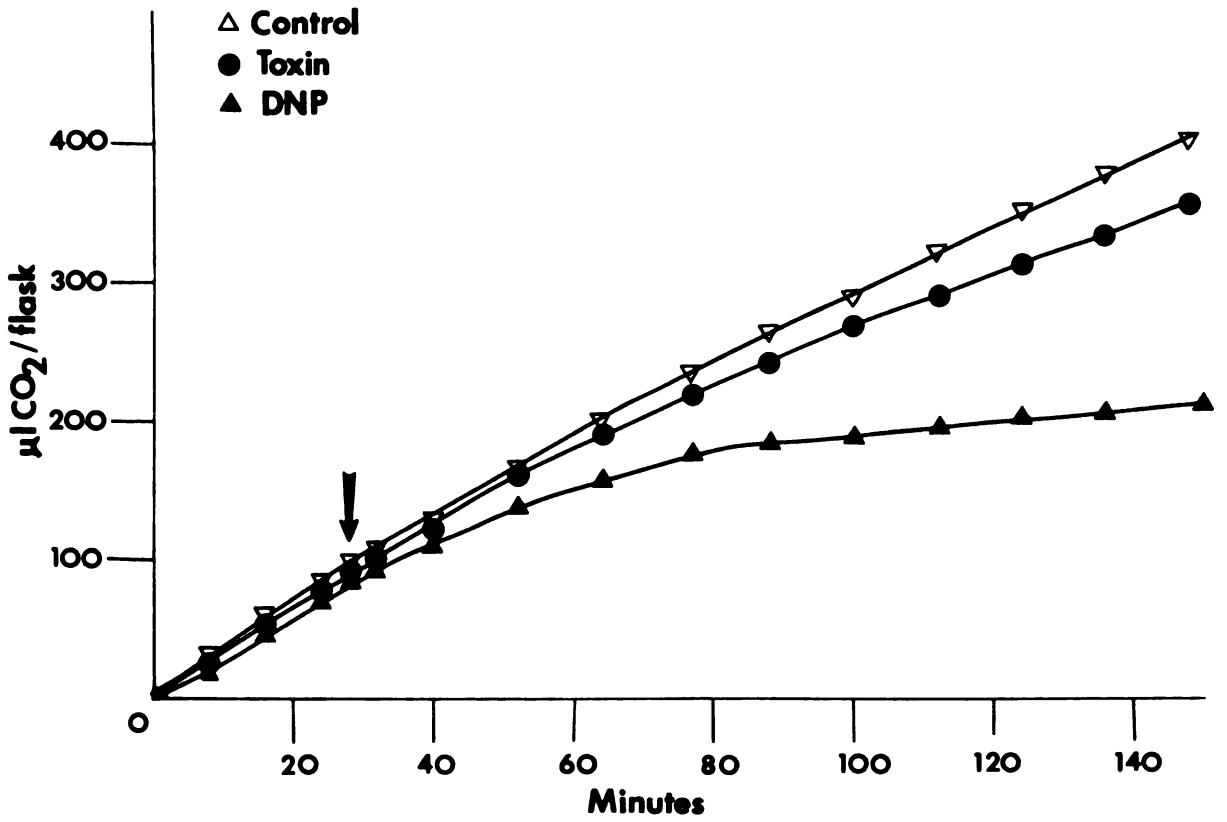



Figure 13. Comparative effects of HM-T toxin and 2,4-dinitrophenol (DNP) on $CO_2$ release from root sections under anaerobic conditions. Four-day-old susceptible root tips (each, 1 cm long; 0.5 g/flask) were placed in Warburg flasks in potassium phosphate buffer (5 mM, pH 5.5) and agitated during a twenty-minute flush with nitrogen. Equilibration time was fifteen minutes at 25.5 C. When linear rates were established, toxin (100 µg/ml) and DNP (2 mM) were added from the side arm, as indicated by arrow ( ↓ ). $CO_2$ evolution was determined manometrically. $ED_{50}$ for the toxin preparation in a root growth assay was 20 µg/ml.

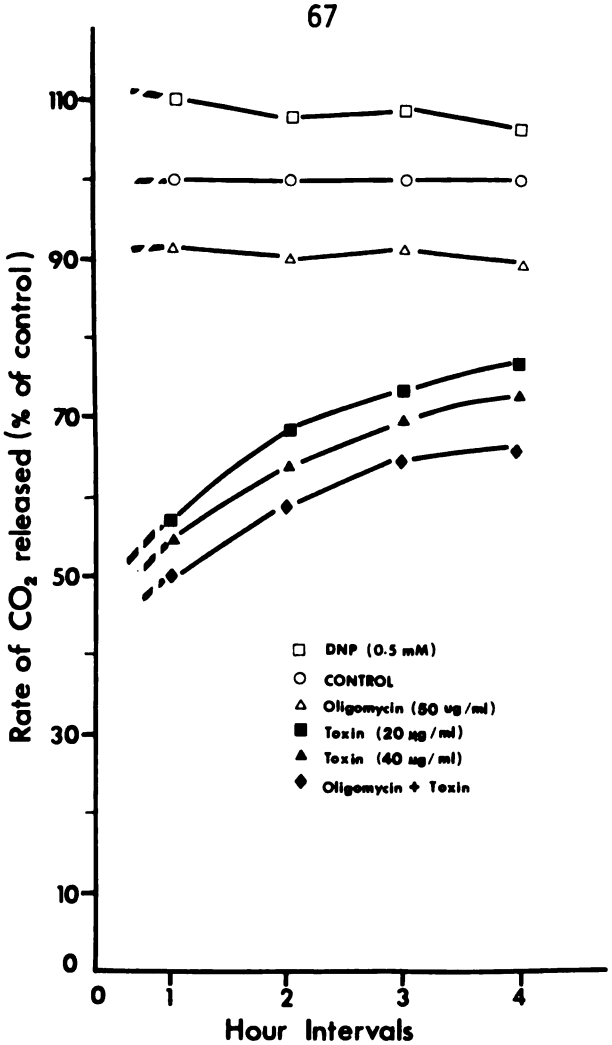



Figure 14. Effects of HM-T toxin, 2,4-dinitrophenol (DNP), and oligomycin on glycolytic $CO_2$ evolution by susceptible tissues. Coleoptile tips (2 cm long) were placed in a solution containing glucose (0.01 M) and phosphate buffer (5 mM, pH 5.8), and vacuum infiltrated for twenty minutes. Toxin (20 and 40 µg/ml), DNP (0.5 mM), or oligomycin (50 µg/ml) were added at the beginning of the infiltration period. Tissue sections were blotted dry, placed in Warburg flasks (0.5 g/flask) on wet paper, and flushed with nitrogen. Equilibration time was fifteen minutes and temperature was 25.5 C. $CO_2$ evolution was then determined manometrically for six hours; changes in rates were recorded for each hour as indicated. Untreated control rate was 35 µl $CO_2$ released/10 mg dry wt·hour. $ED_{50}$ for the toxin preparation in a root growth assay was 60 µg/ml.

alone caused a 40 per cent decrease. The possible additive effect of oligomycin may mean that the substances have different modes of action. Effects of toxin on mitochondrial adenosine triphosphatase, to be discussed later, support this suggestion.

