Plant Physiol. (1984) 76, 508–517
0032-0889/84/76/0508/10/$01.00/0

Effects of Methomyl and *Helminthosporium maydis* Toxin on Matrix Volume, Proton Motive Force, and NAD Accumulation in Maize (*Zea mays* L.) Mitochondria[1]

Received for publication April 15, 1983 and in revised form June 9, 1984

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ABSTRACT

Methomyl and *Helminthosporium maydis* race T toxin block oxidative phosphorylation in mitochondria isolated from maize plants with Texas male sterile cytoplasm (T) but not in mitochondria isolated from those with Normal cytoplasm (N) (Bednarski, Izawa, Scheffer 1977 Plant Physiol 59: 540–545). Moreover, they have been reported to cause specific swelling in T mitochondria (Miller, Koeppe 1971 Science 173: 67–69; Koeppe, Cox, Malone 1978 Science 201: 1227–1229). We could not detect, by direct volume measurements, any change induced by these compounds in the mitochondrial matrix space. We show here that the proton motive force, which in maize mitochondria is composed of a large transmembrane potential and of a low transmembrane pH difference, is absent in T mitochondria incubated in the presence of methomyl or of *Helminthosporium maydis* race T toxin, while it is unchanged in N mitochondria. Methomyl and *Helminthosporium maydis* race T toxin induce, independently of the collapse of the proton motive force, a release of the cofactors NAD and coenzyme A from the mitochondrial matrix space. In particular, we show that NAD is transported in maize mitochondria, and that this transport, which is not dependent on the proton motive force, is inhibited by methomyl or *Helminthosporium maydis* race T toxin.

The fungi *Dreschlera maydis* authority race T (*Helminthosporium maydis* Nisikado and Miyake) and *Phyllosticta maydis* Arny and Nelson severely attack maize (*Zea mays* L.) plants carrying the Texas male sterile cytoplasm (Texas plant), causing the diseases known as Southern and Yellow corn leaf blight, respectively (16, 35, 36). The host-specific pathotoxins responsible for the symptoms of these diseases, T-toxin from *H. maydis* and Pm-toxin from *P. maydis*, have been isolated from culture filtrates and T-toxin has been structurally characterized (20). Carbamate methomyl, an insecticide whose molecular structure is quite different from that of T-toxin, has a similar effect when sprayed on Texas plants (1, 17). Plants carrying one of the other cytoplasms, Normal (N), Charrua (C), or S, are insensitive to T-toxin, Pm-toxin, and methomyl.

Mitochondria isolated from Texas plants are sensitive to all three compounds (T-toxin, Pm-toxin, and methomyl) whereas N, C, and S mitochondria are not, at the same concentrations (19, 25). Furthermore, methomyl and the pathotoxins have no effect on mitochondria isolated from animals (rat liver and pig heart) and other plants (potato, wheat, sunflower, field bean, sorghum) mitochondria (Bervillé, unpublished data). The pathotoxins and methomyl have been shown to cause various effects on T mitochondria (for a recent review, see 6 and 11), and in particular they block oxidative phosphorylation (3). It is not yet clear whether methomyl and the pathotoxin effects on mitochondria are the only cause of the observed corn leaf blight symptoms. It can, however, be expected to be one of its important components. Moreover, since mutants from T cytoplasm plants, resistant to T-toxin, are also revertant for male fertility and behave as maintainers (8–10, 13), the understanding of the mechanism of action of methomyl and toxin on mitochondria may shed some light on the basic biochemical events underlying cytoplasmic male sterility.

Although methomyl and the pathotoxins have often been described as uncouplers of oxidative phosphorylation in T mitochondria (3, 5, 19), no attempt has been made so far, to substantiate this statement from a chemiosmotic point of view.

It is now generally accepted that the chemiosmotic theory provides an adequate framework for the understanding of oxidative phosphorylation (7). According to this theory, proton pumps driven by electron transport generate a difference of electrochemical potential of protons across energy conserving membranes. This difference of electrochemical potential ($\Delta\sim\mu_H^+$) or the PMF[3] (equal to $\Delta\sim\mu_H^+/F$), which is composed of a membrane potential component ($\Delta\Psi$) and of a pH difference (ΔpH), is the obligatory link between respiration and phosphorylation (29). The reentry of protons through the ATPase is an exergonic reaction which drives the endergonic reaction of ATP synthesis. Thus, inhibition of phosphorylation may have several causes: (a) inhibition of the PMF building up at the level of the respiratory chain; (b) dissipation of the PMF by an increase in the membrane permeability to protons (*e.g.* action of uncouplers); (c) inhibition of ATP, ADP, or Pi transport; or (d) direct blocking of ATPase (*e.g.* action of oligomycin).

We present here measurements of the PMF in maize mito-

[1] Supported in part by grants from the Centre National de la Recherche Scientifique (ATP 8070-033 608) and from Institut National de la Recherche Agronomique.

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[3] Abbreviations: PMF, proton motive force; $\Delta\Psi$, difference of electrical membrane potential; ΔpH, pH difference; EGTA, ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 2-4 DNP, 2-4 dinitrophenol; Ph_4P^+, tetraphenyl phosphonium ion.

chondria which show that, in the presence of T-toxin or methomyl, the PMF is absent in T mitochondria, whereas it is virtually unchanged in N mitochondria. However, the absence of a PMF in T mitochondria in the presence of methomyl or of T-toxin, which can account for the lack of oxidative phosphorylation does not explain *per se* previously published data nor the observations reported in this paper concerning the action of these compounds on NADH, succinate, malate, and α-ketoglutarate-sustained respiration. Some of these observations can be readily explained by a release of the cofactors NAD and coenzyme A from the mitochondrial matrix space, induced by methomyl or T-toxin. In particular, we show that NAD is transported in N and T maize mitochondria, and that this transport, which is not dependent on the PMF, is selectively inhibited in T mitochondria by methomyl and T-toxin.

MATERIALS AND METHODS

Plant Material. The french maize (*Zea mays* L.) line F7 with normal cytoplasm (F7N) and with Texas cytoplasm (F7T) was used. Kernels (50 g) were surface sterilized by soaking 10 min in 50% commercial bleach and subsequently washed three times with deionized sterile H_2O. They were placed onto nine sheets of sterile filter paper and covered by one sheet in a black tray (0.26 × 0.42 m); 250 ml $CaCl_2$ (0.1 mM) were added per tray and the trays stored at 25°C for 7 d in the dark at 100% RH.

Isolation of Mitochondria. All operations took place at 0 to 2°C. Shoots were rapidly harvested, rinsed with cold sterile H_2O, cooled in ice for 10 min, and then ground with 3 g of Fontainebleau sand, using a mortar and a pestle, with 30 ml of medium containing: 0.4 M sucrose, 50 mM KH_2PO_4, 1 mM EGTA, 50 mM Tris (pH 7.6), and 0.1% (w/v) BSA. After homogenization, 130 ml of the same medium were added and the suspension filtered through a sheet of Miracloth into a chilled beaker. The filtrate was centrifuged for 15 s at 10,000*g* (rotor SS 34, Sorvall RC 2B). The supernatant was poured into fresh tubes and recentrifuged for 2 min at 38,000*g*. The mitochondrial pellets were resuspended using a fine paint brush in 0.2 M sucrose, 10 mM Tes (pH 7.2), 0.1% (w/v) BSA at a concentration of 10 to 15 mg protein/ml, and kept on ice until used. For all subsequent measurements the mitochondria were diluted at the desired concentration in the following reaction medium: 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.2), 10 mM KH_2PO_4, 0.1% (w/v) BSA, adjusted to pH 7.2 with 10 mM KOH.

