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# Cyanide-resistant Respiration of Sweet Potato Mitochondria[1]

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## ABSTRACT

**The oxidation of malate and succinate by sweet potato mitochondria (*Ipomoea batatas* [L.] Lam.) was blocked only partly by inhibitors of complexes III (2-heptyl-4-hydroxyquinoline-N-oxide) and IV (cyanide and azide). The respiration insensitive to inhibitors of complexes III and IV was inhibited by salicylhydroxamic acid. Essentially complete inhibition was obtained with inhibitors of complex I (rotenone, amytal, and thenoyltrifluoroacetone) and complex II (thenoyltrifluoroacetone). The observations indicated that electrons were transferred to the cyanide-resistant pathway from ubiquinone or from nonheme iron (iron-sulfur) proteins of complexes I and II before reaching the *b* cytochromes. In contrast, the oxidation of exogenous NADH did not involve the alternate pathway, as indicated by complete inhibition by inhibitors of complexes III and IV and the absence of an effect of inhibitors of complexes I and II. Hence, electrons from exogenous NADH appear to pass directly to complex III in sweet potato mitochondria.**

In contrast to that of animal mitochondria, the respiration of plant mitochondria from some sources is not inhibited completely by cyanide (12). The degree of resistance to cyanide varies with species and treatment of the tissue. For example, $O_2$ uptake of mitochondria freshly isolated from white potato tubers (*Solanum tuberosum* L.) was inhibited completely by cyanide, whereas the respiration of mitochondria extracted from hypocotyls of legumes (*Pisum sativum* L., *Phaseolus aureus* Roxb.), sweet potato roots (*Ipomoea batatas* [L.] Lam.), and skunk cabbage spadices (*Symplocarpus foetidus* [L.] Nutt.) was inhibited approximately 80, 50, and 30%, respectively, by cyanide (1 mM or less) with succinate as substrate (1, 2, 4, 10, 31). Cyanide had no effect on the oxidation of succinate by mitochondria isolated from spadices of *Arum maculatum* L. (21). Although the respiration of mitochondria isolated from intact white potato tubers was completely sensitive to cyanide, resistance to cyanide was observed in mitochondria extracted from slices of tubers following exposure to air or running water for several hours (16, 20).

The effect of cyanide on the oxidation of malate and NADH has not been studied as extensively as its effect on the oxidation of succinate. With sweet potato mitochondria, the sensitivity of malate oxidation to cyanide paralleled that of succinate oxidation (6). In contrast, the oxidation of malate by *Arum* mitochondria was partly cyanide-resistant, whereas the oxidation of succinate, as indicated above, was entirely resistant to cyanide (21). The oxidation of exogenous NADH by sweet potato mitochondria was inhibited strongly by cyanide (3, 6), whereas only partial inhibition was obtained with *Arum* mitochondria (27).

The site at which the cyanide-resistant (alternate) pathway branches from the cyanide-sensitive electron transport system has not been identified precisely. Electron transport involving complexes I and II is inhibited by TTA[3] (30), an inhibitor of nonheme iron proteins (22, 24); hence, branching of the cyanide-resistant pathway must occur at or subsequent to complexes I and II, but prior to the *b* Cyt (2, 4, 6, 12). The cyanide-resistant pathway is inhibited by hydroxamic acids such as SHAM (23).

The objectives of the experiments reported herein were to characterize the participation of the alternate pathway during the oxidation of malate, succinate, exogenous NADH, and reduced TMPD in mitochondria isolated from sweet potato roots.

## MATERIALS AND METHODS

Sweet potato roots (variety Jewel) were harvested in the fall of 1972 and 1973, cured for 1 week at 29.5 C and 90 to 95% relative humidity, and then stored in ventilated chambers at 14.0 ± 0.5 C. The roots were used between 3 and 9 months after harvest.

