OXIDATIVE PATHWAYS OF ISOLATED PLANT MITOCHONDRIA

a thesis submitted to the University of Adelaide as a requirement for the degree of

DOCTOR OF PHILOSOPHY

by

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July, 1975

CONTENTS

SUMMARY

1. Mitochondria isolated from a variety of plant tissues oxidized NADH via three distinct pathways. These were: (a) a dehydrogenase located on the outer membrane and linked to exogenous cytochrome *c* reduction by cytochrome b_{555}. This pathway was insensitive to respiratory inhibitors. (b) A dehydrogenase probably located on the outside of the inner membrane and linked to the respiratory chain in the ubiquinone - cytochrome *b* region. This pathway was insensitive to rotenone (but inhibited by antimycin A) and coupled to two phosphorylation sites. Both 'external' dehydrogenases were characteristically inhibited by pCMB, but could be protected from mercurial attack by preincubation of the mitochondria with NADH. (c) A dehydrogenase accessible only to NADH generated within the matrix, e.g. by oxidation of malate. This pathway was coupled to three phosphorylation sites and was sensitive to rotenone.

An exception to the above were mitochondria isolated from beetroot (*Beta vulgaris* L.), which appeared to lack the dehydrogenase associated with the outer surface of the inner membrane, and in this respect resembled animal mitochondria. However, exogenous NADH oxidation could be induced in these mitochondria by slicing the beetroot into discs and aging them in an aerated $CaSO_4$ solution, prior to isolation of the mitochondria.

Exogenous NADPH was also oxidized, in a rotenone-insensitive manner, by isolated cauliflower bud mitochondria, but this oxidation was slow compared to that of external NADH.

2. The outer membrane of isolated plant mitochondria was found to be impermeable to cytochrome *c*. Added cytochrome *c* was not accessible to inner membrane enzymes (succinate - cyt. *c* reductase and cyt. *c* - oxidase) unless the outer membrane was disrupted by digitonin.

3. Low concentrations of digitonin fragmented the outer membrane but left the inner membrane of isolated plant mitochondria relatively intact. This enabled the outer membrane of turnip mitochondria to be isolated and purified, free from contamination by inner membrane enzymes. Unlike the microsomes of turnip, the outer mitochondrial membrane displayed little NADPH - cytochrome *c* reductase activity, although very active NADH - cytochrome *c*, Fe CN and DCPIP reductases were measured. An acid phosphatase and NADPH - Fe CN reductase were found associated with the outer membrane, but the latter enzyme was only loosely bound and could be washed off.

   In contrast to the external NADH dehydrogenase of the inner membrane, which was strongly inhibited by low (less than 10 μM) dicoumarol concentrations, the outer membrane NADH dehydrogenase was only inhibited by high (50 μM) concentrations of dicoumarol, and then only partially.

The outer membrane dehydrogenase was not affected by $CaCl_2$ (0.5 mM) or EGTA (1 mM).

4. In the presence of added NAD^+, the oxidation of malate (and other NAD-linked substrates) displayed properties usually associated with exogenous NADH oxidation. That is, malate oxidation became insensitive to rotenone and associated ADP/O ratios were lower. The addition of malate to isolated cauliflower and beetroot mitochondria, in the presence of antimycin A, caused a reduction of exogenous NAD^+. The kinetics of this reduction, together with the observation that it (and the oxidation of malate in the presence of exogenous NAD^+ and rotenone) were dependent on added phosphate and inhibited by n-butylmalonate, suggest that extramitochondrial malate oxidation was not responsible. Exogenous NAD^+ reduction was not observed with isocitrate or ∝-ketoglutarate as substrates, although rotenone inhibition of the oxidation of these substrates was relieved by the addition of NAD^+.

   Since exogenous NADH was not oxidized by beetroot mitochondria, and NADH oxidation by other plant mitochondria was insensitive to rotenone, it appears that the inner membrane was impermeable to pyridine nucleotides. Hence a transmembrane transhydrogenase was postulated to catalyse the transfer of reducing equivalents from intramitochondrial NADH to external NAD^+. Exogenous $NADP^+$ reduction was not observed.

5. Malate and succinate oxidation by isolated plant mitochondria was dependent on inorganic phosphate and inhibited by n-butylmalonate, showing that this oxidation was dependent on the dicarboxylate transporter. Malate oxidation in the absence of added glutamate was severely inhibited by oxaloacetate accumulation, even though this substance was shown to readily penetrate the inner membrane of isolated plant mitochondria. Tentative evidence was obtained for the restriction of isocitrate and ∝-ketoglutarate oxidation by their respective dehydrogenase complexes in isolated mitochondria.

   No NADP-linked malate or isocitrate oxidation was observed by intact or disrupted plant mitochondria.

6. Swelling of isolated beetroot and cauliflower mitochondria in ammonium glutamate was activated by inorganic phosphate and malate (or malonate), and inhibited by n-butylmalonate, suggesting that glutamate entered the mitochondria via a carrier similar to that for tricarboxylate ions.

7. Isolated beetroot mitochondria oxidized exogenous NADH when malate and malate dehydrogenase were added. This oxidation was inhibited by glutamate and did not recover upon addition of glutamate-oxaloacetate transaminase. This suggests that a malte-oxaloacetate, but not the malate-aspartate, shuttle was operative. Beetroot cells may rely on such a shuttle for the oxidation of extramitochondrial NADH.

DECLARATION

The investigations described in this thesis were performed in the Botany Department, University of Adelaide, from March, 1972 to July, 1975. The following papers were written during the period of study:

1. The Oxidation of Malate and Exogenous NADH by Isolated Plant Mitochondria, by D.A. Day and J.T. Wiskich, Plant Physiol. (1974) 53 : 104 - 109

2. The Effect of Exogenous NAD on the Oxidation of NAD-linked Substrate by Isolated Plant Mitochondria, by D.A. Day and J.T. Wiskich. Plant Physiol. (1974) 54 : 360 - 363

3. Isolation and Properties of the Outer Membrane of Plant Mitochondria, by D.A. Day and J.T. Wiskich. Arch. Biochem. Biophys. (in press).

A fourth paper entitled "NADH Oxidation by Mitochondria Isolated from *Beta vulgaris* (L) is in preparation.

To the author's belief and knowledge, this thesis contains no material previously submitted for a degree in any University by the author or by any other person, except where due reference is made in the text.

D.A. DAY

ACKNOWLEDGEMENTS

I would like to sincerely thank the following people: Dr. J.T. Wiskich for his supervision, guidance and encouragement throughout the course of this work; Mr. S.P. Robinson for helpful discussion; Professor P.G. Martin and Dr. R.T. Lange for use of laboratory facilities; Mrs. C. Evans for typing this thesis; and Misses S. Hill and C. Wilkins for their help with diagrams.

I also dearly thank my wife, Judith, without whose love and support this work may not have been possible.

This work was carried out during the tenure of a Commonwealth Postgraduate Award.

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ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
APAD(H)	3 - acetyl pyridine adenine dinucleotide (reduced)
asc	ascorbate
ATP	adenosine triphosphate
BM	2 - n - butylmalonate
BSA	bovine serum albumen
cit.	citrate
cyt.	cytochrome
CoA	coenzyme A
DCPIP	2, 6 - dichlorophenol indophenol
DIC	dicoumarol
DNP	2, 4 - dinitrophenol
D9o	Decon 90
E	Extinction
E_m	midpoint redox potential
Eo'	oxidation - reduction potential
EDTA	ethylene diaminetetra-acetate
Fe CN	potassium ferricyanide
FP	flavoprotein
glut.	glutamate
GOT	glutamate - oxaloacetate - transaminase
isocit.	isocitrate
K_M	Michaelis constant
KG	∝-ketoglutarate
mal.	malate
malon.	malonate

MDH	malate dehydrogenase
M_W	washed mitochondria
M_D	disrupted mitochondria
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
OAA	oxaloacetate
pCMB	para - chloromercuribenzoate
Pi	inorganic phosphate
pmf	proton motive force
PMS	phenazine methosulphate
pNPP	para - nitrophenylphosphate
roten.	rotenone
succ.	succinate
tes	tris (hydroxymethyl) methylethanesulphonic acid
TMPD	tetra methyl-p-phenylene diamine

CHAPTER 1 *GENERAL INTRODUCTION*

Since Millerd et al (1951) demonstrated that isolated mungbean mitochondria oxidized organic acids of the Kreb's cycle, and that ATP was formed concommitantly, mitochondria from a large number of different plant species have been isolated and studied in some detail. The respiratory processes and related reactions, especially those pertaining to the electron transport chain, of plant and animal mitochondria have been extensively reviewed (Azzone, 1973; Chappell, 1968; van Dam and Meyer, 1971; Greville, 1969; Hackett, 1963; Ikuma, 1972; Klingenberg, 1970; Lehninger, 1972; Mitchell, 1966 and 1972; Ohnishi, 1973; Racker, 1970; Senior, 1973; Skulachev, 1972; Slater, 1971; Wainio, 1970; Wikstrom, 1973).

1. *The Respiratory Chain*

Figure 1.1 depicts a currently accepted scheme of the mammalian mitochondrial respiratory chain (van Dam and Meyer, 1971). Reducing equivalents produced by the oxidation of organic acids or fatty acids enter the chain via dehydrogenases; electrons flow down the chain, through ubiquinone and the cytochromes to reduce oxygen; three sites of phosphorylation are coupled to this flow of electrons. These sites of phosphorylation are termed site I, II and III and their respective positions on the respiratory chain are shown in figure 1.1.

The dehydrogenase contains flavin and several iron-sulphur centres (Ohnishi, 1973; Ohnishi et al, 1972), the function of

which remains obscure although they appear to be involved in site I energy conservation (Ohnishi, 1973). The flavin prosthetic group is probably FMN and functions catalytically (Kumar et al, 1968). Both rotenone and piericidin A are very potent inhibitors of the NADH dehydrogenase and act at the same site, somewhere on the oxygen side of the flavin component (Gutman et al, 1970; Ohnishi et al, 1969). Studies with yeast grown under iron - and sulphate - limited conditions (Light and Garland, 1971; Clegg and Garland, 1971; Ragan and Garland, 1971) suggest that piericidin A sensitivity and site I energy conservation are conferred on the mitochondria by two distinct iron-sulphur components of the NADH dehydrogenase. Chance et al (1967) proposed that another flavin component of high potential and low fluorescence was present in the NADH dehydrogenase region of the chain. That is, that two flavoproteins exist (FP1 and FP2) and that the site of action of rotenone was between the two species. However, this proposal seems doubtful due to the finding that the high potential FP was in the soluble fraction of sonicated mitochondria and was not detected in all animal mitochondria (Ragan and Garland, 1969).

The position that ubiquinone occupies on the chain has been a controversial matter, but studies with ubiquinone depleted mitochondrial particles (Ernster et al, 1969) indicate that it transfers electrons between the dehydrogenase systems and cytochrome *b*, and it appears to be the point at which the NADH and succinate systems converge (Klingenberg and Kroger, 1970).

Extraction of ubiquinone inhibits reduction of cytochrome *b* by NADH and succinate, and the measured redox potential of ubiquinone suggests that it lies on the substrate side of cytochrome *b* (Kroeger and Klingenberg, 1966). Since ubiquinone has a low molecular weight, is lipophilic and is present in the chain in excess quantities (with respect to the flavoproteins and cytochromes), it may function as a diffusible hydrogen pool (Klingenberg and Kroger, 1970).

Three cytochrome *b* components have been found in mammalian mitochondria, *b*562, *b*566 and *b*558 (Slater, 1973; Wikstrom 1973), although the latter two may be identical (Sato et al, 1971). These *b* type cytochromes appear to be grouped into two functionally different complexes with different kinetic and spectrophotometric properties (Wikstrom, 1973). It has been suggested that one of these cytochromes is intimately involved in site II energy conservation since ATP seems to have a direct affect on cytochrome *b*566 (or *b*568), inducing a shift in its redox potential (Chance et al, 1970). Chance et al (1970) renamed the high potential *b*566, cytochrome bT, and the low potential *b*562, cytochrome bK (since it did not seem to be involved in energy transduction). These results have recently been reinterpreted (Wikstrom and Lambowitz, 1974) and doubt has been cast on the direct involvement of *b* cytochromes in energy conservation (see discussion on oxidative phosphorylation). It has also been pointed out that the different absorption peaks observed could simply be due to different forms of a single *b* cytochrome (Wikstrom, 1971; Yu et al, 1972).

Two closely associated *c* cytochromes (*c* and c_1) can be distinguished in the mammalian respiratory chain (van Dam and Meyer, 1971) and the antibiotic, antimycin A, inhibits electron transfer between cytochrome *b* and cytochromes *c* + c_1 (Berden and Slater, 1970). The actual site of inhibition of antimycin A is not known but appears to be cytochrome *b*562 because its absorption band and redox potential appears to change upon binding antimycin (Berden and Opperdoes, 1972; Dutton et al, 1972). Inhibition by antimycin may be allosteric under certain conditions since it shows cooperative binding with substrate-reduced (succinate + cyanide treated) submitochondrial particles (Berden and Slater, 1972). Cytochrome *c* oxidase, the terminal portion of the respiratory chain, is a complex consisting of two haem molecules and two copper molecules, all of which are essential for activity (Beinert et al, 1970) although the role of copper is not yet clear. Estabrook (1961) showed that cytochrome *c* was located near the outer surface of the inner membrane, and was accessible from the intermembrane space. Hinkle and Mitchell (1970) have suggested that the cytochrome *c* oxidase complex is orientated across the membrane, cytochrome *c* being on the outer surface, cytochrome *a* embedded in the middle of the membrane and cytochrome a_3 close to the matrix. Such an arrangement of this system has important implications for oxidative phosphorylation theories (see next section).

As in most areas of biochemical research, less is known about the electron transport chain of plant mitochondria than that of animal mitochondria. Unfortunately, much of the work on the plant respiratory chain components has been done by one laboratory; obviously a more diversified approach is desirable. Little is known about the internal NADH dehydrogenase (FP1 in Fig 1.2) except that it is not as sensitive to rotenone as its mammalian counterpart. (Ikuma and Bonner, 1967; Wilson and Hanson, 1969). This may not reflect a difference in the dehydrogenases themselves however, since the binding of rotenone is noncovalent and involves the phospholipid environment of the membrane-bound enzyme (Singer and Gutman, 1971). The low sensitivity to rotenone may reflect the presence of an alternative pathway for NADH oxidation, since plant mitochondria oxidize added NADH while animal mitochondria do not, indicating the presence of an additional, respiratory-linked NADH dehydrogenase in plants (Coleman and Palmer, 1972; Cunningham, 1964; Ikuma and Bonner, 1967; Miller and Koeppe, 1971; Wilson and Hanson, 1969; Wiskich and Bonner, 1963). Douce et al (1973) have recently shown this flavoprotein (FP3 in Fig 1.2) to be located on the outer surface of the inner membrane (see later). Fluorescence measurements of mung bean and skunk cabbage mitochondria have shown that four flavoproteins can be discerned (Erecinska and Storey, 1970; Storey, 1970). Storey (1970) named these components FPha, FPm, FPhf and FP1f. Two species FPha and FPhf can be reduced by succinate and added NADH (under anaerobic conditions) suggesting that these flavoproteins are on that part of the respiratory chain shared by NADH and succinate.

Studies on the time sequence of oxidation of electron carriers suggest that FPha lies between ubiquinone and cytochrome *b* (Storey and Bahr, 1972) and corresponds to FP4 in Fig. 1.2, but beasurements of redox potentials of plant mitochondrial components suggest that FPhf and PFlf are identical(Storey, 1971) and the position this component occupies on the chain (if any) is obscure. FPm is reduced only by malate and corresponds to FPl in Fig. 1.2. The flavoproteins associated specifically with the oxidation of exogenous NADH have not been determined. Storey (1973) suggested that FPhf was associated with the cyanide insensitive pathway, but its highly negative redox potential makes this unlikely (Ikuma, 1972).

Three *b*-type cytochrome peaks can be distinguished in plant mitochondria (Bonner, 1965; Lance and Bonner, 1968). The room temperature α-absorption peaks of these cytochromes are 556nm, 560nm and 566nm (Lambowitz and Bonner, 1974); there has been one report of an additional peak at 558 nm (Lambowitz and Bonner, 1974). Cytochromes *b*558 and *b*566 seem to be analogous to the cytochromes with idential peaks found in animal mitochondria in that their reduction is enhanced by treatment with antimycin A plus oxidant (Lambowitz and Bonner, 1974). Cytochrome *b*-560 appears to correspond to *b*-562 in animal mitochondria since its α-peak shifts slightly to the red upon addition of antimycin A (Bonner and Slater, 1970; Storey, 1973). However, cytochrome *b*-556 seems to be unique to plants. The positioning of these cytochromes is uncertain but kinetic studies prompted Storey (1973) to place

cytochrome *b*-556 on the substrate side of *b*-560, despite the finding that the Em of cytochrome *b*560 is lower than that of cytochrome *b*556 (Dutton and Storey, 1971; Lambowitz and Bonner, 1974). The position of cytochrome *b*-566 has not been determined.

Two *c*-type cytochromes with α-absorption peaks at 549 and 547 nm can be distinguished in plant mitochondria, corresponding to cytochromes *c* and c_1 in animal mitochrondria (Ikuma, 1972). Since cytochrome *c*549 is reduced together with cytochromes *a* + a_3 in the presence of cyanide and the absence of substrate (Lance and Bonner, 1968; Storey, 1970) and cannot be extracted as easily as cytochrome *c*549 by salt solutions (Lance and Bonner, 1968) it appears that cytochrome *c*547 is analagous to cytochrome c_1, and cytochrome *c*549 analagous to *c*.

Cytochrome oxadise in plant mitochondria, as in animal mitochondria, consists of cytochromes *a* and a_3 (Storey, 1970; Chance et al, 1968) and displays similar inhibitor sensitivity as its animal counterpart (Bendall and Bonner, 1971; Wiskich and Bonner, 1963).

The studies outlined above can be summarized by a scheme such as that shown in Fig. 1.2. It should be noted that not all the flavoproteins shown have been identified, and not all the *b*-type cytochromes are shown. Ubiquinone, FP4 and the *b* cytochromes are grouped because their redox potentials and their time sequence of oxidation are similar (Storey, 1970; 1973; Lambowitz and Bonner, 1974).

Rat liver mitochondria will not oxidize added malate unless glutamate (or another compound) is present to transaminate (or remove) oxaloacetate and prevent product inhibition of malate dehydrogenase (Lehninger, 1964). However, some plant mitochondria oxidize malate rapidly in the absence of glutamate (Ikuma and Bonner, 1967) although often a progressive decrease in the rate of malate oxidation has been observed (e.g. see Wiskich et al, 1964) and the presence of a transaminase has been demonstrated (Wiskich et al, 1964). Recently Macrae (1971a, b and c) has shown that many plant mitochondria contain both malate dehydrogenase and malic enzyme which oxidatively carboxylates malate to pyruvate. Macrae (1971) has shown that the pH of the assay medium has a profound effect on which enzyme activity predominates in isolated cauliflower mitochondria oxidizing malate.

Although Macrae and Moorehouse (1970) envisaged malic enzyme to be located in the matrix, Coleman and Palmer (1973) have suggested that it lies in the intermembrane space, reducing equivalents being transferred to the respiratory chain via the external NADH dehydrogenase on the outside of the inner membrane (Douce et al, 1973). More recently Hatch and Kagawa (1974) have reported evidence supporting Macrae's idea that malic enzyme is located in the matrix. The greater ease of malate oxidation by plant mitochondria may also be due to a greater permeability of the inner membrane to oxaloacetate (Douce and Bonner, 1972), hence preventing a high intramitochondrial concentration of it. Rat liver mitochondria appear to be impermeable to oxaloacetate (Borst, 1962; Chappell, 1968).

Perhaps the most striking difference between the respiratory chains of plant and animal mitochondria is that the former may contain (or develop) an alternative cyanide insensitive electron transport pathway (Ikuma, 1972). The degree of sensitivity of plant mitochondria to cyanide varies from species to species; skunk cabbage mitochondria are highly cyanide resistant (Bendall and Bonner, 1971; Simon, 1957; Yocum and Hackett, 1957), Mung bean mitochondria are only partially resistant to cyanide (Ikuma and Bonner, 1967), while most other plant mitochondria are very sensitive to cyanide (Bendall and Bonner, 1971). Mitochondria isolated from cyanide resistant species contain both the cyanide resistant, non phosphorylating pathway and the cyanide sensitive pathway coupled to phosphorylation (Storey and Bahr, 1969). The former pathway branches from the main chain between substrate and cytochrome *b* and does not contain cytochromes, although a flavoprotein is involved (Lance and Bonner, 1968; Storey and Bahr, 1969). The alternative pathway contains an oxidase which transfers electrons from the flavoprotein to oxygen and is inhibited by m-CLAM (Schonbaum et al, 1971). Storage tissues which are usually sensitive to cyanide have been found to develop the alternative pathway upon slicing and aging (Beevers, 1961; Hackett, 1963). It also appears that there is a substrate specificity for the cyanide-insensitive chain, since exogenous NADH oxidation is inhibited almost completely in mitochondria from cultured sycamore, while malate and succinate oxidation are not (Wilson, 1971).

The function of this pathway *in vivo* is not immediately apparent, but it may serve to control adenylate concentrations in the cell without altering metabolic flux (Ikuma, 1972).

2. *Oxidative Phosphorylation*

Three main hypotheses are currently used to explain how ATP synthesis is coupled to electron flow through the respiratory chain.

(a) *The Chemical Hypothesis*

This hypothesis, as originally stated by Slater (1953), can be represented as;

$$AH_2 + B + I \longrightarrow A \sim I + BH_2 \quad \text{----- (1)}$$

$$A \sim I + ADP + Pi \longrightarrow A + ATP + I \quad \text{----- (2)}$$

$A \sim I$ represents a high energy intermediate in which the redox energy (liberated during the electron transfer processes of the respiratory chain) is conserved in a covalent bond between the specific electron carrier and a ligand, I (Azzone, 1972). This intermediate then reacts with ADP and Pi to form ATP, a reaction catalysed by the ATPase of the inner mitochondrial membrane and which releases A and I to participate in further redox reactions. This hypothesis was later elaborated to include an intermediate ($X \sim I$) common to all three phosphorylation sites (Ernster et al, 1967).

This form of the hypothesis can be represented as;

$$AH_2 + B + I \longrightarrow A\sim I + BH_2 \quad \text{-----} \quad (3)$$

$$A\sim I + X \longrightarrow A + X\sim I \quad \text{-----} \quad (4)$$

$$X\sim I + P \longrightarrow X\sim P + I \quad \text{-----} \quad (5)$$

$$X\sim P + ADP \longrightarrow X + ATP \quad \text{-----} \quad (6)$$

Often equations (5) and (6) are replaced by a single, concerted reaction by some proponents of the chemical scheme (Cooper, 1965; Boyer, 1968; Slater, 1971);

$$X\sim I + ADP + Pi \longrightarrow X + I + ATP \quad \text{-----} \quad (7)$$

Advocates of this hypothesis envisage uncouplers as hydrolysing A$\sim$ C or X$\sim$I (Slater, 1971), preventing ATP formation and recycling A and C (or X and I).

The chemical hypothesis has proved to be of relatively little use experimentally and has been strongly criticized on these grounds by Azzone (1971). The main experimental prediction of the hypothesis is the existence of a high energy intermediate. The strongest evidence for such an intermediate has come from studies that indicate that the α-absorption bands of cytochrome *b*, and the midpoint potentials of *b* and *a*, shift upon energization of the mitochondrial membrane (Chance et al, 1970; Slater et al, 1970; Wilson and Dutton, 1970). The midpoint potential of cytochrome *b*566 is the primary high energy intermediate of site II phosphorylation. However, some doubt has been cast on the techniques used to measure

these changes in potential, and on their interpretation (Wikstrom and Lambowitz, 1974). The technique employed by Wilson and Dutton (1970) and Chance et al (1970) depends on added redox mediators (such as PMS) to bring the membrane-bound cytochromes into equilibrium with the potential of the electrode used (De Vault, 1971; Caswell, 1971). That is, upon addition of ATP reversed electron transport from cytochrome *c* to cytochrome *b* occurs, and the PMS should recycle electrons to cytochrome *c*, thus 'clamping' the redox potential on both sides of site II energy conservation (Wilson et al, 1973). However, if the redox mediator reacted rapidly with cytochrome $c + c_1$ but slowly with cytochrome *b*, the apparent midpoint potential shift in energized mitochondria could have been due to reversed electron transport from cytochrome $c + c_1$ to cytochrome *b* without the latter being directly involved in energy transduction (Wikstrom and Lambowitz, 1974). Wikstrom and Lambowitz (1974) showed that the concentrations of PMS used by Wilson and Dutton (1970) and Chance et al (1970) were not high enough to react efficiently on both sides of site II phosphorylation and therefore did not establish redox equilibrium during potentiometric titrations. Wikstrom and Lambowitz (1974) therefore concluded that the ATP-induced potential shifts observed were a consequence of reversed electron transport and cannot be considered evidence for the role of cytochrome *b* as a high energy intermediate. Dutton and Storey (1971) and Lambowitz et al (1974) have shown that mung bean mitochondria do not possess a *b*-type cytochrome which undergoes a shift in midpoint potential upon addition of ATP.

Even if valid, these potential shifts can be equally well explained by any hypothesis that allows for energized membranes.

(b) *The Conformational hypothesis*

Another hypothesis proposed by Boyer (1965) suggested that energy from redox reactions of the respiratory chain is conserved in a change in the conformation of some protein and then utilized in some manner for ATP formation. This hypothesis can be symbolized (Slater, 1971) as;

$$AH_2 + B \longrightarrow A^* + BH_2 \quad (1)$$

$$A^* + ADP + Pi \longrightarrow A + ATP \quad (2)$$

$$\text{Sum: } AH_2 + B + ADP = Pi \longrightarrow A + BH_2 + ATP \quad (3),$$

where A* represents a high energy state of a redox carrier. When represented in this way there is very little difference between the conformational and chemical hypothesis. More recently however, Boyer (1975) has adapted this idea to account for ATP synthesis driven by proton translocation; protein conformational changes could be induced by a proton gradient across the membrane, an idea proposed by Azzone (Azzone and Massari, 1973) and Papa et al (1973). This concept however, still represents the 'black box' type of approach to oxidative phosphorylation.

(c) *The Chemiosmotic hypothesis*

Lundegardh (1945) suggested that an anisotropic organization of the cytochrome chain across a membrane would lead to protons being produced on one side and taken up on the other. This difference in proton concentration across the membrane represents an osmotic potential which could be used for ATP synthesis, if coupled to an ATPase (Mitchell, 1961).

This idea has been developed by Mitchell (1961, 1966) who envisaged a reversible anisotropic ATPase system positioned across the inner mitochondrial membrane such that it could couple ATP synthesis to a backflow, across the membrane, of protons produced by the redox reactions of the respiratory chain. This hypothesis requires the membrane involved to be relatively impermeable to protons; if it also had a low permeability to other ions, the electron transport and ATPase systems could be coupled by the sum of the osmotic pressure (pH) difference and the electrical potential difference (Mitchell, 1966). These combined potentials have been termed 'proton motive force' (pmf; Mitchell, 1966). In short, the chemiosmotic theory proposes that "oxidation and phosphorylation are coupled via the transmembrane electrochemical potential of hydrogen ions ($\Delta\bar{\mu}\ H^+$):

oxidation $\longrightarrow \Delta\mu\ H^+ \longrightarrow$ phosphorylation"

(Skulachev, 1974).

The four basic postulates of the chemiosmotic hypothesis are (Mitchell and Moyle, 1967):

(a) An ion impermeable coupling membrane, which contains,
(b) the proton - translocating respiratory chain,
(c) the proton - translocating reversible ATPase, and
(d) exchange diffusion systems which couple proton translocation to that of anions and other cations.

Mitchell and Moyle (1965; 1967) have shown that not only does the cristae membrane have a low permeability to protons (this permeability can be increased by uncouplers) but that protons are translocated out of the matrix during respiration or ATP hydrolysis, with H^+/O ratios close to 4 for succinate and 6 for β-hydroxy butyrate oxidation (i.e. corresponding to P/O ratios of 2 and 3 respectively). These studies support the postulate (Mitchell 1961, 1966) that the respiratory chain is arranged in proton translocating loops (see Fig. 1.3) and that uncouplers act by causing protons to flow back through the inner membrane, destroying the pH gradient. Much of the criticism of Mitchell's hypothesis has been directed at his proposed arrangement of the electron transport chain (see Slater, 1971, for a review), particularly the positioning of ubiquinone. (See Fig. 1.3).

However, it has been shown that flavins and cytochrome *c* are on opposite sides of the membrane, so that reducing equivalents flowing from flavins to *c* must transverse the membrane (Grinius et al, 1971). Hinkle and Mitchell (1970) and Racker (1973) have also obtained evidence suggesting that cytochrome a_3 is on the matrix side, and *a* in the middle, of the inner membrane; electron transfer in cytochrome oxidase vesicles results in formation of an electrical gradient across the membrane (Hinkle, 1974). It has also been shown that energization of the coupling membrane leads to the production of a transmembrane electrical potential, and/or a pH gradient, which is large enough to drive ATP synthesis, and that an artificially induced membrane potential (using synthetic ions) can be used for ATP synthesis (Skulachev, 1971 and 1974).

One of the main problems in the field of oxidative phosphorylation has been the structure and mechanism of the ATPase system coupled to electron transfer. Figure 1.4 illustrates the most recently proposed chemiosmotic model (Mitchell,1974). The lipophilic Fo component contains a specific proton conducting pathway which can be blocked by oligomycin (Mitchell, 1973) and it is assumed that H_{20} can also migrate through Fo. The component F_1 catalyses ATP hydrolysis and closes the proton - conducting pathway through Fo (Mitchell, 1973). Mitchell (1974) has therefore postulated that the active centre is situated at the Fo-Fi interface such that during synthesis the phosphorus centre

of Pi (or ATP if hydrolysis is occurring) becomes accessable only on the right side (see Fig. 1.4) to protons at a relatively high potential in the aqueous phase when a pmf is applied across the membrane. Protonation of phosphate will then allow ATP synthesis to occur by a nucleophilic substitution mechanism (Mitchell, 1974).

Evidence for exchange diffusion systems for anion-proton exchange across the cristae membrane has come from a large number of studies (for reviews see Chappell, 1968, and Klingenberg, 1970). For example, it has been demonstrated that Pi enteres rat liver mitochondria in exchange for hydroxyl ions (Chappell, 1968). Transport systems such as this enable ions to be transported across the inner membrane without disrupting any pmf present, and will be discussed in detail in a later section.

Evidence in support of the chemiosmotic hypothesis has also been provided by studies on photophosphorylation in green plants and photosynthetic bacteria. Hind and Jagendorf (1963) showed that preiltuminated chloroplasts would synthesize ATP in the dark if supplied with ADP and Pi immediately after the light was switched off. Apparently light driven electron transport produced an intermediate (termed X_E) which could support ATP synthesis in the dark. This dark phosphorylation was dependent on the presence of a coupling factor (Avron, 1963). Since the amount of ATP formed was large compared to the amounts of electron carriers present, it was concluded that X_E could not be a high energy form of these carriers (Jagendorf and Uribe, 1967).

It has been shown that light driven electron transport is accompanied by proton translocation, suggesting that X_E is a pH gradient (Jagendorf, 1967). This proton translocation appears to be into the grana sac and can be measured as an increase in the pH of the surrounding medium (Jagendorf, 1967). That a pH gradient can drive ATP formation is indicated by the demonstration of "acid-bath" ATP synthesis in the dark (Jagendorf and Uribe, 1967). Chloroplasts in the dark and in the presence of electron transport inhibitors were found to synthesize ATP if they were first incubated at pH 4, followed by a rapid adjustment of the medium to pH 8.5 (as ADP, Pi and Mg^{2+} were added). This acid-base ATP synthesis was inhibited by uncouplers (Jagendorf and Uribe, 1967) and an antibody specific for the chloroplast ATPase (McCarty and Racker, 1966). Apparently protons penetrate the thylakoid membrane slowly at pH 4 and some cations leak out; when the medium is adjusted to pH 8.5 an artificial pH gradient is set up, which can drive ATP formation (Jagendorf, 1967). The light-triggered Mg^{2+} - dependent ATPase of chloroplasts (Petrack and Lipman, 1961) can also be triggered by the formation of a pH gradient in the dark (Kaplan et al, 1967).

Despite the wealth of evidence produced by proponents of the chemiosmotic hypothesis, final proof (or disproof of other hypotheses) has yet to be demonstrated, and much of the data collected can be interpreted in terms of the chemical or conformational hypotheses (Slater, 1971).

Therefore these hypotheses remain viable alternatives and must be considered when discussing energy transduction. However the studies outlined above (particularly those with isolated chloroplasts) do show conclusively that pH and/or electrical gradients across membranes are intimately associated with energy transduction and must be incorporated into any hypothesis dealing with oxidative phosphorylation.

3. *Oxidation of extramitochondrial NAD(P)H*

(a) *NADH oxidation by animal mitochondria*

The final oxidation of the end products of lipid, protein and carbohydrate metabolism produced in the cell occurs in the mitochondria (van Dam and Meyer, 1971) and the reducing power of these compounds must be delivered to the respiratory chain. However Lehninger (1951) had shown that exogenous NADH was not accessible to the respiratory chain of isolated rat liver mitochondria, an observation confirmed by studies with other tissues (Sacktor and Dick, 1962; Borst, 1962). Klingenberg and Pfaff (1966) have since shown that the inner mitochondrial membrane is impermeable to added NADH, although it freely penetrates the outer membrane. Since direct oxidation pathways are not readily available, attention has been focussed on substrate shuttles involving a reaction between NADH and oxidized substrate in the cytoplasm, followed by penetration of the reduced substrate

into the mitochondria, and subsequent oxidation. In other words, the substrate (eg malate) serves as a carrier of reducing equivalents from cytoplasmic NADH to the respiratory chain. Since a considerable portion of the Kreb's cycle pathway is duplicated in the cytoplasm (Greville, 1966), a variety of enzymatic reactions and intermediates are theoretically available to provide a number of shuttles and cycles for the transfer of reducing equivalents to the mitochondria. Examples of proposed shuttles include, (i) the ∝-glycerol-phosphate cycle (Estabrook and Sacktor, 1958), (ii) the β-hydroxybutyrate cycle (Devlin and Bendell, 1960) and (iii) the malate-asparte cycle (Delbruck et al, 1959; Borst, 1962 and Chappell, 1969).

External NADH can also be oxidized directly by liver mitochondria but not via the respiratory chain. Ernster et al (1955, 1956) found that added cytochrome *c* was reduced by rat liver mitochondria in the presence of NADH, but this reduction was insensitive to amytal and antimycin A and was not coupled to phosphorylation. Ernster et al (1956) proposed that an NADH-cytochrome *c* reductase, similar to that found in microsomes, resided on the outer membrane. This prediction was later confirmed by studies with isolated outer membrane fragments (Sottocasa et al, 1967; Parsons et al, 1967) which were found to possess a rotenone-insensitive NADH-cytochrome *c* reductase.