Glycolytic responses to toxin by roots and coleoptiles were compared. The roots used in this set of experiments were not vacuum infiltrated; instead, the tissue was incubated for thirty minutes on the reciprocating shaker. Glycolysis was inhibited in root tissue, as it was in coleoptiles; similar responses to several toxin concentrations were evident. Maximum inhibition in roots was evident at about 10 μg toxin/ml (Figure 15). The experiment was repeated, using susceptible tissues, resistant tissues, and DNP controls. Results (Figure 16) were similar to those previously obtained with coleoptiles under anaerobic conditions.

## III. Determination of Acid-Labile Phosphate and Phosphatase Activity

### Effect of HM-T toxin on phosphate levels in root tissue

Phosphate levels in toxin-treated and control tissues were compared, in an attempt to correlate the inhibition of glycolysis induced by toxin with the *in vitro* effect on mitochondria. Root tissues were treated with toxin, with DNP, or with water (controls). Tissues were placed in Warburg flasks under anaerobic conditions for determination of $CO_2$ release over a one-hour period. The tissues were then quickly frozen with dry ice and later homogenized in cold 0.5 N

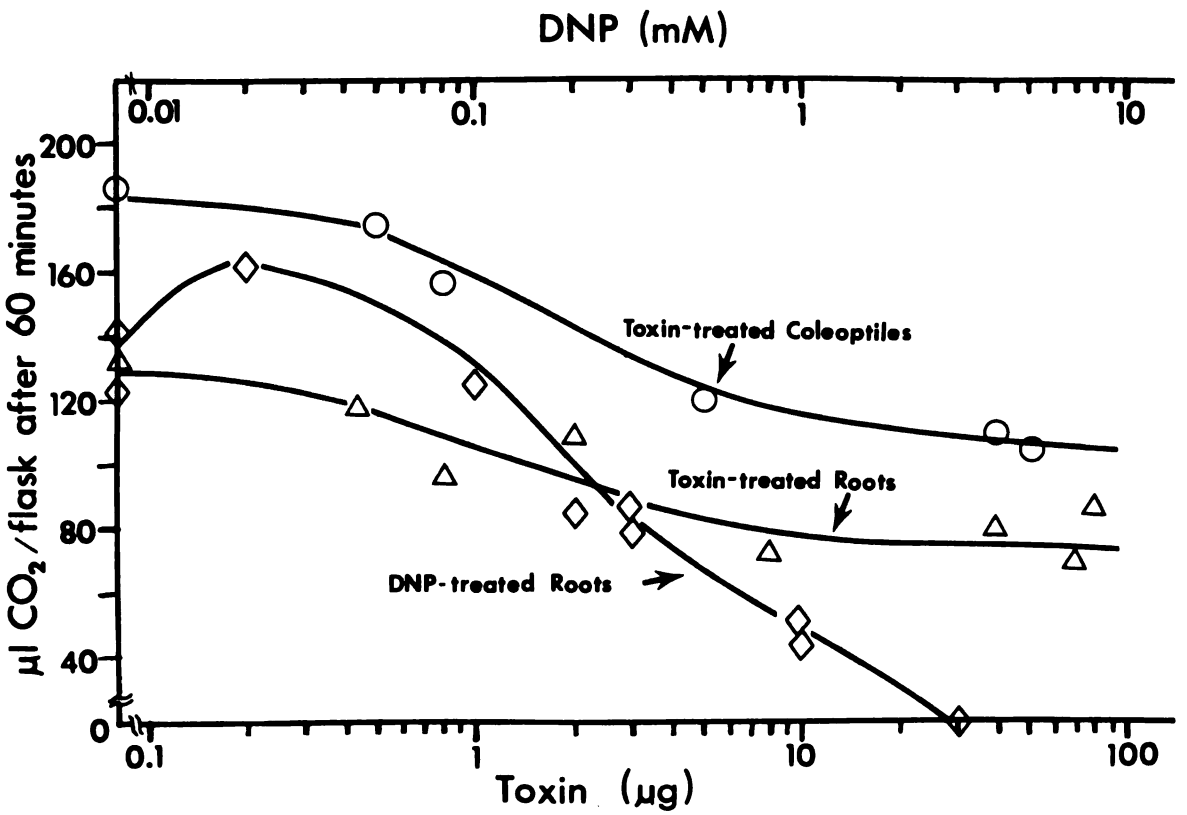



Figure 15. Comparative effects of HM-T toxin on glycolysis by roots and coleoptiles, and effects of 2,4-dinitrophenol (DNP) on roots. Coleoptile tips (2 cm long) and root tips (1 to 1.5 cm long) were placed in a solution containing glucose (0.01 M) and phosphate buffer (5 mM, pH 5.8) with or without toxin. Coleoptile tips (○) were vacuum infiltrated for twenty minutes; root tips (△) were incubated twenty minutes on the reciprocal shaker (120 strokes/min). Tissues were then blotted dry, placed in Warburg flasks (0.5 g tissue/flask) on wet paper, and flushed with nitrogen. Equilibration time was fifteen minutes and temperature was 25.5 C. Evolution of $CO_2$ was determined manometrically. These are composite data for four different experiments. A comparable procedure was used with the DNP-treated roots (◇).

