ABSTRACT

SELECTIVE EFFECTS OF THE HOST-SPECIFIC TOXIN FROM HELMINTHOSPORIUM CARBONUM ON CELLULAR ORGANELLES AND ON SOLUTE ABSORPTION

by

Olen C. Yoder

Helminthosporium carbonum, race 1, causes a leaf spot disease of certain corn (Zea mays) lines. The fungus produces a toxin (HC-toxin) in culture which specifically affects susceptible corn. Both fungus and toxin cause the same physiological changes in susceptible tissue. An attempt was made to determine those cellular functions most closely related to the site of toxin action. The first detectable effects of toxin on susceptible corn tissues were stimulatory; inhibition became evident later. Experiments were designed to determine whether or not toxin action is: a) hormone-like or related to that of hormones; b) involved in energy production by the cell; c) related to synthesis or degradation of enzymes; or d) associated with inter- or intracellular solute movement.

Toxin promotes seedling growth under certain conditions, suggesting gibberellin-like activity. Therefore, toxin and gibberellic acid (GA) were compared in several standard assays. Toxin did not promote dark germination of tobacco seeds, cucumber hypocotyl elongation, or α-amylase synthesis by embryoless barley half-seeds; GA was

active in all these tests. Thus, there was no evidence that toxin had a hormone-like action on tissues. In contrast, helminthosporol, a bioactive metabolite produced by certain Helminthosporium spp., has gibberellin-like activity.

Toxin was tested for possible effects on isolated mitochondria and chloroplasts. Toxin did not uncouple phosphorylation, inhibit electron transport, alter phosphorylation capacity, inhibit the Krebs cycle, or affect conformational changes whether or not electron transport was occurring.

The effect of toxin on *in vivo* activity of nitrate reductase (NR), a substrate inducible enzyme, was determined. Treatment with toxin for 4 hr before induction (that is before exposure to NO_3^-), caused an increase in NR *in vivo* activity which was apparent from the beginning of the induction period. This difference between toxin-treated and control tissues persisted but did not change throughout the induction period, indicating that toxin did not enhance the rate of NR induction. Toxin had no effect on NR activity when the enzyme was extracted from tissue and assayed *in vitro*. This also supports the conclusion that toxin had no effect on NR induction.

Toxin-treated tissue accumulated NO_3^- up to 3 times faster than did control tissue. This observation could explain the increased NR *in vivo* activity. The absorption of other substances (Na^+, Cl^-, 3-o-methylglucose and leucine) was also stimulated by toxin-treatment. There was no effect on uptake of NO_2^-, K^+, Ca^{2+}, Pi, SO_4^{2-}, or glutamic acid.

Toxin-enhanced permeability was not caused by a general derangement of cell membranes. Cells had an increased capacity to absorb and retain certain solutes. Substances accumulated in 30 min by toxin-stimulated tissues could not be washed out of such tissues in 30 min desorption. Toxin-treatment enhanced active influx, but had no effect on active efflux of 3-o-methylglucose.

Toxin-stimulated ion absorption did not require the presence of other mineral ions. Thus, toxin enhanced the uptake rate of NO_3^- from a tris-NO_3^- solution; toxin enhanced the uptake rate of NO_3^- but not Ca^{2+} from a $Ca(NO_3)_2$ solution. There was no apparent effect of toxin on H movement.

For the following reasons it is suggested that toxin causes specific changes in the characteristics of the plasmalemma. 1) Toxin-stimulated uptake is highly temperature sensitive, which suggests an active uptake process. 2) Toxin-treated tissues accumulate ions against a concentration gradient, and develop a steeper gradient with some ions than do control tissues. Mechanism 1, the mechanism of ion uptake which operates at low ion concentrations, is generally thought to be in the plasmalemma and is stimulated by toxin. 3) Negative results from tests of organelles support the hypothesis that increased uptake is caused by a change at the cell surface. 4) All other physiological effects of toxin can be explained on the basis of stimulated uptake.

Toxin concentrations which enhanced the absorptive capacity of H. carbonum-susceptible tissue had no effect on H. carbonum-resistant tissue. When the toxin concentration was increased 500 times, resistant toxin-treated tissue accumulated solutes at a faster rate than did resistant control tissue.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (Continued) Page

LIST OF TABLES (Continued) Page

LIST OF FIGURES

LIST OF FIGURES (Continued) Page

LIST OF ABBREVIATIONS

BSA	bovine serum albumin
Ca	calcium ion
Ck	control
Cl	chloride ion
DNP	2,4-dinitrophenol
EDTA	disodium ethylenediaminetetraacetic acid
Fecy	potassium ferricyanide
GA	gibberellic acid
GD	gramicidin D
H	hydrogen ion
HC-toxin	Helminthosporium carbonum-toxin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HV-toxin	Helminthosporium victoriae-toxin
K	potassium ion
MeG	3-o-methylglucose
MES	2-(N-morpholino)ethanesulfonic acid
Na	sodium ion
NADH	reduced nicotinamide adenine dinucleotide
NO_2	nitrite ion
NO_3	nitrate ion
NR	nitrate reductase
O. D.	optical density
PCA	perchloric acid
Pi	phosphate ions ($H_2PO_4^-$ and HPO_4^{2-})
P:O	moles ATP formed:atoms oxygen consumed
PO_4	phosphate ions ($H_2PO_4^-$ and HPO_4^{2-})
Rb	rubidium ion
RCR	respiratory control ratio = respiratory rate + Pi / respiratory rate - Pi
SO_4	sulfate ion
TCA	trichloroacetic acid
Tox	toxin-treated
Tricine	N-tris (hydroxymethyl)methylglycine
tris	tris (hydroxymethyl)aminomethane
uc	microcurie
ueq	microequivalent

INTRODUCTION

Helminthosporium leaf spot is a fungus disease of certain inbred corn (Zea mays) lines and corn hybrids (119). The causal organism, Helminthosporium carbonum Ullstrup (119), has a sexual stage known as Cochliobolus carbonum Nelson (79). A host-specific toxin produced by H. carbonum race 1 was first described in 1965 by Scheffer and Ullstrup (104). This toxin (known as HC-toxin) selectively affects corn lines that are susceptible to H. carbonum (57). The toxin is a low MW peptide whose structure is not fully known because of problems with lability (88, 90, 92). Several lines of evidence show that HC-toxin is required by the fungus for initial colonization of susceptible corn, and for development of disease symptoms (102, 103, 105). Furthermore, resistance of corn to H. carbonum is the same as insensitivity to HC-toxin (102, 105). Therefore, susceptibility and resistance can be studied on the level of individual chemical reactions without the complication of 2 interacting metabolic systems. The basic problem with such a model is to determine the characteristics of plant cells that convey sensitivity or resistance to toxin. The initial interaction between toxin and a sensitive site in the susceptible cell can be considered the key to understanding disease development.

This study was undertaken to determine which physiological functions of susceptible cells are most likely to be associated with the initial site of toxin action. The earliest physiological effects of toxin described to date are stimulatory (54, 56, 57); toxin at low concentrations can also cause increased growth (57). Therefore, stimulatory effects are probably more closely associated with initial toxin action than are inhibitory effects (118). Several potential modes-of-action were considered: 1) Possible hormone-like effects of toxin were examined by comparison with effects of gibberellic acid and of helminthosporol. 2) Possible effects on energy metabolism or intracellular membrane systems were observed with tests of isolated mitochondria and chloroplasts. 3) Possible effects on synthesis or degradation of proteins were tested with inducible enzymes, amylase and nitrate reductase. 4) Effects on inter- and intracellular solute movement were studied by observing absorption of exogenous solutes by excised and intact roots, by observing changes in intracellular nitrate pools, and by observing ion fluxes across membranes of isolated organelles.

All experiments with toxin gave negative results except those designed to measure effects on solute absorption. The data indicate that toxin-treatment altered the characteristics of the plasmalemma of susceptible cells such that active uptake and retention of certain substances was enhanced. Uptake of several other substances was not affected by toxin-treatment. Toxin at concentrations which affected susceptible cells had no effect on resistant cells.

LITERATURE REVIEW

There is a large literature on metabolic changes which occur in plants after infection by microorganisms (129). The objective of most of this work has been to understand the mechanisms by which pathogens cause disease. Generally, the remarkable host-specificity of many plant pathogens has not been considered in this literature. Direct effects of pathogens on host cells have been proposed at various levels. There are reports on changes in respiration (78), nucleic acid metabolism (38), levels of regulatory hormones (108), protein synthesis (120), cell walls (1), photosynthesis (13), and cell permeability (124). Most of these disease-induced changes occur long after the pathogen has established a parasitic relationship with the host. Therefore, they cannot be considered as primary events, essential to the success of the pathogen. Even when a physiological aberration occurs relatively soon after infection, it is difficult to establish such a change as the initial event which leads to disease development.

In spite of all the efforts, we have a poor understanding of disease at the molecular level. However, the diseases known to involve host-specific toxins offer exciting possibilities for studies of the chemical interactions between host and pathogen. To date, 8

substances which affect only hosts of the producing fungus have been discovered (105). Only 4 of these have been investigated in sufficient detail to give some understanding of disease relationships. The four are: 1) HV-toxin produced by Helminthosporium victoriae, specific for certain varieties of oats; 2) HC-toxin produced by H. carbonum, race 1, specific for certain corn lines; 3) HM-toxin produced by H. maydis, race T, specific for corn with certain types of male sterile cytoplasm; and 4) PC-toxin produced by Periconia circinata, specific for certain lines of sorghum. Compound names derived from the initials of the producing fungus have been assigned to these toxins to signify their special biological roles as genotype-specific molecules (92). Trivial names become confusing in differentiating host-specific toxins from the large group of nonspecific toxic metabolites produced by microorganisms.

HC-toxin is the subject of this investigation. Several lines of evidence are outlined below, which show that HC-toxin is necessary for pathogenicity of H. carbonum race 1 and is required for initial establishment of the fungus in host tissue. Similar data are available for HV and PC-toxins (102, 103, 105).

1. The most obvious indication of a role in disease is host-specificity. Corn genotypes which are susceptible to H. carbonum are also sensitive to HC-toxin. Plants which are resistant are insensitive to the toxin. Plants with intermediate susceptibility to H. carbonum have a corresponding intermediate sensitivity to the toxin (57).

2. All isolates of H. carbonum race 1 which produce toxin are also pathogenic to susceptible corn. All isolates which do not produce HC-toxin are nonpathogenic to corn. This is true for a large group of wild type isolates collected from all over the world (100), for mutants which have lost ability to produce HC-toxin (unpublished), and for ascospore progeny of crosses of a pathogen with a nonpathogen (100). H. carbonum is sexually compatible with H. victoriae. Progeny of such crosses segregate in a ratio of 1:1:1:1 for ability to produce HC-toxin, HV-toxin, both toxins, or neither toxin. One gene pair controls production of HC-toxin by H. carbonum, whereas another gene pair controls production of HV-toxin by H. victoriae. Again, toxin producing ability is correlated with ability to cause disease in specific host plants. Progeny which produce both toxins are pathogenic to both oats and corn, whereas progeny which produce neither toxin are not pathogenic to either oats or corn (100).

3. HC-toxin is required by H. carbonum for successful colonization of susceptible host tissue. When exogenous HC-toxin is added to spores of H. victoriae (nonpathogenic to corn) it colonizes H. carbonum-susceptible corn tissue just as does H. carbonum (105).

4. HC-toxin causes the same physiological changes that are found in H. carbonum-infected tissues. These include increases in respiration, dark fixation of CO_2 (56), and electrolyte leakage (54).

There are several reviews which discuss the biological significance of host-specific toxins in more detail (102, 103, 105). The

literature on physiological changes in H. carbonum-infected tissue has also been reviewed (54). Therefore, I am summarizing the known effects of HC-toxin on susceptible corn. These effects are compared with effects of HV-toxin on susceptible oats.

Uptake and/or activity of HC-toxin on susceptible corn is dependent on time, temperature and cellular metabolism (55). Roots of growing seedlings require at least 4 hr exposure to toxin to accumulate enough toxin to inhibit later growth. As toxin exposure time is increased, the degree of later root growth inhibition increases. If toxin exposure time is held constant, later inhibition becomes progressively greater as temperature is increased from 5 C to 37 C. To test the need of oxygen for toxin uptake and/or activity, susceptible seedlings were placed in toxin solution under nitrogen for 20 hr, then were rinsed and transferred to solutions without toxin in air for 4 days. Toxin had no effect on seedling root growth when seedlings were exposed to toxin under anaerobic conditions (55). In similar experiments susceptible seedlings were exposed to toxin in the presence of metabolic inhibitors (DNP, KCN, or NaN_3) for 20 hr, then transferred to solutions without toxin or inhibitors. There was no inhibition of growth when tissues were exposed to toxin under such conditions (55), which suggests that the inhibitors interferred with uptake of toxin.

To date, there is no direct evidence that HC-toxin is actually taken up by corn tissue (54, 55). This statement is based on data which show that toxin cannot be recovered from either susceptible or resistant

tissue. Cuttings were exposed to a high concentration of toxin (500 μg/ml) for 20 hr, then homogenized and extracted with water. Water extracts were assayed for host-specific toxicity. None was found. In another experiment, 3 large masses of roots were each exposed in turn for 12 hr to the same small volume of toxin solution. Bioassays of the residual solution showed no loss in host-specific toxicity, even though roots had accumulated toxic doses (54, 55). A similar experiment with ^{14}C-labelled toxin showed that susceptible and resistant plants removed equal amounts of radioactivity from solution (54).

There have been attempts to identify the site of toxin action by use of ^{14}C-labelled toxin (54). Susceptible tissues were exposed to HC-toxin, then tissues were homogenized and subcellular particles were separated by differential centrifugation. Most of the radioactivity (80%) remained in the 105,000 g supernatant. Small amounts of radioactivity were in ribosomes (11%), mitochondria (8%), and nuclei and chloroplasts (1%). The results were inconclusive as to the site of toxin action (54). There are inherent problems in such an experiment. It is difficult to obtain a homogeneous toxin preparation which is free of nonhost-specific peptides and breakdown products produced by spontaneous toxin inactivation (88, 92). In addition, both resistant and susceptible tissues completely inactivate the toxin. Thus, even if a pure toxin preparation is used, labelling of cellular sites could be caused by toxin breakdown products.

The ability of HC-toxin to induce electrolyte leakage from corn tissue was tested (54). Leaves infiltrated with toxin and incubated for 2 hr were then rinsed and leached in distilled water. No toxin-induced electrolyte leakage was evident before 8 hr after initial exposure to toxin. Toxin exposure of 8-10 hr resulted in 30-70% increase in leakage of electrolytes from toxin-treated leaves, as compared to controls.

HC-toxin was tested for its ability to stimulate respiration of susceptible leaves (56), as HV-toxin is known to do. Cuttings were allowed to take up toxin for varying periods, then oxygen uptake by leaf sections was measured. Toxin exposures up to 4 hr had no effect on respiration; 8 hr exposure caused 30% increase in oxygen uptake, as compared to nontreated controls.

The potential of corn tissues to fix CO_2 in the dark is known to be increased after infection with H. carbonum (54). The ability of HC-toxin to duplicate this effect was tested. Cuttings were allowed to take up toxin for 4 hr, then leaf sections were exposed to $^{14}CO_2$ for an additional 4 hr. Toxin-treated tissues fixed as much as 200% more CO_2 than did control tissues. In a second experiment, toxin-treated and control tissues were homogenized, centrifuged, and the supernatants were used as sources of CO_2 fixing enzymes. When ribose-5-phosphate was the substrate, enzymes from toxin-treated tissues fixed up to 99% more CO_2 than did control enzymes. Toxin-treatment had no effect on cell-free activity when other substrates involved in dark CO_2 fixation were used. When toxin was added directly to enzymes extracted

from nontreated tissues, there was no effect on CO_2 fixation by the ribose-5-phosphate system. Therefore, stimulation of CO_2 fixation in the dark is a secondary effect of toxin (56).

The effects of toxin on the ability of corn tissues to fix ^{14}C-labelled amino acids and uridine into TCA-insoluble cellular components (54) were examined. Incorporation was not affected by toxin exposures up to 6 hr. When tissues were treated with toxin for 8 hr, then exposed to ^{14}C-amino acids for 4 hr, there was a 23% increase in incorporation by susceptible tissues and a 43% increase by resistant tissues. Similar results were obtained with uridine. Longer toxin exposures (22 hr) inhibited incorporation by susceptible tissues 45% but stimulated incorporation by resistant tissues 20% (54).

HV-toxin, produced by H. victoriae and specific to certain varieities of oats, has been used as a model with which to compare other host-specific toxins (102, 103, 105). HV and HC-toxins are in the same chemical family but there are differences. HV-toxin is a sesquiterpenoid complex ($C_{17}H_{29}NO$) (89) combined with a peptide consisting of aspartic acid, glutamic acid, glycine, valine, and leucine (105). The MW is between 800 and 2000. HC-toxin is a cyclic peptide ($C_{32}H_{50}N_6O_{10}$) containing 2 molecules of alanine, 1 of proline (88), an unsaturated analog of leucine (2-amino-2,3-dehydro-3-methylpentanoic acid) which has an unstable double bond, and an unknown hydroxyamino acid (90). It has a MW of approximately 679 (88). HV-toxin causes complete inhibition of root growth of susceptible oats at 0.0002 µg/ml

(105). Resistant oats tolerate >400,000 times higher concentrations with no effect (105). HC-toxin causes 50% inhibition of root growth of susceptible corn at 0.2 μg/ml (57); resistant corn is affected to the same degree at approximately 100 times this concentration (92). Neither toxin was recovered from susceptible or resistant plants (55, 102) which took up known amounts of toxin. Any effective concentration of HV-toxin invariably inhibits root growth; HC-toxin, at low concentrations, stimulates root growth (57, 105).

The physiological effects of HC-toxin on corn, described above, differ from those of HV-toxin on oats in several categories. In contrast to HC-toxin, uptake and/or activity of HV-toxin is not dependent on time, temperature, or cellular metabolism. HV-toxin has an immediate effect on susceptible cells (98, 105), is not affected by temperature during the exposure time, and acts in the presence of metabolic inhibitors (101). Thus, HV-toxin appears to act by a simple physical process. HV-toxin causes rapid leakage of electrolytes from susceptible cells immediately after exposure (103, 105). In contrast, HC-toxin causes only slight leakage after 8-10 hr exposure. When susceptible tissues are exposed to HV-toxin, there is a rapid rise in the respiration rate (102), whereas the respiratory response to HC-toxin is small and is delayed. HV-toxin has no effect on isolated mitochondria (102). Under certain conditions, HC-toxin stimulates incorporation of ^{14}C-labelled amino acids and uridine into TCA insoluble material. HV-toxin invariably causes a rapid loss of ability to incorporate amino acids and uridine (102).

The two toxins also have physiological effects in common. When tissues were exposed to ^{14}C-labelled HV-toxin, the same type of inconclusive results were obtained as described above for HC-toxin (Samaddar, unpublished). Both HV and HC-toxins stimulate dark fixation of CO_2 by their respective susceptible tissues (56).

Most of the physiological effects of these toxins are probably secondary (105). The site-of-action of HV-toxin is thought to be in the plasmalemma because it quickly alters many characteristics of the plasmalemma (103, 105). There is some evidence that a toxin receptor site is associated with a membrane protein (25). There are no clues to date to the site-of-action of HC-toxin. Data in this thesis suggest that HC-toxin alters characteristics of the plasmalemma, but in a different way than does HV-toxin.

Part of my research included a study of the effects of toxin on protein synthesis, using indirect methods with an inducible enzyme, nitrate reductase (NR). A brief summary of NR literature for background purposes is sufficient, because there are detailed reviews (3, 50). Much of the nitrogen absorbed by plants is in the form of nitrate (NO_3), which must be reduced before it is incorporated into organic materials. This requires at least 3 steps: reduction of NO_3 to NO_2 is catalyzed by NR; reduction of NO_2 is catalyzed by nitrite reductase (a complex of enzymes); and incorporation of NH_3 into α-ketoglutarate to form glutamic acid is catalyzed by glutamic acid dehydrogenase (24). NR appears to regulate this pathway because it is: a) the first enzyme in the

sequence; b) the rate limiting step; c) substrate inducible; and d) relatively unstable with a high turnover rate (3). Although NR has not been completely purified, some of its characteristics are known. It has a MW of 500,000-600,000; a requirement for Mo, Fe, and FAD as cofactors and NADH or NADPH as electron donors; is unstable both in vivo and in vitro; and is induced by NO_3 and by Mo (3). Regulation of NR levels in vivo is not solely dependent on levels of NO_3; concentrations of NO_3 can be high in tissues with low NR activity (3). This could be attributed to effects of light (4) or regulation by endogenous metabolites (23, 106).

Results of many studies indicate that NR is found in all living plant cells. The main evidence for this is that tissues can be grown in media which contain NO_3 as the sole nitrogen source (3). Induction of NR has been reported in green leaves (3, 4), stems and petioles (51), roots (51, 99), germinating embryos of wheat (113) and rice (110), barley aleurone cells (20, 21), corn scutella (3), and tobacco pith cultures (23). Green leaves usually contain the highest levels of NR. In corn, roots contain only 20% of the activity found in leaves (3). The amount of NR in corn leaves increases in light and decreases in darkness (4), suggesting an association of NR with chloroplasts. However, several workers have reported that NR is not a chloroplastic enzyme but probably is located either free in the cytoplasm or in cytoplasmic particles (74, 96, 115).

Development of an in vivo NR assay (21) has made possible greater experimental manipulation. The technique is based on the fact that under anaerobic conditions NO_3 is converted to NO_2 but NO_2 is not further reduced (53) and can be recovered from tissue (21, 22). In green leaf tissue, darkness is required in addition to anaerobiosis for maximum NO_2 production (94). In vivo NR activity is directly related to the rate of NO_2 production.

A major portion of my work involved studies of ion uptake by corn roots. There is a vast literature including a number of reviews (17, 59, 61, 72) on salt absorption by plants. In brief, ion accumulation is generally considered to be dependent upon metabolic energy (61). This conclusion is based on observations that ion uptake is affected by temperature, anaerobiosis, and metabolic inhibitors (67, 68, 117). In addition, ions are transported by roots against concentration (70) and electrochemical gradients (39). Active transport of many ions is dependent on the presence of Ca (16). The role of Ca is unknown, but it probably stabilizes the configuration of the plasmalemma and thus keeps ion carriers in an active form (93). Uptake of most ions is mediated by at least 2 mechanisms; one operates at low ion concentrations and is called mechanism 1, the other operates at high ion concentrations and is called mechanism 2 (17, 59, 61, 72). Uptake rates by both mechanisms lend themselves to kinetic analysis (18). With increasing ion concentrations, maximum rates are attained. The dual mechanisms of uptake have been described for several ions, including K, Rb, Cs, NH_4,

Na, Mg, Sr, Cl, Br, SO_4, and choline sulfate. At least 11 plant species including both monocots (corn among them) and dicots are known to have dual uptake mechanisms (17).

MATERIALS AND METHODS

Plant material. -- H. carbonum-resistant (PR 1 x K 61) and susceptible (Pr x K61) corn hybrids were used in all experiments involving corn. Seedlings were grown from seeds planted in vermiculite and incubated at 24 C under Sylvania Gro-Lux lamps (18 hr light/day). They were watered with White's inorganic nutrient solution (125) or nitrate-less modified White's solution containing the following in mg/l: $CaCl_2 \cdot 2H_2O$, 176; NH_4Cl, 74.2; KCl, 80; $NaH_2PO_4 \cdot H_2O$, 18.7; $MnSO_4 \cdot H_2O$, 5; $ZnSO_4 \cdot 7H_2O$, 2.6; H_3BO_3, 1.5; KI, 0.75; $MgSO_4$, 360; Fe (EDTA chelate), 5; $Na_2MoO_4 \cdot 2H_2O$, 0.025. Green leaf tissue was obtained from tertiary leaves of 15-day-old plants. Mitochondria were isolated from 7 to 8-day-old etiolated shoots.

Roots used in ion uptake experiments were grown as follows: 250 seeds were soaked 8-12 hr in water, washed 10 times in water and placed embryo down on cheesecloth stretched over a ring of stiff polyethylene tubing. Seeds were covered with a second layer of cheesecloth, and suspended over 4000 ml 0.2 mM $CaCl_2$ or $CaSO_4$ in a 4000 ml beaker. The beaker was covered with Saran Wrap and the solution was aerated with filtered air which passed through a gas dispersion tube suspended in the solution. After 3 days, primary roots had grown into the solution and Saran Wrap and top cheesecloth covering were

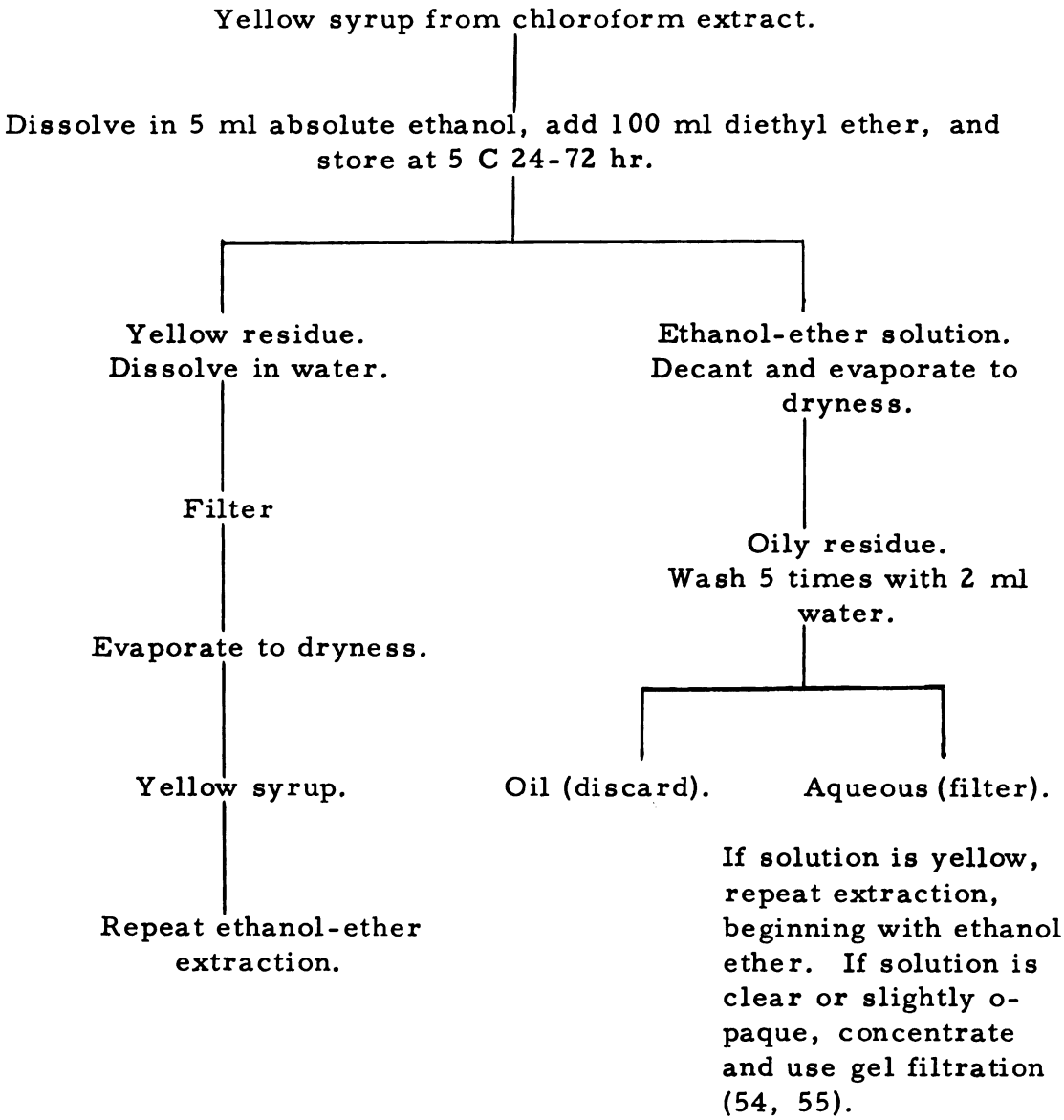
removed. Primary roots were used after 4 days when they were 8-12 cm long; 90-95% of seeds produced usable roots.