Determination of the Matrix Volume, ΔΨ, ΔpH, and NAD Accumulation. *Determination of the Matrix Volume.* The internal water volume was determined according to Rottenberg (32). Mitochondria were incubated 2 min (unless otherwise specified) in the reaction medium (final concentrations ranging from 2.8 to 18.8 mg protein/ml) in the presence of 5 mM NADH, 10 mM succinate, 30 mM malate, or 10 mM α-ketoglutarate, and when necessary, methomyl, T-toxin, or FCCP, at the desired concentration. The suspension was further incubated 1 min with tritiated water (10 μCi/ml) as a marker of the pellet water content and D-[1-^{14}C]mannitol (final concentration, 3.3 μM; 555 mCi/mmol) as a marker of the nonosmotic space. One-ml samples were transferred to microcentrifuge tubes containing 10 μl of 0.2 M H_2O_2 to prevent anaerobiosis and centrifuged for 2 min on a microfuge TH 12. An aliquot (100 μl) of the supernatant was removed and transferred to a counting vial containing a similar pellet of nonradioactive mitochondria. The remaining supernatant was removed and the pellet was cut off with a razor and transferred to a counting vial. Vials were vigorously shaken until complete dissolution of the pellets and counted for radioactivity on the preset $^3H/^{14}C$ program of a liquid scintillation counter. The total mitochondrial volume was determined with tritiated water and (hydroxy-[^{14}C]methyl)inulin (final concentration, 10 μM; 15.6 mCi/mmol) as a marker of the external water content.

Determination of ΔpH. ΔpH was estimated from the accumulation of acetate (32). The experimental procedure was the same as that for the matrix volume determination except that the mitochondria were incubated 5 min (final concentration about 3 mg protein/ml) with [1-^{14}C]acetate (final concentration, 5.2 μM; 47.9 mCi/mmol) and either [6,6′-(n)-^{3}H]sucrose (final concentration, 0.5 μM; 9.8 Ci/mol) as a marker of the nonosmotic space or tritiated water as a marker of the total water content. When necessary, methomyl, or T-toxin was added at the desired concentration, at the beginning of incubation. ΔpH was calculated using a parallel determination of the matrix volume, performed on the same preparation of mitochondria.

ΔΨ Determination. ΔΨ was estimated from the accumulation of the lipophilic cation Ph_4P^+ measured by centrifugation (32) or flow dialysis (31).

(a) Centrifugation. The experimental procedure was the same as for ΔpH determination except that mitochondria were incubated 5 min (final concentration about 3 mg protein/ml) in the presence of [G-^{3}H] Ph_4P^+ (final concentration, 30 nM; 33 mM Ci/mmol) and when necessary, methomyl, or T-toxin, at the desired concentration. Because of the high nonspecific binding of Ph_4P^+ to the membranes and of its high accumulation ratio, no marker of the external water was used, since contamination by Ph_4P^+ dissolved in the external water of the pellet is, under these conditions, negligible as compared to bound and accumulated Ph_4P^+ (see below).

(b) Flow dialysis. Flow dialysis experiment were performed according to Ramos *et al.* (31). The upper chamber contained 1 ml of reaction medium and [U-^{14}C]pH_4P^+ (final concentration, 140 μM; 175 mCi/mmol). The reaction medium minus BSA was pumped through the lower chamber at a flow rate of 2.5 ml/min and its radioactivity measured directly by a radioactive flow detector (Flo-one). Once the steady state was reached, mitochondria were added to the upper chamber at an approximate final concentration of 3 mg protein/ml, together with the appropriate energy source, 3 mM NADH, 10 mM succinate, or 30 mM malate. Oxygen was bubbled through the upper chamber to prevent anaerobiosis. When required, methomyl (final concentration, 10 mM) or T-toxin (91 nM), was added directly in the upper chamber.

Using either technique, the addition of 25 to 50 μM FCCP, which supposedly collapses $\Delta\psi$, does not result in a total release of Ph_4P^+. This phenomenon has already been reported for Ph_4P^+ or triphenylmethylphosphonium in other systems such as mammalian mitochondria (2, 15, 33) or bacteria (14, 24), and it is probably due to unspecific binding of the probe to the membrane due to its lipophilic nature. The accumulation of Ph_4P^+ was calculated in all experiments (centrifugation assay or flow dialysis) by substracting the amount of Ph_4P^+ bound in the presence of FCCP (equivalent to 20% of the total cpm). ΔΨ was calculated from the Nernst equation, using a parallel determination of the matrix volume performed on the same preparation of mitochondria. Because of the logarithmic dependence of ΔΨ on the probe accumulation, high potential determination is relatively accurate (180 mv corresponding to an accumulation of a factor 1000 for the probe) while low potential determination is much more delicate (40 mv corresponding to an accumulation of 5), especially in the presence of a high unspecific binding of the probe. Consequently, no attempt was made to distinguish between 0 and 40 mv.

NAD Transport. Mitochondria were incubated 2 min in the reaction medium at a concentration of 10 mg/ml, with 10 mM succinate or 30 mM malate as an energy source, and, when required, methomyl, T-toxin, or FCCP at the desired concentration. Carbonyl-[U-^{14}C]NAD (final concentration, 0.34 or 1 μM; 287 mCi/mmol), and ^{3}H sucrose as a marker of the nonosmotic space (final concentration, 0.5 μM; 9.8 Ci/mol) were then added to the suspension. In other experiments [adenylate-^{32}P]NAD was

used instead of [^{14}C]NAD (final concentration, 1 μM; 300 mCi/mmol) with tritiated water as a marker of the total water content. One-ml aliquots were removed at intervals, centrifuged, and treated as described above. The radioactivity found in the pellet at time zero (centrifugation performed immediately after addition of NAD) was considered as unspecific binding and substracted from all subsequent measurements. The matrix volume was determined in the conditions of time zero, and in the presence of methomyl or FCCP, on the same preparation of mitochondria.

Measurements of Respiratory Activities. Measurements of respiratory activities were performed with a Clark oxygen electrode, at 27°C in a closed vessel (Gilson medical Corp) containing 3.5 ml of the reaction medium at approximatively 0.6 mg protein/ml. The suspension was kept homogeneous by an air-pulsed magnetic stirrer and the vessel was closed by a lid through which reagents were added.

Absorbance Measurements. Changes in absorbance were recorded at 520 nm using a DB-GT Beckman spectrophotometer (1 cm path). Mitochondria (2 mg protein, corresponding to an O.D. of 1.5) were incubated 2 min with NADH in a 3-ml cell before addition of methomyl or T-toxin.

