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Reduction of Adenosine Triphosphate Levels in Susceptible Maize Mesophyll Protoplasts by *Helminthosporium maydis* Race T Toxin[1]

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ABSTRACT

Helminthosporium maydis **race T (HMT) toxin caused a reduction in the steady-state ATP levels when leaf mesophyll protoplasts isolated from maize containing Texas male-sterile (T) but not male-fertile (N) cytoplasm were incubated in the dark. At a toxin concentration 10 times the mean effectived dose for inhibition of root growth, the ATP levels began to fall in 30 to 90 seconds, fell by 50% in about 4 minutes, and reached 23% of the original levels in 100 minutes. This is faster than any previously observed response of whole cells or tissues to HMT toxin. In protoplasts incubated in the light, ATP levels were 25% higher than in the dark and were either unaffected or only slightly diminished by toxin. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosynthetic electron transport, overcame the effect of light on both toxin-treated and control protoplasts. Oligomycin, an inhibitor of mitochondrial ATP synthesis, mimicked the effects of toxin in the dark, in the light, and in the light plus DCMU, but it was not specific for T cytoplasm. During the first 24 hours of culture, ATP levels in control protoplasts increased in both the light and dark. In the dark, ATP was not detectable after 24-hour incubation in the presence of toxin, whereas in the light a substantial amount of ATP remained. Our results are compatible with the hypothesis that mitochondria *in vivo* are inhibited by HMT toxin. Other responses of cells and tissues to toxin can be explained in terms of reduced ATP levels.**

Helminthosporium maydis Nisikado and Miyake (*Bipolaris maydis* Shoemaker) [race T] produces a toxin (HMT toxin[3]) which selectively inhibits maize (*Zea mays* L.) lines containing Texas male-sterile (T) or closely related cytoplasms. Although the toxin causes many changes in the metabolism of T cytoplasm maize (12), the primary site of action is controversial. Evidence supporting two frequently proposed sites, the mitochondrion and the plasma membrane, has been reviewed in detail (12).

Mitochondria isolated from T but not male-fertile (N) maize are functionally and physically disrupted almost immediately after toxin treatment (1, 4, 23). Toxin causes swelling and increases the rate of NADH oxidation, inhibits malate-pyruvate oxidation strongly, and has variable effects on succinate oxidation. Oxidative phosphorylation by isolated mitochondria is inhibited and hydrolysis of exogenous ATP is stimulated (4). *In vivo*, mitochondria of T but not N cells show ultrastructural changes within 5 to 15 min after exposure to toxin; the mitochondria swell, the cristae disappear, and the matrix stains less densely (1, 31).

Attempts to show that toxin causes loss of mitochondrial activity *in vivo*, however, has been inconclusive, and other evidence suggests a plasma membrane site of action. In one study, maize tissue treated with toxin showed no change in respiration and only a small drop in ATP levels (3), but Bednarski *et al.* (5) later found a 20 to 60% stimulation of O_2 uptake in leaves and coleoptiles, and a 50% decrease in acid-labile phosphate in roots, in toxin-treated T but not N maize. Uptake of certain ions was selectively inhibited, K^+ being most sensitive and PO_4^{3-} the least (11, 21). The membrane potential in maize root epidermal cells began to depolarize in 2 to 5 min after exposure to toxin, putatively due to an effect on an electrogenic pump (20, 22). These results led these authors to postulate a plasma membrane site of toxin action, alone or in addition to a mitochondrial site.

Our experiments were undertaken to determine whether mitochondrial activity is selectively inhibited by HMT toxin *in vivo*. We chose to measure steady-state ATP levels because the mitochondrion is a major site of ATP production. ATP has a high turnover rate in cells and thus its levels should respond quickly to mitochondrial damage, and ATP production is dependent on the integrity of the inner mitochondrial membrane, which has been shown (1) to undergo changes in morphology after toxin treatment. Isolated maize leaf mesophyll protoplasts were used because they can be manipulated easily, they circumvent problems of toxin permeability through intact tissue, and their response to toxin shows the same cytoplasm specificity seen in whole tissues (10, 24).

We report here that HMT toxin causes a rapid reduction in the steady-state cellular ATP concentration in T cytoplasm protoplasts. The results of experiments with light, DCMU, and oligomycin are consistent with a mitochondrial site of HMT toxin action in intact cells.

