Helminthosporium maydis T Toxin Decreased Calcium Transport into Mitochondria of Susceptible Corn

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ABSTRACT

The effects of purified Helminthosporium maydis T (HmT) toxin on active Ca\(^{2+}\) transport into isolated mitochondria and microsomal vesicles were compared for a susceptible (T) and a resistant (N) strain of corn (Zea mays). ATP, malate, NADH, or succinate could drive \(4^\text{Ca}^{2+}\) transport into mitochondria of corn roots. \(4^\text{Ca}^{2+}\) uptake was dependent on the proton electrochemical gradient generated by the redox substrates or the reversible ATP synthetase, as oligomycin inhibited ATP-driven \(4^\text{Ca}^{2+}\) uptake while KCN inhibited transport driven by the redox substrates. Purified native HmT toxin completely inhibited \(4^\text{Ca}^{2+}\) transport into T mitochondria at 5 to 10 nanograms per milliliter while transport into N mitochondria was decreased slightly by 100 nanograms per milliliter toxin. Malate-driven \(4^\text{Ca}^{2+}\) transport in T mitochondria was frequently more inhibited by 5 nanograms per milliliter toxin than succinate or ATP-driven \(4^\text{Ca}^{2+}\) uptake. However, ATP-dependent \(4^\text{Ca}^{2+}\) uptake into microsomal vesicles from either N or T corn was not inhibited by 100 nanograms per milliliter toxin. Similarly, toxin had no effect on proton gradient formation (\(14^\text{C}\)methylamine accumulation) in microsomal vesicles. These results show that mitochondrial and not microsomal membrane is a primary site of HmT toxin action. HmT toxin may inhibit formation of or dissipate the electrochemical proton gradient generated by substrate-driven electron transport or the mitochondrial ATPase, after interacting with a component(s) of the mitochondrial membrane in susceptible corn.

The fungus HmT\(^1\) causes leaf blight in corn with Texas male- sterile cytoplasm (T) while maize with nonsterile or normal (N) cytoplasm and the same nuclear background is resistant to the disease (4; 14, 15). H. maydis race T produces a toxin (HmT toxin) which causes multiple effects in susceptible (T) plants (1, 4, 5, 8, 9, 20, 23, 24, 27). Most of the effects suggest that the primary site of action of the HmT toxin is the mitochondrion (4), but the plasma membrane may also be directly affected. For example, the HmT toxin depolarizes the membrane potential (19) and inhibits ion uptake into T corn roots (18).

The mode of toxin action in T mitochondria is puzzling as the toxin can exert uncoupling effects (1) and also inhibit electron transport (8). For example, in the presence of the HmT toxin, malate-dependent respiration (\(O_2\) uptake) in T mitochondria is inhibited, while NADH (20, 23, 24) or succinate-dependent (23, 24) respiration is increased and uncoupled. Inhibition by toxin of malate-dependent DCPIP reduction in isolated mitochondria suggested that the HmT toxin specifically inhibits the electron transport chain at the first complex associated with NADH dehydrogenase activity (8).

Recently, Kono and Daly (13) have purified and characterized the toxin produced by \(H. \text{maydis}\) race T. They reported that race T toxin consists of a mixture of \(C_{35}\) to \(C_{45}\) polyketol each with apparently identical host-selectivity and toxicity. The empirical formula of the principal component is \(C_{69}H_{58}O_{13}\) (768 D). Availability of such a purified toxin preparation allows comparisons of data from several laboratories using standardized toxin concentrations. Furthermore, a purified toxin preparation is crucial for elucidating the toxin mode of action.

To understand the toxin mode of action and determine its primary site of action, we have examined the effect of purified HmT toxin on energy-dependent ion transport in isolated mitochondria and microsomal vesicles. We wanted to determine whether the toxin affected various membrane systems capable of generating ion or proton gradients in susceptible corn as suggested by Daly and Barna (5). We also wanted to determine the site of toxin action in mitochondria. This paper illustrates that an \textit{in vitro} approach and direct ion transport assays can provide some answers to these questions. HmT toxin at low concentrations decreased active \(\text{Ca}^{2+}\) transport into isolated mitochondria of T but not N corn. A primary site of toxin action appears to be the mitochondrial membrane as active ion transport across the microsomal vesicles from susceptible corn was not affected by the toxin. A preliminary report of these findings has been presented (12).

