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The Oxidation of Malate and Exogenous Reduced Nicotinamide Adenine Dinucleotide by Isolated Plant Mitochondria[1]

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ABSTRACT

Exogenous NADH oxidation by cauliflower (*Brassica oleracea* L.) bud mitochondria was sensitive to antimycin A and gave ADP/O ratios of 1.4 to 1.9. In intact mitochondria, NADH-cytochrome *c* reductase activity was only slightly inhibited by antimycin A. The antimycin-insensitive activity was associated with the outer membrane. Malate oxidation was sensitive to both rotenone and antimycin A and gave ADP/O values of 2.4 to 2.9. However in the presence of added NAD^+, malate oxidation displayed similar properties to exogenous NADH oxidation. In both the presence and absence of added NAD^+, malate oxidation was dependent on inorganic phosphate and inhibited by 2-*n*-butyl malonate.

Previous studies with plant mitochondria (4, 11, 13, 14) have indicated the presence of two NAD^+-linked enzymes involved in the oxidation of malate; *viz.* malate dehydrogenase and "malic" enzyme. Plant mitochondria also appear to possess an alternative system for the oxidation of exogenous NADH. The oxidation involves an NADH dehydrogenase, apparently situated on the outside of the inner membrane (8, 19), which is linked to the respiratory chain and coupled to two sites of phosphorylation (5, 9, 15, 19, 24). Coleman and Palmer (4) suggested that the malic enzyme of plant mitochondria was situated in the intermembrane space and that the oxidation of malate by this enzyme was linked to the respiratory chain via the above mentioned NADH dehydrogenase.

Douce *et al.* (8) and Moreau and Lance (16) have recently confirmed earlier suggestions (7, 26, 27) that an additional pathway of NADH oxidation exists on the outer mitochondrial membrane, similar to that associated with rat liver mitochondria (19).

The aim of this study was to reinvestigate the different pathways of malate and NADH oxidation and the ways in which these systems interact in isolated cauliflower bud mitochondria.

MATERIALS AND METHODS

Fresh cauliflower (*Brassica oleracea* L.) was purchased locally. Bovine serum albumin was obtained from the Commonwealth Serum Laboratories (Melbourne, Australia), enzymes from Boehringer and Soehne (Mannheim, Germany), and other biochemicals from Sigma Chemical Company (St. Louis, Mo.).

Preparation of Mitochondria. Young buds (200 g) were disrupted in 200 ml of a medium containing 0.3 M sucrose, 0.1% bovine serum albumin, 75 mM tris-HCl buffer (pH 7.2), 15 mM EDTA, and 10 mM TES buffer (pH 7.2), in a Waring Blendor for 30 sec. The homogenate was strained through a double layer of cheesecloth and centrifuged at 2500*g* for 10 min in a Sorvall RC2 refrigerated centrifuge. The supernatant was decanted and centrifuged at 12,000*g* for 12 min to yield mitochondria. The mitochondrial pellet was washed by resuspending in 0.3 M sucrose + 0.1% bovine serum albumin, and recentrifuging at 12,000*g* for 15 min. The final precipitate was resuspended in 3 to 4 ml of 0.3 M sucrose. All operations were carried out at about 2 C, using chilled solutions and apparatus.

Oxygen Consumption. Oxygen uptake was measured polarographically in a sealed Perspex vessel with a circulating water bath, using a Clarke electrode (Yellow Springs Instrument Co., Cleveland, Ohio) connected to a 1 mv recorder (Electronik, Honeywell Controls Ltd., Great Britain). A standard reaction medium of 0.25 M sucrose containing 10 mM phosphate buffer (K salts, pH 7.2), 5 mM $MgCl_2$, 0.5 mM EDTA and 10 mM tris-HCl buffer (pH 7.2) was used. A 1.5 to 2 mg mitochondrial protein was used per assay. The total volume of the assay mixture was 3.2 to 3.4 ml and was maintained at 25 C.

ADP/O and Respiratory Control Ratios. These were determined from the oxygen electrode traces obtained upon addition of ADP, according to the method of Chance and Williams (1).

Protein Determinations. Protein content was estimated using the procedure of Lowry *et al.* (10).