MATERIALS AND METHODS

H. maydis **Race T Toxin Preparation.** *H. maydis* race T toxin, the same preparation used by Earle *et al.* (10), caused 50% inhibition in the seedling root growth bioassay (30) at a concentration of 0.65 μg/ml, in the dark CO_2 fixation bioassay (6, 30) at 0.09 μg/ml, and 100% collapse of protoplasts at 0.13 μg/ml (10).

Protoplast Preparation and Treatment. Maize plants of inbred W64A in T and N cytoplasm were grown for 9 to 17 days and protoplasts were isolated from young leaves by the method of Earle *et al.* (10). After isolation, protoplasts were suspended in a

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[3] Abbreviations: SCM buffer: 0.5 M sorbitol, 10 mM $CaCl_2 \cdot 2H_2O$, 3 mM MEs-KOH (pH 6.0); HMT toxin: toxin from *Helminthosporium maydis* race T; ED_{50}: mean effective dose.

filter-sterilized solution (SCM buffer) containing 0.5 M sorbitol, 10 mM $CaCl_2 \cdot 2H_2O$, and 3 mM Mes, (pH 6.0) with KOH, counted on a Fuchs-Rosenthal hemocytometer, and their concentration adjusted to 1.5 to 1.8 $\times 10^5$ protoplasts/ml. Most experiments were done at a concentration of 1.71×10^5 protoplasts/ml. W64A N and W64A T protoplasts behaved similarly in all respects except for sensitivity to toxin.

For experiments involving treatment times of 2 h or less, 1 or 2 ml of protoplast suspension was incubated in either 40-ml Nalgene polyethylene centrifuge tubes or 25-ml Nalgene polycarbonate flasks. Protoplasts were allowed to equilibrate in the containers for 1 h prior to the first ATP measurement. Protoplasts to be incubated in the light were placed in loosely capped 25-ml transparent polycarbonate flasks, set on white blotter paper, and exposed to either normal laboratory light from fluorescent fixtures 2.5 m overhead (1,000 lux, 2.05×10^3 ergs cm^{-2} s^{-1}, or 15 μE m^{-2} s^{-1} between 400 and 700 nm), or to normal laboratory light supplemented with a G.E. "Daylight" 58-cm fluorescent lamp 40 cm above the protoplasts (1,300 lux, 20 μE m^{-2} s^{-1}).

For experiments involving treatment times greater than 2 h, protoplasts were prepared aseptically (10) and 0.5-ml aliquots plus an equal amount of either SCM buffer or filter-sterilized toxin in SCM buffer placed in 3.5-cm plastic Petri plates. The plates were sealed with Parafilm and incubated in the light (1,300 lux) or the dark. At each sampling time one plate of each treatment or control was opened and immediately used.

ATP Extraction Assay. To extract ATP, 100 μl of protoplast suspension was pipetted into an equal volume of fresh ice-cold 10% (w/v) trichloroacetic acid (Sigma Chemical Co.) in a test tube (125 × 16 mm) and immediately mixed using a Vortex mixer. ATP was assayed by a modification of the luciferin-luciferase technique of Kimmich *et al.* (17). Vials containing soluble extract from 50 mg of firefly lanterns, $MgSO_4$, and arsenate buffer (Sigma) were reconstituted with 5 to 7 ml of water. To one polyethylene scintillation vial was added 4 ml of ATP assay medium (25 mM Hepes and 25 mM $MgSO_4$ [pH 7.5] with 1 N NaOH), 25 or 50 μl of the protoplast-acid mixture, and finally 50 μl of reconstituted firefly lantern extract. The vial was capped, shaken thoroughly for 10 s, and placed in a Beckman model LS-100C scintillation counter set on the noncoincident mode. Thirty s after addition of the firefly lantern extract, the vial was counted for 30 s. Duplicate samples of each trichloroacetic acid-protoplast mixture were counted and the values averaged. Variation between counts of duplicates was less than 7%.

Reconstituted firefly lantern extract lost activity over time. The rate of loss was often nonlinear and varied between experiments. Reconstituting the firefly lantern extract on the day of use, leaving it at room temperature for 1 to 2 h, and filtering it 1 to 2 h before use reduced loss of activity to 3 to 4%/h. Standard ATP solutions were measured at regular intervals alternating with the unknowns, and values were corrected for loss of firefly lantern extract activity.