NAD Determination. The mitochondrial suspension was divided into two samples (10 ml, 5 mg protein/ml). Methomyl (final concentration 10 mM) was added to one sample, the remaining sample serving as control. The tubes were immediately centrifuged at 48,000*g* for 2 min. The supernatant was decanted and deproteinized. The proteins were precipited with $HClO_4$ (final concentration, 0.5 M) then centrifuged for 10 min at 15,000*g* and the final supernatant (S) was kept for NAD determination. The mitochondrial pellet was carefully dried with blotting paper, resuspended in 5 ml deionized H_2O to burst the mitochondria, and then deproteinized. After centrifugation, the supernatant (M) was decanted. The two supernatants (S) and (M) were adjusted to pH 6.5 prior to NAD determination according to Klingensberg's method (18) using alcohol NAD oxidoreductase. The percentage of NAD released by the mitochondria was calculated as NAD in (S) × 100/NAD in (S) + NAD in (M).

Protein Determination. Protein concentration was determined by the method of Lowry *et al.* (22) using BSA as a standard.

Materials. [^{14}C]Acetate, ^{14}C and [^{3}H]Ph_4P^+, and tritiated water were from C.E.A. (Saclay) France. [^{3}H]sucrose, [^{14}C]mannitol, [^{14}C]hydroxymethyl-inulin, and [^{14}C] and [^{32}P]NAD were from Amersham. T-toxin was kindly provided by J. M. Daly (University of Nebraska, Lincoln) and its concentration was calculated assuming a mol wt of 789 (20). Methomyl (lannate commercial solution) was recrystallized according to Aranda *et al.* (1). All other chemicals were of reagent grade and obtained from commercial sources.

RESULTS

Action of Methomyl and T-Toxin on the Matrix Volume of Maize Mitochondria. Addition of methomyl or the pathotoxins to a suspension of T mitochondria induces a decrease in absorbance which has been interpreted as swelling (12, 19). However, when matrix volume was systematically determined for each ΔΨ and ΔpH measurement, in the presence or absence of methomyl or T-toxin, no significant difference was found. To clarify this point, series of volume measurements were performed with high concentration of proteins (up to 18.8 mg/ml). The results shown in Table I clearly show that, for the concentrations tested, incubation of T mitochondria for 2 min, in the presence of methomyl or T-toxin, does not induce any significant change in the matrix volume. In addition, it can be seen that methomyl has no action on the total volume determined by [^{14}C]hydroxymethyl-inulin and tritiated water. We have checked (Table I) that, under the same conditions, addition of methomyl or T-toxin to a suspension of T mitochondria does induce a decrease in absorbance, as previously described (12, 19) and this takes place in less than 30 s. Addition of these compounds to a suspension of N mitochondria has no effect on absorbance for the concentrations tested.

Table I. *Comparison of Maize Mitochondrial Volumes and Changes in Absorbance in the Presence or in the Absence of Methomyl or T-Toxin*

Volume in μl/mg protein of the osmotic space or of the total volume of mitochondria from T plants or from N plants. Mannitol was used as a probe of the nonosmotic space, other experimental conditions are described in the text. Each value is the mean of 10 repetitions ± SE calculated according to Student's test.

Genotypes	Control	10 mM Methomyl	91 nM T-Toxin
		μl/mg protein	
F7T matrix	0.75[a] ± 0.07	0.86[a] ± 0.08	0.80[d] ± 0.09
F7N matrix	0.90[b] ± 0.11	0.94[b] ± 0.10	0.85[e] ± 0.10
F7T total volume	2.44[c] ± 0.19	2.22[c] ± 0.19	ND[f]
F7T variation of absorbance	0	−0.040 ± 0.005	−0.025 ± 0.005
F7N variation of absorbance	0	0	0

[a,b,c,d,e] Experiments were carried out, respectively, with 16, 18.8, 2.7, 5.7, 11.2 mg protein.
[f] Not determined.

PMF in Maize Mitochondria. Upon addition of a substrate—NADH, succinate, malate, or α-ketoglutarate—N and T mitochondria maintain a PMF of some 200 mv mainly under the form of a ΔΨ (Table II).

Membrane Potential (Δψ). Figure 1, A and B, shows typical flow dialysis experiments performed with N and T mitochondria, respectively, energized by succinate. It can be seen that addition of FCCP, which is supposed to reduce the PMF to zero, does not lead to a total release of the fixed Ph_4P^+. ΔΨ are calculated from accumulation ratios of Ph_4P^+ corrected for binding (see "Materials and Methods").

The results obtained by the centrifugation method, for N and T mitochondria, in the presence of the different substrates, are given in Table II. The values obtained by flow dialysis (Fig. 1) or centrifugation (Table II) are very similar (about 200 mv). There is no marked difference in the ΔΨ values between N and T mitochondria, nor between the different substrates, for each of the two strains.

pH Difference. ΔpH is very low in every case (0–0.3) and corresponds to an accumulation ratio of [^{14}C]acetate at the limit of detection (Table II).

Methomyl and T-Toxin Action on the PMF. Figure 1B shows that addition of methomyl (10 mM final concentration) to a suspension of T mitochondria in the presence of succinate completely abolishes the ΔΨ-dependent accumulation of Ph_4P^+. Subsequent addition of FCCP has no further effect. In contrast (Fig. 1A), addition of methomyl to a suspension of N mitochondria has no effect on the Ph_4P^+ accumulation. Figure 1, A and B, also shows that the response to methomyl is slower than that to FCCP. Similarly, Figure 1D shows that addition of T-toxin (final concentration, 91 nM) to a suspension of succinate-energized T mitochondria induces a collapse of the ΔΨ-dependent accumulation of Ph_4P^+, while it has no significant effect on a suspension of N mitochondria (Fig. 1C).

The effects of increasing concentrations of methomyl on ΔΨ in succinate- or malate-energized N and T mitochondria is illustrated by Figure 2A. While N mitochondria respond to increasing concentrations of methomyl by a small but noticeable decrease in Δψ, the response of T mitochondria is completely different: the phenomenon presents a threshold (between 1 and

Table II. *Effect of Methomyl or T-Toxin on Components of the Proton Motive Force of N and T Mitochondria*

Experimental conditions are described in the text. Parallel determination of $\Delta\Psi$, ΔpH, and matrix spaces were done. Three mg protein corresponding to 2.5 μl matrix volume were used for N and T mitochondria. Methomyl: 10 mM; T-toxin = 91 nM.

Conditions	$\Delta\Psi$	ΔpH (pH out− pH in)	$-Z^a$ ΔpH	PMF ($\Delta\Psi$ − ZΔpH)
	mv			*mv*
A: N mitochondria				
5 mM NADH[d]	206[abc]	0	0	206
10 mM Succinate[d]	197	−0.21	+12	209
30 mM Malate[d]	188	−0.15	+9	197
10 mM α-Ketoglutarate	211	0	0	211
5 mM NADH[d] + methomyl	194	0	0	194
10 mM Succinate[d] + methomyl	185	−0.11	+6	191
30 mM Malate[d] + methomyl	175	−0.11	+6	181
10 mM α-Ketoglutarate[d] + methomyl	200	0	0	200
10 mM Succinate[d] + T-toxin	185	−0.11	+6	191
30 mM Malate + T-toxin	185	ND[e]		
B: T mitochondria				
5 mM NADH[d]	210	0	0	210
10 mM Succinate[d]	196	−0.14	+8	204
30 mM Malate[d]	194	−0.24	+14	208
10 mM α-Ketoglutarate	212	0	0	212
5 mM NADH[d] + methomyl	0	0	0	0
10 mM Succinate[d] + methomyl	0	−0.1	+6	6
30 mM Malate[d] + methomyl	0	−0.1	+6	6
10 mM α-Ketoglutarate + methomyl	0	0	0	0
10 mM Succinate[d] + T-toxin	0	ND		
30 mM Malate + T-toxin	0	ND		

[a] $-Z = 2{,}3\frac{RT}{F}$ at 20°C; +Z = 59 mv.