Roots for other types of experiments were grown as follows. Seeds were placed embryo down in 30 ml White's solution (40-50 seeds/ 15 cm petri dish) at 24 C for 60-84 hr, when roots were 4-8 cm long. Each day the seedlings were placed in clean petri dishes containing fresh solution. In later experiments, agar (0.9%) was added to the nutrient solution, and 100 ml/15 cm petri dish was used; this made the daily change of solutions unnecessary.

Toxin preparation. -- Toxin was isolated from filtrates (8 l) of cultures grown for 23 days at 24 C in Fries medium plus 0.1% yeast extract (91). Toxicity of each fraction obtained in the purification procedure was assayed against susceptible and resistant seedlings as previously described (100). The isolation procedure was a modification of that described previously (54, 55). Pringle's (92) procedures were followed through chloroform extraction. The chloroform extract was evaporated in vacuo to a thick yellow syrup. The flow diagram in Figure 1 describes separation of yellow pigment from the toxin.

There were about equal amounts of toxin in ethanol-ether soluble and insoluble fractions. Extraction of the yellow syrup was repeated until most of the toxin was removed. Toxic Bio-Gel fractions were pooled and evaporated to dryness, leaving a tan residue of 1.25 g. This material was dried in vacuo over $CaCl_2$ at 4 C for 24 hr. It was stored under nitrogen in the presence of $CaCl_2$ at -20 C, and used for all

Figure 1. Extraction of toxin from chloroform residue.



experiments described here. The preparation caused 50% inhibition of root growth of susceptible corn at 0.2 μg/ml. The probable molecular weight of this toxin is 679 (88). Thus, activity was evident at $>2.95 \times 10^{-7}$ M, which is as good as the best crystalline preparations (88, 92). Under the storage conditions used, this dry preparation did not lose activity or host-specificity in 2 yr. Lyophilized toxin is reported to be more stable than crystalline preparations (90). For experiments, 20-30 mg was dissolved in water (1 mg/ml), stored at -20 C in 1 and 2 ml aliquots and used as needed. One such aliquot was thawed and refrozen at least 6 times without losing activity or host-specificity. A small amount of crystalline toxin was prepared following Pringle's procedure (92). One toxic Bio-Gel fraction produced a clear residue when evaporated to dryness. This residue was dissolved in 5 ml absolute ethanol, 100 ml diethyl ether was added and the solution was placed at 5 C for several days. A small amount of fluffy, colorless, needle-shaped particles precipitated; this preparation caused 50% inhibition of susceptible corn root growth at 0.2 μg/ml.

In most cases, toxin-containing solutions were assayed against susceptible and resistant seedlings (100) before and after completion of the experiment. This was done for all experiments which showed little or no effect of toxin. In all cases, there was 50% inhibition of susceptible and no effect on resistant seedling root growth at 0.2 μg/ml, indicating no loss of toxin activity during the experiment. Inactivated toxin was prepared as described for HV-toxin (98) or the toxin solution was

sealed in a glass tube and autoclaved 1 hr on each of 10 consecutive days. This destroyed essentially all toxic activity.

Amylase induction. -- α-amylase induction in embryoless barley half-seeds was determined by a procedure modified after that of Varner et al (7, 46). Barley seeds were placed in 50% H_2SO_4, stirred vigorously for 1 hr to remove husks (9), and washed 10 or more times with distilled water. Seeds were cut transversely with a razor blade and embryoless halves were surface sterilized 20 min in 1% sodium hypochlorite, then washed 3 times in sterile distilled water and incubated in a beaker of sterile water at 24 C for 24 hr. Half-seeds were placed aseptically in sterile 50 ml flasks (10/flask) containing 2 µmoles sodium acetate (pH 4.8), 40 µmoles $CaCl_2$, 20 µg chloramphenicol with or without 2 nmoles gibberellic acid (GA) or varying amounts of toxin in a total volume of 2 ml. Acetate and $CaCl_2$ were sterilized by autoclaving; other components were sterilized by filtration (0.22 µ Millipore filters) and added to the medium. Flasks were placed on a reciprocal shaker (100 oscillations/min) at 24 C for 24 hr. The medium from each flask was decanted into a 15 ml glass centrifuge tube, and half-seeds were rinsed 3 times with 1 ml volumes of water. The water from each rinse was added to the medium. Half-seeds were ground in a mortar with a pinch of sand and 0.8 ml grinding medium (44 mM KH_2PO_4, 2 mM Ca Cl_2). One ml grinding medium was added and the slurry was poured into glass centrifuge tubes. The mortar was rinsed twice with a total of 3 ml grinding medium and rinsings were added to homogenate. Tubes

containing incubation medium or seed homogenate were centrifuged 10 min at 2000 g; supernatants were assayed for α-amylase. Aliquots (0.02-0.2 ml) were diluted to 1 ml with water and incubated with 1 ml starch solution (46) for 5 min. One ml iodine solution (46) was added, then colored solutions were diluted with water and the color intensity was measured using a Klett-Summerson colorimeter with a #62 filter. Enzyme activity was quantified by defining 1 unit α-amylase as a change of 10 Klett units in 1 min.

Amylase activity in corn endosperm was determined by a method modified from that used for barley half-seeds. Corn seeds were incubated in water 3 hr, then embryos were dissected and discarded. Endosperms were rinsed in distilled water, surface sterilized in 1% sodium hypochlorite for 20 min, rinsed 4 times in sterile water, and incubated 24 hr in sterile water at 24 C. Endosperms were then transferred aseptically to 4 ml incubation medium (described for barley half-seeds) in 50 ml flasks and incubated on a shaker (120 oscillations/min) for 48 hr. Amylolytic activity was determined in endosperms and medium as described for barley. The type of amylolytic activity was not ascertained; Dure (12) has shown that corn endosperm contains primarily β-amylase.

Nitrate reductase induction. -- Nitrate reductase (NR) was induced in aseptic corn embryonic axes (axis = embryo minus scutellum) dissected with a sterile scalpel from corn seeds which were surface sterilized 15 min in 1% sodium hypochlorite, and washed 4 times in sterile

water. Axes were rinsed in sterile water, then allowed to imbibe in petri dishes on filter paper wetted with sterile water or nutrient solution containing filter-sterilized chloramphenicol (20 μg/ml) or a combination of penicillin, mycostatin, and streptomycin (10 μg of each/ml). The nutrient solution was modified after that of Dure (11) and contained the following in mg/l: $CaCl_2 \cdot 2H_2O$, 190; KCl, 65; $NaH_2PO_4 \cdot H_2O$, 16.5; $MgSO_4 \cdot 7H_2O$, 36; iron citrate, 30; $MnSO_4 \cdot H_2O$, 0.3; and glucose, 20 g/l. Induction of NR was initiated by transferring axes to similar solutions containing nitrate (NO_3) and shaking at 200 oscillations/min. Experimental results were not affected when antibiotics were omitted from sterile media. Solutions from all experiments were plated on potato-dextrose agar to detect possible microbial contamination. None was found. Procedures for induction under nonsterile conditions in corn roots and leaves are described in a later section.

The in vivo method of Ferrari and Varner (21) and the in vitro method of Filner (23) were modified to determine NR activity in corn tissues. For the in vivo assay, axes were placed in 1.7 ml assay medium in 15 ml glass centrifuge tubes. The assay medium contained 0.1 M phosphate buffer (pH 7.5), 0.01 M KNO_3, and 5% (v/v) ethanol. Nitrogen was bubbled through the solution for 1 min and the vial containing axes was stoppered. Axes were then incubated 15-30 min at 24 C on a wrist action shaker at high speed. After incubation, the reaction was stopped by adding an equal volume of each color reagent (1% sulfanilamide in 3 N HCl and 0.02% N-1-naphthylethylenediamine

dihydrochloride). Color was allowed to develop for at least 15 min. Turbid solutions were centrifuged 5 min at 5000 g. The color intensity was measured with a Klett-Summerson colorimeter using a #54 filter. The procedure for root tips was similar to that for axes except the volume of the assay medium was 0.5 ml, anaerobic incubation was in 3 ml glass vials, and after incubation contents of vials were boiled briefly before color reagents were added.

For the in vitro assay, 5 mm root tips were excised, rinsed in ice cold water, and homogenized in 1 ml ice cold medium in a 2 ml glass homogenizer (10 tips/replicate). The homogenizing medium contained 25 mM K_2HPO_4, 5 mM disodium ethylenediaminetetraacetic acid (EDTA), and 10 mM cysteine-HCl, pH 8.8 (4). The homogenizer was rinsed twice with 0.5 ml aliquots of homogenizing medium; homogenate and rinsings were centrifuged in a 15 ml glass centrifuge tube at 2 C for 10 min at 15,000 g. Enzyme activity was determined in a reaction mixture which contained 0.5 ml 0.1 M phosphate buffer (pH 7.5), 0.1 ml 0.1 M KNO_3, 0.1 ml 0.001 M NADH, and 0.3 ml supernatant. The reaction was started by transferring tubes from ice to a 28 C water bath. The reaction was stopped after 0 and 30 min by adding 1 ml of each color reagent; color was developed for at least 15 min and optical density (O. D.) at 540 nm was determined with a Coleman spectrophotometer. NR activity was determined from a standard nitrite (NO_2) curve, and was expressed as nmoles NO_2 produced/unit of tissue/hr.

NO_3 and NO_2 determinations. -- The quantitative bacterial assay of Lowe and Hamilton (65) was used to determine NO_3 content of boiled tissue extracts. Boiling released soluble intracellular materials into the suspending medium. Reaction mixtures contained 0.02 M sodium succinate (pH 6.8), 0.1-0.2 ml soybean root nodule bacteroids (65), and NO_3-containing extract in a total volume of 1.0-1.7 ml. Reaction was allowed to go to completion (30-60 min), then an equal volume of each color reagent was added. Color reagents were added directly to boiled and nonboiled tissue extracts to determine NO_2 content. Color intensity of solutions was measured with a Klett-Summerson colorimeter. Concentrations of NO_2 were determined from NO_2 standards.

The metabolic NO_3 pool (22) was demonstrated in tertiary leaves of 17-day-old corn seedlings grown in vermiculite watered with White's solution containing 10 mM KNO_3 + Fe and Mo. Leaf sections (5 mm^2) were placed in 2 ml 0.1 M phosphate buffer (pH 7.5) in 50 ml flasks (100 mg/flask). Nitrogen was bubbled through the solution for 1 min in dim light; flasks were then stoppered and incubated in total darkness at 30 C (94). To demonstrate the capacity of the metabolic NO_3 pool, flasks were opened at intervals, 2 ml phosphate buffer was added (to keep final volumes constant), contents were boiled, and NO_2 in 1 ml of boiled extract was determined by adding 1 ml of each color reagent. To demonstrate refilling of the metabolic pool, flasks were opened after metabolic pool NO_3 was exhausted. Buffer (2 ml) with and without 0.1 M KNO_3 was added, flasks were aerated 1 min, incubated aerobically

15 min, flushed with nitrogen 1 min, and returned to darkness at 30 C. Periodically, contents of flasks were boiled and NO_2 determinations were made. In experiments with toxin, metabolic pool NO_3 was exhausted, and 2 ml buffer with or without toxin (40 μg/ml) was added to the flasks. Contents were aerated 1 min and incubated aerobically 4 hr. Flasks were flushed with nitrogen 1 min and returned to darkness at 30 C. After 2 hr contents of flasks were boiled and NO_2 determinations were made.

The size of the metabolic NO_3 pool was estimated in roots. Tissues (grown in NO_3 solution) were placed in 0.4 ml 0.1 M phosphate buffer (pH 7.5) in a 3 ml glass vial. The vial was flushed with nitrogen 1 min and stoppered. At intervals contents were boiled, cooled, and amount of NO_2 produced was measured.

Isolation of chloroplasts. -- Corn chloroplasts were isolated at 4 C by a method modified after that of Miflin and Hageman (75). Two-week-old seedlings were held in darkness 24 hr prior to use to eliminate starch from leaves. Tertiary leaves (10 g) were cut in 1 cm pieces, chilled and homogenized 15-20 sec in a Waring blender in 80 ml homogenizing medium containing 0.01 M NaCl, 0.05 M Tricine-NaOH (pH 7.8), 0.002 M $MgCl_2$, 0.4 M sucrose, 0.01 M glutathione and 12 mg polyethylene glycol (Carbowax 4000)/ml. Glutathione was added to the medium just prior to blending. Homogenate was squeezed through 8 layers of cheesecloth and centrifuged 4 min in 50 ml centrifuge tubes at 2500 g. The supernatant was discarded; each pellet was gently

dislodged with a camel's hair brush and suspended in 40 ml washing medium which contained 0.01 M NaCl, 0.01 M Tricine-NaOH (pH 7.5), 0.002 M $MgCl_2$, and 0.4 M sucrose. Tubes were centrifuged slowly 30 sec in a clinical centrifuge to remove large debris. Supernatants were decanted into clean tubes and centrifuged 3 min at 2500 g. Supernatants were discarded, pellets were suspended in <10 ml washing medium and chlorophyll content was adjusted by a method modified from that of Mackinney (71). An aliquot of the unadjusted stock suspension was diluted 1:100 with 80% acetone and centrifuged 5 min at 5000 g. The O. D. of the supernatant was determined at 663 nm (chlorophyll a), 645 nm (chlorophyll b) and 652 nm (the wavelength at which absorption spectra of chlorophylls a and b intersect). Chlorophyll content was determined from Mackinney's extinction coefficients:

$$O.D._{663}\ (12.7) - O.D._{645}\ (2.7) = \mu g \text{ chlorophyll a/ml acetone}$$

$$O.D._{645}\ (23) - O.D._{663}\ (4.7) = \mu g \text{ chlorophyll b/ml acetone}$$

The combined equations, $O.D._{663}\ (8) + O.D._{645}\ (20.3)$, give the total μg chlorophyll/ml acetone. To check accuracy of the determination, $O.D._{652}\ (28.5)$ should also equal total μg chlorophyll/ml acetone. The ratio of chlorophyll a:b was about 3:1 for corn. The stock chloroplast suspension, which contained 0.4 mg chlorophyll/ml, was stored on ice.

The reaction medium for chloroplasts contained 0.1 M sucrose, 0.01 M KCl, 0.05 M Tricine-NaOH (pH 7.8), 0.001 M $MgCl_2$, 20 μg chlorophyll/ml, and other components as indicated in Results in a total volume of 2 ml. Changes in light-scattering were estimated by measuring

changes in the apparent O. D. (small angle light-scattering) of a chloroplast suspension at 540 nm in a 1 cm cuvette. Electron transport was induced with a beam of actinic light (>600 nm) at right angles to the detecting beam. The rate of electron transport was measured by following the decrease in O. D. of potassium ferricyanide at 420 nm. The method is similar to that of Izawa and Good (45).

Isolation of mitochondria. -- Mitochondria were isolated and tested following the general procedures of Miller et al (77). All manipulations were done in the cold room (4C) and mitochondria were stored on ice. Seven-day-old etiolated shoots (75 g) were cut in 1 cm segments and chilled. They were ground 30 sec in a mortar on ice with 150 ml grinding medium which contained 0.4 M sucrose, 0.03 M HEPES, 0.005 M EDTA, 0.1% bovine serum albumin (BSA), 0.05% cysteine. The pH was adjusted to 7.5 with NaOH. Cysteine was added just prior to grinding. Homogenate was squeezed through 8 layers of cheesecloth and centrifuged in 50 ml tubes for 5 min at 28,000 g. The supernatants were removed with an aspirator; each pellet was gently dislodged with a camel's hair brush, suspended in 40 ml grinding medium minus cysteine, and centrifuged 3-4 min at 2500 g. The pellets were discarded and supernatants were centrifuged 5 min at 28,000 g. In some experiments the pellets were washed once more in grinding medium minus cysteine to remove all endogenous substrates. Supernatants were aspirated; pellets were dislodged with a brush (leaving behind as much starch as possible) and suspended in 1 ml 0.4 M sucrose. An aliquot

of the mitochondrial suspension was diluted 1:40 with 0.4 M sucrose. The O. D. was measured at 520 nm, and protein content was determined from a standard curve plotting $O.D._{520}$ vs. protein. The stock mitochondrial suspension was adjusted to 5 mg protein/ml with 0.4 M sucrose.

The standard curve was plotted from protein and $O.D._{520}$ determinations made on a series of mitochondrial dilutions. After $O.D._{520}$ measurements were taken, 1 ml 20% (w/v) trichloroacetic acid (TCA) was added to 1 ml mitochondrial suspension in 15 ml centrifuge tubes. After 24 hr, 8 ml 10% TCA was added and suspensions were centrifuged 10 min at 20,000 g. The pellets were washed in 10 ml 95% ethanol, air dried, and dissolved in 1 ml 1 N NaOH (>1 hr required). Protein content was determined by the method of Lowry et al (66) using BSA in 1 N NaOH as the standard.

The reaction medium used in experiments with mitochondria contained 0.2 M KCl, 0.02 M HEPES (pH 7.5 with NaOH), 0.002 M $MgCl_2$, 0.1% BSA, 250 μg mitochondrial protein/ml, and other components as indicated in Results in a total volume of 2 ml. Volume changes in mitochondria suspended in a 1 cm cuvette were determined by changes in O. D. at 520 nm using a Bausch and Lomb Spectronic 505 spectrophotometer. Respiration rates were measured by inserting a Clark oxygen electrode into a special temperature-controlled cuvette (28 C) containing 2 ml reaction medium and monitoring oxygen loss from the solution with a recorder. Phosphorylation was determined by including ADP and

^{32}Pi in the reaction mixture. After 5-10 min reaction time, 1 ml of the radioactive reaction mixture was quickly frozen in ethylene glycol at -20 C. Incorporated Pi was determined by the method of Saha and Good (97) as described later in this section. Corrections were made for volume changes in reaction mixtures caused by additions during the course of the experiment.

Absorption of exogenous solutes by roots. -- A modified standard procedure (19) was used to determine absorption of NO_3, NO_2, K, Na, Cl, Ca, PO_4, SO_4, leucine, glutamic acid, and 3-o-methylglucose (MeG) by roots. Four-day-old seedlings were placed in 15 cm petri dishes containing 50 ml 0.5 mM $CaCl_2$ or $CaSO_4$ with or without toxin (10 µg/ml), and incubated for 4 hr. Toxin-treated and control seedlings were rinsed in separate 4 l beakers containing 3000 ml 0.5 mM $CaCl_2$, unless otherwise indicated. Root tips 6 cm long (0.3-0.5 g/ 6-10 roots) were placed in a cheesecloth bag, rinsed again in 0.5 mM $CaCl_2$, unless otherwise indicated, and twirled rapidly in the air to spin out excess solution. Bags were incubated for 30-45 min in vigorously aerated solutions containing the appropriate solute under test. Absorption periods were terminated by rinsing roots 6 times in 1500 ml 0.5 mM $CaCl_2$, unless otherwise indicated. The amounts of each test solute in tissue were then determined by methods described below. Possible effects of toxin on efflux were tested by desorption (described in Results) in aerated solutions at 24 C. Removal of ions from free space by desorption is usually done at 5 C (22). Variations in the usual procedure are indicated where appropriate.

Absorption of all solutes except NO_3 and NO_2 was detected by use of isotopes. These were: $^{86}RbCl$, $Na_2H^{32}PO_4$, $Na_2{}^{35}SO_4$, $^{22}NaCl$, $^{45}CaCl_2$, and $Na^{36}Cl$, all of which were carrier free. Organic compounds had the following specific activities: 3-o-methyl-^{14}C-D-glucose, 10 mc/mmole; ^{14}C-1-L-leucine, 12.2 mc/mmole; and ^{14}C-1-L-glutamic acid, 20 mc/mmole. None of the isotopes made a significant contribution to the solute concentration of the experimental solution.

To extract NO_3, NO_2 and Cl, roots were cut in 3 cm sections, placed in 5 ml water in a 50 ml flask, boiled and frozen. To determine Cl content, 2 ml aliquots were placed in aluminum planchets, dried and counted in a gas-flow detector. Amounts of NO_3 and NO_2 in the extracts were determined as described in the previous section. Quantities of NO_3 (or NO_2) in tissue are expressed as "accumulated" rather than "absorbed." This is because some of the NO_3 taken up during the absorption period was reduced, and only the "accumulated" NO_3 was detected in the boiled extracts. For all solutes other than NO_3 and NO_2, "absorption" indicates total solute taken up during the absorption period. Total NO_3 uptake could be determined by observing NO_3 loss from the external solution, or by suppressing NR activity with tungstate, which would eliminate NO_3 metabolism (37).

To determine content of other ions in roots, 1-2 cm root sections were placed on aluminum planchets, dried, and ashed 2-3 hr at 500 C. Ash was spread with 0.5 ml 1% Triton X-100, dried, and counted. Correction was made for self-absorption, which occurred only with ^{45}Ca and ^{14}C.

Labelled organic materials were extracted from roots cut into 1 cm sections. Sections were placed in 3 ml vials containing 1.5 ml of the appropriate solvent. Roots containing MeG were placed in 95% ethanol and held 24 hr at -20 C (31). Leucine was extracted in 95% ethanol at 24 C or in boiling water. Glutamic acid was extracted in boiling water. Aliquots were dried in aluminum planchets and counted.

To determine efficiency of amino acid extraction, roots were homogenized in ethanol or water. The homogenate was dried in planchets, counted, and counts were corrected for self-absorption of the root residue. Boiling water extracted 64% of the glutamic acid and 42% of the leucine from roots. Ethanol extracted 66% of the leucine from roots. Since a significant portion of the labelled amino acids remained in the roots, total uptake was calculated as the sum of the counts in the soluble and insoluble fractions.

Phosphorous absorbed by roots was separated into organic and inorganic fractions. Roots (0.5 g) were homogenized in 2 ml 10% perchloric acid (PCA) at 5 C. Homogenate and 1 ml 10% PCA rinse were placed in a 15 ml glass centrifuge tube and centrifuged at 5 C for 10 min at 20,000 g. The pellet was washed in 7 ml 10% PCA, suspended in 1 ml water, placed in an aluminum planchet with 1 drop of 1% Triton X-100 and counted without drying to prevent explosion of residual PCA.

Organic and inorganic phosphate in the combined supernatants was separated and quantified by a procedure modified from that of Saha and Good (97). One ml 10% ammonium molybdate was added to the supernatant to complex Pi. Acetone (2.2 ml) was added and phosphomolybdate

was extracted with 2 volumes (6.5 ml each) of 10% PCA-saturated butanol-benzene (1:1, v/v). The aqueous phase (containing organic phosphate) and the organic phase (containing phosphomolybdate) were counted in a Geiger-Muller immersion tube (97).

Starting times were adjusted so that all toxin-treated seedlings were exposed to toxin 4 hr $\pm$ 15 min. After toxin-treatment there was a 15-30 min handling period and 30-45 min exposure to experimental solutions. Elapsed time from beginning of toxin-treatment to termination of experiment was approximately 5 hr. Short absorption periods have several advantages (19) including a brief post-toxin-treatment period.

The volume of experimental solutions varied, depending on the solute under examination and solute concentration (19). There was often approximately 1 g tissue/500 ml solution. Loss of solute from experimental solutions never exceeded 5-10%.

Some workers report little change in pH of experimental solutions during short term experiments (15, 93), although others (70, 85) have observed alterations in pH. My experimental solutions were prepared at pH 5.8. In some experiments the pH changed from 5.8 to a final value of 4.8-5.3, regardless of the solute used or solute concentration. There was no pH change when the volume of experimental solution was large compared to the amount of tissue used. The pH change was not caused by aeration, presence of cheesecloth, string, or solutes, or cut ends of excised roots. Extensive washing of roots during preparation

did not prevent pH change. Ion uptake is known to be affected by pH, but not in the range of pH 5-7 (68). Furthermore, similar results were obtained in nonbuffered solutions and in solutions buffered at pH 5.8 with 2 mM MES. There was no difference in pH change caused by toxin-treated and control roots. There is a report that H release in small volumes of solution (100-500 ml/g roots) is related to total salt uptake (85). The efflux of H was proportional to salt concentration (above 0.5 mM) and to the rate of salt uptake. Thus, the pH change observed in the present study could be related to salt uptake but cannot be explained on the basis of charge balance or effect of toxin (see experiments with $Ca(NO_3)_2$).

All glassware used in uptake experiments was washed with detergent and rinsed in tap water, distilled water, 0.5 N HCl, and then 6-8 times in distilled water. Cheesecloth and string were boiled in 0.05 N HCl and rinsed many times in distilled water before use. Similar results were obtained with tap distilled (>1,000,000 ohms) or glass distilled (>900,000 ohms) water.

All experiments were repeated, with or without variations, one or more times except where indicated. Ranges, indicated by $\pm$ in tables, are the result of 2 replicates.

RESULTS

Effect of toxin on seed germination and seedling root growth. -- Samaddar found that oat seeds exposed to HV-toxin for 1 hr will not germinate (98), which indicates that metabolically inactive tissues are sensitive to toxin. Thus, it seemed important to determine the effect of HC-toxin on the germination process in corn seeds. In the first experiment, susceptible and resistant seeds were exposed to White's solution with or without toxin (25 seeds/25 ml/15 cm petri dish), for 24, 48, and 72 hr, washed 1 hr in running tap water, and placed in fresh White's solution without toxin in clean petri dishes. After 4 days incubation, % germination was determined and roots were measured. Exposure of seeds to toxin for 24 and 48 hr had little or no effect on germination or root growth of susceptible and resistant seedlings at any concentration. Exposure for 72 hr did not affect % germination, but root growth of susceptible seeds was inhibited 95, 82, and 35% at 20, 2, and 0.2 μg toxin/ml, respectively. There was no effect on resistant seedlings at any concentration used.

In a similar experiment, toxin at 20 μg/ml had no effect on susceptible root growth after 24-40 hr exposure times but caused 54% inhibition after a 48 hr exposure. Results of these 2 experiments were uncertain because germination and root growth were reduced in the

controls by the washing procedure. Even brief washing in tap or distilled water was found to cause a decrease in germination and growth. Washing was omitted in later experiments; instead, seeds were blotted on paper towels before transfer to fresh solutions.

Results of a third experiment show that toxin had no effect on seed germination (Table 1). Exposure of seeds to toxin for 40 hr caused slight inhibition of the root growth which occurred after removal of seeds from toxin. Exposure times longer than 40 hr caused greater inhibition of later root growth. Inhibition was more obvious at 3 days than at 1 day after treatment.

A fourth experiment was designed to determine whether or not seedlings can recover from a toxin exposure capable of causing about 50% inhibition of later root growth. Seeds were exposed to White's solution with or without toxin for 32, 44, or 56 hr. These exposures gave no inhibition, partial, and complete inhibition, respectively, of later root growth (Table 2). Immediately after treatment, seeds exposed to toxin for 32 hr had not germinated, whereas seeds exposed for 44 hr had germinated normally, and seeds exposed for 56 hr had germinated but further growth was inhibited. Roots of seedlings exposed to toxin for 32 hr grew normally throughout the experimental period, indicating that seeds did not accumulate a toxic dose in that time. When 44 hr toxin exposure was used, root lengths and dry wts 1 and 2 days after the treatment were less than for controls. Four days after treatment, the roots of toxin-treated and control plants were equal in length; dry

Table 1. Effect of Toxin Exposure Time on Germination and Root Growth of Susceptible Corn Seeds

Seeds were placed in White's solution with or without toxin (20 µg/ml) for times indicated, then removed, blotted, and placed in fresh White's solution. There were 25 seeds/treatment, with 5 seeds/10 ml in each of 5 petri dishes (10 cm). Roots were measured 1 and 3 days later. A seed was considered germinated if it had a 5 mm root.