Plastic pipettes and containers seemed to cause less disruption of protoplasts than glass. Toxin residues adhered to plastic, so the containers were washed with detergent and rinsed in water, 1 N HCl, and 95% ethanol. It was critical to wash the scintillation vials twice with detergent between experiments.

Oligomycin (Sigma) was dissolved in 95% ethanol; DCMU (gift of P. Gregory, Dept. of Plant Breeding, Cornell Univ.) was dissolved in 95% ethanol + SCM buffer (1 + 16). Standard solutions of ATP, ADP, and AMP (Sigma) were made fresh each day in ATP assay medium.

RESULTS

Measurement of ATP in Protoplasts. Several methods of extracting ATP from protoplasts were compared (29). The method finally chosen, pipetting protoplasts into acid, was convenient, very reproducible, and gave the highest yields. The ATP in the protoplast-acid mixture was stable for at least 4 h. Trichloroacetic acid inhibited the assay significantly and so an appropriate amount was added to each standard.

Light emission was linearly proportional to the amount of ATP up to at least 12.5 pmol/vial. With this technique, the ATP in as few as 200 healthy protoplasts could be measured. Quenching due to protoplast debris was negligible, as determined with internal ATP standards.

This assay gave no response to either ADP or AMP alone. However, Slayman (27) has reported that ADP retards the exponential light emission due to ATP, and thus tends to increase the apparent ATP content of a sample when counted by the technique of Kimmich *et al.* (17). We also found that ADP increased the light emitted by a particular amount of ATP. Increasing the ADP/ATP ratio from 0 to 15 produced a 15% increase in cpm. AMP had no effect at similar ratios. Observed values of ADP/ATP ratios in highly inhibited plant tissues range from 0.4 to 6.0 (8, 9, 18, 26); therefore, it is unlikely that interference by ADP significantly alters our results or conclusions.

ATP Levels in Protoplasts. Steady-state ATP concentrations in protoplasts kept in the dark were constant or decreased slightly over an incubation period of 2 h (5–7 h after leaf excision). ATP levels in the light (1,000 or 1,300 lux) increased slightly over the same time period (Figs. 3–5). ATP levels within any given experiment were always higher in the light than in the dark (average increase in the light over dark; 25%, range 18–36%). A light intensity 10 times higher (14,000 lux) did not increase the ATP levels in the light further.

For comparisons between experiments, we attempted to find a suitable basis for quantification of steady-state ATP concentrations in protoplasts. We calculated ATP concentrations as fmol (10^{-15}mol)/protoplast, nmol/mg protein, and nmol/mg total Chl. Values per protoplast were the most reliable (29). Average values (± one SD, n = no. of samples) in dark-incubated protoplasts were 3.90 ± 1.48 fmol ATP/protoplast (n = 23), and in light-incubated protoplasts 5.04 ± 1.63 fmol/protoplast (n = 16). Protoplasts from plants younger than 12 days had more variable ATP levels than those from 12- to 17-day-old plants, when ATP was expressed as fmol/protoplast. For protoplasts from 12- to 17-day-old plants, average ATP values were 3.54 ± 0.60 fmol/protoplast (n = 14) in the dark and 4.43 ± 0.44 fmol/protoplast (n = 10) in the light.

For comparisons with published values, we estimated ATP on a molar basis. Assuming a typical protoplast volume of 1.3×10^{-8} ml (10) and confinement of the ATP to the cytoplasm, and a cytoplasm volume equal to 10% of the total cell volume, the average ATP concentration in freshly isolated protoplasts was 3.00 mM in the dark and 5.04 mM in the light. Published values range between 1.4 mM and 5.8 mM in maize, wheat, and barley leaves or roots (8, 9, 15, 18, 19, 26), assuming the cytoplasm contributes 0.04 ml/g fresh weight (18).