Cytochrome *c* Reduction. A Beckman spectrophotometer (Model DB), connected to a Beckman linear-log 5 inch strip-chart recorder, was used for the measurement of Cyt *c* reductases. Activity was determined by following absorbance changes at 550 nm at room temperature, using cuvettes with a 1-cm light path.

The reaction mixture consisted of 0.1 ml of mitochondrial suspension, 0.05 mM Cyt *c* and 10 mM KCN in 3.0 ml of the standard reaction medium described above. The reaction was initiated by the addition of 0.5 mM NADH, 15 mM malate, or 15 mM succinate to the cuvette. A molar extinction coefficient (reduced minus oxidized) of 19.8×10^3 cm^{-1} (17) was used.

Malate Dehydrogenase. Malate dehydrogenase activity was measured spectrophotometrically by following the oxidation of NADH at 340 nm, in the presence of oxaloacetate and malate (18).

Fractionation of the Mitochondrial Membranes. A 1%

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stock solution of digitonin was prepared by adding 0.3 M sucrose to recrystallized digitonin and heating gently until the solution was water clear. All such solutions were prepared immediately before use. Aliquots of mitochondrial suspensions in 0.3 M sucrose were placed in an ice bath, and equal aliquots of cold digitonin solutions were added with continuous stirring. The digitonin concentration of the final solution was adjusted to give a digitonin-protein ratio of 3:10. The suspension was incubated at 0 C for 30 min, diluted by adding 3 volumes of cold 0.3 M sucrose, and gently homogenized. The homogenate was centrifuged at 8,500*g* for 20 min to yield a solid precipitate which was washed by resuspending in 0.3 M sucrose and recentrifuging at 8,500*g* for 20 min. The final pellet was resuspended in a small volume of 0.3 M sucrose; the two supernatants were pooled and centrifuged at 37,000*g* for 30 min. The resultant pellet was resuspended in 0.3 M sucrose, and the supernatant was centrifuged at 144,000*g* for 90 min (Spinco Model L, Ti50 rotor) to yield a firm, brown pellet which was resuspended in 2 ml of 0.3 M sucrose. The final supernatant was retained. All procedures were carried out in the cold, and the resuspensions were stored at 0 C until assayed.

RESULTS

Respiratory Control and Inhibition of Oxygen Consumption. Malate and NADH were oxidized rapidly and with good respiratory control (Fig. 1). ADP/O ratios for malate oxidation approximated to 3 (2.4–2.9), while those for NADH oxidation were less than 2 (1.4–1.9). Figure 2 shows the effect of varying rotenone concentration on both malate and NADH oxidation. While malate oxidation was inhibited up to 65%, NADH oxidation was largely unaffected; on the other hand,