(b) *NADH oxidation by plant mitochondria*

Mitochondria isolated from a variety of plant tissues have been shown to readily oxidize exogenous NADH via the respiratory chain (Baker and Lieberman, 1962; Bonner and Voss, 1961; Cunningham, 1964; Hackett, 1961; Ikuma and Bonner, 1967; Miller and Koeppe, 1971; Wilson and Hanson, 1969) with ADP/O values between 1.0 and 1.5 (Cunningham, 1964; Ikuma and Bonner, 1967; Wiskich and Bonner, 1963) suggesting that only two phosphorylation sites are involved. This, together with the fact that exogenous NADH oxidation is insensitive to rotenone (Miller and Koeppe, 1971; Wilson and Hanson, 1969), suggested that the dehydrogenase involved in NAD-linked substrate oxidation was bypassed. Since antimycin A inhibited this exogenous NADH oxidation (Wilson and Hanson, 1969; Miller and Koeppe, 1971), reducing equivalents must enter the respiratory chain on the substrate side of cytochrome *b*. Palmer and Passam (1971) and Coleman and Palmer (1971) suggested that a specific NADH dehydrogenase, situated on the outside of the inner membrane, is responsible for the oxidation of exogenous NADH. This idea was later confirmed by the work of Douce et al (1973) with mung bean mitochondria. Wilson and Hanson (1969) demonstrated that the addition of excess cytochrome *c* to isolated corn mitochondria would partly relieve the antimycin A inhibition of NADH oxidation, suggesting that an alternative NADH-cytochrome *c* reductase, insensitive to respiratory inhibitors, may be present in plant mitochondria.

More recently Douce et al (1972 and 1973) and Moreau and Lance (1972) showed that the outer membrane of mung bean and cauliflower bud mitochondria contained an antimycin A-insensitive NADH-cytochrome *c* reductase similar to that found in animal mitochondria. This reductase consisted of a flavoprotein (with a different stereospecificity for NADH than that of the inner membrane), cytochrome b_{555} and b_{555} reductase.

Plant mitochondria thus possess two external NADH dehydrogenases, one located on the outer membrane and the other on the outersurface of the inner membrane, linked to the respiratory chain. This latter enzyme appears to require a divalent cation for maximum activity since it is specifically stimulated by low (less than 1 mM) Ca Cl_2 and other cations (Miller et al, 1970; Miller and Koeppe, 1971). Coleman and Palmer (1971) suggested that exogenous NADH oxidation has an absolute requirement for Ca^{++} since, in the presence of EGTA, no oxygen uptake was observed unless Ca^{++} was added to the reaction medium.

(c) *NADPH oxidation*

There has been only one report of significant rates of exogenous NADPH oxidation by intact plant mitochondria. Koeppe and Miller (1972) showed that corn mitochondria would oxidize added NADPH in a coupled manner with ADP/O values similar to those for NADH.

This oxidation was dependent on Ca^{++} and phosphate, and was insensitive to rotenone; amytal on the other hand inhibited NADPH oxidation almost completely, although it had little effect on NADH oxidation. These authors concluded that a specific flavoprotein was involved in exogenous NADPH oxidation. The outer membrane of both plant (Douce et al, 1973; Moreau and Lance, 1972) and animal (Parsons et al, 1967; Sottocasa et al, 1967) does not appear to possess an enzyme capable of oxidizing NADPH, and in this aspect differs from the microsomal fraction (Rungie and Wiskich, 1972a, Ernster et al, 1962).

4. *Enzyme distribution and the separation of mitochondrial membranes*

The existence of two mitochondrial membranes, an outer limiting membrane and a folded inner membrane, has been long established (Palade, 1953), but the distribution of enzymes within these membranes was not elucidated until reliable methods were developed to separate and isolate the two membranes. The first preparations of apparently pure inner and outer membranes were obtained by Parsons et al (1966), using what is known as the "swelling-shrinking" technique. This method is based on the observation (Wtodower et al, 1966) that swelling of isolated liver mitochondria in hypotonic phosphate buffer resulted in breakage of the outer membrane, while the inner one simply unfolded without rupturing. By centrifuging swollen mitochondria on a suitable sucrose gradient Parsons et al (1966) obtained small but relatively pure fractions of inner and outer membrane from the bulk of unbroken mitochondria.

Sottocasa et al (1967) improved on this method by contracting the swollen inner membrane in the presence of hypertonic sucrose, Mg^{++} and ATP. Gentle sonication was used to facilitate removal of the broken outer membrane, and after centrifugation on a discontinuous density gradient, high yields of pure inner and outer membranes were obtained. In fact almost 90% of the outer membrane, free from contamination by the inner membrane, was recovered by this technique (Sottocasa et al, 1967). Levy et al (1967) introduced another method based on the selective action of low concentrations of digitonin on the outer membrane. Schnaitman et al (1967) developed this technique further and obtained high yields of outer membrane fragments and relatively intact inner membrane 'ghosts'. Levy et al (1969) have shown that digitonin complexes cholesterol *in vitro* and proposed that it has a similar effect on mitochondrial membranes. Since cholesterol is five times more concentrated in the outer membrane (Levy and Sauner, 1968), digitonin has a more pronounced effect on the outer membrane than on the inner membrane.

As a result of these studies a detailed insight into the intramitochondrial localization of various enzymes has been gained. Sottocasa et al (1967) obtained three fractions upon gradient centrifugation, namely a "heavy", a "light" and a "soluble" fraction. From electron microscopic examination the heavy fraction consisted of inner membranes and some matrix, and the light fraction contained outer membrane vesicles; the soluble fraction was therefore expected to contain the intermembrane components and some matrix.

On the basis of this investigation and many others since, the pattern of enzyme distribution shown in table 1.1 was obtained (Ernster and Kuylenstierna, 1969). It is now widely accepted that monoamine oxidase, cytochrome oxidase and malate dehydrogenase are markers for the outer membrane, inner membrane and matrix respectively, in animal mitochondria.

In contrast to the work done with animal mitochondria, only a few attempts have been made to separate and isolate the membranes of plant mitochondria, and most of these have involved adaptations of the method of Parsons et al (1967), using hypotonic swelling to selectively break the outer membrane (Douce et al, 1973; Moreau and Lance, 1972). Malate dehydrogenase has been found to be located in the matrix (Douce et al, 1973) and an antimycin A-insensitive NADH-cytochrome *c* reductase on the outer membrane (Douce et al, 1973; Moreau and Lance, 1972). This latter enzyme appears to be identical to that found on the outer membrane of animal mitochondria and on plant and animal microsomes (Rungie and Wiskich, 1972). No NADPH-cytochrome *c* reductase has been found associated with the outer membrane fractions, showing that they were not contaminated with microsomes. As well as the respiratory chain components, the inner membrane has been shown to possess an enzyme system which synthesizes CDP-diglyceride (Douce et al, 1973b). Moreau et al (1974) have studied the phospholipid and fatty acid composition of the membranes of cauliflower mitochondria and found that they differ considerably from each other and from microsomes.

In contrast to those of the inner membranes, the fatty acids of the outer membrane were largely saturated, and the authors suggested that this may account for the difference in plasticity between the two membranes.

5. *Metabolite transport*

It has been shown that the outer membrane of rat liver mitochondria is freely permeable to low molecular weight compounds (Klingenberg and Pfaff, 1966) and impermeable to large molecules (Wojtczak and Zaluska, 1969). In particular, the outer membrane forms a permeability barrier to external cytochrome *c*, preventing it from reacting with inner membrane enzymes such as cytochrome oxidase and succinate - cytochrome *c* reductase (Wojtczak and Zaluska, 1969; Wojtczak and Sottocasa, 1972). Similar studies with mung bean mitochondria (Douce et al, 1972) have shown that the outer membrane of plant mitochondria is also impermeable to cytochrome *c*, although Palmer and Kirk (1974) have questioned these results, claiming that the swelling technique used by Douce et al (1972), to break the outer membrane, induced conformational changes in the inner membrane and thus stimulated succinate-cytochrome *c* reductase activity.

On the other hand, the inner membrane is impermeable to many metabolites (Chappell and Haarhoff, 1967); some compounds, such as ADP and Krebs cycle intermediates, move across the membrane on specific carrier systems (Chappell, 1968; Chappell and Crofts, 1966; Chappell and Haarhoff, 1967).

These exchange diffusion systems have been thoroughly investigated in mammalian mitochondria, but only a few studies with plant mitochondria have been published.

Most of the studies of anion transport have used a swelling technique developed by Chappell and Haarhoff (1967). Isolated mitochondria suspended in iso-osmotic solutions of ammonium salts of Kreb's cycle intermediates were found to swell and this swelling could be monitored spectrophotometrically. Apparently, NH_3 enters the matrix down a concentration gradient; the NH_3 associates with a proton within the matrix, leaving an excess of hydroxyl ions which can then exchange for a diffusible anion, such as phosphate (Chappell and Haarhoff, 1967). As the osmotic pressure increases the mitochondria swell. As a result of these studies, and others using radioactive isotope exchange techniques (eg Meyer and Vignais, 1973), the scheme shown in figure 1.5 has been proposed for substrate transport in rat liver mitochondria (Chappell, 1968). This scheme represents the malate-aspartate cycle (Borst, 1962) which can lead to rapid rates of NADH oxidation *in vitro*. This cycle is probably the main mechanism for the transport of reducing power between cytosol and mitochondrion (Krebs, 1967) and relies upon the presence of malate dehydrogenase and aspartate amino-transferase in both cell compartments, since oxaloacetate does not readily penetrate the inner membrane of mammalian mitochondria (Borst, 1962).

If such a system is allowed to proceed to equilibrium, the redox potential of NAD in the cytosol and mitochondria will also be equilibrated (Borst, 1963; Chappell, 1969).

However, measurements of NAD/NADH ratios in liver (Krebs, 1967; Williamson et al, 1967) have indicated that the redox potential of free NAD is much more negative in the mitochondria than in the cytosol, over a wide range of metabolic conditions. If the malate-aspartate cycle operates *in vivo*, there must be an asymmetric distribution of the substrate anions between the two compartments , to maintain the difference in NAD redox state (Chappell, 1969). Chappell (1969) demonstrated that when energy was supplied to isolated rat liver mitochondria, they would accumulate malate (and glutamate) and export aspartate and ∝-ketoglutarate, thus keeping the mitochondrial NAD in a reduced state.

The carriers postulated in fig. 1.5 all seem to operate on an exchange-diffusion mechanism. Phosphate enters the mitochondrion in exchange for hydroxyl ions on a specific carrier that is sensitive to SH poisons such as mersalyl (Tyler, 1969); the phosphate can then exchange for a dicarboxylate anion, such as malate, via the dicarboxylate transporter which is competively inhibited by certain dicarboxylate analogues, such as 2-n-butyl-malonate (Robinson and Chappell, 1967). Both these transporters are capable of accumulating their substrates against a concentration gradient (Chappell, 1969). The dicarboxylate carrier can also carry out dicarboxylate-dicarboxylate and phosphate-phosphate exchange (Chappell, 1969; Johnson and Chappell, 1969). The tricarboxylate transporter, also present in the inner membrane of liver mitochondria, catalyses the exchange of dicarboxylate and tricarboxylate acids. (Chappell and Haarhoff, 1967; Palmieri and Quagliariello, 1968).

Butylmalonate inhibits this exchange by preventing entry of malate via the dicarboxylate transporter (Robinson and Chappell, 1967; Chappell and Robinson, 1968). Glutamate can enter the mitochondrion either via its own specific transporter in exchange for hydroxyl ions (Meijer et al, 1972; McGivan and Chappell, 1970), or in exchange for aspartate (Azzi et al, 1967). Glutamate is also an activator of aspartate transport which occurs via a separate carrier (Chappell, 1969). Oxoglutarate enters the mitochondrion in exchange for malate or an analogue such as malonate (Robinson and Chappell, 1967); this entry is inhibited by aspartate although the efflux of oxoglutarate isn't (Chappell, 1969). This effect may help to maintain an asymmetric distribution of oxoglutarate in the cell and thus influence the NAD/NADH ratio in the cytosol and mitochondria (Chappell, 1969).

A number of differences in anion transport have been observed with mitochondria isolated from different sources (Chappell, 1968), but the adenine nucleotide transporter seems to be common to mitochondria from all animal tissues studied. The existence of this system was detected using atractyloside, a potent inhibitor of adenine nucleotide transport (see Chappell, 1968, for a review of its effects). Investigations of the exchange of exogenous and endogenous adenine nucleotides in isolated mitochondria have shown that an ADP and ATP exchange carrier operates and is sensitive to atractyloside. (Klingenberg and Pfaff, 1966; Heldt et al, 1965; Vignais et al, 1966).

Winkler et al (1968) have shown that this system operates by an obligatory exchange diffusion process, the entry of one molecule of external nucleotide being coupled to the exit of one molecule of internal nucleotide. In this way a constant mitochondrial pool size is maintained.

The picture of metabolite transport in plant mitochondria is not as clear, with only a few investigations having been undertaken. Phillips and Williams (1973) and Wiskich (1974), using the technique of swelling in ammonium salts, have shown that mitochondria from potatoe and cauliflower possess a phosphate-hydroxyl exchange carrier, a dicarboxylate and a tricarboxylate carrier. These systems appear to be similar to their animal counterparts; mersalyl inhibits the phosphate transporter and butyl-malonate inhibits the dicarboxylate system as well as citrate entry (Phillips and Williams, 1973). Succinate, malate and malonate, but not fumarate, can enter the mitochondrion via the dicarboxylate carrier in exchange for phosphate (Wiskich, 1974). Both phosphate and malate were necessary to observe swelling of cauliflower mitochondria in ammonium citrate, but only phosphate was necessary with beetroot mitochondria (Wiskich, 1974), showing that not all tissues are identical in this respect. Lee and Wilson (1972) observed a passive and active swelling of bean-shoot mitochondria incubated with a series of potassium salts of organic anions, including acetate, pyruvate, α-ketoglutarate and β-hydroxybutyrate as well as those already discussed.

The presence of an uncoupler (DNP), or an ATP generating system, inhibited the active (substrate dependent) swelling (Lee and Wilson, 1972). The mechanism of entry of these anions is not known. The physiological significance of these anion transport systems in plants is not immediately apparent, since as discussed previously, plant mitochondria can oxidize exogenous NADH directly; substrate shuttles are therefore not needed for this purpose. The operation of the malate-aspartate cycle has not been demonstrated in plants, and since oxaloacetate movement across the mitochondrial membranes of plants does not appear to be controlled (Douce et al, 1972) such a cycle would be "short-circuited". However, to maintain the pH and electrical gradients of the chemiosmotic scheme of oxidative phosphorylation (Mitchell 1961, 1966), the movement of substrates across the membrane must be controlled. In view of this, it seems strange that oxalacetate should be freely permeable.

Jung and Hanson (1973) have shown that in intact cauliflower mitochondria, ATPase activity is inhibited by atractylate, although this compound had little effect on sonicated mitochondria. State 3 oxygen uptake was also inhibited by atractylate and this inhibition was competive with respect to ADP (Jung and Hanson, 1973). Isolated corn mitochondria display similar properties (Hanson et al, 1972; Bertagnolli and Hanson, 1973). This suggests the presence of an adenine nucleotide transporter similar to that of liver mitochondria. Passam et al (1973) reported that atractyloside did not inhibit adenine nucleotide dependent reactions in Jerusalem artichoke mitochondria, but, as Jung and Hanson (1973) have pointed out, adenine nucleotide transport in some plant

mitochondria may have to be activated by 'priming', which involves a short burst of respiration in the presence of phosphate and magnesium (Carmelli and Biale, 1970).

6. *The Present Study*

This work characterizes the oxidation of both extra - and intramitochondrial NADH by isolated plant mitochondria, the control of these oxidations,and interactions between the different pathways involved. The study was restricted to two tissues, cauliflower buds and turnip tubers, for most of the studies, but comparisons were made with red-beet tubers to clarify certain points. The investigation was carried out with the following aims in mind:

(a) *NAD(P)H oxidation*

A comparison of the inner membrane and outer membrane pathways, and their interactions, was made. This led to a study of the effects of digitonin on mitochondrial membranes, and on cytochrome *c* penetration in particular. Mitochondria from red beet were found not to oxidize exogenous NADH readily, and this tissue was compared with cauliflower buds. Preliminary investigations of the induction of NADH oxidation by red beet mitochondria were made.

(b) *Isolation of the outer membrane*

A method for obtaining pure fractions of outer membrane in relatively large quantities was developed and a detailed investigation of the enzyme systems located on this membrane was undertaken. Comparisons with the microsmal fraction were made.

(c) *Control of substrate oxidation*

It was hoped to determine the controlling factors of NAD-linked substrate oxidation, and attention was focussed on the permeability of the inner membrane to these anions and the products of their oxidation. An investigation was made on the penetration of pyridine nucleotides in an attempt to determine their influence on NAD-linked substrate oxidation and to study interactions between the external and internal pathways of NADH oxidation.

It should be noted that the work outlined above was undertaken prior to the publications of Douce et al (1972 and 1973b), Moreau and Lance (1972) and Coleman and Palmer (1972).

TABLE 1.1 DISTRIBUTION OF ENZYMES WITHIN MITOCHONDRIA

RAT LIVER MITOCHONDRIA		PRESENT IN PLANT MITOCHONDRIA
COMPARTMENT	ENZYME	
Outer	NADH - cyt. *c* reductase	yes
membrane	cyt. b_5	yes
	kynurine hydroxylase	
	monoamine oxidase ----------	no
	ATP dependent fatty acid synthet.	
	fatty acid elongating system	
	phospholipase A2	
	nucleoside diphosphkinase	
Inner	cyt. a, a_3, b, c_1, c	yes
Membrane	NADH dehydrogenase	yes (two)
	succinate dehydrogenase	yes
	oxidative phosphorylation	yes
	pyr. nuc. transhydrogenase	yes
	fatty acyl CoA dehydrogenase	
	ferrochelatase	
	choline dehydrogenase	
Inter	adenylate kinase	
Membrane		
Space		
Matrix	NAD - linked dehydrogenases	yes
	cit. synthetase	yes
	aconitase	yes
	amino - transaminase	yes
	RNA and protein synth. systems	yes
	GTP dependent fatty acyl CoA synthetase	

Figure 1.1 The mammalian respiratory chain.

CoA	=	Coenzyme A
FP	=	Flavoprotein
FeS	=	Iron - sulphur protein
Q	=	Ubiquinone
---------		represents inhibition
⟶		represents electron flow

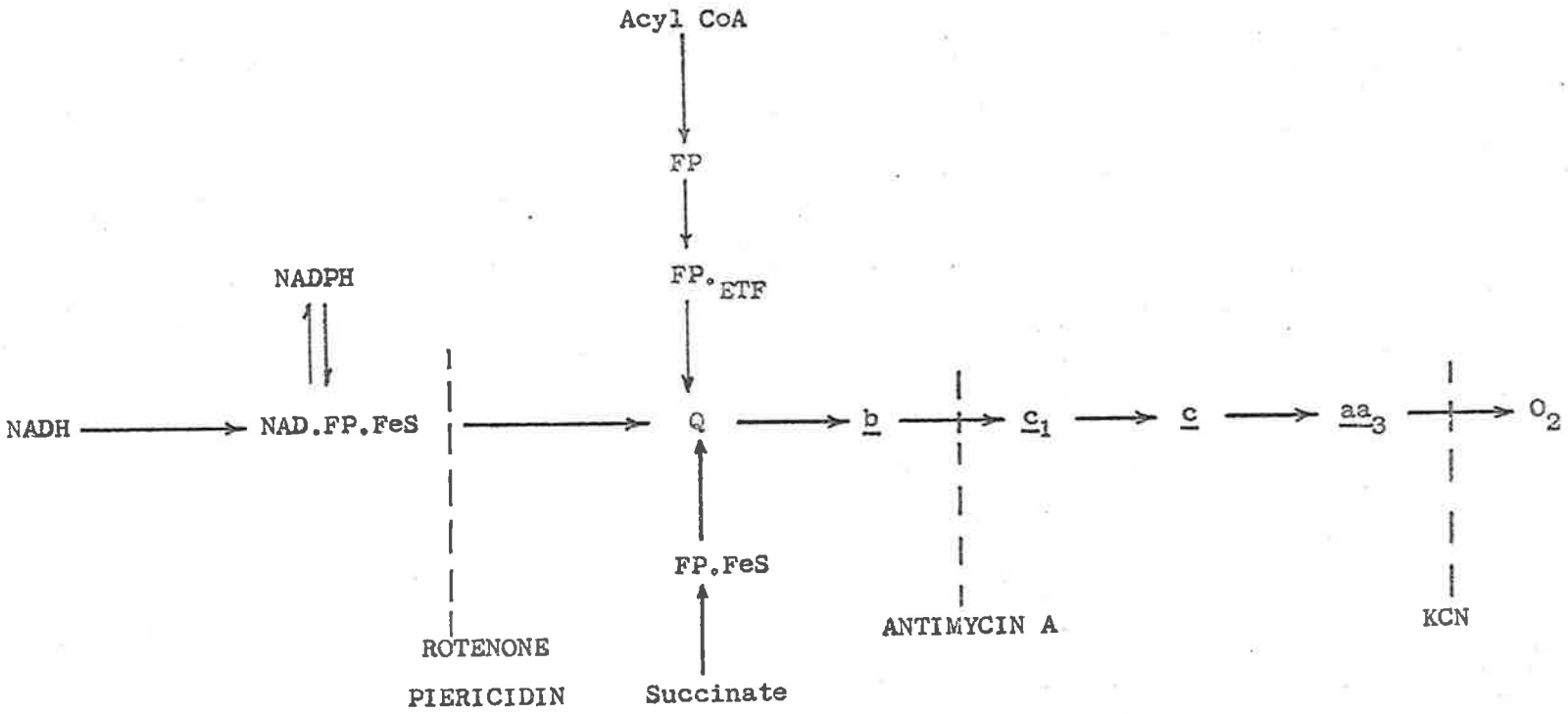


FIGURE 1.1 The mammalian respiratory chain. (van Dam and Meyer, 1971)

Figure 1.2 The plant respiratory chain

cyt.	=	cytochrome
FP	=	Flavoprotein
Q	=	Ubiquinone
mCLAM	=	m-chlorobenzhydroxamic acid
X	=	the alternative (cyanide-insensitive) oxidase
-------	=	inhibition
⟶	=	electron flow

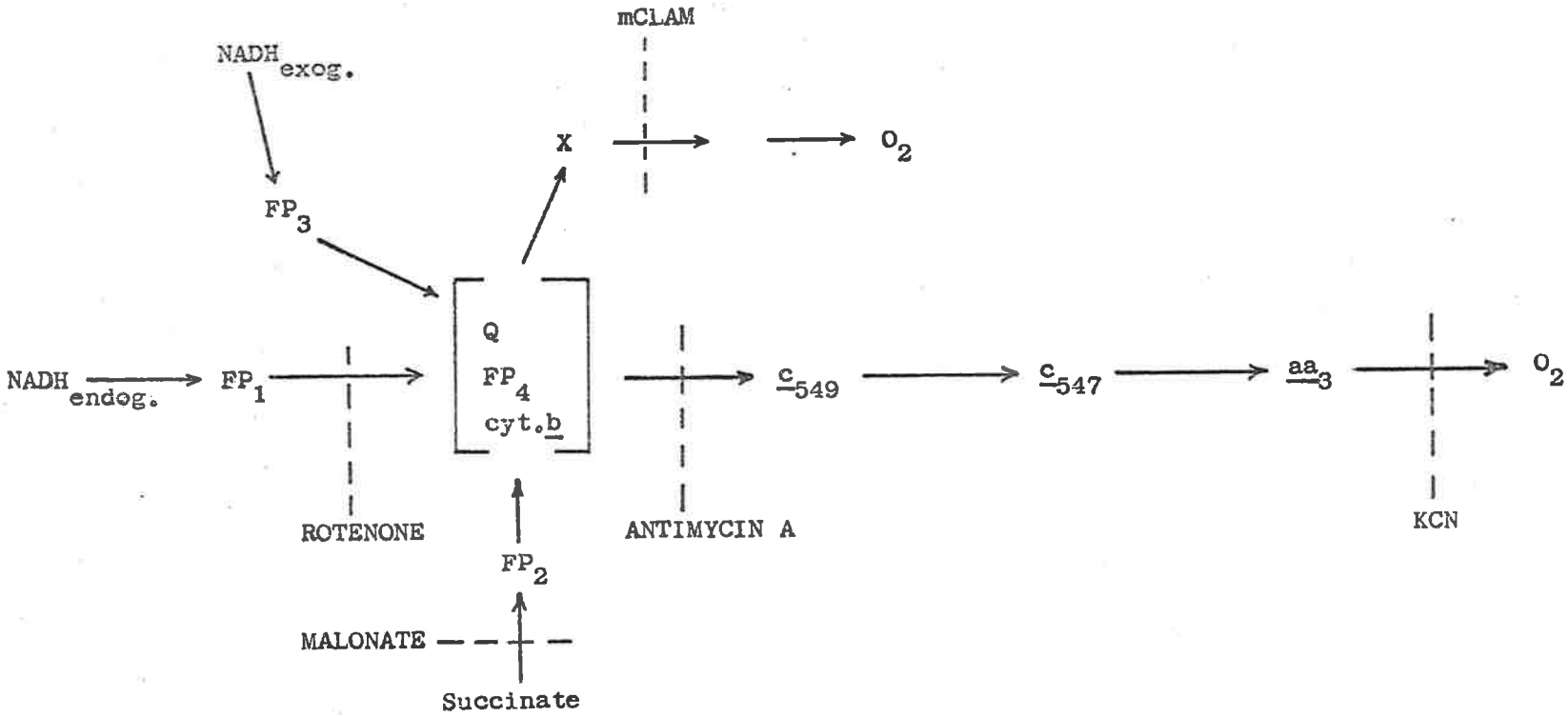


Figure 1.2 Proposed sequence of electron transport carriers of plant mitochondria.

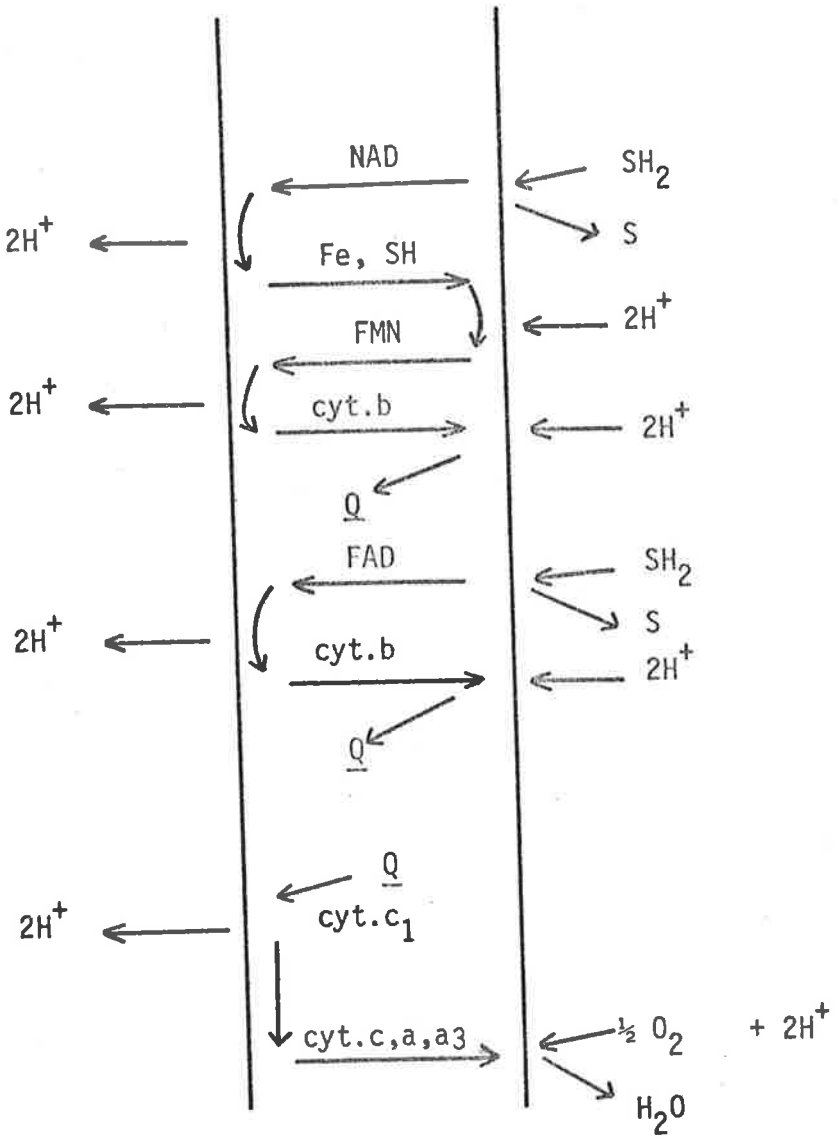


Figure 1.3 Proposed sequence of respiratory chain components (Mitchell, 1966).

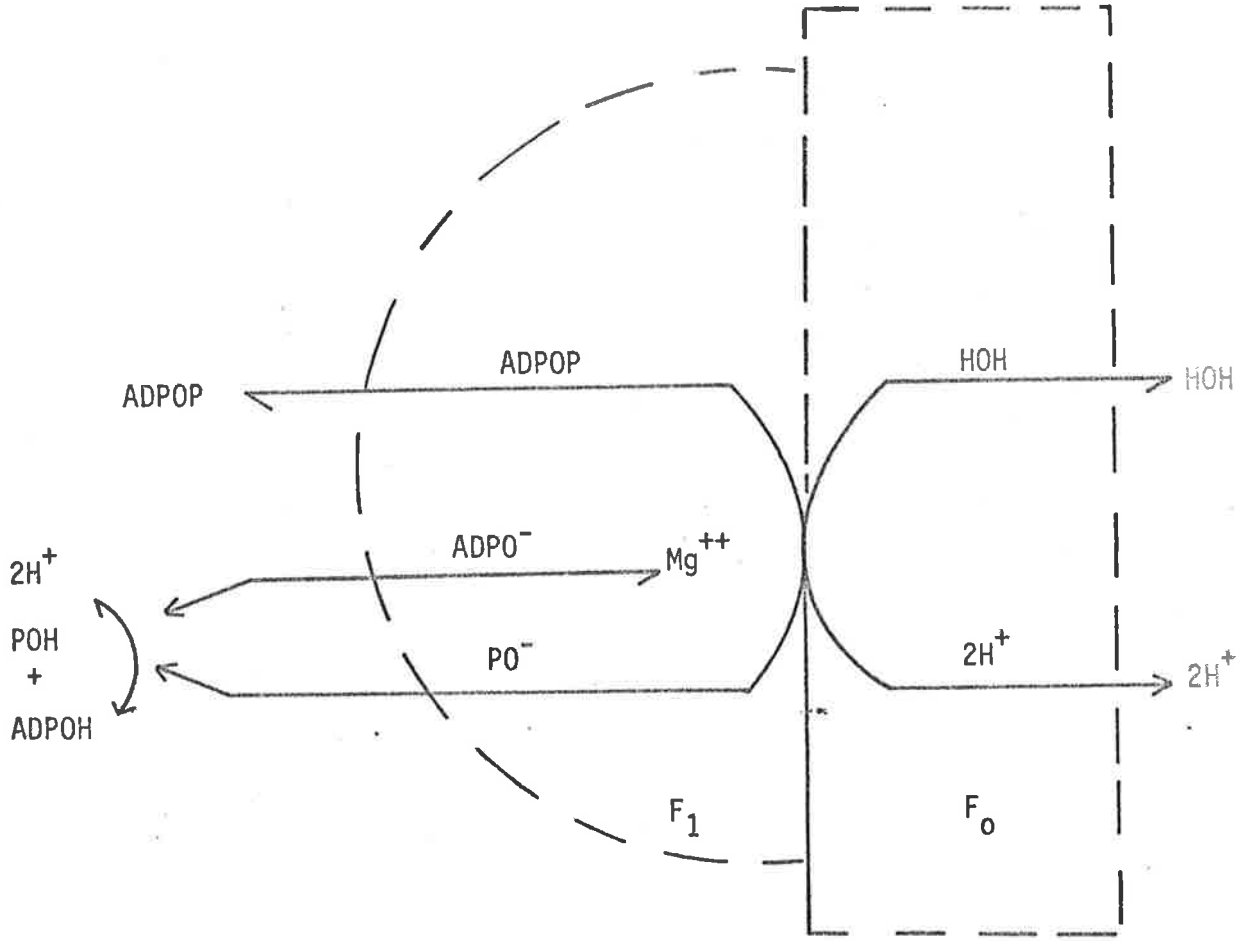


Figure 1.4 Proposed scheme of reversible ATP'ase (Mitchell, 1974)

(see text for explanation of terms)

Figure 1.5 The Aspartate - Malate cycle of rat liver

I = dicarboxylate carrier.
II = glutamate carrier.
III = Aspartate carrier.
IV = oxoglutarate carrier.
1 = malate dehydrogenase.
2 = aspartate amino-transferase.

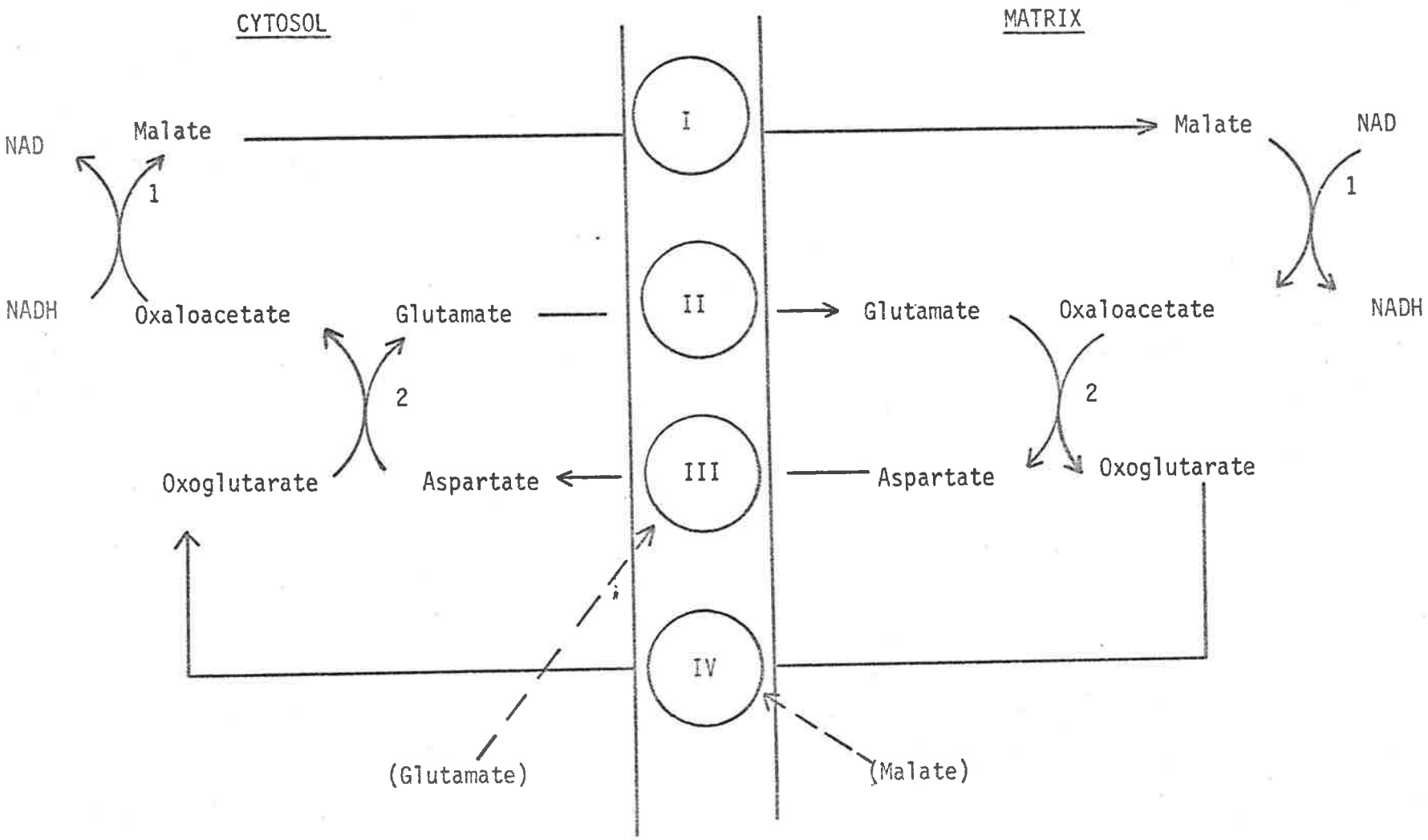


Figure 1.5. The aspartate-malate cycle of rat liver. (Chappell, 1969)

CHAPTER II *MATERIALS AND METHODS*

1. *Materials*

All plant tissues were purchased locally and used when fresh. All reagents were analytical grade. Bovine serum albumen was obtained from the Commonwealth Serum Laboratories (Melbourne, Australia) and enzymes from Boehringer and Soehne (Mannheim, Germany). Antimycin A was obtained from Nutritional Biochemical (Cleveland, Ohio), pCMB from British Drug Houses Ltd. (Poole, U.K.) and amytal from Eli Lilly and Co. Ltd. (Basingstoke, England). 2 - n - butylmalonate was prepared by the Organic Chemistry Department, Adelaide University (Adelaide, South Australia). Other biochemicals used were obtained from Sigma Chemical Company (St. Louis, U.S.A.), and Calbiochem. Inc. (San Diego, California).

