The Effect of Respiratory Inhibitors on NADH, Succinate and Malate Oxidation in Corn Mitochondria

R. H. Wilson and J. B. Hanson
Department of Botany, University of Illinois, Urbana, Illinois 61801
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Abstract. The effect of a series of respiratory inhibitors on the oxidation of NADH in state 4 and state 3 conditions was studied with corn shoot mitochondria. Comparisons were made using malate and succinate as substrates. The inhibitors, rotenone, amytal, antimycin A and cyanide, inhibited oxidation of NADH in state 3 but rotenone and amytal did not inhibit oxidation in state 4. The inhibition by antimycin A was partially overcome by the presence of cytochrome c. The results indicate the presence of alternative pathways available for NADH oxidation depending on the metabolic condition of the mitochondria. Under state 4 conditions, NADH oxidation bypasses the amytal and rotenone sensitive sites but under state 3 conditions a component of the NADH respiration appears to be oxidized by an internal pathway which is sensitive to these inhibitors. Still a third pathway for NADH oxidation is dependent on the addition of cytochrome c and is insensitive to antimycin A. Succinate oxidation was sensitive to cyanide and antimycin A under both state 4 and state 3 conditions as well as amytal and rotenone under state 3 conditions but was not inhibited by amytal and rotenone under state 4 conditions. Malate oxidation was inhibited by cyanide, rotenone and amytal under both state 4 and state 3 conditions. Antimycin A inhibited state 3 but did not appreciably alter state 4 rates of malate oxidation. With all substrates tested inhibition by antimycin A was greatly facilitated by preswelling the mitochondria for 10 min. This was interpreted to indicate that swelling increases the accessibility of antimycin A to the site of inhibition.

Previous studies with plant mitochondria have shown many similarities with animal mitochondria regarding substrates which are oxidized as well as the action of respiratory chain inhibitors (4, 24, 27, 31). One exception is the capacity of plant mitochondria to oxidize extramitochondrial NADH (2, 5, 12, 22, 23, 25, 26). Animal mitochondria only slowly oxidize or lack the capacity to oxidize externally added NADH (3, 9, 13, 29, 36) unless cytochrome c is added to the reaction mix or the mitochondria are subjected to treatments which facilitate the movement of NADH across the membrane (14, 29, 30, 34). With higher plant mitochondria oxidation of external NADH is rapid with P/O values between 1.0 to 1.4 (2, 12, 22, 26, 45), indicating that only 2 coupling sites are utilized. Cunningham has suggested from the 1.2 P/O value with corn root mitochondria that NADH oxidation may bypass the flavoprotein dehydrogenase of the electron transport chain (12). Similar to higher plant mitochondria, yeast mitochondria oxidize externally-added NADH by a pathway which is insensitive to rotenone (38), an inhibitor which acts on the substrate side of cytochrome b (6, 8, 17, 32). The yeast NADH oxidase also lacks the mercurialsensitive group functionally situated between NADH and flavoprotein as well as the characteristic electron paramagnetic spin signal at g = 1.94. These results as well as the low P/2e suggest that in yeast the first phosphorylation site may not be operative with NADH as substrate (35, 38, 39, 43).

Sottocasa et al. (40) recently have shown that rat liver mitochondria contain an active NADH-cytochrome c reductase which catalyzes the oxidation of exogenous NADH by added cytochrome c or ferricyanide in a reaction that is insensitive to respiratory chain inhibitors including rotenone, amytal, and antimycin A. The system, which is similar to but distinct from the microsomal NADH-cytochrome

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2 Present address: Department of Botany, University of Texas, Austin, Texas 78712.
3 Abbreviations: BSA, Bovine Serum Albumin; ADP, adenosine diphosphate; P₃, inorganic phosphate; NAD, NADH, nicotinamide adenine dinucleotide and its reduced form.
b₂ reductase system, is associated with the mitochondrial outer membrane and is not coupled to phosphorylation.

Inasmuch as we have been using NADH as substrate in studying active influx and efflux transport in corn shoot mitochondria (44), it became necessary to have data on NADH oxidation and its inhibition, particularly with KCl as osmoticant. This study reports the effect of rotenone, amytal, antimycin A and cyanide on state 4 and state 3 rates of NADH oxidation by corn shoot mitochondria. Comparisons were made using malate and succinate as substrate. The results show that amytal and rotenone do not inhibit state 4 NADH oxidation but partially inhibit state 3 rates. Antimycin A and cyanide completely inhibited both state 4 and state 3 rates, but the antimycin A inhibited respiration was partially restored by the addition of cytochrome c. From these results it is concluded that portions of the pathway of NADH oxidation differ depending on the metabolic state and the environment of the corn mitochondria.