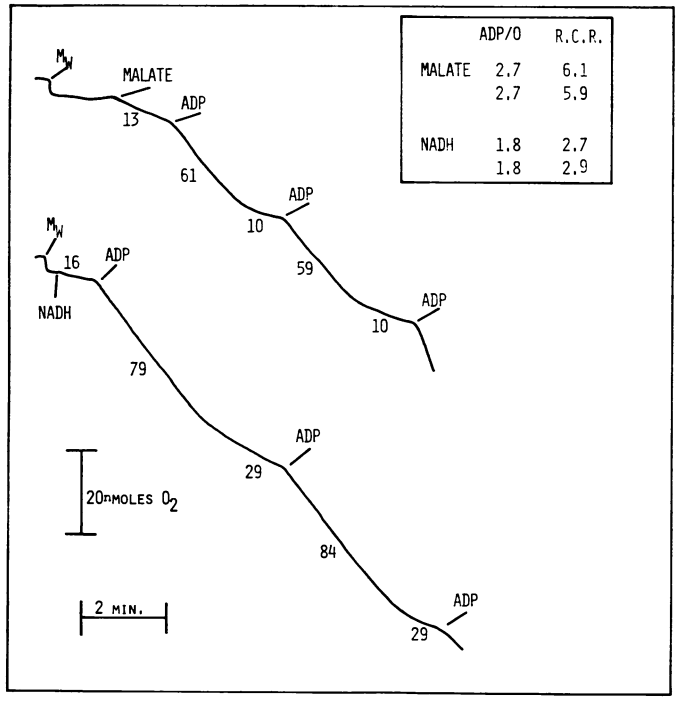

FIG. 1. Malate and exogenous NADH oxidation by cauliflower bud mitochondria. Mitochondria (1.5 mg of protein) were added to 3 ml of standard assay medium as described under "Materials and Methods." Additions as indicated were 20 mM malate, 1 mM NADH, and 0.29 mM ADP. Oxygen uptake is expressed as nmoles O_2/min·mg protein. Glutamate (50 mM) was added prior to malate. M_W washed mitochondria.

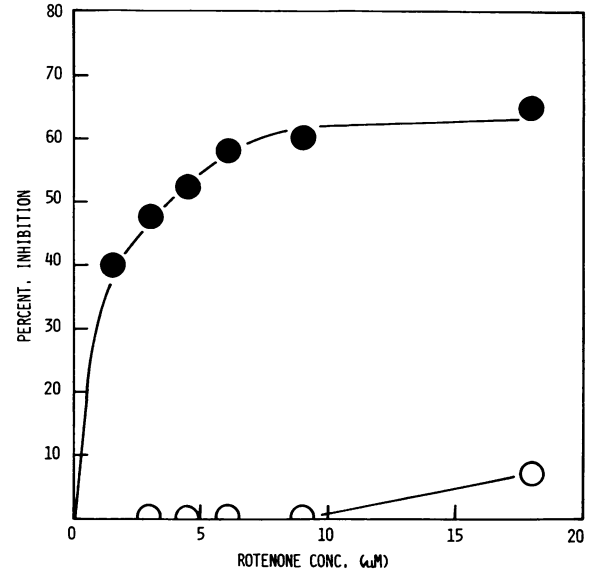

FIG. 2. Effect of rotenone concentration of NADH (○) and malate (●) oxidation by cauliflower bud mitochondria. Assay conditions are described in Figure 1. Initial state 3 rates of oxygen uptake were 88 (for malate) and 119 (for NADH) nmoles O_2/min·mg protein.

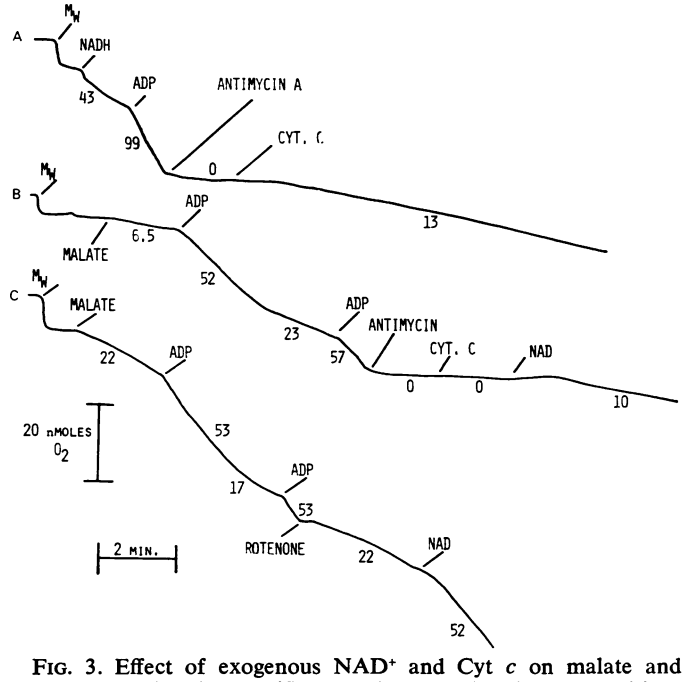

FIG. 3. Effect of exogenous NAD^+ and Cyt *c* on malate and NADH oxidation by cauliflower mitochondria. Assay conditions were as described in Figure 1, except that 1.4 mg of protein were used in each experiment. Additions as indicated were, 20 mM malate, 1 mM NADH, 0.26 mM ADP, 0.5 mM NAD^+, 5 μM antimycin A, 7 μM rotenone, and 5.5 mM Cyt *c*. Rates are expressed as nmoles O_2/min·mg protein.

the oxidation of both substrates was completely inhibited by low concentrations (0.1 μM) of antimycin A (Fig. 3). These results support those of others (8, 15, 19, 26) and indicate that the oxidation of exogenous NADH bypasses the first site of phosphorylation and the rotenone-sensitive site. The sensitivity to antimycin A would suggest that reducing equivalents enter the chain at a point on the substrate side of Cyt *b*.