2. *Preparation of Mitochondria*

(a) *Cauliflower (Brassica oleracea* L.)

Young florets (200g) were disrupted in 200 ml of a medium containing 0.3 M sucrose, 0.5% (W/V) BSA, 50 mM tris - HC1 buffer (pH 7.2), 15 mM EDTA and 20 mM TES buffer (pH 7.2), using either a Moulinex liquidizer or a Waring Blender (for about 30 sec).

The homogenate was strained through a double layer of 'miracloth' and centrifuged at 1500g for 10 min. in a Sorvall RC2 refrigerated centrifuge (rotor no. SS-34). The supernatant was decanted and centrifuged at 12000g for 15 min to pellet the mitochondria. The mitochondrial pellet was washed (sometimes twice) by resuspending in 0.3 M sucrose and recentrifuging at 12000g for 15 min. The final precipitate was resuspended in approximately 3 ml of 0.3 M sucrose and 0.5% BSA. Only the firm pellet was resuspended; if any semi-solid material was present it was decanted. All operations were performed at about 2^{o}C, using chilled solutions and apparatus.

(b) *Turnip (Brassica rapa* L.)

Turnip tubers were peeled, cut into sections and chilled. The tissue (400 - 1000g) was homogenised, using a Braun juice extractor, into a medium of 0.3 M sucrose (40 - 100 ml) containing 8 mM EDTA, 100 mM TES buffer (pH 7.2), 30 mM Tris, 1% (W/V) BSA and 0.4% (W/V) cysteine. The homogenate was strained through 'miracloth' and centrifuged at 1500g for 5 min. The mitochondria were pelleted, washed and resuspended as described for cauliflower. The volume of the final suspension was adjusted to give a protein concentration of approximately 10 mg/ml.

(c) *Red Beet (Beta vulgaris L.)*

The method of Millard et al (1965) was used. Chilled beetroot tissue (400g) was disrupted in a Braun juice extractor into a medium of 0.4 M sucrose (60 ml) containing 15 mM EDTA, 25 mM TES buffer (pH 7.2) and 30 mM Tris. The homogenate was centrifuged and the mitochondria washed as described above, with the final pellet resuspended in 0.4 M sucrose.

(d) *Maize root mitochondria (Zea mays L.)*

One hundred grams of roots were harvested from one to two week old plants growing in vermiculite. The tissue was chilled and then disrupted in a medium (200 ml) of 0.4 M sucrose, 50 mM TES buffer (pH 7.2), 20 mM glutamate, 5 mM EDTA, 5 mM cysteine and 0.5% (W/V) BSA, using a 'Waring' blendor. The final pH of the homogenate was adjusted to 7.7 using KOH, and the homogenate was strained through 'miracloth' and centrifuged at 1500g for 8 min. The supernant was decanted and the pellet washed and resuspended as described above, using 0.4 M sucrose.

3. *Preparation of Microsomes*

Microsomes were prepared by the method of Rungie and Wiskich (1972a). The supernatant obtained after mitochondria had been precipated was centrifuged at 30,000g for 20 min. The supernant

thus obtained was recentrifuged at 150,000g for 45 min. in a Beckman Model L-2 centrifuge (Ti50 rotor), to sediment the microsomes. The microsomal pellet was washed by resuspending in 0.4 M sucrose and recentrifuging at 150,000g for 45 min. The final pellet was resuspended in 5 - 10 ml 0.4 M sucrose. All apparatus was pre-chilled and all operations were performed at about 2°C.

4. *Fractionation of Mitochondria*

Mitochondria were disrupted by incubating them in digitonin solutions. A 1% 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 the desired digitonin-protein ratio (usually 3 : 10 was used). 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 8,500g for 20 min. to yield a solid precipitate which was washed by resuspending in 0.3 M sucrose and recentrifuged at 8,500g 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 37,000g for 30 min.

The resultant pellet was resuspended in 0.3 M sucrose, and the supernatant was centrifuged at 144,000g for 90 min. (Spinco Model L-2, 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 $0^{o}C$ until assayed.

5. *Solubilization of Mitochondrial Enzymes by Digitonin*

Isolated, washed mitochondria (60 - 100 mg protein) were divided into aliquots and incubated, for 30 min. at $0^{o}C$, with equal aliquots of digitonin solution. The concentration of each solution was adjusted to give the desired digitonin : protein ratio. The suspensions were then centrifuged for 30 min. at 30,000g in a Sorvall RC2 centrifuge (rotor no. SS-34), and the supernatants were assayed for the various enzyme activities. These activities were also assayed in the disrupted, but unfractionated, mitochondria.

6. *Isolation of the Outer Mitochondrial Membrane*

The mitochondrial suspension (approximately 10 mg protein/ml) was incubated with an equal volume of digitonin solution whose concentration had been adjusted to give a digitonin : protein ratio of 2 : 10 (for turnip) or 3 : 10 (for cauliflower), for 30 min. at $0^{o}C$ with continuous stirring. The digitonin : protein ratios were selected on the basis of the solubilization experiments described above (see Chapter 4 for details).

The suspension was then diluted by adding 4 volumes of 0.3 M sucrose and centrifuged at 30,000g for 30 min. (Sorval RC2 centrifuge, SS - 34 rotor) to remove inner membrane fragments and unbroken mitochondria. The supernatant was decanted, and centrifuged for 5 hours at 144,000g (Beckman Model L-2 centrifuge ; Ti50 rotor). The resultant light-brown pellet was resuspended in about 1 ml of 0.3 M sucrose (5 - 10 mg protein/ml).

7. *Slicing and Aging Root Storage Tissue*

Disks (1 mM thick) were cut, using a hand microtome, from cylinders (1 cm diameter) of beetroot tissue. The disks were aged in 10^{-4} M $CaSO_4$ aerated with a stream of filtered air at about $20^{o}C$. The aging solution was changed several times in the first hour and thereafter about three times per day. $CaSO_4$ was used in the aging solution to prevent loss of turgidity of the disks. The disks were rinsed at least three times with distilled water and chilled before homogenizing. Mitochondria were prepared from disks in the same way as described above except that a larger volume of isolating medium was used (100 ml per 200g disks) and the disks were homogenized with a 'polytron' (model PT 35) blendor.

8. *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, or Varian, Model G-14).

A standard reaction medium of 0.25 M sucrose containing 10 mM phosphate buffer (K salts, pH 7.2), 5 mM Mg Cl_2, 0.5 mM EDTA and 10 mM tris-HCl or TES buffer (pH 7.2) was used. Mitochondria (1 - 3 mg protein) and reagents were added via a plunger fitted to the vessel. The total volume of the assay mixture was 3.0 to 3.4 ml and was maintained at 25°C.

9. 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 (1956).

10. Enzyme Assays

(a) *Cytochrome c reductase*

Cytochrome *c* reductase activity was measured by following the reduction of cytochrome *c* at 550 nm in either a Beckman Acta CIII or Beckman Model DB spectrophotometer, at room temperature using cuvettes of 1-cm light path. The reaction mixture consisted of 0.1 ml mitochondrial suspension (or 0.05 ml outer membrane suspension), 0.05 mM cytochrome *c* and 10 mM KCN in 3.0 ml of the standard reaction medium described above. The reaction was initiated by adding 0.5 mM NAD(P)H, 15 mM malate or 15 mM succinate to the cuvette. A molar extinction coefficient (reduced minus oxidized) of 19.8×10^3 cm^{-1} (Morton, 1958) was used.

(b) *Fe CN and DCPIP Reductases*

The activity of Fe CN - and DCPIP - reductases was measured by following the reduction of Fe CN (at 420 nm) and DCPIP (at 600 nm) spectrophotometrically. The reaction mixture was the same as that used for cytochrome *c* reductase, except that cytochrome *c* was replaced by 1 mM K_3 Fe $(CN)_6$ or 0.05 mM DCPIP. A molar extinction coefficient of 15.5 x 10^3 cm^{-1} was used for DCPIP (Strittmatter and Velick, 1956) and 1.05 x 10^3 cm^{-1} for Fe CN (Morton and Sturtevant, 1964).

(c) *NAD(H) oxidation - reduction*

NADH oxidation, or NAD^+ reduction, was measured spectrophotometrically by following changes in absorption at 340 nm. The reaction medium for NAD^+ reduction contained 0.1 ml of mitochondrial suspension, 0.25 to 1.0 mM NAD^+ and 10 µM antimycin A in 3 ml of standard reaction medium. The assay mixture for NADH oxidation was the same except that antimycin A was omitted and 0.25 mM NADH replaced the NAD^+. In the latter case, the reaction was started by adding the NADH to the cuvette, but NAD^+ reduction was initiated by the addition of 10 mM malate or isocitrate. A molar extinction coefficient of 6.22 x 10^3 cm^{-1} was used (Pabst Laboratories, 1961).

(d) *Malate Dehydrogenase*

Malate dehydrogenase activity was measured spectrophotometrically by following the oxidation of NADH at 340 nm in the presence of oxaloacetate (Ochoa, 1955).

The reaction mixture contained 0.01 ml mitochondrial suspension (about 50 µg protein), 0.25 mM NADH and 10 µM antimytin A in 3 ml of standard reaction medium. The reaction was started by the addition of 0.5 mM oxaloacetate.

(e) *Acid phosphatase*

Acid phosphatase was assayed by the method of Linhardt and Walter (1963) by estimating p-nitrophenyl formed from p-nitrophenyl phosphate hydrolysis. The assay medium contained 5 mM p-nitrophenyl phosphosphate ; 50 mM sodium citrate (pH 4.1 - 5.0), MES (pH 6.5 - 7.0) or Tris-HCl (pH 7.5 - 8.0) buffer; and 0.05 ml outer membrane or 0.02 ml mitochondrial suspension, in a total volume of 1 ml. After 30 min. at 25°C, 2 ml of 0.5 N NaOH was added and the absorbance read at 405 nm against a control (enzyme omitted), using p-nitrophenyl standards.

(f) *Transhydrogenase*

Pyridine nucleotide transhydrogenase activity was measured by following the increase in absorbance of APAD(P) at 375 nm using a molar extinction coefficient of 5.2×10^3 cm^{-1} (Ragland and Hackett, 1964). The reaction mixture consisted of 0.1 ml mitochondrial suspension or 0.05 ml of outer membrane, 10 mM KCN, 0.2 mM NAD(P)H , 0.5 mM APAD(P) in 3 ml of standard reaction medium.

(g) *Monoamine Oxidase*

Monoamine oxidase activity was measured by following the formation of benzaldehyde (at 250 nm) from benzylamine hydrochloride. The reaction mixture consisted of 2.5 mM benzylamine hydrochloride, 0.1 ml mitochondria in 3 ml of standard reaction medium. Absorbance was read against a blank which did not contain benzylamine. A molar extinction coefficient of 0.012×10^3 cm^{-1} was used (Tabor et al, 1954).

(h) *Cytochrome oxidase*

Cytochrome oxidase activity was measured either by (1) following the oxidation of ferrocytochrome *c* spectrophotometrically at 550 nm in a medium containing 0.05 mM cytochrome *c* 5 μM antimycin A, 0.1 ml mitochondria (added to start the reaction) in 3 ml of standard reaction medium, or (2) polarographically using a Clarke oxygen electrode to measure oxygen uptake in the presence of 5 mM ascorbate and 1 mM TMPD.

(11) *Chemical assays*

(1) pCMB was routinely assayed by the method of Boyer (1954).

(2) Antimycin A was assayed by the method of Strong et al (1960).

(3) Protein was estimated by the method of Lowry et al (1951) with the Cu SO_4 solution in 1% citrate (rather than tartrate). Standards were run with BSA (fraction V).

(12) *Mitochondrial Swelling*

Swelling was measured as a decrease in absorbance at 520 nm upon addition of mitochondria (about 1 mg protein) to the reaction mixture, using a Beckman Acta CIII spectrophotometer.

(13) *Absorption Spectra*

Absorption spectra (of outer membrane and microsomes) were measured with a Shimadzu recording spectrophotometer at $77^{o}K$ using cuvettes with a 2 mm light path. Samples were reduced with sodium dithionite and read against oxidized samples for difference spectra.

CHAPTER III

NAD(P)H OXIDATION BY ISOLATED MITOCHONDRIA

The aim of this section is to describe the pathways of exogenous NADH oxidation by mitochondria isolated from turnip tubers and cauliflower buds, and to compare these pathways to the oxidation of intramitochondrial NADH (generated by the oxidation of malate). The effect of various inhibitors, particularly pCMB and dicumarol, is described and discussed with reference to previously published data, and interactions between the outer membrane and inner membrane systems are considered. Studies on the effect of digitonin on NADH and succinate oxidation led to an investigation of the penetration of the outer membrane by cytochrome *c*. After these studies had been completed, a report on the effect of mitochondrial swelling on cytochrome *c* penetration appeared in the literature (Douce et al, 1972), and the results presented here are considered in the light of this report. Finally, an investigation into the oxidation of exogenous NADPH was undertaken and comparisons made to the oxidation of NADH.

RESULTS

1. *Respiratory control and electron transport inhibitors*

Malate and NADH were oxidized rapidly and with good respiratory control (Fig. 3.1). 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 3.2 shows the effect of

of varying rotenone concentration on malate and NADH oxidation by cauliflower bud mitochondria. While malate oxidation was inhibited up to 65%, NADH oxidation was largely unaffected in intact mitochondria. However, when the mitochondria were disrupted by sonication NADH oxidation became sensitive to rotenone (Fig. 3.3). Both malate and NADH oxidation were inhibited completely by low concentrations (0.1 μM) of antimycin A (Fig. 3.4). These results support those of others (Douce et al, 1972; Ikuma and Bonner, 1967; Miller and Koeppe, 1971; Palmer and Passam, 1970; Wilson and Hanson, 1969; Wiskich and Bonner, 1963), 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 on the substrate side of cytochrome *b*. Palmer and Passam (1970) and Coleman and Palmer (1971) have suggested that a specific NADH dehydrogenase, situated on the outside of the inner membrane, is responsible for the oxidation of exogenous NADH. This suggestion has been tentatively confirmed by Douce et al (1973a). The inhibition of NADH oxidation by antimycin A was partially relieved by the addition of large amounts of cytochrome *c* to the medium (Fig. 3.5); cytochrome *c* alone had no such affect on the inhibited malate oxidation (Fig. 3.5). This effect was also observed by Wilson and Hanson (1969), 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 cytochrome *c* was only slight.

2. *Cytochrome c and Ferricyanide reduction*

Table 3.1 shows the rate of cytochrome *c* reduction with succinate, malate and NADH as substrates, and the effect of rotenone and antimycin A. These results reflect those obtained with the oxygen electrode, although cytochrome *c* reduction by malate and succinate was much slower than that by NADH. The small inhibition of NADH-cytochrome *c* reductase by antimycin A again suggests an alternative, inhibitor-insensitive pathway of NADH oxidation, probably involving an electron transport chain on the outer membrane (Douce et al, 1973a; see chap. IV) similar to that of rat liver mitochondria (Sottocasa et al, 1967). Such a pathway would account for the difference in rates of cytochrome *c* reduction by malate and NADH, since the outer membrane appears to be largely impermeable to the high molecular weight cytochrome *c* (Douce et al, 1972; Wojtczak and Sottocase, 1972; Wojtczak and Zaluska, 1969). 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 cytochrome *c* reductase, and antimycin A-sensitive NADH-cytochrome *c* reductase activities, but did not affect the antimycin A-insensitive cytochrome *c* reduction by NADH (Table 3.1). These results are in agreement with those of Douce et al (1972). It is unlikely that the antimycin A-insensitive reductase represents an alternative oxidase activity (Storey, 1969) of the inner membrane, since succinate cytochrome *c* reductase was completely inhibited by antimycin A (Table 3.1).

Further support for the postulate of the outer membrane limiting cytochrome *c* reductase activities is provided by studies with Fe CN as electron acceptor. In contrast to succinate-cytochrome *c* reductase, succinate - Fe CN reductase activity decreased slightly upon incubation with digitonin (Table 3.2). Antimycin A-sensitive NADH - Fe CN reductase activity decreased even more markedly, while the antimycin-insensitive activity increased (Table 3.2). Similar results with mung bean mitochondria have been reported (Douce et al, 1973a) and indicate that Fe CN readily penetrates the outer membrane; hence little change in succinate - Fe CN reductase activity occurred upon rupture of the outer membrane. Douce et al (1973a) suggested that the decrease in antimycin-sensitive, and the increase in antimycin-insensitive, Fe CN reductases, upon disruption of the mitochondria, were due to the flavoprotein on the outside of the inner membrane becoming more accessible to Fe CN. In other words, Fe CN accepts electrons directly from the NADH-dehydrogenase, rather than the cytochrome *c* position of the respiratory chain, in digitonin-treated mitochondria. This implies that in intact mitochondria the respiratory-linked external NADH-dehydrogenase is not accessible to Fe CN.

3. *Cytochrome oxidase activity*

The effect of incubating turnip mitochondria with low concentrations of digitonin (0.2 mg/mg. protein) increased cytochrome *c* oxidase, and succinate-cytochrome *c* reductase, several-fold (Table 3.3), again indicating a permeability barrier

(of the outer membrane) to added cytochrome *c*, preventing it from reacting with inner membrane enzymes. Similar results were obtained by Wojtczak and Zaluska (1969) with rat liver mitochondria. The higher rates of cytochrome *c* oxidation, compared with cytochrome *c* reduction (Table 3.3), probably reflects the high activity of cytochrome oxidase in the broken mitochondria in the preparations used.

On the other hand, oxygen uptake with ascorbate plus TMPD as substrate was dramatically inhibited by digitonin (Table 3.3). When cytochrome *c* was added to the digitonin treated mitochondira, however, oxygen uptake was restored to its rate in untreated mitochondria (Fig. 3.6). These results imply that endogenous cytochrome *c* was released from the inner membrane by digitonin; by adding back cytochrome *c*, respiratory chain activity was restored. This explanation may account for the decrease in antimycin A-sensitive Fe CN reductase activities (at least that with succinate as substrate; Table 3.2). The results shown in Fig. 3.7 support this interpretation; cauliflower mitochondria incubated with digitonin (0.2 mg/mg. protein) would not oxidize succinate unless cytochrome *c* was added, although succinate oxidation by untreated mitochondria was not affected by cytochrome *c*, suggesting that endogenous cytochrome *c* is removed by digitonin. If correct, this interpretation means that TMPD can only transfer reducing equivalents to the respiratory chain via endogenous cytochrome *c*, as found with animal mitochondria (Jacobs et al, 1965).

4. *Effect of pCMB*

The respiratory-linked external NADH oxidase and the outer membrane antimycin A-insensitive NADH-cytochrome *c* reductase showed similar patterns of inhibition by pCMB (Figs. 3.8 and 3.9). Both systems were severely inhibited (greater than 90%) by 2×10^{-5} M pCMB when it was added to the mitochondria before NADH. When the mitochondria were preincubated with NADH, the dehydrogenases were largely protected from pCMB inhibition, at least initially (Fig. 3.8 and 3.9). NADH dehydrogenase protection has also been observed in both animal microsomes (Stritmatter, 1965) and plant microsomes (Rungie and Wiskich, 1972a). The effect has been discussed by these authors; NADH binds to its dehydrogenase via a sulphydryl group in such a way that bound NADH prevents the binding of pCMB. When NADH and pCMB are added to the mitochondria together they compete for sites on the dehydrogenase, resulting in an intermediate inhibition (Figs. 3.8 and 3.9). These results differ from observations made with mammalian mitochondria; the respiratory chain NADH dehydrogenase of rat liver mitochondria was only inhibited significantly by pCMB when pretreated with NADH or substrate (Tyler et al, 1965) and was not inhibited to the same extent as the outer membrane NADH-cytochrome *c* reductase (Ragan and Garland, 1969). The rotenone-insensitive NADH-dehydrogenase from pig liver mitochondria was severely inhibited by pCMB whether the enzyme was preincubated with NADH or not (Mahler et al, 1958) although a similar enzyme isolated from sarcosomes was only slightly sensitive to pCMB (Avi-Dor et al, 1958). Sottocasa et al (1967) found that the outer membrane electron transport chain of rat liver mitochondria was much less sensitive to pCMB than was its micro-

somal counterpart.

Malate oxidation was also severely inhibited by pCMB but substrate protection was not observed (Table 3.4). Since malate dehydrogenase was not affected by pCMB (Table 3.4) the site of pCMB inhibition could be either the internal NADH dehydrogenase or the phosphate transporter on the inner membrane. Swelling of plant mitochondria in ammonium phosphate is inhibited by mercurials (Phillips and Williams, 1973; Wiskich, 1974). Inhibition of phosphate transport into the mitochondria would prevent malate-phosphate exchange across the inner membrane and hence inhibit malate oxidation.

However, pCMB inhibited malate both in the presence and absence of added inorganic phosphate (Fig. 3.10). The phosphate-stimulated and the state 3 rates of oxygen consumption were completely inhibited by 10 μM pCMB (Fig. 3.10 A and B). In untreated mitochondria, there was no malate oxidation unless phosphate was added (Fig. 3.10A), but when the mitochondria were swollen in water they became leaky to malate and oxidized it readily in the absence of added phosphate (Fig. 3.10 C). This oxygen uptake by swollen mitochondria was also inhibited completely by pCMB (Fig. 3.10 C).

That is, pCMB appears to inhibit both malate penetration (by preventing phosphate transport) and the internal NADH dehydrogenase.

5. *Effect of dicoumarol*

Malate, succinate and NADH oxidation by cauliflower and turnip mitochondria were inhibited by dicoumarol, but NADH oxidation was inhibited much more severely by low concentrations (5 - 10 μM) than were succinate and malate oxidation (Fig. 3.11 and Table 3.5). On the other hand, the oxidation of ascorbate plus TMPD, and malate dehydrogenase activity, were not inhibited by dicoumarol (Table 3.5) suggesting that it acts on the flavoproteins of the respiratory chain. Fifty percent inhibition of malate and succinate oxidation was only observed when the dicoumarol concentration was greater than 25 μM (Fig. 3.11); lower concentrations uncoupled malate oxidation (Fig. 3.12), when added during state 4 respiration. Dicoumarol inhibition of succinate and malate dehydrogenases has been observed in rat liver mitochondria (van Dam, 1967; Wilson and Merz, 1969), but at higher concentrations than those used here.

The high sensitivity of NADH oxidation to low concentrations of dicoumarol (5 μM caused 73% inhibition; Fig. 3.11) suggests a DT-diaphorase-like activity. DT-diaphorases of mammalian tissues are almost completely sensitive to 1 μM dicoumarol (Ernster et al, 1962). The extent of dicoumarol inhibition did not change much when mitochondrial concentration was increased 4-fold (Table 3.6); the dicoumarol sensitivity of rat liver DT-diaphorase was also independent of enzyme concentration (Ernster et al, 1962).

6. *Effect of Ca Cl_2 on NADH oxidation*

In contrast to the results of Coleman and Palmer (1971), cauliflower mitochondria isolated and assayed in the presence of EGTA readily oxidized exogenous NADH (Table 3.7). Although NADH oxidation in the presence of EGTA was not completely dependent on Ca Cl_2, the addition of low (1 mM) Ca Cl_2 to the medium markedly stimulated NADH oxidation (Table 3.7). Malate oxidation was only slightly stimulated by the same concentration of Ca Cl_2 (Table 3.7). Stimulation of NADH oxidation by divalent cations has been observed by several other workers and discussed at some length (Hanson et al, 1965; Koeppe and Miller, 1971; Miller et al, 1970).

7. *NADPH oxidation*

Exogenous NADPH was oxidized by cauliflower bud mitochondria, but oxygen uptake was very slow compared to NADH oxidation (Fig. 3.13). This oxidation was stimulated by low concentrations of Ca Cl_2 and was coupled to phosphorylation, although respiratory control was poor (Fig. 3.13). ADP/O values of less than 2 (1.2 - 1.6) were obtained suggesting the first phosphorylation site was bypassed. NADPH oxidation was insensitive to rotenone but strongly inhibited by antimycin A (Fig. 3.13). The addition of cytochrome *c* did not relieve this inhibition although subsequent addition of NADH stimulated oxygen uptake slightly (Fig. 3.13). Similar results to these were obtained by Koeppe and Miller (1972) although faster rates of oxygen consumption were observed.

It should be noted that cauliflower bud mitochondria did not consistently oxidize NADPH; significant rates were only observed with cauliflowers grown during the winter months. Significant oxidation rates were rarely obtained with mitochondria isolated from turnip tubers. Therefore, these results must remain tentative. It is unlikely that the NADPH solutions used had been contaminated with NADH, since small amounts of NADH gave a brief burst of rapid oxygen uptake.

DISCUSSION

1. *NADH oxidation*

The results presented in this chapter provide further evidence of alternative pathways of NADH oxidation in isolated plant mitochondria. These pathways are represented in Fig. 3.14. The antimycin A-insensitive oxidation of NADH (measured by cytochrome *c* reduction) occurs on the outer membrane of the mitochondria as shown by fractionation studies. (Tables 3.1 and 3.2). Douce et al (1973a) and Moreau and Lance (1972) have shown this pathway to be similar to that of animal mitochondria (Sottocasa et al, 1967), consisting of a flavoprotein and *b*-type cytochrome (see also chapter IV).

Another pathway of NADH oxidation occurs via a dehydrogenase apparently located on the outside of the inner membrane and coupled to two sites of phosphorylation.

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 cytochrome *b* and bypass phosphorylation site I. Although this dehydrogenase remains to be identified, it could involve one of the flavoproteins detected by Storey (1969; 1970).

Apparently these outer and inner membrane pathways can interact as suggested by the relief of antimycin A-inhibited NADH oxidation by added cytochrome *c* (Fig. 3.5). Presumably, cytochrome *c* reduced by the outer pathway moves to the inner chain; the recovery of NADH oxidation is small because cytochrome *c* has difficulty in penetrating the outer membrane.

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 (Chapter VI).

The external and internal NADH dehydrogenases are also distinguished by their different responses to the inhibitor dicoumarol; only the flavoprotein on the outer surface of the inner membrane is inhibited significantly by low concentrations of dicoumarol (i.e. less than 10 μM). The internal dehydrogenase, and that on the outer membrane (see chapter IV), are virtually uninhibited by these concentrations.

2. *Site I energy conservation and rotenone inhibition*

The lack of site I phosphorylation during exogenous NADH oxidation can best be explained in terms of the chemiosmotic hypothesis (Mitchell, 1961). If NADH is oxidized by a dehydrogenase on the outside of the inner membrane and reducing equivalents are transferred directly to cytochrome *b*, then the site I proton-translocating loop would be bypassed (Fig. 3.15); hence external NADH oxidation is coupled to only two sites of phosphorylation.

Yeast mitochondria were found to lose their sensitivity to rotenone (and piericidin A) and their first phosphorylation site when grown under iron and sulphur limited conditions (Light and Garland, 1971); this was shown to be associated with a loss of non-haem iron from these mitochondria (Ragan and Garland, 1971). Ragan and Garland (1971) suggested that iron-sulphur proteins were essential for piericidin A inhibition, and site I energy conservation, and constituted part of the internal NADH dehydrogenase. The fact that oxidation of exogenous NADH by plant mitochondria is insensitive to rotenone (and coupled to only two phosphorylation sites) suggests that the external NADH dehydrogenase lacks these non-haem iron components.

3. *The effect of digitonin on mitochondrial membranes: cytochrome c penetration*

The results presented in tables 3.1, 3.2 and 3.3 suggest that the outer membrane forms a permeability barrier to cytochrome *c*, preventing it from reacting with inner membrane enzymes.

Incubation with low concentrations of digitonin apparently removed this barrier. The same conclusions have been made from similar studies with mung bean (Douce et al, 1973a) and rat liver mitochondria (Wojtczak and Zaluska, 1969). This interpretation, however, has been challenged recently by Palmer and Kirk (1974). These authors claimed that hypotonic swelling of mitochondria induced conformational changes in the inner membrane rather than rupture of the outer membrane. That is, lack of succinate-cytochrome *c* reductase activity in untreated mitochondria was not due to impermeability of the outer membrane to cytochrome *c*, but due to the inability of added cytochrome *c* to interact (accept electrons from) with the inner membrane. In support of this proposal Palmer and Kirk (1974) showed that, even in untreated mitochondria, antimycin A-sensitive NADH-cytochrome *c* reductase activity was higher than that of succinate-cytochrome *c* reductase. In addition, succinate-cytochrome *c* reductase showed the same pattern of activation (by ATP) in intact and in swollen mitochondria. The antimycin-sensitive NADH-cytochrome *c* reductase activity of intact cauliflower mitochondria was also faster than succinate-cytochrome *c* reductase activity (Table 3.1). Palmer and Kirk (1974) interpreted their results to show that the outer membrane did not limit cytochrome *c* reduction. Obviously, these authors considered their preparations to be completely intact. However, if the small amount of succinate-cytochrome *c* reductase activity observed was due to the presence of some damaged or broken mitochondria in the preparations used, then the different antimycin-sensitive rates with succinate and NADH probably reflect different activities of the two enzymes in the broken mitochondria.

In support of this interpretation, oxygen uptake with NADH as substrate was faster than that with succinate, (Palmer and Kirk, 1974), and the antimycin-sensitive NADH-cytochrome *c* reductase activity of digitonin-treated mitochondria was greater than the succinate-cytochrome *c* reductase (Table 3.1). Similarly, one might expect succinate oxidation by damaged mitochondria to require activation by ATP.(Since Oestreicher et al (1973) observed activation of succinate dehydrogenase in frozen and sonicated cauliflower mitochondria, by ATP and ADP.)

When viewed in this manner, the results of Palmer and Kirk (1974) are not inconsistent with the idea that the outer membrane of isolated plant mitochondria is impermeable to the high molecular weight cytochrome *c*. However, the effect of digitonin on ascorbate plus TMPD oxidation (Fig. 3.6) shows that disruption of mitochondria may lead to removal of endogenous cytochrome *c* from the inner membrane. Addition of cytochrome to the medium reconstitutes electron transfer in digitonin-treated mitochondria and restored ascorbate plus TMPD, and succinate, oxidation (Figs. 3.6 and 3.7). Reconstitution by exogenous cytochrome *c* has been observed in rat liver mitochondria (Jacobs and Sanadai, 1960).

4. NADPH oxidation

Respiratory linked oxidation of exogenous NADPH showed a similar response to rotenone and antimycin A, and yielded similar ADP/O values, as NADH oxidation and may involve the same dehydrogenase.

However, Koeppe and Miller (1972) suggested that a separate enzyme was involved in corn mitochondria, on the basis of different effects of amytal on NADH and NADPH oxidation. The fact that added cytochrome *c* did not relieve antimycin A inhibition (Fig. 3.13) suggests that NADPH is not oxidized by the outer membrane electron transport chain.

Table 3.1. The effect of inhibitors and digitonin on cytochrome c reductase activity in isolated cauliflower mitochondria

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

Substrate	Untreated Mitochondria			Mitochondria pre-incubated with digitonin[1]		
	Control	+Rotenone (7 μM)	+Antimycin A (5 μM)	Control	+Rotenone (7 μM)	+Antimycin A (5 μM)
	nmoles cytochrome 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] 0.1mg/mg protein, for 30 min. at 0°C.

Table 3.2 Effect of digitonin on NADH and succinate - Fe CN reductase activity of turnip mitochondria

Fe CN reductase was assayed as described in Materials and Methods. Substrates used were 0.5 mM NADH and 10 mM succinate.

Enzyme	Untreated Mitochondria	*digitonin treated mitochondria
	nmoles Fe CN reduced/min. mg protein	
antimycin-sensitive NADH - Fe CN reductase	1020	394
antimycin-insensitive NADH - Fe CN reductase	2508	3135
succinate - Fe CN reductase	823	735

* 0.2 mg/mg protein, for 30 min. at 0°C.

Table 3.3 Effect of digitonin on cytochrome c oxidase activity and ascorbate plus TMPD oxidation by turnip mitochondria.

Assay conditions are described in Materials and Methods. Substrates used were 10 mM succinate, 10 mM ascorbate plus 1 mM TMPD and 0.05 mM cytochrome *c*. Rates are expressed as nmoles O_2 consumed/min. mg (for Ascorbate + TMPD) and nmoles cytochrome *c* reduced or oxidized/min. mg protein.

Enzyme	Untreated mitochondria	*Digitonin treated mitochondria
succinate-cyt.*c* reductase	10	281
cyt. *c* oxidase	198	1304
Ascorbate + TMPD oxiation	422	48

* 0.2 mg/mg protein, for 30 min. at $0^{o}C$.

Table 3.4. Effect of pCMB on malate oxidation and Malate dehydrogenase activity in cauliflower mitochondria

Assay conditions are described in Materials and Methods. The final concentration of pCMB was 20 μM, and 3 mg (for malate oxidation) or 0.15 mg (for MDH assay) of mitochondrial protein were used.

	Malate oxidation	Malate dehydrogenase
	nmoles O_2/min. mg. protein	*nmoles NADH oxidized/ min. mg.*
Control	60	5.805
pCMB added before substrate	0	5.810
pCMB added after substrate	0	5.805

Table 3.5. Effect of dicoumarol on various activities of isolated cauliflower mitochondria.