Effects of HMT Toxin on ATP Levels. A decrease in ATP concentrations in toxin-treated W64A T protoplasts was observable within 1.5 min (range for all experiments, 0.5 to 1.5 min, n = 6), and the ATP levels fell to 24% of the original levels (average for all experiments, 23 ± 4%, n = 12) in 100 min (Fig. 1). The time required for a 50% decrease in ATP levels was approximately 4 min (average for all experiments, 4.2 ± 1.6 min, n = 8).

Figure 2 is a dosage response curve for reduction of ATP levels in W64A T protoplasts exposed for 2 h to toxin in the dark. In this regime maximum inhibition was 77% and the ED_{50} was 0.18 μg/ml. A sample of purified HMT toxin (gift of J. M. Daly, Univ. of Nebraska; see 12) was tested in the same regime for ability to reduce ATP levels in protoplasts. This toxin preparation was three to six times more active on a dry weight basis than our partially purified toxin.

Toxin had little or no effect on ATP levels when the protoplasts were incubated in light (1,000–14,000 lux). In three experiments (Fig. 3), toxin had no effect in the light, whereas in five experiments it caused a 10 to 25% reduction in ATP levels. Our experi-

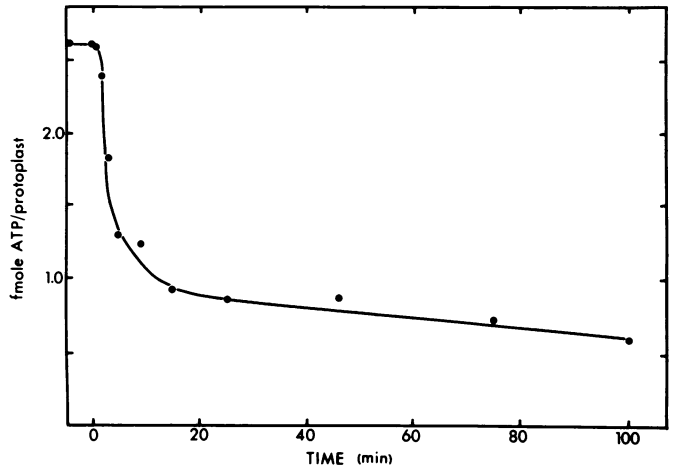

FIG. 1. Time course of ATP concentration in a suspension of W64A T maize leaf mesophyll protoplasts (1.71×10^5/ml) incubated in the dark in the presence of 6.2 μg HMT toxin/ml. Toxin was added at time 0.

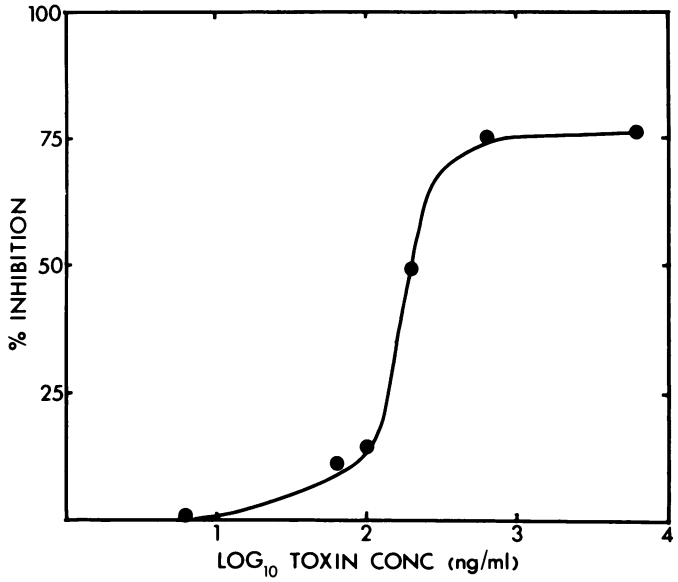

FIG. 2. Dosage response curve for W64A T protoplasts and HMT toxin. Protoplasts were incubated in the dark in 40-ml Nalgene centrifuge tubes and sampled before and after treatment for 2 h with different toxin concentrations. This graph incorporates the results of two experiments. The ATP concentrations in the control tubes were 3.20 and 3.80 fmol/protoplast and the protoplast concentrations were 1.36 and 1.50×10^5 protoplasts/ml, respectively.

ments were not able to resolve this variability; it was not correlated with age of the plants or light intensity. DCMU (2 μM) inhibited the ability of protoplasts in the light to maintain high cellular ATP levels in the presence of toxin (Fig. 3). DCMU alone caused the ATP levels in the light to fall to those in the dark.

