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The Effect of Calcium and Inhibitors on Corn Mitochondrial Respiration[1]

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RAYMOND J. MILLER AND D. E. KOEPPE
Department of Agronomy, University of Illinois, Urbana, Illinois 61801

ABSTRACT

The effects of KCN, antimycin A, malonate, rotenone, and amytal on the oxidation of malate, succinate, and extramitochondrial reduced nicotinamide adenosine dinucleotide (NADH) by corn mitochondria were studied. Potassium cyanide and antimycin A inhibited the oxidation of all three substrates. Rotenone and amytal inhibited only the oxidation of malate, and malonate inhibited only the oxidation of succinate. Rotenone, amytal, and malonate did not inhibit the oxidation of extramitochondrial NADH. The calcium stimulation of the oxidation of extramitochondrial NADH was prevented by KCN and antimycin A but not by amytal, rotenone, or malonate. It is suggested that corn mitochondria possess a flavoprotein specific for extramitochondrial NADH and that this flavoprotein is sensitive to divalent cations.

Divalent cations appear to affect the respiration of isolated corn mitochondria in two ways (10). First, divalent cations stimulate the oxidation of extramitochondrial NADH, a stimulation not found when corn mitochondria are oxidizing the Krebs cycle intermediates malate or succinate. The second divalent cation effect is the promotion of respiration in the presence of phosphate by Ca^{2+}, Ba^{2+}, and Sr^{2+} which is the result of phosphate transport and occurs when extramitochondrial NADH, malate, or succinate is being oxidized.

The cause of the divalent cation stimulation of the oxidation of extramitochondrial NADH is not known. Hackett (6) has suggested that it may be due to increased membrane permeability or to the release of a rate-limiting step in the respiratory chain. Since the effect is not found with substrates other than NADH, the permeability effect is unlikely. If there is a flavoprotein for the oxidation of extramitochondrial NADH, as proposed by Bonner (1), and Storey (16), and if it is divalent cation-sensitive, it could account for the cation stimulation of the oxidation of extramitochondrial NADH. Such a divalent cation requirement has been suggested by Miller *et al.* (10). But the divalent cation effects could also be explained if an enzyme, such as the DT diaphorase (4, 11), was present to catalyze the oxidation of extramitochondrial NADH.

Detailed spectrophotometric studies of the electron transport chain of animal mitochondria have been conducted. Similar studies have recently been performed with mung bean and skunk cabbage mitochondria (12–18), clarifying the characteristics of the cytochromes of the electron transport chain. Various inhibitors of electron transport have also been used to help elucidate the electron transport chain components of animal mitochondria (2, 5), and to a limited extent those of plant mitochondria (7, 18, 19). But inhibitors have not been used to study systematically the oxidation of extramitochondrial NADH by plant mitochondria.

Three substrates—malate, succinate, and NADH—and the inhibitors KCN, antimycin A, malonate, rotenone, and amytal were used to characterize further the electron transport of corn mitochondria as it pertains specifically to extramitochondrial NADH oxidation. The experiments were not designed to study all of the effects of the inhibitors. The results of these experiments, plus the divalent cation effects, suggest that there is a specific flavoprotein for the oxidation of extramitochondrial NADH in corn mitochondria.

METHODS

Corn seedlings (*Zea mays* L., Wf9 × M14) were grown on paper toweling saturated with 0.1 mM $CaCl_2$ (29 ± 0.5 C). Mitochondria were isolated from 3-day-old etiolated shoots by the procedure of Miller *et al.* (10).

For all experiments a filled 4.0-ml glass temperature-controlled (27 ± 0.2 C) reaction cell was equipped with a Clark oxygen electrode (Yellow Springs Instrument Co.), and the oxygen concentration was measured polarographically. Cell contents were stirred with a magnetic stirrer. Reaction solutions contained 300 mM sucrose; 20 mM tris-HCl, pH 7.5; 1 mg/ml BSA[2]; and 0.6 to 0.9 mg of mitochondrial protein, or as indicated in the figures and tables. Protein was determined by the procedure of Lowry *et al.* (9) with bovine serum standards. Rotenone and antimycin A were dissolved in 95% ethyl alcohol and all other chemicals in double distilled water.

Rotenone and antimycin A were obtained from Sigma Chemical Co., St. Louis, Missouri, and amytal from Eli Lilly Co., Indianapolis, Indiana.