Palmer and Passam (19) and Coleman and Palmer (3) have suggested that a specific NADH dehydrogenase, situated on the outside of the inner membrane, is responsible for the oxidation of exogenous NADH. The inhibition of NADH oxidation by antimycin A was partially relieved by the addition of large amounts of Cyt *c* to the medium (Fig. 3A); Cyt *c* alone had no such effect on the inhibited malate oxidation (Fig. 3B). This effect was also observed by Wilson and Hanson (26), and suggests the presence of an alternative, antimycin A-insensitive route for the oxidation of NADH. However, it should be noted that the recovery of antimycin A-inhibited NADH oxidation by Cyt *c* was only slight.

Effect of NAD on Malate Oxidation. In the presence of exogenous NAD^+, the properties of malate oxidation were altered and resembled those usually associated with NADH oxidation. Upon addition of NAD^+, rotenone inhibition was almost completely relieved (Fig. 3C) and ADP/O ratios were lowered (Table I). When both rotenone and NAD^+ were present, ADP/O values were lower than when NAD^+ alone was added (Table I). This together with the faster rates of oxygen uptake observed when NAD^+ was present (Table I), suggests that an alternative route of malate oxidation was operating in addition to the "normal" pathway (*i.e.* no added NAD^+ present), whenever NAD^+ was added. When rotenone was also added, flow of reducing equivalents through the first energy conservation site would be restricted, leading to the lower respiratory rates observed (Table I). In the presence of gross amounts of Cyt *c*, the addition of NAD^+ partly relieved antimycin A inhibition (Fig. 3B), again indicating the operation of an alternative pathway.

Transport of Malate into the Mitochondria. The relief of rotenone inhibition by exogenous NAD^+ was also observed by Coleman and Palmer (4) who attributed it to the action of a malic enzyme (11, 13, 14) which they postulated to be situated in the intermembrane space. In support of their hypothesis, they reported that butyl-malonate, a specific inhibitor of the phosphate-malate exchange carrier in both animal (2, 20) and cauliflower bud mitochondria (Wiskich, unpublished results), did not inhibit malate oxidation in the presence of rotenone and NAD^+. Contrary to this, we found that butyl-malonate inhibition of malate oxidation was independent of added NAD^+ (Fig. 5). Furthermore, addition of NAD^+ (and rotenone) had little effect on malate oxidation in the absence of inorganic phosphate (Fig. 4), suggesting that the entry of malate into the matrix (via the phosphate-malate carrier, (2, 20)) is a prerequisite for its oxidation.

Table I. *Effect of Exogenous NAD^+ and Rotenone on Malate Oxidation by Cauliflower Bud Mitochondria*

Conditions of assay are described in Figure 4, with 10 mM malate as substrate.

Additions	Oxygen Uptake		Respiratory Control	ADP/O
	State 3	State 4		
	nmoles/min·mg protein		*ratio*	
None (1)[1]	70	18	3.9	2.7
(2)	64	16	4.0	2.7
0.5 mM NAD^+ (1)	99	22	4.5	2.3
(2)	97	21	4.6	2.4
0.5 mM NAD^+ (1)	48	29	1.6	1.8
+7 μM Rotenone (2)	44	24	1.8	1.8

Numbers in parentheses indicate separate experiments.