MATERIALS AND METHODS

Isolation of Mitochondria and Microsomal Vesicles. Corn seeds (Zea mays W64AN and W64AT) were surface sterilized in 0.1% NaClO\(_2\), rinsed in distilled H\(_2\)O, and germinated in the dark on moist cheesecloth over 0.5 mm CaSO\(_4\). The entire roots of 3- to 4-d-old seedlings were excised and homogenized using a medium to tissue ratio of 10 ml/g fresh weight tissue. The homogenization medium was 250 mm sorbitol, 25 mm Hepes-BTP (pH 7.3), 3 mm EGTA, 0.5% BSA, 1 mm phenylmethylsulfonyl fluoride, and 1 mm DTT. The homogenate was filtered through two layers of wetted cheesecloth and was centrifuged at 1500g for 15 min. The pellet of cell walls, nuclei, and cell debris was discarded and the supernatant was centrifuged at 6000g for 15 min. The pellet from this centrifugation was en-
riched in mitochondria and was gently resuspended in 250 mM sorbitol, 2.5 mM Hepes-BTP (pH 7.4), and 1 mM DTT. The 6000g supernatant was centrifuged at 60,000g for 30 min to yield a pellet of crude microsomal membranes. The microsomal pellet which includes tonoplast, ER, Golgi, and plasma membrane vesicles was resuspended in the resuspension buffer described above.

Roots were used in our experiments since there is more information about their membrane transport properties than shoots. Comparisons of our results with those in the literature (23, 24) show that mitochondria isolated either from roots or shoots of Texas male-sterile corn seedlings show similar sensitivity to the Hmt toxin.

Protein Determinations. Proteins were precipitated with cold 10% TCA and quantified using the Lowry method, with BSA as the standard (16).

Calcium Transport. Calcium uptake was measured by determining the relative amount of 45Ca2+ association with either mitochondria or microsomal vesicles using a method similar to that of Dieter and Marme (6). The standard mitochondria-containing reaction mixture was 25 mM Hepes-BTP (pH 7.0), 5 mM K2HPO4, 0.2 mM 45CaCl2, (1–1.5 μCi 45Ca), 0.25 mg/ml mitochondrial protein, plus either 4 mM succinate, malate, or 0.1 mM NADH. ATP-dependent Ca uptake included 3 mM ATP-Tris and 3 mM MgSO4. For microsomal vesicles, the reaction mixture was 25 mM Hepes-BTP (pH 7.0), 3 mM MgSO4, 3 mM ATP, 1 to 1.5 mg protein/ml, 10 μM CaCl2, (1–1.5 μCi 45Ca), and 5 μg/ml oligomycin. Total volume of reaction mixtures was 0.5 or 1.0 ml.

Purified native Hmt toxin was dissolved in DMSO and aliquots added to yield final toxin concentrations of 1 to 1000 ng/ml. Equivalent amounts of DMSO were added to controls. The final DMSO concentration was less than 0.5% which had no effect on Ca2+ or H+ transport. Disposable glassware was used in the assays to prevent cross contamination of toxin (23).

Reactions were conducted at 21°C to 23°C and were started by adding mitochondria or vesicles. Reaction times usually varied between 10 and 15 min, after which duplicate 0.1-ml aliquots were filtered directly onto 0.45-μm pore size Millipore filters moistened with rinse solution (250 mM sucrose, 2.5 mM Hepes-BTP (pH 7.4), plus either 2 mM CaCl2 for mitochondria or 0.1 mM CaCl2 for microsomal vesicles). After the aliquots were filtered, the filters were rinsed with 2 ml of cold rinse solution. The amount of 45Ca2+ taken up was determined by liquid scintillation counting. Active calcium transport was the difference between uptake with and without an exogenous energy source and was expressed on a nmol/mg protein basis.