RESULTS

The stimulatory effects of $CaCl_2$ on the oxidation of extramitochondrial NADH, succinate, and malate by isolated corn mitochondria in the presence of inorganic phosphate has been reported by Miller *et al.* (10), in a KCl reaction media. Of these three substrates, only the oxidation of extramitochondrial NADH was stimulated by $CaCl_2$ in the absence of P (10). When the effects of different substrates and inhibitors are correlated with the $CaCl_2$ stimulation of substrate oxidation, it is

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[2] Abbreviation: BSA: bovine serum albumin.

Table I. *Inhibition of Oxidation of Three Substrates by KCN and Antimycin A*

Reaction media contained 300 mM sucrose; 20 mM tris-HCl, pH 7.5; and 1 mg/ml BSA for acceptorless conditions. State 4 media contained in addition 4 mM KH_2PO_4, and state 3 contained 4 mM KH_2PO_4 and 1.2 μM ADP. Additions of substrate and the inhibitors antimycin A (1.2 ηmoles) and KCN (4 μmoles) were made as indicated in Figure 1. Data are reported as respiration rate (ηmoles O_2/min·mg protein) before and after inhibitor addition and as percentage inhibition.

	NADH			Malate			Succinate		
	Initial rate	Inhibitor rate	Inhibition	Initial rate	Inhibitor rate	Inhibition	Initial rate	Inhibitor rate	Inhibition
			%			%			%
Acceptorless									
Antimycin A	76	38	50	90	47	48	137	38	72
KCN	78	23	71	91	29	68	106	16	85
State 4									
Antimycin A	126	51	59	95	32	67	114	27	76
KCN	134	23	82	90	16	83	118	24	79

	Initial rate	ADP	Inhibitor rate	Inhibition	Initial rate	ADP	Inhibitor rate	Inhibition	Initial rate	ADP	Inhibitor rate	Inhibition
				%				%				%
State 3												
Antimycin A	152	402	42	89	129	201	58	71	127	188	29	85
KCN	154	355	20	94	139	211	38	82	114	188	20	89

necessary to conduct experiments with mitochondria oxidizing substrate in state 3, state 4, and under acceptorless conditions (no P). Since the $CaCl_2$ stimulation of extramitochondrial NADH oxidation is greater in a sucrose medium than in KCl (8), these experiments were conducted in sucrose. The same qualitative effects were found when a KCl reaction medium was used.

The concentrations of antimycin A (0.3 μM) and KCN (1 mM) used were chosen from the state 4 and state 3 inhibitions reported by Wilson and Hanson (19). Wilson and Hanson (19) did not report acceptorlesss inhibitions, and since all their work was with KCl reaction media rather than sucrose, it was necessary for our purpose to determine the percentage inhibition of NADH, succinate, and malate oxidation by several inhibitors in state 3, in state 4, and under acceptorless conditions in sucrose. Table I reports the inhibitions of substrate oxidation affected by antimycin A and KCN. The state 4 and state 3 inhibitions, in sucrose, were qualitatively similar to those in KCl reported by Wilson and Hanson (19). The KCN and antimycin A acceptorless inhibitions (Table I) were in all instances less than those affected in either state 4 or state 3. Similarly, state 4 inhibitions were less than those affected in state 3, which is an agreement with the data of Wilson and Hanson (19). None of the three substrates tested exhibited respiratory control after the addition of KCN or antimycin A (unpublished results). In contrast, Storey and Bahr (18) found an ADP/O ratio of 0.7 with malate in the presence of KCN in mitochondria isolated from skunk cabbage.

Because of their inhibitory specificity, rotenone, amytal, and malonate were also tested for their effects on the acceptorless rate of oxidation of NADH, succinate, and malate. The effective concentrations of amytal (5 mM) and rotenone (30 μM) were again chosen from the work of Wilson and Hanson (19). The concentration of malonate (1.3 mM) employed was determined on the basis of several experiments (unpublished results) and literature reports including that of Ikuma and Bonner (7). As expected, malonate inhibited only the oxidation of succinate under acceptorless conditions (Table II). Amytal and rotenone under similar conditions inhibited only the oxidation of malate (Table II). Both rotenone and amytal were found to stimulate the oxidation of NADH and succinate to varying

Table II. *Percentage Inhibition of Oxidation of Three Substrates by Various Inhibitors*

Experimental conditions were the same as given in Figure 2. Data are reported as respiration rate (ηmoles O_2/min·mg protein) before and after inhibitor addition and as percentage inhibition or stimulation.