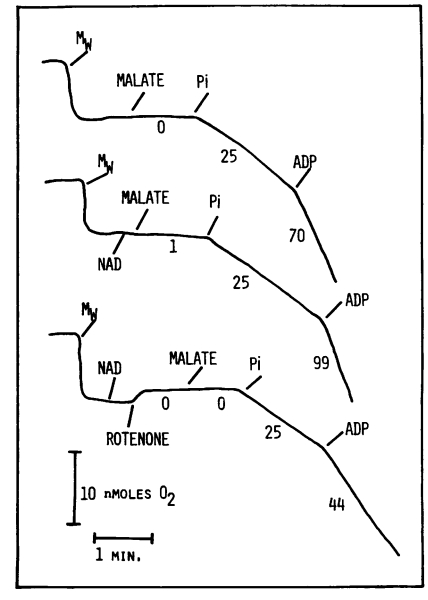

FIG. 4. Dependence of malate oxidation on inorganic phosphate, and the effect of rotenone and exogenous NAD^+. Assay conditions were described in Figure 1, except that 2.4 mg of protein were used in each experiment. Additions as indicated were 10 mM malate, 10 mM phosphate, 0.26 mM ADP, 0.25 mM NAD^+, and 7 μM rotenone.

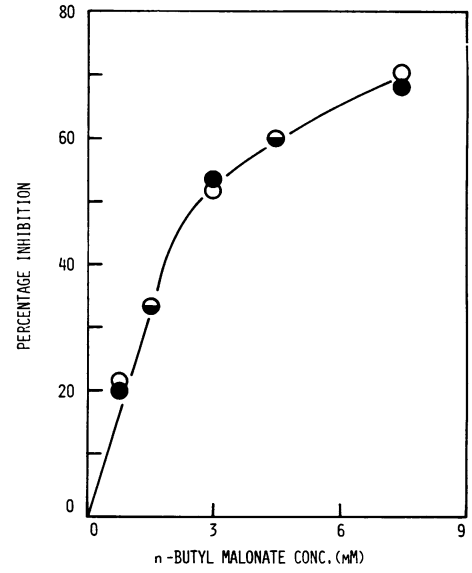

FIG. 5. Effect of *n*-butyl malonate concentration on malate oxidation by cauliflower bud mitochondria. Oxygen consumption was measured upon addition of mitochondria (1.6 mg of protein) to 3 ml of standard reaction medium which included 0.5 mM ADP and up to 7.5 mM *n*-butyl malonate. 10 mM malate as substrate (○); 10 mM malate plus 0.25 mM NAD^+ and 12 μM rotenone (●). In all experiments, all reagents were added to the reaction vessel prior to the addition of mitochondria. The initial rate of oxygen consumption was 48 nmoles O_2/min·mg protein.

It should be noted that glutamate was present in the reaction medium whenever malate oxidation was studied. In the absence of glutamate, the rate of malate oxidation decreased quite rapidly with time, presumably due to accumulation of oxaloacetate.

Cytochrome *c* Reduction. Table II shows the rate of Cyt *c* reduction with succinate, malate, and NADH as substrates

Table II. *Effect of Inhibitors and Digitonin on Cyt c Reductase Activity in Isolated Cauliflower Mitochondria*

Cyt *c* reductase was assayed as described under "Materials and Methods." Substrates used were 0.5 mM NADH, 10 mM malate, and 10 mM succinate.

Substrate	Untreated Mitochondria			Mitochondria Preincubated with Digitonin[1]		
	Control	+ 7 μM Rotenone	+ 5 μM Antimycin A	Control	+ 7 μM Rotenone	+ 5 μM Antimycin A
	nmoles Cyt. c. reduced/min·mg protein					
NADH	41.9	41.7	31.7	78	71.4	33.9
Malate	2.3	1.2	0	19.4	8.9	0
Succinate	5.6		0	29.2		0

[1] 0.1 mg/mg protein.

and the effect of rotenone and antimycin A. These results reflect those obtained with the oxygen electrode, although Cyt *c* reduction by malate and succinate was much slower than that by NADH. The partial inhibition of NADH-Cyt *c* reductase by antimycin A again suggests an alternative, inhibitor-insensitive pathway of NADH oxidation, possibly involving an electron transport chain on the outer membrane (8), similar to that of rat liver mitochondria (20). Such a pathway would account for the difference in rates of Cyt *c* reduction by malate and NADH, since the outer membrane is thought to be largely impermeable to the high molecular weight Cyt *c* (28, 29). Support for these ideas comes from studies with disrupted mitochondria. Incubation of the mitochondria with low concentrations of digitonin prior to assay resulted in an increase in succinate and malate Cyt *c* reductase and antimycin A-sensitive NADH-Cyt *c* reductase activities, but did not affect the antimycin A-insensitive Cyt *c* reduction by NADH (Table II). These results are in agreement with the observations of Douce *et al.* (7).