Materials and Methods

Mitochondria were isolated by differential centrifugation in 0.4 mM sucrose, 20 mM tris-Cl buffer (pH 7.6) and 5 mM EDTA by previously described procedures but with the omission of ADP during washing (28). Oxygen was measured polarographically with a Clark oxygen electrode in a 2.6 ml controlled temperature chamber. The value for dissolved O₂ in solution was corrected for temperature (27°) and dissolved KCl. In experiments in which state 4 rates were measured, the mitochondria were added to a reaction mixture containing substrate, 0.2 mM KCl, 4 mM MgCl₂, 4 mM P₄, 1 mg/ml BSA, 20 mM Tricine buffer (pH 7.4), and were passed through one state 4—state 3—state 4 cycle by addition of 0.25 μmoles ADP. After the second state 4 was established 1 μg/ml oligomycin was added to block any increment of respiration due to ADP formed by ATPase. Inhibitor was then added. State 3 rates were measured in the same medium but containing a hexokinase trap of 0.6 mg hexokinase (Sigma) and 50 mM glucose to maintain ADP levels. State 3 rates were initiated by addition of 0.25 μmoles ADP with inhibitor added subsequently.

Rotenone (K and K, Inc.) and antimycin A (California Corporation for Biochem. Research) were dissolved in ethanol. Amytal (Eli Lilly and Co.) was made up fresh daily in lightly buffered solution pH 7.0. ADP concentration was determined spectrophotometrically based on an extinction coefficient of 15.4 at 260 μM. All other chemicals were reagent grade and were dissolved in glass distilled water.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>State 3 rates</th>
<th>Respiratory control</th>
<th>ADP:O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O₂ nmol/min</td>
<td>per mg protein</td>
<td>ratio</td>
</tr>
<tr>
<td>NADH</td>
<td>177</td>
<td>2.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Malate</td>
<td>62</td>
<td>3.2</td>
<td>2.4</td>
</tr>
<tr>
<td>Succinate</td>
<td>135</td>
<td>2.8</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Results

Coupling Characteristics. Table I summarizes the state 3 respiration rates, the respiratory control ratios and the ADP:O ratios of corn mitochondria with KCl as osmoticant. Chloride solutions are not preferred osmoticants since the mitochondria will swell unless respiring, in which case volume is regulated by an efflux pump (44). However, the values in table I are not too different from those obtained with plant mitochondria in sucrose.

The ADP:O ratio of 1.3 with NADH is in good agreement with results from other plant mitochondria and suggests oxidation by a pathway entering after Site I.

Effects of Rotenone. The effect of rotenone on both state 4 and state 3 rates of oxidation with externally added NADH, succinate and malate is shown in Fig. 1. Consistent with the role of rotenone in blocking NADH-linked substrate oxidation (6,17) malate oxidation was inhibited 85% and 100% in state 4 and state 3 respectively. On a protein basis the half-maximal inhibition for rotenone with malate as substrate was 5.9 nmoles/mg protein for state 4 rates and 3.0 nmoles/mg protein for state 3 rates. An identical value was obtained with mung bean mitochondria in state 3 (27). As pointed out by Ikuma and Bonner (27) this value is considerably higher than those reported for rat liver mitochondria (17). With both NADH and succinate, rotenone slightly enhanced state 4 oxidation (Fig. 1A). In contrast state 3 respiratory rates were inhibited, although never completely so. With succinate half-maximal inhibition was attained at 10.1 nmoles/mg protein. NADH oxidation fell off slowly with increasing concentration of rotenone and a maximum inhibition was not attained.

Effects of Amytal. With few exceptions rotenone and oxybarbiturates have been found similar in their effects on electron transport with animal mitochondria (6,8). Similarly, with corn mitochondria the effects of amytal on substrate oxidation (Fig. 2) are very like the effects with rotenone (Fig. 1). Malate oxidation was completely inhibited in both state 4 and state 3, with a half-maximal inhibition at 3.0 mM and 0.7 mM respectively. With NADH state 4 oxidation was slightly stimulated by amytal.
while maximum inhibition of state 3 was 40% at 6 mM. Amytal showed little effect on state 4 succinate oxidation but almost completely inhibited respiration in state 3 at 8 mM. The concentration of half-maximal inhibition was 3 mM. These results in general agree with the values for half-maximal inhibition and degree of inhibition reported for mung bean mitochondria (27) with the exception of the greater sensitivity of malate oxidation in corn mitochondria. Amytal inhibition of succinate oxidation has been reported in animal mitochondria (8,37) but it was not as extensive as found here. The reason for the almost complete inhibition at high concentrations is not clear.