NADH, malate and ascorbate plus TMPD oxidation were measured polarographically, and malate dehydrogenase spectrophotometrically, as described in Material and Methods. Substrates used were 0.5 mM NADH, 10 mM malate and 5 mM ascorbate plus 1 mM TMPD. Oxygen uptake rates shown are state 3 rates, and 2 mg protein was used.

Dicoumarol concentration	NADH oxidation	malate oxidation	asc. + TMPD oxidation	malate dehydrogenase
	nmoles O_2 / min. mg protein			*nmoles NADH/ min. mg.*
0	110	102	330	4.85
10 µM	22	76	-	-
50 µM	7.5	25	330	4.7
100µM	0	8	310	4.6

Table 3.6. Effect of mitochondrial concentration on dicoumarol inhibition of NADH oxidation

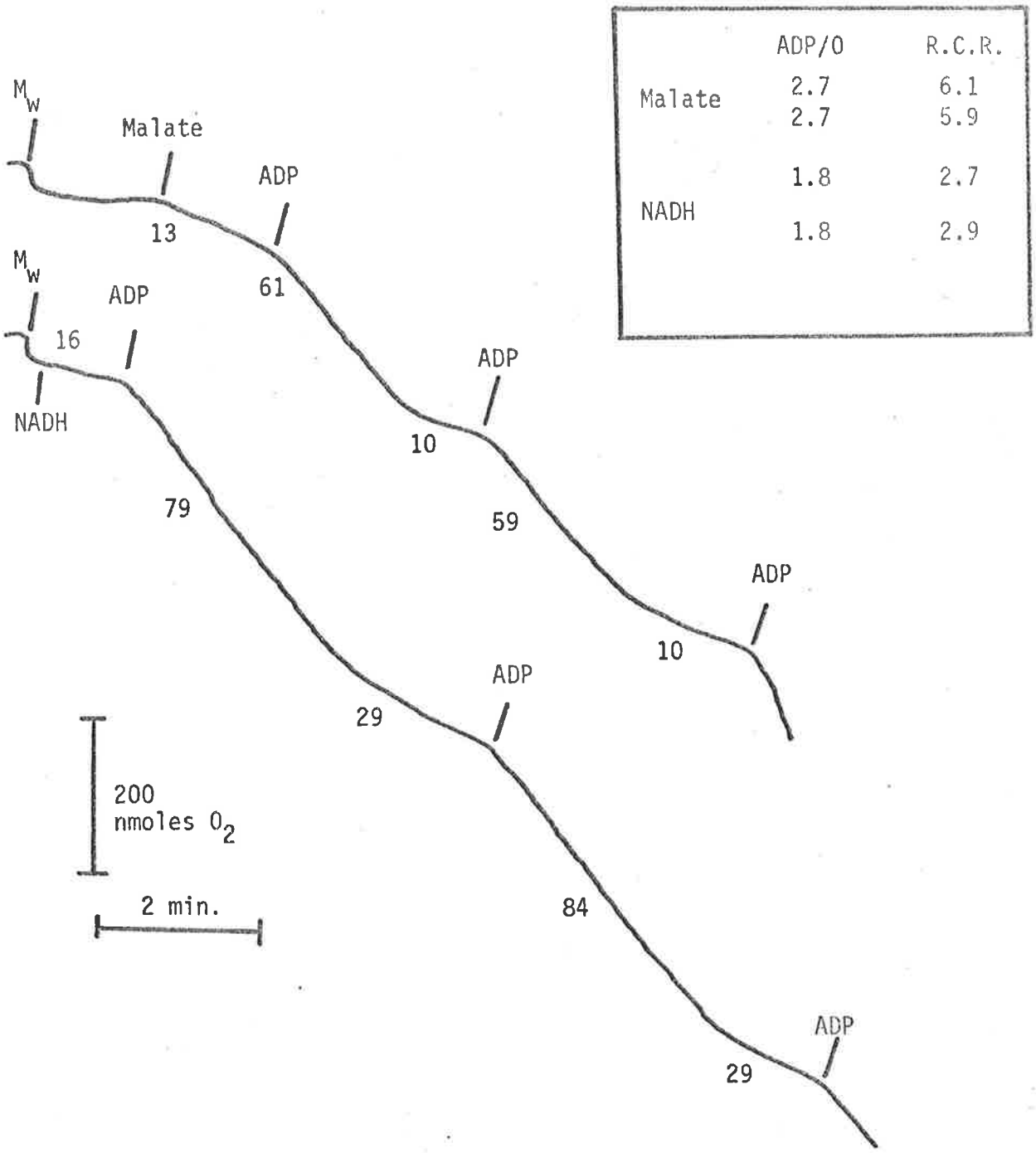
NADH oxidation was measured polarographically as described in Materials and Methods. Control (state 3) rate = 110 nmoles O_2/ min. mg protein.

mg protein	Percent inhibition of S_3 rate	
	25 μM dicoumarol	50 μM dicoumarol
0.75	97	100
1.5	89	92
3.0	85	91

Table 3.7. Effect of $CaCl_2$ on NADH and malate oxidation by cauliflower mitochondria

Mitochondria were isolated in the presence of 5 mM EGTA. Details of assays are given in Materials and Methods, except that 1 mM EGTA was included in the reaction medium. Substrates used were 1 mM NADH and 15 mM malate (plus 15 mM glutamate).

Substrate		State 2 rate	State 3 rate	RCR
		nmoles O_2 / min.mg protein		ratio
NADH	control	45	150	2.7
	+ 0.5 mM $CaCl_2$	135	240	2.5
malate	control	45	142	3.2
	+ 0.5 mM $CaCl_2$	60	180	3.3



	ADP/O	R.C.R.
Malate	2.7	6.1
	2.7	5.9
NADH	1.8	2.7
	1.8	2.9

Figure 3.1. Malate and NADH oxidation by cauliflower mitochondria.

Mitochondria (1.5mg protein) were added to 3 ml of standard reaction medium as described in Materials and Methods. Additions as indicated were 20 mM malate, 1 mM NADH and 0.29 mM ADP. Oxygen uptake is expressed as nmoles/min.mg.protein. Glutamate (15 mM) was added prior to the addition of malate. Mw = washed mitochondria.

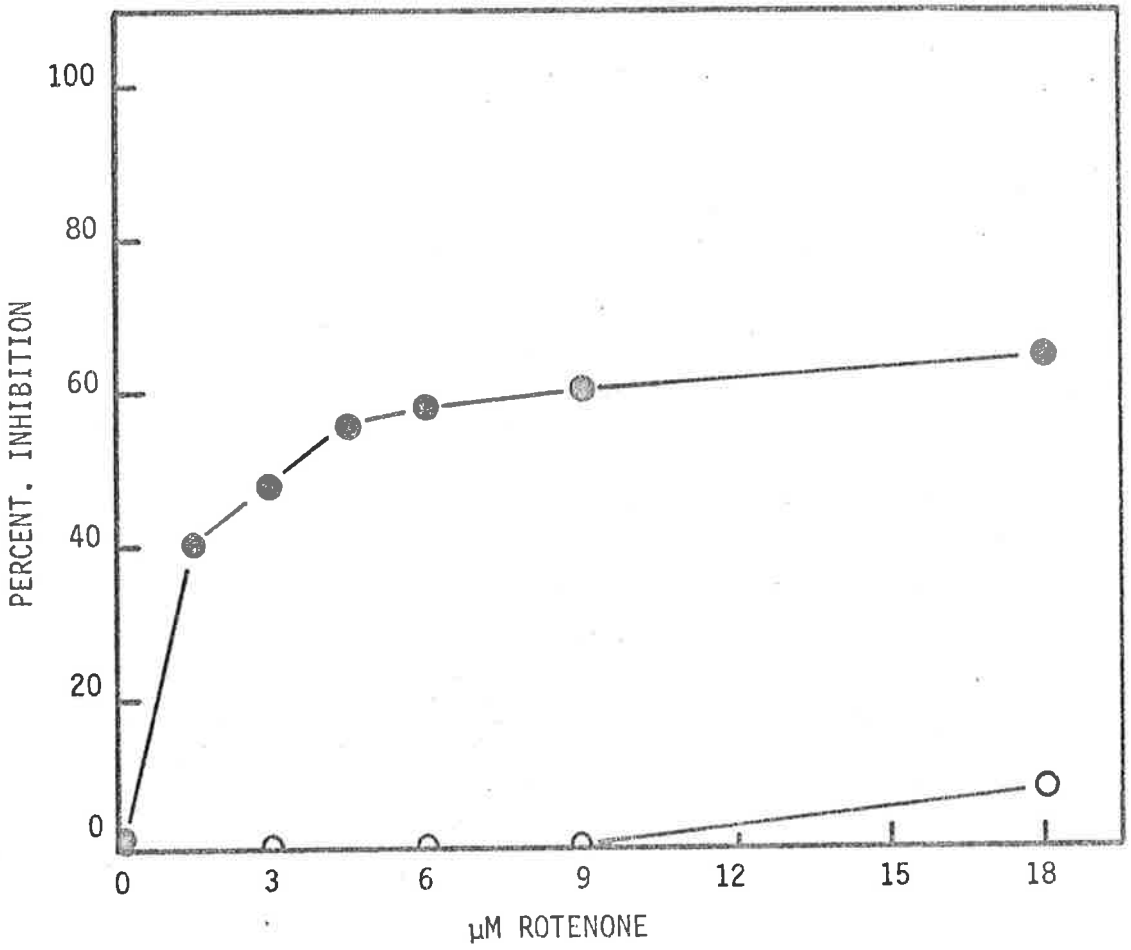


Figure 3.2. Effect of rotenone concentration on NADH and malate oxidation by cauliflower mitochondria.

O — O NADH oxidation; ● — ● malate oxidation. Assay conditions are described in Figure 3.1. Initial state 3 rates of oxygen uptake were 88 (malate) and 119 (NADH) nmoles O_2/min.mg protein.

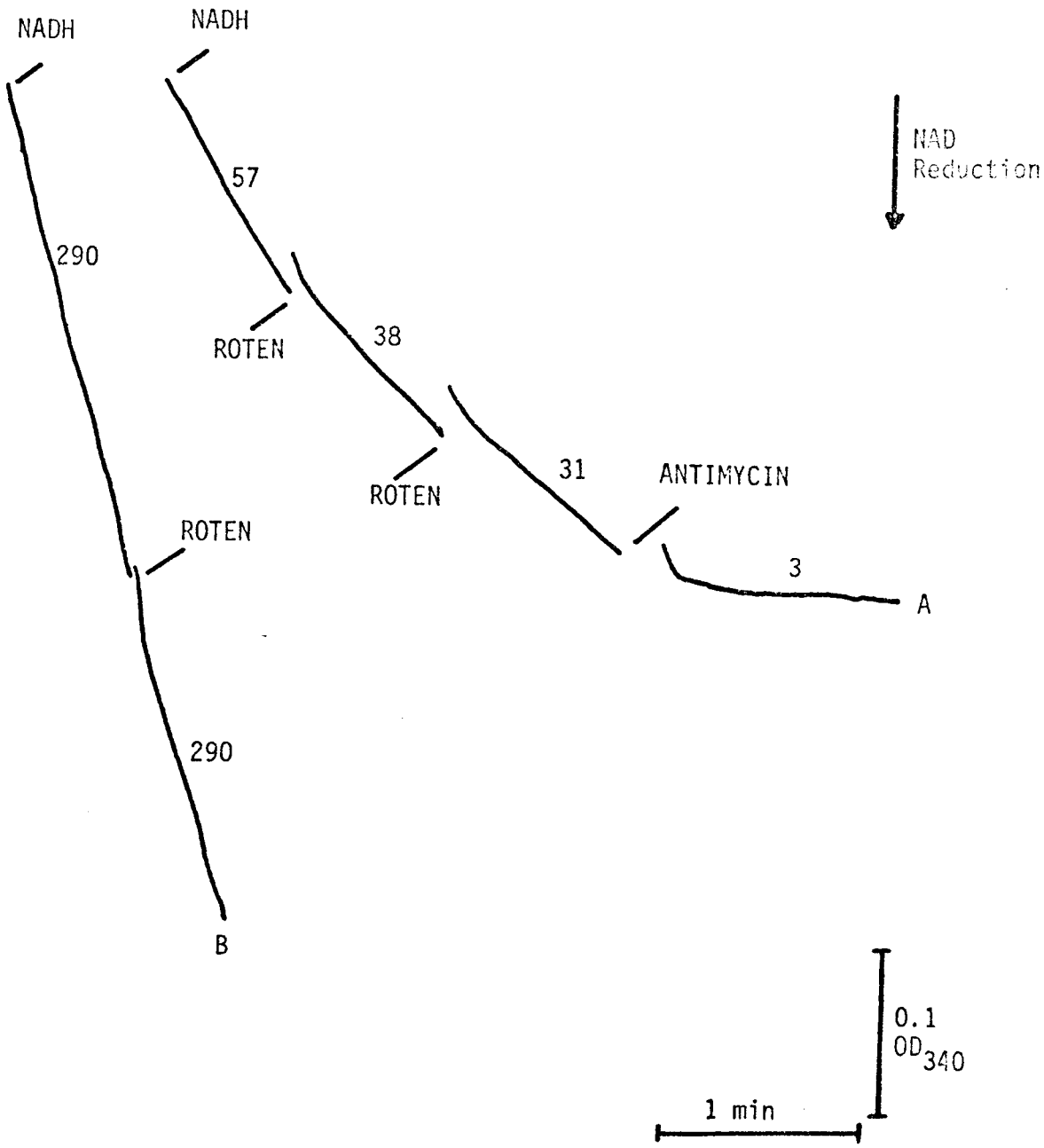


Figure 3.3. NADH oxidation by intact and sonicated cauliflower mitochondria.

A = sonic particles (1.7 mg protein); B = untreated mitochondria (0.85 mg protein). NADH oxidation was measured spectrophotometrically as described in Materials and Methods. Additions as indicated were 0.5 mM NADH, 15 µM rotenone and 5 µM antimycin A. Rates are expressed as nmoles NADH oxidized/min.mg. protein. ADP (0.5mM) was also included in the reaction medium.

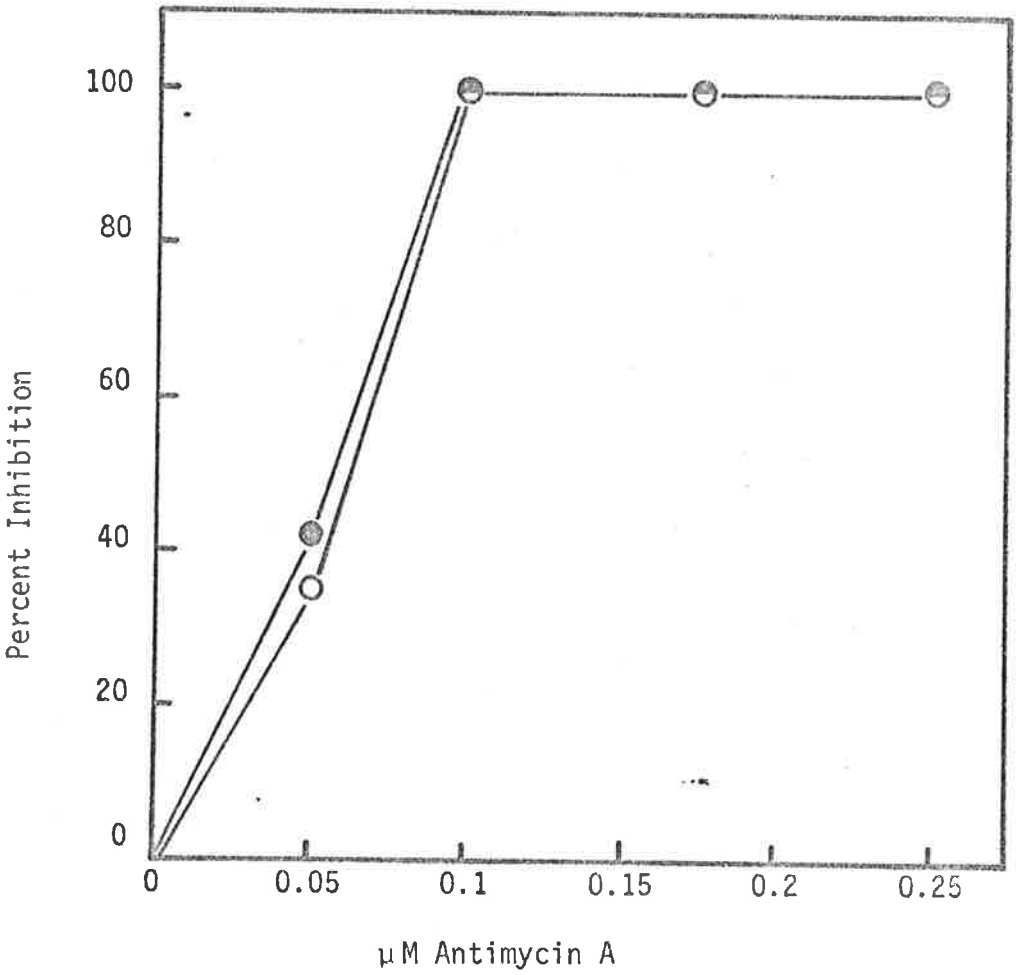
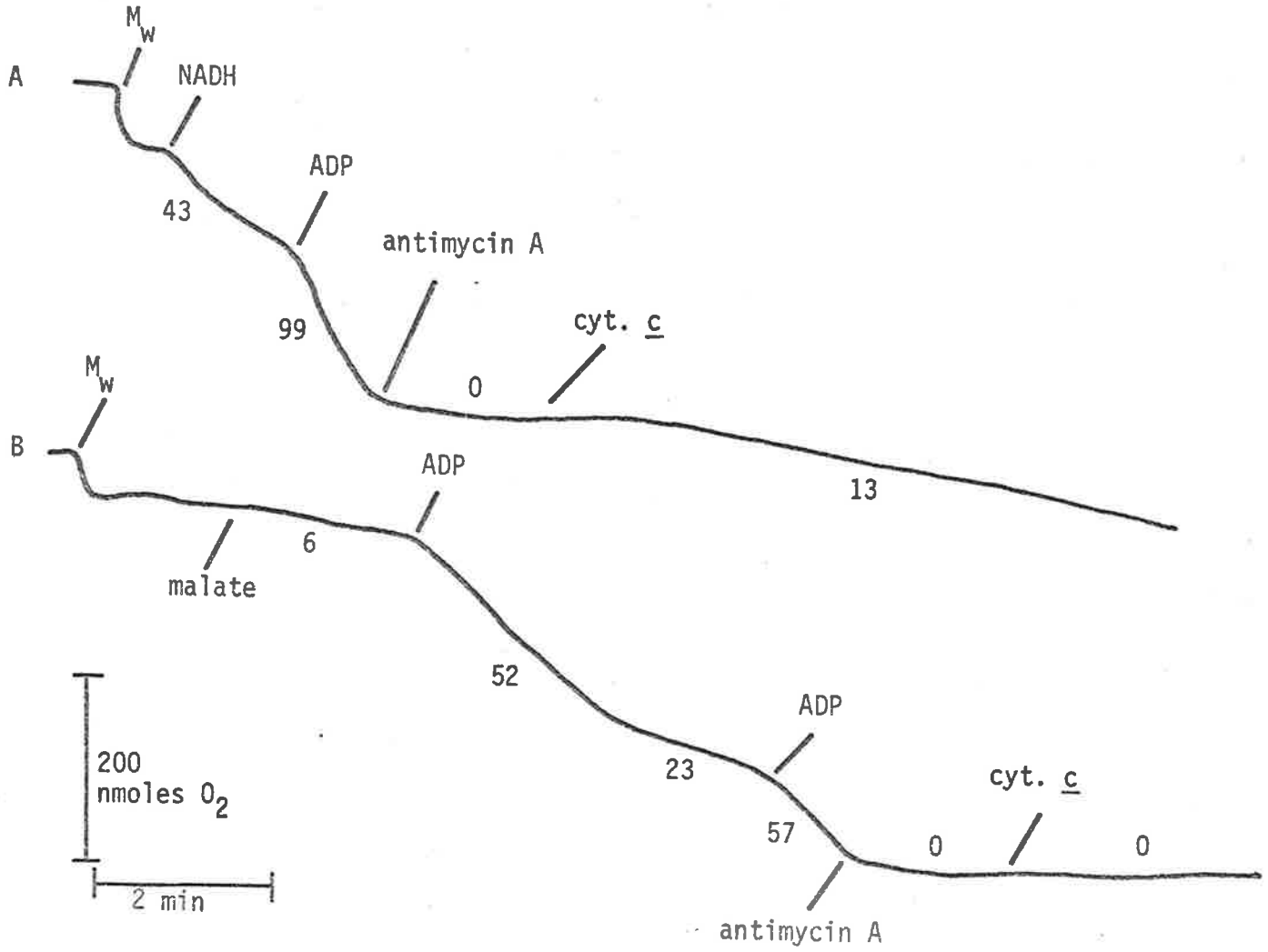


Figure 3.4 Effect of antimycin A concentration on NADH and malate oxidation by cauliflower mitochondria.

O — O NADH, ● — ● malate. Assay conditions and initial rates were the same as for Figure 3.2.

Figure 3.5 Effect of antimycin A and cytochrome c on malate and NADH oxidation by cauliflower bud mitochondria.

Assay conditions were the same as those in Figure 3.1, except that 1.4 mg protein was used in each experiment. Additions as indicated were, 20 mM malate, 1 mM NADH, 0.26 mM ADP, 5 µM antimycin A, 7 µM rotenone and 0.05 mM cytochrome c. Rates are expressed as nmoles O_2/min. mg. protein.


A
M_w
NADH
ADP
43
antimycin A
99
cyt. c
0
13
B
M_w
ADP
6
malate
52
ADP
23
cyt. c
57
0
0
antimycin A
200
nmoles O_2
2 min

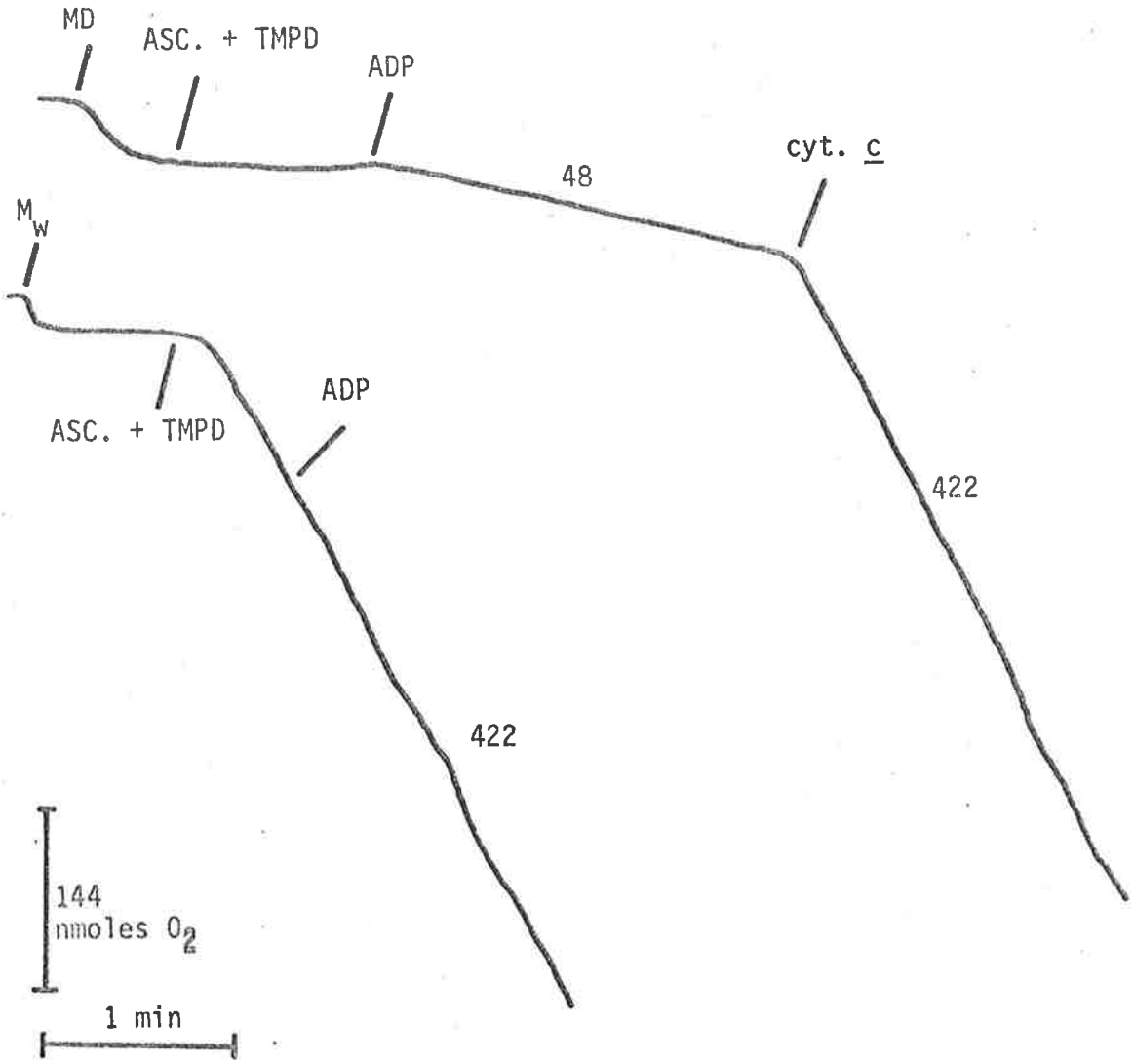


Figure 3.6 Effect of digitonin on the oxidation of ascorbate plus TMPD by turnip mitochondria.

Assay conditions are described in Materials and Methods. Additions as indicated were 0.1 mg mitochondrial protein, 5 mM ascorbate + 1 mM TMPD, 0.2 mM ADP, 0.04 mM cytochrome c. MD = mitochondria preincubated with digitonin (0.2 mg/mg. protein, for 30 min. at 0°C); M_w = washed mitochondria. Rates are expressed as nmoles O_2/min.mg. protein.

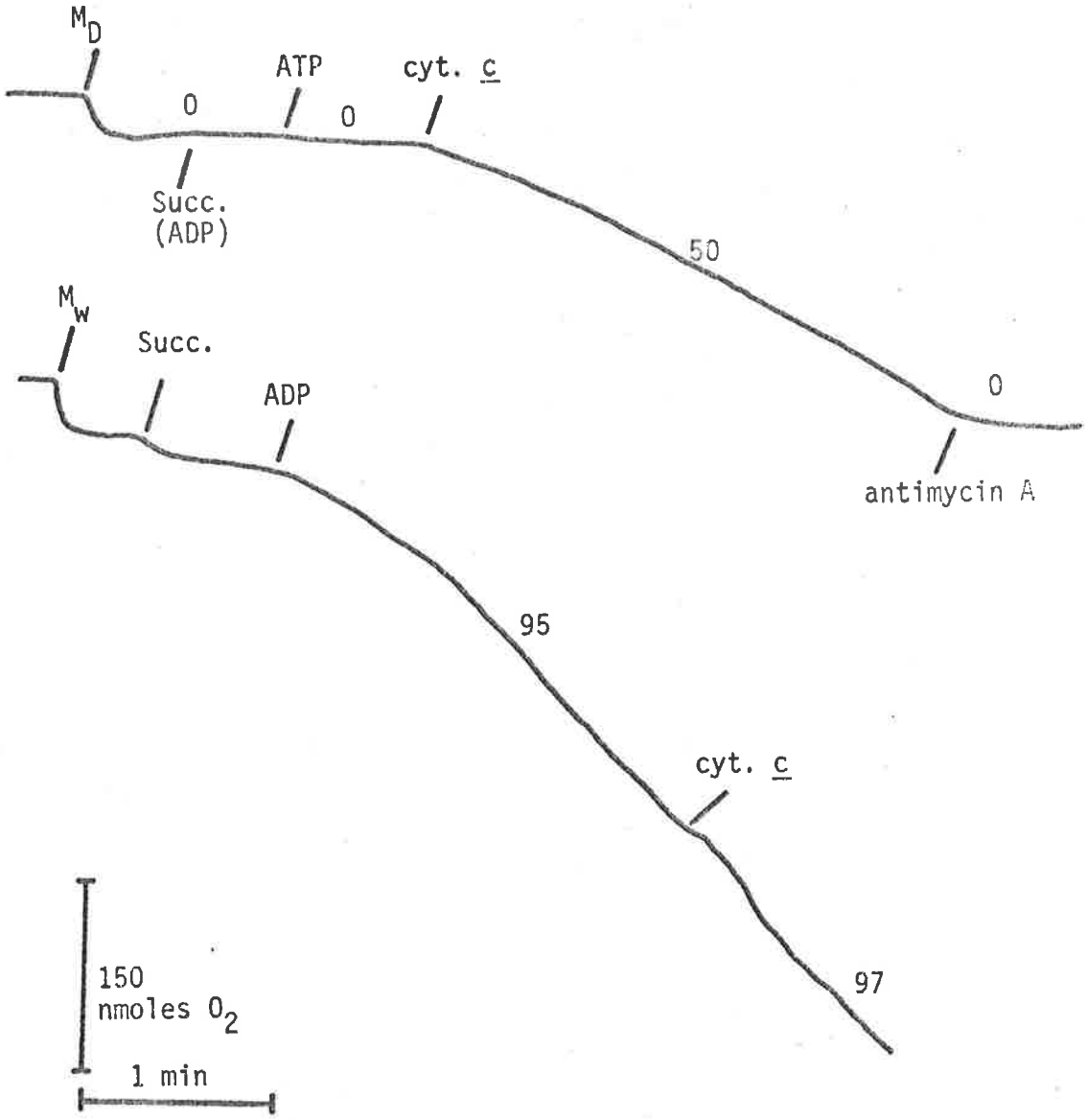


Figure 3.7 Effect of digitonin and cytochrome c on succinate oxidation by cauliflower mitochondria.

Assay conditions are described in Materials and Methods. Additions as indicated were 10 mM succinate, 0.3 mM ADP, 0.2 mM ATP, 0.04 mM cytochrome c and 5 µM antimycin A. M_D = mitochondria preincubated with digitonin (0.2 mg/mg protein); M_W = washed mitochondria. Rates expressed as nmoles O_2/min.mg.protein.

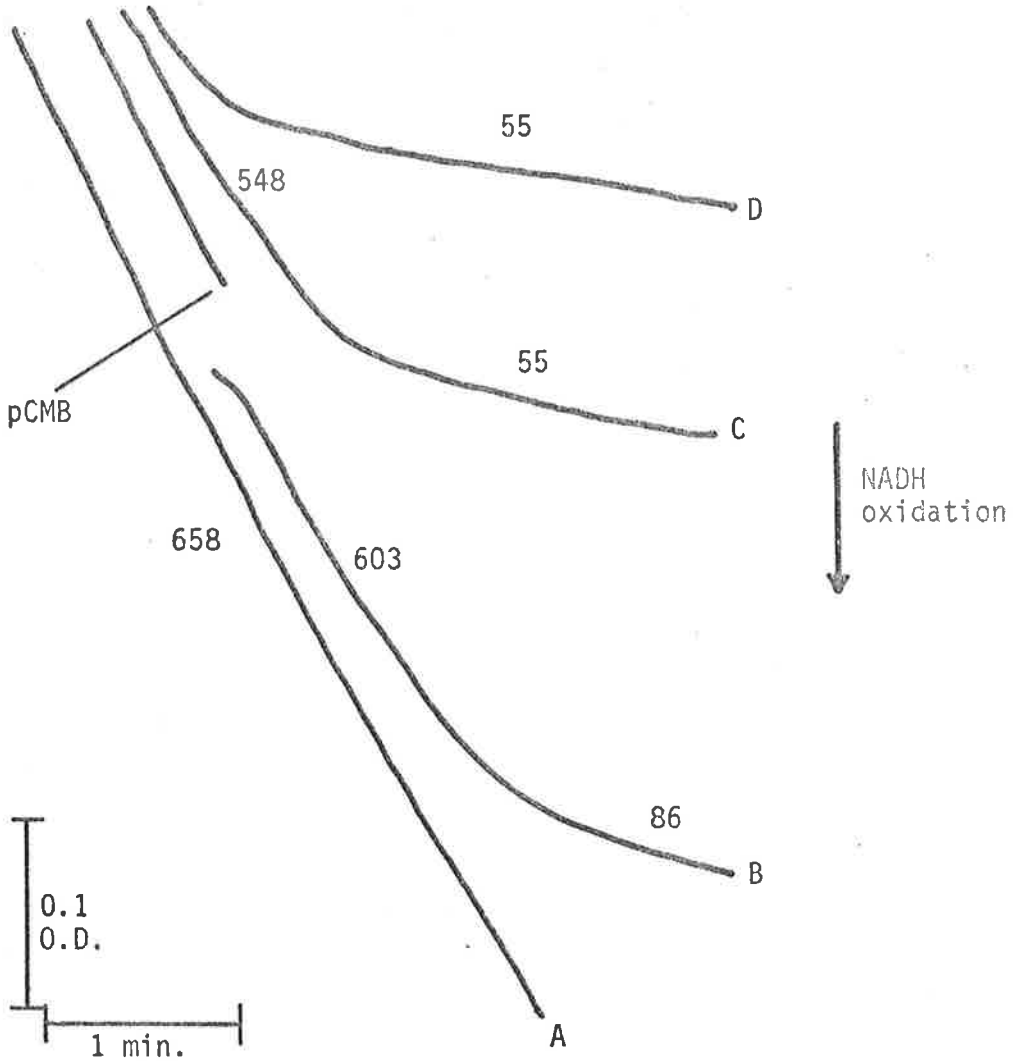


Figure 3.8 Effect of pCMB on the external NADH-oxidase of turnip mitochondria.

NADH oxidation was measured spectrophotometrically as described in Methods, except that 0.5 mM ADP was included in the reaction medium. The final concentration of pCMB was 20 µM and 0.2 mg mitochondrial protein was used in each assay. Rates are expressed as nmoles NADH oxidized $min.^{-1} mg.protein^{-1}$.

A : control

B : mitochondria preincubated with NADH. pCMB added as indicated.

C : NADH and pCMB added together.

D : mitochondria preincubated with pCMB.