[b] Each value is the average of at least two repetitions.

[c] $\Delta\Psi$ is negative inside.

[d] Substrate state.

[e] Not determined.

3 mM), above which depolarization is complete. T-toxin has no effect on $\Delta\Psi$ in succinate- or malate-energized N mitochondria, for the concentration range tested (1 nM to 1 μM); but, between 30 and 60 nM, it causes the depolarization of T mitochondria (Fig. 2B). As can be seen in Table II (results obtained by the centrifugation method), methomyl (10 mM) or T-toxin (91 nM) causes a collapse of $\Delta\Psi$ in T mitochondria in the presence of each of the substrates tested (NADH, succinate, malate, α-ketoglutarate) and has no action on N mitochondria. In the case of ΔpH, the results are less clearcut due to the lack of precision for such low accumulation ratios.

At this point, it should be stressed that the values of the membrane potential reported above correspond to an accumulation of more than 1000-fold for the probe Ph_4P^+. Consequently, any change in volume which was not revealed by our technique, could not account for the observed release of Ph_4P^+.

Action of Methomyl and the T-Toxin on NADH, Succinate, Malate, and α-Ketoglutarate-Sustained Respiration. *NADH Oxidation.* Addition of methomyl (Fig. 3A; final concentration, 10 mM) or T-toxin (Fig. 3B; final concentration, 91 nM) to a suspension of T mitochondria induces, in the presence of NADH, an increase in the respiratory rate, as does FCCP (Fig. 3C), whereas it has no effect on N mitochondria (Fig. 3D). Rotenone added after methomyl induces a decrease of about 50% in the respiratory rate (Fig. 3A). In contrast, practically no decrease in the respiratory rate is observed when rotenone is added after FCCP addition (Fig. 3C). Figures 4 and 5 show the percentage stimulation of NADH oxidation with increasing concentrations of methomyl and T-toxin, respectively. The action of methomyl is detectable at 0.5 mM and is fully effective at 3 mM. In the case of T-toxin, stimulation occurs for concentrations as low as 0.5 nM and reaches a maximum between 10 and 100 nM.

Succinate Oxidation. In the presence of methomyl (Fig. 6A; final concentration, 10 mM) or T-toxin (Fig. 6B; final concentration, 91 nM), succinate oxidation in T mitochondria is first stimulated and then decreases back to its original level. FCCP stimulates succinate oxidation in T (Fig. 6C) and in N mitochondria (Fig. 6D) while methomyl and T-toxin have no effect on N mitochondria (Fig. 6D).

Malate Oxidation. Both methomyl (Fig. 7A; final concentration, 10 mM) and T-toxin (Fig. 7B; final concentration, 91 nM) completely inhibit malate oxidation in T mitochondria but have no effect on N mitochondria (Fig. 7E). FCCP added after methomyl (not shown) or after T-toxin (Fig. 7C) has no effect whatsoever. The effect of methomyl and T-toxin on T mitochondria is in striking contrast with that of FCCP which induces the usual release of the respiratory control (Fig. 7, D and E). In addition, we have shown here that respiration is restored when 0.5 mM NAD is added to the medium after methomyl, or T-toxin (Fig. 7, A, B, D). We have verified that, in this case, $\Delta\Psi$ is still zero

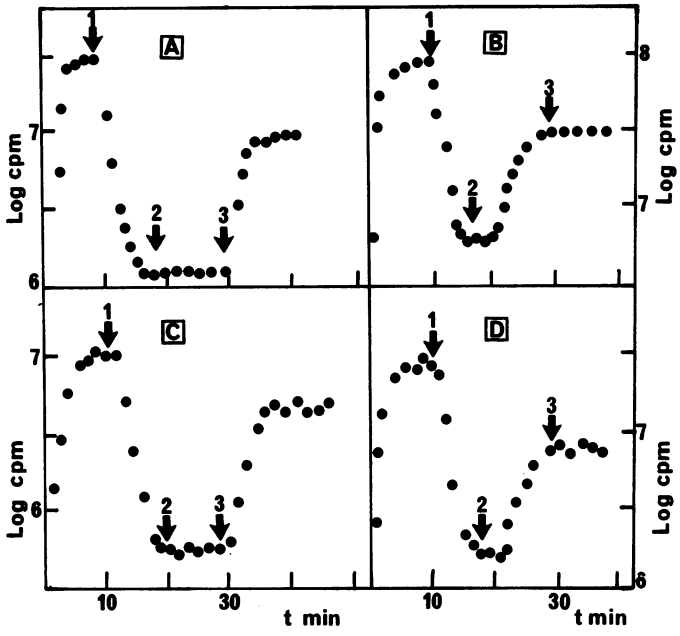

FIG. 1. Accumulation of radioactive Ph_4P^+ monitored by flow dialysis. A and C, N mitochondria (2.64, 2.10 mg protein corresponding to a measured volume of 1 μl); reaction medium and experimental conditions as described in the text. Calculation led to a $\Delta\Psi$ value of 182 and 186 mv. B and D, T mitochondria (2.54, 2.82 mg protein corresponding to a measured volume of 1 μl). In the presence of methomyl and T-toxin, $\Delta\Psi$ is zero. In a parallel experiment, $\Delta\Psi$ calculation led to values of 177 and 182 mv. Sequential additions as indicated by arrows: ↓1, mitochondria plus succinate, final concentration 10 mM; ↓2 (A and B), methomyl, final concentration 10 mM, or ↓2 (C and D), T-toxin, final concentration 91 nM; ↓3, FCCP, final concentration 50 μM.

(data not shown); moreover, the ability to phosphorylate ADP is definitively lost, since addition of ADP has no further effect (Fig. 7, A and B). Addition of NAD alone has no effect on the oxidation of malate in T mitochondria (Fig. 7F). Figures 4 and 5 give the percentage of inhibition of malate oxidation by methomyl and T-toxin, respectively, as a function of increasing concentrations. Inhibition of malate oxidation by methomyl in T mitochondria occurs between 1 and 3 mM and is complete above this concentration. In N mitochondria, methomyl slightly inhibits malate oxidation for concentrations above 10 mM. T-toxin, which has no action in N mitochondria up to 1 μM, induces inhibition of malate oxidation in T mitochondria between 1 and 100 nM; above this concentration, inhibition is total.

α-Ketoglutarate Oxidation. Methomyl (Fig. 8A; final concentration, 10 mM) and T-toxin (Fig. 8B; final concentration, 91 nM) completely inhibits α-ketoglutarate oxidation in T mitochondria. FCCP added after methomyl (Fig. 8A) or T-toxin (Fig. 8B) has no effect. In contrast, FCCP induces a release of the respiratory control in N and T mitochondria (Fig. 8, C and E). Addition of NAD after methomyl or after T-toxin increases slightly the respiratory rate while subsequent addition of CoA fully restores the respiratory rate (Fig. 8D). NAD or CoA alone do not modify the α-ketoglutarate sustained respiration in T min T mitochondria (Fig. 8F).