Toxin-Treatment Time	Interval After Removal from Toxin	Control		Toxin		Inhibition of Root Growth
		Germination	Avg Root Length	Germination	Avg Root Length	
hr	days	%	mm	%	mm	%
40	1	100	34	96	27	19
44		100	40	92	20	51
48		96	40	88	13	68
52		100	43	92	9	98
56		96	33	96	9	97
40	3		100		63	37
44			114		50	57
48			115		34	71
52			110		20	82
56			111		10	91

Table 2. Recovery of Susceptible Corn From Effects of Toxin on Root Length and Dry Weight

Seeds were placed in toxin solutions (20 µg/ml) or in control solutions for 32, 44, or 56 hr. Seeds were then placed on cheesecloth suspended over 150 ml White's solution in glass staining jars. There was a 12 hr difference in age of seedlings of the 3 treatments. Thus, each treatment can be compared only with its own control. There were 10 roots/treatment. Between 4 and 10 days after treatment, primary and secondary roots formed a tangled mat and seedlings could no longer be removed individually. Therefore, the entire mat was excised, dried and weighed. Avg wt is total wt divided by the number of seeds contributing roots to the mat. This number varied from 13-20.

Interval After Toxin Exposure	Toxin Treatment Time	Avg Length		Toxin-Induced Change	Avg Wt		Toxin-Induced Change
		Control	Toxin		Control	Toxin	
days	hr	mm	mm	%	mg	mg	%
0	32	0	0	...	0	0	...
	44	3.4	3.9	+15	0.70	0.81	+16
	56	23.0	5.1	-78	2.86	1.04	-64
1	32	13.3	13.7	+3	1.66	1.57	-5
	44	29.1	17.0	-42	3.20	1.90	-41
	56	46.7	7.0	-85	4.57	1.11	-76
2	32	28.5	31.7	+11	3.08	3.27	+6
	44	45.4	33.5	-26	4.74	2.64	-44
	56	60.6	7.4	-88	5.19	0.86	-83
4	32	75.4	65.1	-13	5.87	4.09	-30
	44	101.8	96.5	-5	8.73	5.78	-34
	56	115.9	17.4	-85	8.86	1.77	-80
10	32	...	...	...	22.20	24.70	+11
	44	...	...	...	24.10	23.00	-4
	56	...	...	...	24.10	18.20	-24

wt of roots indicated partial recovery by toxin-treated seedlings. By 10 days there was no difference between dry wts of the control root mat and the root mat from seeds exposed to toxin 44 hr, which suggests complete recovery. However, a small effect on dry wt of primary roots could have been masked by the large mass of secondary roots in the mat. Exposure of seeds to toxin for 56 hr resulted in essentially complete inhibition of primary root growth throughout the experimental period, indicating that roots cannot recover from the effect of toxin if a large dose is accumulated. The apparent increase in growth of 56 hr primary roots at 4 days is due to 2 roots which grew abnormally long (73 and 89 mm). The average length of the other 8 roots was 9 mm, indicating complete inhibition. Primary roots from seeds exposed to toxin 56 hr had a brown discoloration throughout the experimental period. Exposure to toxin for 56 hr had little or no effect on growth of secondary roots, as shown by the large root mat at 10 days. The mat from toxin-treated seedlings weighed less than that of the control, probably because all primary roots in the mat were dead and shriveled by that time.

It was concluded that intact, resting corn seeds do not accumulate a toxic dose of HC-toxin in the same way that intact, resting oat seeds accumulate a toxic dose of HV-toxin.

In contrast to its inhibitory effect on root growth, toxin can also stimulate root growth if used at the proper concentration (57). My data on this subject were published previously (57). The experiments were

with a toxin preparation which caused 50% inhibition of growth of susceptible and resistant roots at 0.2 and 20 μg toxin/ml, respectively. This same toxin preparation stimulated growth of susceptible and resistant roots at 0.025 and 1.0 μg/ml, respectively. Further results (Table 3) show that there is a corresponding increase in dry wt of resistant roots, which was statistically significant at the 10% level. There was no significant increase in dry wt of susceptible roots, perhaps because of the small sample size used. There were 25 observations/treatment for root length but only 5 observations/treatment for dry wt (roots were handled in groups of 5 to facilitate the dry wt procedure). As with root length analysis (57), a large number of observations would be required to show that toxin-stimulation of dry wt is highly significant. Increased dry wt could mean that toxin enhanced movement of materials from seed to root.

The toxin preparation which inhibited root growth of H. carbonum-susceptible corn at 0.2 μg/ml was found to inhibit root growth of radish, oats, and tomato at 3 μg/ml; barley, cucumber, sorghum, wheat, and H. carbonum-resistant corn were inhibited at 45 μg/ml. Tomato root growth was stimulated 15% by toxin at 0.2 μg/ml, barley roots were stimulated 15% by 3 μg/ml, and sorghum roots were stimulated 34 and 40% by 0.2 and 3 μg toxin/ml, respectively (57). Stimulation of sorghum root growth was much greater than stimulation of either susceptible or resistant corn.

Table 3. Effect of Toxin on Growth of Corn Seedling Roots

Roots were measured after 87 hr exposure to White's solution with or without toxin, then placed in groups of 5, dried and weighed. Avg length is an average for 25 roots; avg wt was determined from 5 observations of 5 roots each. Susceptible and resistant seedlings were exposed to 0.025 and 1.0 μg toxin/ml, respectively.

Corn Hybrid	Avg Length/Root		Avg Wt/5 Roots	
	Control	Toxin	Control	Toxin
	mm	mm	mg	mg
Susceptible (Pr x K61)	115	133[a]	42.5	45.5
Resistant (Prl x K61)	123	134[a]	37.5	40.5[b]

[a]Significantly different ($P < .01$) from control.

[b]Significantly different ($.05 < P < .10$) from control.

The stimulatory effects of toxin evidently precede the inhibitory effects (54); thus, they may be related to the site of toxin action. Further experiments were built upon stimulatory effects in an attempt to understand the earliest effects of toxin on plant cells. Several possibilities were considered: 1) Toxin may act as a growth hormone; 2) toxin may interfere in energy metabolism of the cell; 3) synthesis or degradation of regulatory substances such as proteins may be affected by toxin; 4) toxin may alter inter- or intracellular solute movement. Experiments were designed to determine whether any of these possibilities is related to toxin action.

Tests for hormone-like activity of toxin. -- Toxin-stimulation of growth of host and nonhost plants, as well as stimulation of certain physiological processes (54, 56, 57), suggested that toxin may have hormone-like activity for plants in general. Such is the case with helminthosporol, a metabolite produced by certain Helminthosporium spp. which has gibberellin-like activity. To test for hormone action, toxin was assayed in three biological systems known to be stimulated by both gibberellic acid (GA) and helminthosporol (36, 48, 81).

Maryland Mammoth tobacco seeds ordinarily require light to germinate, but will germinate in darkness in the presence of GA or helminthosporol (36). My data (Table 4) show that GA, but not toxin, stimulated dark germination of tobacco seeds. After the experimental period, all nongerminated seeds were incubated in the light for 1 week. Seeds in all categories listed in Table 4, except those exposed to >1.0 µg toxin/ml, germinated and grew.

Table 4. Effects of Toxin and Gibberellic Acid on Germination of Tobacco Seeds in the Dark

Maryland Mammoth tobacco seeds (50/treatment) were placed on 3 filter paper discs saturated with 2 ml White's solution with or without GA or toxin in 5 cm petri dishes (25 seeds/dish). They were incubated in total darkness or in diffuse light on the laboratory bench for 6 days.

Treatment	Concentration	Germination
		%
Control - Dark	...	0
Control - Light	...	100
GA - Dark	10^{-3} M	88
	10^{-4} M	86
	10^{-5} M	4
Toxin - Dark	1000.0 µg/ml	0
	100.0 µg/ml	0
	10.0 µg/ml	0
	1.0 µg/ml	0
	0.1 µg/ml	0
	0.01 µg/ml	0

The rate of cucumber hypocotyl elongation is stimulated by GA or by helminthosporol (48). For comparison, GA or toxin was applied to apices of cucumber seedlings. Data in Table 5 show that GA, but not toxin, stimulated cucumber hypocotyl elongation.

Production of α-amylase in embryoless barley half-seeds is induced by GA and by helminthosporol (81). An experiment was designed to determine whether or not toxin can induce α-amylase production, or affect GA-induced α-amylase production in barley half-seeds. Two cultivars of barley (cv. Betzes and cv. Hudson) were used. Toxin alone did not induce α-amylase production and had little or no effect on GA-induced α-amylase production by half-seeds of cv. Betzes (Table 6). However, toxin at 1 and 0.1 μg/ml caused striking stimulation of GA-induced α-amylase production by half-seeds of cv. Hudson. Control seeds of cv. Hudson had much less amylase production than did control seeds of cv. Betzes. A germination test showed that all Betzes seeds were viable, but $<20\%$ of Hudson seeds were viable. Toxin (0.5 μg/ml), GA (10^{-6} M), or GA (10^{-6} M) + toxin (0.5 μg/ml) had no effect on germination of Hudson seeds, compared with control seeds in water.

Another batch of Hudson seeds which gave 99% germination was used to test the effect of toxin on α-amylase production. Results were similar to those obtained with barley cv. Betzes (Table 6). Thus, toxin has no effect on α-amylase production in normal barley seeds, but it can enhance the effect of GA in certain seeds with low viability.

Table 5. Effects of Toxin and Gibberellic Acid on Cucumber Hypocotyl Elongation

The procedure of Katsumi et al (48) was followed. National Pickling cucumber seedlings (10/treatment) were treated with 0.01 ml 95% ethanol with or without GA or toxin, and incubated 3 days in light.

Treatment	Dosage	Avg Hypocotyl Elongation
	µg/plant	mm
Control (nontreated)	...	2
Control (ethanol)	...	2
GA	10.0	11
	1.0	7
Toxin	1000.0	0 (dead)
	100.0	0 (alive)
	10.0	2
	1.0	2
	0.1	2
	0.01	2
	0.001	2

Table 6. Effect of Toxin and Gibberellic Acid on ∝ -Amylase Production by Embryoless Barley Half-Seeds

One unit ∝-amylase is defined as a change of 10 Klett units/min. GA concentration was 10^{-6} M.

Barley cv.	Treatment	Toxin Concn.	∝-Amylase/Half-Seed		
			Medium	Seeds	Total
		µg/ml	units	units	units
Betzes	Control	...	0	70	70
	GA	...	117	105	222
	Toxin	0.1	0	67	67
	GA & Toxin	10.0	101	103	205
		1.0	140	108	248
		0.1	109	95	205
		0.01	113	99	212
		0.001	99	98	198
Hudson	Control	...	1.5	4.5	6.0
	GA	...	9.0	7.5	16.5
	GA & Toxin	100.0	7.0	6.5	13.5
		10.0	5.0	6.5	11.5
		1.0	16.5	13.5	30.0
		0.1	19.0	8.0	27.0

The production of soluble sugar by embryoless corn endosperms can be stimulated by exposure to GA (43). This implies an increase in amylase activity, which according to Dure (12) is primarily β-amylase. Toxin at 10 and 1 μg/ml was found to inhibit GA-induced amylase activity in susceptible corn endosperms (Table 7). Toxin at low concentrations had little or no effect on amylase activity.

Experiments with toxin and isolated mitochondria. -- Toxin-enhanced growth and metabolism should require increased expenditure of cellular energy. Both toxin and infection are known to increase the respiration rate of intact tissues (56). The possibility of an effect of toxin on energy metabolism or on other intracellular sites was tested with isolated organelles.

Respiration rates and phosphorylation by corn mitochondria in the presence of exogenous NADH were measured in a series of experiments. Results (Figure 2) show that toxin did not affect electron transport rates of either phosphorylating or nonphosphorylating mitochondria, nor did it affect the respiratory control ratio (RCR) or the P:O ratio. A known uncoupler, 2,4-dinitrophenol (DNP), caused an increase in the rate of electron transport. These data agree well with published results using corn mitochondria (76).

Oxidation and phosphorylation after preincubation of mitochondria in the presence and absence of toxin were determined in another series of experiments using 2 different substrates (Table 8). With NADH as the substrate, mitochondria which were not preincubated had normal

Table 7. Effect of Toxin on Gibberellic Acid-Induced Amylase Production by Susceptible Corn Endosperm

One unit amylase is defined as a change of 10 Klett units/min. GA concentration was 10^{-5} M.

Treatment	Toxin Concn.	Amylase/Endosperm		
		Medium	Endosperm	Total
	µg/ml	units	units	units
Control	...	80	392	472
GA	...	164	652	816
GA & Toxin	10.0	40	220	260
	1.0	95	528	623
	0.1	180	600	780
	0.01	158	712	870
	0.001	192	632	824

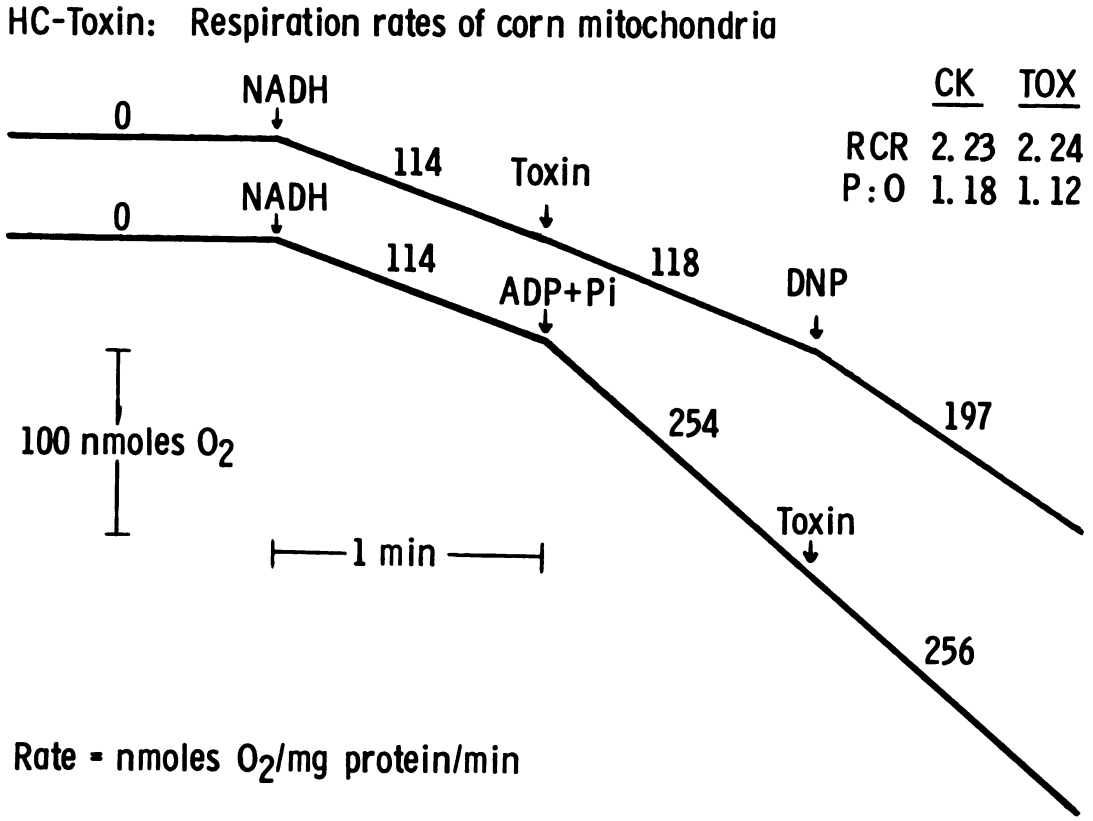


Figure 2. Respiration rates and phosphorylation of corn mitochondria in the presence and absence of toxin. The reaction medium contained 0.2 M KCl, 0.02 M HEPES (pH 7.5), 2 mM $MgCl_2$, 1 mg BSA/ml and 250 µg mitochondrial protein/ml in a final volume of 2ml. The following additions were made as indicated: 1 µmole NADH, 2 µmoles ADP, 9 µmoles Na_2HPO_4 labelled with $Na_2H^{32}PO_4$, 0.08 µmole DNP, and 40 µg toxin.

Table 8. Respiration and Phosphorylation by Corn Mitochondria Preincubated in the Presence and Absence of Toxin

Reaction conditions and additions are given in Figure 2. ADP was present in all reaction mixtures; Pi was added as indicated. Malate-pyruvate was 20 µmoles each/2 ml reaction mixture. Preincubated mitochondria were held 1 hr at 24 C in 0.3 ml 0.38 M sucrose containing 750 µg mitochondrial protein with or without 6 µg toxin or inactive toxin. To measure respiration and phosphorylation, 0.2 ml of this mixture was added to reaction mixture (See Figure 2). RCR = respiratory control ratio.

Substrate	Treatment at 24 C	O_2 Uptake		RCR	P:O
		-Pi	+Pi		
		nmoles/mg protein/min			
NADH	None	127	280	2.21	1.63
	1 hr Control	143	170	1.19	0.96
	1 hr Toxin	204	214	1.05	1.46
	1 hr Inactive Toxin	164	218	1.33	1.50
Malate-Pyruvate	None	45	69	1.53	2.33
	1 hr Control	48	39	0.81	2.24
	1 hr Toxin	40	36	0.90	2.24
	1 hr Inactive Toxin	57	36	0.63	2.06

respiratory rates, RCR, and P:O ratios. Mitochondria incubated for 1 hr at 24 C had slightly higher nonphosphorylating respiratory rates than did mitochondria stored on ice, but the phosphorylating rates, RCR, and P:O ratios were below the values for mitochondria stored on ice. Preincubation of mitochondria for 1 hr in the presence of toxin or inactive toxin at 24 C caused slight stimulation of respiratory rates, but had essentially no effect on RCR and P:O. The P:O of the preincubated control was unusually low in this experiment, for unknown reasons. With malate-pyruvate as the substrate, respiratory rates of fresh mitochondria were relatively low but P:O ratios were comparable to those reported for similar experiments (76). Preincubation of mitochondria for 1 hr in the presence of toxin or inactive toxin at 24 C had no effect on mitochondrial functions.

The mitochondrial functions just described are dependent on the integrity of the mitochondrial membrane for normal operation (76, 126). No change in these functions after toxin treatment suggests that the mitochondrial membrane is immune to toxin. However, negative evidence is not conclusive and another parameter of membrane integrity was used. Light-scattering is a direct measure of volume changes in mitochondria (64); volume changes reflect water flow caused by solute fluxes across the membrane (82, 114). If toxin affects the mitochondrial membrane, it should affect the light-scattering properties of mitochondria.

Corn mitochondria swell spontaneously when placed in a buffered salt solution (76, 114). Toxin had no effect on this spontaneous swelling (Figure 3) but gramicidin D increased the swelling rate, as others have found (76). Addition of NADH caused swelling to stop and mitochondria contracted slightly, as they normally do (76). Toxin had no effect on stabilized mitochondria, but gramicidin D reversed the contracted state.

Experiments with toxin and isolated chloroplasts. -- Chloroplasts were used in experiments similar to those with mitochondria. Results (Figure 4) show that toxin had no effect on electron transport by phosphorylating or by nonphosphorylating chloroplasts. Ammonium sulfate completely uncoupled electron transport, indicating a normal response of chloroplasts to an uncoupling agent (52).

Toxin had no effect on light-scattering by chloroplasts in the presence or absence of actinic light (Figure 5, left). This indicates that toxin did not induce active or passive swelling of chloroplasts. Ammonium sulfate was included as a control to confirm that chloroplasts can be induced to swell in light and shrink in darkness (45). Toxin had no effect on light-scattering when added during the experiment in the absence of actinic light (Figure 5, right). This eliminates the possibility of an instantaneous effect of toxin on passive swelling by chloroplasts. Triton X-100, which affects chloroplast membranes in the dark (44), was included as a control to show induced passive swelling. Chloroplast functions and membrane integrity were not affected by toxin. At

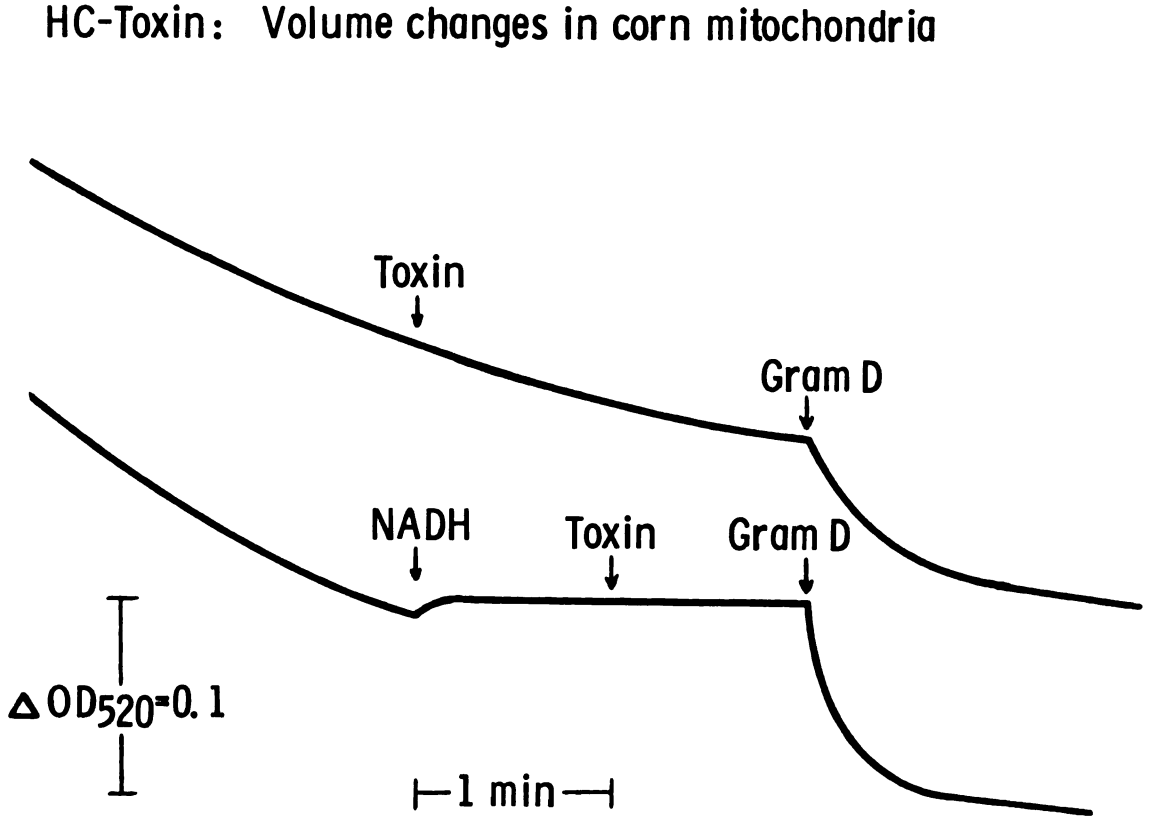


Figure 3. Volume changes in toxin-treated and control mitochondria. The reaction medium contained 0.2 M KCl, 0.02 M HEPES (pH 7.5), 2 mM $MgCl_2$, 1 mg BSA/ml, and 250 µg mitochondrial protein/ml in a total volume of 2 ml. Additions of 1 µmole NADH, 40 µg toxin, or 5 nmoles gramicidin D were made as indicated.

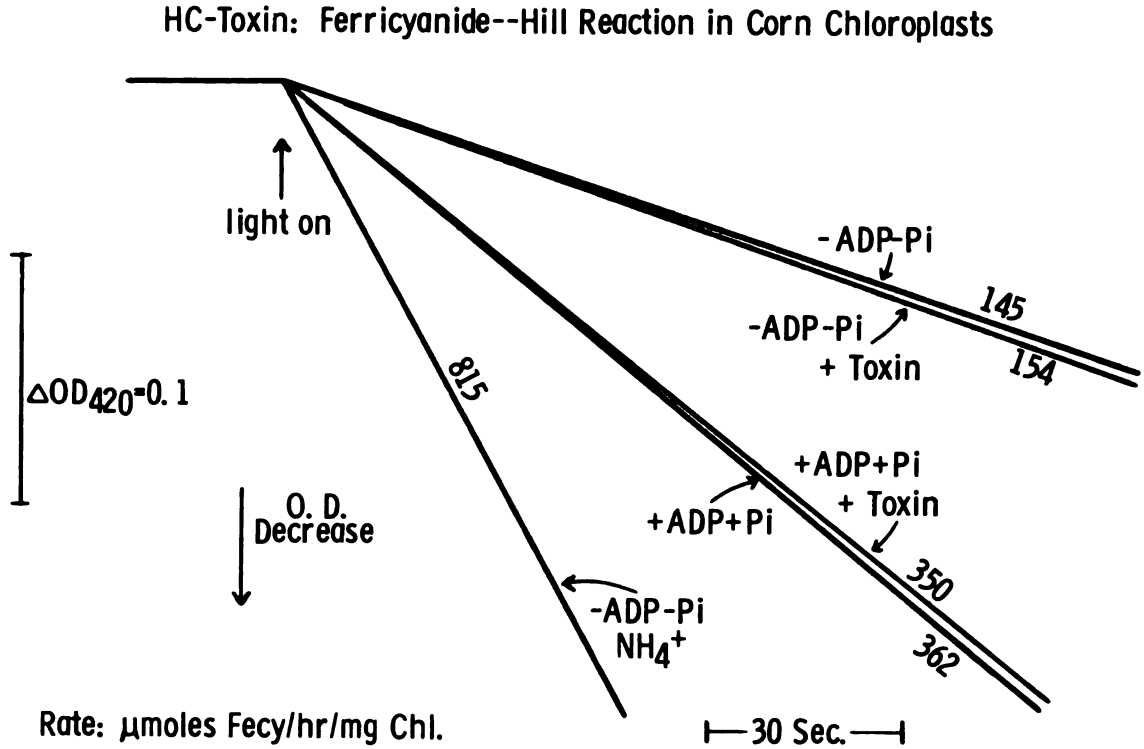


Figure 4. Electron transport and phosphorylation by corn chloroplasts in the presence and absence of toxin. Chloroplasts were suspended in a reaction mixture containing 0.1 M sucrose, 0.01 M KCl, 0.05 M Tricine-NaOH (pH 7.8), 0.001 M $MgCl_2$, 0.4 mM potassium ferricyanide, 20 μg chlorophyll/ml and the following components where indicated: 4 mM $(NH_4)_2SO_4$, 1 mM ADP, 4.5 mM Na_2HPO_4, or 20 μg toxin/ml.

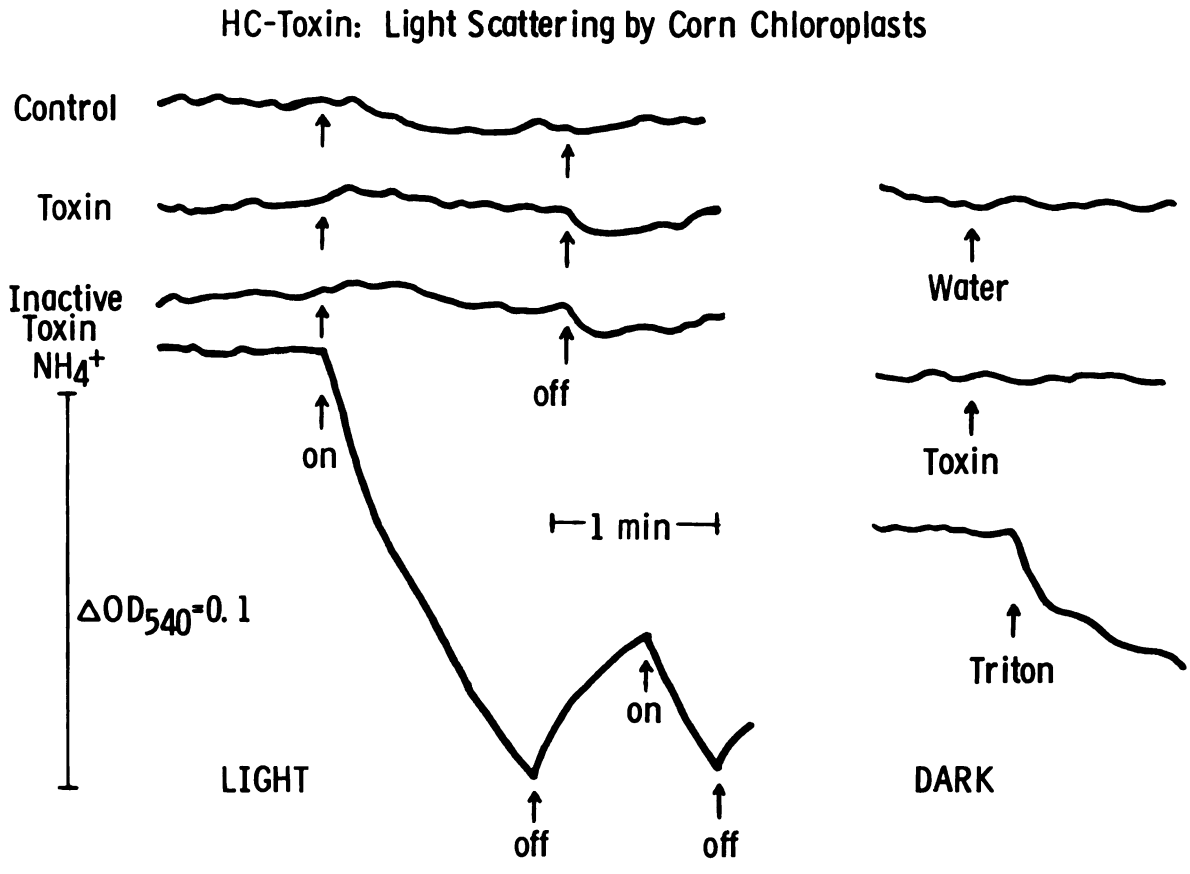


Figure 5. Light-scattering by corn chloroplasts in the presence and absence of toxin. Conditions for experiment on left were as described in Figure 4, except KCl was 0.1 M. Conditions for experiment on right were as those on the left except ferricyanide was omitted and 0.00025% Triton X-100 was added where indicated.

the end of each experiment with chloroplasts and mitochondria, toxicity of the toxin preparation was bioassayed. Full toxicity and host-specificity was retained in all cases.