	NADH			Malate			Succinate		
	Initial rate	Inhibitor rate	Inhibition	Initial rate	Inhibitor rate	Inhibition	Initial rate	Inhibitor rate	Inhibition
			%			%			%
Rotenone	80	110	+37	62	18	−71	147	231	+58
Amytal	46	50	+10	56	27	−51	90	132	+46
Malonate	78	77	0	72	72	0	118	30	−74

degrees (Table II). The stimulation is without explanation. In general, the inhibitions we report here are in agreement with those reported for other plant mitochondrial systems (7, 18, 19).

The experiments reported in Figures 1 and 2 were designed to test whether the observed Ca^{2+} stimulation of O_2 uptake was due to a stimulation of an alternate pathway(s) of electron flow, or if it was releasing a rate-limiting step in the passage of electrons. Stimulations due to Ca^{2+} were observed after the ineffective additions of rotenone, amytal, and malonate to mitochondria oxidizing NADH (Fig. 2). The Ca^{2+} stimulation was found to be less after the addition of malonate than after the addition of amytal or rotenone. Calcium did not stimulate the oxidation of NADH over the inhibition of KCN or antimycin A (Fig. 1). When Ca^{2+} was added to mitochondria oxidizing succinate or malate, no stimulation of the rate of O_2 uptake was recorded over any of the ineffective inhibitions (Fig. 2).

DISCUSSION

On the basis of this inhibitor study, it can be concluded that the $CaCl_2$ stimulation of the oxidation of extramitochondrial

NADH is via normal electron transport pathways and not the result of the mobilization of different paths for electron flow such as the DT diaphorase suggested by Ernster, Danielson, and Ljunggren (4) and Sottocasa *et al.* (9). This DT diaphorase was reported to be uninhibited by antimycin A. If an enzyme complex similar to the DT diaphorase were functioning in isolated corn mitochondria, then Ca^{2+} should have stimulated the rate of oxidation of NADH after the addition of antimycin A, which it did not.

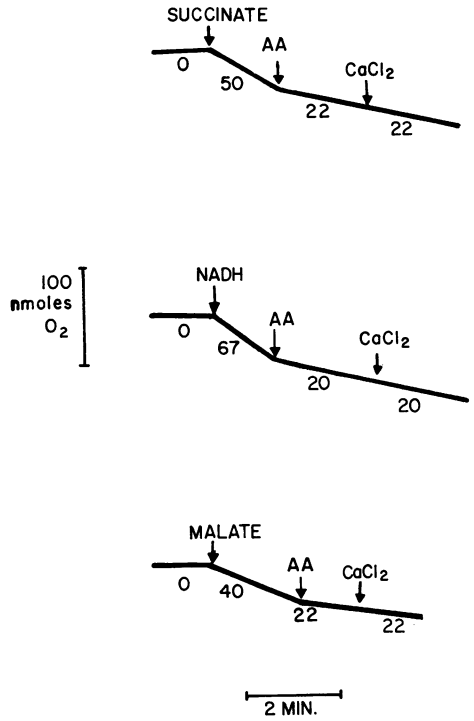

FIG. 1. The effect of antimycin A and $CaCl_2$ on the oxidation of succinate, extramitochondrial NADH, and malate by isolated corn mitochondria. The reaction was carried out in 4 ml of 300 mM sucrose; 20 mM tris-HCl, pH 7.5; and 1 mg/ml BSA. One micromole of NADH, 40 μmoles of succinate, 40 μmoles of malate, 12 μmoles of $CaCl_2$ or 1.2 ηmoles of antimycin A (AA) were added as indicated.

Hackett (6) presented evidence which he interpreted to mean that Ca^{2+} either was increasing the permeability of the mitochondrial membrane to NADH or was releasing a rate-limiting step in the respiratory chain. The data of Miller *et al.* (10) support the latter suggestion. In their case the hypothesis that the cation effect was possibly associated with a flavoprotein was substantiated when in the absence of P the oxidation of NADH, but not that of succinate or malate-pyruvate, was stimulated by the addition of Ca^{2+}. In our studies, the oxidation of all three substrates was inhibited 48 to 89% by antimycin A depending on the substrate. The inefficiency of this inhibition poses somewhat of a problem. Is antimycin A simply inefficient, or is there an alternate pathway for electron flow that is insensitive to antimycin A? Our research does not resolve this question. It does, however, show that there is no Ca^{2+} stimulation of the oxidation of the three substrates after the antimycin A inhibition site, or, if there is a bypass to O_2 around the antimycin A block, that this bypass is not stimulated by Ca^{2+}. All of this suggests that the active Ca^{2+} site could be associated with the extramitochondrial NADH flavoprotein.