**Effects of Antimycin A.** Studies on the effect of antimycin A at low concentrations (<$10^{-7}$ M) were complicated by a pronounced lag between the time the inhibitor was added and the time its effect was seen. During this lag time, which generally lasted 10 min or longer, the inhibition of respiration gradually increased but the O$_2$ in the medium was depleted before respiration was completely inhibited. The effect of higher concentrations (>10$^{-7}$ M) on substrate oxidation in which the maximum effect was observed in less than 5 min after the addition of the antimycin A is shown in Fig. 3. Complete inhibition was observed with NADH and succinate in both state 4 and state 3. Malate oxidation was almost completely inhibited in state 3 but state 4 respiration was remarkably resistant.

A short lag time in respiratory inhibition with antimycin A has been reported with animal mitochondria (17, 20). One suggested explanation for the lag time was the accessibility of antimycin A to its site of inhibition (20). To examine this possibility corn mitochondria were incubated without

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**Fig. 1.** Effect of rotenone concentration on NADH, succinate and malate oxidation. The reaction media is given in Materials and Methods section. Concentration of substrates were 1 mM NADH, 10 mM succinate, and 8 mM malate. Protein was 0.88 mg Temperature 27ºC. A) second state 4 rates; B) state 3 rates.

**Fig. 2.** Effect of amyta l on NADH, succinate and malate oxidation. Conditions are identical with Fig. 1. Protein was 0.89 mg. A) second state 4 rates; B) state 3 rates.
substrates (state 1) for a period of 10 min during which passive swelling is known to occur (41). Table II shows that the presence of antimycin A during the passive swelling or its addition after 10 min passive swelling eliminated the lag time and resulted in maximal inhibition immediately after the addition of the substrate. In comparison, without preswelling, the extent of respiratory inhibition was less than 15 % of the maximal inhibition obtainable. An apparent additional effect of preswelling was a lack of complete respiratory inhibition by antimycin A. Between 10 to 25 % of the respiration with each of the 3 substrates was insensitive to concentrations of antimycin A as high as 3.0 μM for periods up to 10 min, the longest tested. It could be that while the swelling of mitochondria increases the accessibility of antimycin A to the site of inhibition it also opens an antimycin-resistant pathway. Cytochrome c partially restored NADH oxidation after inhibition by antimycin A but did not restore either malate or succinate oxidation (Table II).

**Effects of Cyanide.** While several plant tissues show a cyanide insensitive respiration (4, 21) the results in Fig. 4 show that respiration with corn mitochondria can be completely inhibited by cyanide. The half-maximal inhibition for NADH with cyanide was 70 μM for both state 4 and state 3 rates. The half-maximal inhibition for succinate was 80 μM in both state 4 and state 3, and for malate was 70 μM in state 4 and 60 μM in state 3. In contrast, Ikuma and Bonner (27) found about 20 % cyanide resistant respiration and half-maximal inhibitory concentrations of 5 to 15 μM. It is not clear why these differences exist but is worth noting that complete inhibition was not instantaneous. On addition of cyanide there was an abrupt decline in oxygen consumption but 30 to 60 sec elapsed before complete inhibition was obtained. It is probably also relevant that cyanide treated mitochondria undergo swelling in KCl (41).

**Discussion**

Under state 4 conditions of NADH oxidation, neither amytal nor rotenone inhibited electron flow with corn mitochondria. However, during state 3 conditions, NADH oxidation was partially inhibited by both amytal and rotenone (Fig. 1 and 2). Thus, coupling of phosphorylation to electron transport appears to affect the sensitivity of NADH oxidation to these inhibitors. One possible explanation for this difference in sensitivity may be the presence of alternative pathways of oxidation in the different states. Based on spectrophotometric studies with yeast mitochondria, it has been proposed that both rotenone and amytal inhibit NADH-linked substrate oxidation at a site between 2 flavoproteins, FPD1 and FPD2 located on the pathway to cytochrome b (6). This pathway does not appear to be utilized by exogenous NADH oxidation which results in the reduction of still another flavoprotein component of