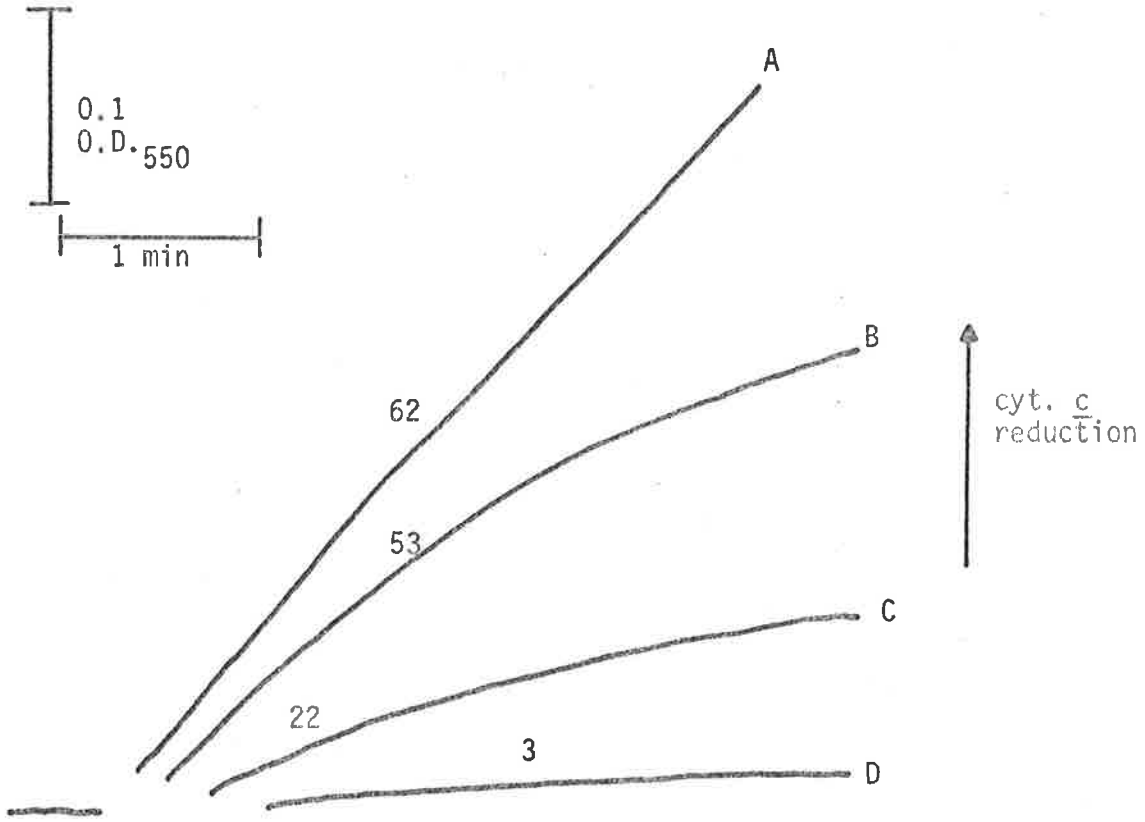


Figure 3.9 Effect of pCMB on the antimycin A-insensitive NADH-cytochrome c reductase of turnip mitochondria.

Details of assay are given in Methods. 0.35 mg mitochondrial protein used per assay. Final concentration of pCMB = 20 μM. Rates are expressed as nmoles cyt. c reduced min^{-1} $mg.protein^{-1}$.

A : control

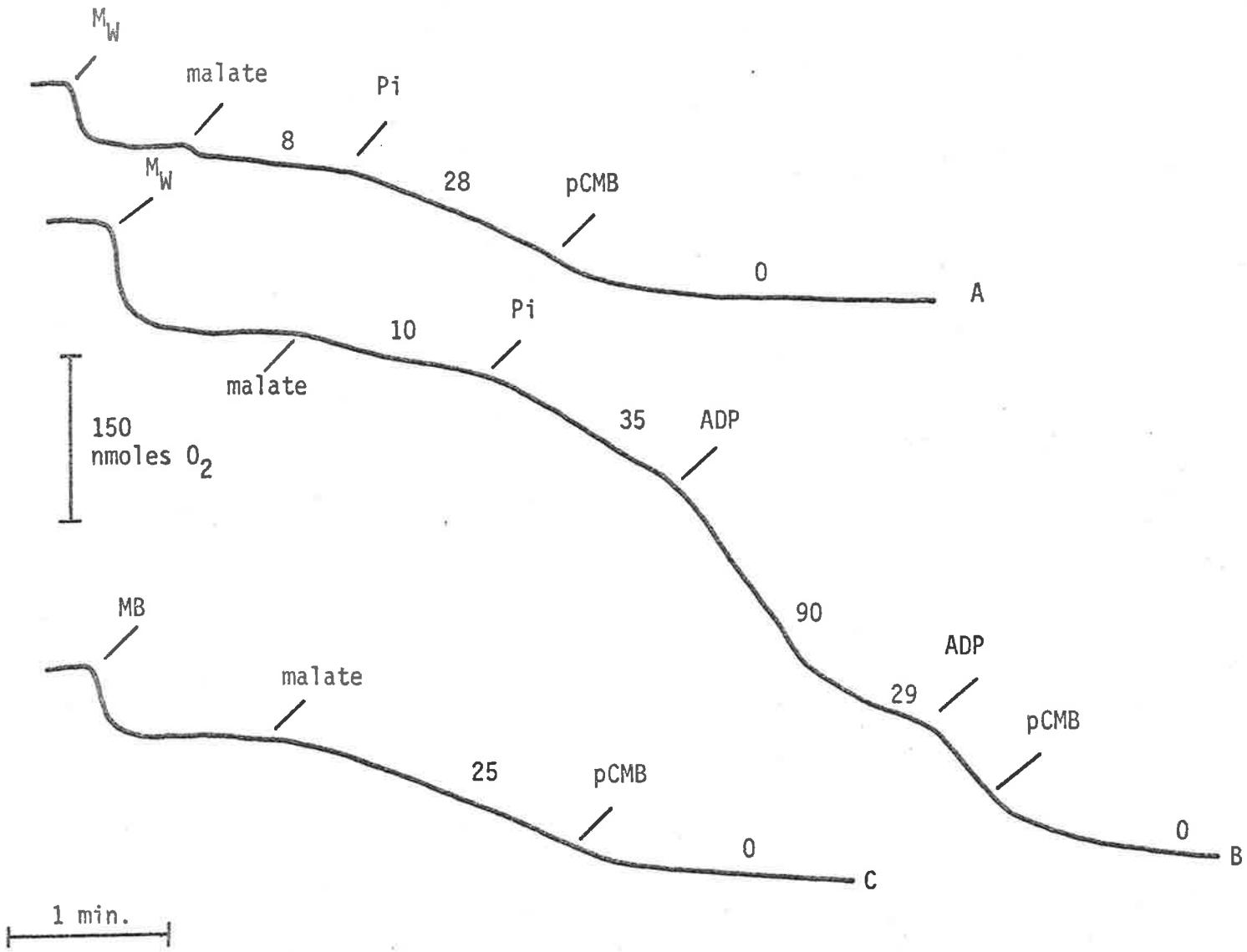
B : mitochondria preincubated with NADH, reaction started with cytochrome c.

C : NADH and pCMB added together.

D : mitochondria preincubated with pCMB.

Figure 3.10 Effect of pCMB on malate oxidation by cauliflower bud mitochondria.

Oxygen uptake was measured as described in Materials and Methods, except phosphate was omitted from the medium and added as shown. Additions as indicated were; A and B: 2.4 mg mitochondrial protein,10 mM malate, 10 mM phosphate, 0.28 mM ADP and 10 µM pCMB; C: 2.4 mg mitochondria pre-incubated in water for 5 min, 10 mM malate, 10 µM pCMB. Glutamate (10 mM) was included in the reaction medium in each case. Oxygen uptake is expressed as nmoles/min.mg protein.


M_W
malate
Pi
8
28
pCMB
0
A
M_W
10
Pi
malate
35
ADP
150
nmoles O_2
MB
malate
90
ADP
29
pCMB
25
pCMB
0
0
B
C
1 min.

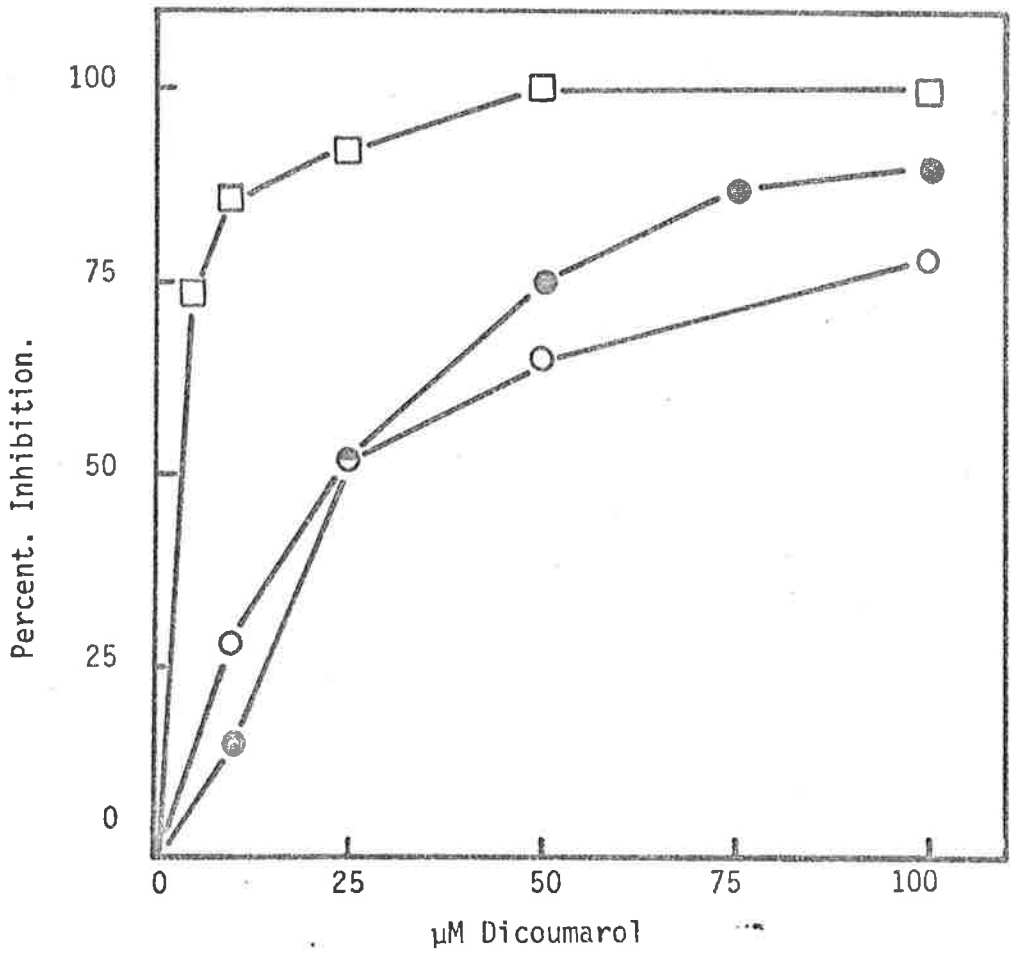


Figure 3.11 Effect of dicoumarol concentration on NADH, malate and succinate oxidation by turnip mitochondria.

Details of assays are given in Materials and Methods. □ —□ ; NADH oxidation, state 3 rate = 130 nmoles O_2/min.mg protein, 1 mM NADH as substrate. ○ — ○; 10 mM succinate, State 3 rate = 155 nmoles O_2/min.mg protein. ● — ●; 10 mM malate, state 3 rate = 100 nmoles/min.mg protein. 1.4 mg mitochondrial protein was used in all assays.

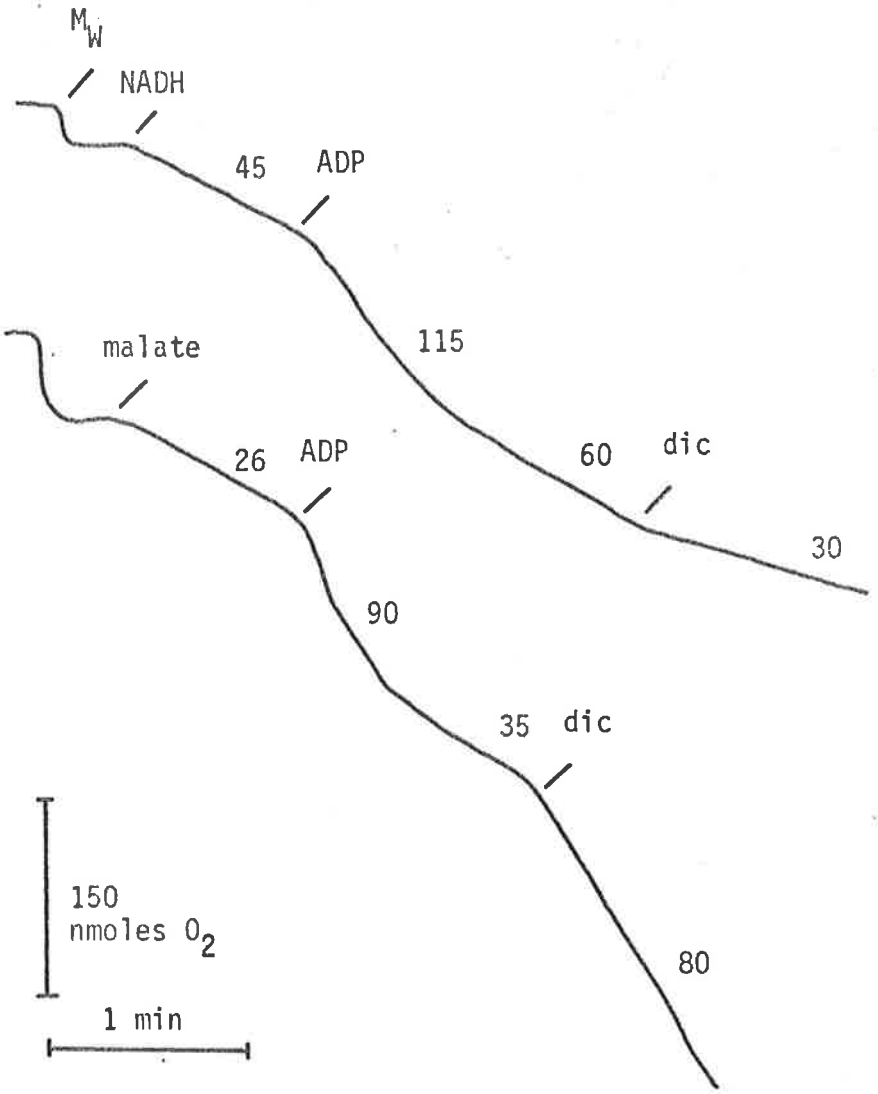
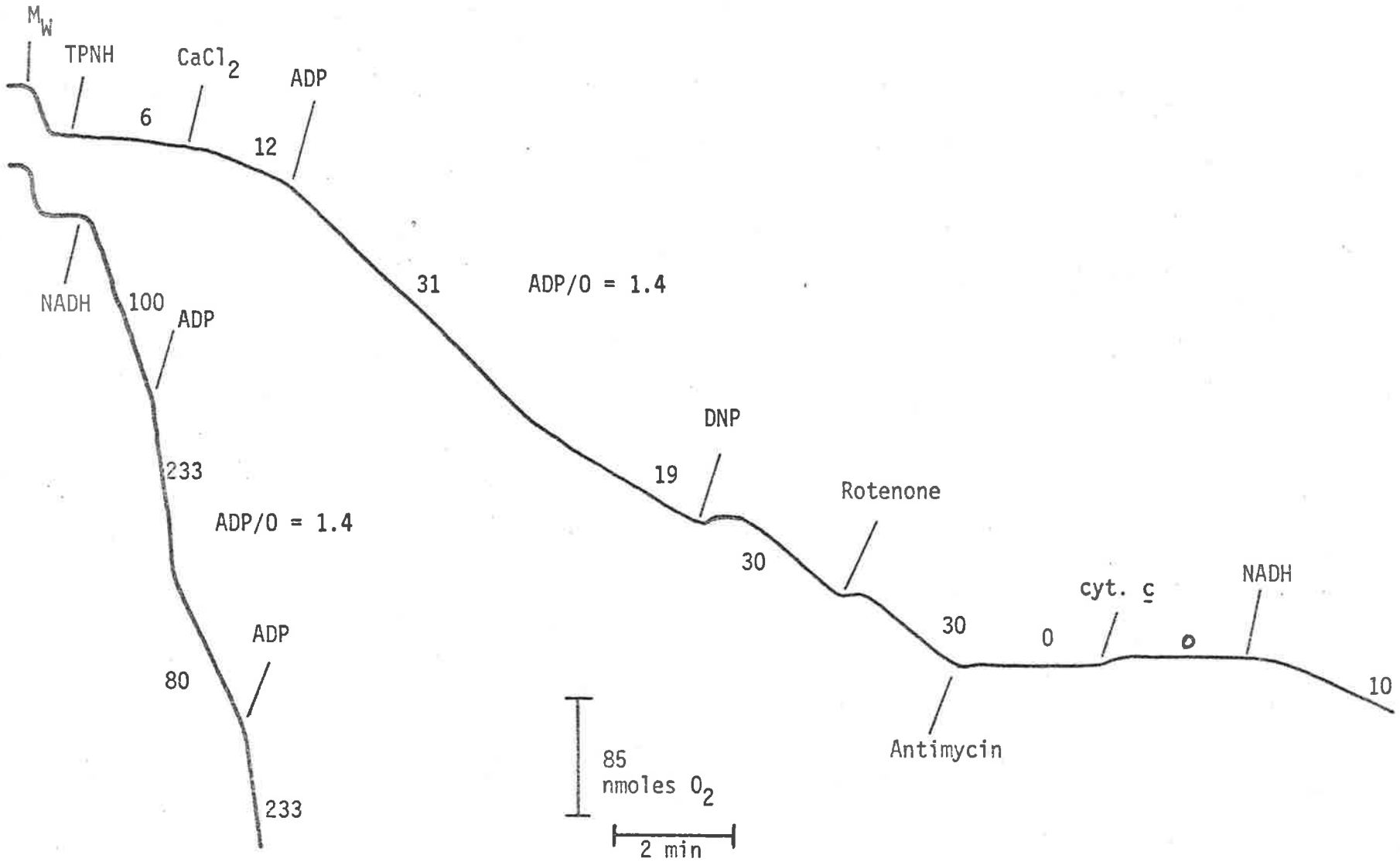


Figure 3.12 Effect of low dicoumarol concentration on NADH and malate oxidation by turnip mitochondria.

Details of assays are given in Materials and Methods. Additions as indicated were, 1.4 mg protein, 1 mM NADH, 15 mM malate, 0.18 mM ADP (for malate) or 0.09 mM ADP (for NADH), and 5 µM dicoumarol. Rates are expressed as nmoles O_2/min. mg protein. Glutamate (15 mM) was included in the reaction mixture when malate was substrate.

Figure 3.13 Exogenous NADPH oxidation by cauliflower bud mitochondria.

Details of assays are given in Materials and Methods. Additions as indicated were mitochondria (1.5 mg protein), 1 mM NADH, 0.14 mM ADP, 0.6 mM $CaCl_2$, 30 µM DNP, 5 µM antimycin A, 7 µM rotenone, 0.025 mM cytochrome c. Rates are expressed as nmoles O_2 consumed/min. mg protein.


M_W
TPNH
$CaCl_2$
ADP
6
12
NADH
100
ADP
31
ADP/O = 1.4
DNP
19
Rotenone
233
ADP/O = 1.4
30
cyt. c
NADH
30
0
ADP
80
10
Antimycin
85
nmoles O_2
233
2 min

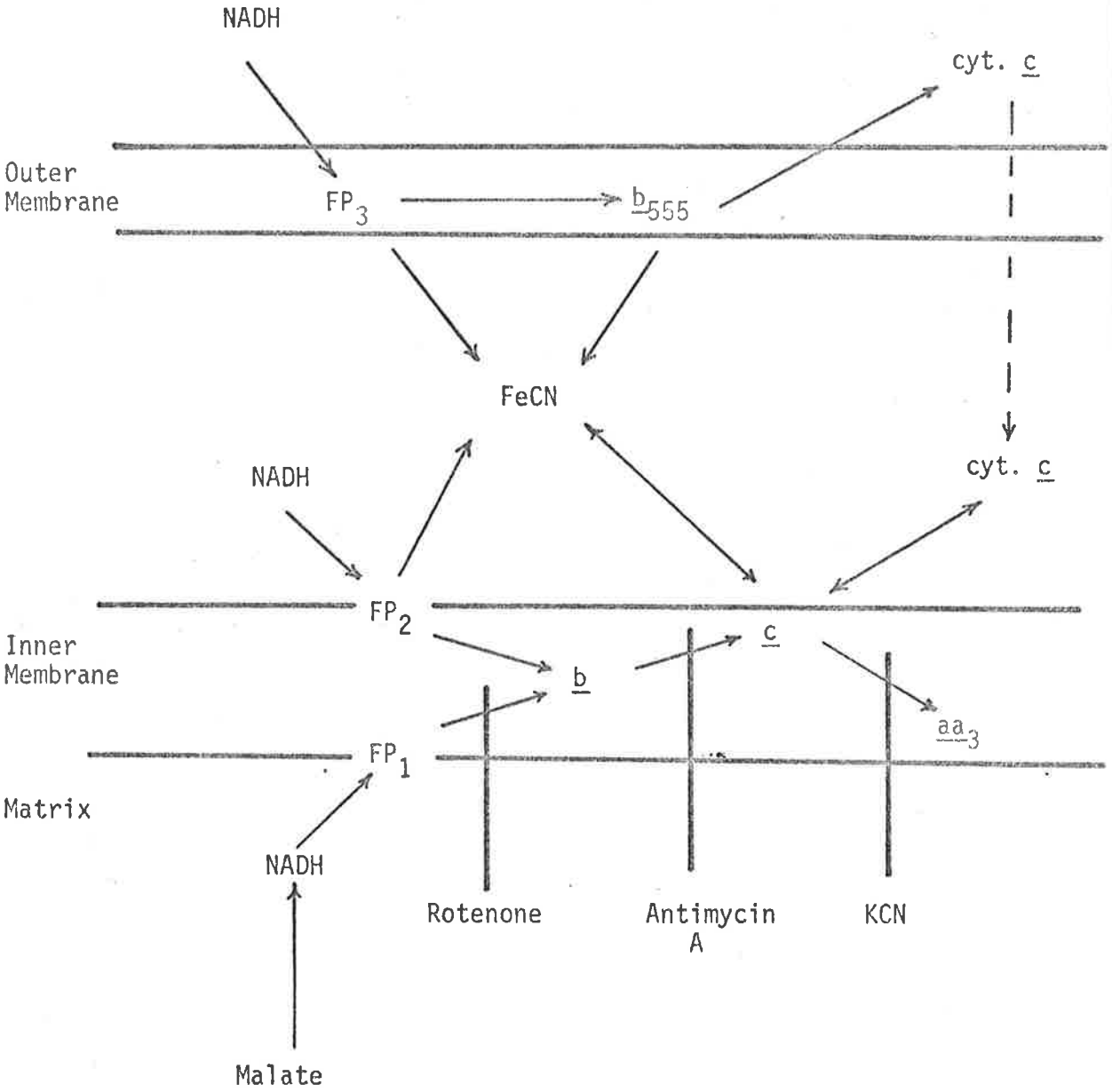
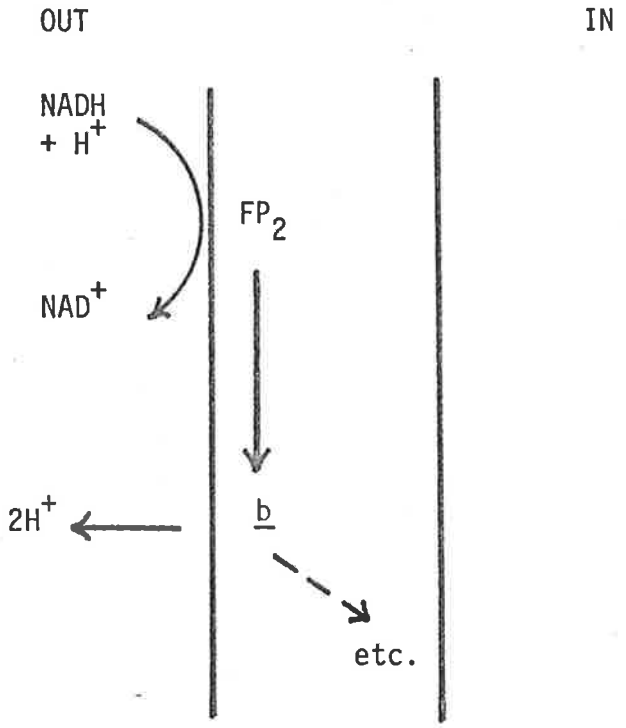


Figure 3.14 Pathways of NADH oxidation in isolated plant mitochondria.

(a) External NADH oxidation



(b) Internal NADH oxidation

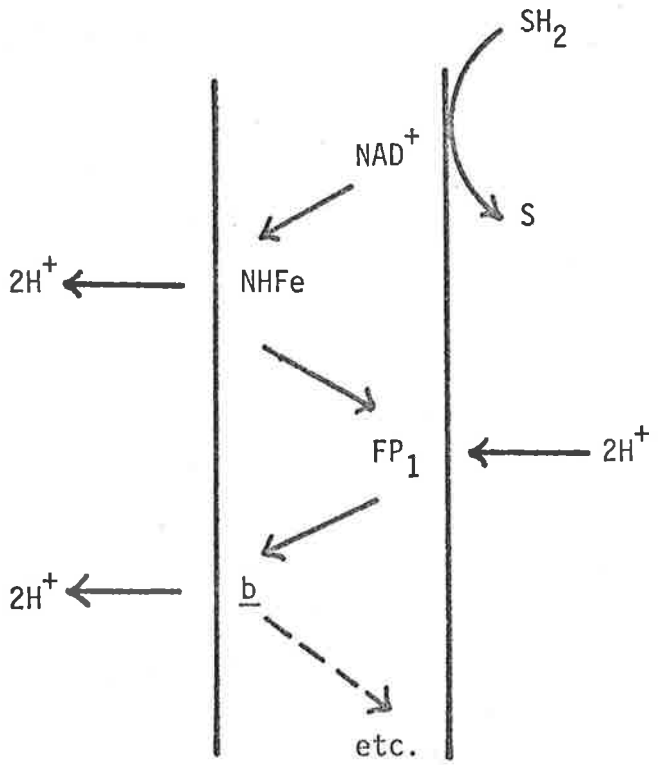


Figure 3.15 Proton translocation during NADH oxidation by plant mitochondria.

CHAPTER IV

ISOLATION AND PROPERTIES OF THE OUTER MEMBRANE

Although the outer membrane of mammalian mitochondria has been isolated and studied extensively, relatively little is known about the outer membrane of plant mitochondria, apart from its spectral properties (Douce et al, 1973b; Moraeu and Lance, 1972).

This chapter describes the enzymic properties of the outer membrane isolated from cauliflower and turnip mitochondria, after incubation with digitonin, with particular exphasis on the associated NAD(P)H reductases. The effect of digitonin on the inner membrane is also described, and the outer membrane is compared to the microsomal fraction.

RESULTS

1. *Solubilization of mitochondrial enzymes by digitonin*

Figures 4.1, 2 and 3 show the degree of solubilization of certain mitochondrial enzymes by digitonin. Malate dehydrogenase was used as a marker for the matrix space, succinate-cytochrome *c* or antimycin-sensitive NADH-cytochrome *c* reductase as a marker for the outer membrane. A similar pattern of solubilization was obtained with cauliflower, turnip and beetroot mitochondria, as the digitonin : protein ratio was increased. Outer membrane activity was released from the mitochondria at a lower digitonin : protein ratio than that at which the inner membrane was broken

(as judged by the release of soluble malate dehydrogenase from the matrix). The inner membrane enzymes were solubilized only to a small degree even by high digitonin protein ratios (Fig. 4.1, 4.2 and 4.3).

2. *Fractions obtained after centrifugation of digitonin - treated mitochondria*

Four distinct fractions could be obtained by centrifuging the digitonin-treated mitochondria at different speeds (Table 4.1). Although the technique employed was rather harsh, resulting in disruption of some inner membranes (the 37,000g pellet probably consisted of fragmented mitochondria and contained a considerable proportion of both inner and outer membrane enzyme activities), a fraction was obtained in which the NADH-cytochrome *c* reductase activity was largely insensitive to antimycin A. This fraction (the 144,000g pellet) was devoid of succinate-cytochrome *c* reductase and was poor in malate dehydrogenase activity (even this low activity could be "washed off" with sucrose- see later). It thus appears that this fraction consists of outer membrane fragments. The 8,500g pellet appears to consist largely of inner membrane vesicles, being enriched in antimycin - sensitive NADH - cytochrome *c* and possessing high succinate - cytochrome *c* reductase and malate dehydrogenase activity (Table 4.1). However, this fraction also contained some antimycin-insensitive NADH-cytochrome *c* reductase activity suggesting that some intact mitochondria were present.

Attempts to purify the inner membrane vesicles by swelling the 8,500g pellet in hypotonic phosphate buffer and then contracting it in 1.5 M sucrose were unsuccessful. Centrifugation of the 8,500g pellet on a suitable density gradient may yield a purer inner membrane fraction.

At the concentration of digitonin used in table 4.1 (0.3 mg/mg. protein), a considerable proportion of enzyme activity was lost; lower concentrations (e.g. 0.15mg digitonin/mg protein; Table 4.2) yielded higher recoveries but separation of the membranes was not as good.

A comparison of figures 4.1 and 4.2 shows that a larger proportion of turnip outer membrane activity was solubilized, by low concentrations of digitonin, than was cauliflower-bud outer membrane activity. Hence turnip mitochondria were used to investigate the outer membrane more thoroughly. Using the technique described in chapter II, approximately 50% of pure outer membrane could be recovered after differential centrifugation of mitochondria incubated with 0.2 mg digitonin/mg. protein (Table 4.3). The specific activity of antimycin-insensitive NADH-cytochrome *c* reductase was approximately 4 times higher in the isolated outer membrane than in the mitochondria, yet no inner membrane enzyme activity was detected in the outer membrane (Table 4.3). Very little (less than 0.3%) of malate dehydrogenase was attached to the outer membrane.

Low temperature ($77^{o}K$) spectra of this fraction (Fig. 4.4) showed that it contained a flavoprotein and *b*-type cytochrome

(with an ∝-absorption peak at 560 nm), confirming the results of Douce et al (1973b) and Moreau and Lance (1972). No cytochrome *a* peak was evident, suggesting that it was devoid of cytochrome oxidase.

3. *NAD(P)H reductases of the outer membrane and microsomes*

The outer membrane fraction was capable of high rates of cytochrome *c*, DCPIP and Fe CN reduction with NADH as substrate (Table 4.4). However, unlike the microsomal fraction, very little NADPH-cytochrome *c* reductase activity was present (Table 4.4). DCPIP reduction was much faster than cytochrome *c* reduction, and approximately equal to the rate of ferricyanide reduction, in the outer membrane fraction (Table 4.4), suggesting that DCPIP and Fe CN were reduced at the same site (or their reduction involved the same limiting step) prior to that at which cytochrome *c* accepted electrons. Studies of the concurrent reduction of DCPIP and cytochrome *c* support this idea. Reduced DCPIP (E_o' = +0.22) reduces oxidized cytochrome *c* (E_o' = +0.26) non-enzymically. DCPIP has a broad absorption peak and absorbs almost as strongly at 550 nm as at 600 nm; therefore in a mixture of the two electron acceptors ΔE_{550} is given by the sum of the increase due to cytochrome *c* reduction and the decrease due to DCPIP reduction. Table 4.5 shows that the initial rate of cytochrome *c* reduction (at 550 nm) was greater in the presence of DCPIP (despite any drop in absorption due to DCPIP reduction).

Figure 4.5 shows that since reduced DCPIP reduced cytochrome *c* non-enzymically there was no net reduction of DCPIP until all the cytochrome *c* had been reduced. These results can be best explained by a rate limiting step between the site of DCPIP (and Fe CN) reduction and the site of cytochrome *c* reduction.

NADH-DCPIP reductase activity of microsomes was considerably slower than that of the outer membrane (Table 4.4). However addition of DCPIP to the reaction mixture stimulated the rate of cytochrome *c* reduction in the microsomes (Table 4.5), suggesting it accepts electrons prior to the site of cytochrome *c* reduction. Microsomal NADH-Fe CN reduction was much faster than DCPIP reduction (Table 4.4) and Rungie and Wiskich (1972a) have shown that Fe CN and DCPIP are reduced at different sites on the microsomal electron transport chain.

The most significant outer membrane rate with NADPH as substrate was with Fe CN as electron acceptor (Table 4.4).

These results indicate that the outer membrane fraction contained very few microsomal membranes, and that the electron transport pathways associated with the two types of particles are different.

4. Effect of inhibitors on NADH reductases

Table 4.6 shows the effect of some electron transport inhibitors on the outer membrane reductases. Significant inhibition was observed only with pCMB and high concentrations of dicoumarol.

These high concentrations (Fig. 4.6) show that the reductase activities cannot be attributed to DT-diaphorase which is completely inhibited by 1 x 10^{-6} M dicoumarol (Ernster et al, 1962). The rotenone-insensitive NADH-cytochrome *c* reductase of pig liver mitochondria was also sensitive to high concentrations of dicoumarol (Raw and Mahler, 1959), but the inhibition observed was not as great as reported here. Mahler et al (1958) demonstrated that dicoumarol inhibition of the same enzyme from pig liver was greater when the enzyme was preincubated with NADH. No such effect was observed with turnip reductases.

The lack of inhibition of the outer membrane NADH dehydrogenase by low concentrations of dicoumarol contrasted with the severe inhibition of the inner membrane enzyme (Fig. 3.10). Unlike NADH oxidation by the inner membrane, the outer membrane reductases were not affected by Ca Cl_2 (Table 4.6).

NADH-cytochrome *c* and Fe CN reductase were both severely inhibited by low concentrations of pCMB (Table 4.6) when it was added before NADH. Preincubation of the outer membrane with NADH protected the dehydrogenase from pCMB inhibition (Table 4.7). The pattern of pCMB inhibition of NADH-cytochrome *c* reductase was identical for the isolated outer membrane and intact mitochondria (Fig. 3.9 and Table 4.7). Similar results were obtained with cauliflower and turnip mitochondria. Preincubating the outer membrane with NADPH did not protect NADH-cytochrome *c* reductase from pCMB inhibition (Table 4.7), suggesting that NADPH is oxidized by a different dehydrogenase.

The NADH : pCMB ratio employed in the present study (12:1) provided considerably greater protection of the mitochondrial enzyme than did the same ratio for the turnip microsomal fraction (Rungie and Wiskich, 1972a). This suggests that the microsomal dehydrogenase contains other essential sulphydryl groups (Strittmatter, 1965) not present in the mitochondrial enzymes.

5. *NADPH - Fe CN reductase*

The NADPH - Fe CN reductase of the outer membrane was not inhibited by either pCMB or dicoumarol (Table 4.6), indicating that it was distinct from the NADH dehydrogenase, but was not a DT-diaphorase. Since pCMB did not inhibit this enzyme, accessible sulphydryl groups cannot be involved in NADH binding. Rungie and Wiskich (1972b) detected an NADPH-Fe CN reductase, with similar properties, in the soluble fraction of turnip ; this soluble enzyme may have adsorbed to the outer mitochondrial membrane during isolation.

Although the specific activity of the NADH reductases increased after washing, some NADPH-Fe CN reductase activity was lost (Table 4.8) indicating that it was more loosely bound to the membrane. This also shows that different enzymes were responsible for NADH and NADPH oxidation, and supports the idea that the NADPH-Fe CN reductase may have originated from the cytosol.