Control values for Ca2+ transport into isolated mitochondria varied from one experiment to another, and sometimes varied between mitochondria isolated from N and T corn roots. Though the variation between N and T mitochondria could be an intrinsic difference, most of the variability appeared to be dependent on the relative population of coupled mitochondria. Since active Ca2+ transport occurred mainly in well-coupled mitochondria, toxin effects on Ca2+ transport reflected activities of those mitochondria. Sometimes mitochondria gradually lost the ability to actively transport Ca2+ 1 h after isolation. Experiments were therefore conducted immediately after mitochondria preparation and activities were expressed as per cent of the control which was measured at the beginning and the end of all experiments.

Proton Gradient Formation Assay. The accumulation of CH3NH3+ (methylamine) inside microsomal vesicles reflects formation of a proton gradient (acid inside). The procedure used was that of Churchill and Sze (3). About 1 to 1.5 mg/ml protein was used per reaction mixture.

Chemicals. 45CaCl2 and [14C]methylamine were obtained from New England Nuclear. Sodium-ATP was purchased from Boehringer-Mannheim and converted to Tris-ATP with Dowex ion exchange resin. Other chemicals were reagent grade.

Corn Seeds and Hmt Toxin. Both the seeds (Zea mays W64ATMS and W64AN) and the purified Hmt toxin were generous gifts of J. M. Daly, University of Nebraska, Lincoln, NE.

RESULTS

Ca2+ Uptake into Mitochondria. Redox Substrates. Initially the time course of malate-dependent Ca2+ transport into mitochondria isolated from N or T corn roots was determined. Ca2+ influx into mitochondria was dependent on the proton electrochemical gradient as explained in "Discussion" (and Fig. 7). Malate oxidation by malate dehydrogenase yields NADH which is oxidized by the internal NADH dehydrogenase of site 1. Malate-induced Ca2+ association with mitochondria reflected Ca2+ accumulation as A23187 (a Ca2+ ionophore) immediately decreased Ca2+ levels in the mitochondria (Fig. 1, inset). Maximum net Ca2+ uptake was reached at 10 to 20 min after which there was a time-dependent decrease in Ca levels (Fig. 1). The decrease of Ca2+ levels after 20 min could be caused by a Ca2+ extrusion pump triggered perhaps by the high internal Ca2+ concentration. In the presence of 5 ng/ml toxin, malate-dependent Ca2+ transport was partially inhibited in mitochondria from T but not N corn (Fig. 1). Since net Ca2+ uptake was maximum around 10 min with or without the toxin, subsequent experiments of Ca2+ uptake driven by redox substrates were measured after a 10-min incubation.

![Figure 1](https://example.com/figure1.png)

FIG. 1. Hmt toxin effect on the time course of malate-dependent Ca2+ uptake into mitochondria isolated from N (top panel) and T (bottom panel) corn roots. Final concentrations of reactants were 0.2 mM 45CaCl2, 25 mM Hepes-BTP at pH 7.2, 5 mM K2HPO4, 0.2 mg mitochondrial protein either without malate (Δ), with 4 mM malate (○), or with 4 mM malate plus 5 ng/ml Hmt toxin (■). Inset shows decrease of net Ca2+ uptake in either N or T mitochondria induced by the ionophore A23187 (5 μM). Data are from one experiment. Two data points at each time are from duplicate samples.
Malate-dependent Ca\(^{2+}\) uptake into T and N mitochondria were differentially sensitive to HmT toxin concentration. At 10 ng/ml, HmT toxin decreased Ca\(^{2+}\) uptake by about 90% in T mitochondria, whereas Ca\(^{2+}\) uptake into N mitochondria decreased less than 20% by 100 ng/ml toxin (Fig. 2).

The effect of HmT toxin on succinate-dependent Ca\(^{2+}\) uptake was examined to determine whether the toxin affected site II of the electron transport chain. Figure 3 shows that succinate-driven Ca\(^{2+}\) uptake into T mitochondria was decreased by HmT toxin preferentially. At high toxin concentration of 1000 ng/ml, Ca\(^{2+}\) uptake into T mitochondria was completely inhibited, whereas Ca\(^{2+}\) uptake into N mitochondria was only 40% inhibited (Fig. 3). Partial sensitivity of N mitochondria to high toxin concentrations has been observed in O\(_2\) uptake studies of Payne et al. (23). Low levels of toxin (e.g., 1-5 ng/ml) sometimes appeared to stimulate Ca\(^{2+}\) uptake in N mitochondria (Fig. 3). The significance of this observation is unclear.