Preliminary experiments by Samaddar (unpublished) indicated that susceptible oat chloroplasts were not affected by HV-toxin. I have used HV-toxin with chloroplasts from susceptible oats (cv. Park) in electron transport and light-scattering experiments. The procedure was the same as described for corn chloroplasts. HV-toxin, from a preparation which completely inhibited susceptible roots at 0.0016 μg/ml, was used at 1.6 and 0.16 μg/ml. Results showed no effect of HV-toxin on oat chloroplasts.

Effect of toxin on nitrate reductase activity. -- Enhanced metabolic activity could result from increased enzyme synthesis. Toxin is known to enhance incorporation of ^{14}C-amino acids into TCA insoluble materials of susceptible corn tissues (54). However, toxin had little or no stimulatory effect on GA-induced amylase activity in barley (a nonhost) or in susceptible corn. Another inducible enzyme was selected to further test the possibility that toxin affects protein synthesis in vivo. Nitrate reductase (NR) is induced by its substrate, is easily manipulated, and has been studied extensively in corn (3). Recent development of an in vivo assay (21) has greatly facilitated NR investigations. Seed tissues were used for the first series of experiments, because they are easy to handle under aseptic conditions. Microbial contamination can be an important source of error in enzyme studies.

Induction of NR in various seed tissues was tested in preliminary experiments. Tissues were allowed to imbibe 24, 48, or 72 hr; NR was then induced by transferring tissues to NO_3-containing solution. The results show that inducibility of intact seeds was low but increased with age whereas inducibility of embryos, scutella, and axes was high and decreased with age (Table 9). There was negligible NR induction in endosperms. Axes were selected for further studies because they are more active than the other tissues on a dry wt basis. More NR was induced in axes which were allowed to imbibe in nutrient solution than in those allowed to imbibe in water (Table 10). Induction of NR in axes was not affected by light, darkness, $(NH_4)_2SO_4$ (500 mg/l), or substitution of White's solution for modified Dure's solution.

NR induction was not affected in axes that imbibed toxin solution for 12 hr. Toxin exposures of 24-72 hr inhibited NR induction but did not eliminate it (Figure 6). In fact, NR activity in toxin-treated axes was higher at 72 hr than at 24 and 48 hr. The same pattern was evident when the data were plotted on a dry wt basis. Other experiments showed that there was less NR induction in embryonic shoots than in embryonic roots. Induction of NR in shoots was inhibited more by toxin than was NR induction in roots. The rate of NR induction was about the same whether embryonic roots were separated from embryonic shoots before or after imbibition.

The toxin exposure time required to affect NR induction was tested by including toxin in the induction medium. Axes were allowed

Table 9. Induction of Nitrate Reductase in Corn Seed Tissues

Seeds were surface sterilized and dissected aseptically. Five tissues/treatment were allowed to imbibe in 12 ml water containing 20 μg chloramphenicol/ml in 10 cm petri dishes. Enzyme was induced aseptically in 4 ml 0.05 M KNO_3 containing 20 μg chloramphenicol/ml in 50 ml flasks shaking at 160 oscillations/min for 7 hr.

Imbibition Time	Tissue	NO_2 Production
hr		nmoles/g dry wt/hr
24	Whole Seed	20
	Endosperm	2
	Embryo	1103
	Scutellum	957
	Axis	6559
48	Whole Seed	146
	Endosperm	4
	Embryo	485
	Scutellum	222
	Axis	2468
72	Whole Seed	261
	Endosperm	4
	Embryo	75
	Scutellum	201
	Axis	1043

Table 10. Effect of Nutrients on Capacity for Nitrate Reductase Induction in Corn Embryonic Axes

Axes (4/treatment) were allowed to imbibe aseptically in 5 cm petri dishes in 2 ml of water or modified Dure's nutrient solution. The induction medium corresponded to the imbibition medium and contained 0.05 M KNO_3. Induction time was 6 hr.

Imbibition Time	Nutrients	NO_2 Production
hr		µmoles/g dry wt/hr
24	-	4.9
	+	6.0
48	-	1.7
	+	10.2
72	-	2.0
	+	13.9

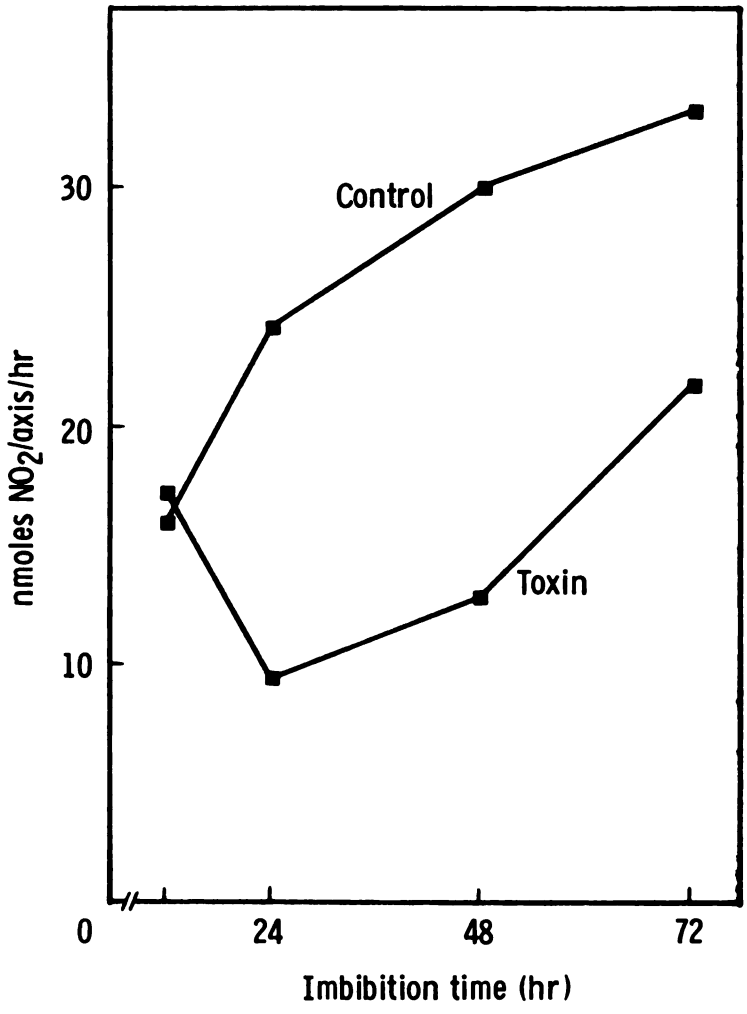


Figure 6. Effect of toxin on induction of NR in corn embryonic axes as affected by imbibition time. Axes imbibed aseptically in 2 ml modified Dure's solution with or without toxin (2 μg/ml) in 5 cm petri dishes (4 axes/dish). Enzyme was induced aseptically in 2 ml imbibition solution containing 0.05 M KNO_3 in 50 ml flasks incubated 8 hr on a shaker (200 oscillations/min).

to imbibe for 36 hr; NR was then induced by placing axes in NO_3-solution with or without toxin. At intervals NR activity in the axes was determined. The data (Figure 7) show no effect of toxin during the first 2 hr of induction. Between 2 and 8 hr of induction NR activity decreased in the control but continued to increase in toxin-treated axes. Thus, the earliest observable effect of toxin was stimulatory rather than inhibitory; it was evident 4 hr after exposure to toxin.

The effect of toxin or of NO_3 on NR induction in axes was examined by varying toxin and NO_3 concentrations in the induction medium. Results in Figure 8 show the effect of toxin concentration on NR induction. Toxin concentrations of 2 μg or more/ml caused maximum stimulation of NR induction. Considering variability between replicates, there was probably no real difference between 2, 20, and 200 μg toxin/ml. A NO_3 concentration of 0.05 M was optimum for both induction and toxin-stimulation of NR activity (Figure 9).

Toxin-stimulated NR induction suggested that toxin may enhance total protein synthesis. To test this possibility, the experiment just described was repeated and total protein in the axes was estimated. Toxin apparently had no significant effect on total protein content (Table 11). Since induced NR is less than 0.1% of the total protein (42), its contribution was negligible. A similar experiment with green corn leaf tissue gave similar results.

The effect of toxin on NR degradation in corn axes was tested. Axes imbibed for 36 hr; NR was induced for 2 hr in the presence or

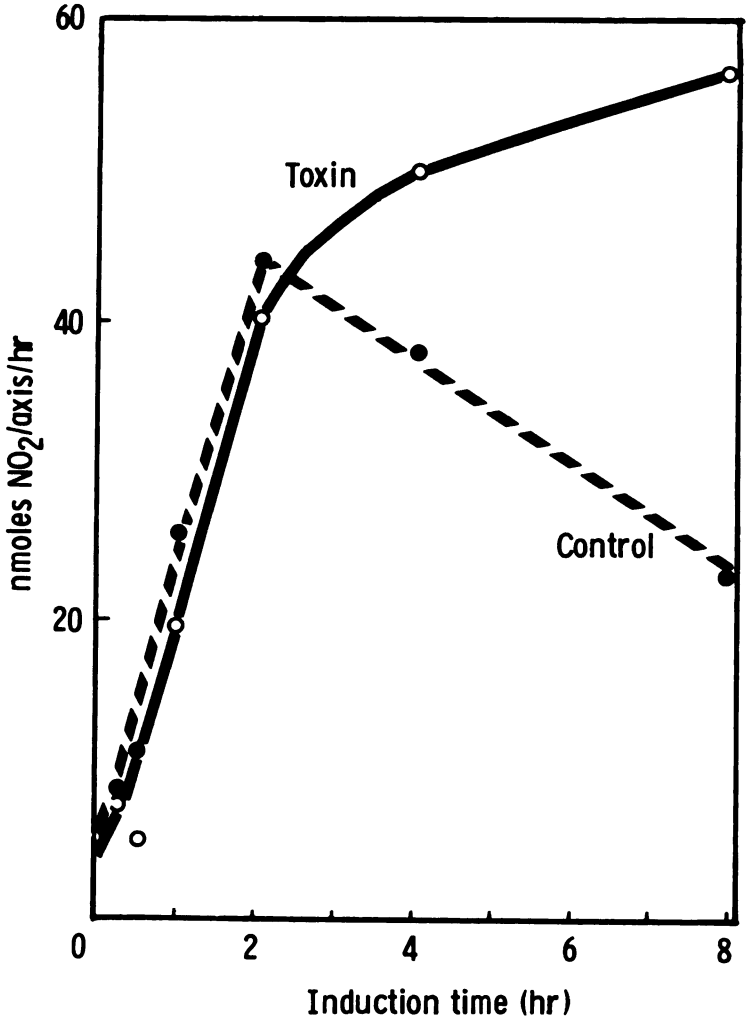


Figure 7. Effect of toxin on induction of NR in corn axes. Axes imbibed aseptically for 36 hr in 15 ml modified Dure's solution on filter paper in a 15 cm petri dish. Enzyme was induced aseptically in 10 ml imbibition solution which contained 0.05 M KNO_3 with or without toxin (20 µg/ml) in 300 ml flasks (3 axes/flask) on a shaker (200 oscillations/min).

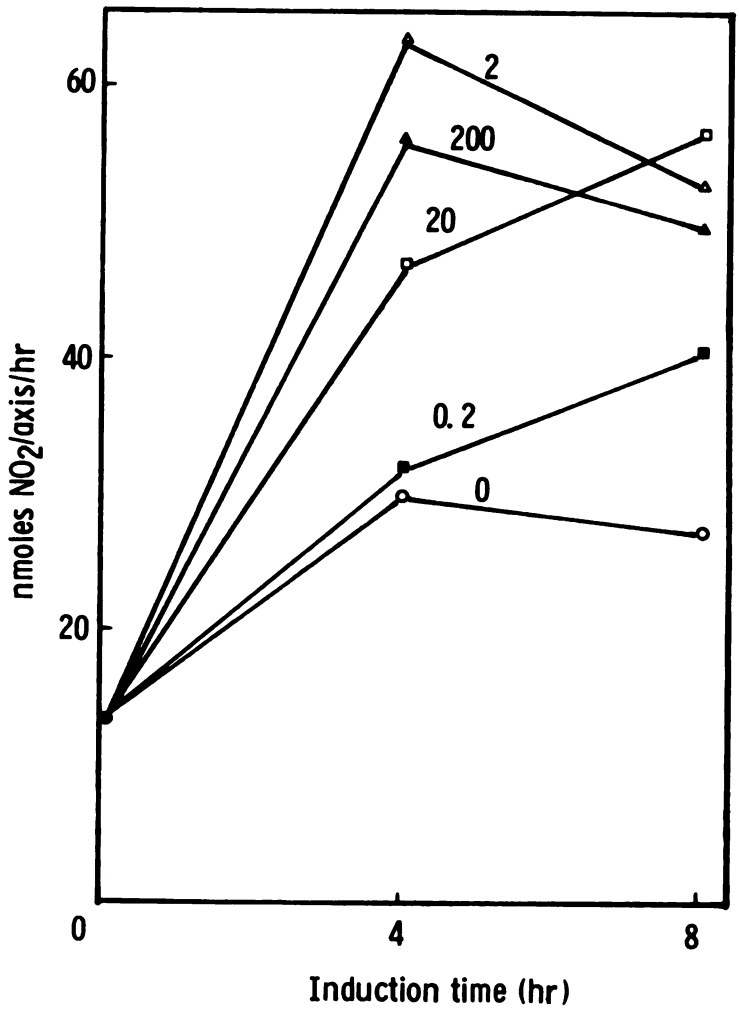


Figure 8. Effect of toxin concentrate on induction of NR in corn axes. Axes imbibed aseptically for 36 hr on filter paper wetted with 15 ml modified Dure's solution in a 15 cm petri dish. Enzyme was induced aseptically in 4 ml imbibition solution containing 0.05 M KNO_3 with or without toxin in 50 ml flasks (3 axes/flask) on a shaker (220 oscillations/min). Toxin concentrations (µg/ml) are indicated.

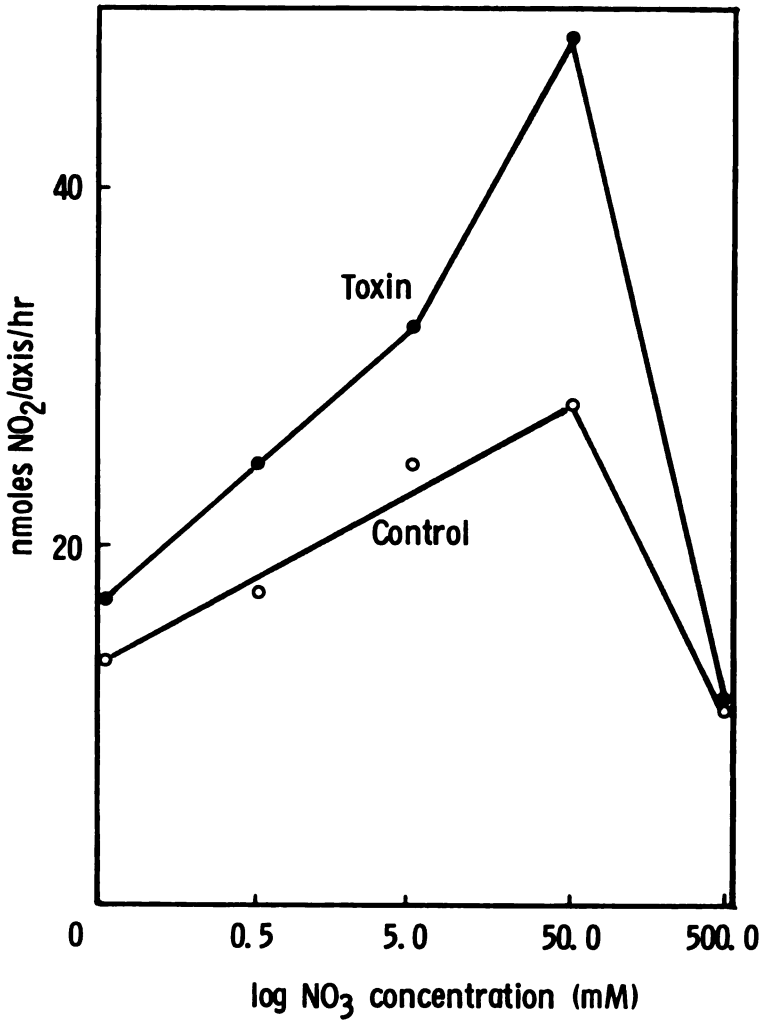


Figure 9. Effect of NO_3 concentration on toxin-stimulated induction of NR in corn axes. Axes imbibed aseptically for 36 hr on filter paper wetted with 15 ml modified Dure's solution in a 15 cm petri dish. Enzyme was induced aseptically in 6 ml imbibition solution containing 0.05 M KNO_3 with or without toxin (2 µg/ml) in 50 ml flasks. Induction was for 4 hr on a shaker (220 oscillations/min).

Table 11. Protein Content of Susceptible Corn Embryonic Axes After Induction of Nitrate Reductase in the Presence or Absence of Toxin

Axes (5/treatment) imbibed aseptically in 15 ml modified Dure's solution on filter paper in 15 cm petri dishes for 36 hr. For induction, axes were incubated in 10 ml imbibition solution + 0.05 M KNO_3 with or without toxin (20 µg/ml) in 300 ml flasks shaking at 200 oscillations/min. Axes were homogenized and protein was extracted and estimated by the methods of Filner (23). Ranges between 2 samples are indicated.

Induction Time	Protein Content	
	Control	Toxin
hr	µg/axis	µg/axis
0	203 ±54	...
4	233 ±17	251 ±1
8	238 ±12	265 ±5

absence of toxin. Then axes were placed in NO_3-less solution with or without toxin and loss of NR activity was observed. Results (Figure 10) show that about half the in vivo NR activity disappeared in both toxin-treated and control axes in 4 hr. NR is reported to have a half-life of 4 hr in corn tissues (107).

Root tips and green leaves were used in experiments similar to those with axes, except that nonsterile conditions were employed. Addition of toxin to NR induction medium of root tips gave results similar to those shown in Figure 7 for axes. Toxin had no effect on NR activity after 2 hr induction. Between 2 and 4 hr, NR activity decreased slightly in the control but continued to increase in toxin-treated tips. This resulted in 88% stimulation by toxin after 4 hr induction.

The effect of toxin on initial kinetics of NR induction in corn root tips was studied. Seedlings were first exposed to toxin 4 hr, then root tips were excised and placed in NR induction medium. Results (Figure 11) show an increase in NR activity of toxin-treated axes which was apparent from the beginning of the induction period. This difference between toxin-treated and control tissues persisted but did not change throughout the experiment. Similar parallel lines were obtained with axes after 4 hr toxin pretreatment.

Stimulation of NR activity by toxin at 0 induction time and the failure of toxin-treatment to increase the slope of the induction curve suggested that the effect of toxin was not on induction of NR. To further check this point, NR activity in root tips was estimated

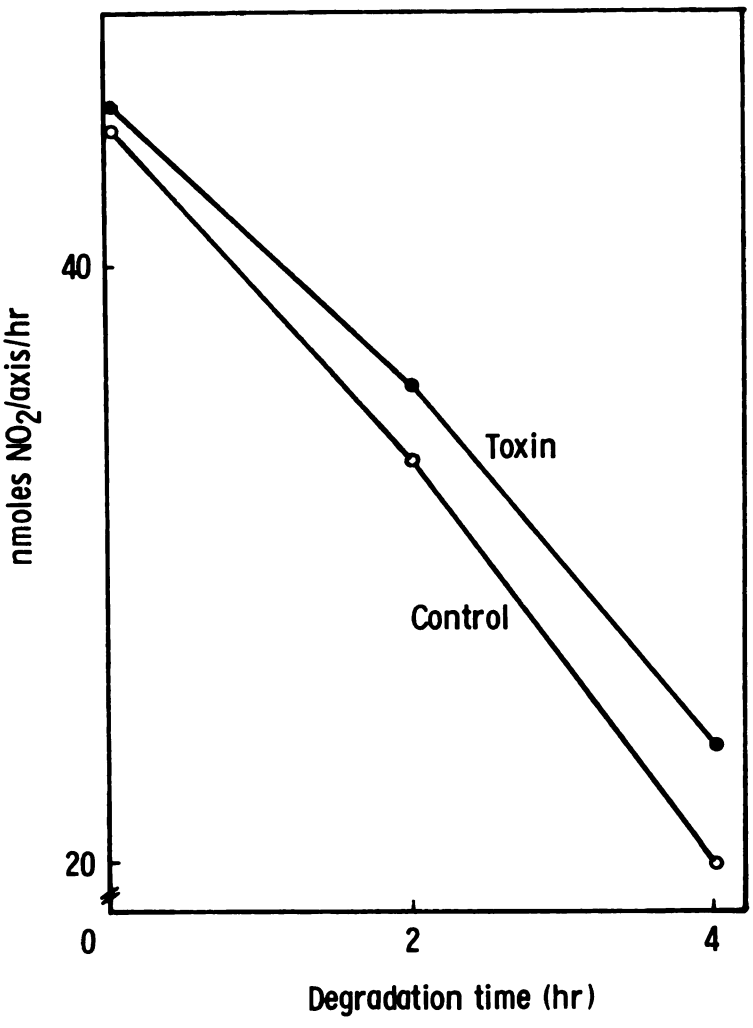


Figure 10. Effect of toxin on degradation of NR in vivo. Axes imbibed aseptically for 36 hr on filter paper wetted with 15 ml modified Dure's solution in a 15 cm petri dish. Enzyme was induced aseptically in 6 ml imbibition solution containing 0.05 M KNO_3 with or without toxin (2 µg/ml) in 50 ml flasks on a shaker (200 oscillations/min). After 2 hr induction, axes were rinsed in sterile water and transferred to sterile NO_3-less modified Dure's solution with or without toxin (2 µg/ml) in 50 ml flasks on a shaker (200 oscillations/min). At intervals (0, 2, and 4 hr), in vivo NR activity was determined.

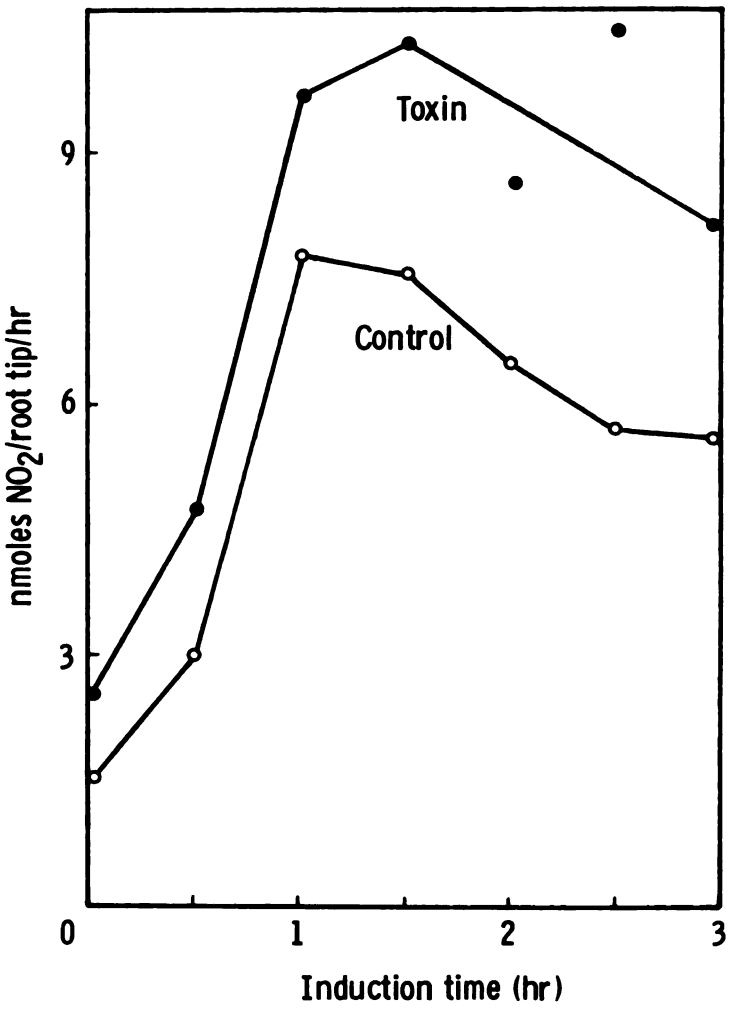


Figure 11. Effect of pretreatment with toxin on induction of NR in corn root tips. Seedlings were grown in petri dishes in NO_3-less modified White's solution. Seedlings were placed in 30 ml of the same solution with or without toxin (20 µg/ml) in 15 cm petri dishes for 4 hr, then NR was induced by placing seedlings in 30 ml White's solution containing 10 mM KNO_3 and 0.104 µM Na_2MoO_4. At intervals 5 mm root tips were excised and assayed for NR activity in 0.5 ml assay medium in 3 ml vials (5 tips/vial). Contents of vials were then boiled and NO_2 production was determined by adding color reagents. Fresh wt of a 5 mm root tip is about 3.5 mg.

simultaneously with both in vivo and in vitro assays. Results of the in vivo assay (Figure 12) showed the usual stimulatory effect of toxin. However, when NR was extracted from the tissue and an in vitro assay was performed, toxin-treatment appeared to have had no effect on, or perhaps inhibited, NR inducibility. Thus, toxin-enhanced NR activity in vivo did not appear to result from an effect of toxin on induction of NR.

Effect of toxin on NO_3 accumulation and compartmentation. -- The tissue extracts which were used for the in vitro assay of NR activity (Figure 12), were tested for NO_3 content. Toxin-treated root tips accumulated NO_3 at a faster rate over the 3 hr induction period than did control root tips (Figure 13). Results of another experiment showed that toxin-induced NO_3 accumulation was affected by temperature (Table 12).

The in vivo assay for NR activity was used in a further test of the effect of toxin on NO_3 uptake. This experiment was based on the fact that NO_3 must enter the cell to be reduced. If NR content is held constant, any increase in NO_3 reduction must be caused by increased NO_3 uptake. Noninduced toxin-treated and control leaf sections were placed directly into in vivo assay medium rather than into induction medium. Within 10 min, more NO_3 was reduced in toxin-treated than in control leaves, and the slope of the curve for toxin-treated leaves was steeper than that for the control (Figure 14). Thus, NO_3 entered toxin-treated cells at a faster rate than it entered control cells.