6. *Acid phosphatase*

An acid phosphatase was also detected in the outer membrane fraction (Table 4.3), although its activity was rather low. This enzyme, which has a pH optimum of 6.0 (Fig. 4.7), may account for the glucose-6-phosphatase activity found by Moreau and Lance (1972) in cauliflower bud mitochondria. Rungie and Wiskich (1973) observed acid phosphatase activity in both the microsomal and soluble fractions of turnip, but these phosphatases had a more acidic pH optimum than the one described here. Phosphatase activity did not decrease when the outer membrane was washed with sucrose (Table 4.8), suggesting that the enzyme was firmly bound to the membrane and hence unlikely to be due to contamination from the soluble fraction. In contrast, some malate dehydrogenase activity (presumably released from the matrix during isolation of the outer membrane) was lost during the washing procedure (Table 4.8).

The significance of this acid phosphatase is not immediately apparent.

7. *Transhydrogenase and Monoamine oxidase*

No tranhydrogenase (NADPH $\longrightarrow$ NAD) or monoamine oxidase activity was observed in the outer membrane fraction of any of the plant tissues studied.

DISCUSSION

1. The methods used in this study enabled the isolation of outer membrane free from inner membrane enzymes, and confirms the existence of two NADH oxidizing pathways in plant mitochondria. The outer membrane was virtually free from microsomal contamination also; on the basis of NADPH-cytochrome *c* reductase activity, only 10% contamination by microsomes was observed (Table 4.4).

The studies with DCPIP and cytochrome *c* reduction, and those of Rungie and Wiskich (1972a), show that the outer membrane and microsomal electron transport chains are not identical. The microsomal pathway appears to contain an additional component, and the two possible schemes of electron transport are depicted in figure 4.8. The outer mitochondrial membrane seems to be devoid of NADPH reductases; the small amount of NADPH-Fe CN activity was not firmly bound to the membrane.

In other aspects the two membrane fractions are similar and may have a common origin during cellular synthesis. Parsons et al (1969) have suggested that the outer membrane is derived from the endoplasmic reticulum in mammalian tissues, since electron-micrographs often show the two membranes in close association, or actually joined, in intact cells.

2. This study also shows that the two dehydrogenases responsible for the oxidation of exogenous NADH in plant mitochondria are not identical.

The inner membrane dehydrogenase was severely inhibited by concentrations of dicoumarol (see chap. III) which had no effect on the outer membrane enzyme. NADH oxidation by the outer membrane did not respond to low concentrations of Ca Cl_2, although the inner membrane dehydrogenase was stimulated by the same concentrations. This suggests that the outer membrane dehydrogenase does not have a divalent cation requirement like that reported for the inner membrane enzyme (Coleman and Palmer, 1972). These differences between the two enzymes may also reflect differences in their phospholipid environment or structural differences between the outer and inner membranes.

Both dehydrogenases showed similar responses to -SH poisons, indicating that they have similar NADH-binding sites. The involvement of an essential sulphydryl group binding NADH has also been reported for plant microsomal and soluble NADH-dehydrogenases (Rungie and Wiskich, 1972a and b). Similar observations have been made with animal microsomes (Strittmatter, 1965), outer mitochondrial membrane (Ragan and Garland, 1969) and lactate dehydrogenase (Holbrook and Stinson, 1970). These findings suggest a possible evolutionary relationship, perhaps indicating a common source or function (Fondy and Holohan, 1971). However, the NADH dehydrogenases of mung bean mitochondria appear to have different stereospecificities for the hydrogen atoms of NADH (Douce et al, 1973b); obviously this does not alter pCMB inhibition.

3. The plant outer mitochondrial membrane appears to be similar to its mammalian counterpart, since both contain a flavoprotein and cytochrome b_{555}. However, the dehydrogenase of the animal membrane

did not show protection from pCMB inhibition by NADH (Mahler et al, 1958), and no acid phosphatase activity has been reported in the animal outer membrane. Unlike animal mitochondria (Schnaitman and Greenwalt, 1968), the outer membrane of plant mitochondria did not possess any monoamine oxidase activity.

4. The involvement of the outer membrane electron transport chain in the functioning of mitochondria *in vivo* has yet to be demonstrated. It was suggested that if small amounts of endogenous cytochrome *c* were located in the intermembrane space then exogenous NADH could be oxidized via the respiratory chain (Nicholls et al, 1969; see Fig. 4.9). Certainly isolated plant mitochondria can oxidize NADH via the respiratory chain, in the presence of antimycin A, if cytochrome *c* is added to the reaction medium (chap. III). However, attempts to demonstrate the presence of cytochrome *c* in the intermembrane space have failed (eg. Nicholls, 1974). Obrien (1971) has shown that a number of different artificial electron acceptors can shuttle electrons between the outer and inner membrane systems of isolated liver mitochondria, and suggested that such shuttles may play a role in the oxidation of extramitochondrial NADH. No such acceptors have been found in isolated mitochondria, but since the outer membrane is permeable to low molecular weight compounds, it might be expected that the shuttle-carriers would leak out of the mitochondria during isolation.

It is unlikely that such intermembrane shuttle systems play a role in plant mitochondria since NADH can be readily oxidized directly by the respiratory chain.

The outer membrane electron transport pathway must therefore be linked to other functions of the outer membrane or cytoplasm ; the role it plays will not become clear until the natural terminal acceptor (s) are determined. It may be that the phosphatase reported in this study is involved, although Rungie and Wiskich (1972a, 1973) could not demonstrate a relationship between the turnip microsomal phosphatase and electron transport activities.

Table 4.1 Fractionation of Digitonin-treated cauliflower mitochondria.

Mitochondria were incubated with digitonin (0.3mg digitonin/mg protein) and subjected to differential centrifugation as described in Materials and Methods. A sample of mitochondria was drawn off prior to centrifugation and used as the control. Assays of enzymes are described in Materials and Methods. Cytochrome *c* reductase is expressed as nmoles cyt. c reduced/min. and malate dehydrogenase as nmoles NADH oxidized/min. 5 μM antimycin A (final conc.) was used in all cases. (- represents no detectable activity)

	Protein (mg)	NADH-Cytochrome c Reductase				Succinate-Cytochrome 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
Control mito.	61.7	5553	90	3702	60	3085	50	0	0	20918	340
8,500xg (P)	14	3629	259	333	24	1411	101	0	0	2800	200
37,000xg (P)	13.3	1423	107	705	33	705	53	0	0	3830	288
144,000xg (P)	9.2	92	10	460	50	-	-	-	-	693	77
144,000xg (S)	17.1	-	-	92	5.4	-	-	-	-	5036	318
Percent recovery	87	90		43		78				59	

(P) denotes pellet (S) denotes supernatant

Table 4.2. Fractionation of digitonin-treated cauliflower mitochondria.

For details of treatments used see legend to the previous table (4.1). 0.15 mg digitonin / mg mitochondrial protein was used. (- represents no detectable activity).

(P) = pellet (S) = supernatant

	PROTEIN CONTENT (mg)	SUCCINATE-CYT.C REDUCTASE			ANTIMYCIN A-SENSITIVE NADH-CYT.C REDUCTASE		ANTIMYCIN A-INSENSITIVE NADH-CYT.C REDUCTASE	
		specific activity (per mg)	total activity	+5 μM Antimycin A	specific activity (per mg)	total activity	specific activity (per mg)	total activity
WHOLE MITOCHONDRIA	74.4	14.1	1049	0	32.5	2418	35.9	2671
18,000g (P)	40.6	33.8	1372	0	83.6	3394	28.4	1153
144,000g(P)	18.38	-	-	-	0	0	37.4	687
144,000g (S)	15.12	-	-	-	0	0	28.3	428
PERCENT RECOVERY	99.5		130.6			140.3		85

Activities expressed in nmoles cytochrome c reduced/min.

Table 4.3. Enzyme activities of turnip mitochondria and outer mitochondrial membrane.

Details of assays are given in Materials and Methods. Rates are expressed as : nmoles cyt. *c* reduced min (cyt. *c* reductases); μmoles NADH oxidized/min. (MDH); nmoles p-nitrophenyl released/min (acid phosphatase). The final concentration of antimycin A was 5 μM.

Enzyme	Mitochondrial Activity		Outer Membrane Activity	
	Total	Specific	Total	Specific
Antimycin-sens. NADH-cyt.*c* reductase	19920	400	0	0
antimycin-insens. NADH-cyt.*c* reductase	4080	68	1746	291
Malate dehydrogenase	1423	23.7	4.2	0.7
Succinate-cyt.*c* reductase	14890	118	0	0
Acid phosphatase	89	1.4	21	3.7

Table 4.4. NAD(P)H reductase activity of turnip outer mitochondrial membrane and microsomes.

Details of assays are given in Materials and Methods. Rates expressed as nmoles NAD(P)H oxidized/min. mg protein.

Reductase	Outer membrane	Microsomes
	nmoles/min. mg. protein	
NADH - cyt. *c*	119	232
NADPH - cyt. *c*	2	20
NADH - DCPIP	2678	511
NADPH - DCPIP	81	98
NADH - Fe CN	2778	1224
NADPH - Fe CN	278	204

Table 4.5. Effect of DCPIP on cytochrome c reduction by turnip outer mitochondrial membrane and microsomes.

Details of assays are given in Materials and Methods.

Fraction	μmoles cyt. *c* reduced/min. mg. protein	
	Control	+ DCPIP (50 μM)
microsomes	0.260	0.447
outer membrane	0.180	2.21

Table 4.6. Effect of electron transport inhibitors on the NAD(P)H reductases of the outer mitochondrial membrane.

Details of assays are given in Materials and Methods.

Inhibitor	Concn.	Percent Inhibition		
		NADH-cyt.*c* reductase	NADH-Fe CN reductase	NADPH-Fe CN reductase
Rotenone	10 µM	0	0	0
Amytal	3 mM	0	0	0
Antimycin A	5 µM	0	0	0
Dicoumarol	10 µM	12	0	0
	100 µM	75	58	10
pCMB	30 µM	95	86	0
EGTA	1 mM	0	0	0
$CaCl_2$	0.5 mM	10	5	0

Table 4.7. Effect of pCMB on outer mitochondrial membrane NADH reductases.

Details of assays are given in Materials and Methods. Final concentration of pCMB was 20 µM, and 0.1 mg protein was used.

	µmoles NADH oxidized / min. mg. protein	
	NADH-cyt. *c*	NADH- Fe CN
Control	0.095	2.17
preincubated with NADH ; + pCMB	0.077	1.83
pCMB and NADH added together	0.058	-
preincubated with pCMB	0.004	0.67
preincubated with NADPH; + pCMB	0.0	-

Table 4.8. The effect of 'washing' the outer membrane fraction on some associated enzyme activities.

The outer membrane suspension (approximately 5mg protein in 2ml) was sonicated for a few seconds, (MSE 100 watt) diluted 10 fold with 0.4M sucrose and centrifuged for 5 hrs at 144,000g in a Spinco Model L centrifuge with a Ti50 rotor. The pellet was resuspended in 0.4M sucrose. The various enzymic assays were carried out before and after washing, as described in Methods. Rates are expressed as: nmoles p-nitrophenyl formed/ min. mg protein (acid phosphatase); nmoles cyt. *c* or Fe CN reduced/min. mg. protein(NAD(P)H - reductases); nmoles NADH oxidized/min. mg protein (MDH).

Enzyme	Activity before washing	Activity after washing
Acid phosphatase	3.7	4.4
NADH - cyt *c* reductase	300	321
NADH - Fe CN reductase	3246	4081
NADPH - Fe CN reductase	287	105
Malate dehydrogenase	484	244

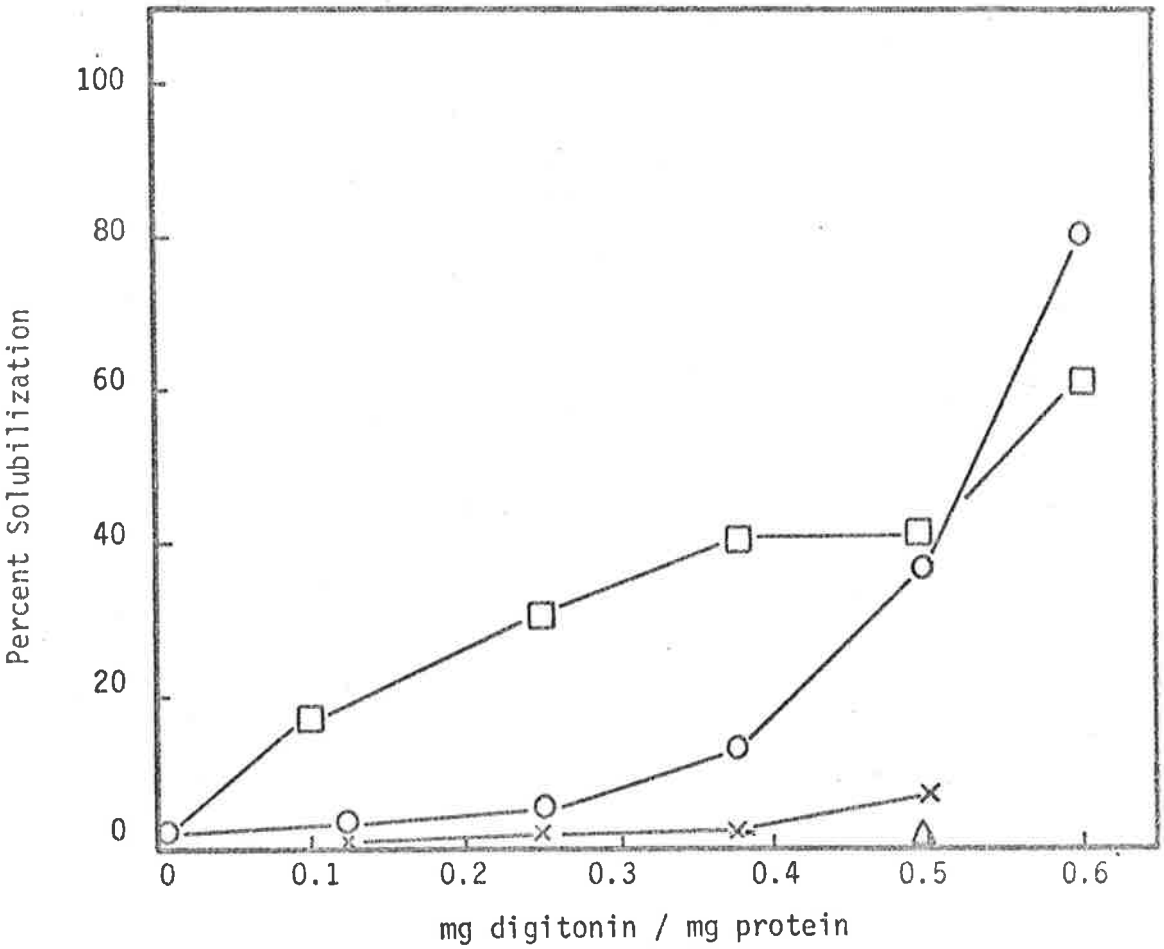


Figure 4.1 Solubilization of cauliflower mitochondrial enzymes by digitonin.

Mitochondria were incubated with digitonin, centrifuged and the supernatants assayed for the various enzyme activities as described in Chapter II. Activities are expressed as percentages of those present in disrupted, but unfractionated, mitochondria.
O — O MDH, □ —□ antimycin insensitive NADH-cyt. c reductase; Δ — Δ antimycin-sensitive NADH-cyt. c reductase; X — X succinate - cyt. c reductase.

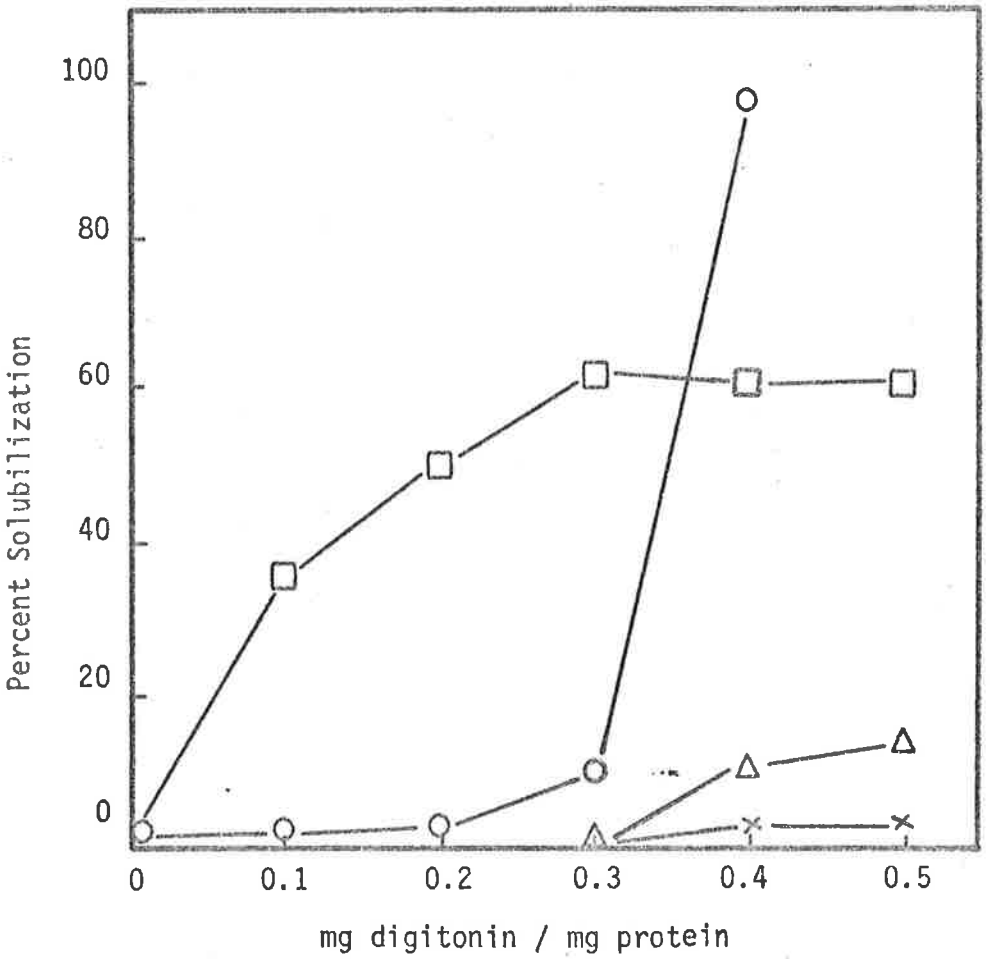


Figure 4.2 Solubilization of turnip mitochondrial enzymes by digitonin.

For details of assays see previous figure (4.1) O — O MDH: □ —□ antimycin-insensitive NADH-cyt.c reductase; Δ — Δ antimycin-sensitive NADH - cyt. c reductase; X — X succinate-cyt. c reductase.

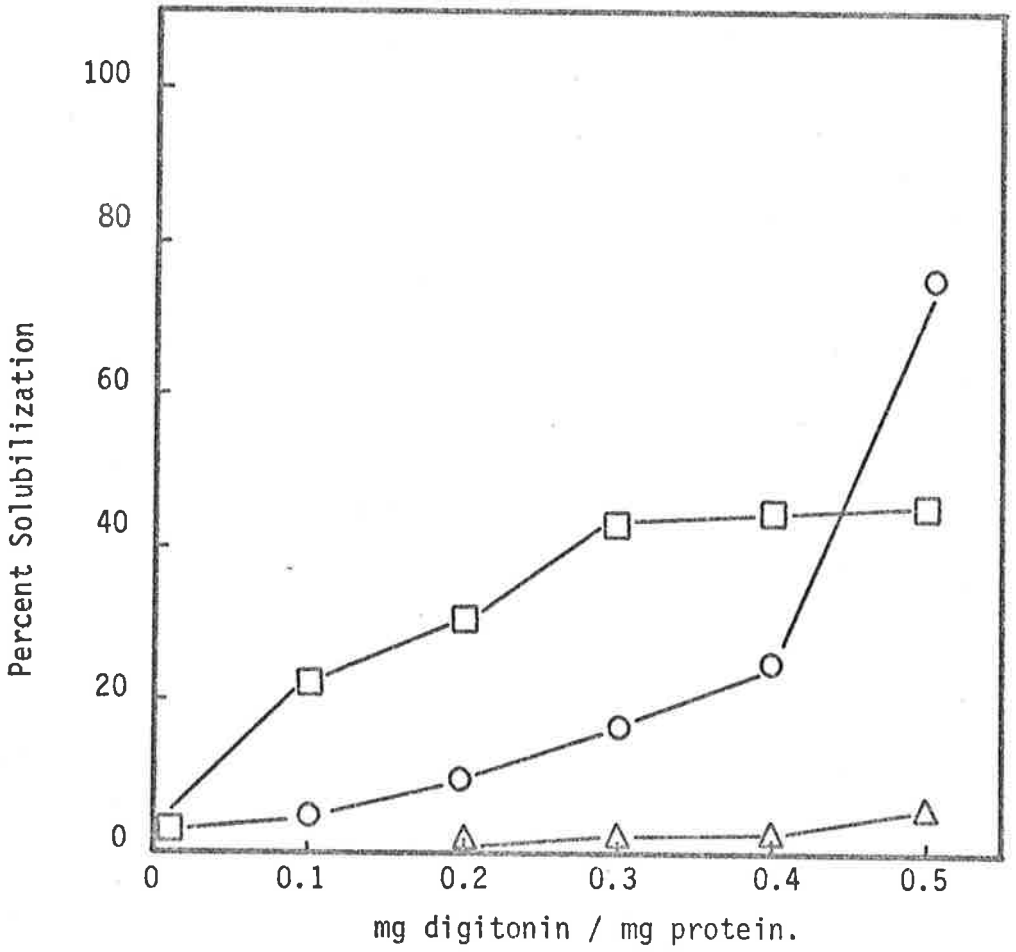


Figure 4.3 Solubilization of beetroot mitochondrial enzymes by digitonin.

Conditions of assay were the same as those given in Figure 4.1. O — O MDH; □ —□ antimycin-insensitive NADH - cyt. c reductase; Δ — Δ succinate - cyt. c reductase.

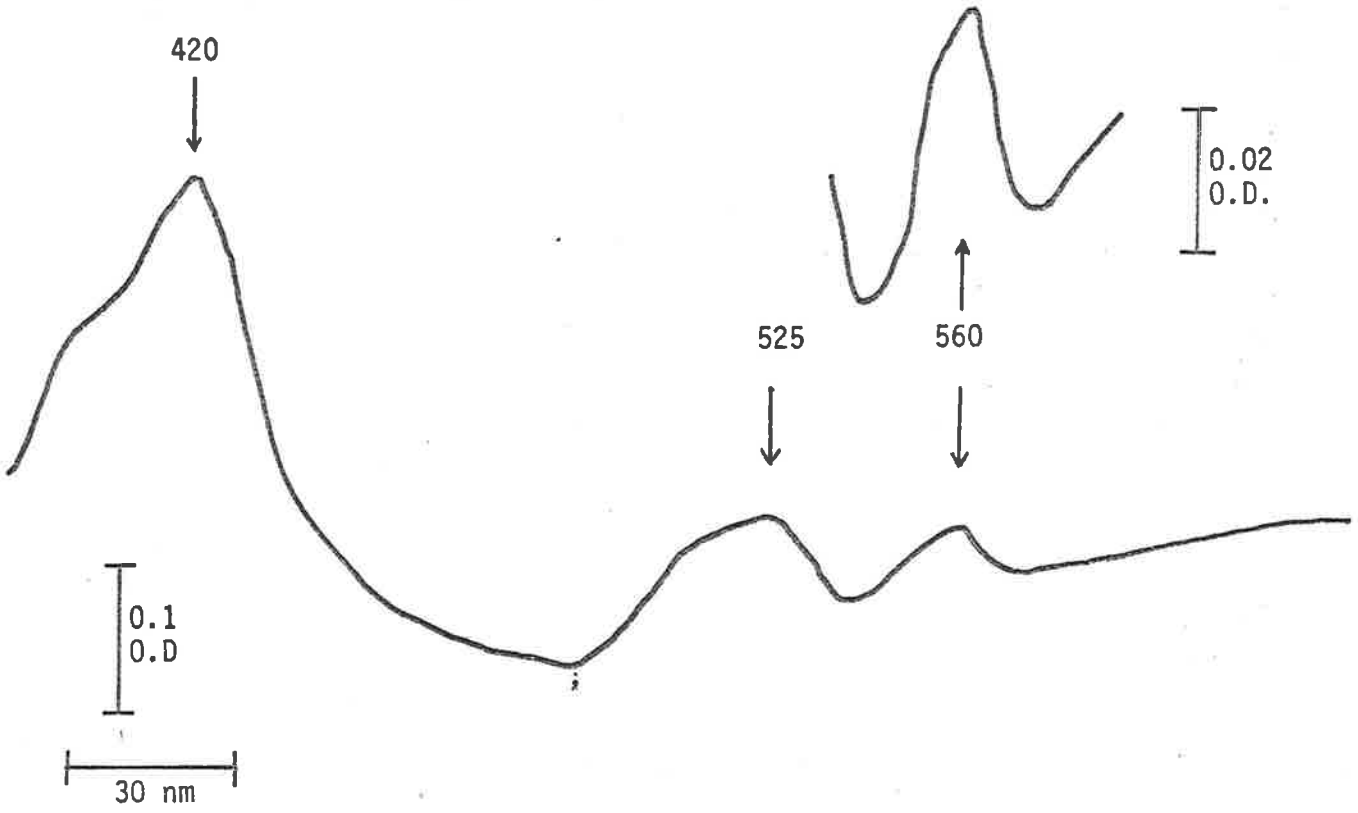
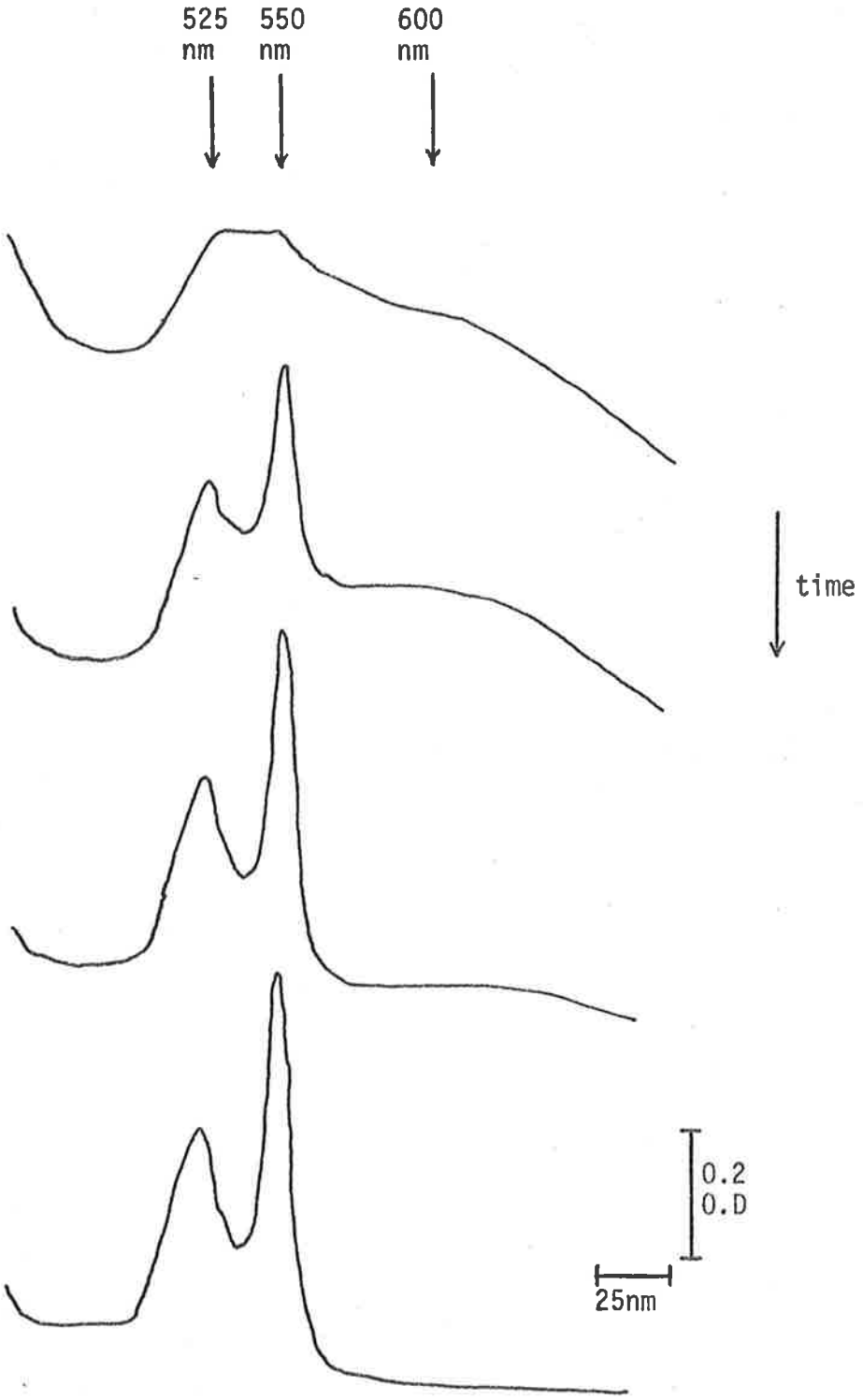


Figure 4.4. Difference spectrum of turnip outer mitochondrial membrane.
Dithionite-reduced minus aerobic. Path length 2 mm, liquid-air temperature, 2 mg protein.

Figure 4.5. Concurrent reduction of DCPIP and cytochrome c by turnip outer mitochondrial membrane.

Repetitive scans of absorbance between 450 and 700 nm were made using a Beckman Acta CIII spectrophotometer. Time taken for each scan was 82.5 seconds. The reaction was started by adding NADH (0.25 mM) to 3 ml of standard reaction medium containing 25 µM DCPIP and 25 µM cytochrome c and 50 µg outer membrane.


525
nm
550
nm
600
nm
time
0.2
O.D
25nm

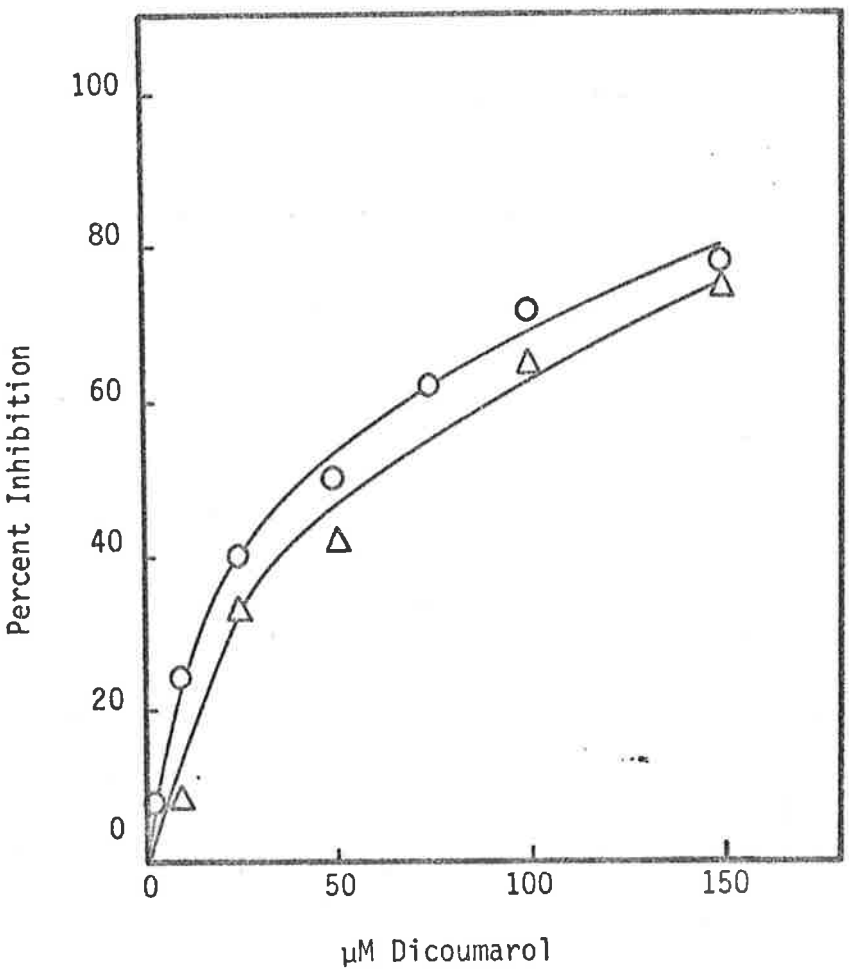


Figure 4.6 Effect of dicoumarol on turnip outer mitochondrial membrane NADH reductases.

Details of assays are given in Materials and Methods. O — O NADH-cytochrome c reductase; Δ — Δ NADH - FeCN reductase.

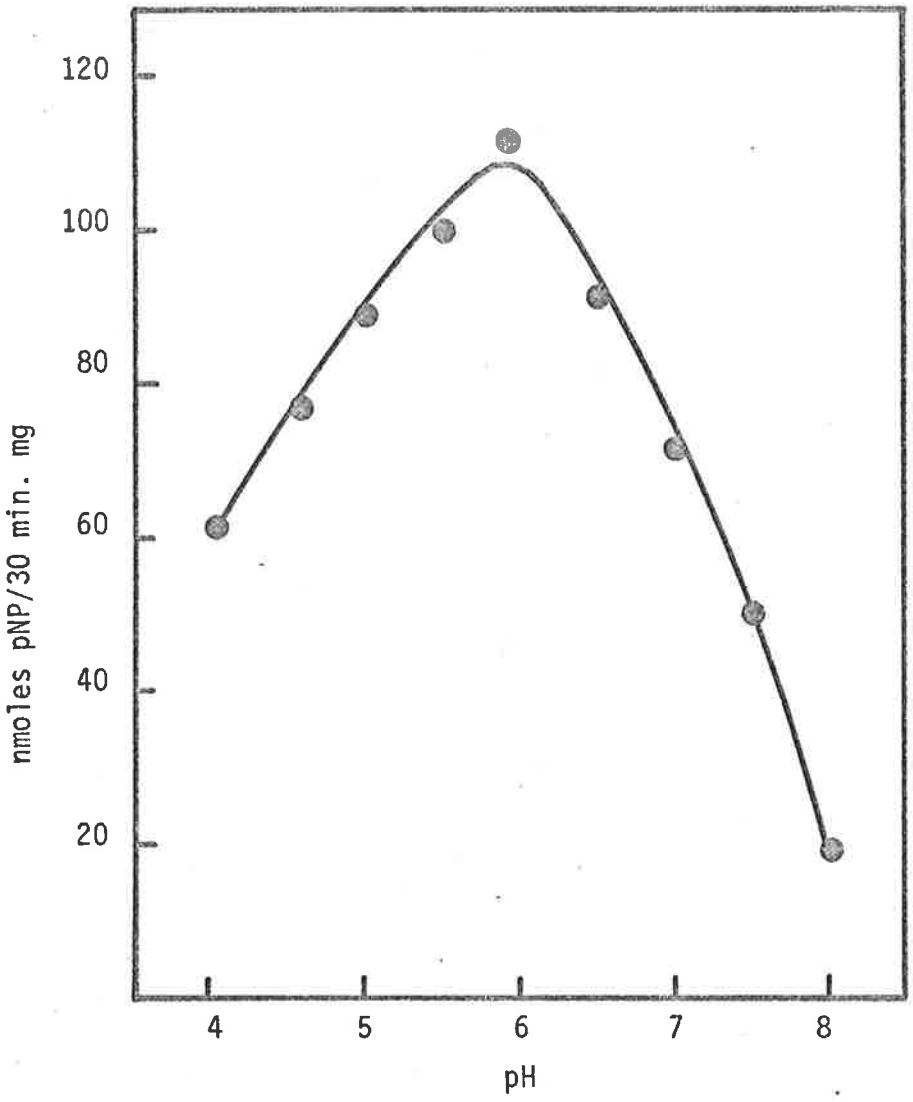
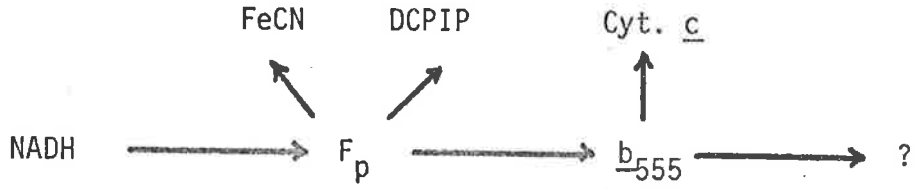


Figure 4.7 Acid phosphatase activity of outer membrane of turnip mitochondria.