When NADH was used to drive Ca\(^{2+}\) uptake, we found similar results. Toxic at 100 ng/ml had no effect on NADH-dependent Ca\(^{2+}\) uptake in N mitochondria but the same level of toxin inhibited completely Ca\(^{2+}\) uptake into T mitochondria (Table I). NADH-driven Ca\(^{2+}\) uptake is dependent on the redox electron transport chain as KCN inhibited completely Ca\(^{2+}\) uptake in both N and T mitochondria (Table I). In contrast to mammalian mitochondria, plant mitochondrion has an NAD(P)H dehydrogenase associated with the outer face of the inner membrane capable of oxidizing exogenous NADH (see Fig. 7). This enzyme is specific for the β-hydrogen of NADH and feeds electrons directly to ubiquinone by passing the rotenone-sensitive site or site I (22).

**ATP.** To test whether toxin affected components other than those of the redox electron transport chain, the effect of toxin on ATP-driven Ca\(^{2+}\) uptake was studied. Initially, the conditions for ATP-dependent Ca\(^{2+}\) transport was determined. Net Ca\(^{2+}\) uptake into mitochondrion was maximal after 20 min incubation (Fig. 4a). However, unlike malate-driven Ca\(^{2+}\) uptake, Ca\(^{2+}\) levels remained steady for at least 60 min, perhaps because Mg prevented Ca\(^{2+}\) efflux (6) and high NAD\(^{+}\) levels promoted Ca\(^{2+}\) influx (2). Ca\(^{2+}\) influx in N or T mitochondria was maximum near 3 mM ATP and inhibited by oligomycin (Fig. 4b). ATP-driven Ca\(^{2+}\) uptake did not show a clear pH dependence (Fig. 4c). Subsequent assays of ATP-dependent Ca\(^{2+}\) uptake were conducted at pH 8.0 with 3 mM ATP.

Assessment of ATP-dependent Ca\(^{2+}\) uptake in N and T mitochondria revealed that toxin at 10 ng/ml immediately decreased Ca\(^{2+}\) uptake into T mitochondria, whereas transport into N mitochondria was slightly decreased over an 8-min assay period (Fig. 5). The effect of HmT toxin concentration on ATP-dependent Ca\(^{2+}\) uptake was similar to those of the redox substrates. At 10 ng/ml toxin, Ca\(^{2+}\) uptake into T mitochondria was inhibited by 80% while transport into N mitochondria was inhibited (Fig. 6). Thus, toxin decreased Ca\(^{2+}\) transport into T mitochondria driven by either redox substrates or ATP.

**Inhibitors.** The effect of inhibitors on Ca\(^{2+}\) transport support the idea that the redox proton pump or the ATPase can separately drive Ca\(^{2+}\) uptake (2). Succinate-driven Ca\(^{2+}\) uptake was completely inhibited by 1 mM KCN, an inhibitor of the electron transport chain, whereas ATP-driven Ca\(^{2+}\) uptake was totally insensitive to cyanide (Table II). However, oligomycin, an inhibitor of the mitochondrial ATPase, inhibited ATP-dependent but not succinate-dependent Ca\(^{2+}\) uptake into either N or T mitochondria (Table II). These results with maize roots are similar to those of Ca\(^{2+}\) transport into mitochondria isolated from maize shoots (7, 10).

Ca\(^{2+}\) or H\(^{+}\) Transport into Microsomal Vesicles. To determine whether the HmT toxin affected active ion pumps in nonmitochondrial membranes from T corn roots, the effect of toxin on ATP-dependent Ca\(^{2+}\) or methylamine uptake into microsomal vesicles was studied. Toxic concentration (100 ng/ml) that inhibited Ca\(^{2+}\) uptake into T mitochondria by 80 to 100% (Figs.