Preliminary experiments (*e.g.* see Table III) on the isolation of the two mitochondrial membranes have confirmed the existence of an outer membrane reductase. Although the techniques employed were rather harsh, resulting in disruption of some inner membranes (the 37,000*g* pellet probably consisted of fragmented mitochondria), a fraction was obtained in which the NADH-Cyt *c* reductase activity was largely insensitive to antimycin A. This fraction (the 144,000*g* pellet) was devoid of succinate Cyt *c* reductase and was poor in malic dehydrogenase activity (even this low activity could be "washed off" with sucrose). This fraction probably consists of outer membrane fragments and is currently under morphological and spectrophotometric investigation. The 8,500*g* pellet appears to consist largely of inner membrane vesicles, being enriched in antimycin-sensitive NADH-Cyt *c* reductase and possessing high succinate Cyt *c* and malic dehydrogenase activity. It is unlikely that the antimycin A-insensitive reductase represents an alternative oxidase activity (23) of the inner membrane, since succinate-Cyt *c* reductase was completely inhibited by antimycin A (Table III).

At the concentration of digitonin used, a large proportion of enzyme activity was lost; lower concentrations (*e.g.* 0.1 mg digitonin/mg protein) yielded higher recoveries but separation of the membranes was not as good. Improvements on this method are under investigation.

The addition of NAD^+, in the presence of malate, to intact mitochondria stimulated the rate of Cyt *c* reduction several fold, and relieved the inhibition by both rotenone and antimycin A (Table IV), in agreement with the oxygen uptake results (Fig. 3).

DISCUSSION

NADH Oxidation. The results presented in this study provide further evidence of alternative pathways of NADH oxida-

Table IV. *Effect of Exogenous NAD on the Malate-Cyt c Reductase Activity of Isolated Plant Mitochondria*

Cyt *c* reductase was assayed as described under "Materials and Methods." Malate (10 mM) was used as substrate.

Additions	Malate-Cyt *c* Reductase Activity		
	Control	7 μM Rotenone	5 μM Antimycin A
	nmoles Cyt c reduced/min·mg protein		
None			
(1)[1]	2.3	1.2	0
(2)	3.3	1.6	0
0.5 mM NAD			
(1)	13.8	12.8	8.1
(2)	28	27	18.9

[1] Numbers in parentheses indicate separate experiments.

Table III. *Fractionation of Digitonin-treated Mitochondria*

Mitochondria were incubated with digitonin (0.3 mg digitonin/mg protein) and subjected to differential centrifugation. A sample of mitochondria was drawn off prior to centrifugation and used as the control. Assays of enzymes are described under "Materials and Methods." Cyt *c* reductase is expressed as nmoles Cyt *c* reduced/min and malate dehydrogenase as nmoles NADH oxidized/min. Antimycin A (5 μM) (final conc) was used in all cases.

	Protein	NADH-Cyt *c* Reductase				Succinate-Cyt *c* Reductase				Malate Dehydrogenase	
		Antimycin A-sensitive		Antimycin A-insensitive		Control		+ Antimycin A		Total	Per mg protein
		Total	Per mg protein	Total	Per mg protein	Total	Per mg protein	Total	Per mg protein		
	mg										
Control mitochondria	61.7	5,553	90	3,702	60	3,085	50	0	0	20,918	340
8,500*g* pellet	14	3,629	259	333	24	1,411	101	0	0	2,800	200
37,000*g* pellet	13.3	1,423	107	705	33	705	53	0	0	3,830	288
144,000*g* pellet	9.2	92	10	460	50	0	0	0	0	693	77
144,000*g* supernatant	17.1	0	0	92	5.4	0	0	0	0	5,036	318