Storey (16) has recently identified this flavoprotein for the dehydrogenation of NADH as Fp_{ha}. This flavoprotein differs from that reduced by malate (15). Succinate, according to Erecinska and Storey (3), reduced both Fp_{ha} and a different high potential flavoprotein, Fp_{hf}. Our results are in general agreement with the flavoprotein designations of Storey and coworkers. But the fact that malonate did not inhibit the oxidation of NADH would point to there being a flavoprotein which is more specific for succinate. Since the oxidation of succinate in the absence of P was not stimulated by Ca^{2+}, as was extramitochondrial NADH (10), this would also point to the uniqueness of the flavoproteins for the oxidation of succinate and NADH. The possibility that the flavoprotein for the oxidation of extramitochondrial NADH is different from that for malate is substantiated by our inhibitor studies where the oxidation of malate is inhibited by rotenone and amytal, but NADH is not.

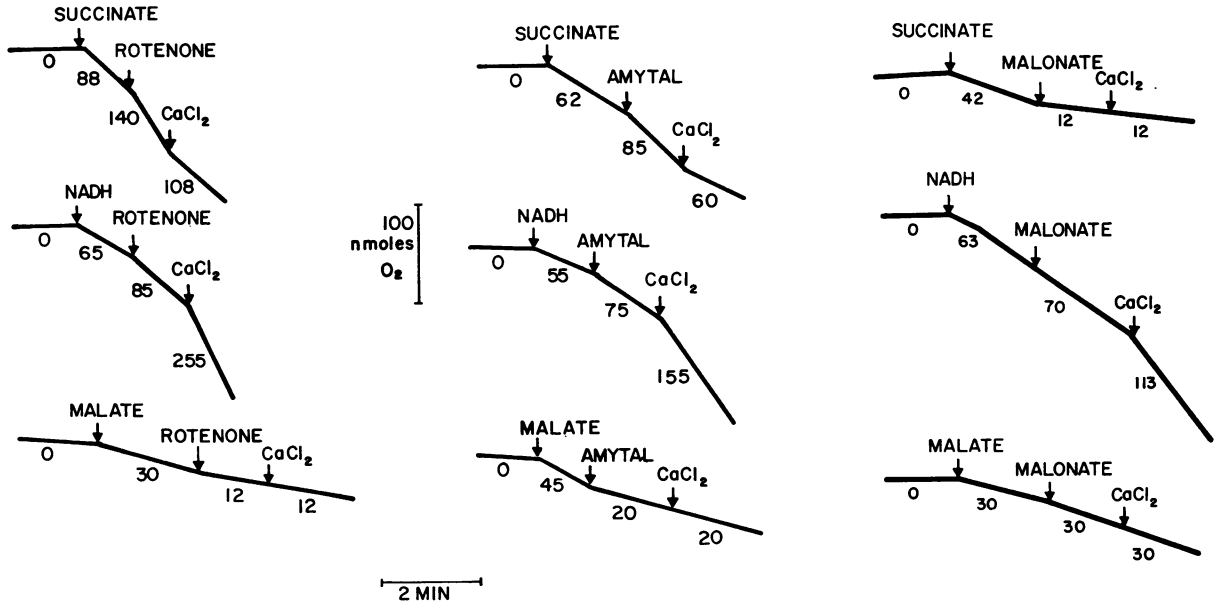

FIG. 2. The effect of rotenone, amytal, malonate, and $CaCl_2$ on the oxidation of succinate, extramitochondrial NADH, and malate by isolated corn mitochondria. The reaction conditions were the same as in Figure 1 except 0.12 μmole of rotenone, 20 μmoles of amytal, 5.2 μmoles of malonate, and 12 μmoles of $CaCl_2$ were added as indicated.

The effects of the addition of Ca^{2+} after the additions of rotenone, amytal, and malonate to mitochondria oxidizing extramitochondrial NADH, succinate, or malate are interesting, but somewhat confusing. The experiments were designed to demonstrate that Ca^{2+} was not shifting the flow of electrons from the flavoprotein specific for extramitochondrial NADH to those specific for succinate and/or malate. If it had been, then these inhibitors would have suppressed the Ca^{2+} stimulation of the oxidation of NADH. Malonate did suppress the Ca^{2+} stimulation to some extent when compared to additions of Ca^{2+} after rotenone or amytal. This might indicate some transfer of electrons between Fp_{ha} and Fp_{hf}, as suggested by Storey (16). The amytal- and rotenone-induced stimulations of the oxidation rate of extramitochondrial NADH and succinate could be due to some effect on the passage of electrons through what is a growing maze of possible electron transfers in the flavoprotein and cytochrome *b* complexes. No explanations are forthcoming for the Ca^{2+} reduction of the amytal- or rotenone-stimulated rate of the oxidation of succinate.