NAD Accumulation in Maize Mitochondria and Action of Methomyl and of T-Toxin. Figures 9 and 10 display the time course of NAD transport in N and T mitochondria, respectively. The accumulation of radioactive NAD increases with time and reaches an accumulation ratio (ratio of the internal matrix concentration to the external concentration) of about 15, demonstrating without ambiguity that NAD is transported into maize mitochondria. Interestingly enough, treatment of both N and T mitochondria with FCCP does not significantly diminish NAD accumulation (Figs. 9 and 10A). Although methomyl (final concentration, 10 mM) has virtually no action on NAD transport in N mitochondria (Fig. 9), it prevents NAD accumulation in T mitochondria (Fig. 10A). Moreover, it induces a release of NAD when it is added once the accumulation of radioactive NAD has occurred. Very similar results are obtained with T-toxin (final concentration, 0.5 μM); this has no action on NAD uptake in N mitochondria (Fig. 9) but prevents NAD accumulation in T mitochondria and induces efflux of accumulated NAD (Fig. 10B). These results are confirmed by direct determination of NAD release by T mitochondria under the action of methomyl. Resuspension of N and T mitochondria in the reaction medium results in the release of about 40% of the NAD content of the mitochondria (corresponding to an initial internal concentration of 4–9 mM); addition of methomyl to a suspension of N mitochondria has no further effect, but when it is added to a suspension of T mitochondria, 88% of the NAD content of the mitochondria is found in the medium.

Figure 11A shows the effect of increasing concentration of methomyl on the accumulation of radioactive NAD. Up to 5 mM, methomyl has no action on NAD accumulation in N mitochondria and above this value, NAD accumulation decreases to about 80% of the control for a methomyl concentration of 20 mM. In contrast, a sharp decrease in NAD accumulation is observed in T mitochondria between 0.5 and 3 mM; this is followed by a slower decrease giving a similar slope to that for N

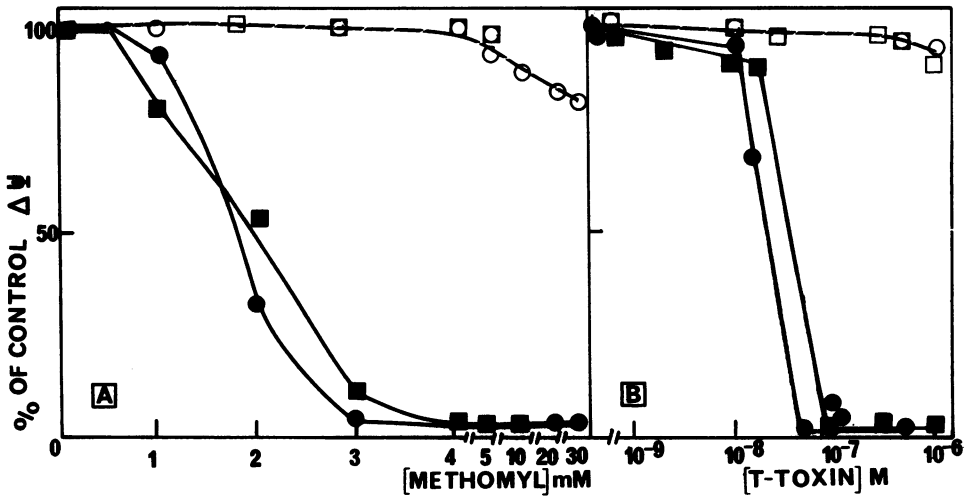

FIG. 2. A, Effect of varying concentrations of methomyl on membrane potential in succinate (10 mM) -energized N (○) and T mitochondria (●). Malate (30 mM) energized N (□) and T mitochondria (■). B, Effect of varying concentrations of T-toxin on membrane potential in succinate (10 mM) -energized N (○) and T mitochondria (●). Malate energized (30 mM) N (□) and T (■) mitochondria.

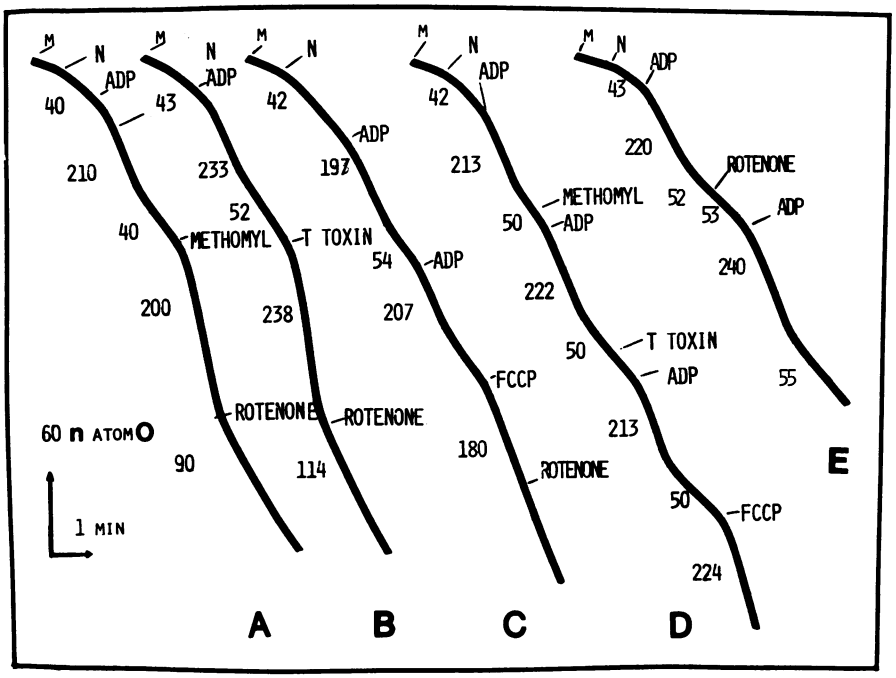

FIG. 3. Traces of maize mitochondria oxidizing NADH. Sequential addition as indicated by arrows: M, mitochondria (2 mg protein); N, NADH, final concentration 1.5 mM; methomyl, final concentration 10 mM; ADP, final concentration 50 μM; FCCP, final concentration 50 μM; rotenone in DMSO, final concentration 50 μM; T-toxin, final concentration 91 nM. Numbers along the traces indicate oxygen uptake in natoms·min^{-1}·mg^{-1} protein. A, B, C, and E: T mitochondria; D: N mitochondria.

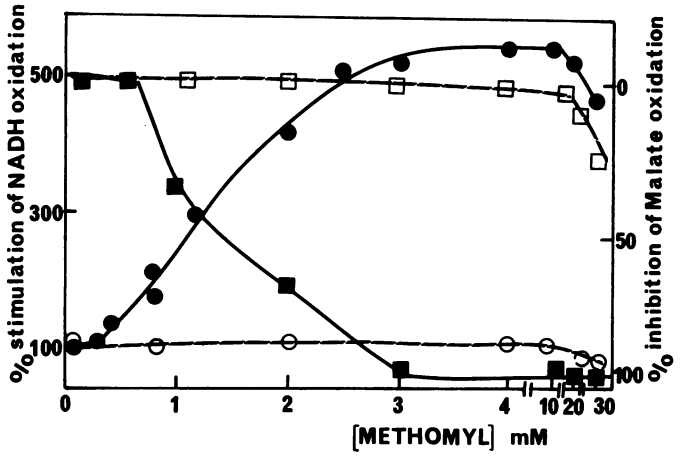

FIG. 4. Effect of varying concentrations of methomyl: on the per cent stimulation of NADH oxidation by N (○) (0.6 mg protein/ml) or T (●) (0.6 mg protein/ml) mitochondria; on the per cent inhibition of state 3 malate oxidation by N (□) (1.2 mg protein/ml) or T (■) (1.2 mg protein/ml) mitochondria.