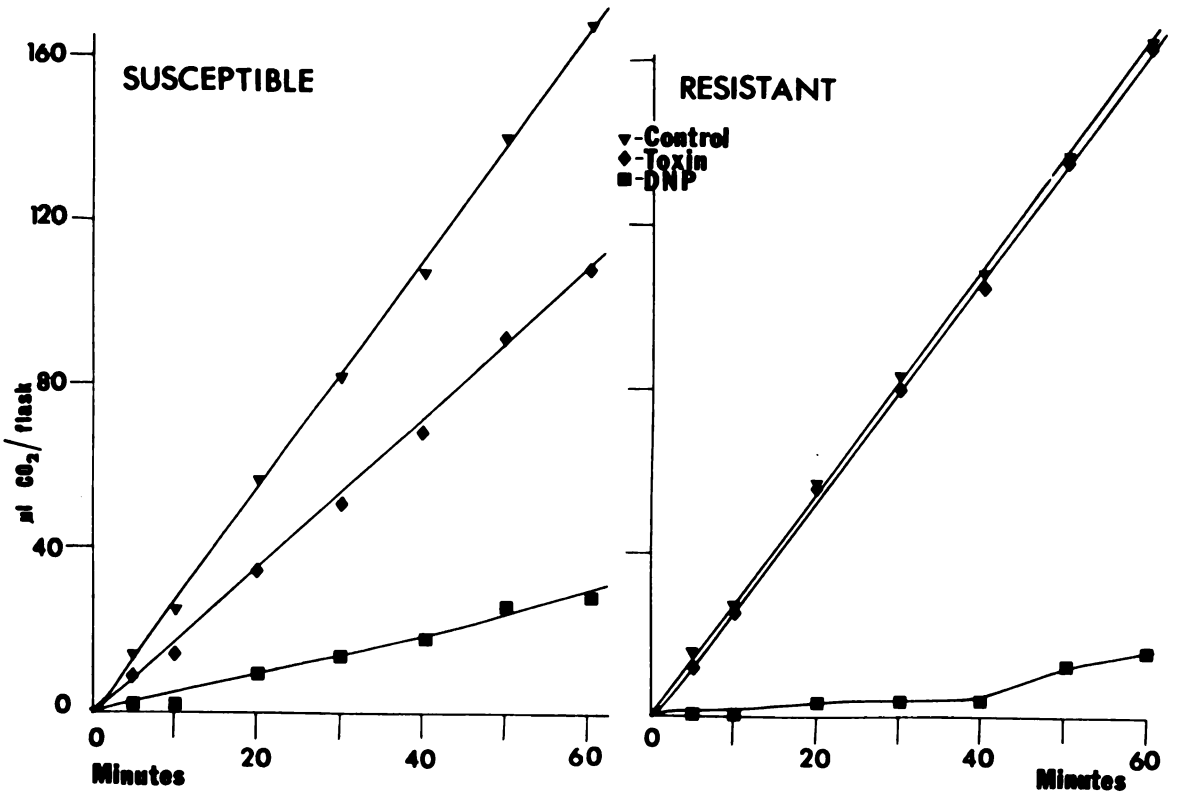



Figure 16. Effect of HM-T toxin on glycolytic respiration by roots from susceptible and resistant corn. Plants were grown for three days in 150 mm petri dishes in 0.2 mM $CaSO_4$. Root sections (2 cm long, 0.5 g) were incubated in water, with or without toxin (9 μg/ml) or 2,4-dinitrophenol (DNP) (3 mM), on a reciprocal shaker (120 strokes/minute) at room temperature for thirty minutes before being placed in Warburg flasks. Equilibration time was fifteen minutes and temperature was 25.5 C. $CO_2$ evolution was determined manometrically. $ED_{50}$ for the toxin preparation in a root growth bioassay was 20 μg/ml.

trichloroacetic acid. The homogenate was centrifuged and the resulting supernatant solution was used to determine content of acid-soluble phosphate. Toxin-treated roots had 46 to 56 per cent lower levels of labile phosphate than did control tissue (Table 9), which could result from decreases in ATP and ADP. DNP-treated tissue had only 30 per cent as much acid-labile phosphates as did the controls. This experiment was repeated and a resistant tissue control was included. Labile organic phosphate in toxin-treated susceptible roots was 44 per cent of the control tissue value (Table 9). The level of labile organic phosphate was not changed in resistant tissue after exposure to toxin. DNP caused a similar decrease in labile organic phosphates in both the susceptible and resistant root sections. The nonspecific effect of DNP was obtained in repeated experiments.

When susceptible tissues were treated with toxin or DNP and frozen quickly (i.e., without incubation under nitrogen for one hour), DNP-treated but not toxin-treated tissues had low levels of labile organic phosphate. Apparently, incubation after exposure to toxin under anaerobic conditions is necessary for toxin to cause a change in ATP and ADP levels.

Arntzen et al. (5) have reported that toxin did not change $^{32}P$ uptake by susceptible corn roots but caused a slight decline in the ATP levels of both resistant and susceptible roots after six hours. My data show no immediate changes in labile organic phosphate levels, but changes were evident in susceptible and not resistant tissues after toxin exposure for one hour or more. There was little or no effect on leakage of electrolytes at that time (34,46).

Table 9. Effect of HM-T toxin and 2,4-dinitrophenol (DNP) on the level of acid-labile phosphate in corn roots. Root sections (2 cm long, 0.5 g replicate treatment) were incubated with toxin or DNP for twenty minutes at 23 C on a shaker (120 strokes/minute) and placed under anaerobic conditions for one hour. Non-treated controls were used. Tissues were then frozen quickly and ground in ten volumes of cold trichloroacetic acid (0.5 N). Acid-soluble phosphate and phosphate esters were determined spectrophotometrically. $ED_{50}$ for the toxin preparation in a root growth bioassay was 20 µg/ml.

| Expt. No. | Corn type | Treatment | Total phosphate (a) | $P_i$ plus acid-labile phosphate (b) | $P_i$ (c) | Acid-labile phosphate (d) | |
|---|---|---|---|---|---|---|---|
| | | | (nmoles/g tissue) | (nmoles/g tissue) | (nmoles/g tissue) | (nmoles/g tissue) | % of Control |
| I | Suscep- | Control | 645 | 136 | 90 | 46 | 100 |
| | tible | Toxin (20µg/ml) | 690 | 117 | 91 | 26 | 56 |
| | | Toxin (50µg/ml) | 610 | 100 | 79 | 21 | 46 |
| | | DNP (3 mM) | 648 | 83 | 69 | 14 | 30 |
| II | Suscep- | Control | 758 | 179 | 129 | 50 | 100 |
| | tible | Toxin (50µg/ml) | 751 | 133 | 111 | 22 | 44 |
| | | DNP (3 mM) | 754 | 68 | 70 | 0 | 0 |
| | Resis- | Control | 789 | 165 | 119 | 46 | 100 |
| | tant | Toxin (50µg/ml) | 802 | 180 | 136 | 44 | 96 |
| | | DNP (3 mM) | 849 | 118 | 112 | 6 | 12 |

(a) Total trichloroacetic acid-soluble phosphate determined as $P_i$ after combustion.
(b) Total $P_i$ plus acid-labile phosphate soluble in trichloroacetic acid.
(c) $P_i$ before acid hydrolysis; soluble in trichloroacetic acid.
(d) Acid-labile phosphate (b less c).

## Adenosine triphosphatase activity in microsomes treated with HM-T toxin

The decrease in acid-labile phosphate levels in tissues after exposure to HM-T toxin does not preclude a possible effect of toxin on membrane-bound adenosine triphosphatase. For this reason, the microsomal vesicles were isolated from susceptible and resistant root tissues and membrane-bound adenosine triphosphatase activity was measured as a release of $P_i$ from ATP (39). The reaction was initiated with the addition of the enzyme preparation.

HM-T toxin caused no change in the rates of ATP hydrolysis by root microsomal fractions isolated from susceptible and resistant tissue; the rates were the same as those for controls (Figure 17). The enzyme preparation was assayed with and without KCl in the medium; toxin caused no change during the first forty minutes after exposure. The experiment was repeated three times, using slightly different experimental conditions each time; in each case, the results were negative. These results do not agree with data reported by Tipton *et al.* (94), which indicated that HM-T toxin inhibited the adenosine triphosphatase activity associated with microsomal membrane fractions from susceptible but not from resistant roots. My experimental conditions were comparable to those of Tipton *et al.* (94).

Tipton *et al.* (94) also proposed that HM-T toxin does not permeate the plasmalemma of plant cells *in vivo*. The implication is that toxin does not affect oxidative phosphorylation directly, but instead inhibits adenosine triphosphatase in the plasmalemma. As a further test of this possibility, I have incubated root sections with