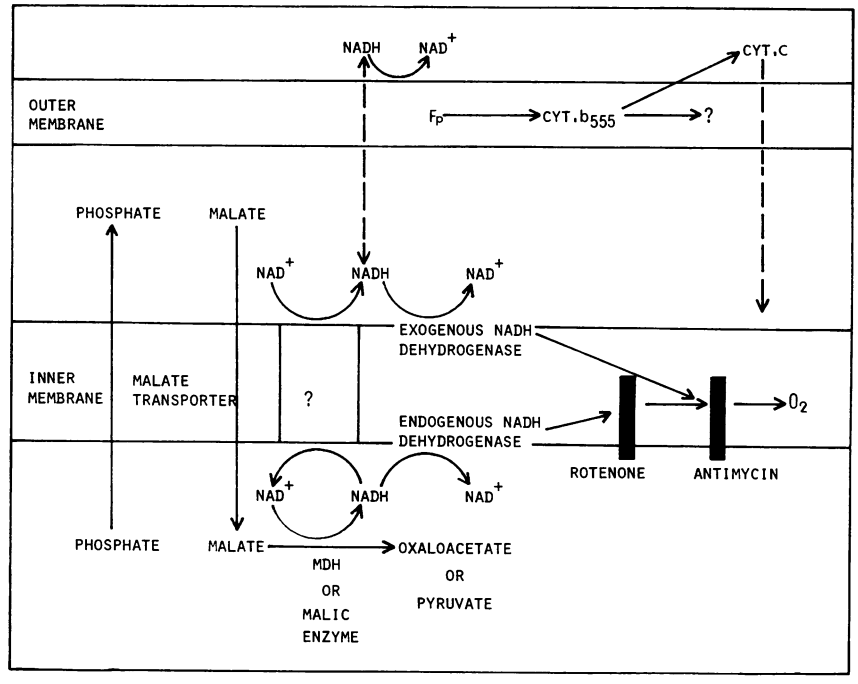

FIG. 6. Proposed pathways of NADH and malate oxidation in isolated cauliflower bud mitochondria. MDH: malate dehydrogenase; Fp: flavoprotein; cyt.: cytochrome. Broken arrows indicate diffusion; solid arrows indicate active transport or transfer of reducing equivalents.

tion in isolated plant mitochondria. The antimycin A-insensitive oxidation of NADH (measured by Cyt *c* reductase) occurs on the outer membrane of the mitochondria as the fractionation studies show (Tables II and III). Douce *et al.* (8) and Moreau and Lance (16) have recently shown this pathway to be similar to that of animal mitochondria (21, 22), consisting of a flavoprotein and *b*-type Cyt.

Another pathway of NADH oxidation (Fig. 6) occurs via a dehydrogenase, apparently located on the outside of the inner membrane (8) and coupled to two phosphorylation sites. This pathway is insensitive to rotenone but is strongly inhibited by antimycin A, indicating that reducing equivalents enter the electron transfer chain on the substrate side of Cyt *b* and bypass phosphorylation site I. Although this dehydrogenase remains to be identified, it could involve one of the flavoproteins detected by Storey (24, 25).

Apparently, these outer and inner membrane pathways can interact as suggested by the relief of antimycin A-inhibited NADH oxidation by added Cyt *c* (Fig. 3A). Presumably Cyt *c* reduced by the outer pathway moves to the inner chain; the recovery of NADH oxidation is small because Cyt *c* has difficulty in penetrating the outer membrane. This explains the slow rates of Cyt *c* reduction by malate and succinate in untreated mitochondria (Table II). Similar results have been reported for mitochondria isolated from mung bean hypocotyls (7), demonstrating that impermeability of the outer membrane to very large molecules is a common characteristic of plant and animal (28, 29) mitochondria.

The third NADH oxidation pathway is inhibited by rotenone, coupled to three phosphorylation sites and is exclusive to NADH generated within the mitochondria. Presumably it is located on the inside of the inner membrane and is not available (in intact mitochondria) to added NADH which cannot penetrate the inner membrane. This internal pathway can interact with the others if NAD^+ is added to the medium (Fig. 3).