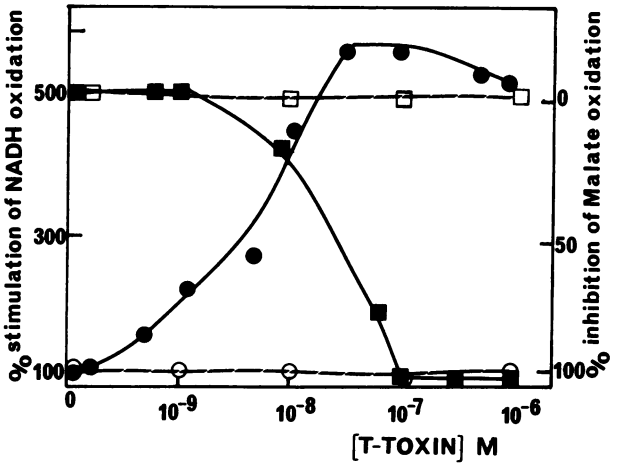

FIG. 5. Effect of varying concentrations of T-toxin: on the per cent stimulation of NADH oxidation by N (○) (0.6 mg protein/ml) or T (●) (0.6 mg protein/ml) mitochondria; on the per cent inhibition of state 3 malate oxidation by N (□) (1.2 mg protein/ml) or T (■) (1.2 mg protein/ml) mitochondria.

mitochondria over the same concentration range. T-toxin has no effect on NAD accumulation in N mitochondria for concentrations up to 1 μM (Fig. 11B). The action of T-toxin in T mitochondria is clearly biphasic: the drug is active for a concentration as low as 1 nM and a plateau is reached between 10 and 100 nM; above this value, NAD accumulation falls rapidly to 30% of the control (Fig. 11B).

DISCUSSION

Although the decrease in absorbance induced by methomyl or T-toxin has been interpreted as swelling (12, 19, 25), we could not observe any change either in the matrix volume or in the total volume of T mitochondria with addition of these compounds. The observed decrease in O.D. is therefore probably related either to a change in volume that is too small to be detected by our method (*i.e.* less than 15%) or, alternatively, to a modification of the membrane structure. Methomyl and T-toxin do not apparently alter significantly the membrane integrity, since they do not cause the membrane to become pervious to mannitol.

The PMF has been intensively studied in mammalian mitochondria (7, 29). Apart from the work of Moore *et al.* (26) and Moore and Bonner (27), few attempts have been made to determine this parameter in plant mitochondria. The values of the PMF reported here for maize mitochondria are in good agreement with values reported in other systems (*i.e.* a PMF of some 200 mv, with $\Delta\Psi$ as the main component, in state 4). The present work shows that, in the presence of methomyl or T-toxin, T mitochondria are unable to maintain a substantial PMF whatever the energy source tested. The dose-response curves of methomyl and T-toxin actions on $\Delta\Psi$ in N and T mitochondria show the high specificity of these compounds for T mitochondria. Nevertheless, an unspecific effect of methomyl is clearly observed for high concentrations (10 mM and above). According to the chemiosmotic theory, this effect of methomyl and of T-toxin on the PMF can account for the observed inhibition of oxidative phos-

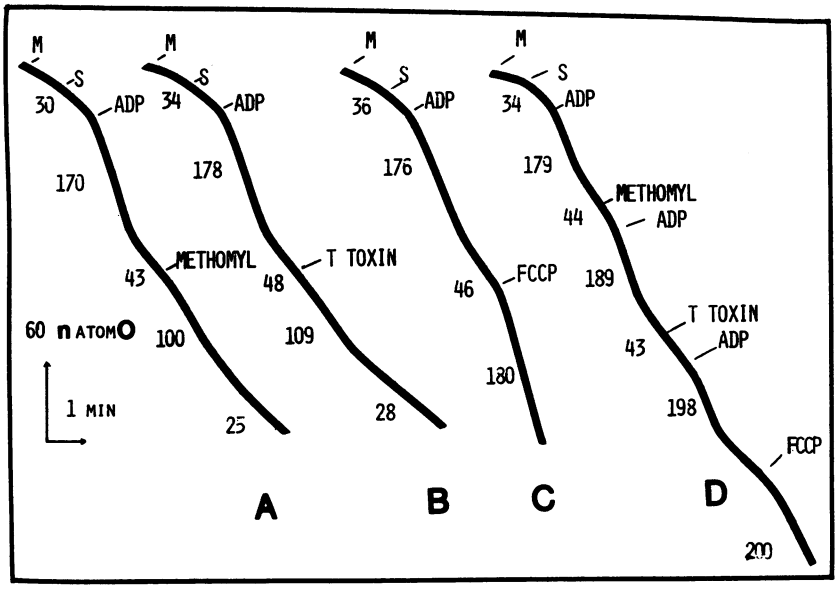

FIG. 6. Traces of maize mitochondria oxidizing succinate. Sequential addition as indicated by arrows: M, mitochondria (2 mg protein); S, succinate, final concentration 10 mM; methomyl, final concentration 10 mM; ADP, final concentration 50 μM; FCCP, final concentration 50 μM; T-toxin, final concentration 91 nM. Numbers along the traces indicate oxygen uptake in natoms·min^{-1}·mg^{-1} protein. A, B, and C: T mitochondria; D: N mitochondria.