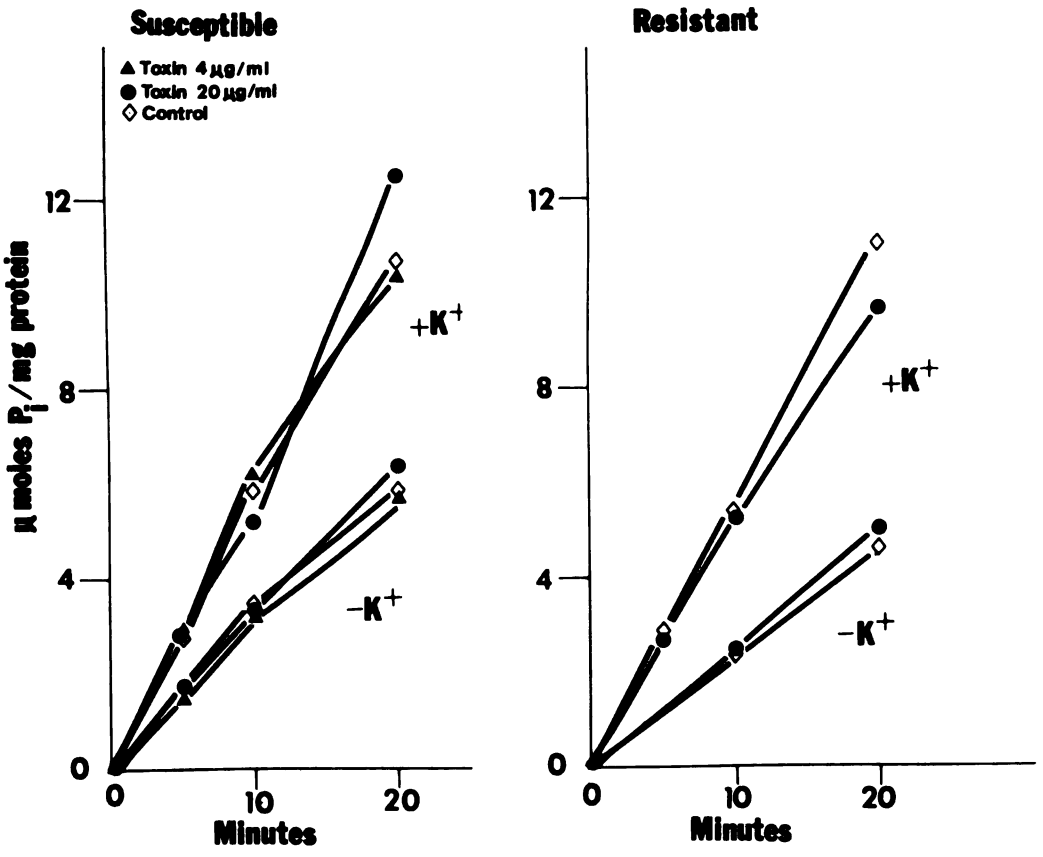



Figure 17. $K^+$-stimulated adenosine triphosphatase activity associated with microsomes from resistant and susceptible corn, in the presence of HM-T toxin. The reaction mixture contained MES-Tris (33 mM, pH 6.0), ATP-Tris (3 mM, pH 6.0), and $MgSO_4$ (1.5 mM). KCl (50 mM) and HM-T toxin (4 (▲) and 20 (●) µg/ml) were added as indicated. The reaction was started with addition of the enzyme (200 µg protein) to the reaction mixture (total volume, 5 ml). Control rates are shown (◇). Root growth was inhibited in susceptible seedlings at 60 µg toxin/ml.

ATP, ADP, glycerol phosphate, or with a buffered control, in the presence and absence of toxin. The concentration of phosphate in the ambient medium was measured several times during a two-hour incubation period. Results showed no difference in phosphate levels in the ambient medium of toxin-treated and control tissues. There does not appear to be a toxin-induced inhibition or stimulation of the cell wall or membrane-bound phosphatases.

### Effect of HM-T toxin on the hydrolysis of ATP by mitochondria in vitro

Isolated mitochondria were exposed to toxin and assayed for an effect on the hydrolysis of ATP. HM-T toxin caused up to 44 per cent stimulation of the adenosine triphosphatase activity in mitochondria from susceptible plants, but did not affect the activity in mitochondria from resistant plants (Figure 18). DNP (0.1 and 0.5 mM) stimulated adenosine triphosphatase activity in both types of mitochondria. The experiment was repeated four times with similar results. This toxin preparation at 0.6 and 6 μg/ml was shown to inhibit incorporation of $P_i$ by mitochondria in the presence of succinate, ADP, and $P_i$.

## IV. Reactions of Chloroplast Lamallae in the Presence of HM-T Toxin

An attempt was made to determine the effect of toxin on electron transport and phosphorylation by isolated chloroplast lamellae. Incorporation of $^{32}P_i$ into ATP by isolated chloroplasts was determined without toxin, and in the presence of toxin at two different