**Table I. Effect of HmT Toxin on NADH-Dependent Ca Uptake into Mitochondria from N or T Corn Roots**

<table>
<thead>
<tr>
<th>HmT Toxin (ng/ml)</th>
<th>NADH-Dependent Ca Uptake (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>94.5 (100)</td>
</tr>
<tr>
<td>10</td>
<td>91.3 (97)</td>
</tr>
<tr>
<td>100</td>
<td>94.2 (100)</td>
</tr>
<tr>
<td>0 + KCN (1 mM)</td>
<td>2.8 (3)</td>
</tr>
</tbody>
</table>

*Ca uptake was measured after 15 min reaction time in a mixture similar to that described in the legend to Figure 1, except 0.1 mM NADH was the redox substrate. Result from one experiment.
Fig. 4. ATP-driven Ca\(^{2+}\) uptake into mitochondria isolated from N and T corn roots. a, Time course of Ca\(^{2+}\) uptake into N mitochondria. Reaction mixture included 25 mM Hepes-BTP (pH 7.0), 3 mM MgSO\(_4\), 0.1 mg protein, 0.2 mM CaCl\(_2\), 5 mM K\(_2\)HPO\(_4\), with or without 3 mM ATP. b, Effect of ATP concentration on Ca\(^{2+}\) uptake into N (○) or T (△) mitochondria at 8 min. Oligomycin (5 μg/ml) inhibited Ca\(^{2+}\) uptake into N (○) and T (△) mitochondria at 8 min.

2, 3, and 6) had little or no effect on Ca\(^{2+}\) uptake into either N or T microsomal vesicles (Table III). Similarly, ATP-driven [\(^{14}\)C]methylamine uptake into either N or T microsomal vesicles was completely insensitive to the HmT toxin at 10 or 100 ng/ml (Table IV). Methylamine accumulates inside vesicles when a pH gradient (acide inside) is generated across the membrane vesicles (3). Churchill and Sze (3) have previously shown that ATP-dependent methylamine uptake reflected activity of a proton-pumping ATPase enriched in vacuolar membranes and perhaps ER. Lack of HmT toxin effect on active H\(^{+}\) or Ca\(^{2+}\) transport across the microsomal vesicles suggests that nonmitochondrial membranes are not sites of toxin action.

**DISCUSSION**

This paper demonstrates an *in vitro* approach and a transport assay aimed at understanding the effect of HmT toxin on the transport function of various subcellular membranes. A very sensitive test of mitochondrial membrane function is active Ca\(^{2+}\) uptake which is a relative measure of the proton electrochemical gradient generated by proton pumping (2). Malate- or succinate-dependent Ca\(^{2+}\) uptake reflects activity of the redox proton pump while ATP-driven Ca\(^{2+}\) uptake reflects activity of proton pump-

Fig. 5. HmT toxin effect on the time course of ATP-dependent Ca\(^{2+}\) uptake in N (upper panel) or T (lower panel) corn mitochondria. Reactions were conducted either in the absence of ATP (○), presence of 3 mM ATP (●), or presence of 3 mM ATP plus 10 ng/ml HmT toxin (△).

Fig. 6. Effect of HmT toxin concentration on ATP-dependent Ca\(^{2+}\) uptake in N and T corn mitochondria. Reaction mixtures were similar to those described in the legend to Figure 4a. In the absence of toxin, Ca\(^{2+}\) uptake into N and T mitochondria was 45.0 ± 9.0 and 17.6 ± 1.7 nmol/mg protein·15 min. Data are the average of three experiments. Bars are se.

ing by the reversible ATP synthetase (or ATPase) (Fig. 7). In support of the Chemiosmotic hypothesis (21), the redox electron transport chain or ATP hydrolysis results in proton extrusion from the mitochondria generating a membrane potential (negative inside) and a pH gradient (basic inside). The electrical gradient drives Ca\(^{2+}\) transport into the mitochondria and, in the presence of phosphate, Ca\(^{2+}\) accumulates as the phosphate salt (2, 10, 28). Phosphate uptake is thought to be driven by the pH gradient. We have used Ca\(^{2+}\) uptake into mitochondria and ATP-dependent proton or Ca\(^{2+}\) transport into microsomal vesicles as assays to study toxin effects on membrane functions. These studies do not imply that toxin specifically alters Ca\(^{2+}\) transport. But changes in Ca\(^{2+}\) transport reflect changes in membrane functions which include activities of active transport mechanisms.
Table II. Effect of Various Substrates and Inhibitors on Ca Uptake into Mitochondria Isolated from N or T Corn Roots