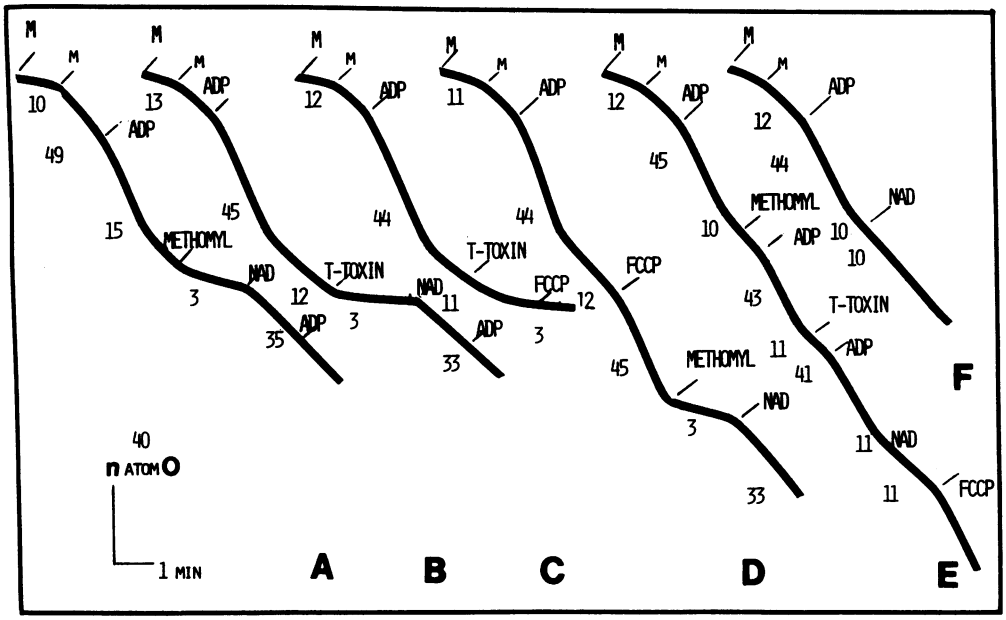

FIG. 7. Traces of maize mitochondria oxidizing malate. Sequential addition as indicated by arrows: M, mitochondria (4 mg protein); malate, final concentration 30 mM; methomyl, final concentration 10 mM; ADP, final concentration 50 μM; FCCP, final concentration 50 μM; T-toxin, final concentration 91 nM; NAD, final concentration 0.5 mM. Numbers along the traces indicate oxygen uptake in natoms·min^{-1}·mg^{-1} protein. A, B, C, D, and F: T mitochondria; E: N mitochondria.

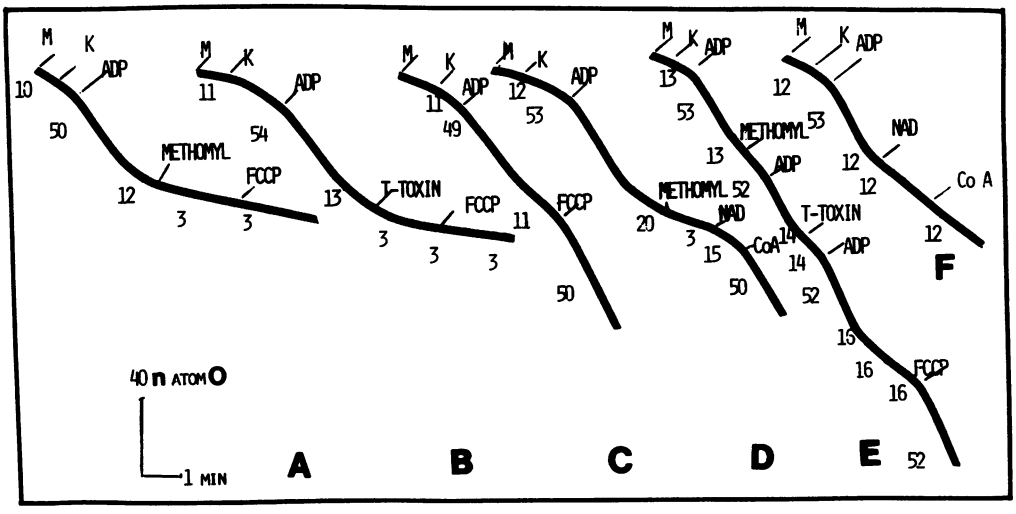

FIG. 8. Traces of maize mitochondria oxidizing α-ketoglutarate. Sequential addition as indicated by arrows: M, mitochondria (2 mg protein); K, α-ketoglutarate, final concentration 10 mM; methomyl, final concentration 10 mM; ADP, final concentration 150 μM; FCCP, final concentration 50 μM; T-toxin, final concentration 91 nM; NAD, final concentration 0.5 mM; CoA, final concentration 0.5 mM. Numbers along the traces indicate oxygen uptake in natoms·min^{-1}·mg^{-1} protein. A, B, C, D, and F: T mitochondria; E: N mitochondria.

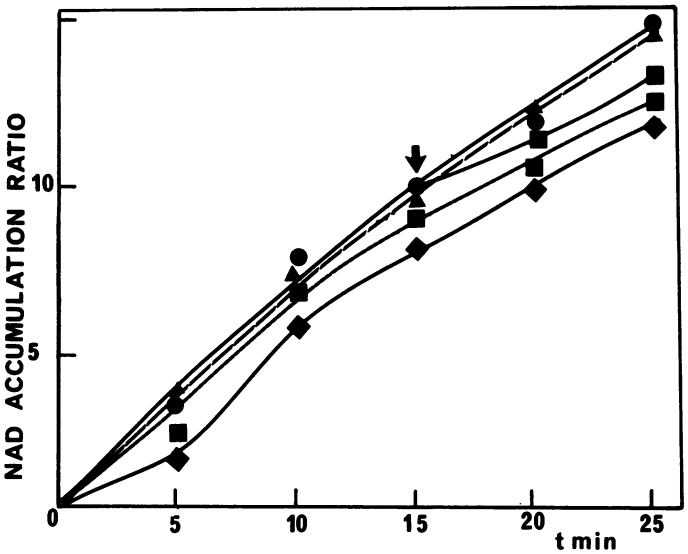

FIG. 9. Effect of methomyl or T-toxin on the time course of [^{14}C]-NAD accumulation (1 μM, external concentration) by N mitochondria (3.85 μl of matrix volume) energized by 10 mM succinate. Each points corresponds to two experiments. Control (●); + 10 mM methomyl (◆); + 5 × 10^{-7} M T-toxin (▲); + 50 μM FCCP (■); ↓, addition of 10 mM methomyl.

phorylation in T mitochondria. If the hypothesis of a direct effect of these compounds on the ATPase or on ATP, ADP, or Pi transport cannot be ruled out, it is at this stage unnecessary.

A possible effect of methomyl or T-toxin could be a permeabilization of the internal mitochondrial membrane to ions, and especially to protons. The mechanism of such an effect could not be identical to that of the unspecific effect of uncouplers, which carry protons across the hydrophobic core of the membrane lipids, since no effect is observed in N mitochondria. It is possible to conceive, nevertheless, that methomyl or T-toxin interacts with a membrane protein, present in T mitochondria and absent (or modified) in N mitochondria, thus leading to the opening of a channel for protons or other ions and subsequently to the observed collapse of the PMF.

The stimulation of NADH oxidation observed previously (3, 12, 19) and in this study could indeed be explained by such an uncoupling effect, since depolarization and stimulation of respiration take place in the same active concentration range for methomyl as well as for T-toxin. However, the increase in respiration caused by methomyl or T-toxin is partly due to oxidation of NADH via the rotenone sensitive pathway, which would indicate that under these conditions NADH can enter the matrix, while in the case of an increased respiration induced by a protonophore such as FCCP, oxidation via this pathway does not occur.

Moreover, the inhibition of malate oxidation (3, 12, 19) and of α-ketoglutarate oxidation (4) by methomyl and T-toxin is in striking contrast with the stimulation induced by uncouplers. The action of methomyl and of T-toxin cannot therefore be explained only in terms of uncoupling (namely membrane permeabilization to protons or to ions).