From these studies, two facets of electron transport in isolated corn mitochondria have been further elucidated: (*a*) the inhibitor data suggest that there is a specific flavoprotein for the oxidation of extramitochondrial NADH, and (*b*) the Ca^{2+} stimulation of the oxidation of extramitochondrial NADH is associated with the NADH-specific flavoprotein and not with alternate pathways of electron flow to oxygen. However, the specific mode of Ca^{2+} action still remains unknown.

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LITERATURE CITED

1. Bonner, W. D., Jr. 1965. Mitochondria and electron transport. *In:* J. Bonner and J. E. Varner, eds., Plant Biochemistry. Academic Press, New York. pp. 89–123.
2. Chance, B. W., W. D. Bonner, Jr., and B. T. Storey. 1968. Electron transport in respiration. Annu. Rev. Plant Physiol. 19: 295–320.
3. Erecinska, M. and B. T. Storey. 1970. The respiratory chain of plant mitochondria. VII. Kinetics of flavoprotein oxidation in skunk cabbage mitochondria. Plant Physiol. 46: 618–624.
4. Ernster, L., L. Danielson, and M. Ljunggren. 1962. DT diaphorase. I. Purification from the soluble fraction of rat-liver cytoplasm, and properties. Biochim. Biophys. Acta 58: 171–188.
5. Garland, P. B., B. Chance, L. Ernster, C. Lee, and D. Wong. 1967. Flavoprotein of mitochondrial fatty acid oxidation. Proc. Nat. Acad. Sci. U.S.A. 58: 1696–1702.
6. Hackett, D. P. 1961. Effect of salts on DPNH oxidase activity and structure of sweet potato mitochondria. Plant Physiol. 36: 445–452.
7. Ikuma, H. and W. D. Bonner, Jr. 1967. Properties of higher plant mitochondria. III. Effects of respiratory inhibitors. Plant Physiol. 42: 1535–1544.
8. Koeppe, D. E. and R. J. Miller. 1971. The effect of reaction media on the coupling and uncoupling of respiration in corn mitochondria. Physiol. Plant. 24: 181–184.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193: 265–276.
10. Miller, R. J., S. W. Dumford, D. E. Koeppe, and J. B. Hanson. 1970. Divalent cation stimulation of substrate oxidation by corn mitochondria. Plant Physiol. 45: 649–653.
11. Sottocasa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand. 1967. An electron-transport system associated with the outer membrane of liver mitochondria. Cell Biol. 32: 415–438.
12. Storey, B. T. 1969. The respiratory chain of plant mitochondria. III. Oxidation rates of the cytochromes *c* and *b* in mung bean mitochondria reduced with succinate. Plant Physiol. 44: 413–421.
13. Storey, B. T. 1970. The respiratory chain of plant mitochondria. IV. Oxidation rates of the respiratory carriers of mung bean mitochondria in the presence of cyanide. Plant Physiol. 45: 447–454.
14. Storey, B. T. 1970. The respiratory chain of plant mitochondria. V. Reaction of reduced cytochromes *a* and *as* in mung bean mitochondria with oxygen in the presence of cyanide. Plant Physiol. 45: 455–460.
15. Storey, B. T. 1970. The respiratory chain of plant mitochondria. VI. Flavoprotein components of the respiratory chain of mung bean mitochondria. Plant Physiol. 46: 13–20.
16. Storey, B. T. 1970. The respiratory chain of plant mitochondria. VIII. Reduction kinetics of the respiratory chain carriers of mung bean mitochondria with reduced nicotinamide adenine dinucleotide. Plant Physiol. 46: 625–630.
17. Storey, B. T. and J. T. Bahr. 1969. The respiratory chain of plant mitochondria. I. Electron transport between succinate and oxygen in skunk cabbage mitochondria. Plant Physiol. 44: 115–125.
18. Storey, B. T. and J. T. Bahr. 1969. The respiratory chain of plant mitochondria. II. Oxidative phosphorylation in skunk cabbage mitochondria. Plant Physiol. 44: 126–134.
19. Wilson, R. H. and J. B. Hanson. 1969. The effect of respiratory inhibitors on NADH, succinate and malate oxidation in corn mitochondria. Plant Physiol. 44: 1335–1341.