<table>
<thead>
<tr>
<th>Additions</th>
<th>Ca Uptake</th>
<th>% (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>T</td>
</tr>
<tr>
<td>Succinate</td>
<td>100 (98.5 ± 12.4)</td>
<td>100 (74.8 ± 5.9)</td>
</tr>
<tr>
<td>Succinate + KCN</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Succinate + oligomycin</td>
<td>100</td>
<td>93</td>
</tr>
<tr>
<td>Succinate + A23187</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ATP</td>
<td>100 (45.0 ± 9.0)</td>
<td>100 (17.6 ± 1.7)</td>
</tr>
<tr>
<td>ATP + KCN</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ATP + oligomycin</td>
<td>8</td>
<td>20</td>
</tr>
</tbody>
</table>

Table III. Effect of HmT Toxin on ATP-Dependent Ca Uptake in Microsomal Vesicles from N or T Corn Roots

<table>
<thead>
<tr>
<th>Addition</th>
<th>ATP-Dependent Ca Uptake</th>
<th>% (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>T</td>
</tr>
<tr>
<td>None</td>
<td>3.02 (100)</td>
<td>3.36 (100)</td>
</tr>
<tr>
<td>HmT toxin (10 ng/ml)</td>
<td>3.59 (119)</td>
<td>3.12 (93)</td>
</tr>
<tr>
<td>HmT toxin (100 ng/ml)</td>
<td>3.16 (105)</td>
<td>2.67 (79)</td>
</tr>
<tr>
<td>A23187 (5 μM)</td>
<td>0.25 (8)</td>
<td>0.31 (9)</td>
</tr>
<tr>
<td>CCCP (5 μM)</td>
<td>2.20 (73)</td>
<td>1.86 (55)</td>
</tr>
</tbody>
</table>

Table IV. Effect of HmT Toxin on ATP-Driven Methylene Accumulation in Microsomal Vesicles from N or T Corn Roots

<table>
<thead>
<tr>
<th>Addition</th>
<th>ATP-Driven MeA Uptake</th>
<th>pmol/mg protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>T</td>
</tr>
<tr>
<td>None</td>
<td>30.5 (100)</td>
<td>13.4 (100)</td>
</tr>
<tr>
<td>HmT toxin (10 ng/ml)</td>
<td>28.2 (93)</td>
<td>15.8 (116)</td>
</tr>
<tr>
<td>HmT toxin (100 ng/ml)</td>
<td>23.5 (77)</td>
<td>13.4 (100)</td>
</tr>
<tr>
<td>CCCP (5 μM)</td>
<td>6.4 (21)</td>
<td>3.1 (23)</td>
</tr>
</tbody>
</table>

as well as membrane integrity. The assay provides more direct information about membrane transport functions than O2 uptake studies of isolated mitochondria. The results presented in this paper will provide a basis for designing future experiments aimed at testing toxin action more specifically. This type of approach is widely applicable to studies where an understanding of toxin, hormone, or herbicide effects on membrane transport is sought.

Our results suggest that a primary site of HmT toxin action is on mitochondrial membranes and not on microsomal membranes (which include tonoplast and plasma membranes) of susceptible corn. HmT toxin decreased active Ca2+ transport into mitochondria isolated from T but not N corn roots (Figs. 2, 3, 6; Table I) or oat roots (data not shown). However, the HmT toxin had little or no effect on either ATP-dependent Ca+ transport (Table III) or H+ transport (Table IV) into microsomal vesicles isolated from T or N corn roots. Our laboratory (3) and Mandalia et al. (17) have shown the ATP-dependent methylamine uptake affected activity of a proton-pumping ATPase enriched in tonoplast vesicles and perhaps some ER vesicles. ATP-dependent Ca2+ transport in microsomal vesicles includes two types of Ca2+ transport mechanisms (25, 26) which could be localized on tonoplast, ER, Golgi, and plasma membrane vesicles (Sze, unpublished results; 6). The physiological finding of mitochondria being a site of toxin action is consistent with the genetic studies showing cytoplasmic inheritance. Molecular studies have revealed differences in the restriction endonuclease analyses of DNA from mitochondria, but not chloroplast, between N and T corn (14, 15). However, the possibility of chloroplast membranes being another site of toxin action (4, 5) is not eliminated.