The fact that malate oxidation can be restored after methomyl or T-toxin treatment by addition of NAD strongly suggests that inhibition of malate oxidation could be due to an efflux of NAD out of the mitochondria, induced by methomyl or T-toxin. Such an efflux, induced by T-toxin, has indeed been reported by Matthews *et al.* (23). Douce and collaborators (28, 34) have shown that, in potato tuber mitochondria, contrary to what happens in mammalian mitochondria (21), NAD is actively transported and that this transport has a direct influence on malate oxidation. We have shown here that NAD is also transported in maize mitochondria. This transport is not dependent on the PMF, since it is unaffected by FCCP. The action of methomyl or of T-toxin, which are shown here to prevent accumulation of NAD or promote efflux of accumulated NAD, is not therefore a consequence of their action on the PMF. The inhibition of NAD transport by methomyl or T-toxin could explain the blocking of malate-sustained respiration, since, in the absence of NAD, malate cannot be oxidized to pyruvate or to oxaloacetate.

The comparison between the effect of methomyl and T-toxin on malate oxidation and on NAD transport, as a function of increasing concentrations, seems to support this interpretation and stresses again the specificity of action of these compounds. In the case of methomyl, both effects (inhibition of malate oxidation and of NAD transport) occur between 1 and 3 mM. The clearly unspecific effect of methomyl on N and T mitochondria above 10 mM should be compared to the similar effect observed on depolarization. Interpretation of the results obtained with T-toxin is less evident: maximum inhibition of malate oxidation is achieved for a concentration of 100 nM while inhibition of NAD accumulation does not change between 10 and 100 nM. However, the dose-response effect of T-toxin on NAD transport has been found to vary with the mitochondria preparation; Figure 11 gives the more constantly observed pattern but, in some cases, inhibition of NAD transport increased steadily up to 1 μM. No effect of T-toxin is observed in N mitochondria at these concentrations. In this respect, it should be noted that an unspecific effect of T-toxin on malate oxidation in N mitochondria has been reported (30), but for concentrations 60 times higher than the maximum concentration tested here. Neverthe-

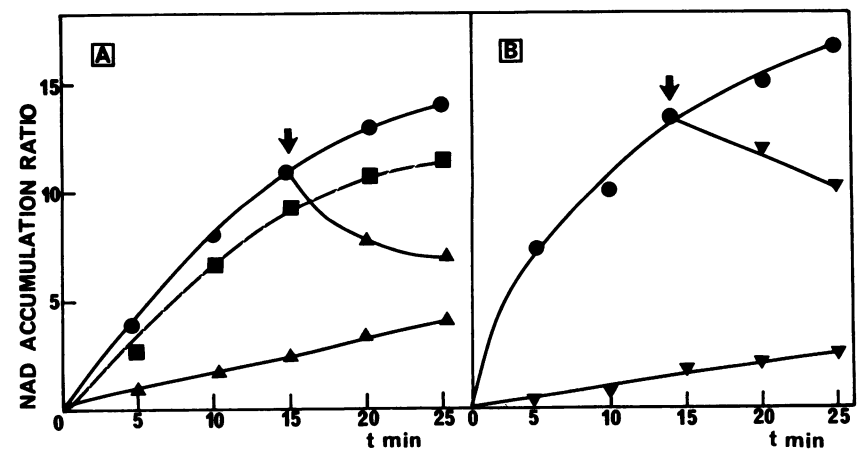

FIG. 10. A, Effect of methomyl on time course of [^{14}C]NAD (1 μM, external concentration) by T mitochondria (3.70 μl of matrix volume). Control (●); + 10 mM methomyl (▲); + 50 μM FCCP (■); ↓.; addition of 10 mM methomyl. B, Effect of T-toxin on time course of [^{32}P]NAD accumulation (1 μM, external concentration) by T mitochondria (2.50 μl of matrix volume). Control (●); + 5 × 10^{-7} M T-toxin (▼); ↓, addition of 5 × 10^{-7} M T-toxin.

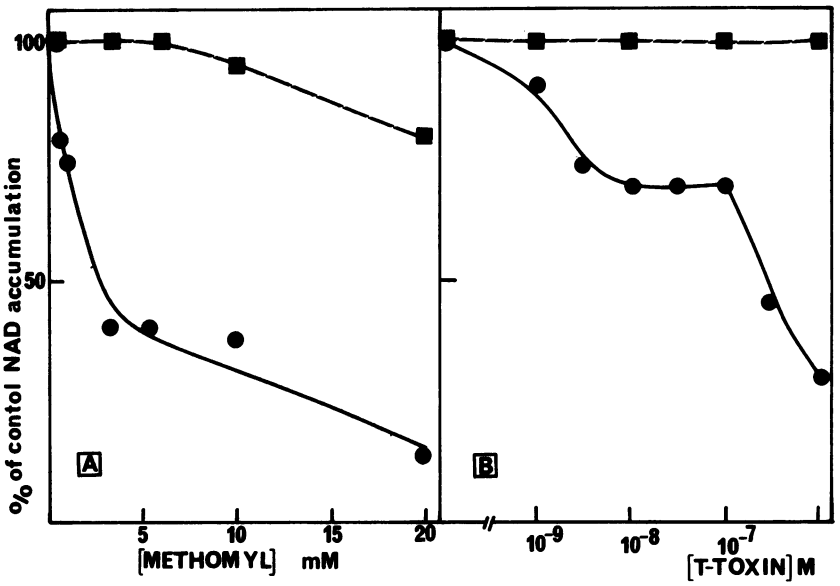

FIG. 11. Effect of varying concentrations of methomyl and T-toxin on [^{32}P]NAD accumulation (1 μM, external concentration) by N (■) and T (●) mitochondria (5.13 and 4.15 μl of matrix volume, respectively). A, Effect of methomyl; the accumulation ratio of NAD by the control was 17 after 25 min incubation. B, Effect of T-toxin; the accumulation ratio of the control was 14 after 25 min incubation.

less, it is apparent from Figures 8 and 10 that inhibition of malate oxidation is complete within 2 min, while significant efflux of NAD induced by the drugs appears to be much slower. Clearly, the relationship between the amount of NAD released from the mitochondria and the induced inhibition of malate oxidation deserves further attention.

The fact that CoA plus NAD can restore α-ketoglutarate sustained respiration previously inhibited by methomyl or T-toxin, strongly suggests that CoA, like NAD, is transported in plant mitochondria and that methomyl and T-toxin cause a release of this cofactor. It should be stressed that this release of cofactors is not the result of unspecific leakiness of the membrane caused by methomyl or T-toxin, since these compounds do not make the membrane permeable to mannitol. Nevertheless, the release of cofactors does not explain the absence of a PMF during oxidation of succinate, NADH, or malate supplemented with NAD.

Finally, the temporary stimulation of succinate oxidation by methomyl or T-toxin is difficult to understand. Stimulation or inhibition of succinate oxidation upon addition of these compounds has been reported, depending on the osmoticum used (sucrose or KCl) (12, 19). The reason for these discrepancies is not clear.

In conclusion, methomyl and T-toxin have apparently at least two different effects in T mitochondria: (a) collapse of the PMF which can account for the lack of oxidative phosphorylation; (b) release of the cofactors NAD and CoA which can account for the inhibition of malate and α-ketoglutarate oxidation.

Further studies should help to decide whether these two effects correspond to different targets for the toxin or methomyl, or whether they are the consequence of a more general effect.

Acknowledgments—We wish to thank Drs. R. Douce, L. Letellier, M. Neuburger, H. Therisod, and E. Shechter for very stimulating discussions and Dr. Gianinazzi-Pearson for correction of the English manuscript.

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