Mitochondria were extracted by a modification of a procedure reported by Verleur (26). Extraction and suspension media are identified in Table I. The outer 0.5 cm of the root was discarded. Of the remaining tissue, 150 g were diced, added to an equal amount of extraction medium (w/v), and homogenized in a Sorvall Omni-Mixer. The homogenate was centrifuged at 1,900*g*, and the mitochondria were collected by centrifugation of the supernatant at 14,500*g*. The pellet was washed and resuspended in suspension medium. All operations were carried out between 0 and 4 C.

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[3] Abbreviations: TTA: thenoyltrifluoroacetone; HOQNO: 2-heptyl-4-hydroxyquinoline-N-oxide; SHAM: salicylhydroxamic acid; TMPD: N,N,N',N'-tetramethyl-*p*-phenylenediamine; RC: respiratory control.

Table I. *Composition of Media Used for Studies with Sweet Potato Mitochondria*

| Component | Extraction Medium | Suspension Medium | Reaction Mixture |
|---|---|---|---|
| Mannitol | 0.5 M | 0.5 M | 0.5 M |
| HEPES | 20 mM | 20 mM | |
| $K_2HPO_4$, $KH_2PO_4$ (pH 7.2) | | | 10 mM |
| EDTA | 1 mM | 0.1 mM | 0.1 mM |
| BSA | 0.1% | 0.1% | |
| Ascorbate | 10 mM | | |
| $MgCl_2$ | | 5 mM | |
| pH | 7.5 | 7.2 | 7.2 |

To the components of the reaction mixture listed in Table I were added either 200 μmoles of malate, 16 μmoles of succinate, 2.2 μmoles of NADH, or 0.3 μmole of TMPD as substrate and an aliquot of the mitochondrial suspension containing 0.7 mg protein, in a final volume of 2 ml. When TMPD was used as the electron donor, 3 μmoles of sodium ascorbate and 10 μM HOQNO were included to maintain TMPD in the reduced form and to force electron flow through Cyt oxidase. Oxygen uptake was measured with a Clark electrode fitted into a 2-ml reaction cell thermostated at 25 C. The $O_2$ content of the air-saturated media was calculated to be 236 nmoles $O_2$/ml. The protein concentration was estimated, after solubilization of the mitochondria with NaOH, by the method of Lowry *et al.* (17) with crystalline BSA as a standard.

Following the terminology of Chance and Williams (7), ADP-stimulated and ADP-limited respiration are referred to as state 3 and state 4 respiration, respectively. The respiratory control ratio is defined as the state 3 rate divided by the state 4 rate of $O_2$ uptake. ADP/O ratios were calculated by dividing the moles of ADP added by the moles of atomic $O_2$ consumed during state 3 respiration.

SHAM, rotenone, amytal, and TTA were dissolved in acetone, and HOQNO was added in ethanol. At the final concentration in the reaction mixture (1%, v/v), neither solvent affected the state 3 oxidation of malate, succinate, or NADH, or the cyanide-resistant oxidation of succinate. With malate as substrate, however, acetone and ethanol inhibited cyanide-resistant respiration approximately 30 and 20%, respectively.

Effects on $O_2$ uptake were measured by adding the test compounds to mitochondrial suspensions that were respiring at a constant rate in either state 3 or state 4. Per cent inhibition was calculated by relating the rate of $O_2$ utilization in the presence of the inhibitor to the state 3 or state 4 control rate. A response curve relating per cent inhibition to the logarithm of the concentration was obtained for each inhibitor. A concentration of each compound was selected from the plateau region of the response curve.

Data were averaged from observations made with three or more separate mitochondrial isolations.

## RESULTS

**Oxidation of Malate.** The respiration of sweet potato mitochondria, with malate as substrate, was stimulated by the addition of ADP, but was coupled loosely to phosphorylation, as indicated by the relatively low RC and ADP/O ratios (Fig. 1, trace A). RC and ADP/O ratios averaged 2.5 and 1.3, respectively. The efficiency of phosphorylation was less than that reported for mung bean mitochondria (13).