To localize the site of toxin action in the mitochondria, we functionally dissected proton pumping driven by the redox electron transport chain and the reversible ATP synthetase by using various substrates. To our initial surprise, we found that Ca2+ transport into T mitochondria was decreased by the HmT toxin regardless of the substrate (i.e. malate, succinate, NADH, or ATP) (Figs. 2, 3, and 6; Table I). One interpretation of these results is that the toxin acts on multiple sites rather than at one major site of the inner mitochondrial membrane, but other interpretations are possible as discussed later.

One criterion to distinguish between primary versus secondary sites of action is to determine the activity affected by the lowest toxin concentration. The effects of toxin concentration on Ca2+ transport driven by either malate, succinate, or ATP were not drastically different (Figs. 2, 3, and 6). The toxin concentration required to decrease Ca2+ uptake by 50% was approximately 2 to 5 ng/ml, similar to purified toxin concentrations required to inhibit various physiological and biochemical effects in susceptible corn (23, 24). Though, the relative amount of inhibition of Ca2+ transport varied for each toxin concentration from one experiment to another, malate-driven Ca2+ uptake (Fig. 2) was frequently more sensitive to toxin than either succinate (Fig. 3) or ATP-dependent Ca2+ uptake (Fig. 6). This observation suggests that there may be a specific toxin effect on the NADH

![Fig. 7. Scheme showing a possible mechanism of active Ca2+ uptake into corn mitochondria according to Mitchell's Chemiosmotic principle (21). ATP hydrolysis by the reversible ATP synthetase (F-F0) or oxidation of redox substrates by the electron transport chain results in H+ extrusion from the mitochondria generating a membrane potential (negative inside) and a pH gradient (basic inside). The electrical gradient drives Ca2+ uptake and, in the presence of phosphate, Ca2+ accumulates as Ca phosphate salt. It is not clear whether Ca2+ uptake is via a uniport or some other mechanism in corn mitochondria. Complex I, II, III, and IV refer to NADH-UQ oxidoreductase, succinate dehydrogenase, UQ-Cyt c oxidoreductase and Cyt c oxidase, respectively. Q and Fp indicate ubiquinones and flavoprotein, respectively.](https://www.plantphysiol.org)
dehydrogenase complex of site I. These results would agree with the conclusion reached by others (4, 8, 20, 23, 24) that the HmT toxin inhibits electron transport at site I.

However, other modes of toxin action have been suggested (4, 9) and need to be explored directly. For example, the toxin may act by one or more of the following ways depending on the specific redox substrate: (a) toxin induces ionophore effect in T mitochondria (1, 9, 23); (b) toxin inhibits the redox electron transport chain at site I (malate oxidation) (4, 9); and (c) toxin directly affects the proton-pumping ATPase of T mitochondria. We are testing these possibilities and have direct evidence of toxin-induced ionophore effect in T mitochondria. We recently reported that HmT toxin dissipated calcium gradients in T mitochondria similar to a calcium ionophore, A23187 (11). Though we do not understand how toxin induced dissipation of Ca\(^{2+}\) gradients, this effect would be consistent with our finding that HmT toxin decreased Ca\(^{2+}\) transport driven by different redox substrates as well as ATP in T mitochondria.

Acknowledgments—We are grateful to Dr. J. M. Daly for many helpful suggestions and generously providing the purified HmT toxin and corn seeds. We also want to thank Mrs. Steve Danko, Anton Novacky, and James Siedow for stimulating discussions.

LITERATURE CITED