ATP levels in W64A N protoplasts in the dark or light did not respond to 6.2 μg toxin/ml, and subsequent treatment with 2 μM DCMU gave the same result as DCMU alone (Fig. 4). Toxin concentrations as high as 46 μg/ml had no effect on W64A N protoplasts.

Toxin was inactivated by adjusting the pH to 12.0 and autoclaving for 1 h on each of 3 consecutive days, followed by readjustment of the pH to 6.0. At 40 μg/ml, the inactivated toxin did not inhibit T maize root growth or decrease ATP levels in T protoplasts.

Effect of Oligomycin on ATP Levels in Protoplasts. Oligomycin, an inhibitor of the mitochondrial ATPase, reduced ATP levels in protoplasts incubated in the dark. The ED_{50} for oligomycin in our 2-h bioassay was 1.2 ng/ml. A concentration of 13.5 ng oligomycin/ml caused the maximum inhibition of 77%. Figure 5 shows that oligomycin mimicked the effects of toxin on ATP levels in protoplasts. It had only a small effect in the light, and oligomycin plus DCMU in the light was equivalent to oligomycin alone in the dark. The average time for a 50% decrease in ATP levels in protoplasts in the dark treated with 0.6 μg oligomycin/ml was 6.3 min (n = 4). W64A T and W64A N protoplasts responded identically to oligomycin.

Long Term Experiments. In both the light and the dark, ATP levels in control protoplasts rose for 24 h before falling (Figs. 6 and 7). Within any given experiment, ATP levels always stayed higher in the light than the dark for the first 24 h. In the dark, ATP levels in toxin-treated protoplasts fell to nondetectable levels in 24 h (Fig. 6). In the light, however, ATP levels rose equally in toxin-treated and control protoplsts for 8 h, after which those in toxin-treated protoplasts began to fall (Fig. 7). After 70 h, toxin-

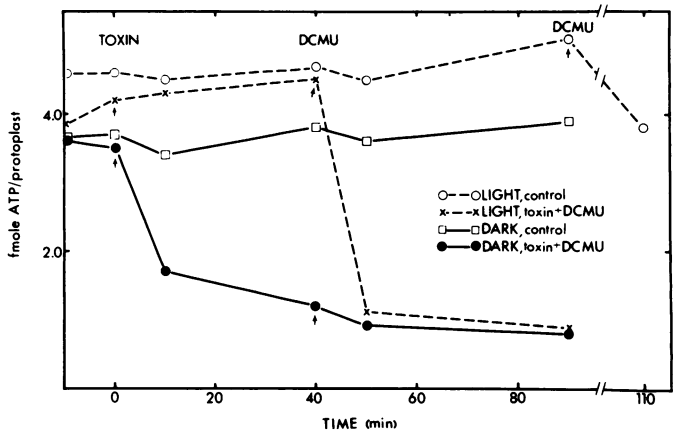

FIG. 3. Effect of light and DCMU on ATP levels in W64A T protoplasts treated with HMT toxin. Aliquots (2 ml) of protoplast suspension (1.71×10^5/ml) were placed in each of four 25-ml transparent polycarbonate flasks. Two flasks were incubated in the dark and two in 1,000 lux light. Arrows indicate addition of toxin or DCMU. After 1 h of equilibration, toxin (final concentration 6.2 μg/ml) was added to one flask incubated in the dark and one in the light. An equivalent amount of SCM buffer was added to the controls. After 50 min, DCMU (final concentration 2.0 μM) was added to the toxin-treated flasks and an equivalent amount of ethanol-SCM buffer to the controls. After 90 min, DCMU (2 μM) was added to the light-incubated control.