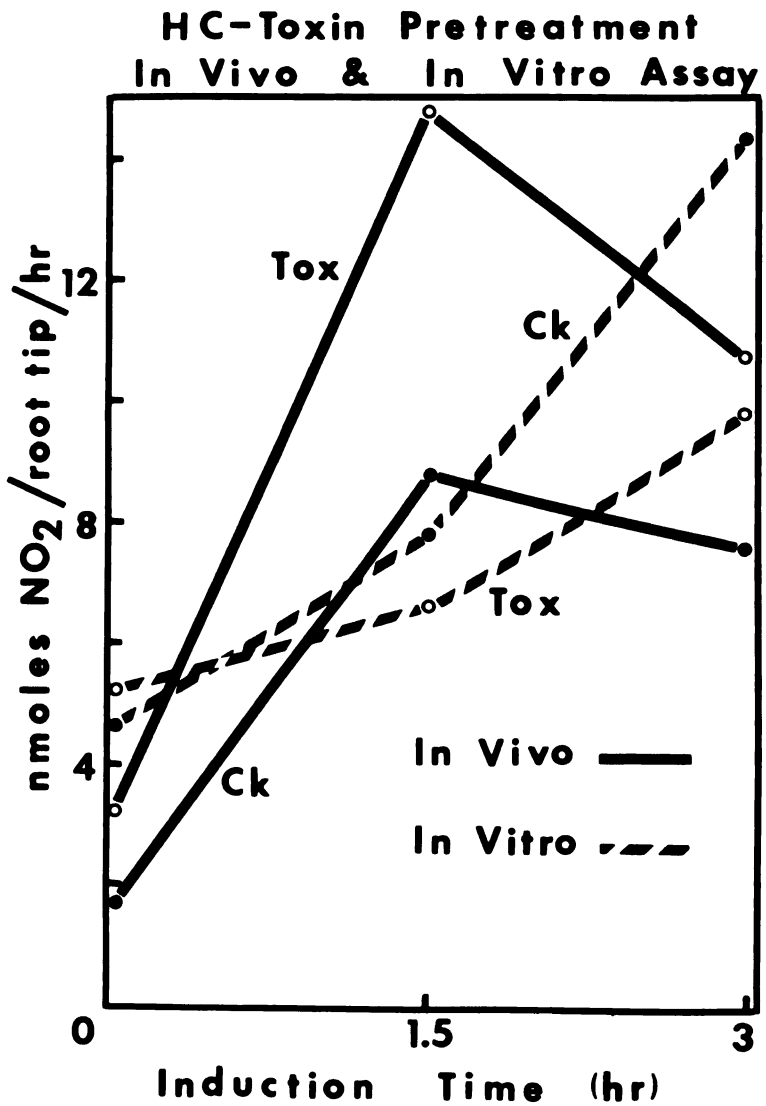


Figure 12. Effect of pretreatment with toxin on induction of NR as measured by in vivo and in vitro assays. Seedlings were grown in NO_3-less modified White's solution, then placed in 30 ml of the same solution with or without toxin (20 µg/ml) in 15 cm petri dishes for 4 hr. Enzyme was induced by placing seedlings in 30 ml White's solution containing 10 mM KNO_3 and 0.104 µM Na_2MoO_4, with or without toxin (20 µg/ml). At intervals, 5 mm root tips (10 tips/replicate) were excised and NR activity was determined by in vivo and in vitro methods.

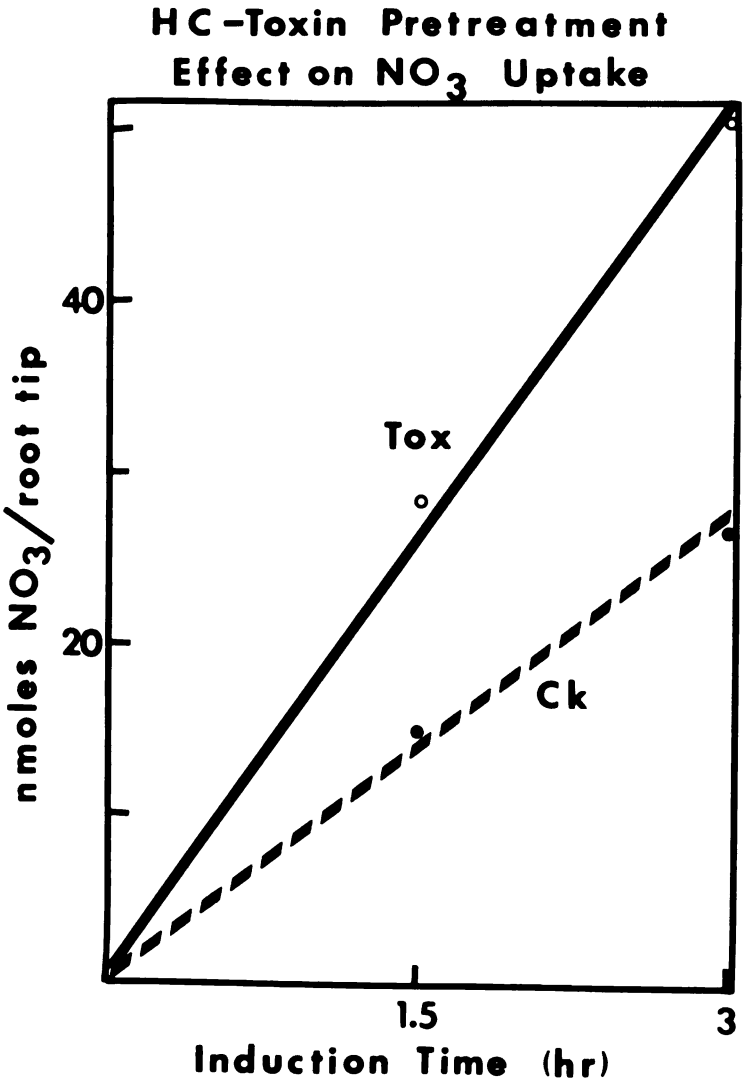


Figure 13. Effect of pretreatment with toxin on NO_3 accumulation by corn root tips. Supernatants from the in vitro assay described in Figure 12 were assayed for NO_3 content.

Table 12. Effect of Temperature on Toxin-Stimulated NO_3 Accumulation by Corn Root Tips

Seedlings, grown in 15 cm petri dishes in NO_3-less modified White's solution, were transferred to the same solution with or without toxin (20 μg/ml) in 15 cm petri dishes for 4 hr. They were then placed in White's solution containing 10 mM KNO_3 at 3 or 24 C. At intervals 5 mm root tips were excised, rinsed and boiled in 0.8 ml water (5 tips/replicate). The NO_3 content of the water was determined.

Time of Exposure to NO_3	NO_3 Accumulation				Q_{10}	
	3C		24 C			
	Control	Toxin	Control	Toxin	Control	Toxin
hr	nmoles/root tip		nmoles/root tip			
1	1.1	1.5	...	...	...	...
2	2.1	3.0	15.8	32.1	2.61	3.09
4	4.4	6.2	...	...	...	...

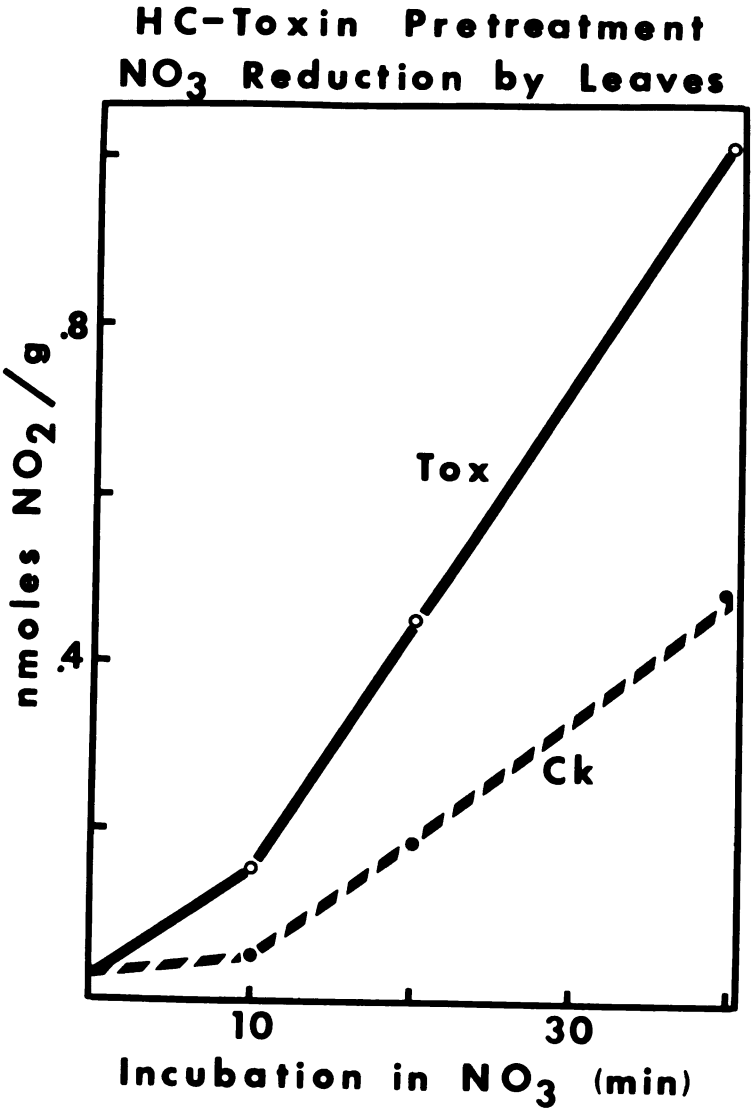


Figure 14. Effect of pretreatment with toxin on NO_3 uptake and reduction in the absence of NR induction. Seedlings were grown for 12 days in NO_3-less modified White's solution. Cuttings were placed in 15 ml water with or without toxin (20 µg/ml) in a 50 ml beaker under Gro-Lux lamps for 4 hr. Tertiary leaves were cut into 5 mm^2 sections, 100 mg was placed in 3 ml 0.1 M phosphate buffer (pH 7.5) + 0.1 M KNO_3 in a 50 ml flask. Nitrogen was flushed through the solution 1 min, flasks were stoppered and incubated in the dark at 30 C. At intervals contents of flasks were boiled and assayed for NO_2.

A recent report (22) indicates that NO_3 is compartmented in plant cells. A small portion of the cellular NO_3 is available for reduction by NR and is known as the metabolic pool; the rest of the cellular NO_3 is not available to NR and is called the storage pool. This compartmentation was demonstrated in corn leaves (Figure 15). Seedlings were grown in the presence of NO_3 so that tissues contained both NO_3 and NR. Leaf sections were placed under anaerobic conditions in the dark. This allows reduction of NO_3 to NO_2, but NO_2 accumulates and is excreted from the cells. Accumulation of NO_2 continues as long as NO_3 is available to NR. Production of NO_2 was linear for 2 hr (Figure 15). Production of NO_2 then stopped, which resulted in a plateau at approximately 2 μmoles NO_3/g tissue. The plateau indicated that all NO_3 available to NR was exhausted, although only 8% of the total cellular NO_3 (24 μmoles/g in this experiment) had been reduced. Reduction of NO_3 was resumed when exogenous NO_3 was added to the incubation medium. This shows that NO_3, not some other factor, was limiting NO_2 production.

The capacity of the metabolic pool in corn leaves is about 2 μmoles NO_3/g leaf tissue (22 and Figure 15). An attempt was made to determine the capacity of the metabolic pool in corn root tips. Results (Table 13) show that the pool in root tips contains about 0.03 μmole NO_3/g and is depleted so rapidly that it is almost nondetectable. Ethanol released NO_3 from the storage pool of root tips, as it does in corn leaves (unpublished), tobacco cells (22), and barley aleurone layers (21).

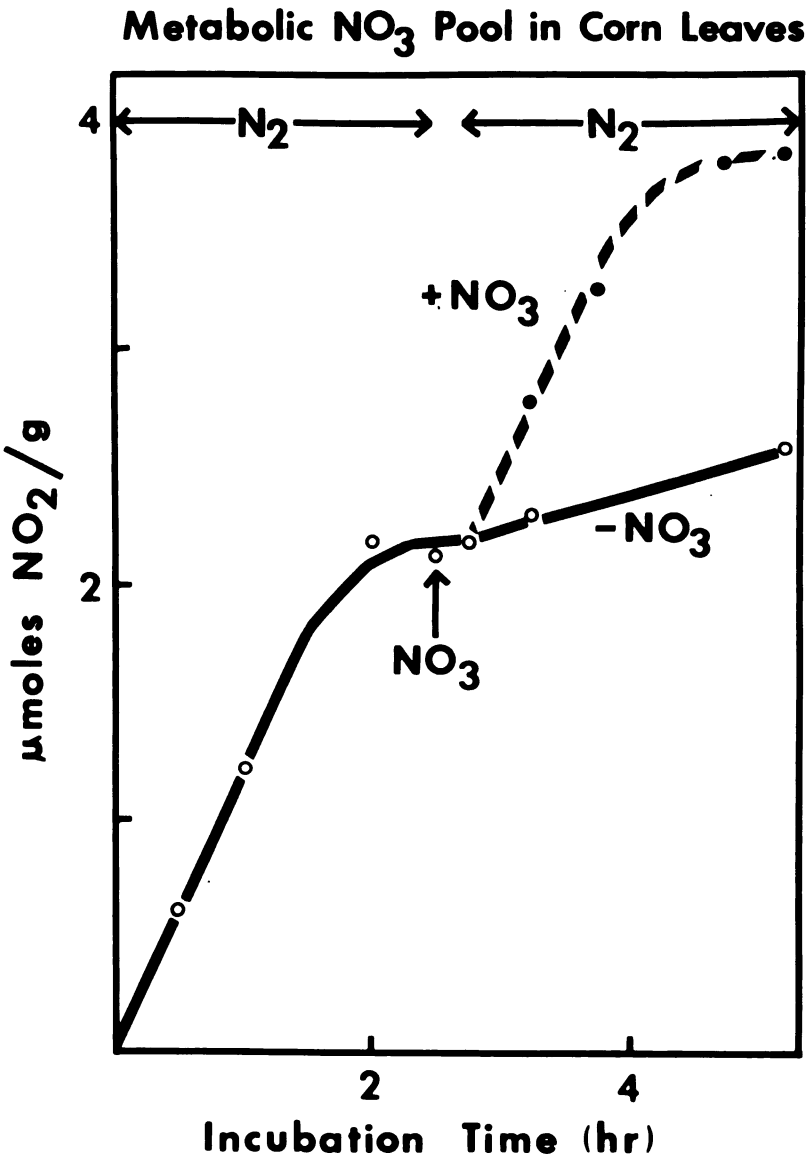


Figure 15. Evidence for a metabolic pool of NO_3 in corn leaves. Total NO_3 in tissue was 24 µmoles/g; the metabolic pool contained approximately 2 µmoles/g.

Table 13. The Metabolic NO_3 Pool in Corn Root Tips

Seedlings were grown in White's solution containing 20 mM KNO_3 + Fe and Mo for 60 hr. Root tips 5 mm long (10 tips/replicate) were placed under anaerobic conditions within 1-2 min after excision. Ethanol (5%) was included in the incubation medium where indicated.

Anaerobic Incubation Time	NO_2 Production
min	nmoles/g
10	27
20	36
40	27
60	27
30 + ethanol	1057

Similar results were obtained with 1 cm root sections taken 5 mm behind the root tip (unpublished).

Since toxin affected movement of NO_3 into cells, it seemed appropriate to determine if toxin affected movement of NO_3 within the cell, i.e., from storage to metabolic pool. Results of an experiment designed to test this possibility are shown in Figure 16. Leaf tissue containing NO_3 and NR was incubated anaerobically for 2 hr to exhaust the metabolic NO_3 pool. Buffer with or without toxin was added and leaves were incubated aerobically 4 hr to give toxin time to act. One group of leaves received exogenous NO_3 15 min before the end of the aerobic period. This was a control to determine if NR was still active 4 hr after metabolic pool NO_3 was exhausted. Leaves were then returned to anaerobic conditions and NO_2 production was monitored. The treatment which received exogenous NO_3 produced NO_2, indicating the presence of active NR. Control and toxin-treated leaves did not produce additional NO_2, indicating that toxin did not release NO_3 from the storage NO_3 pool.

Characteristics of toxin-stimulated NO_3 accumulation by roots. -- A toxin-induced change in the plasmalemma could lead to enhanced NO_3 uptake. This is the most plausible explanation considering that mitochondria, chloroplasts, and storage pools of NO_3 are not affected by toxin. The nature of the stimulatory effect was explored in further experiments with NO_3 accumulation. One experiment showed that NO_3 accumulation from a 10 mM solution was linear with time for at least

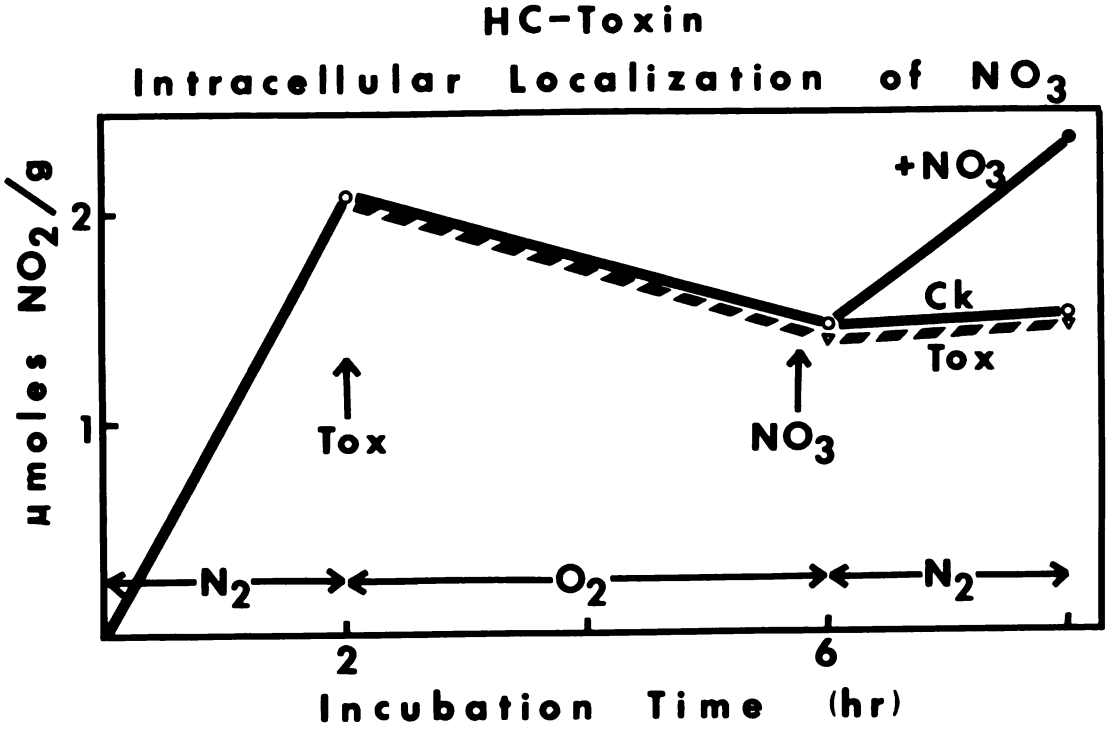


Figure 16. The metabolic pool of NO_3 in toxin-treated and control corn leaves. Ck = control without addition of exogenous NO_3; Tox = toxin-treated without addition of exogenous NO_3.

4 hr (Figure 17). The results of the experiment with corn root tips pretreated with toxin (Figure 13) suggest that toxin-stimulated NO_3 accumulation is also linear with time.

The condition of roots during the toxin-treatment period affected the rate of NO_3 accumulation and toxin-stimulated NO_3 accumulation (Table 14). Excised roots and roots of intact seedlings were exposed to solutions with or without toxin for 4 hr. This resulted in a 4 hr aging period for excised roots. Excised roots were aerated as they aged. Roots of intact seedlings were excised after the toxin-treatment period and can be considered fresh tissue. All roots were placed in NO_3 solution and the rates of NO_3 accumulation were determined. Aged control roots accumulated NO_3 twice as fast as fresh control roots (Table 14). Toxin-treatment caused a large stimulation of NO_3 accumulation by fresh roots but had only a small stimulatory effect on aged roots.

An additional treatment included excised roots which were not aerated, but floated on the surface of a solution during the toxin-treatment period. Such roots had a reduced rate of NO_3 accumulation, perhaps because of poor aeration. Toxin-treatment caused a marked increase in the rate of NO_3 accumulation by aged, nonaerated roots.

There was evidence that NO_3 accumulation is an active process. One such indication was the effect of temperature on NO_3 accumulation and toxin-stimulated NO_3 accumulation (Table 12). Accumulation of NO_3 against a concentration gradient by both toxin-treated and control

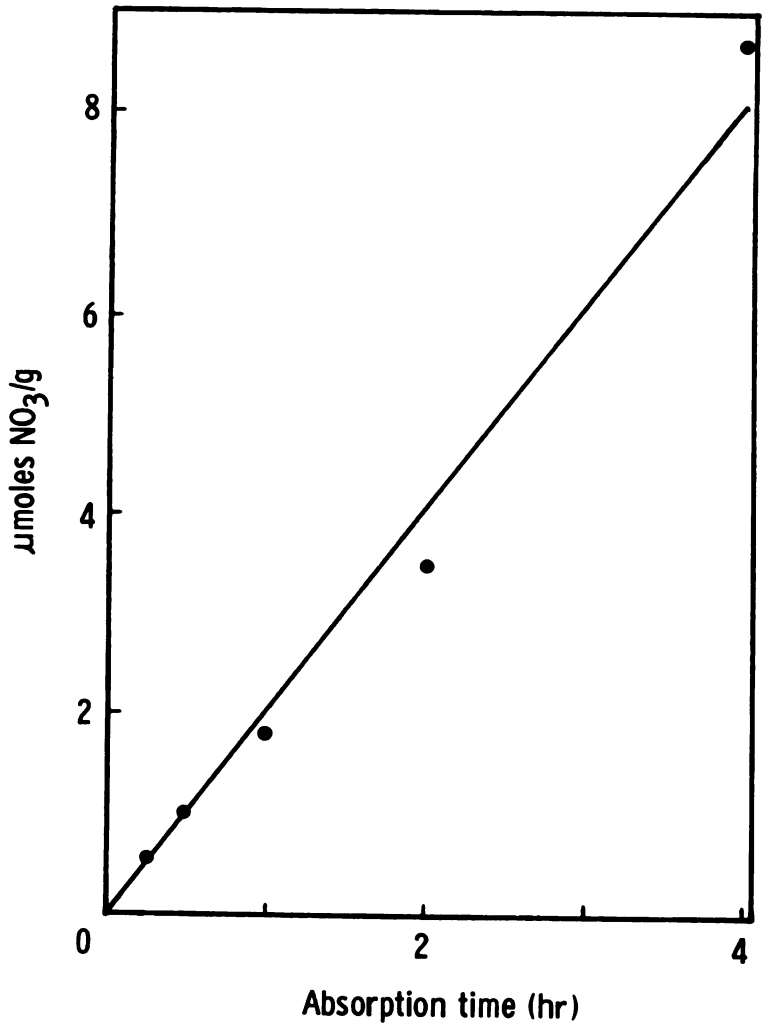


Figure 17. Accumulation of NO_3 by corn roots. Seedlings were grown over 4000 ml 0.2 mM $CaCl_2$. They were then placed in 50 ml 0.5 mM $CaCl_2$ + 10 mM KNO_3 in 15 cm petri dishes for the times indicated, and rinsed 3 times in 0.5 mM $CaCl_2$. Roots were excised, blotted, weighed (0.5 g/replicate), and NO_3 content was determined.

Table 14. Effect of Toxin on NO_3 Accumulation by Aged and Fresh Corn Roots

Fresh roots were prepared as follows. Intact seedlings were incubated 4 hr in 50 ml 0.5 mM $CaCl_2$ with or without toxin (10 µg/ml) in 15 cm petri dishes. Roots were excised after incubation. For aged tissues, roots were excised and incubated 4 hr in 50 ml 0.5 mM $CaCl_2$ with or without toxin (10 µg/ml) under one of the following conditions: a) aerated in a 100 ml beaker; b) shaken at 150 rpm in a 300 ml flask; or c) floated in a 15 cm petri dish. Fresh and aged roots were allowed to absorb NO_3 from solutions containing 0.5 mM $CaCl_2$ and 0.4 mM KNO_3.

Condition of Tissue	Condition for Aging	NO_3 Accumulation		Toxin-Induced Stimulation
		Control	Toxin	
		nmoles/g/hr		%
Fresh	...	265	383	45
Aged	Aerated	510	623	22
	Shaken	575	642	12
	Floated	172	401	133

roots is another indication of an active process. Toxin-treated roots exposed to a 0.4 mM NO_3 solution for 50 min acquired an internal NO_3 concentration of 1.12 mM. Control roots did not establish a concentration gradient under these conditions. However, control roots exposed to a 0.04 mM NO_3 solution for 45 min accumulated an internal concentration of 0.068 mM. The rate of NO_3 accumulation by control roots was less than that by toxin-treated roots. This explains the failure of control roots to establish a concentration gradient in 0,4 mM NO_3 during a brief exposure. The rate of NO_3 accumulation by control roots (ca 300 nmoles/g/hr) in 0.4 mM NO_3 indicates that they begin to accumulate NO_3 against a concentration gradient within 1-2 hr. Accumulation of NO_3 is linear for at least 4 hr (Figure 17).

There were several experiments to test the effect of other ions (K, Ca, Cl, Na, SO_4) in the experimental solution on NO_3 accumulation. Seedlings were exposed to solutions with or without toxin for 4 hr. Roots were excised and placed in NO_3-containing solutions in the presence of varying concentrations of other ions. The data (Table 15) suggest the following. 1) There was considerable variation in the rates of NO_3 accumulation by toxin-treated and control roots in different experiments and also between similar treatments in the same experiment. 2) These experiments, and experiments to be described later, indicated that Ca is required for the maximum rate of NO_3 accumulation but not for toxin-stimulation of NO_3 accumulation (See Table 17). 3) Presence or absence of other ions or combinations of ions had no

Table 15. Effect of Exogenous Ion Content and Concentration on Toxin-Stimulated NO_3 Accumulation by Corn Roots

Seedlings were exposed to 0.5 mM $CaCl_2$ with and without toxin (10 µg/ml) for 4 hr. Roots were excised and placed in experimental solutions for 30-45 min.

Expt. No.	Ion Concentrations				NO_3 Accumulation		Stimulation by Toxin
	K	Ca	NO_3	Cl	Control	Toxin	
	mM	mM	mM	mM	nmoles/g/hr		%
1	0.4	0.5	0.4	1.0	218	470	115
	...	0.5	1.0	...	338	600	78
	0.4	0.5[a]	0.4	...	232	495	114
	...	0.7	0.4	1.0	286	423	48
	0.4	0.05	0.4	0.1	148	240	62
2	0.04	0.4	0.04	0.8	90	110	22
	0.4	0.4	0.4	0.8	316	437	39
	4.0	0.4	4.0	0.8	930	1160	25
	0.4	...	0.4	...	181	288	59
	...	0.4	0.4	0.4	320	455	42
	...	...	0.4[b]	...	171	238	39
3	0.4	0.5	0.4	1.0	249	550	120
	10.0	...	10.0	...	1488	1780	20
	9.9	0.05	10.0	...	1760	2000	14
	9.0	0.5	10.0	...	1660	2040	23
	...	5.0	10.0	...	1520	1975	30
	...	0.5	0.4	0.6	276	404	46

[a] Ca was added as SO_4 salt.

[b] NO_3 was added as Na salt.

consistent effect on the rate of NO_3 accumulation or on toxin-stimulation of NO_3 accumulation. 4) The percent stimulation by toxin may have been less at high than at low NO_3 concentrations.