Acid phosphatase assayed using p-nitrophenyl phosphate as substrate as described in Methods. 0.1 mg outer membrane protein used per assay.

(a) Outer membrane.



(b) Microsomes. (Rungie and Wiskich, 1972a)

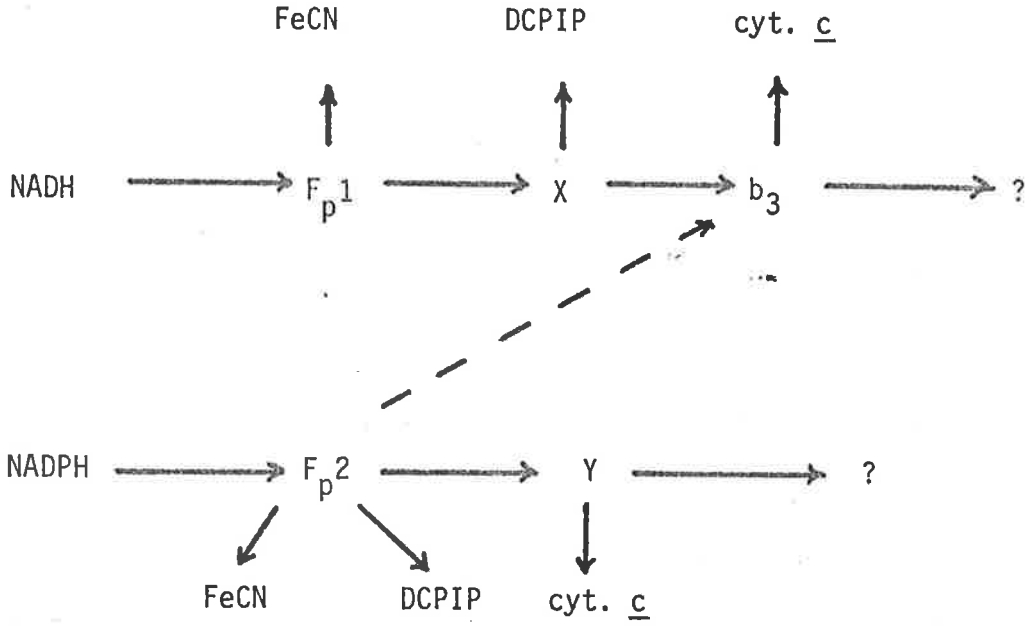


Figure 4.8 Proposed schemes of electron transport in plant microsomes and outer mitochondrial membranes.

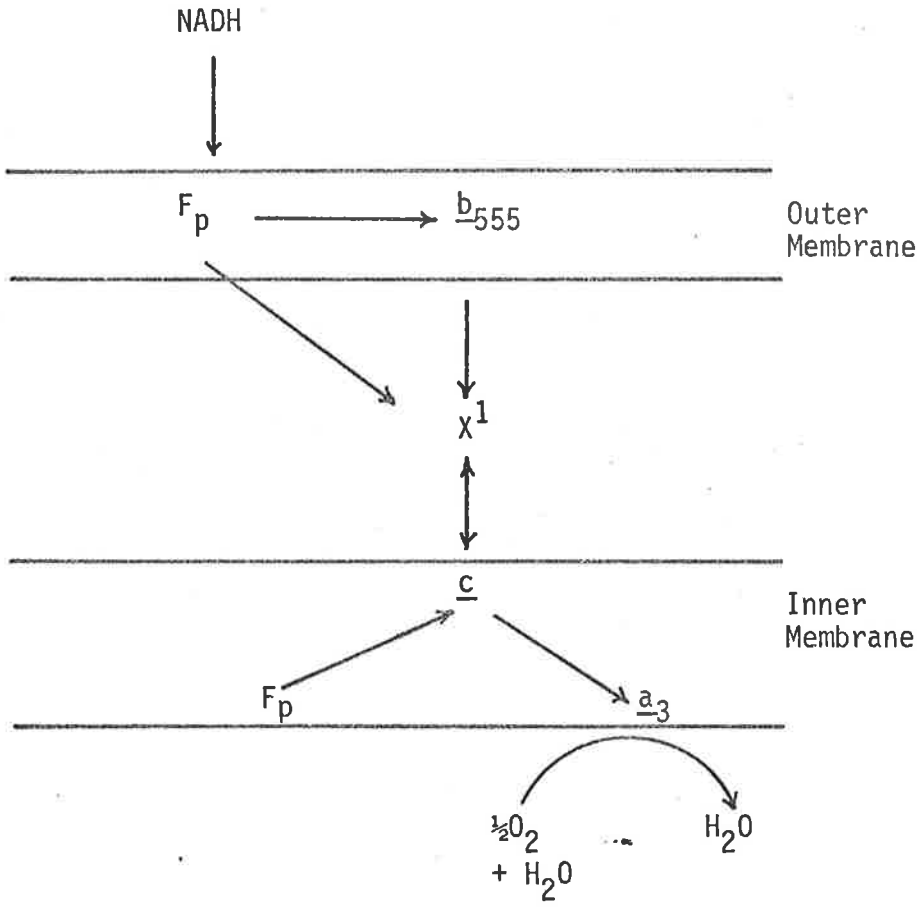


Figure 4.9 Possible intermediate electron transport shuttles in mitochondria.

1. X = electron acceptor and could be endogenous cytochrome c

CHAPTER V

REGULATION OF SUBSTRATE UTILIZATION BY ISOLATED PLANT MITOCHONDRIA

Control of the citric acid cycle in isolated animal mitochondria by oxidative phosphorylation has been demonstrated (Stuart and Williams, 1966; McElroy and Williams, 1968), and control of oxygen uptake accompanying oxidation of specific citric acid cycle intermediates by the concentration of ADP has been investigated in detail in both plant and animal mitochondria. Substrate oxidation by isolated mitochondria can also be partly controlled by anion penetration of the inner membrane. For example, Klingenberg and Pfaff (1968) have shown that substrate oxidation by rat liver mitochondria can be limited by the rate of entry of adenine nucleotides into the matrix.

Williams et al (1972) have demonstrated that flow of carbon through the Kreb's cycle can be regulated by the intramitochondrial malate concentration; hence the malate transporting systems may play a role in control of the cycle. Quagliariello and Palmieri (1971) have shown that succinate oxidation by isolated heart mitochondria can be limited by the rate of succinate transport across the inner membrane; this transport was dependent on a pH gradient across the membrane. Similar observations have been made with rat adrenal mitochondria oxidizing malate (Sauer and Park, 1973).

Plant mitochondria also possess specific anion transporters on their inner membrane (Phillips and Williams, 1973b; Wiskich, 1974) and under certain conditions these transporters can be expected to control the oxidation of their substrates. In fact, Wiskich (1975) has shown that malate and citrate entry, and therefore their oxidation, is dependent on the presence of phosphate in cauliflower bud mitochondria. In the present study, control of malate, succinate, citrate and ∝-ketoglutarate oxidation by isolated plant mitochondria was reinvestigated, with emphasis on anion transport, product inhibition and the effect of exogenous NAD^+.

RESULTS

1. *Succinate oxidation*

Succinate was readily oxidized by isolated cauliflower-bud and beetroot mitochondria, with ADP/O values between 1.4 and 1.9. ATP stimulated both state 2 and state 3 rates of oxygen consumption with succinate as substrate (Fig. 5.1). This effect has been observed with several other plant tissues (Wiskich and Bonner, 1963; Oestreicher et al, 1973; Palmer and Kink, 1974) as well as with mammalian mitochondria (Gutman et al, 1971). ATP is apparently an allosteric activator of succinate dehydrogenase and as such may play an important role in regulation of the Kreb's cycle (Gutman et al, 1971).

Phosphate stimulated succinate oxidation in the presence and absence of ATP and ADP (Fig. 5.1) and in some cases was an absolute requirement for succinate oxidation (Fig. 5.2). Phosphate probably has its effect via the dicarboxylate exchange carrier of the inner membrane, succinate entering the matrix in exchange for phosphate (Chappell and Haarhoff, 1967; Phillips and Williams, 1973b; Wiskich, 1974); this idea is supported by the fact that n-butylmalonate, an inhibitor of the dicarboxylate transporter (Robinson and Chappell, 1967; Wiskich, 1974), inhibited succinate oxidation (Fig. 5.2). This inhibition has also been observed with mung bean mitochondria (Phillips and Williams, 1973a). The different rates of succinate oxidation by different preparations of mitochondria in the absence of phosphate (compare Figs. 5.2 and 5.1), presumably reflect varying degrees of intactness and the amount of endogenous phosphate (and substrates) present.

Wiskich (1975) has observed similar effects of phosphate on malate oxidation by cauliflower bud mitochondria, and has shown that the stimulation is not due to salt respiration or an effect on oxidative phosphorylation (oligomycin did not inhibit the phosphate-stimulated rate in the absence of ADP).

2. *Malate oxidation*

Malate was oxidized readily by cauliflower bud and beetroot mitochondria in the presence of substrate amounts of glutamate (Figs. 5.3B and 5.4B), but when glutamate was omitted from the reaction medium the state 3 rate decreased with time (Figs. 5.3A and 5.4A); subsequent addition of glutamate restored malate

oxidation almost to its maximum rate (Figs. 5.3A and 5.4A). This effect has been observed previously (Macrae, 1971a; Wiskich et al, 1964) and has been interpreted as an accumulation of oxaloacetate which inhibits malate oxidation; when glutamate is present oxaloacetate is transaminated to ∝-ketoglutarate and aspartate via aspartate aminotransferase (Wiskich et al, 1964). However, when the mitochondria were disrupted by the addition of a detergent, oxaloacetate inhibition of malate oxidation was not as marked (Fig. 5.5). In these experiments NAD^+ and PMS were included in the reaction mixture. It was assumed that rupture of the mitochondrial membranes by the detergent ('Decon 90') released malate dehydrogenase which, in the presence of malate, would reduce NAD^+. The NADH thus formed reduced PMS directly, which in turn reduced oxygen to water. The slow rate of oxygen uptake prior to the addition of detergent (Fig. 5.5) suggests that PMS was not reacting with the respiratory chain and that malate dehydrogenase activity was measured upon disruption.

i.e. $\text{malate} + NAD^+ \rightleftharpoons OAA + NADH + H^+$ ----- (1)

$NADH + H^+ + PMS \longrightarrow PMSH_2 + NAD^+$ ----- (2)

$PMSH_2 + O_2 \longrightarrow PMS + H_2O_2$ ----- (3)

$H_2O_2 \longrightarrow H_2O + \frac{1}{2}O_2$[1] ----- (4)

$\text{malate} + \frac{1}{2}O_2 \longrightarrow OAA + H_2O$ ----- (5)

---

[1] The addition of catalase assured that reaction (4) took place.

This result suggests that oxaloacetate accumulates within the mitochondria when the inner membrane is intact. However, addition of oxaloacetate to the external medium severely inhibited malate oxidation; no oxygen uptake occurred until glutamate was added and this rate was slower than that when oxaloacetate was omitted (Fig. 5.6). Clearly, oxaloacetate can readily penetrate the inner membrane of isolated plant mitochondria, a conclusion also reached by Douce and Bonner (1972). Since malate oxidation is inhibited in intact mitochondria (Figs. 5.3A and 5.4A), not all of the oxaloacetate formed can leave the mitochondria; only a small quantity of oxaloacetate would be necessary to inhibit malate oxidation due to the unfavourable equilibrium constant for equation (1). Thus malate dehydrogenase, and oxaloacetate movement across mitochondrial membranes, may play an important role in the regulation of Kreb's cycle activity in plants.

3. *Citrate and ∝-ketoglutarate oxidation*

Citrate was readily oxidized by isolated cauliflower bud mitochondria, but ∝-ketoglutarate oxidation required the addition of ADP to the reaction mixture (Fig. 5.7). ADP/O ratios observed with citrate were close to three (2.7 - 2.9) but ∝-ketoglutarate oxidation gave ADP/O values of 3.3 to 3.6 (Fig. 5.7). The ADP activation of ∝-ketoglutarate oxidation, and the high ADP/O values, were due to the associated substrate-level phosphorylation (Chance and Baltcheffsky, 1968; Wiskich et al, 1964).

State 3 rates of oxygen uptake and respiratory control ratios associated with citrate and ∝-ketoglutarate oxidation were less than those observed when malate was substrate (Figs. 5.7 and 5.6), and often the mitochondria had to be incubated in the presence of substrate and cofactors (ADP, and thiamine pyrophosphate) before ∝-ketoglutarate oxidation was observed (Fig. 5.8). The length of the lag period varied from 1 to 10 minutes depending on the time of the year (a shorter incubation was needed with mitochondria isolated from plants grown during the winter months) and was observed with both cauliflower bud and beetroot mitochondria. Even when ∝-ketoglutarate oxidation had been initiated, an induction period (one S_3 - S_4 transition) was necessary before maximal rates of oxygen uptake were observed (Fig. 5.7). Similar results have been obtained by others (Chance and Baltcheffsky, 1958; Wakiyama and Ogura, 1970; Wiskich et al, 1964). The addition of NAD^+, PMS and detergent during the lag period did not stimulate ∝-ketoglutarate oxidation very much (Fig. 5.8), suggesting that penetration of substrate and cofactors was not limiting. State 3 rates of ∝-ketoglutarate oxidation were generally higher in beetroot mitochondria than in cauliflower bud mitochondria (Figs. 5.7 and 5.8) but this rate also varied with the season.

Citrate oxidation was dependent on the presence of inorganic phosphate (Fig. 5.9B), a result also obtained and discussed by Wiskich (1975). This author also showed that dicarboxylic acids stimulated the entry and oxidation of citrate by cauliflower bud mitochondria; hence malonate was always included in the reaction medium when citrate (and ∝-ketoglutarate) oxidation was studied, to aid its entry into the mitochondria via the tricarboxylate

exchange carrier, and to prevent succinate oxidation. The effect of inorganic phosphate on ∝-ketoglutarate oxidation was not investigated (because of the lengthy incubation period required), but the oxidation of this substrate by beetroot mitochondria was slightly inhibited by n-butylmalonate (Fig. 5.9) suggesting that the dicarboxylate carrier was involved. Although the actual mechanism of ∝-ketoglutarate entry has yet to be determined, it seems plausible that it exchanges for a dicarboxylate ion in a similar manner to the exchange reaction of mammalian mitochondria (Chappell, 1968).

Table 5.1 shows that oxygen uptake coupled directly to the malate and isocitrate dehydrogenases via NAD^+ and PMS was considerably faster than respiratory linked oxygen consumption, suggesting that the oxidation of these substrates in intact mitochondria is restricted by the electron transport chain or by compartmentation of the enzymes and their substrates and products. Little oxygen uptake was observed when $NADP^+$ replaced NAD^+ (Table 5.1) indicating that malate and isocitrate dehydrogenases of plant mitochondria are NAD^+-specific.

4. Glutamate oxidation and transport

Glutamate was not oxidized by intact cauliflower bud mitochondria, and very little NAD-linked glutamate dehydrogenase activity was detected upon disruption of the mitochondria (Fig. 5.10). The effect of glutamate on malate oxidation (Figs. 5.3 and 5.4) must therefore be due solely to aspartate aminotransferase activity.

However arsenite, an inhibitor of ∝-ketoglutarate dehydrogenase, had no effect on malate oxidation in the presence of glutamate (Figs. 5.3 and 5.6), suggesting that the ∝-ketoglutarate formed by the transaminase reaction was not oxidized. This is not surprising in view of the lengthy preincubation necessary to observe ∝-ketoglutarate oxidation in isolated mitochondria; alternatively the ∝-ketoglutarate may have been transported out of the matrix (in exchange for malate) as it was produced.

Transport of glutamate into the matrix was investigated by following swelling of the mitochondria in ammonium glutamate (see Chapter I). Cauliflower and beetroot mitochondria suspended in ammonium glutamate solutions did not swell until both phosphate and a dicarboxylate anion (malate or malonate) were added (Figs. 5.11 and 5.12). Although the extent of this swelling was not great, it was comparable to that in ammonium malate (Figs. 5.11C and 5.12C) and was inhibited by n-butylmalonate (Fig. 5.11B). Presumably, glutamate exchanges across the inner membrane for malate which had entered the matrix in exchange for phosphate. Butylmalonate, by inhibiting malate-phosphate exchange, would also inhibit glutamate - malate exchange. The glutamate transporter of plant mitochondria thus resembles the tricarboxylate carrier (Phillips and Williams, 1973; Wiskich, 1974) and differs from glutamate transport in mammalian (Chappell, 1969; Meijer et al, 1972) and yeast (Chateaubodeau et al, 1974) mitochondria where glutamate exchanges either for hydroxyl ions or aspartate. Wiskich (unpublished results) found that phosphate and malate were necessary to observe swelling of plant mitochondria in ammonium ∝-ketoglutarate solutions.

The transport of citrate, glutamate and ∝-ketoglutarate across the inner membrane of plant mitochondria appears to occur by similar exchange-diffusion mechanisms.

The lack of glutamate oxidation in intact cauliflower bud mitochondria (Fig. 5.10B) cannot be attributed to the absence of a dicarboxylate anion requirement since the addition of malonate did not stimulate oxygen uptake (Fig. 5.10C).

5. *The effect of exogenous NAD^+ on NAD-linked substrate 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. 5.13) and ADP/O ratios were lowered (Table 5.2). When both rotenone and NAD^+ were present, ADP/O values were lower than when NAD^+ alone was added (Table 5.2). This, together with the faster rate of oxygen uptake observed when NAD^+ was present (Table 5.2), 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 exogenous cytochrome *c*, the addition of NAD^+ partly relieved antimycin A inhibition of malate oxidation (Fig. 5.14) indicating that reducing equivalents were transferred

to the outer membrane reductase. Measurements of cytochrome *c* reduction support the oxygen uptake results; the addition of NAD^+, in the presence of malate, to intact mitochondria stimulated the rate of cytochrome *c* reduction several fold, and relieved the inhibition by both rotenone and antimycin A (Table 5.3).

These results indicate that in the presence of added NAD^+ (and rotenone),reducing equivalents from malate oxidation enter the respiratory chain via the external NADH dehydrogenases (see Chapter IV). Two explanations can be forwarded to account for this; (a) malate is oxidized in the intermembrane by malic enzyme (Coleman and Palmer, 1972) or (b) malate is oxidized in the matrix by malate dehydrogenase, and the reducing equivalents transferred to the intermembrane space. Either possibility would explain the lack of rotenone sensitivity and the dependence on added NAD^+.

The relief of rotenone inhibition by exogenous NAD^+ has also been reported by Coleman and Palmer (1972) who attributed it to the action of malic enzyme (Macrae, 1971) in the intermembrane space. In support of their hypothesis, they reported that butylmalonate did not inhibit malate oxidation, by Jerusalem artichoke mitochondria, in the presence of NAD^+ and rotenone. Contrary to this, n-butyl-malonate inhibition of malate oxidation by cauliflower bud mitochondria was independent of added NAD^+ (Fig. 5.15). Furthermore, addition of NAD^+ (and rotenone) had little effect on malate oxidation in the absence of inorganic phosphate (Fig. 5.16), suggesting that the entry of malate into the matrix (via the phosphate-malate carrier) is a prerequisite for its oxidation.

These results suggest that alternative (b) above is the most likely explanation. Further support comes from studies with citrate and ∝-ketoglutarate. The oxidation of both these substrates was inhibited by rotenone, but this inhibition was relieved to varying degrees by the addition of 1 mM NAD^+ (Table 5.4). Table 5.5 shows that ADP/O ratios and respiratory control ratios associated with citrate oxidation were lowered when NAD^+ and rotenone were added to the medium, in a similar manner to malate oxidation (Table 5.2). These results suggest that transfer of reducing equivalents across the inner membrane can occur with all NAD-linked substrates under certain conditions.

6. Concurrent oxidation of more than one substrate

When a combination of any two of malate, succinate and NADH was used, oxygen uptake rates were greater than those associated with the oxidation of a single substrate (Table 5.6). Upon the addition of malonate to mitochondria respiring NADH plus succinate, or malate plus succinate, oxygen consumption rates reverted to those found with malate or NADH alone (Table 5.6). Similarly, the addition of rotenone during oxidation of NADH plus malate resulted in oxygen uptake rates similar to those observed when only NADH was substrate (Table 5.6). That is, electrons entered the respiratory chain at different sites during the oxidation of more than one substrate. ADP/O values associated with the oxidation of malate plus succinate, and malate plus NADH, were intermediate between values usually indicative of two and three sites of phosphorylation (Table 5.6).

On the other hand, oxygen uptake with malate plus citrate was no faster than that with malate alone as substrate (Table 5.6).

When NADH oxidation was monitored spectrophotometrically, it was found that the addition of malate or succinate caused an inhibition (Table 5.7). This inhibition was more pronounced when both malate and succinate were present (Table 5.7).

Investigations with mammalian mitochondria have shown that NAD(P)H can activate succinate dehydrogenase either directly (Rassmussen, 1971) or via ubiquinone reduction (Gutman et al, 1971). However, studies with sub-mitochondrial particles from heart and liver (Gutman and Silman, 1972; Davis et al, 1969) indicate that succinate oxidation can inhibit NADH oxidation, perhaps by keeping the ubiquinone pool reduced so that it cannot receive reducing equivalents from NADH (Gutman and Silman, 1972).

DISCUSSION

1. *Anion transport in isolated plant mitochondria*

The results presented in this chapter (Figs. 5.1 and 5.2) show that the oxidation of succinate, like that of malate and citrate (Wiskich, 1975), can be limited by the rate of substrate entry into the matrix. In intact mitochondria this entry is governed by the presence or absence of phosphate, whose transport in turn depends on a pH gradient across the inner membrane (Chappell and Haarhoff, 1967; Phillips and Williams, 1973).

Figure 5.17 illustrates some of the probable anion transporting systems of plant mitochondria.

At the present time it appears that transport of glutamate, α-ketoglutarate and tricarboxylate acids involves a similar mechanism. Transporters III and IV in Figure 5.17 could well be identical. Hence, complex substrate shuttles such as occur in animal mitochondria (See Chapter I) may not operate in plant cells. This suggestion is supported by the apparent ease with which oxaloacetate moves across the inner membrane of plant mitochondria (Fig. 5.6; see also Douce and Bonner, 1972). This movement would 'short-circuit' a shuttle such as the malate-aspartate cycle of rat liver. Control of anion penetration is essential, however, to preserve membrane potentials and gradients. Oxaloacetate movement must therefore be controlled in some manner, not only to maintain membrane gradients, but also to keep the Kreb's cycle operating. Perhaps during turnover of the TCA cycle, when acetyl CoA is in sufficient supply, high intramitochondrial oxaloacetate levels would not occur, minimizing efflux of carbon skeletons from the mitochondrial matrix.

Obviously it is the higher permeability of plant mitochondrial membranes to oxaloacetate which allows malate oxidation to occur in isolated plant mitochondria, in contrast to mammalian mitochondria. This permeability cannot be attributed to a general 'leakiness' of the inner membrane since the movement of other intermediates is strictly controlled in isolated plant mitochondria (Figs. 5.2, 5.9 and 5.16; Wiskich, 1975).

The importance of the anion movements discussed above to Kreb's cycle activity *in vivo* may not be very great, since flow of carbon through the cycle ultimately depends on influx of carbon skeletons from glycolysis, either via acetate or pyruvate.

Transport of these two substances has not been studied extensively in plant mitochondria, although Lee and Wilson (1972) observed active swelling of bean mitochondria with potassium acetate. This swelling was inhibited by DNP and an ATP-generating system, suggesting that acetate transport is controlled by the pmf across the inner membrane. Studies with rat liver mitochondria (Halestrap and Denton, 1974) suggest that a specific carrier for pyruvate exists on the inner membrane of animal mitochondria. A similar carrier may be present in plant mitochondria, since pyruvate-induced swelling of bean-shoot mitochondria was inhibited by DNP, although to a less extent than acetate-induced swelling (Lee and Wilson, 1972). The acetate and pyruvate transporters can be expected to play a greater role in the regulation of Kreb's cycle turnover rates *in vivo* than the dicarboxylate and tricarboxylate carriers.

However, the transporting systems shown in Figure 5.17 are obviously essential to maintain ion and electrical gradients across the mitochondrial membrane, and may also be important during gluconeogenesis. In plants which store organic acids (such as malate) in their vacuoles, these carriers may also regulate mitochondrial oxidations. For example, during malate oxidation in the presence of glutamate, the malate transporter may regulate (at least partially) the respiration rate and energy production.

The role of anion transporting systems in the oxidation of cytoplasmic NADH by animal mitochondria has been discussed in Chapter I.

Since plant mitochondria can oxidize exogenous NADH directly (see Chapter III), these transporters are probably not involved in oxidation of cytoplasmic reducing power in plant cells.

2. *Control of substrate oxidation by dehydrogenases*

The activity of individual dehydrogenases may also regulate substrate oxidation by isolated plant mitochondria. Isocitrate oxidation by intact and disrupted mitochondria was considerably slower than malate oxidation (Table 5.1), suggesting lower levels or lower activity of isocitrate dehydrogenase. Citrate and isocitrate oxidation in intact mitochondria may also be restricted by the equilibrium of the aconitase reaction (Lehninger, 1970) and by NADH levels within the matrix (Cox and Davies, 1967). Unlike the mammalian enzyme (Lehninger, 1970) the plant mitochondrial isocitrate dehydrogenase does not appear to be allosterically regulated by adenine nucleotides (Cox and Davies, 1967).

The lengthy lag period observed before isolated mitochondria oxidized ∝-ketoglutarate (Fig. 5.9) is probably due to deactivation of the ∝-ketoglutarate dehydrogenase complex during isolation (e.g. by depletion of endogenous substrate and cofactors) and may not reflect the *in vivo* situation.

The results shown in Table 5.6 suggest that the rate limiting steps involved in oxygen uptake, upon oxidation of malate or succinate, are the internal NADH and succinate dehydrogenases of the respiratory chain.

This idea is supported by the faster rate of malate oxidation by disrupted than by intact mitochondria (Table 5.1) which indicates that malate dehydrogenase activity was not rate limiting. The fact that oxygen uptake with malate as substrate was stimulated by the addition of NAD^+ also suggests that the internal NADH dehydrogenase limits the rate of malate oxidation (at least in the presence of excess malate, glutamate and phosphate), in intact mitochondria. Oxygen uptake with malate plus succinate was less than the combined rates with the single substrates (Table 5.1), suggesting that succinate and malate may compete for the dicarboxylate carrier, when added simultaneously.

Since citrate oxidation was slower than malate oxidation (Tables 5.1 and 5.6), NADH dehydrogenase activity obviously was not limiting (see above). The presence of citrate did not lead to faster rates of oxygen uptake with malate (Table 5.6), suggesting that reducing equivalents from these two substrates enter the electron transport chain via a common NADH dehydrogenase.

However, exogenous NADH oxidation *per se* was inhibited by the addition of succinate and malate (Table 5.7). This implies a competition for a component common to the oxidation pathways of all three substrates (perhaps ubiquinone), and explains why the oxygen uptake rate with two or more of these substrates was less than the aggregate of the rates associated with the oxidation of the substrates when they were added singly (Table 5.6).

3. *Pyridine nucleotide specificity of dehydrogenases*

The lack of NADP-linked isocitrate dehydrogenase activity

(Table 5.1) suggests that isocitrate oxidation by plant mitochondria is not involved in producing reducing power for biosynthetic pathways (e.g. fatty acid biosynthesis), in contrast to mammalian mitochondria (Lehninger, 1970). However, there is some confusion over the presence of NADP-linked isocitrate dehydrogenase in different plant mitochondria. For example, Ragland and Hackett (1964) reported no significant NADP-linked activity in pea stem mitochondria, while Yamamoto (1969) claimed that bean cotyledon mitochondrial isocitrate dehydrogenase showed a higher activity with $NADP^+$ than with NAD^+. Cox and Davies (1967) found that 95% of NADP-linked isocitrate dehydrogenase activity was located in the soluble fraction of pea shoots, while NAD^+-specific activity was confined to the mitochondria.

Production of NADPH by plant mitochondria may be restricted to the energy-linked NAD(P)H-transhydrogenase reaction (if no NADP-linked dehydrogenases are present), although only tentative evidence for the existence of this enzyme in plant mitochondria has been provided (Wilson and Bonner, 1970).

4. *Alternative pathways for NAD-linked substrate oxidation*

The results presented here show that in isolated cauliflower bud mitochondria, malate is oxidized only within the matrix; in the presence of NAD^+ reducing equivalents are transferred across the inner membrane. 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 phos-

phate. Since phosphate is necessary for malate penetration across the inner membrane via the malate transporter, which is inhibited by n-butyl-malonate (Robinson and Chappell, 1967; Wiskich, 1975), one 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 mitochondria (Macrae and Moorehouse, 1970), is either localized in the matrix or is not functional in the mitochondria studied here.

The major discrepancy between these results (Fig. 5.15) and those of Coleman and Palmer (Fig. 3, 1972) is the differing effects observed with butylmalonate. Coleman and Palmer's results are difficult to interpret since they used high concentrations of malate (100 mM) and had $CaCl_2$ present. Oxygen uptake by cauliflower bud mitochondria was not strongly inhibited by butylmalonate when high malate concentrations were used, and the presence of $CaCl_2$ caused an even smaller inhibition (Table 5.8). Butylmalonate did not inhibit as much when it was added during malate oxidation as it did when added prior to malate (compare Fig. 5.15 and Table 5.7). Coleman and Palmer (1972) did not say at what stage butylmalonate was added to their mitochondria fron Jerusalem artichoke. The conflicting results may also reflect the different tissues (cauliflower and artichoke) used, although the present author observed similar effects to those reported here, when turnip roots were used as a mitochondrial source.

Hatch and Kagawa (1974) found that NAD-specific malic enzyme activity of *Atriplex spongiosa* bundle sheath cells showed a similar distribution to fumarate hydratase activity, a marker for the matrix of mitochondria. These authors also found that malic enzyme of isolated mitochondria was not available to exogenous NAD^+; very little activity was observed unless digitonin was added to the mitochondria. Hatch and Kagawa (1974) therefore concluded that NAD malic enzyme was located in the matrix. Sauer and Park (1973) and Sauer (1973) reached the same conclusion from their studies on NAD-dependent malic enzyme activity in rat liver and adrenal mitochondria, since phosphate (which stimulated malate penetration) was necessary to observe malate oxidation by malic enzyme.

Although Phillips and Williams (1973a) reported that butylmalonate inhibited malate dehydrogenase in mung bean mitochondria, Wiskich (1975) has shown that butylmalonate did not affect malate oxidation by disrupted cauliflower and beetroot mitochondria, nor the reduction of oxaloacetate to malate. The results obtained in the present study, in the absence of inorganic phosphate (Fig. 5.16), also suggestthat butylmalonate has its site of action at the level of malate entry into the mitochondria, rather than at malate dehydrogenase itself.

The fact that exogenous NAD^+ relieved the rotenone inhibition of citrate and ∝-ketoglutarate oxidation also suggests that a transmembrane transfer of reducing equivalents is involved. If the NAD^+ effect was attributable to intermembrane enzyme activity, then enzymes capable of oxidizing citrate and ∝-ketoglutarate must also be present in this outer compartment. However, there is no

evidence to support this. Glyoxysomes, which are capable of oxidizing malate and reducing NAD^+ (Breidenhach et al, 1968; Marcus and Velasco, 1960), cannot be implicated since these organelles do not possess enzymes which can oxidize citrate and ∝ketoglutarate (Breidenbach et al, 1968).

The oxidation of NAD-linked substrates by isolated plant mitochondria, and their interactions with exogenous NAD^+, are summarized in Figure 5.18. How intramitochondrial NADH communicates with extramitochondrial NAD^+ is not clear. It could be either by (1) a transhydrogenase across the inner membrane, capable of transferring reducing equivalents from within the matrix to the intermembrane space upon oxidation of NAD-linked substrates in the matrix, or (2) transport of NAD^+ and NADH across the inner membrane. Once NADH is present in the outer compartment, it can be oxidized by the external NADH dehydrogenase located on the outside of the inner membrane (see Chapter III).

The observations reported here could also be due to leakage of pyridine nucleotides across the inner membrane, particularly if the mitochondria had been damaged or swollen during isolation. This does not seem likely, however, since malate oxidation was dependent on added phosphate and inhibited by butylmalonate. That is, the mitochondria were not 'leaky' to organic acids. Other integrity assays are reported in the following chapter.

In summary, the results presented in this chapter suggest that in isolated plant mitochondria; (a) In the absence of inorganic phosphate substrate oxidation is limited by the inner membrane transporting systems. (b) When phosphate is present malate, exogenous

NADH and succinate oxidation are limited by the activity of the respiratory-linked dehydrogenases; citrate and ∝-ketoglutarate oxidation are restricted by isocitrate and ∝-ketoglutarate dehydrogenase activity. (c) Malate oxidation is inhibited by oxaloacetate accumulation although oxaloacetate can readily penetrate the inner membrane. Glutamate relieved oxaloacetate inhibition. (d) Glutamate entry into plant mitochondria is stimulated by phosphate and dicarboxylate ions, and inhibited by n-butylmalonate. (e) Malate and isocitrate dehydrogenases are NAD-specific. (f) Malate-oxidizing enzymes are confined to the matrix. (g) In the presence of exogenous NAD^+, reducing equivalents are transferred out of the matrix upon oxidation of NAD-linked substrates.

The significance of the above findings to *in vivo* control of Kreb's cycle activity is not clear and they may only represent secondary control mechanisms. The observation that oxidative phosphorylation (i.e. ADP levels) limits respiration in fresh storage tissue (e.g. see Ap Rees and Beevers, 1960), suggests that primary control mechanisms involve the energy charge of the cell (i.e. adenine nucleotide concentrations and allosteric effects on cytoplasmic and mitochondrial enzymes, as well as oxidative phosphorylation).

Table 5.1 Malate and isocitrate oxidation by cauliflower bud mitochondria.