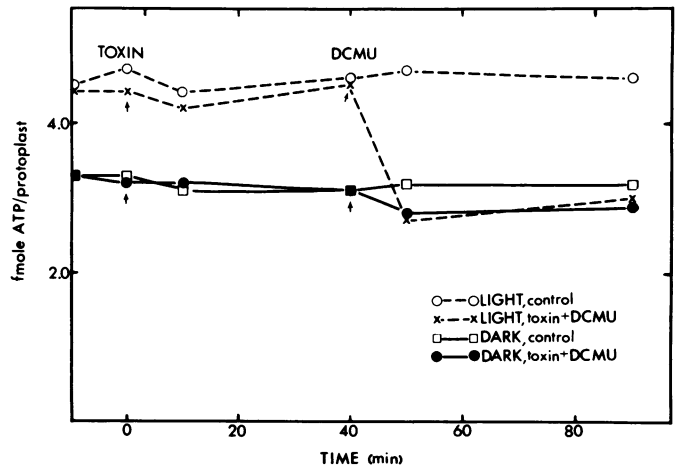

FIG. 4. Lack of effect of toxin on ATP levels in W64A N protoplasts. Protoplast concentration was 1.71×10^5/ml. Protocol was identical to that of Figure 3 except that DCMU was not added to the light-incubated control.

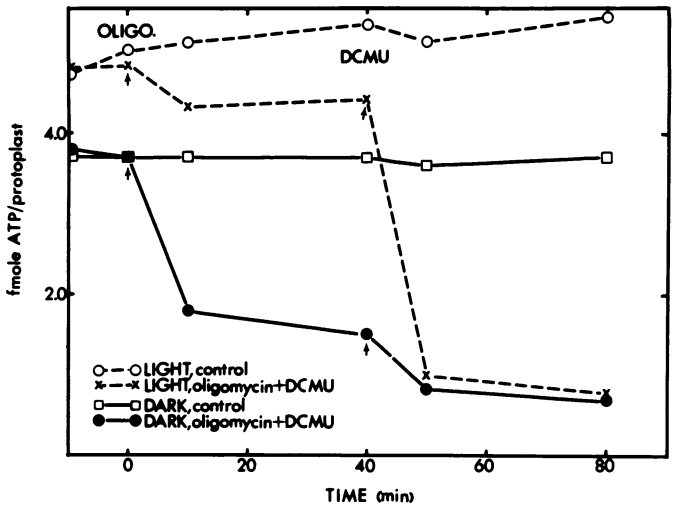

FIG. 5. Effect of oligomycin on ATP levels in W64A N protoplasts. Protoplasts (1.71×10^5/ml) were incubated and treated as described in Figure 3, except that oligomycin (final concentration 60 ng/ml) was added instead of toxin at time 0, and the light intensity was 1,300 lux.

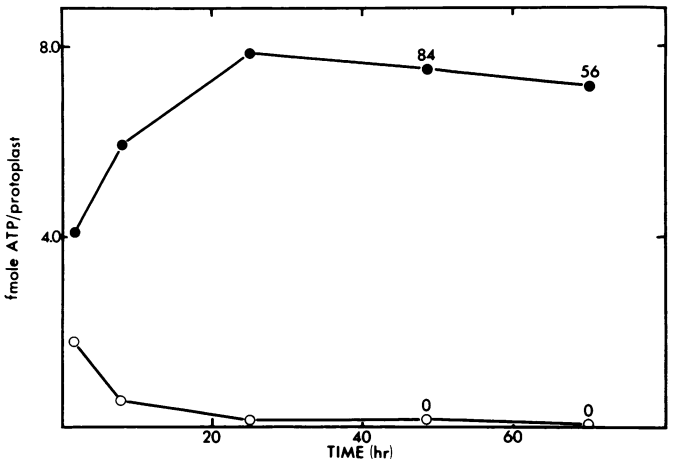

FIG. 6. Long term effect of HMT toxin on ATP levels in W64A T protoplasts incubated in the dark. Protoplasts (1.71×10^5/ml) were prepared aseptically and incubated. Toxin (final concentration 0.65 μg/ml) was added at time 0. The figures by the data points at 48 and 70 h represent per cent protoplast survival as determined by microscopic observation (10). ATP concentrations are expressed on the basis of original numbers of protoplasts, not surviving protoplasts. Control (●——●); toxin-treated (○——○).

treated protoplasts incubated in the light still maintained significant concentrations of ATP. The dashed lines at 24 h in Figure 7 show the changes in ATP levels which occurred when protoplasts were removed from the light and put in the dark for 1 h. The ATP levels in the control and toxin-treated protoplasts both fell rapidly, but the toxin-treated ones fell proportionately more and to a lower final value. ATP measurements, but not survival counts, (10) could reliably distinguish between control and toxin-treated protoplasts in the light (Fig. 7). Control protoplasts survived better in the dark than in the light over 70 h (Figs. 6, 7), as Earle *et al.* (10) also observed. ATP measurements were consistent with this differential survival (Figs. 6 and 7).