The oxidation of malate was inhibited only partly (approximately 40%) by cyanide (Fig. 1, trace A; Fig. 2). As indicated by the shape of the response curve (Fig. 2), the Cyt system became saturated when the concentration of cyanide reached 40 μM, and increasing the concentration of cyanide to 1 mM had little additional effect. Inhibition of state 3 respiration by $NaN_3$ and HOQNO was as incomplete as the inhibition by cyanide (Table II). Response curves obtained with $NaN_3$ and HOQNO were similar in shape to the curve obtained with cyanide.

In contrast to compounds that affect sites in complexes III and IV, inhibitors of complex I (TTA, rotenone, and amytal) (11) blocked a large percentage of the total state 3 oxidation of malate (Table II).

SHAM (0.75 mM) had a slight effect (approximately 30% inhibition) on the ADP-stimulated oxidation of malate (Table II). The low RC and ADP/O ratios referred to above appear

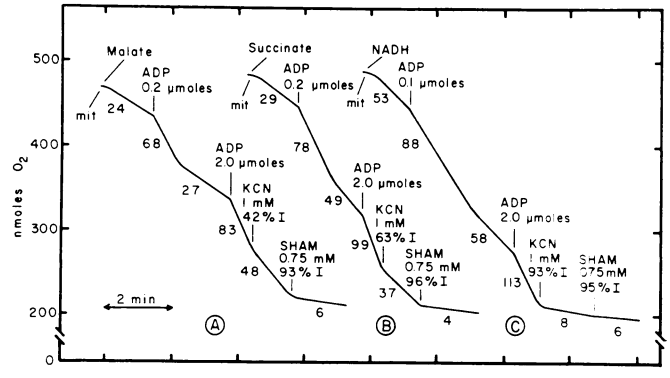


FIG. 1. Representative polarographic traces depicting $O_2$ utilization obtained with sweet potato mitochondria during the oxidation of malate (trace A), succinate (trace B), and NADH (trace C). Respiratory control is shown in the first cycle, and inhibitors were added after the introduction of excess ADP to regenerate state 3 respiration. Rates of $O_2$ utilization (nmoles $O_2$/min·2 ml) are indicated below the traces. Mitochondria (mit) containing 0.7 mg protein, 200 μmoles of malate, 16 μmoles of succinate, 2.2 μmoles of NADH, ADP, KCN, and SHAM were added at the points indicated. Concentrations of components are shown as μmoles added (ADP) or as the final molarity (KCN, SHAM) in the 2-ml reaction medium. Per cent inhibition of state 3 respiration produced by cyanide alone and in combination with SHAM is shown.

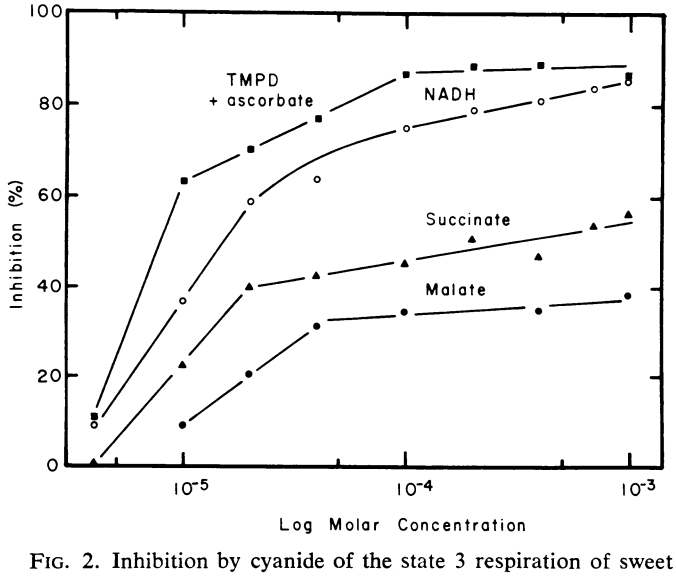


FIG. 2. Inhibition by cyanide of the state 3 respiration of sweet potato mitochondria with malate, succinate, NADH, and reduced TMPD as substrates.