Malate Oxidation. In the absence of inhibitors and exogenous NAD^+, malate is oxidized within the mitochondrial matrix generating NADH which, in turn, is oxidized endogenously via the electron transfer chain. This malate oxidation, coupled to three phosphorylation sites, is sensitive to rotenone and antimycin A. However in the presence of exogenous NAD^+ and rotenone, reducing equivalents from malate appear to enter the chain via the NADH dehydrogenase on the outside of the inner membrane.

It has been proposed (4) that under these conditions malate is oxidized either in the intermembrane space by malic enzyme or in the matrix by malate dehydrogenase and the reducing equivalents transferred to the outside of the inner membrane. Either possibility would explain the lack of rotenone sensitivity and the dependence on added NAD^+. Coleman and Palmer (4) concluded that in mitochondria isolated from Jerusalem artichoke malate oxidation (in the presence of NAD^+ and rotenone) occurs via malic enzyme in the intermembrane space.

The results presented here show that in isolated cauliflower bud mitochondria, malate is oxidized within the matrix of the mitochondria and that reducing equivalents are transferred across the inner membrane, perhaps via a transmembrane transhydrogenase. This conclusion is based on the following evidence: (*a*) *n*-butyl malonate inhibits malate oxidation to the same extent with or without rotenone and NAD^+; (*b*) NAD^+ has no effect on malate oxidation in the absence of exogenous phosphate.

Since phosphate is necessary for malate penetration across the inner membrane via the malate transporter, which is inhibited by *n*-butyl malonate (2, 20, and Wiskich, unpublished results), we must conclude that malate oxidation requires the operation of the malate transporter and is confined to the inner compartment of isolated cauliflower bud mitochondria. Malic enzyme, which is known to exist in cauliflower bud mitochondria (11–13), is either localized in the matrix or is not functional in the mitochondria studied here.

The major discrepancy between our results (Fig. 5) and those of Coleman and Palmer (see Fig. 3 in ref. 4) is the differing effects observed with *n*-butyl malonate. This discrepancy is under further investigation.

Although the possibility of *n*-butyl malonate inhibition of malate dehydrogenase cannot be ruled out, the results obtained in the absence of inorganic phosphate (Fig. 4) suggest that this inhibitor has its site of action at the level of malate entry into the mitochondria. The results of Coleman and Palmer (4) also suggest this. Observations made with substrates other than malate (to be published) exclude the involvement of nonmitochondrial organelles (such as glyoxisomes) in our preparations.

Since glutamate was found to be necessary to maintain fast rates of malate oxidation, it appears that malate dehydrogenase is mainly responsible for malate oxidation under the conditions present in our experiments. It is assumed that the decrease in the rate of malate oxidation is due to the formation of oxaloacetate which is removed by transamination with glutamate. This suggests that oxaloacetate accumulates within the mitochondrial matrix, in contrast to the results of Douce and Bonner (6). However, Macrae (12) also observed product inhibition of malate oxidation by cauliflower bud mitochondria at a pH of 7 or above.

Integrity of Mitochondria. The level of succinate-Cyt *c* reductase activity can be used as an indicator of mitochondrial integrity. Fully intact mitochondria should not display such activity (7); therefore, very low activity indicates that a large proportion of the mitochondria have intact membranes. Douce *et al.* (7) have used the ratio of succinate-Cyt *c* reductase activity in purified mitochondria to that in disrupted mitochondria as an indication of the percentage of mitochondria with intact outer membranes. For the mitochondria used here, this ratio is 5 (Table II), suggesting that approximately 80% of the mitochondria had intact outer membranes. However, it should be noted that this value depends on the degree to which both the outer membrane and inner membrane have been disrupted. Partial disruption of the outer membrane will lead to lower values while any breakage of the inner membrane may lead to higher values. Therefore, a more valid indication of outer membrane integrity may be that based on the level of succinate-Cyt *c* reductase activity in the untreated mitochondria, providing that the penetration of the outer membrane by the added Cyt *c* is the only limiting factor in the assay. On the other hand, an estimation of inner membrane integrity may be gained from its permeability to substrates. Fully intact mitochondria should display complete dependence on the inner membrane substrate transport systems. For example, malate penetration should be strongly inhibited by butyl malonate and stimulated by phosphate (Figs. 4 and 5).

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