DISCUSSION

ATP in Isolated Protoplasts. ATP turnover rates in plant cells range between 2 and 20 s (7). Cellular ATP levels as measured by us are thus steady-state concentrations, *i.e.* a result of balanced rates of ATP regeneration and ATP utilization.

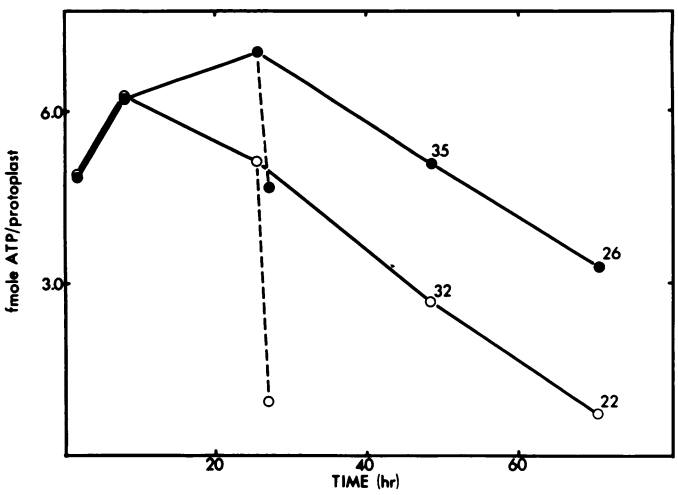

FIG. 7. Long term effect of HMT toxin on ATP levels in W64A T protoplasts incubated in continuous 1,300 lux light. The remainder of the protocol was identical to that described in Figure 6; control (●——●); toxin-treated, (○——○). After sampling at 24 h, the Petri plates were put in the dark for 1 h and sampled again. Dashed lines show changes in ATP levels; control (●- - -●); toxin-treated (○- - -○).

We found a 25% stimulation of ATP levels by light in fresh protoplasts (Figs. 3–5). Although a light-stimulated increase in ATP levels has been observed in several algae and higher plants (*e.g.* 14, 19), Sellami (26) and Bomsel and Pradet (9), with wheat leaves, and Lüttge *et al.* (19), with maize leaves, found no significant difference between light and dark ATP levels. The possibility that the light-induced increase observed by us is an artifact peculiar to protoplasts cannot be excluded; however, it is consistent with reports of stimulation of ATP-dependent processes by light (16). Protoplasts should prove useful for studying the relationship between ATP and processes such as solute transport in higher plant cells. The fact that ATP levels in protoplasts incubated in a light intensity of 1,300 lux were not increased further by 10-fold higher light intensity suggests that this ATP increase is due to cyclic photophosphorylation, which saturates at approximately 1,000 lux. DCMU blocked the light-induced rise in ATP levels (Figs. 3 and 4). indicating the involvement of PSII.

Over a period of 24 h, ATP levels in aseptic protoplasts incubated in Parafilm-sealed Petri plates rose by 50 to 100% (Figs. 6 and 7). If the values for ATP concentration are plotted per surviving protoplast rather than per initial protoplast the rise is even more striking. We have no simple explanation for this phenomenon, but it may reflect RNA breakdown as the protoplasts undergo senescence. Measurements of ADP and AMP would clarify this.

Effect of HMT Toxin on ATP Levels in Protoplasts. HMT toxin caused a rapid drop in steady-state ATP concentrations in protoplasts (Fig. 1). The kinetic data are comparable to the fastest response of whole cells previously observed, depolarization of the membrane potential (12, 20, 22). Our results indicate an inhibition of oxidative phosphorylation by toxin as the cause of this drop in ATP. Isolated thylakoid lamellae (2) and glycolysis *in vitro* (5) are unaffected by toxin. In toxin-treated protoplasts incubated in the light, photophosphorylation by chloroplasts can maintain high cellular ATP levels for several hours despite damage to mitochondra by toxin (Figs. 3 and 7). When photophosphorylation is also inhibited, by DCMU, ATP levels fall (Fig. 3).