Table II. *Effects of Inhibitors on State 3 Respiration of Sweet Potato Root Mitochondria during Oxidation of Malate, Succinate, and NADH*

Data are presented as arithmetic means. The concentrations (mM) of the inhibitors were: amytal, 4; HOQNO, 0.01; KCN, 1; $NaN_3$, 1; rotenone, 0.04; SHAM, 0.75; and TTA, 1.

| Inhibitor | Substrate | | |
|---|---|---|---|
| | Malate | Succinate | NADH |
| | % inhibition | | |
| KCN | 39 | 63 | 87 |
| $NaN_3$ | 36 | 44 | 64 |
| HOQNO | 42[1] | 53 | 72 |
| TTA | 62 | 64 | 0 |
| Rotenone | 70 | 0 | 0 |
| Amytal | 73 | 0 | 0 |
| SHAM | 28 | 0 | 0 |
| KCN + SHAM | 88 | 89 | 93 |
| $NaN_3$ + SHAM | 72 | 73 | 54 |
| HOQNO + SHAM | 71 | 85 | 60 |
| KCN + TTA | 92 | 92 | |
| KCN + rotenone | 90 | 70 | |
| KCN + amytal | 90 | 83 | |

[1] Corrected for solvent effect.

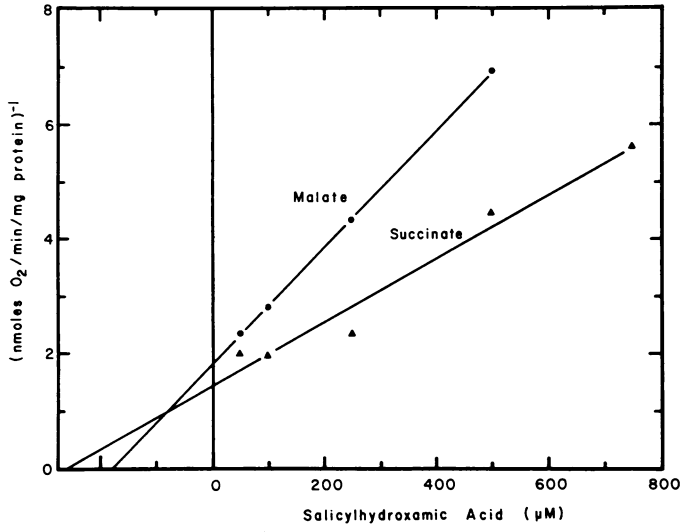


FIG. 3. Effect of SHAM on the cyanide-resistant oxidation of malate and succinate by sweet potato mitochondria. The reciprocals of the rates of $O_2$ uptake in the presence of 1 mM cyanide and SHAM are plotted as a function of the molar concentration of SHAM. The molarity of SHAM associated with the intercept on the abscissa is equal to $-Ki$.

to reflect the contribution of the cyanide-resistant respiration to the total state 3 respiration. The addition of 0.75 mM SHAM in combination with 1 mM cyanide arrested almost completely the residual $O_2$ uptake (Fig. 1, trace A; Table II). SHAM also inhibited the respiration that was resistant to $NaN_3$ and HOQNO (Table II). Inhibition by cyanide also was enhanced by the addition of 1 mM TTA, 40 μM rotenone, or 4 mM amytal, which, like SHAM, prevented respiration through the alternate pathway (Table II). The components that participated in the limited uptake of $O_2$ that was resistant to combinations of SHAM plus cyanide, azide, or HOQNO have not been identified. The dissociation constant, $Ki$, calculated by the method of least squares, for the effect of SHAM on the alternate pathway was 176 μM (Fig. 3).