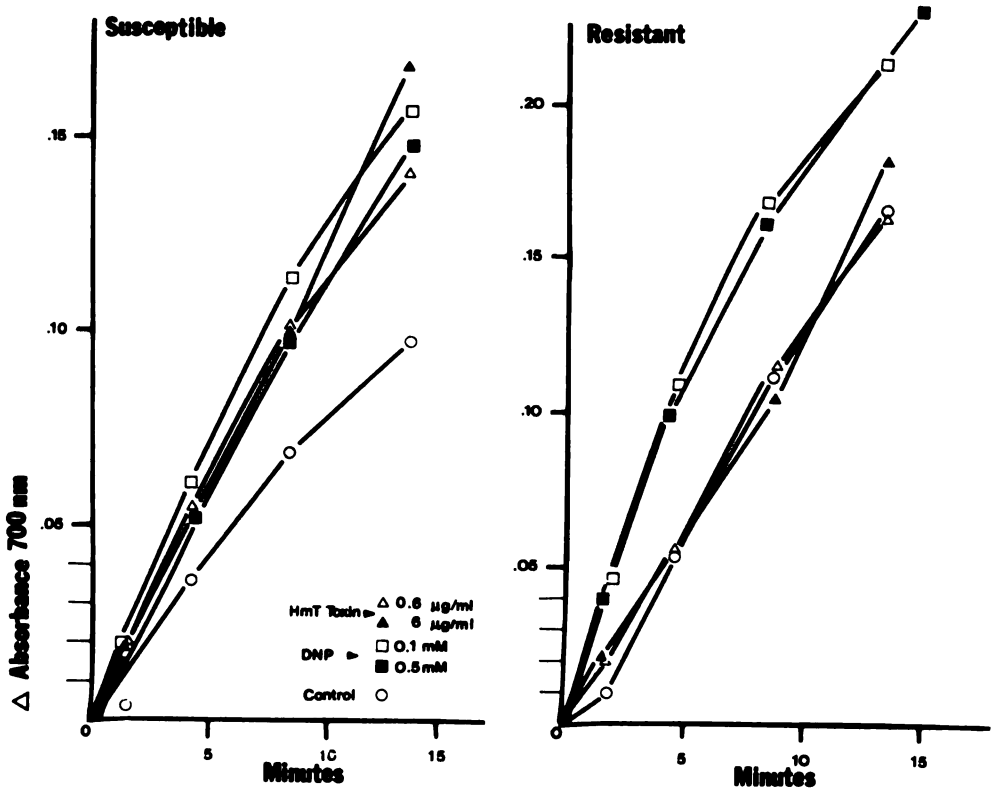


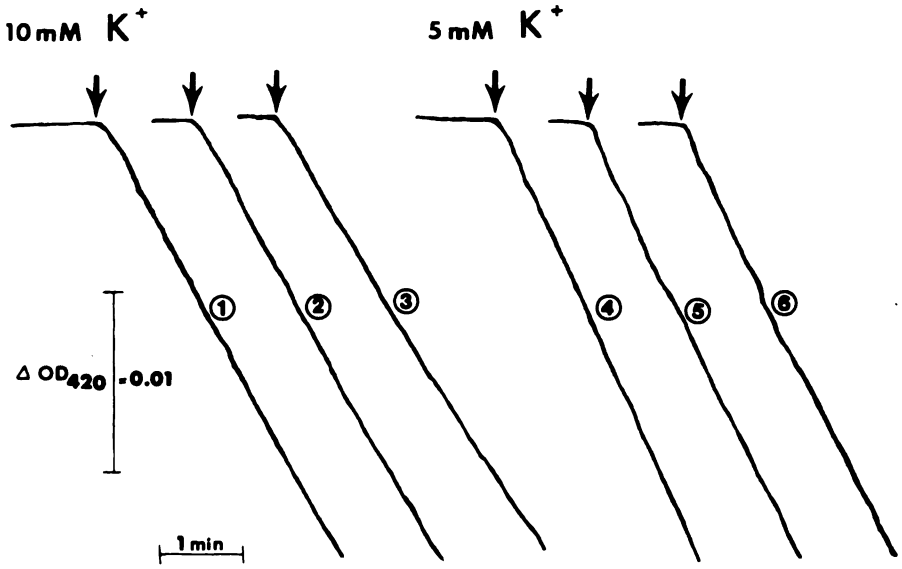

Figure 18. Effect of HM-T toxin and 2,4-dinitrophenol (DNP) on adenosine triphosphatase activity associated with mitochondria from susceptible and resistant corn. The reaction was started by adding mitochondria (1 mg protein) to a reaction medium containing KCl (0.1 M), HEPES-NaOH (0.01 M, pH 7.5), $MgCl_2$ (1 mM), BSA (1.5 mg), and $Na^+$-ATP (3 mM), at 30 C. Two concentrations of DNP (0.1, □ ; and 0.5 mM, ■ ) and toxin (0.6, Δ ; and 6 µg/ml, ▲ ) were used. Control mitochondria are indicated by ( ○ ). To stop the reaction, an aliquot was pipetted into six volumes of cold trichloroacetic acid (0.5 M). Mitochondrial protein was removed by centrifugation and $P_i$ was measured spectrophotometrically. $ED_{50}$ for toxin preparation in a root growth bioassay was 60 µg/ml.

concentrations. The reduction of ferricyanide was used to determine nonphosphorylating electron transport. When chloroplasts were suspended in a reaction medium with 5 mM $K^+$, transported electrons reduced 285 μmoles ferricyanide/mg chlorophyll·hour. High concentrations of toxin (10 and 30 μg/ml) did not cause a change in the electron transport rate (Figure 19).

Some uncoupling agents affect rates of electron transport only in the presence of alkali metal cations at concentrations greater than those present in the experiments described above. Therefore, electron transport rates were measured in chloroplasts suspended in a reaction medium containing 10 mM $K^+$. Electron transport in control preparations reduced 199 μmoles ferricyanide/mg chlorophyll·hour. When toxin (10 or 30 μg/ml) was added, chloroplasts reduced 223 and 217 μmoles ferricyanide/mg chlorophyll·hour, respectively (Figure 19). Thus, toxin had no effect on electron transport. Chloroplasts isolated from resistant plants were not tested.

ATP formed by illuminated chloroplasts during the transfer of a pair of electrons from water to ferricyanide ($P/2e^-$) was measured in these experiments. Toxin caused no change in the $P/2e^-$ ratio by chloroplasts (Table 10).

Finally, toxin (25 μg/ml) was examined for effects on light scattering by chloroplasts. Changes in volume of chloroplast lamellae cause changes in the amount of light scattering and this can be measured. Volume changes depend on electron transport; uncouplers or molecules which cause a general disruption of the membrane will effect swelling or shrinking of chloroplasts lamellae. The corn chloroplasts were




Rate: μmoles $Fe_3(CN)_6^{++}$ reduced/mg chl·hr·2 ml

| $K^+$ | 10 mM | 5 mM |
|---|---|---|
| CONTROL | ① 199 | ④ 285 |
| TOXIN (10 μg) | ② 223 | ⑤ 277 |
| (30 μg) | ③ 217 | ⑥ 251 |

Figure 19. Electron transport by chloroplasts isolated from susceptible corn seedlings in the presence and absence of HM-T toxin. The basic reaction mixture (2 ml) contained sucrose (0.1 M), Tricine-NaOH (50 mM, pH 7.8), $MgCl_2$ (1 mM), $K_3Fe(CN)_6$ (0.4 mM), and chloroplasts (40 μg). Additional components added were ADP (1 mM), $Na_2H^{32}PO_4$ (5 mM), KCl (5 and 10 mM), and toxin (10 and 30 μg). Arrows indicate the time actinic light was applied. Deflections in the tracings represent the decrease in absorbancy of ferricyanide. $ED_{50}$ of toxin preparation in a root growth assay was 2 μg/ml.

Table 10. Phosphorylation by chloroplasts from susceptible corn in the presence of HM-T toxin. Experimental conditions are given in Figure 20. Organic $^{32}P$ was estimated from a 1 ml sample of the reaction mixture which was quickly frozen and from which unincorporated $P_i$ was extracted.

| Toxin (μg/ml) | $^{32}P$ incorporation per $2e^-$, in the presence of | |
|---|---|---|
| | 5 mM $K^+$ | 10 mM $K^+$ |
| 0 | 0.95 | 0.81 |
| 10 | 0.96 | 0.80 |
| 30 | 0.91 | 0.81 |

suspended in reaction media with $K^+$ (2.5 mM) or without $K^+$. In the absence of $K^+$, toxin did not cause a change in light scattering by chloroplasts. In the presence of $K^+$, an endogenous contraction by the chloroplasts was observed, but toxin did not alter this response (Figure 20). Triton X-100 (0.1 per cent) caused the chloroplasts to swell. Thus, HM-T toxin had no significant effect on this membrane system.

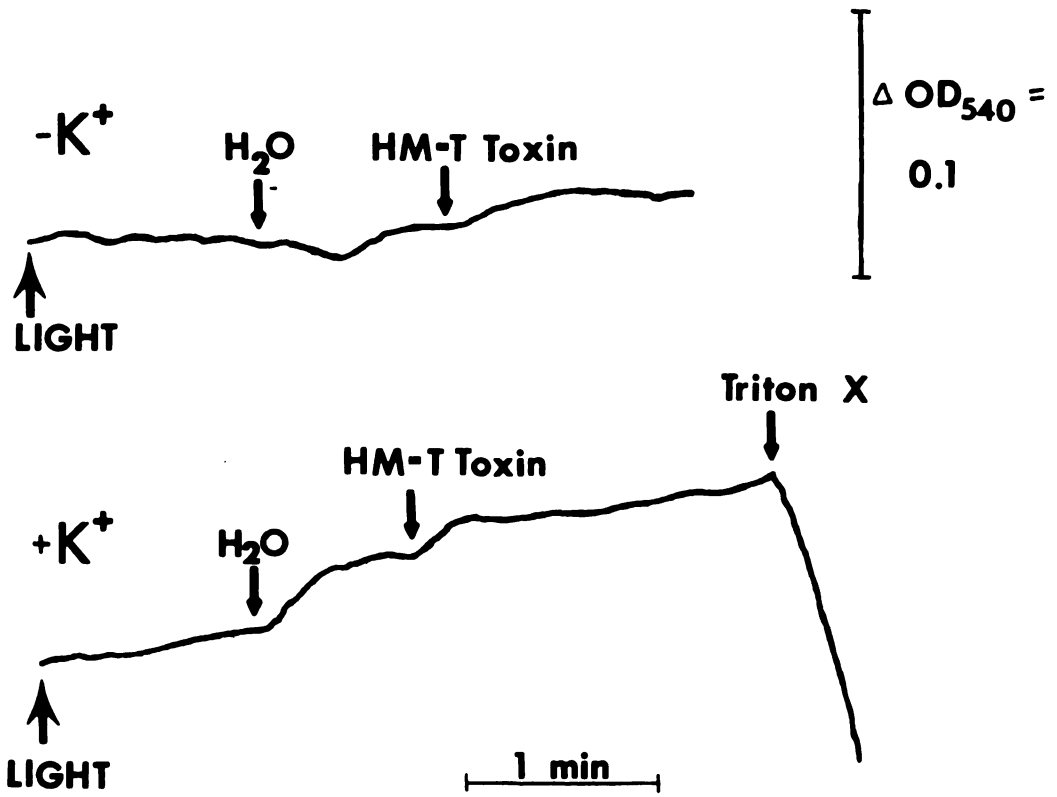



Figure 20. Light scattering by chloroplasts from susceptible corn, in the presence of HM-T toxin. The reaction mixture (2 ml) contained sucrose (0.05 M), Tricine-NaOH (25 mM, pH 7.8), $MgCl_2$ (0.5 mM), chloroplasts (40 μg chlorophyll), with or without KCl (2.5 mM) and toxin (50 μg). Triton X-100 (0.1 per cent) was added to show that chloroplasts respond by swelling. $ED_{50}$ for the toxin preparation in a root growth bioassay was 2 μg/ml.