Variability in NO_3 accumulation between identical experiments run simultaneously and between replicates of treatments within experiments was tested. Maximum variability between replicates and between experiments was 14 and 13%, respectively (Table 16). Root tips 3 cm long accumulated NO_3 faster, on a fresh wt basis, than did whole roots (Table 16). In subsequent experiments, 5-6 cm root tips were used as a compromise between efficient use of tissue and use of the most active portion of the root.

Several experimental conditions were evaluated for their effect on NO_3 accumulation and toxin-stimulated NO_3 accumulation. Seedlings were exposed to solutions with or without toxin for 4 hr. Conditions for the NO_3-absorption period were varied as follows. 1) Toxin-treated and control roots were excised and placed in identical experimental solutions in separate flasks. 2) Toxin-treated and control intact seedlings were placed in identical experimental solutions in separate flasks. 3) Toxin-treated and control roots were excised and placed in the same experimental solution, buffered at pH 5.8 with MES. 4) Toxin-treated and control roots were excised and placed in the same experimental solution containing tris as the counterion for NO_3. Results (Table 17) indicate the following. 1) The same results were obtained whether toxin-treated and control roots were in the same or

Table 16. Variation in Rates of NO_3 Accumulation by Corn Roots Under Standard Experimental Conditions

Seedlings were placed in 0.5 mM $CaCl_2$ with or without toxin (10 µg/ml) for 4 hr. Whole roots or 3 cm root tips were excised and placed in flasks containing 0.5 mM $CaCl_2$ and 0.4 mM KNO_3. Each flask contained 2 toxin-treated and 2 control bags of roots.

Tissue	Flask No.	NO_3 Accumulation						Stimulation by Toxin
		Control	Range		Toxin	Range		
			Replicates	Flasks		Replicates	Flasks	
		nmoles/g/hr			nmoles/g/hr			%
Whole Roots	1	248	± 1	±13	468	± 35	±39	89
	2	264	±15		530	± 2		100
Root Tips	1	283	±40	±39	915	± 5	±97	224
	2	336	± 2		810	±115		141

Table 17. Effect of Experimental Conditions on Toxin-Stimulation of NO_3 Accumulation by Corn Roots

Seedlings were placed in 0.5 mM $CaCl_2$ with or without toxin (10 μg/ml) for 4 hr. Roots were exposed to NO_3 solutions under the following conditions. A) Toxin-treated and control roots were excised and placed in separate flasks. B) Toxin-treated and control intact seedlings were placed in separate flasks. C) Toxin-treated and control roots were excised and placed in the same flasks, which contained solution buffered at pH 5.8 with 0.2 mM MES. D) Toxin-treated and control roots were excised and placed in the same flask, containing 0.4 mM HNO_3 adjusted to pH 6.0 with tris. Tissues for flasks A, B, and C were rinsed in 0.5 mM $CaCl_2$ and exposed to experimental solutions containing 0.5 mM $CaCl_2$ and 0.4 mM KNO_3. Roots for flask D were rinsed well with water before exposure to the experimental solution.

Conditions for NO_3 Absorption	Expt. No.	NO_3 Accumulation		Stimulation by Toxin
		Control	Toxin	
		nmoles/g/hr		%
A. Toxin-treated and control excised roots in separate flasks.	1	286	785	175
	2	398	815	105
B. Toxin-treated and control intact seedlings in separate flasks.	1	559	1570	181
	2	504	1083	116
C. Toxin-treated and control excised roots in the same flask, solution was buffered.	1	382	570	50
	2	270	567	110
	3	282	645	128
D. Toxin-treated and control excised roots in the same flask containing tris-NO_3.	1	160	392	146

different flasks. 2) Roots of intact seedlings accumulated NO_3 at a faster rate than did freshly excised roots. Toxin-treatment caused the same percentage stimulation of NO_3 accumulation rates in excised roots and intact seedling roots. Entry of NO_3 through cut ends of toxin-treated and control roots were the same in buffered and non-buffered solutions. 4) Accumulation of NO_3 occurred in the absence of other mineral ions, i.e., when tris was the counterion. The accumulation rate was low, probably because Ca was absent. Toxin-treatment caused the same percentage stimulation of accumulation that would be expected from a conventional NO_3 solution.

A change in membrane characteristics resulting in either increased influx or decreased efflux could result in an increase in NO_3 accumulation. To test these possibilities, the rate of NO_3 loss from roots was determined. When a 30 min absorption period was followed by a 30 min desorption period there was little or no detectable loss of NO_3 from roots (Table 18). This indicates that efflux of NO_3 is minimal in short term experiments with roots. In all 3 experiments (Table 18) toxin-treated roots lost about the same proportion of NO_3 during desorption as did control roots.

The absorption rates of many ions from a range of ion concentrations provide evidence for dual mechanisms of uptake (17, 59). The possibility that corn roots absorb NO_3 by more than one mechanism was investigated. After the series of NO_3 experiments was concluded, NO_3 accumulation rates were collected from each different NO_3

Table 18. Loss of NO_3 From Toxin-Treated and Control Corn Roots During Desorption

Seedlings were placed in 0.5 mM $CaCl_2$ with or without toxin (10 µg/ml) for 4 hr. Roots were excised and exposed to solutions containing 0.5 mM $CaCl_2$ and 0.4 mM KNO_3. Roots were desorbed in 3 l aerated 0.5 mM $CaCl_2$. One min desorption is the time required to remove NO_3 from free space with 6 rinses of 0.5 mM $CaCl_2$.

Expt. No.	Desorption Time	NO_3 Content				Toxin-Stimulation of NO_3 Content
		Control	Δ NO_3 Content	Toxin	Δ NO_3 Content	
	min	nmoles/g	%	nmoles/g	%	%
1	1	119 ± 2	...	144 ± 21	...	21
	30	112 ± 5	-6	156 ± 24	+8	39
2	1	128 ± 4	...	249 ± 15	...	95
	30	90 ± 13	-29	198 ± 9	-21	120
3	1	179 ± 14	...	407 ± 7	...	127
	30	212 ± 6	+18	398 ± 25	-2	88
	60	211 ± 11	+17	343 ± 41	-15	62

concentration in each NO_3 experiment. These data were plotted on a linear scale and are shown in Figure 18. Surprisingly, data from a diverse group of experiments, done over a period of several months, show evidence of dual mechanisms of NO_3 uptake. The pattern is qualitatively similar to that described for other ions (93). Mechanism 1 has a high affinity for NO_3 and operates at concentrations up to about 1 mM, with logarithmic increases in accumulation rates with increased NO_3 concentration. Mechanism 2 has a low affinity for NO_3 and operates at concentrations above 1 mM, with linear increases in accumulation rates with increased NO_3 concentration. Toxin-treatment appears to increase the maximum velocity (Vm) and decrease the Km of mechanism 1. Little can be said about mechanism 2 except that toxin appears to increase the slope of the curve, which suggests greater affinity of a carrier for NO_3. The data in Figure 18 were analyzed with double reciprocal and Woolf-Hofstee (10) plots. Results supported the suggestion that NO_3 is absorbed by 2 mechanisms. These plots also supported the possibility, mentioned above, that toxin-treatment increased Vm and decreased Km. However, points were too few and too scattered for firm conclusions. There is evidence that mechanism 2 is sensitive to temperature before and after toxin-treatment (Table 12). The data given in Figure 18 must be confirmed by experiments designed specifically to test for dual uptake mechanisms.

Effect of toxin on uptake of selected cations. -- The implications from experiments with NO_3 were that toxin causes changes in the

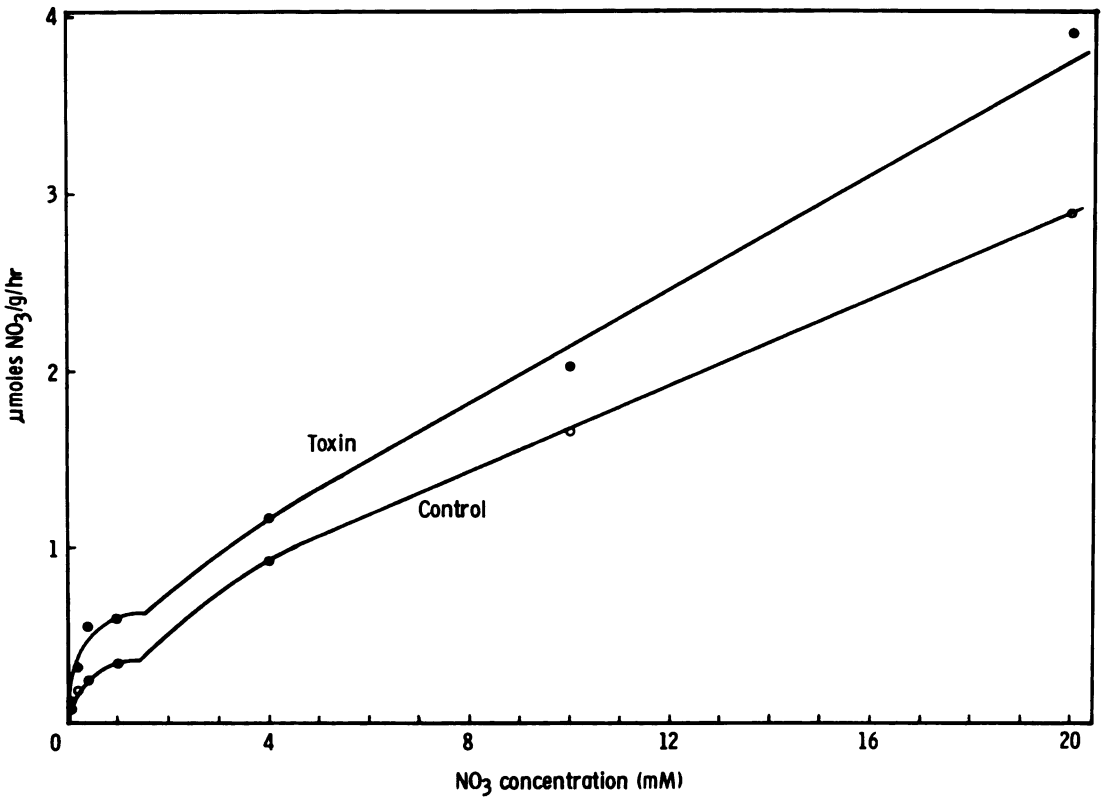


Figure 18. Rates of NO_3 accumulation in toxin-treated and control corn roots as a function of NO_3 concentration. Rates shown are for 7 NO_3 concentrations collected from 5 different experiments over a period of several months. The stimulation percentages are: 0.04 mM, 22%; 0.2 mM, 125%; 0.4 mM, 120%; 1.0 mM, 78%; 4.0 mM, 25%; 10.0 mM, 23%; 20.0 mM, 35%.

plasma membrane which increase the efficiency of NO_3 uptake. A survey of selected solutes was made to determine if stimulation of uptake is a general phenomenon.

Potassium (K) or rubidium (Rb) is actively accumulated in corn roots by at least 2 mechanisms (117). Mechanism 1 has a high affinity for K and operates at K concentrations <0.5 mM. Mechanism 2 has a low affinity for K and operates when K concentration is between 1 and 50 mM.

The effect of toxin on both mechanisms was tested using ^{86}Rb as a label to estimate K absorption. Some workers have questioned the validity of this technique (69), but recent results with corn roots show that when proper conditions are used, ^{86}Rb gives only a slight overestimate of actual K absorption (60). I used conditions required for a valid estimate, and based my experiments on those of other workers (15, 60). Seedlings were exposed to solutions with or without toxin for 4 hr. Roots were excised and placed in solutions containing K. Results (Table 19, experiment 1) show that toxin-treatment did not increase the rate of K absorption at any K concentration used. Exposure to toxin for 0.5 to 8.0 hr did not stimulate the K absorption rate (Table 19, experiment 2). The decrease in uptake rate of control roots after 0.5 hr could be caused by lack of aeration of roots during the toxin-treatment period. In experiment 3 (Table 19), K solutions were prepared with the NO_3 rather than the Cl salt and both K and NO_3 accumulation rates were measured simultaneously. Toxin-treatment enhanced

Table 19. Absorption of K by Toxin-Treated and Control Corn Roots

Seedlings were placed in 0.5 mM $CaCl_2$ with or without toxin (10 μg/ml) for 4 hr. Roots were excised and exposed to the following experimental solutions: experiment 1, KCl and 0.5 mM $CaCl_2$; experiment 2, KCl and 0.2 mM $CaCl_2$; experiment 3, KNO_3 and 0.5 mM $CaCl_2$. Uptake of both K and NO_3 was determined in experiment 3. Solutions were labelled with 0.01 μc ^{86}Rb/μmole K.

Expt. No.	Toxin-Treatment Time	K Concn.	K or NO_3 Accumulation		Toxin-Induced Change
			Control	Toxin	
	hr	mM	nmoles/g/hr		%
1	4.0	0.02	556 ± 122	384 ± 34	- 31
	4.0	0.2	816 ± 36	648 ± 103	- 21
	4.0	20.0	4760 ± 95	4832 ± 260	+ 1
2	0.5	0.02	428 ± 41	416 ± 27	- 3
	2.0	0.02	270 ± 42	276 ± 76	- 2
	4.0	0.02	310 ± 3	310 ± 27	0
	8.0	0.02	305 ± 31	231 ± 61	- 24
3	4.0	0.2	1700 ± 1	1140 ± 185	- 33
	4.0	0.2[a]	1220 ± 270	905 ± 145	- 26
	4.0	0.2[b]	131 ± 14	149 ± 1	+ 14
	4.0	0.2[c]	192 ± 19	433 ± 73	+125

[a]Roots were desorbed 30 min in 1500 ml aerated solution containing 0.5 mM $CaCl_2$ and 5 mM RbCl.

[b]Experimental solution and rinsing solution were held at 5 C.

[c]Data from this treatment are for NO_3 uptake.

the accumulation of NO_3 but not that of K. Desorption caused 28% and 21% loss of K from treated and control roots, respectively, indicating that toxin did not affect K efflux.

Sensitivity of K absorption to low temperature (experiment 3, Table 19) is characteristic of active accumulation (61). Further evidence for active uptake is movement against a concentration gradient (70). After 30 min absorption in experiment 1 (Table 19), control roots in 0.02, 0.2, and 20 mM K solutions had internal K concentrations of 0.278, 0.408, and 2.38 mM, respectively. Ion movement against a gradient occurred in 0.02 and 0.2 mM solutions but not in the 20 mM solution. More than 30 min may be required for roots to reach an internal K concentration of 20 mM.

Sodium (Na) is not known to be an essential element in higher plants, but is thought to be actively absorbed (32, 34) by both K uptake mechanisms (93). However, Na uptake by mechanism 1 is almost eliminated in the presence of K and Ca. Na is preferred over K by mechanism 2 (93). This information was interpreted to mean that a Ca-stabilized plasma membrane in the presence of physiological concentrations of Na and K will discriminate against the nonessential element Na. Mechanism 2, which operates at abnormally high concentrations, transports Na more efficiently than K.

The effect of toxin on Na uptake by both mechanisms was tested using modifications of previously described procedures (32, 34). Seedlings were exposed to solutions with or without toxin for 4 hr, then

placed in solutions containing Na. The results of 3 experiments (Table 20) show that toxin-treatment caused an increase in the rate of Na absorption by both absorption mechanisms in the presence and absence of Ca. In each experiment, some of the experimental solutions contained NO_3. Toxin-treatment stimulated NO_3 accumulation to a greater degree than Na absorption. A 30 min desorption period indicated that toxin-treatment did not cause leakage of Na from roots.

Experiments with plant roots have shown that Na uptake is inhibited by the presence of Ca (32, 93). My data show the same thing; mechanism 1 operating alone was more sensitive (about 80% inhibition) than both mechanisms operating together (about 40% inhibition). Corn roots absorbed Na equally well from NO_3 and chloride (Cl) solutions (experiments 2 and 3, Table 20).

Results of individual experiments with Na and K indicated that toxin-treatment distinguished between these 2 ions. To re-evaluate this, accumulation rates of Na, K, and NO_3 from the same solution under identical conditions were measured. Toxin-treated roots selectively accumulated Na and NO_3 at increased rates with no change in the rate of K absorption (Table 21). This phenomenon was observed for both uptake mechanisms.

Plant cells require Ca to maintain membrane integrity and absorption mechanisms (16, 60, 93). Actual uptake of Ca occurs slowly in most plants and uptake is mostly nonmetabolic (68). However, corn roots absorb Ca at rates similar to those of actively absorbed ions.

Table 20. Effect of Toxin on Absorption of Na by Corn Roots

Seedlings were exposed to 0.5 mM $CaCl_2$ with or without toxin (10 µg/ml) for 4 hr. Roots were excised and placed in experimental solutions. All rinsing was with water. Solutions were labelled with 0.03-0.1 µc Na/ml.

Expt. No.	Test Ion	Ion Concentrations				Na or NO_3 Accumulation		Toxin-Induced Stimulation
		Na	Ca	Cl	NO_3	Control	Toxin	
		mM	mM	mM	mM	nmoles/g/hr		%
1	Na	20.0	...	20.0	...	2920 ± 220	4360 ± 210	49
	Na	0.2	...	0.2	...	371 ± 26	390 ± 4	5
	Na	0.2	0.5	1.2	...	55 ± 6	94 ± 20	71
	Na	0.2	0.5	0.8	0.4	66 ± 3	87 ± 17	32
	NO_3	0.2	0.5	0.8	0.4	368 ± 12	720 ± 105	96
2	Na	20.0	...	20.0	...	3020 ± 380	3480 ± 120	15
	Na	20.0	0.5	21.0	...	1422 ± 62	2320 ± 30	63
	Na	0.2	...	0.2	...	335 ± 22	449 ± 42	34
	Na	0.2	0.5	1.2	...	68 ± 4	102 ± 13	50
	NO_3	0.2	0.5	0.8	0.4	225 ± 8	520 ± 10	131
3	Na	20.0	...	...	20.0	3200 ± 140	4290 ± 270	34
	Na	20.0	0.5	1.0	20.0	2520 ± 130	2910 ± 140	15
	Na	20.0	0.5	21.0	...	1900 ± 330	2800 ± 40	42
	Na	0.2	...	...	0.2	316 ± 2	662 ± 40	97
	Na	0.2	0.5	1.0	0.2	90 ± 7	137 ± 31	52
	Na	0.2	0.5	1.2	...	113 ± 35	143 ± 2	27
	NO_3	0.2	0.5	1.0	0.2	388 ± 38	655 ± 27	69
	NO_3	0.2	...	...	0.2	158 ...	265 ± 5	68

Table 21. Effect of Toxin on Simultaneous Accumulation of Na, K, and NO_3 by Corn Roots

Seedlings were exposed to 0.5 mM $CaCl_2$ with or without toxin (10 µg/ml) for 4 hr. Roots were excised and placed in experimental solutions which contained 0.3 mM $CaCl_2$, 0.2 mM $Ca(NO_3)_2$, and chlorides of Na and K at indicated concentrations. Solutions were labelled with ^{22}Na or ^{86}Rb at 0.1 µc/ml.

Test Ion	Ion Concn.	Ion Accumulation		Toxin-Induced Change
		Control	Toxin	
	mM	nmoles/g/hr		%
Na	0.2	74 ± 4	116 ± 16	+ 57
K	0.2	420 ± 20	390 ± 36	- 7
NO_3	0.4	368 ± 16	748 ± 8	+104
Na	20.0	1954 ± 114	2430 ± 90	+ 24
K	20.0	3020 ± 160	2880 ± 1	- 5
NO_3	0.4	254 ± 9	668 ± 63	+163

There are indications that absorption is dependent on cell metabolism (33, 68). In addition, Ca uptake by corn roots is mediated by dual mechanisms which operate in the concentration ranges found for other ions (68). The effect of toxin on Ca uptake was studied because of this peculiarity of corn, and because it provides an opportunity to observe simultaneous absorption, by a Ca-stabilized membrane, of both ions from a simple one-salt solution, $Ca(NO_3)_2$.

Experiments with Ca were patterned after those described elsewhere (33, 68). Seedlings were exposed to solutions with or without toxin. Roots were excised and placed in Ca-containing solutions. Toxin-treatment had no effect on the rate of Ca absorption by either of the dual mechanisms (Table 22). Desorption in water caused about 15% loss of absorbed Ca from both toxin-treated and control roots. Similar results were obtained in another experiment when roots were desorbed in 0.5 mM $CaCl_2$, except Ca losses ranged from 30-50%. This indicates that ^{45}Ca influx was due in part to exchange.

The following additional observations were made. Roots exposed to 0.2 mM Ca for 40 min had accumulated Ca to a concentration of 0.59 mM, indicating movement of external Ca against a concentration gradient. Absorption of Ca by mechanism 1 was slightly inhibited by K (Table 22). The accumulation rate of the counterion, NO_3, was stimulated $>100\%$ by toxin-treatment (Table 22). High or low Ca concentrations did not affect NO_3 uptake or toxin-stimulated NO_3 uptake (Table 22). In another experiment, NO_3 was accumulated from a 10 mM

Table 22. Absorption of Ca by Toxin-Treated and Control Corn Roots

Seedlings were exposed to 0.5 mM $CaCl_2$ with or without toxin (10 µg/ml) for 4 hr. Roots were excised and placed in experimental solutions containing $Ca(NO_3)_2$ with or with ^{45}Ca (0.05 µc/ml). All rinsing was with water.

Expt. No.	Test Ion	Ion Concn.	Special Conditions	Ca or NO_3 Accumulation		Toxin-Induced Change
				Control	Toxin	
		mM		nmoles/g/hr		%
1	Ca	10.0	...	1618 ±53	1490 ±150	- 8
	Ca	0.2	...	828 ± 2	782 ± 39	- 6
	Ca	0.2	KCl[a]	592 ...	662 ± 80	+ 12
	NO_3	0.4	High Ca[b]	382 ±18	850 ±114	+122
	NO_3	0.4	Low Ca[c]	394 ±48	790 ± 25	+101
2	Ca	0.2	...	1110 ±20	1030 ± 22	- 7
	Ca	0.2	Desorbed[d]	887 ±35	855 ± 35	- 4
	Ca	0.2	KCl[e]	959 ±29	927 ± 59	- 3
	NO_3	0.4	...	282 ± 4	645 ± 15	+128

[a]KCl (0.1 mM) was included in experimental solution.

[b]Experimental solution contained 0.2 mM $Ca(NO_3)_2$ + 9.8 mM $CaCl_2$.

[c]Experimental solution contained 0.2 mM $Ca(NO_3)_2$.

[d]Roots were desorbed for 35 min in 1000 ml aerated water.

[e]KCl (0.2 mM) was included in experimental solution.

$Ca(NO_3)_2$ solution (20 mM NO_3) at 2900 nmoles/g/hr and toxin caused 35% stimulation.

Differential absorption from a solution containing only Ca and NO_3 provided an opportunity to determine if the pH change of the experimental solution, observed occasionally (see Methods), was related to unequal absorption of charged particles. During one 40 min absorption period control roots removed 0.708 uequivalents (ueq) of Ca and 0.326 ueq of NO_3 from the experimental solution. The pH changed from 5.8 to 4.8 indicating that 15.9 ueq of H were added to the solution. Excess Ca uptake required H loss of 0.382 ueq to balance the charges. Thus, H loss from tissue was much greater than that required to compensate for excess Ca uptake.

Toxin-treated roots under similar conditions removed 0.662 ueq of Ca (no stimulation) and 0.672 ueq of NO_3 (101% stimulation) while the pH changed from 5.8 to 4.8. In this case there was slightly more NO_3 than Ca accumulation, which would require an increase in pH (0.01 ueq of H uptake) to balance charges. Instead the pH dropped the same as with control roots. Values for NO_3 accumulation are conservative estimates of total NO_3 uptake, because some of the NO_3 taken up was reduced during the absorption period.

Thorough washing of roots for 1 hr before the experiment in 4 l aerated 0.5 mM $CaCl_2$ did not affect the pH. The pH of a salt solution containing cheesecloth and string was not changed after aeration. It was concluded that the pH change, which occurred only when the volume

of the experimental solution was small compared to the amount of tissue in it, was caused by the tissue, as reported by others (85), and was not related to toxin-stimulation of ion uptake.

Effect of toxin on uptake of selected anions. -- Absorption of Cl by corn roots is dependent on cell metabolism and is mediated by dual uptake mechanisms similar to those described for other ions (67, 70). The effect of toxin on both mechanisms was tested. Seedlings were exposed to solutions containing toxin for 4 hr. Roots were excised and placed in solutions containing Cl. Toxin-treatment caused a small stimulation of Cl absorption rates by both mechanisms (Table 23). Progressively faster rates occurred when solutions contained 0.1 and 0.5 mM Ca. Toxin-stimulated Cl absorption occurred in the presence and absence of Ca. Roots in 0.2 mM Cl solution contained 0.95 mM Cl after a 40 min absorption period, indicating Cl movement against a concentration gradient. Another experiment showed that desorption for 30 min in 4 l of 0.2 mM NaCl caused 4-7% loss of Cl from toxin-treated roots and 13-20% loss from control roots. Variation between duplicate samples was great enough to indicate that the difference in losses was not significant. Some of the experimental solutions also contained NO_3 (Table 23). Uptake of Cl by control roots was faster than accumulation of NO_3, although toxin stimulated NO_3 accumulation more than Cl uptake. The NO_3 accumulation rate was low when experimental solutions contained no Ca.

Table 23. Effect of Toxin on Absorption of Cl by Corn Roots

Seedlings were grown in 0.2 mM $CaSO_4$ solution. They were exposed 4 hr to 0.5 mM $CaSO_4$ with or without toxin (10 µg/ml). Roots were excised and placed in experimental solutions. All rinsing was with water. Solutions were labelled with 0.01 µc ^{36}Cl/ml.

Test Ion	Ion Concentrations					Cl or NO_3 Accumulation		Toxin-Induced Stimulation
	Na	K	Ca	Cl	NO_3	Control	Toxin	
	mM	mM	mM	mM	mM	nmoles/g/hr		%
Cl	20.0	...	...	20.0	...	3300 ± 130	4430 ± 85	34
Cl	0.2	...	...	0.2	...	711 ± 25	1010 ± 25	42
Cl	...	...	0.1	0.2	...	970 ± 70	1185 ± 155	23
Cl	0.2	...	0.5[a]	0.2	...	1035 ± 35	1488 ± 108	43
Cl	0.2	0.4	...	0.2	0.4	796 ± 76	970 ± 145	22
NO_3	0.2	0.4	...	0.2	0.4	187 ...	474 ± 4	153

[a]Ca was added as SO_4 salt.

Of the ions discussed so far, K, Na, and Cl are not known to be metabolized by the cell. Metabolism does not appear to be a significant factor in toxin-stimulated uptake of NO_3 under my experimental conditions (see Discussion) and Ca is required primarily for stabilization of cell membranes (16, 60, 93). Therefore, it was advisable to test the effect of toxin on uptake of an essential ion such as phosphate (PO_4) which is rapidly metabolized by the cell. Corn roots absorb PO_4 by 2 mechanisms, both of which require Ca for maximum PO_4 absorption rates (6). The high affinity mechanism operates at PO_4 concentrations up to 10 μM and the low affinity mechanism operates at concentrations from 20-200 μM (6).

Experiments on PO_4 uptake were similar to those described for previously discussed ions, except that PO_4 was included in experimental solutions. Toxin-treatment did not stimulate PO_4 absorption at any PO_4 concentration (Table 24). Absorption of NO_3 from a PO_4 solution was enhanced by toxin-treatment. Roots in 2 and 20 μM PO_4 contained 41 and 91 μM internal PO_4 concentrations, respectively, after a 30 min absorption period. Thus, PO_4 moved against a concentration gradient. In one experiment, excised roots and roots of intact seedlings were exposed to solutions with or without toxin for 4 hr, which resulted in 4 hr aging of excised roots. All roots were then placed in solutions containing PO_4. Aged control roots absorbed PO_4 about twice as fast as did fresh control roots. Toxin-treatment had no effect on PO_4 uptake by aged or fresh roots (Table 24).