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**Table II. The Effect of Passive Swelling on Substrate Oxidation in the Presence of Antimycin A and Cytochrome c**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Substrate</th>
<th>Conc. of antimycin A</th>
<th>State 3 rates1</th>
</tr>
</thead>
<tbody>
<tr>
<td>No preswelling</td>
<td>NADH</td>
<td>0.0</td>
<td>155.5</td>
</tr>
<tr>
<td>No preswelling</td>
<td>NADH</td>
<td>0.5</td>
<td>135.9</td>
</tr>
<tr>
<td>Swelling in the presence of antimycin A²</td>
<td>NADH</td>
<td>0.5</td>
<td>14.0</td>
</tr>
<tr>
<td>Swelling in the absence of antimycin A³</td>
<td>NADH</td>
<td>0.5</td>
<td>14.0</td>
</tr>
<tr>
<td>Swelling in the absence of antimycin A plus cytochrome c⁴</td>
<td>NADH</td>
<td>0.5</td>
<td>35.5</td>
</tr>
<tr>
<td>No preswelling</td>
<td>Malate</td>
<td>0.0</td>
<td>66.8</td>
</tr>
<tr>
<td>No preswelling</td>
<td>Malate</td>
<td>0.5</td>
<td>62.6</td>
</tr>
<tr>
<td>Swelling in the presence of antimycin A²</td>
<td>Malate</td>
<td>0.5</td>
<td>17.2</td>
</tr>
<tr>
<td>Swelling in the absence of antimycin A³</td>
<td>Malate</td>
<td>0.5</td>
<td>12.0</td>
</tr>
<tr>
<td>Swelling in the absence of antimycin A plus cytochrome c⁴</td>
<td>Malate</td>
<td>0.5</td>
<td>11.1</td>
</tr>
<tr>
<td>No preswelling</td>
<td>Succinate</td>
<td>0.0</td>
<td>125.1</td>
</tr>
<tr>
<td>No preswelling</td>
<td>Succinate</td>
<td>0.5</td>
<td>105.2</td>
</tr>
<tr>
<td>Swelling in the presence of antimycin A²</td>
<td>Succinate</td>
<td>0.5</td>
<td>10.2</td>
</tr>
<tr>
<td>Swelling in the absence of antimycin A³</td>
<td>Succinate</td>
<td>0.5</td>
<td>11.1</td>
</tr>
<tr>
<td>Swelling in the absence of antimycin A plus cytochrome c⁴</td>
<td>Succinate</td>
<td>0.5</td>
<td>12.0</td>
</tr>
</tbody>
</table>

1 Rates immediately after adding substrate.
2 Antimycin A added immediately after mitochondria and before swelling.
3 Antimycin A added after mitochondria had swollen and immediately before addition of substrate.
4 Same as 3 plus 8 × 10⁻⁶ M cytochrome c added with the antimycin A.
yeast, $F_{o25}$, associated with an additional pathway of oxidation. It has also been shown with yeast mitochondria that NADH oxidation has lower $P/2e$ ratios, lacks a mercurial sensitive functional group situated between NADH and flavoprotein as well as an electron paramagnetic resonance signal at $g = 1.94$, and is insensitivity to rotenone (35, 38, 39, 40). Thus, the first coupling site appears to be bypassed by NADH oxidation. In our study under state 4 rates the lack of inhibition by amytal and rotenone as well as the low ADP:O ratios (1.3, table I) support the conclusion that the first coupling site with NADH is bypassed (12). A similar pathway appears to exist in liver mitochondria with glutamate oxidation in the presence of vitamin K$_3$.
which is required for activation. This pathway is not sensitive to amytal (11) or rotenone (17) but is sensitive to antimycin A and has P:O ratio of 1.3 to 1.7.

The partial inhibition of NADH oxidation under state 3 conditions suggests a change in the early portion of the pathway of NADH oxidation. In some fashion NADH has gained access to rotenone and amytal sensitive oxidation sites. With animal mitochondria, it has been shown that NADH is oxidized in appropriately treated mitochondria by an internal pathway which is coupled to phosphorylation and is sensitive to amytal as well as antimycin A (15, 18, 30, 33). Lehninger (29, 30) showed that hypotonic swelling facilitated this pathway of oxidation. Under state 3 conditions corn mitochondria have been shown to swell somewhat (44) which might provide for accessibility of NADH to an internal oxidative pathway. There may even be some active inward transport of NADH to the internal rotenone-sensitive flavoproteins. Inhibition of NADH respiratory activity by amytal in liver mitochondria is greater in the presence of ADP and P1 than in the presence of an uncoupler, indicating an inhibition of energy transfer by amytal in addition to an inhibition of electron transfer. Thus the inhibition of NADH oxidation when ADP and P1 are present is attributed to an effect of energy transfer (8). Additional studies are necessary to clarify these possibilities.

A number of studies with liver mitochondria have shown an additional path for NADH oxidation which requires externally added cytochrome c, is not coupled to phosphorylation and is insensitive to both amytal and antimycin A (1.7, 30, 40). Studies by Sottocasa et al. (40) have shown that rat liver mitochondria contain an active NADH-cytochrome c reductase which catalyzes the oxidation of exogenous NADH by added cytochrome c in a reaction that is insensitive to respiratory chain inhibitors including rotenone, amytal, and antimycin A. The partial insensitivity of NADH oxidation by corn mitochondria to antimycin A in the presence of cytochrome c may be due to a similar NADH-cytochrome c reductase (table II). Neither malate nor succinate oxidation was restored by addition of cytochrome c to the antimycin A inhibited respirations.

Acknowledgment

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Literature Cited