## DISCUSSION

The effects of toxin on tissue respiration were determined as a basis for a comparison of the effects of toxin *in vitro* and *in vivo*. HM-T toxin caused at least a 20 per cent increase in respiration of intact tissues. Other fungi and their host-specific toxins are also known to cause respiratory increases in host tissue; examples are *Helminthosporium victoriae*, *H. carbonum*, and *Periconia circinata*, and their host-specific toxins (50,60,81). Furthermore, respiratory increases are known to accompany most plant infections (18,49,66). For example, an increase in respiration and a decrease in the Pasteur effect was observed in safflower hypocotyls after infection by *Puccinia carthami* (18).

Arntzen *et al.* (5) reported that HM-T toxin is an exception in that it did not affect respiration by susceptible tissue. Such negative results may be related to the methods used. In the experiments of Arntzen *et al.*, corn coleoptiles were submerged in relatively high concentrations of toxin and oxygen uptake was monitored with an oxygen electrode. I did not observe a toxin-induced increase in respiration when root tissues were used; the respiration rates of root tissue from Tms corn treated with HM-T toxin were comparable to rates for untreated controls. Root tissue appears to differ in some way from leaf tissue; there may be alternate pathways in roots which cope with the enhanced

phosphate turnover, or there may be limiting factors other than phosphate acceptors.

Toxin-induced increase in respiration by coleoptiles was compared with DNP-induced responses. DNP at $10^{-5}$M causes an increase in respiration by tissues of many plant species; higher concentrations inhibit respiration. DNP does not affect oxygen uptake when lower concentrations are used (8). HM-T toxin at relatively high concentrations caused an increase in respiration; no inhibitory effects were observed when several toxin concentrations and exposure times were used in experiments with coleoptile tissue. Thus, there appear to be some differences between DNP and toxin-induced changes in oxygen uptake.

Uncoupling as a possible cause of increased respiration in infected tissue was first suggested by Allen (2). However, prior to the work with HM-T toxin, uncoupling had never been established as a significant causal factor in disease development (10,75,86). Clearly, uncoupling is not involved in the action of host-specific toxins from H. victoriae, H. carbonum and P. circinata. These toxins have no effect on mitochondria from susceptible plants (84,101). In contrast, the toxin from H. maydis race T uncoupled oxidative phosphorylation in isolated mitochondria (65). Thus, the increase in the respiration rate which I found for leaf and coleoptile tissues might be caused by the uncoupling effect of toxin on mitochondria in vivo. Other workers have taken electron micrographs of mitochondria in H. maydis race T-infected tissue and in toxin-treated tissues; the condition of the mitochondria suggests that both infection and toxin-treatment cause uncoupling in vivo (48).

Miller and Koeppe (65) have shown that HM-T toxin can stimulate or inhibit mitochondrial respiration and reduce both respiratory control and ADP/O ratios. The degree of such responses depends in part on the substrate. I have extended this work to show a decrease in the $^{32}P_i$ incorporated into ATP and to compare the effects induced by toxin with the effects of other uncoupling agents. My data with isolated susceptible mitochondria oxidizing exogenous NADH show an effect of toxin on nonphosphorylating electron transport and on ATP formation; the effect is correlated with toxin concentration. A plot of the response gave a hyperbolic curve, suggesting that the effect of toxin on mitochondria is reversible.

A reversible effect of toxin on coupling in mitochondria was confirmed by "wash-out" experiments. Susceptible toxin-treated mitochondria recovered normal phosphorylating capacity when toxin was removed. Comparable recovery of oxygen uptake rates and P/O ratios was obtained with NADH, succinate, and malate-pyruvate as respiratory substrates. This indicates that toxin is not firmly bound; it dissociates readily and free toxin is in equilibrium with bound toxin. A further indication of the lack of firm binding was evident in data that show mitochondria do not remove detectable amounts of free toxin from the ambient solution. These results are compatible with those of Arntzen et al. (5), who showed that growth of roots was inhibited by one hour exposure to toxin, but growth resumed about six hours after toxin was removed. My data and Arntzen's data appear to eliminate the possibility of firm binding of HM-T toxin to sites in the susceptible cell, and suggest that the action is in some ways similar to that of DNP.

The host-specific toxin from H. sacchari appears to bind firmly to a receptor site in the plasmalemma (91). The toxin, helminthosporoside from H. sacchari, reportedly forms a complex with a host protein in situ; toxin also binds with a protein extracted from the host. Other workers have examined possible binding of host-specific toxins to host components. Data on HV-toxin from H. victoriae suggest that the site of "toxin recognition" is in the plasma membrane of the oat cell (83). Scheffer and Pringle (82) and Damann (19) incubated resistant and susceptible oat tissues in solutions containing known concentrations of toxin. Tissues were then removed, and the ambient solution was assayed for toxin content. No binding or removal of toxin by resistant or susceptible tissue was evident. In subsequent experiments, Damann (19) isolated membrane vesicles from susceptible and resistant oats, treated the vesicles with HV-toxin, solubilized the preparation with detergent, and fractionated it on Sephadex-G-50 columns. Equal amounts of toxin were present in protein-containing fractions from both resistant and susceptible membranes, and also in detergent solutions without membranes. Experiments with a toxin from Alternaria kikuchiana gave similar results (personal communication from S. Nishimura). To date, there are no indications that H. victoriae or A. kikuchiana toxins are bound firmly to receptor sites, although the characteristics of the plasmalemma are changed drastically.

The indications thus far are that the mitochondrion contains the primary site of action for HM-T toxin. Mitochondria from susceptible tissues are extremely sensitive to toxin; mitochondria from resistant tissues will tolerate hundred times higher toxin concentrations.

The effect of HM-T toxin on susceptible mitochondria oxidizing different substrates was examined in a number of experiments. The toxin appears to cause uncoupling more effectively with NADH, rather than with succinate or malate-pyruvate, as the substrate. Effects of toxin on oxygen uptake by nonphosphorylating mitochondria also indicate differences in sensitivity, depending on the substrate. Toxin caused 60 per cent increase in nonphosphorylating electron transport when NADH was the substrate, but little or no increase with succinate. Toxin inhibited oxidation of malate-pyruvate by about 40 per cent in the absence of $P_i$. Relatively high concentrations of toxin did not affect oxidation of succinate, but completely inhibited oxidation of malate-pyruvate.