Table 24. Absorption of PO_4 by Toxin-Treated and Control Corn Roots

Fresh roots were prepared as follows. Intact seedlings were incubated 4 hr in 0.5 mM $CaCl_2$ with or without toxin (10 µg/ml). After incubation roots were excised. For aging, roots were excised and aerated 4 hr in 0.5 mM $CaCl_2$ with or without toxin (10 µg/ml). Fresh and aged roots were placed in solutions containing 0.5 mM $CaCl_2$ and Na_2HPO_4 (pH 4.8) labelled with $Na_2{}^{32}PO_4$ such that the 200 µM solution contained 12,000 cpm/ml. One solution in experiment 2 also contained 0.4 mM KNO_3. Roots placed in this solution were analyzed for NO_3 content.

Expt. No.	Test Ion Concn.	Type of Tissue	PO_4 or NO_3 Accumulation		Toxin-Induced Change
			Control	Toxin	
	µM		nmoles/g/hr		%
1	200	Aged	230 ± 2	256 ± 6	+11
	20	Aged	181 ± 1	138 ± 12	-24
	2	Aged	81 ± 1	70 ± 5	-13
	20	Fresh	93 ± 10	96 ± 9	+ 3
2	400	Fresh	208 ± 13	200 ± 12	- 4
	400[a]	Fresh	248 ± 40	438 ± 33	+72

[a]Data from this treatment are for NO_3 uptake.

Failure of toxin to affect total PO_4 uptake did not rule out a possible effect on a specific phase of PO_4 metabolism. Such an effect could be masked by the large amount of total PO_4 absorbed. However, data show that toxin-treatment did not affect the PO_4 content of PCA-soluble organic or inorganic fractions or the PCA-insoluble residue (Table 25). Under identical conditions NO_3 accumulation was enhanced by toxin-treatment. Accumulation of NO_3 by control roots was faster than usual and toxin-stimulation was small, because aged roots were used in this experiment (see Table 14).

The effect of toxin on sulfate (SO_4) uptake by corn roots was determined by modification of described procedures (35, 62). Toxin-treatment either had no effect on, or was slightly inhibitory to SO_4 absorption at all concentrations used (Table 26). Accumulation of NO_3 from the same solutions was stimulated by toxin-treatment. The usual amount of NO_3 stimulation was observed in experiment 3 (Table 26), but stimulation was abnormally small in experiments 1 and 2, for unknown reasons. Desorption removed about the same amount (10-25%) of SO_4 from both toxin-treated and control roots. Roots in 0.002 and 0.02 mM SO_4 solutions contained 0.023 and 0.066 mM internal SO_4 concentrations, respectively, after a 40 min absorption period. Thus, SO_4 was accumulated against a concentration gradient.

The effect of toxin on NO_2 accumulation was compared with the effect on NO_3 accumulation. Seedlings were placed in solutions with or without toxin for 4 hr. Roots were excised and placed in solutions

Table 25. Uptake and Incorporation of PO_4 by Toxin-Treated and Control Corn Roots

Aged roots were used for all treatments. Roots were excised, aerated 4 hr in 0.5 mM $CaCl_2$ with or without toxin (10 µg/ml), and placed in solutions containing 0.5 mM $CaCl_2$, 0.4 mM KNO_3 and 0.4 mM Na_2HPO_4 (pH 4.9) labelled with $Na_2H^{32}PO_4$ to give 53,000 cpm/ml. Organic and inorganic P was separated.

Test Ion	Fraction	PO_4 or NO_3 Accumulation		Toxin-Induced Change
		Control	Toxin	
		nmoles/g/hr		%
PO_4	Orthophosphate	126	128	+ 2
	Organic Phosphate	224	197	-12
	Residue	6	5	-16
	Total	356	330	- 7
NO_3	Total	554	820	+48

Table 26. Absorption of SO_4 by Toxin-Treated and Control Corn Roots

Seedlings were exposed 4 hr to 0.5 mM $CaCl_2$ with or without toxin (10 μg/ml). Roots were excised and placed in experimental solutions. In experiment 1, experimental solutions contained 0.2 mM $Ca(NO_3)_2$ and Na_2SO_4 labelled with $Na_2{}^{35}SO_4$ such that 0.2 mM solution contained 2200 cpm/ml. Roots were desorbed 30 min in 3000 ml aerated 0.5 mM $CaCl_2$ and 0.2 mM Na_2SO_4. In experiments 2 and 3, experimental solutions contained 0.5 mM $CaCl_2$, 0.4 mM KNO_3, and Na_2SO_4 labelled as in experiment 1. Roots were desorbed as in experiment 1, except Na_2SO_4 concentration was 0.02 mM.

Expt. No.	Test Ion	Test Ion Concn.	Desorption	SO_4 or NO_3 Accumulation		Toxin-Induced Change
				Control	Toxin	
		mM		nmoles/g/hr		%
1	SO_4	0.2	+	97 ± 11	96 ± 3	- 1
	SO_4	0.2	-	109 ± 1	121 ± 3	+ 11
	NO_3	0.4	-	334 ± 5	386 ± 6	+ 15
2	SO_4	0.02	+	49 ± 4	40 ± 2	- 18
	SO_4	0.02	-	66 ± 4	42 ± 1	- 36
	SO_4	0.002	+	34 ± 3	27 ± 1	- 19
	NO_3	0.4	-	199 ± 4	240 ± 16	+ 21
3	SO_4	0.02	+	57 ± 10	43 ± 5	- 24
	NO_3	0.4	-	204 ± 4	500 ± 25	+145

containing NO_2 or NO_3 or both NO_2 and NO_3. After absorption, roots were analyzed for NO_2 and NO_3 content. The usual stimulatory effect on NO_3 accumulation was observed, but toxin had no effect on NO_2 accumulation (Table 27). This differential stimulation was observed when roots were exposed to NO_3 and NO_2 in separate solutions and when exposed to both ions simultaneously in the same solution. Desorption caused 35% NO_2 loss from control roots and 22% NO_2 loss from toxin-treated roots. This differential loss of NO_2 was also observed after roots absorbed NO_2 and NO_3 simultaneously. Loss of NO_2 during a 30 min desorption period could be caused by leakage through cell membranes damaged by NO_2 poisoning, or the loss could indicate NO_2 metabolism within the cells. The latter possibility seems feasible because NO_2 is very reactive with many substances, including amines. In one experiment NO_2 and NO_3 were absorbed simultaneously. During desorption some NO_2 was lost but NO_3 was not, indicating that membranes were not damaged by NO_2 poisoning.

Roots accumulated NO_2 about 10 times faster than NO_3. At the pH used for this experiment (5.8), a significant amount of NO_2 is undissociated and could passively diffuse into cells at a fast rate as HNO_2. A high intracellular pH would prevent rapid HNO_2 leakage during desorption. The effect of pH was tested in solutions buffered at pH 7.5 with 2 mM HEPES. The rate of NO_2 accumulation was reduced 95% at pH 7.5, as compared with pH 5.8. The rate of NO_3 accumulation was also reduced (about 80%) at pH 7.5, for unknown reasons. Although

Table 27. Accumulation of NO_2 and NO_3 by Toxin-Treated and Control Corn Roots

Seedlings were placed 4 hr in 0.5 mM $CaCl_2$ with or without toxin (10 µg/ml). Roots were excised and placed in experimental solutions containing 0.5 mM $CaCl_2$ and 0.4 mM KNO_2 or KNO_3 or both KNO_2 and KNO_3 in the same solution.

Test Ion	Ion Content	NO_2 or NO_3 Accumulation		Toxin-Induced Change
		Control	Toxin	
		nmoles/g/hr		%
NO_3	NO_3	222 ± 3	642 ± 77	+190
NO_2	NO_2	2400 ± 90	2050 ± 245	- 14
NO_2[a]	NO_2	1565 ± 45	1595 ± 65	+ 2
NO_3	NO_3 + NO_2	394 ± 5	617 ± 10	+ 57
NO_2	NO_3 + NO_2	1950 ± 30	1765 ± 20	- 10

[a]Roots were desorbed 30 min in 1000 ml aerated 0.5 mM $CaCl_2$.

accumulation rates for both ions were slower at pH 7.5 than at pH 5.8, toxin-treatment still had no effect on NO_2 accumulation but increased the NO_3 accumulation rate by 270%. Estimation of NO_3 content in the presence of NO_2 was inaccurate and quite variable because there was much more accumulation of NO_2 than of NO_3.

Selective stimulation of uptake by toxin-treatment in resistant and susceptible corn was tested in several experiments. In one experiment, accumulation of NO_3 and Na by resistant corn roots was determined because uptake of these ions by susceptible corn is relatively sensitive to toxin. Results (Table 28) show that the rate of NO_3 accumulation by susceptible roots was enhanced by as little as 0.2 μg toxin/ml. The rate of NO_3 accumulation by resistant roots was not affected by 2 and 10 μg toxin/ml but was stimulated 138% by 100 μg/ml. Uptake of Na by resistant roots was not stimulated by toxin at any concentration used. The experiment comparing the effects of toxin on NO_3 and Na accumulation by resistant corn was not repeated; it should be confirmed before concluding that toxin-treatment has a differential effect on NO_3 and Na accumulation by resistant roots.

Effect of toxin on uptake of selected organic molecules. -- Selective toxin-stimulation of ion uptake raised the possibility that this effect is specific to ions. This possibility was tested by studying uptake of a noncharged organic molecule, 3-o-methylglucose (MeG). Use of MeG (an analog of glucose) simplifies study of sugar uptake because it is not metabolized by higher plant cells (31, 95), although it is

Table 28. Effect of Toxin Concentration on NO_3 or Na Accumulation by Susceptible and Resistant Corn Roots

Seedlings were exposed 4 hr to 0.5 mM $CaCl_2$ with or without toxin. Roots were excised and placed in experimental solutions which contained 0.5 mM $CaCl_2$ and 0.4 mM KNO_3 or 0.2 mM $NaNO_3$ labelled with ^{22}Na (0.1 μc/ml).

Corn Hybrid	Toxin Concn.	NO_3 or Na Accumulation			
		NO_3	Toxin-Induced Change	Na	Toxin-Induced Change
	μg/ml	nmoles/g/hr	%	nmoles/g/hr	%
Resistant	0	442	....	102	...
	2.0	351	- 20	74	-27
	10.0	550	+ 24	62	-39
	100.0	1050	+138	98	- 4
Susceptible	0	260	...		
	0.2	802	+208		
	2.0	1235	+395		
	10.0	745	+186		

actively accumulated by the glucose transport mechanism (95). In addition, efflux of MeG in the presence of glucose is reported to require cellular energy (95). This provides a system for studying the effect of toxin on active efflux.

Experiments with MeG were similar to those with ions; toxin-treated and control roots were placed in solutions containing MeG. After absorption, MeG was extracted from roots and quantified. Toxin-treatment caused 25-35% stimulation of the MeG absorption rate and 105% stimulation of NO_3 accumulation rate from identical solutions (Table 29). Desorption for 30-60 min in 0.5 mM $CaCl_2$ caused no loss of MeG from toxin-treated or control roots. Both toxin-treated and control roots lost 25% of their MeG when desorbed 60 min in the presence of glucose. Thus, toxin-treatment enhanced active influx but had no effect on active efflux of MeG. Glucose competitively inhibits MeG uptake (95). My data showed that presence of 10 mM glucose in the experimental solution inhibited the rate of MeG uptake by 30% in both toxin-treated and control roots.

Amino acids were used in further experiments to determine effects of toxin on uptake of organic molecules. Toxin-treated and control roots were exposed to amino acid-containing solution for 30-45 min. The amounts of amino acids in roots were then determined. Results (Table 30) show that toxin had a selective effect on amino acid uptake. Leucine uptake was stimulated about 25% by toxin-treatment, glutamic acid uptake was not affected, and the NO_3 control showed

Table 29. Effect of Toxin on Absorption and Desorption of 3-o-Methylglucose (MeG) by Corn Roots

Seedlings were exposed 4 hr to 0.5 mM $CaCl_2$ with or without toxin (10 µg/ml). Roots were excised and placed in solutions containing 0.5 mM $CaCl_2$, 0.4 mM KNO_3 and 10 mM MeG labelled with ^{14}C-MeG (0.01 µc/µmole MeG). Roots were desorbed in 3000 ml aerated 0.5 mM $CaCl_2$ with 10 mM glucose where indicated. One min desorption was time required to remove MeG from free space with 6 rinses of 0.5 mM $CaCl_2$.

Test Substance	Desorption Time	MeG or NO_3 Content		Toxin-Stimulation of MeG or NO_3 Content
		Control	Toxin	
	min	nmoles/g/hr		%
MeG	1	1832 ± 82	2285 ± 60	25
MeG	30	1805 ± 135	2440 ± 85	35
MeG	60	1850 ± 45	2400 ± 35	30
MeG	60 (in glucose)	1400 ...	1800 ...	28
NO_3	1	398 ± 2	815 ± 45	105

Table 30. Leucine and Glutamic Acid Accumulation by Toxin-Treated and Control Corn Roots

Seedlings were exposed 4 hr to 0.5 mM $CaCl_2$ with or without toxin (10 µg/ml). Roots were excised and placed in experimental solutions containing 0.5 mM $CaCl_2$ and 0.4 mM KNO_3 with or without 0.1 mM leucine or glutamic acid or both. The corresponding ^{14}C-1-labelled amino acids were included at 0.01 µc/ml. All solutions were adjusted to pH 5.8. Specific activities of ^{14}C-1-leucine and glutamic acid were 12.2 and 20.0 mc/mmole, respectively.

Test Substance	Supplements to Expt'l. Solution	Total Amino Acid or NO_3 Accumulation		Toxin-Induced Change
		Control	Toxin	
		nmoles/g/hr		%
Leucine	Leucine	823 ± 35	1030 ± 77	+ 25
Glutamic Acid	Glutamic Acid	199 ± 5	200 ± 4	0
Leucine	Leucine and Glutamic Acid	879 ± 10	1091 ± 31	+ 24
Glutamic Acid	Leucine and Glutamic Acid	65 0	64 ± 1	0
NO_3	Leucine and Glutamic Acid	288 ± 40	745 ± 32	+159

159% stimulation. Glutamic acid did not affect leucine uptake, but leucine inhibited glutamic acid uptake by 67%.

In another series of experiments using different experimental conditions (see Appendix), leucine uptake and incorporation by corn root sections were studied. Toxin-treatment caused an increase in the rates of uptake and incorporation, and an increase in the size of the soluble leucine pool (Figure 19). Thus, there is agreement between the 2 sets of data on leucine uptake.

A summary of results of experiments on solute absorption by susceptible corn roots is presented in Table 31.

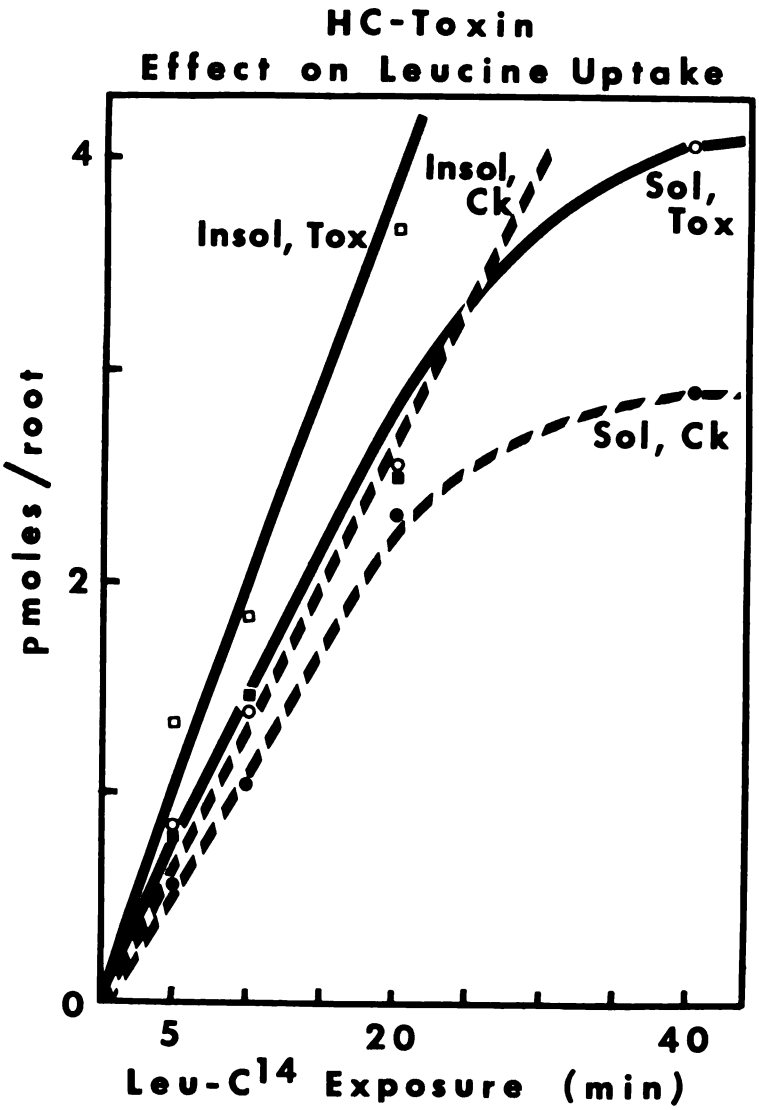


Figure 19. Effect of toxin on leucine uptake, incorporation, and soluble pool size. Seedlings were grown in 15 cm petri dishes in White's solution. They were placed in 30 ml White's solution with or without toxin (2 µg/ml) for 4 hr. Roots were then rinsed, 1 cm sections were excised 2 mm behind the root tip and placed in 10^{-6} M ^{14}C-l-leucine in White's solution. At intervals, sections were removed, rinsed in ^{12}C-leucine in White's solution, blotted and placed in 0.5 ml 95% ethanol (10 sections/replicate) for 12 hr. Activity in aliquots was counted to determine the size of the soluble pool. Sections were then rinsed 3 times in 95% ethanol and hydrolyzed in 0.5 ml 6 N HCl at 95 C 1 hr. Activity in aliquots was counted to determine leucine incorporation. Tox = toxin, Ck = control, Sol = ethanol-soluble leucine, Insol = ethanol-insoluble residue.

Table 31. Selective Stimulation of Solute Accumulation in Susceptible Corn Roots by Toxin

Solute	Toxin-Induced Stimulation[a]
	%
NO_3	100
NO_2	0
Na	50
K	0
Cl	30
Ca	0
PO_4	0
SO_4	0
3-o-methylglucose	25
leucine	25
glutamic acid	0

[a]Values are estimates of the average stimulation observed in several experiments.

DISCUSSION

The effect of HC-toxin on corn seed germination was compared with the immediate lethal effect which HV-toxin has on resting susceptible oat seeds (98). The 2 systems are strikingly different. Corn seed germination was not affected by massive doses of HC-toxin (Table 1). Intact seeds required 36-40 hr exposure to toxin before an effect on later root growth was observed. Kuo (55) found that growing corn seedlings required at least 4 hr toxin exposure to accumulate a toxic dose.

The long toxin exposure necessary for toxin uptake by seeds is due in part to inability of toxin to penetrate the seedcoat. Thus, only 24 hr imbibition in the presence of toxin was sufficient to reduce the capacity for NR induction in excised embryonic axes. Inhibition of NR induction is a late effect of toxin-treatment and an insensitive parameter. Additional studies are necessary to determine the sequence for activation of metabolic systems during germination, and the point in this sequence at which tissues can absorb effective doses of toxin. It is possible that toxin does not prevent germination of intact seeds because the initial stages of germination are stimulated rather than inhibited.

The earliest physiological effects of toxin observed to date are stimulatory and only later does inhibition become evident (54). It is generally agreed that the earliest observable effect of a biologically active molecule is closer to the site of action than effects observed later (118). Therefore, it seemed logical that mode-of-action studies should involve the stimulatory activity of toxin. Experiments were designed to determine whether stimulatory effects could be related to a particular aspect of cell metabolism.

Toxin-stimulated growth of susceptible and resistant corn and nonhost plants suggested that toxin may have nonhost-specific hormone action. An analogy could be drawn with the gibberellin-like activity of helminthosporol (36, 48, 81). Possible hormone-like activity may or may not be related to host-specific activity. Toxin, when applied over a wide concentration range, did not promote hypocotyl growth, seed germination, or enzyme induction. Both helminthosporol and GA are active in all these tests. This indicates that toxin does not have GA-like activity. In addition, stimulatory effects are not caused by contaminants in the toxin preparation which have GA-like activity. The apparent toxin-stimulation of GA-induced α-amylase production by nongerminable barley half-seeds is difficult to interpret. Further experiments are required to establish the validity of this phenomenon. It is possible that α-amylase production by poor quality barley seeds is directly related to the number of viable seeds/treatment. Toxin at low concentrations did not enhance GA-induced

amylase activity in susceptible corn endosperm. High toxin concentrations inhibited amylase activity. This may be an indirect effect because 48 hr exposure caused only partial inhibition. However, the amylase results with corn are not conclusive because most of the amylolytic activity in corn endosperm results from β-amylase (12) which may not be synthesized de novo in seeds (121).

Results of experiments with NR support the suggestion that toxin-inhibition of enzyme synthesis is an indirect effect. When axes imbibed in the presence of toxin for 24 hr, the capacity for NR induction was decreased but not eliminated. By comparison with diseased plants, there is a report that Fusarium infection causes decreased NR activity in roots and increased activity in shoots of rice plants (112). Inducibility on a per axes and on a dry wt basis actually increased with longer toxin exposures. Results suggest that toxin did not directly inhibit induction but rather inhibited some other function on which induction depends. If long exposure to toxin resulted in disruption of intracellular transport systems, materials for enzyme synthesis would not be available.

The possibility that toxin causes direct stimulation of protein synthesis was attractive because toxin stimulates growth, and increased growth should require increased synthesis. In addition, there is evidence that toxin stimulates incorporation of ^{14}C-labelled amino acids (54). This question could be approached directly by preparing a cell-free amino acid-incorporating system from corn and testing the

effect of toxin *in vitro*. However, results of experiments with such systems from plants are not always conclusive because of problems in achieving net protein synthesis (73). Therefore, experiments were designed to detect possible effects of toxin on protein synthesis by indirect means. Enzyme induction was examined using a substrate-induced enzyme, NR, as a model. There is evidence that NR is synthesized *de novo* in tobacco cells (132).

Toxin-treatment during induction enhanced NR activity *in vivo*, which at first suggested stimulation of NR induction. However, when tissues were pretreated with toxin, then exposed to inducer (NO_3), there was increased activity by endogenous NR at 0 induction time. Furthermore, the rate of toxin-stimulated induction did not differ significantly from the control rate. These observations suggested that toxin had no direct effect on induction of the enzyme. When NR from toxin-treated and control tissues was assayed *in vitro*, little or no difference was observed, providing additional evidence that toxin did not stimulate NR induction by NO_3. It also showed that toxin itself did not induce NR activity.

Several experiments indicated an increased rate of NO_3 accumulation after toxin-treatment. When toxin-treated and control roots were excised and placed in NO_3 solutions, treated roots accumulated NO_3 as much as 3 times faster than did control roots. Toxin-treatment stimulated NO_3 accumulation to different degrees in different experiments. However, the effect was observed often enough to provide

convincing evidence that it is real. In 30 different experiments, 86 duplicated treatments were compared with 86 duplicated controls (172 individual comparisons). Toxin-stimulation was observed in all cases.

The NO_3 accumulated by toxin-treated roots was retained through 30-60 min desorption, which suggests that the tissues were not leaky to NO_3. However, these data must be considered within the context of NO_3 pools in corn roots (Table 13). The metabolic NO_3 pool is assumed to be in the cytoplasm because NR is considered to be a cytoplasmic enzyme (74, 96, 115). The most likely location for the storage NO_3 pool is the vacuole. These assumptions form the basis for analyzing data which relate to NO_3 pools. When roots are exposed to NO_3, NR induction begins within 30 min (Figure 11). The amount of NR present after 30 min is capable of reducing NO_3 at the rate of 850 nmoles/g/hr. Therefore, all NO_3 taken into the very small metabolic pool in roots during a 30 min absorption period was reduced. When roots were removed from NO_3 solution, any NO_3 remaining in the metabolic pool was reduced within a few min (Table 13), i.e., before roots were killed to terminate the experiment. Thus, measurements of NO_3 content of roots before and after desorption reflected the NO_3 content of the storage pool (probably in the vacuole), not the total NO_3 taken up during 30 min absorption. The fact that little or no NO_3 was lost from toxin-treated tissues during 30-60 min desorption does not mean that toxin did not affect NO_3 metabolism or NO_3 leakage across the plasmalemma. It means that NO_3 did not leak from the storage pool, probably in the vacuole.

In addition to the circumstantial evidence just discussed, experimental results also indicate that toxin did not cause NO_3 leakage from the storage pool (Figure 16). After the metabolic NO_3 pool in leaves was exhausted, toxin did not cause translocation of NO_3 from the storage to the metabolic pool, as certain other chemicals are known to do (22). Although this point needs further investigation to be conclusive, the data suggest that toxin has no effect on efflux across the tonoplast.

One aspect of the NO_3 pool experiment needs clarification. It is possible that toxin released NO_3 from the storage pool and all NO_3 was reduced prior to the end of the 4 hr aerobic toxin-treatment period. This possibility is negated by the large amount of NO_3 known to be in the storage pool (24 μmoles/g tissue in the experiment described) and by the rate of NO_3 reduction under these conditions (1 μmole/g tissue/hr). NR has a half-life of 4 hr; toxin does not affect NR degradation. Assuming no degradation of NR during the 4 hr aerobic period, a maximum of 20% of total storage NO_3 could have been reduced in 4 hr, had it been released from the storage pool. Thus, both NR and NO_3 were present in the cell under these conditions, but toxin did not cause them to interact.

There are no data which indicate whether or not toxin affects the NO_3 pathway between NO_2 and NH_4. However, toxin apparently does not inhibit NO_3 reduction to NO_2 (Figures 7-14). This rules out the possibility that toxin blocks NO_3 metabolism, diverts NO_3 from the metabolic to the storage pool and thus increases the NO_3 content of the

storage pool. A possible effect of toxin on NO_2 reduction is not important with respect to stimulation of NO_3 uptake. Even if toxin blocked NO_2 reduction, NO_3 in the metabolic pool would still be converted to NO_2. The NO_2 would probably be excreted from the cell (24) or otherwise dissipated. Therefore, the increased uptake of NO_3 into the storage pool is a result of increased uptake across the plasmalemma, not the result of a block in NO_3 metabolism.

Increased movement of NO_3 into the storage NO_3 pool suggested that toxin may alter ion movements across membranes of other organelles as do certain antibiotics (76, 87, 109). However, results of experiments with mitochondria and chloroplasts indicated that these organelles are immune to toxin. The negative data may rule out several possible sites of toxin action. Toxin did not uncouple, inhibit electron transport, affect phosphorylation, inhibit the Krebs cycle, or affect the conformational changes of mitochondria or chloroplasts whether or not electron transport occurred. Each of these sites is known to be affected by one or more biologically active molecules. Thus, toxin appears to be in a different category, at least with respect to specificity if not mode-of-action.

The isolated chloroplasts were actually chloroplast lamellae, that portion of the chloroplasts responsible for light absorption, electron transport, and ATP formation. The effect of toxin on intact chloroplasts was not tested. It would be of particular interest to test the effect of toxin on CO_2 fixation in the dark by chloroplasts, because

toxin stimulates dark fixation of CO_2 by whole tissues (56). Methods for isolating a high proportion of intact chloroplasts are available (122).

Evidence of toxin-stimulated NO_3 accumulation and the concept of NO_3 pools offered an alternative explanation for toxin-stimulated NR activity in vivo. More NO_2 was produced because more substrate was available to NR. For this to be true, NO_3 in the metabolic pool must be the limiting factor in NO_2 production. This is easily rationalized with the small metabolic NO_3 pool in corn root tips, which is exhausted in less than 10 min (Table 13). The rate of NO_3 reduction is thus dependent on the rate at which NO_3 enters the metabolic pool. If toxin stimulates entry of NO_3 into the pool, the rate of reduction would increase until the point at which NR activity would be the rate-limiting factor. Since toxin does not stimulate NR induction, this would result in a systematic increase in NO_2 production which would be constant throughout the experimental period. The plot of toxin data would then parallel the plot of the control data as seen in my graphs.