State 4 respiration was inhibited 25% by 1 mM cyanide and 19% by 0.75 mM SHAM. As with skunk cabbage mitochondria (1), state 4 respiration was more resistant to cyanide than state 3 respiration. A combination of 1 mM cyanide and 0.75 mM SHAM inhibited state 4 respiration by 77%. Hence, the state 4 oxidation of malate also appeared to involve the participation of both pathways.

**Oxidation of Succinate.** The oxidation of succinate, like that of malate, was coupled loosely to phosphorylation (Fig. 1, trace B). RC and ADP/O ratios averaged 1.2 and 0.95, respectively. The response curve obtained for the effect of cyanide on the oxidation of succinate was similar in shape to the curve shown for malate (Fig. 2), but the oxidation of succinate was slightly more sensitive to cyanide (approximately 60% inhibition) than the oxidation of malate (Table II). $NaN_3$, HOQNO, and TTA also inhibited only a part of the total oxidation of succinate (Table II). However, inhibition was enhanced when either cyanide, $NaN_3$, or HOQNO was combined with SHAM (Table II). Rotenone, amytal, and SHAM by themselves did not affect the oxidation of succinate (Table II). When TTA or amytal was combined with KCN, both the cyanide-sensitive and the cyanide-resistant pathways were inhibited strongly (Table II). Carmeli and Biale (6) also reported that amytal inhibited the cyanide-resistant oxidation of succinate in sweet potato mitochondria.

The dissociation constant, $Ki$, calculated for the effect of SHAM on the cyanide-resistant oxidation of succinate, was estimated to be 250 μM (Fig. 3), slightly less than the $Ki$, 260 μM, reported for skunk cabbage mitochondria (23). The larger $Ki$ associated with the oxidation of succinate indicates that the affinity of the catalyst of cyanide-resistant respiration for SHAM was lower with succinate as substrate than with malate as substrate. A similar situation was observed with *Arum* mitochondria (27).

**Oxidation of Exogenous NADH and Reduced TMPD.** With NADH as substrate, RC and ADP/O ratios averaged 1.5 and 0.72, respectively. Unlike the oxidation of malate and succinate, the oxidation of NADH was inhibited almost completely by cyanide (Fig. 1, trace C; Fig. 2). $NaN_3$ and HOQNO, but not TTA, also inhibited strongly the oxidation of NADH (Table II).

With reduced TMPD as substrate, $O_2$ utilization, calculated from polarographic traces obtained under conditions identified in Figure 1, averaged 119 nmoles $O_2$/min. Oxygen uptake was reduced to 11 nmoles/min by 1 mM KCN, which is close to the effect expressed on the oxidation of NADH (Fig. 1, trace C). Oxidation of reduced TMPD was the most sensitive of the four substrates to cyanide (Fig. 2).

## DISCUSSION

Partial resistance of electron transport to cyanide, azide, and HOQNO; essentially complete inhibition by SHAM when combined with inhibitors of complexes III and IV; and loose coupling to phosphorylation support evidence for the presence of an alternate pathway that participates in the respiration of sweet potato mitochondria. A scheme of electron transport that is consistent with results obtained with sweet potato mitochondria and reported herein is presented in Figure 4.

The strong inhibition of the oxidation of malate and succinate by inhibitors of complexes I and II indicates that electrons do not enter the alternate pathway, in sweet potato mitochondria, prior to the sites of action of rotenone, amytal, and TTA. In agreement with the spectrophotometric evidence