Such substrate effects are not unusual; several uncouplers have comparable, differential effects, depending on the substrate. Ikuma and Bonner (43), working with mitochondria from mung bean, found that DNP at low concentrations increased the oxidation of succinate but not of malate in the presence of ADP. They also found that 80 μM DNP caused maximum uncoupling when succinate was the substrate, whereas 150 μM DNP was required for maximum uncoupling with malate as substrate. Bottrill and Hanson (11) found that concentrations of DNP greater than 20 μM inhibited malate dehydrogenase in mitochondria from corn roots.

In my experiments, HM-T toxin inhibited oxidation of malate-pyruvate in the presence of ADP and $P_i$ more than in the absence of these factors. This suggests an inhibition of energy transfer in addition to inhibition of electron transport. Comparable differences were seen in studies with ionophores. Nigericin inhibited mitochondrial oxidation

of malate and other NAD-linked substrates but did not inhibit oxidation or phosphorylation of succinate (33). The effects of nigericin on substrate oxidation are apparently secondary to the loss of cations, which occur without inhibition of oxidation or phosphorylation of succinate. Further, the inhibition of malate oxidation by nigericin can be reversed by a high concentration of alkali-metal cations in the medium (33). There are also qualitative differences in the effects of gramicidin on oxidation of substrates by mitochondria. Miller, et al. (63) found that gramicidin stimulated oxidation of NADH but did not affect the oxidation of malate-pyruvate; there was a slight stimulation in oxidation of succinate.

Valinomycin, gramicidin, and nigericin cause uncoupling and increase the rate of substrate oxidation by selectively affecting the permeability of mitochondrial membranes to cations (13). The actions of most uncoupling agents are related to ion transport (13). Does uncoupling by HM-T toxin depend on monovalent or divalent metal ions? When $K^+$ was missing from the medium, both toxin and DNP caused 100 per cent increases in endogenous oxygen uptake under nonphosphorylating conditions with NADH as the substrate. In contrast, $K^+$ was necessary for stimulation of oxidation by valinomycin (Figure 6). Phosphorylating mitochondria were then treated with HM-T toxin, in the presence or absence of $K^+$. Toxin caused uncoupling, with or without $K^+$ (Table 4). Toxin-induced uncoupling was not affected by other monovalent cations ($Li^+$, $Na^+$, and $NH_4^+$). Uncoupling of oxidative phosphorylation by gramicidin and ionophorous (ion-carrying) antibiotics requires monovalent cations (36,71). Thus, the uncoupling action of HM-T toxin appears to

be comparable to that of DNP, which uncouples via proton translocation, rather than to that of the three antibiotics.

I have examined the sensitivity of Tms mitochondria to uncoupling agents, by monitoring nonphosphorylating oxygen uptake and changes in P/O ratios. Nigericin at $10^{-7}$ M stimulated oxygen uptake by susceptible (Tms) but had no effect on the oxygen uptake by resistant (N) mitochondria (Table 5). Nigericin at $5.0 \times 10^{-7}$ M caused 50 per cent inhibition of phosphorylation by susceptible mitochondria, and a 20 per cent decrease for resistant mitochondria. Thus, nigericin, which catalyzes $K^+/H^+$ exchange across the mitochondrial membrane, seems to affect susceptible mitochondria more than it affects resistant mitochondria. Nigericin at 0.5 to $2.0 \times 10^{-6}$ M caused animal mitochondria to lose intramitochondrial $K^+$ and inhibited respiration of some substrates (33).

Other workers have compared the sensitivity of normal and Tms mitochondria to uncoupling agents (28). Susceptible mitochondria were reported to be more sensitive to both gramicidin and DNP than were resistant mitochondria.

The quantitative difference between resistant and susceptible mitochondria in response to nigericin suggested that there may be differences in the permeability properties of the membrane. Permeability characteristics of intact organelles can be determined by influx studies. Mitochondrial suspensions were monitored with a $K^+$-sensitive electrode; results suggest that susceptible mitochondria do not take up $K^+$ as rapidly as do resistant mitochondria during NADH oxidation. This

may indicate another quantitative difference between the two types of mitochondria.

Another possible difference between resistant and susceptible mitochondria is evident from the effects of $Ca^{++}$ reported by Gengenbach et al. (28). In the absence of $P_i$, $Ca^{++}$ caused a 100 per cent increase in oxygen uptake by mitochondria from resistant seedlings, and a 50 per cent increase in mitochondria from susceptible plants. The difference was not apparent in the presence of $P_i$, indicating a difference in anion permeability.

In my experiments, $Ca^{++}$ lowered P/O ratios to the same extent in both resistant and susceptible mitochondria with NADH as substrate (Table 7). Calcium and toxin-induced uncoupling of susceptible mitochondria was additive, indicating no interference between the two agents. However, $Ca^{++}$ uptake studies might be better for showing whether or not there is an interaction between toxin and $Ca^{++}$-induced uncoupling. The results might indicate differences between the action of HM-T toxin and the action of membrane-disruptive agents.

There is a report that toxin inhibits $K^+$-dependent adenosine triphosphatase activity in microsomal preparations from susceptible roots (94). I could not confirm this report. No inhibition or change in the endogenous or in the $K^+$-stimulated adenosine triphosphatase activity associated with isolated membrane vesicles was observed; toxin-treated preparations had linear rates equal to control rates (Figure 17). There was no immediate change in ion transport after toxin treatment of susceptible roots (5). The differences in results are hard to reconcile, because membrane-bound adenosine triphosphatase from isolated root

microsomes was associated with ion transport (25). Furthermore, I found that phosphatase activity associated with plant cell walls was not affected by toxin. Other host-specific toxins are thought to act on the plasmalemma (83), but my data seem to eliminate the plasmalemma as a site for the action of HM-T toxin.

Arntzen et al. (4) found that HM-T toxin induced stomatal closure in susceptible corn leaves. The $K^+$-transport associated with stomatal functioning was affected in susceptible epidermal strips. Stomatal opening is associated with $K^+/H^+$ exchange (77), but $K^+$ uptake in exchange for $H^+$ is not unique to guard cells; it is seen also in mitochondria and in root cells. DNP disrupts $K^+$ transport in mitochondria; toxin has features in common with DNP, although $H^+$ release from mitochondria by toxin has not been examined. Further, functional -SH groups are involved in the control system for ion uptake across mitochondrial membranes (12,85). It is possible that HM-T toxin affects the uptake system by acting on such groups. This possibility has not been examined. Sulfhydryl-binding reagents are known to protect susceptible oat tissues from HV-toxin, which is thought to have an initial effect on the plasmalemma (27).

The question remains: does toxin affect mitochondria in vivo? If so, are there sites of action in addition to the mitochondrial site? I have attempted to answer these questions, first by an examination of the effects of toxin on the glycolytic system in vivo. Tissues were treated with toxin, held under anaerobic conditions, and $CO_2$ evolution was measured. HM-T toxin caused a decrease in $CO_2$ evolution by susceptible corn tissues, as compared to untreated control tissues. The

effects of toxin on glycolysis differed from the effects of DNP. The inhibition did not exceed 50 per cent of the control rate even when very high concentrations of toxin were used. Exogenous $P_i$, ADP, ATP, glucose, fructose, or $NAD^+$ did not counteract the inhibition by toxin. Uncoupling should have no direct effect on the enzymes involved in glycolysis; therefore, it seems reasonable to assume that toxin-induced inhibition of glycolysis is a secondary effect. This is strongly supported by the experiments with cell-free preparations containing glycolytic enzymes. Toxin did not affect glycolysis by these preparations.