The explanation must be modified to account for results with leaves. Corn leaves have a large metabolic NO_3 pool which requires 2 hr for complete reduction (Figure 15). If this pool was full or even partially full, the rate of NO_3 entry into the pool would not limit the rate of NO_2 production. Thus, toxin-stimulated NO_3 uptake would not be detected by the in vivo assay. However, toxin did stimulate NO_2 production in leaves. This can be explained by the fact that the experiments were started when the pool was empty. The initial rate of NO_3

reduction (by endogenous NR) was limited by the rate of NO_3 movement into the pool. Since tissues were given a 4 hr pretreatment with toxin, the initial rate of NO_3 uptake was enhanced and thus NO_2 production was increased as in root tips.

This explanation is supported by the fact that the metabolic pool in corn leaves fills slowly, compared to the time course of my experiments. After 9 hr exposure to NO_3, the NO_3 content of the metabolic pool in leaves was only 25% of the pool capacity (22). Furthermore, NO_2 production becomes nonlinear as the final portion of the pool is reduced (22). Below a certain critical NO_3 concentration, the rate of NO_3 reduction is dependent on NO_3 concentration in the pool. Thus, even after NO_3 enters the pool faster than it is reduced, a time interval is required before the NO_3 concentration in the pool is sufficient to eliminate the rate of NO_3 entry as the factor limiting NO_2 production. Since the maximum length of experiments on NR induction in leaves was 2 hr (the experiment shown in Figure 14 was terminated at 40 min), it is feasible to propose that the rate of NO_3 entry into the metabolic pool limits NO_2 production during the time course of the leaf experiments.

The rationale presented above predicts that toxin causes an increased rate of NO_3 entry from extracellular sources into the metabolic pool. Data in Figure 14 are evidence that this is true. The initial rate of NO_3 uptake was observed in noninduced tissues under anaerobic conditions in the dark. Uptake of NO_3 could be observed under

anaerobic conditions because leaf tissue does not go anaerobic immediately. In leaves, production of NO_2 is more dependent on darkness than on anaerobiosis (94). Endogenous NR was used to detect NO_3 as it entered the metabolic pool. At 0 time there was essentially no NO_3 in either toxin-treated or control tissues. Within 10 min after exposure to NO_3, toxin-treated tissues took up and reduced more NO_3 than did control tissues. An increased rate of NO_3 uptake was seen throughout the experimental period. Toxin-induced uptake went into the metabolic NO_3 pool, because NR would not have reduced it at any other location (22). As noted earlier, toxin-treatment also caused increased uptake of NO_3 into the storage pool. It is not likely that the linear increase in NO_2 production was complicated by an increase in NR activity due to induction. There are reports of a lag phase of up to 30 min before NR induction begins (20, 110). Since this experiment was terminated in 40 min, NO_2 production was probably caused by a constant amount of endogenous NR. It would be possible to do long term NO_3 uptake studies in the absence of induced NR activity by suppressing NR induction with tungstate (37).

The effect of toxin on the capacity of the metabolic NO_3 pool was not tested, but it could be by a procedure similar to that described in Figure 16. Cuttings from plants grown in the presence of NO_3 should be exposed to toxin and NO_3 for 4 hr. Leaves placed under anaerobic conditions should produce more total NO_2 if the capacity of the pool had been expanded by toxin-treatment. This would fit with data on the

capacity of the soluble leucine pool, which is increased by toxin-treatment (Figure 19 and Appendix).

An apparent anomaly in the NO_3 metabolism data is that toxin stimulated NO_3 accumulation but not NR induction. Increased accumulation of inducer without a corresponding increase in induction can be interpreted as evidence for an "inducer" NO_3 pool. Such a pool could be defined as that amount of NO_3 which is capable of inducing NR. When the pool is full, additional NO_3 uptake has no effect on induction. If such a pool exists, toxin apparently did not affect it. There are other reports of tissues which contained high NO_3 concentrations but low NR activity (3).

An attempt should be made to explain the decreased NR activity in control tissues after 2 or more hr of induction followed by the *in vivo* assay (Figures 7, 8, 11, 12). I observed this phenomenon many times in both embryonic axes and root tips. Ferrari (personal communication) made the same observation using corn root tips. Induction of NR in apple leaves shows a similar pattern (51). The decreased activity in my experiments was not caused by exhaustion of NO_3 in the induction medium. Results of the *in vitro* assay and results of several other workers (20, 110) suggest that NR content increases for several hr. In addition, NO_3 uptake is linear at least 4 hr (Figure 17). If both NO_3 concentration and NR activity increase, the decrease in NO_2 production may result from their failure to interact. Perhaps after brief exposure to NO_3, the cell's immediate need for nitrogen is

satisfied and some NO_3 is diverted from the metabolic pool to the storage pool. The kinetics of induction were followed only in root tips (which have a very small metabolic pool) and embryonic axes (which have a metabolic pool of unknown size). If NO_3 was partially diverted from a small metabolic pool such as that in root tips, a decrease in the rate of NO_2 production would soon be evident, regardless of the amount of NR present. There are other possible explanations (3).

An increased rate of NO_3 accumulation by whole tissues and negative results with all other experiments suggested that the cell surface was the most promising area to examine for a site of toxin action. Uptake of several solutes by corn roots was observed for possible effects of toxin-treatment. The data indicate that 4 hr toxin-treatment causes a change in the characteristics of the plasmalemma. This change results in increased capacity of the plasmalemma to actively transport certain substances (NO_3, Na, Cl, MeG, and leucine) into the cell and retain them within the cell. Transport of certain other substances (NO_2, K, Ca, PO_4, SO_4, and glutamic acid) is not affected by toxin-treatment. The evidence can be summarized as follows: 1) Toxin-stimulated uptake is temperature sensitive. 2) Toxin-treated tissues accumulate ions against a concentration gradient, and develop a steeper gradient with some ions than do control tissues. 3) Mechanism 1 of ion transport is generally thought to be in the plasmalemma (17, 59). Toxin-treatment stimulates uptake of certain ions by mechanism 1. 4) Substances accumulated by toxin-stimulated tissues cannot

be washed out of such tissues in short desorption periods. 5) Toxin-treatment enhanced active influx but had no effect on active efflux of MeG. 6) Accumulation of only one ion (NO_3) from a single-salt solution ($Ca(NO_3)_2$) was stimulated by toxin-treatment. Therefore, the altered plasmalemma had a greater affinity for NO_3 itself. 7) Negative results from tests of intracellular sites support the conclusion that increased uptake is due to a change at the cell surface. 8) There is a change in electrical potential difference across the plasmalemma within 2 min after exposure to toxin (25).

Data on desorption are of particular importance. Toxin-treatment stimulated uptake of NO_3, Na, Cl, MeG, and leucine by roots. There was no evidence that toxin-treatment either stimulated or inhibited leakage of any substance, although variability was great enough to have masked small differences. In addition, toxin did not affect active efflux of MeG. These results contrast with those for HV-toxin and susceptible oats. HV-toxin causes immediate and massive leakage of materials from cells, which indicates that mechanisms-of-action of the 2 toxins are distinctly different.

Of the ions tested with whole tissues, only the accumulation of NO_3, Na, and Cl was stimulated by toxin. By comparison, reaction mixtures for experiments with isolated organelles contained high concentrations of Cl and small amounts of Na (pH was adjusted with NaOH). Light-scattering and storage NO_3 pool data indicate that toxin had no effect on ion fluxes across organelle membranes. This suggests that

toxin specifically alters certain characteristics of the plasmalemma, but has no effect on other cellular membrane systems.

The ion uptake experiments were modeled after experiments done by other workers. I have tried to relate my results to those already published.

Absorption of most ions, including K, is enhanced in the presence of Ca (16). However, there is a report that Ca inhibits K uptake by corn roots in short term experiments (15). This report has been refuted by other workers (60, 69). Thus, my experimental solutions contained Ca unless Ca was a variable. Attempts were made to test tissues under physiological conditions; Ca apparently is required for normal functioning of the plasmalemma (16, 60, 93). In barley, Na uptake by mechansim 1, which has high affinity for ions, is essentially eliminated by the presence of K and Ca (93). Uptake of Na by mechanism 1 in corn roots was also greatly reduced in the presence of K and Ca. Mechanism 2 in barley roots, which has low affinity for ions, prefers Na over K (93). In contrast, under my experimental conditions there was more absorption of K than Na by mechanism 2 in corn roots.

There was greater loss of ^{45}Ca from corn roots when they were desorbed in $CaCl_2$ than when they were desorbed in water. This indicates that at least part of the ^{45}Ca entered the root by exchange. Since roots were grown in the presence of Ca, it is not surprising that ^{45}Ca should exchange for unlabelled Ca. However, Maas reported that corn roots grown in the presence of Ca linearly absorb net Ca for

several hr when placed under the described experimental conditions (68). Maas also reported that mechanism 2 of Ca transport is greatly inhibited in the presence of K (68). My results indicate that mechanism 1 is slightly inhibited by the presence of K. There are reports that Cl is absorbed equally well in the presence and absence of Ca (70). My data indicate slight stimulation of Cl uptake when Ca is present. Uptake of Cl was said to be faster from NaCl or KCl solutions than from a $CaCl_2$ solution (117). My data show the reverse to be true.

One of the most lively controversies in plant physiology concerns the location of the low affinity ion transport system, or mechanism 2 (17, 59, 72, 123). Laties and his group claim that mechanism 1 is in the plasmalemma and that mechanism 2 is in the tonoplast, i.e., the mechanisms are in series (59, 117). Epstein and co-workers hold that both mechanisms are in the plasmalemma and thus are in parallel (123). Both groups have evidence for their claims, but MacRobbie is not convinced by the claims of either (72).

This controversy is of special interest with respect to the site of toxin action. If mechanism 2 is convincingly shown to be in the plasmalemma, it would support the hypothesis that toxin-treatment affects the plasmalemma. If mechanism 2 is in the tonoplast, then toxin affects the tonoplast because it affects mechanism 2. Alternatively, mechanism 2 would appear to be in the plasmalemma if toxin is shown to have no effect on the tonoplast.

It is possible that toxin enhances transport by binding directly with the solute to form a lipid soluble complex which can easily pass across the plasmalemma. Certain antibiotics, called ionophores, are thought to act in this way (87, 109). This possibility was not eliminated, but there are several reasons to think it unlikely. 1) It does not seem feasible that toxin would specifically complex with species as different from each other as NO_3, Na, Cl, MeG, and leucine. 2) Toxin does not complex with K. This was tested by monitoring K concentration in buffered solution with a K sensitive electrode. Addition of toxin to the solution did not affect the K concentration. Of course, toxin does not affect K transport either. Similar experiments should be tried with ions whose movement is affected by toxin. 3) Toxin does not interfere in the bacterial NO_3 assay, but because of the nature of the assay, this does not eliminate the possibility that toxin binds reversibly with NO_3. 4) Toxin does not require exogenous ions of any kind to kill susceptible corn roots in the standard seedling bioassay. This implies that toxin does not act directly on the substance being transported, but rather causes a change in the plasmalemma which increases the affinity of certain carriers for their substrates. The argument is weakened by the fact that endogenous ions are present in tissue. 5) Toxin is host-specific and appears to have at least a degree of specificity for the plasmalemma of susceptible plants. Ionophores generally transport ions across any lipid-water interface (87).

Gramicidin D (GD) is known to stimulate ion fluxes across membranes of higher plants (40, 76). It is interesting to compare the effects of GD on plant roots (40) with those of toxin. 1) GD affects membranes of both mitochondria and intact roots. Toxin is specific for membranes of intact roots. 2) GD-enhanced influx does not occur through cut ends of roots; the same is true for toxin (Table 17). 3) The percentage stimulation of influx by GD is dependent on the portion of the root used; the same is true for toxin (Table 16). GD stimulates ion uptake by squash, cucumber, pea, corn, barley, and oats; oats are by far the most sensitive. Effects of toxin are specific for susceptible corn (57; Table 28). 4) GD stimulates uptake of Na and Cs but not to the degree observed for K and Rb. Other solutes were not tested. Toxin stimulation is most striking for NO_3, but stimulation is evident for Na, Cl, MeG, and leucine. There was no effect of toxin on any other substance tested (Table 31). 5) GD-stimulated influx is inhibited by anaerobiosis and respiratory inhibitors. Toxin-stimulated influx is inhibited by low temperature (Table 12). 6) GD enhanced transport of K by mechanism 1 more than that by mechanism 2. Toxin enhanced NO_3 accumulation by mechanism 1 more than by mechanism 2 (Figure 18), but it caused about equal stimulation of uptake of Na and Cl by both mechanisms (Tables 20 and 23). 7) GD appears to stimulate ion movement across all types of cell membranes (40, 76, 87). Negative results with organelles suggest that toxin is specific for the plasmalemma.

The mechanisms of stimulation are unknown for both GD (40) and toxin. Since they both show certain degrees of specificity for plant species, ions, and (in the case of toxin) membranes, their sites of action may involve the extremely variable protein or lipid components of membranes. Chemically similar ionophores differ with respect to membrane specificity (40). Some antibiotics act only on specific lipid components of the membrane (40). It is now recognized that membranes are not just lipid-containing permeability barriers, but rather are dynamic units which contain catalytic proteins (72). In contrast to HV-toxin, which acts independently of cell functions (101), any model for HC-toxin action must accommodate known effects on metabolic functions of membranes.

Antibiotics other than GD have been reported to have ion specificity similar to that of toxin (29). Modes-of-action of 2 synthetic antibacterial compounds were compared with those of gramicidin and valinomycin against bacterial protoplasts. "Synthetic 1" increased permeability to NO_3; "synthetic 2" to NO_3 and Cl; gramicidin to NO_3, NH_4, K, and Na; valinomycin to NO_3, NH_4, and K. None of the antibiotics affected membrane permeability to Ca, Mg, Mn, SO_4, or H_2PO_4. It is notable that resistance of some bacteria to these antibacterials is located in the cell wall; when the wall is removed bacteria become sensitive (29). This is in contrast to the case of HV-toxin and oats. Susceptible cells are sensitive and resistant cells are insensitive whether cell walls are present or absent (103, 105).

Tissues which are excised and allowed to age have altered metabolic capabilities (28, 31, 80). Several experiments demonstrated this phenomenon and tested the effect of toxin on altered tissues. Aged corn roots absorbed PO_4 at a rate twice as fast as that of freshly excised roots (Table 24). The same thing is known to happen in aged potato slices (28) and sweet potato roots (41). Toxin-treatment did not affect PO_4 absorption by either fresh or aged corn roots (Table 24). The rate of NO_3 accumulation was about twice as fast in aged as in fresh corn roots (Table 14). The percent stimulation of NO_3 accumulation by toxin was reduced in aged roots. It should be noted that excised roots were aerated during the aging period, whereas comparable intact roots were not. The effect of aeration was not tested, but it seems unlikely that increased uptake is due to aeration because data agree with those from similar experiments by others (28, 41).

The rate of leucine uptake was not measured in aged corn roots. However, the size of the soluble leucine pool in nonaerated aged roots was doubled as compared with that in nonaerated fresh roots (Table 35, Appendix). Data of Oaks also show more ^{14}C-leucine in the soluble pool of aged than of fresh corn roots (Figures 1 and 3 in citation 84). Toxin-treatment expanded the size of the soluble leucine pool in fresh roots but had no effect on the size of the pool in aged roots (Figure 19 and Table 35).

The data suggest that cell components have maximum capacities beyond which there is little or no stimulation by toxin. Certain systems

under normal cellular control in intact tissues can be stimulated by toxin-treatment. An analogous situation has been reported in squash hypocotyls infected with Hypomyces solani (31). Disease caused 96% stimulation of the MeG uptake rate when hypocotyls were freshly excised. As hypocotyls were aged from 1-28 hr, the rate of uptake increased in both healthy and diseased tissues. By 28 hr, there was only 3% stimulation of uptake rate in diseased hypocotyls when compared with the control rate (31).

Based on kinetics of MeG uptake by healthy and diseased squash hypocotyls, Hancock (31) proposed that infection induced a high affinity MeG carrier which was not present in healthy tissue. Since toxin also stimulated MeG uptake, similar analysis should be performed on the kinetics of MeG uptake by corn roots. Rates of accumulation of NO_3, Na, Cl, and leucine by toxin-treated corn roots could also be analyzed for evidence of possible induced carriers. Perhaps toxin activates new uptake systems instead of enhancing the affinity of constitutive carriers for their substrates. Such a study would require individual analysis of both known uptake mechanisms where applicable. Woolf-Hofstee plots of data on proposed mechanism 1 of NO_3 uptake (Figure 18) showed no evidence for a toxin-induced system but rather suggested toxin-enhancement of the normal carrier. However, this is speculative and additional experiments are required.

Permeability changes are characteristic of plants infected with fungi, bacteria, or viruses (124). The most common observation is

increased leakiness of tissues after infection. Some examples: 1) Sweet potato roots develop an increased capacity to absorb ^{32}P, ^{35}S, and ^{86}Rb as they age. Infection by Ceratocystis fimbriata inhibits the capacity for increased uptake (41). 2) Mung bean hypocotyls infected with Rhizoctonia solani lose electrolytes at a faster rate than do healthy tissues (58). 3) For the first 24 hr after inoculation of cucumber leaves with Pseudomonas lachrymans, the rate of electrolyte loss was slower than that from healthy leaves. (One possible explanation is presence of a stimulatory pathogen-produced substance with action similar to that of HC-toxin.) After 24 hr, infected leaves lost more electrolytes than did healthy controls (127). 4) Pepper leaves infected with Xanthomonas vesicatoria lost electrolytes faster than did healthy leaves (8). 5) When ^{32}P-containing carnation leaves were incubated in a bathing solution with Pseudomonas caryophylli, a pathogen, more ^{32}P was lost than when leaves were incubated in the presence of Corynebacterium spp., a nonpathogen (5). 6) When leaves of pepper plants were inoculated with tobacco etch virus, roots, but not leaves, lost more electrolytes than healthy controls or than pepper varieties which were susceptible but did not express the wilt symptom (27). 7) Bacteriophage infection of Escherichia coli caused ^{42}K and ^{28}Mg to leak from cells within a few minutes (111).

In addition to infective entities, many microbial toxins purported to be involved in plant disease are thought to alter cell membrane permeability (2, 83). Most toxic metabolites are nonspecific with respect

to plant species (2, 83) and possible roles in disease development have not been demonstrated. However, HV-toxin specifically disrupts membranes of only those oat varieties which are susceptible to the producing fungus (103, 105). There is abundant evidence that the toxin is required for disease initiation and disease development (103, 105).

In contrast to disease-induced leakiness, there are several reports of increased uptake or translocation in infected plant tissues. The most clearly defined case of increased uptake in diseased tissue is that of the MeG-squash hypocotyl system (31) described above. Tissues adjacent to lesions contained no fungus. They were apparently healthy as measured by apparent free space, plasmolysis, leakage of amino acids, permeation of urea, and uptake of glycerol (30). However, as noted above, these same tissues had an increased capacity to accumulate MeG (31). Another well-known case is crown gall, in which tumor cells accumulated twice as much K, PO_4, and atabrine from the growth medium as did normal cells growing at the same rate (128). Under the conditions used, uptake rates by both cell types were insensitive to temperature. Still other examples of disease-induced stimulation of uptake are known: 1) There was greater accumulation of ^{35}S, ^{32}P, and ^{14}C-sucrose from external media by diseased leaves than by healthy leaves in 19 different diseases (131). In 6 diseases accumulation was decreased in diseased leaves and in 6 others there was no change. 2) Infection of tomatoes with curly-top virus caused an increase in Ca uptake and a decrease in P and S uptake.

Efflux was not affected by disease (84). 3) Several reports indicate that there is rapid translocation of organic materials from healthy to infected tissues (14, 49, 63). There is also evidence that rust-infected leaves accumulate more ^{32}P and ^{35}S by long distance transport than do healthy leaves (26, 86). It is possible that at least some of these effects are caused by the presence of stimulatory pathogen-produced substances such as HC-toxin. In none of these cases has such a substance been found.

A question not considered in this thesis is whether the stimulatory effects of toxin are links in the chain of events which lead to cell death or whether stimulatory effects are independent and are later masked as inhibition becomes evident. It should be noted that stimulation (at low toxin concentrations) is specific to susceptible corn and that enhanced physiological activity is characteristic of most plant infections (105). This lends credibility to the suggestion that stimulatory effects of toxin are an important, and perhaps critical or even initial, part of the disease syndrome.

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APPENDIX

APPENDIX

Effect of Toxin on the Soluble Leucine Pool in Corn Roots

There were several early experiments to determine the effects of HC-toxin on uptake of ^{14}C-labelled leucine by corn roots, in addition to experiments described in the main body of the thesis. Results of these early experiments were confirmed by later data (Figure 19 and Table 30). Several of the early experiments have little or no meaning in relation to the main body of work. However, some of the data are of interest, and are included in an appendix to preserve their record.

In all these experiments, roots were exposed to toxin for 4 hr, then were incubated in ^{14}C-leucine for 2-3 hr. Ethanol soluble materials were extracted and counted. Thus, these experiments measured the size of the soluble leucine pool, as defined by Oaks (80). Results show that 4 hr toxin-treatment caused an increase in uptake of leucine into ethanol-soluble and insoluble root fractions (Table 32). A 2 hr treatment may have caused slight stimulation in the size of the leucine pool. A toxin-induced increase in the size of the soluble leucine pool has been shown kinetically (Figure 19). The data do not show whether increased soluble leucine results from increased uptake from an

Table 32. Effect of Toxin on Leucine Content in Ethanol-Soluble and Insoluble Fractions from Corn Roots

Seedlings were placed in 30 ml White's solution with or without toxin in 15 cm petri dishes. Toxin preparation gave 50% inhibition of susceptible root growth at 1.0 μg/ml. Root sections (1 cm) were excised 2 mm behind the root tip and incubated 2.5 hr in 1 mM leucine labelled with 0.25 μc ^{14}C-1-leucine/ml. There were 5 replicates of each treatment. Time required for leucine treatment and handling was 3.5 hr, which resulted in totals of 5.5 and 7.5 hr from beginning of toxin-treatment until experiment was terminated.

Fraction	Toxin Concn.	Toxin-Treatment Time			
		2 hr		4 hr	
		Activity/ 5 roots	Toxin-Induced Stimulation	Activity/ 5 roots	Toxin-Induced Stimulation
	μg/ml	cpm	%	cpm	%
Ethanol-Soluble	0	1316	...	1420	...
	1	1379	4	1832	23
	5	1469	12	2280	61
Ethanol-Insoluble	0	274	...	278	...
	1	274	0	376	35
	5	244	...	360	29

extracellular source or from increased release from an intracellular source. Increased leucine content of the ethanol-insoluble fraction could result from an affect on protein synthesis or from higher concentration of ^{14}C than ^{12}C-leucine in the soluble pool after toxin treatment. Later experiments suggest that increased pool size results from an effect of toxin on uptake rate (Table 31). Increased incorporation of ^{14}C-amino acid is probably not caused by a direct effect of toxin on protein synthesis (Figure 12), but rather by a higher concentration of ^{14}C-amino acid in the protein precursor pool. Certain growth regulators are known to enhance amino acid incorporation by increasing the size of the precursor pool (116).

Data in Table 33 are additional evidence that 2 hr toxin-treatment increases the size of the soluble leucine pool. Note that 3.5 hr elapsed from the time that roots were removed from toxin until the roots were placed in ethanol. Exposure of roots to toxin for 16 hr resulted in a 93% increase in amount of ethanol-soluble leucine present. This increase does not necessarily result from active accumulation because roots were given only 1 rinse in ^{12}C-leucine before being placed in ethanol. Brief rinsing may not remove all leucine absorbed passively across a leaky membrane. Later experiments showed that increased absorptive capacity of roots after 4 hr toxin-treatment resulted from active accumulation.

Root sections (1 cm long) taken 2 mm behind the root tip were used for all these experiments because this is the most active portion

Table 33. Effect of Toxin-Treatment Time on Size of the Leucine Pool in Corn Roots

Seedlings were placed in White's solution with or without toxin. Root sections (1 cm) were excised and held in ^{14}C-leucine 2.5 hr. The toxin preparations used in experiments 1 and 2 caused 50% inhibition of susceptible corn roots at 1.0 and 0.2 μg/ml, respectively. In experiment 1, concentrations of 5, 25, and 125 μg/ml gave essentially the same results for all treatment times. In experiment 2, toxin concentration was 2 μg/ml. There were 4 replicates of each treatment. Time required for handling and leucine treatment was 3.5 hr, giving total elapsed times from initial toxin exposure of 4.0-19.5 hr.

Expt. No.	Toxin-Treatment Time	Activity/5 Root Sections		Stimulation by Toxin
		Control	Toxin	
	hr	cpm	cpm	%
1	0.5	1099	1071	...
	1.0	1196	1301	8
	2.0	1125	1409	25
2	4.0	903	1274	41
	8.0	701	1093	56
	16.0	694	1339	93

of the root (130). It is not likely that the physiological state or toxin-sensitivity of this area of the root would change with seedling age, but these possibilities were checked experimentally, with the results shown in Table 34. Seedling age did not affect the percentage stimulation of leucine taken up by toxin-treated roots.

As plant tissues age, they are known to become more active in uptake and other physiological functions (28, 31, 80). Infection has been reported to have a similar effect (31). Results of an experiment (Table 35) show that control roots excised prior to toxin-treatment time contained twice as much soluble leucine than did roots excised just prior to leucine exposure. These roots had aged 4-5 hr before exposure to ^{14}C-leucine. Toxin-treatment did not increase the soluble leucine content of aged roots, but did increase the leucine content of freshly excised roots. Increased activity after aging was also observed in relation to uptake of NO_3 (Table 14) and PO_4 (Table 24).

Results in Table 35 suggest that a signal from the seed or shoot suppresses the aging process in intact roots and that when this signal is removed by excision, the aging process begins. Such a signal could be required for toxin action. Results of another experiment (Table 36) suggest that the effect of removing the coleoptile or seed is qualitatively but not quantitatively the same in the root as excising the root. Roots of seedless or coleoptileless seedlings contained more leucine but were stimulated less by toxin than roots of intact seedlings. It is notable that gibberellins (GA_3 and GA_4), which normally stimulate

Table 34. Age of Seedlings in Relation to Size of the Leucine Pool in Toxin-Treated and Control Corn Roots

Seedlings were placed 4 hr in White's solution with or without toxin (2 μg/ml). Toxin ED_{50} was 0.2 μg/ml. Root sections (1 cm) were held in ^{14}C-leucine 2.5 hr.

Seedling Age	Activity/5 Root Sections		Stimulation by Toxin
	Control	Toxin	
hr	cpm	cpm	%
48	856	1253	47
60	827	1267	53
72	1031	1605	56

Table 35. Effect of Toxin on Size of the Leucine Pool in Aged and Fresh Corn Roots

Tissues were placed 4 hr in White's solution with or without toxin. Toxin ED_{50} was 1.0 µg/ml. Aged roots were excised before toxin-treatment; fresh roots were excised after toxin-treatment. Root sections were held in ^{14}C-leucine 2.5 hr.

Toxin Concn.	Aged		Fresh	
	Activity/ 5 Roots	Toxin-Induced Stimulation	Activity/ 5 Roots	Toxin-Induced Stimulation
µg/ml	cpm	%	cpm	%
0	3704	...	1624	...
5	3719	0	2463	52
25	3754	1	2557	57

Table 36. Effect of Coleoptile or Seed Removal on Toxin Stimulation of the Leucine Pool in Roots of Corn Seedlings

Seedlings were placed 4 hr in White's solution with or without toxin (2 μg/ml). Toxin ED_{50} was 0.2 μg/ml. Where indicated, seeds or coleoptiles were removed prior to toxin-treatment. Roots (1 cm) were excised and held in ^{14}C-leucine 2.5 hr.

Seedling Preparation	Activity/5 Root Sections		Stimulation by Toxin
	Control	Toxin	
	cpm	cpm	%
Intact	1151	1469	29
Coleoptile Removed	1402	1606	14
Seed Removed	1333	1532	15

cucumber hypocotyl elongation, fail to do so if cotyledons are removed (47). The degree of GA-stimulation was directly proportional to the area of the cotyledon.