Since the protoplasts were incubated in a simple osmoticum with no metabolizable substrate, glycolysis would be expected to be functioning slowly. It might account for the residual ATP levels present after treatment with toxin or oligomycin for 2 h (Fig. 2). Over longer times, glycolysis is also inhibited by toxin *in vivo* (5), and ATP levels fall to zero (Fig. 6).

Effect of Oligomycin on ATP Levels in Protoplasts. Oligomycin was chosen for comparison with HMT toxin for several reasons. Its mode of action as an inhibitor of mitochondrial electron transport and ATP synthesis has been well characterized at the molecular level, and at low concentrations it is specific for the mitochondrion. Oligomycin had the same effects as toxin on ATP levels under all conditions (Fig. 5). However, work with isolated mitochondria and coleoptile sections (5) makes it clear that toxin and oligomycin do not have the same mitochondrial site of action.

Functional Damage in Relation to Structural Damage. Aldrich *et al.* (1) and York (31) showed that ultrastructural damage to mitochondria in W64A T root cells and leaf protoplasts occurred 5 to 15 min after treatment with a toxin concentration identical to the one used here. Our results show that functional damage occurs in less than 2 min and thus precedes observable ultrastructural damage to mitochondria exposed to toxin *in vivo*. Bednarski *et al.* (5) found that toxin decreased levels of acid-labile phospate in T but not N maize roots, which is consistent with our findings.

Our results differ from those with excised roots (3) where toxin caused only a 30% drop in ATP levels. Lin and Hanson (18) reported that oligomycin, too, had little effect on ATP levels in maize roots, probably due to poor penetration of the tissue. Mertz and Arntzen (21) have supplied evidence that toxin, like oligomycin, penetrates roots poorly. The use of protoplasts avoids any problems of tissue permeability, and both oligomycin and toxin have a dramatic effect on ATP levels (Figs. 3 and 5).

Reduction of ATP Levels in Relation to Other Responses of Cells to HMT Toxin. Mitochondrial damage and a concomitant rapid decrease in ATP levels can explain most other physiological responses of whole cells or tissues to HMT toxin. The depolarization of the membrane potential in maize roots by toxin (20, 22) can be explained by the effect of the reduced ATP level on an electrogenic pump. In cyanide-treated *Neurospora*, *e.g.* depolarization of the membrane potential and decrease in the cellular ATP concentration had identical kinetics (28). Stimulation of ion leakage has been observed in toxin-treated roots (13), possibly reflecting an inability of the cells to maintain membrane integrity in the absence of an energy source. Arntzen *et al.* (2) reported that toxin induced closure of stomata and inhibited K^+ uptake by guard cells. Guard cells contain numerous mitochondria and the energy for stomatal movement probably comes primarily from oxidative phosphorylation despite the presence of chloroplasts in guard cells (25).

Several workers have found that toxin inhibits K^+ uptake in excised maize roots (11, 21) as would be expected if ATP synthesis were inhibited. Arntzen *et al.* (3) and Mertz and Arntzen (21), however, found no inhibition of phosphate uptake by toxin, although active phosphate uptake is driven by ATP (7). We do not think that these reports represent a serious argument against the mitochondrion as the primary site of toxin action, because problems of the effects of tissue aging on phosphate uptake rates (18), separation of passive from active uptake, and the metabolic fate of phosphate in healthy *versus* toxin-treated cells were not satisfactorily resolved (3, 21). Mertz and Arntzen (21) found that inhibition by toxin of K^+ uptake by leaf discs was greater in the light than in the dark, opposite to what one would predict from our results. The effect was slight, however, and requires further clarification.

Dark CO_2 fixation is very sensitive to toxin (6, 30), although P-enolpyruvate carboxylase is not inhibited by toxin (6). Inhibition of dark CO_2 fixation might be explained by lack of ATP for the regeneration of P-enolpyruvate from pyruvate by pyruvate, Pi dikinase.

The results in this paper support a mitochondrial site of HMT toxin as the simplest explanation for the experimental data. Although it cannot be unequivocally stated that HMT toxin directly affects only the mitochondrion, it is not necessary to postulate additional sites of action to explain most observed responses to HMT toxin.

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