A major factor in control of glycolytic rates is thought to be the levels of ATP, ADP, and $P_i$ present in tissues (9). Mitochondrial respiration competes with glycolysis for ADP and $P_i$. Hence, the uncoupling effects of HM-T toxin on susceptible tissue should stimulate release of $CO_2$ via glycolysis. To test this possibility, the levels of acid-soluble phosphate were determined in toxin-treated and control tissues. Toxin treatment resulted in significantly lower levels of acid-labile phosphate. In contrast, DNP lowered the levels of acid-labile phosphate in both resistant and susceptible tissues. The decrease in the acid-labile phosphates in susceptible tissue following exposure to HM-T toxin is compatible with the hypothesis that toxin penetrates the plasmalemma and acts on mitochondria.

Finally, my data show that phosphorylation and electron transport in chloroplasts were not affected by HM-T toxin. No uncoupling effect was found and no conformational changes were observed in chloroplasts from susceptible maize. Chloroplasts are known to be affected by most of the uncouplers of oxidative phosphorylation. Thus, toxin

does not have a site of action in the chloroplasts. The action of toxin differs in this respect from the action of DNP.

My data show at least one sensitive site for HM-T toxin; the toxin causes specific and rapid changes in isolated mitochondria from susceptible but not resistant plants. Uncoupling by toxin can cause respiratory increases in tissues. There is also evidence for an effect on mitochondria in vivo. Acid-labile phosphates are decreased in susceptible but not in resistant tissues following toxin treatment; and mitochondrial adenosine triphosphatase activity is stimulated. Others have suggested that there is a primary site of toxin action in the plasmalemma, and that $K^+$-stimulated adenosine triphosphatase is inhibited. These claims were not confirmed. Furthermore, HM-T toxin does not cause rapid disruption or other immediate changes in characteristics of the plasma membrane, as are induced by several other host-specific toxins (83). To date, only the mitochondrial site is known to be affected directly by HM-T toxin.

## SUMMARY

Susceptibility or resistance to H. maydis race T is based on sensitivity or lack of sensitivity to the fungal product, HM-T toxin. This toxin was shown to stimulate respiration (oxygen uptake) and decrease glycolysis in susceptible but not in resistant tissues. Toxin affected susceptible mitochondria in vitro, causing uncoupling of oxidative phosphorylation, stimulation of mitochondrial adenosine triphosphatase activity, and swelling. Mitochondria from resistant corn were not affected by toxin at 100 times higher concentration than that which affected mitochondria from susceptible corn. Toxin-induced uncoupling did not appear to be dependent on monovalent cations. Oxygen uptake by mitochondria was either stimulated or inhibited, depending on the substrate. The effect of toxin on mitochondria was reversible, indicating lack of firm binding to the site. Resistant and susceptible mitochondria differed in several ways, in addition to sensitivity to HM-T toxin; these included differences in $K^+$ transport and in sensitivity to nigericin. Electron transport by chloroplasts from susceptible plants was not affected by toxin. It was concluded that the mitochondria contain the only significant site of action for toxin in intact tissue.

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# APPENDIX

# APPENDIX

## INSTABILITY OF HM-T TOXIN

Some of my preliminary experiments were of value for future work and are included for the record, even though they have no direct bearing on my conclusion.

Comstock (17) indicated that HM-T toxin is stable under acid conditions (pH 3.5). However, I have noticed that diluted toxin lost activity, even when stored at pH 3.5. Effects of toxin on mitochondrial activities are correlated quantitatively with toxin concentration (Figures 6 and 10); the response of mitochondria was used to assay toxin activity over a five-hour period. The pH of toxin solutions was adjusted to 3.8 with HCl, or dilutions were made with 0.02 M Tris-acetate buffer at pH 3.8. The solutions were incubated at room temperature or on ice for various times, then were assayed for their effects on P/O ratios of mitochondria. Results showed that toxin lost activity after several hours (Figure 21). Mitochondria treated with toxin solutions that had been maintained on ice or at room temperature after dilution were not uncoupled as drastically as were mitochondria treated with freshly diluted toxin. As a result of these experiments, all toxin preparations were diluted and used at once.

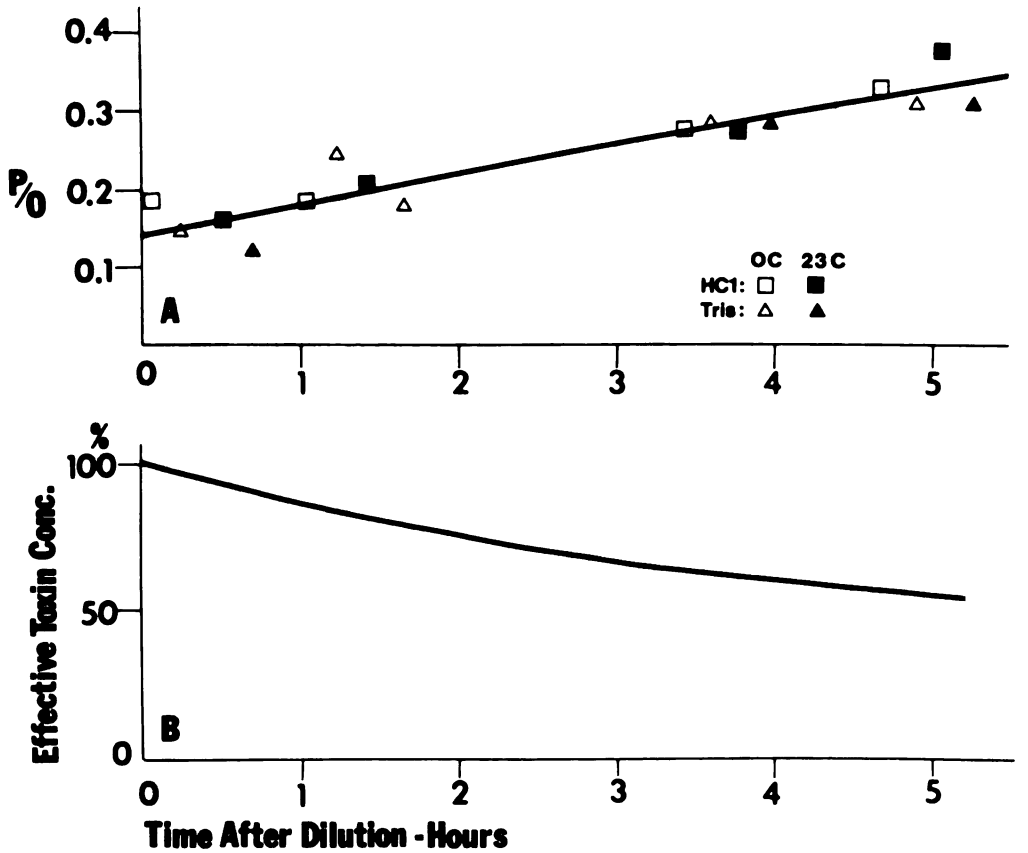


Figure 21. Loss of activity of HM-T toxin in diluted solutions, over a five-hour period. The pH of toxin solutions was adjusted to 3.8 with dilute HCl ( □ , ■ ) or with 0.02 M Tris-acetate ( △ , ▲ ). Toxin solutions were kept at 0 C (open symbols) or at room temperature (23 C) (solid sombols), and periodically assayed with susceptible mitochondria oxidizing NADH in the presence of $P_i$. Mitochondria without toxin had a P/O ratio of 0.95. Final toxin concentration in all reaction solutions was 0.5 µg/ml. A = effect of toxin on P/O ratio; B = relative activity of toxin remaining in solutions, estimated from data in curve A.

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