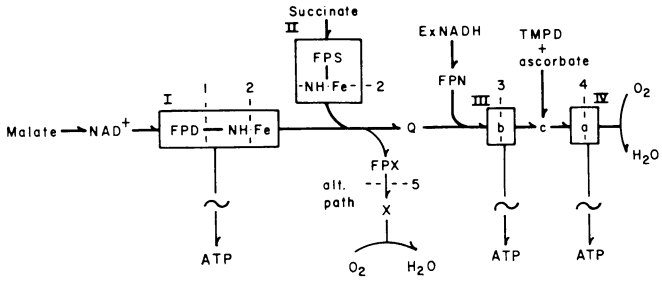


FIG. 4. Suggested arrangement of electron carriers in mitochondria extracted from roots of sweet potatoes. Abbreviations used: I, II, III, and IV: complexes I, II, III, and IV, respectively; alt. path: terminal components of the alternate (cyanide-resistant) pathway; ExNADH: exogenous NADH; $NAD^+$: the coenzyme associated with mitochondrial malate dehydrogenase; FPD: flavoprotein associated with complex I; FPS: flavoprotein associated with complex II; FPX: flavoprotein postulated to be associated with the alternate pathway; FPN: flavoprotein postulated to be associated with oxidation of exogenous NADH; NHFe: nonheme iron proteins; Q: ubiquinone; b: *b* Cyt; c: Cyt *c;* a: Cyt oxidase; X: postulated terminal component of the alternate pathway. Sites of action of selected inhibitors identified as: 1, rotenone and amytal; 2, TTA; 3, HOQNO; 4, KCN and $NaN_3$; 5, SHAM.

of Bendall and Bonner (4), complex III does not appear to be involved in cyanide-resistant respiration, because inhibition was incomplete with HOQNO alone, but became complete upon the subsequent addition of SHAM. Cyt *c* and complex IV do not appear to participate in cyanide-resistant respiration, because the oxidation of TMPD, which transfers electrons directly to Cyt *c* (25), is inhibited completely by cyanide as reported herein and by Bendall and Bonner (4), Passam and Palmer (21), and Wilson (30). Consequently, the evidence presented is consistent with the suggestion that components of the cyanide-resistant pathway accept electrons from nonheme iron proteins or ubiquinone prior to complex III in sweet potato mitochondria.

The site(s) through which electrons from exogenous NADH enter the transport system of plant mitochondria has not been resolved completely (8, 12, 19). The postulated point of entry shown in Figure 4 is consistent with results reported herein for sweet potato mitochondria. ADP/O ratios and essentially complete inhibition by HOQNO and antimycin A indicate that electrons from exogenous NADH are passed to complex III and travel through only two phosphorylation sites in plant mitochondria (5, 6, 8, 15, 19, 21, 28).

Oxidation of exogenous NADH by plant mitochondria may involve more than a single pathway (9, 28). There is no evidence for the participation of complex I or the alternate pathway in the oxidation of NADH by mitochondria from sweet potato as reported herein and by others (3, 6), mung bean (9, 19), sycamore maple (*Acer pseudoplatanus* L.) (30), and cauliflower (*Brassica oleracea* L.) (8). However, sensitivity to rotenone suggests that complex I participates in the cyanide-sensitive oxidation of exogenous NADH in corn mitochondria (28). Oxidation of exogenous NADH in mitochondria from *Arum* (21) and *Neurospora crassa* (mutant strain *poky* $NSXf^+$) (14) may involve the alternate pathway, because weak inhibition was obtained with cyanide.

There has been considerable speculation concerning the function of the cyanide-resistant electron transport pathway in plants (1, 12, 15, 27, 29). Cyanide-resistant respiration has been associated with the generation of heat in spadix tissue (1), but the role of the alternate pathway in the metabolism of other tissues is less clear. Sweet potato roots are storage organs that remain dormant for extended periods of time. A controlled rate of electron transport through the alternate pathway may permit respiration to continue when intracellular ATP and NADH concentrations limit electron transport through the Cyt system (2). Consequently, regeneration of substrates of the glycolytic pathway and the citric acid cycle would be continued (1, 15). Such metabolites would be available for synthetic reactions required for the maintenance of cellular integrity (15, 18).

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