Oxygen uptake was measured as described in Materials and Methods. 10 mM malate, 10 mM isocitrate, 0.5 mM NAD^+ and 0.03 mM PMS were used. The mitochondria were disrupted by adding 30 µl of 'Decon 90' detergent concentrate. Rates shown are averages of three experiments, and are expressed as nmoles O_2 consumed/min. mg protein.

	Intact mitochondria	Disrupted mitochondria	
Substrate	State 3 rate	NAD+PMS	NADP+PMS
malate	86	160	19
isocitrate	27	74	7

Table 5.2 The effect of exogenous NAD^+ and rotenone on malate oxidation by cauliflower bud mitochondria

Conditions of assay are described in Materials and Methods. Final concentrations were; 10 mM malate, 10 mM glutamate, and 0.26 mM ADP.

Other Additions		Oxygen uptake State 3	State 4	R.C.R.	ADP/O
		nmoles / min.mg protein		*Ratio*	
None	(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^+ and 7 µM rotenone	(1)	48	29	1.6	1.8
	(2)	44	24	1.8	1.8

* Numbers in parenthesis indicate separate experiments

Table 5.3 The effect of exogenous NAD^+ on malate cytochrome c reductase activity of isolated cauliflower mitochondria

Cytochrome *c* reductase was assayed as described in Materials and Methods. 10 mM malate was used as substrate. (1) and (2) indicate separate experiments.

Other Additions		Malate - cytochrome *c* reductase activity		
		Control	7 µM Rotenone	5 µM Antimycin A
		nmoles cyt. *c* reduced / min.mg protein		
None	(1)	2.3	1.2	0
	(2)	3.3	1.6	0
0.5 mM NAD^+	(1)	13.8	12.9	8.1
	(2)	28	27	18.9

Table 5.4 The effect of exogenous NAD^+ on malate citrate and ∝-ketoglutarate oxidation

Oxygen uptake was measured as described in Materials and Methods. 20 mM malate, 20 mM citrate, 10 mM ∝-ketogultarate, 0.15 mM ADP and 2 mg of mitochondrial protein were used. In addition, 20 mM glutamate was added to the reaction mixture when malate was substrate, and 10mM malonate when citrate or ∝-ketoglutarate were used. (1) and (2) indicate separate experiments.

Substrate		State 3 oxygen uptake		
		Control	+12 μM rotenone	+12 μM rotenone and 1 mM NAD^+
		nmoles O_2/min. mg. protein		
malate	(1)	63	9	48
	(2)	78	12	50
citrate	(1)	29	7	27
	(2)	31	10	33
∝ keto-glutarate	(1)	24	6	18
	(2)	29	9	18

Table 5.5 The effect of NAD^+ and rotenone on citrate oxidation

Conditions of assay are described in Materials and Methods. 20 mM citrate was used and 5 mM malonate was included in the reaction mixture. (1) and (2) indicate different experiments. 2.5mg mitochondrial protein was used per assay.

Other Additions		Rate O_2 uptake State 3	State 4	R.C.R.	ADP/O
		nmoles/min.mg. protein		*Ratio*	
None	(1)	53	26	2.0	2.7
	(2)	59	28	2.1	2.7
1 mM NAD^+ and 12 µM rotenone	(1)	40	30	1.3	1.6
	(2)	45	33	1.36	1.8

Table 5.6 Concurrent oxidation of more than one substrate by cauliflower bud mitochondria.

Oxygen consumption was measured as described in Materials and Methods. 10 mM malate, 5 mM malonate, 10 mM succinate, 10 mM citrate, 15 µM rotenone, 1 mM NADH and 0.25 mM ADP were used. Rates shown are averages of at least two experiments. 10 mM glutamate was included in the reaction medium when malate was used, and 5 mM malonate when citrate was used. Otherwise malonate (and rotenone) was added during state 3 respiration. 2.4 mg mitochondrial protein was used.

Table 5.6 Concurrent oxidation of two or more different substrates by cauliflower bud mitochondria.

Substrate (s)	State 3 rate	State 4 rate	+ rotenone	+malonate	ADP/O
	nmoles O_2 / min. mg. protein				*Ratio*
malate	69	26	27	-	2.74
succinate	90	43	-	19	1.9
malate + succinate	120	41	-	60	2.2
NADH	118	51	-	-	1.25
NADH + succinate	167	85	-	112	1.6
NADH + malate	160	62	100	-	1.7
citrate	28				
malate + citrate	61	26	-	-	2.4
malate + succinate + NADH	173	64	-	-	1.45

Table 5.7 NADH oxidation by cauliflower bud mitochondria in the presence of succinate and malate.

NADH oxidation was followed spectrophotometrically as described in Materials and Methods. 0.5 mM NADH, 10 mM succinate, 10 mM malate and 0.2 mM ADP were used. Rates are expressed as nmoles NADH oxidized / min. mg. protein. 0.6 mg mitochondrial protein was used per assay.

	NADH oxidation	
Substrate	+ ADP	preincubated with ADP and succinate
NADH	258	208
NADH + malate	209	177

Table 5.8 Effect of malate concentration on n-butylmalonate inhibition of malate oxidation by cauliflower bud mitochondria

Malate oxidation was measured polarographically as described in Materials and Methods. 2mg mitochondrial protein was used and butylmalonate was added during state 3 respiration. Control (state 3) rate of oxygen uptake was 120 nmoles/min. mg protein.

malate concentration	n-butylmalonate concentration	percent inhibition
10 mM	8 mM	50
	16 mM	60
17 mM	8 mM	42
	16 mM	54
100 mM	8 mM	30
	16 mM	40
100 mM + 0.5 mM $CaCl_2$	8 mM	20
	16 mM	33

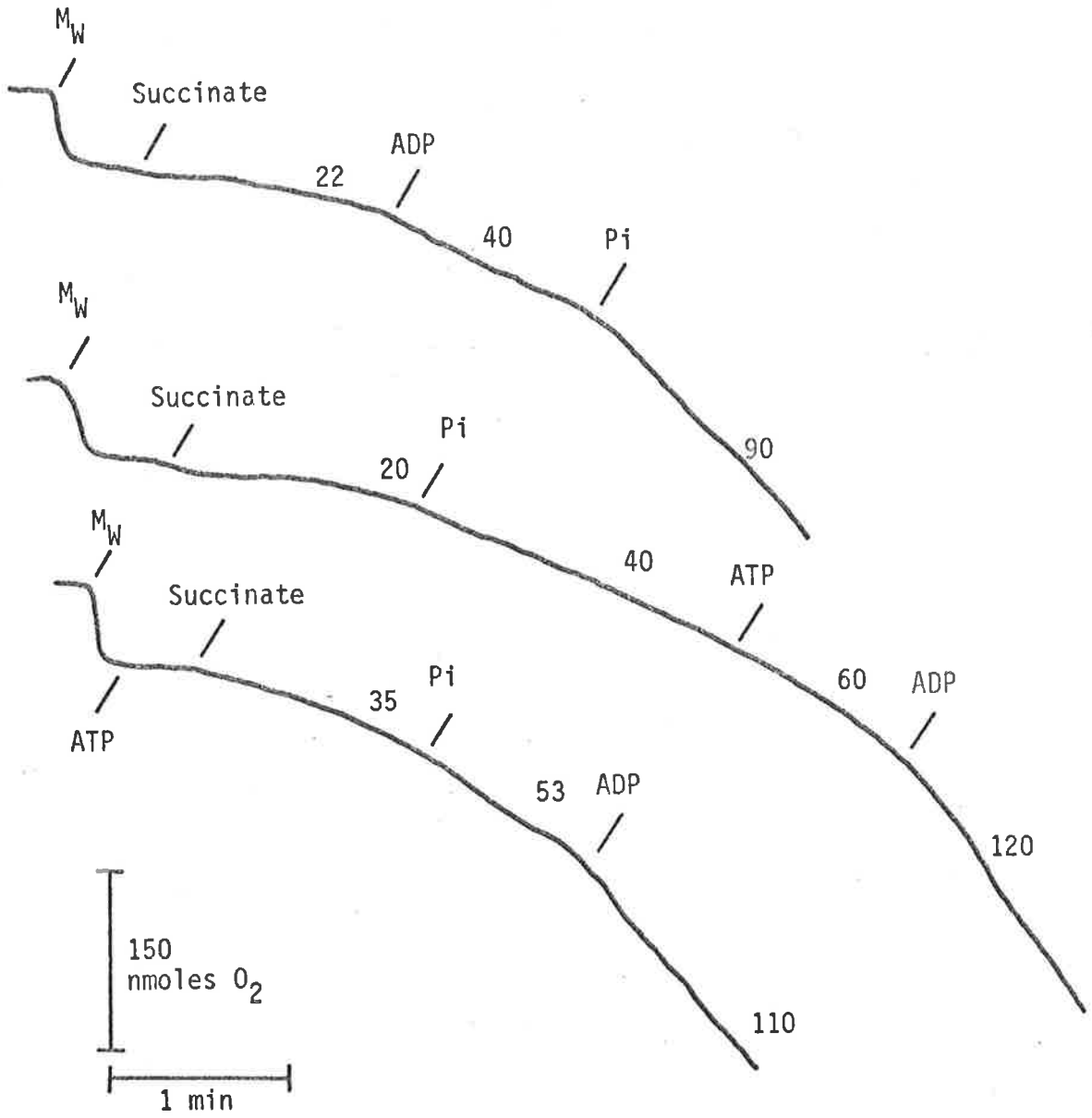


Figure 5.1 Effect of phosphate and adenine nucleotides on succinate oxidation by cauliflower bud mitochondria.

1.7 mg mitochondrial protein was added to 3 ml of standard reaction medium as described in Materials and Methods. Additions as indicated were: 10mM succinate, 0.17 mM ADP, 0.35 mM ATP, 10 mM phosphate. Rates are expressed as nmoles O_2 consumed/min. mg protein. Phosphate was omitted from the reaction medium and added as shown.

Figure 5.2 Effect of phosphate on succinate oxidation by beetroot mitochondria.

Assay conditions are given in Figure 5.1. In addition 15 mM butylmalonate (BM) was added as indicated. Rates are expressed as nmoles O_2/min.mg protein. In trace A, phosphate was omitted from the reaction medium and added as shown.

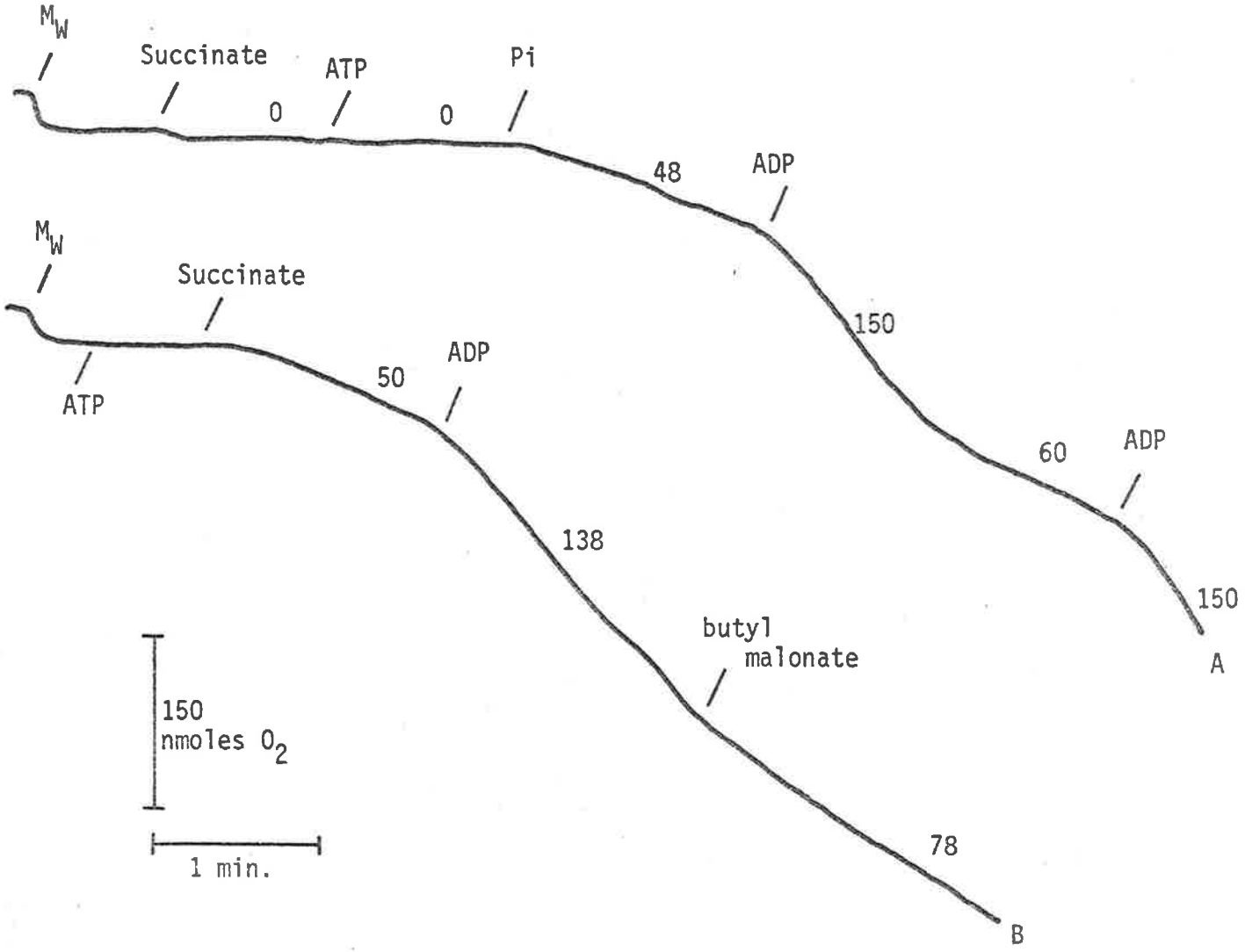
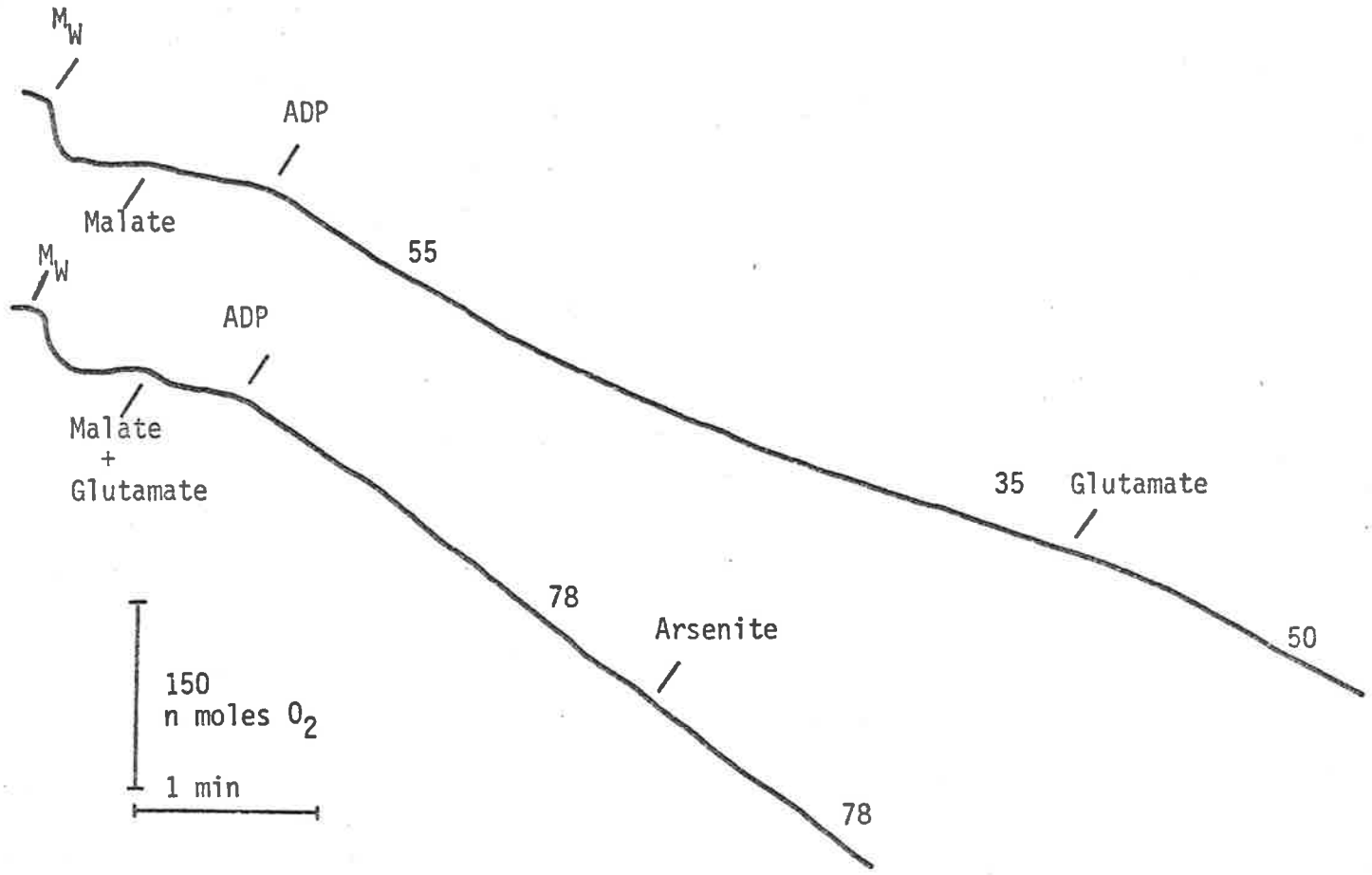

M_W
Succinate
ATP
Pi
0
0
48
ADP
150
60
ADP
150
A
M_W
Succinate
ATP
50
ADP
138
butyl
malonate
78
B
150
nmoles O_2
1 min.

Figure 5.3 The effect of glutamate on malate oxidation by intact cauliflower mitochondria

1.6 mg mitochondrial protein was added to 3ml of standard reaction mixture as described in Materials and Methods. Additions as indicated were, 16 mM malate, 16 mM glutamate, 0.75 mM ADP and 6 mM arsenite. Rates are expressed as nmoles O_2 consumed/min.mg protein.


M_W
ADP
Malate
55
M_W
ADP
Malate
+
Glutamate
35
Glutamate
78
Arsenite
50
150
n moles O_2
1 min
78

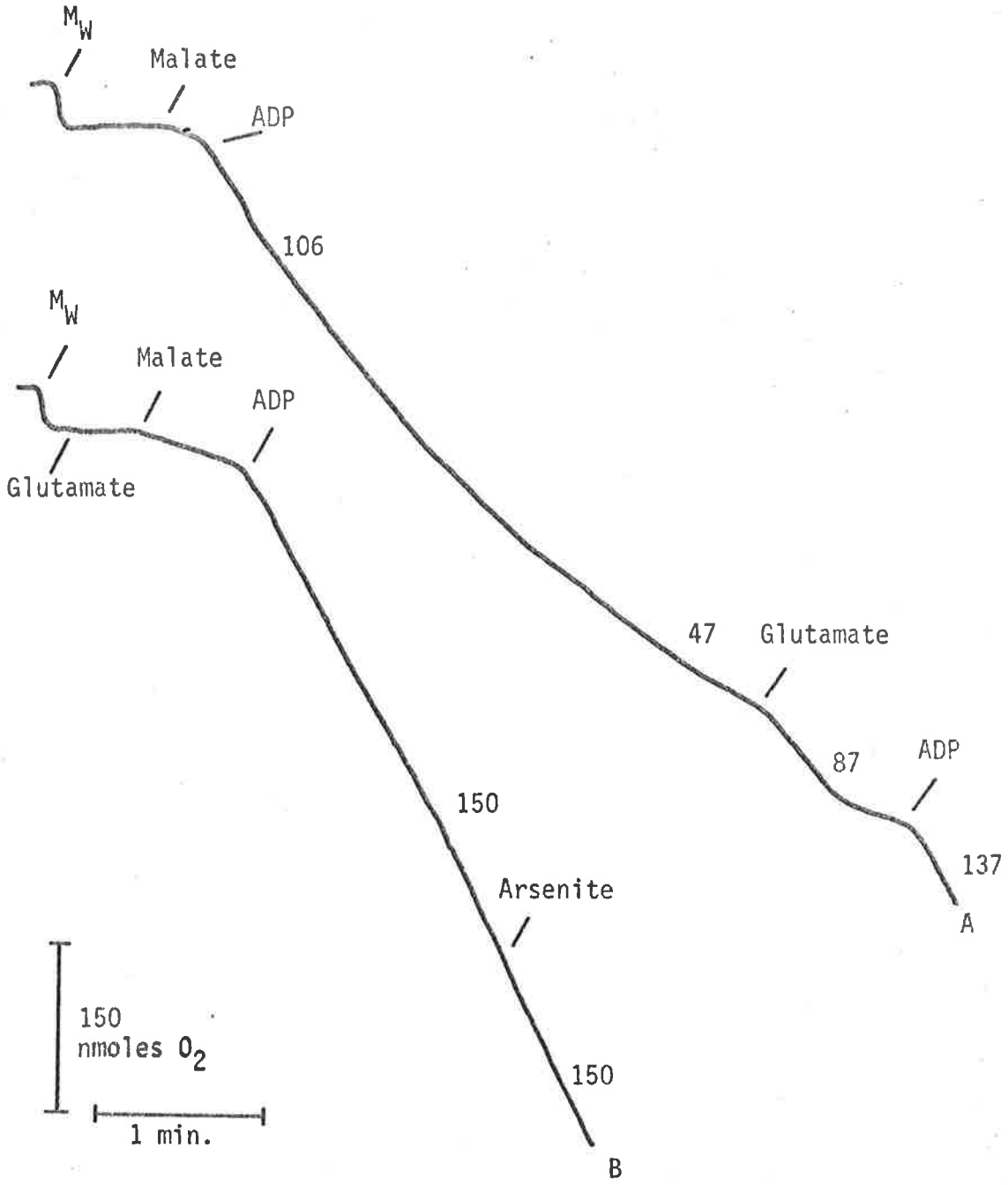


Figure 5.4 The effect of glutamate on malate oxidation by intact beetroot mitochondria.

Oxygen uptake was measured as described in Materials and Methods. Additions as indicated were, 2 mg mitochondrial protein (M_W), 16 mM malate, 16 mM glutamate, 0.75 mM ADP. Rates are expressed as nmoles O_2 consumed/min. mg protein.

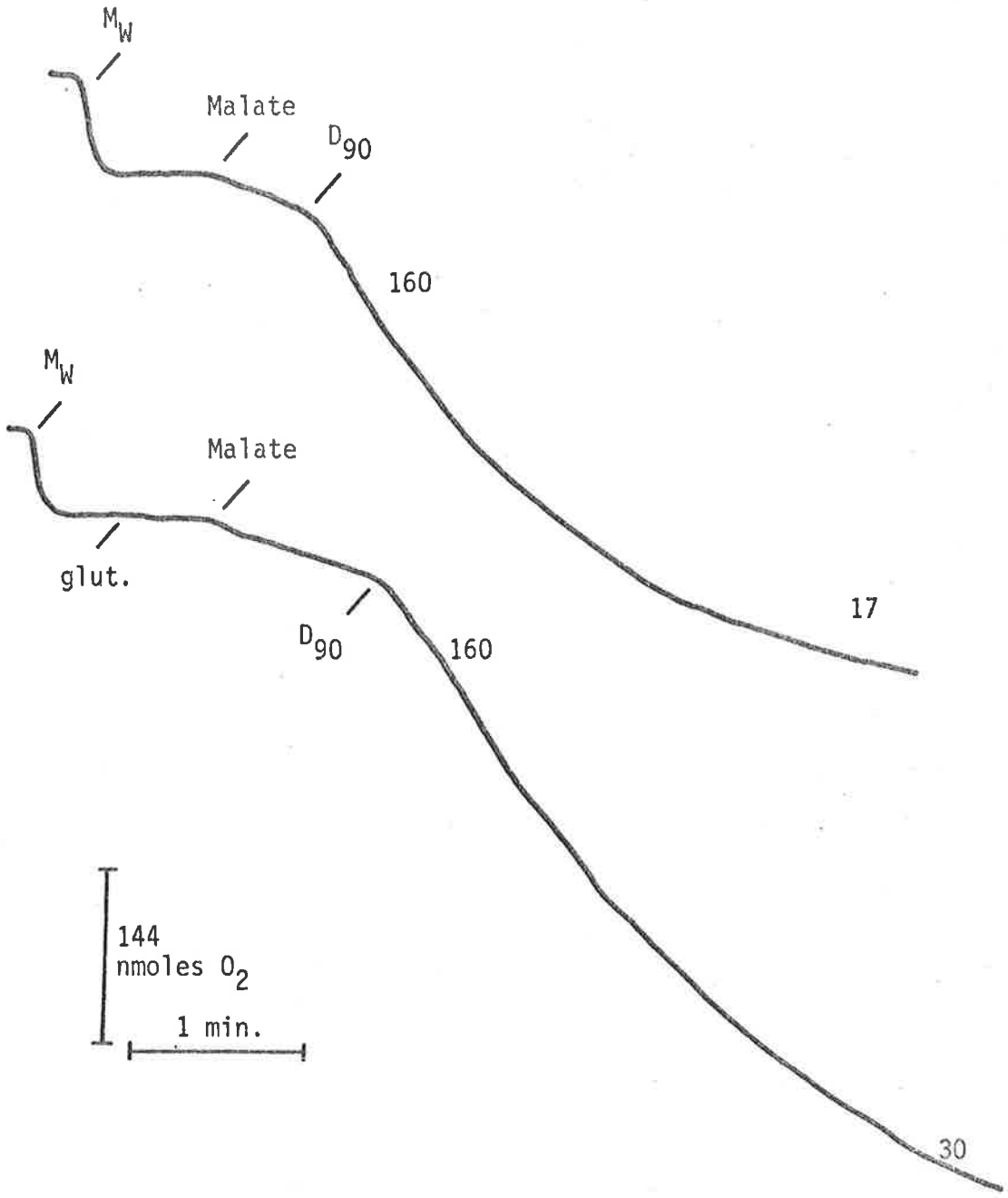


Figure 5.5 Malate oxidation by disrupted cauliflower bud mitochondria.

Conditions of assay are given in Figure 5.3, except 0.5 mM NAD^+ and 0.025 mM PMS were included in the reaction mixture, and 25 µl of 'Decon 90' (D_{90}) detergent concentrate was added as shown. Rates are expressed as nmoles O_2/min.mg protein.

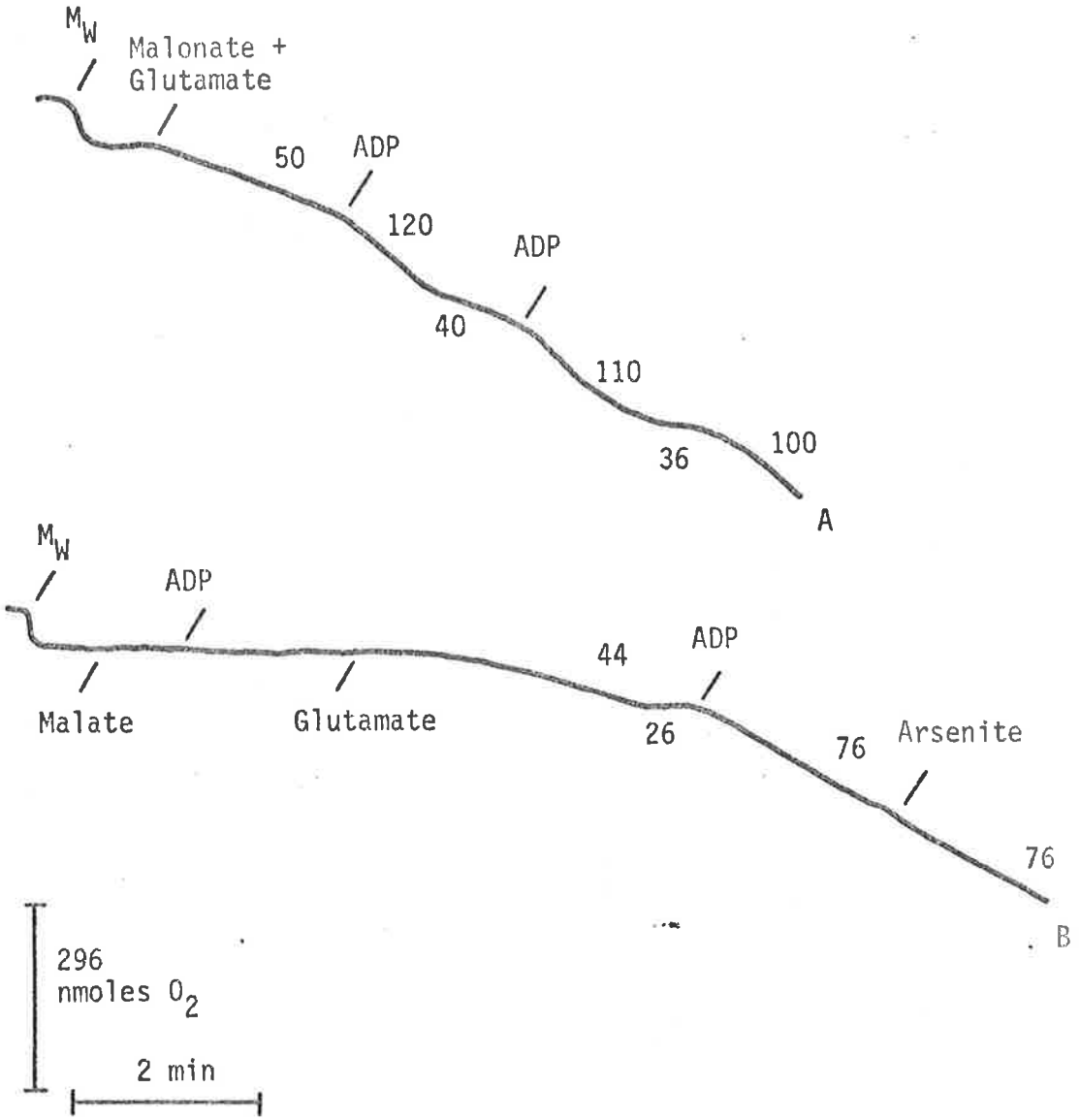
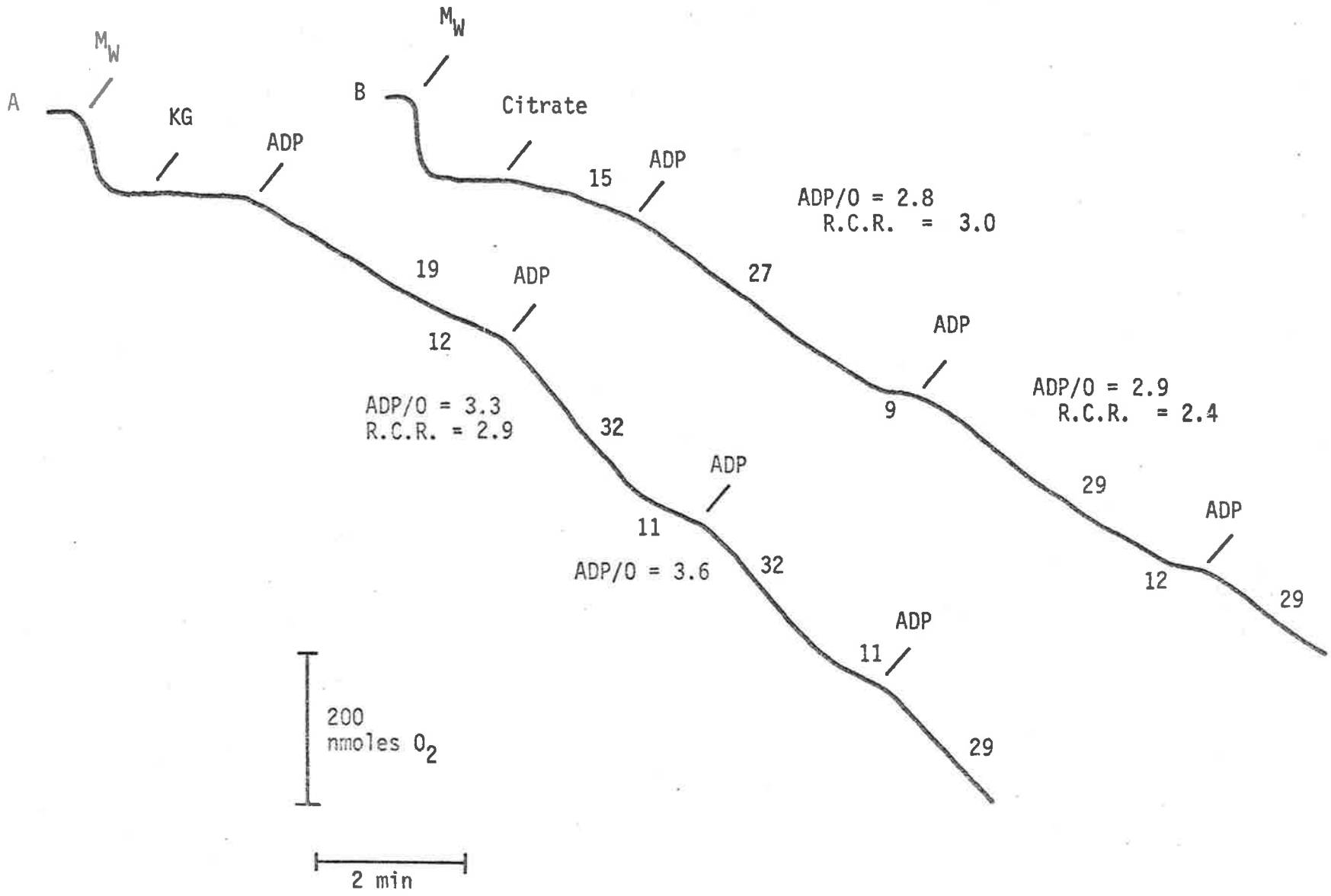


Figure 5.6 Effect of oxaloacetate on malate oxidation by cauliflower bud mitochondria.

Oxygen uptake was measured as described in Materials and Methods. Additions as indicated were, 10 mM malate, 16 mM glutamate, 0.30 mM (trace A) or 0.5 mM (trace B) ADP, 5 mM arsenite and 2 mg of mitochondrial protein (M_W). In trace B, 0.5 mM oxaloacetate was included in the reaction medium. Rates expressed as nmoles O_2/min.mg protein.

Figure 5.7 Citrate and α-ketoglutarate oxidation by cauliflower bud mitochondria.

Oxygen uptake was measured as described in Materials and Methods. Additions as indicated were; A: 2.52 mg of mitochondrial protein, 10 mM α-ketoglutarate and 0.15 mM ADP; B: 2 mg mitochondrial protein, 20 mM citrate and 0.15 mM ADP. In addition, 10 mM malonate was included in the reaction medium in both A and B, and 0.03 mM TPP in A. Rates are expressed as nmoles O_2/min.mg protein.


A
M_W
KG
ADP
B
M_W
Citrate
15
ADP
ADP/O = 2.8
R.C.R. = 3.0
19
ADP
12
ADP/O = 3.3
R.C.R. = 2.9
27
ADP
9
ADP/O = 2.9
R.C.R. = 2.4
32
ADP
11
ADP/O = 3.6
29
ADP
12
29
32
ADP
11
29
200
nmoles O_2
2 min

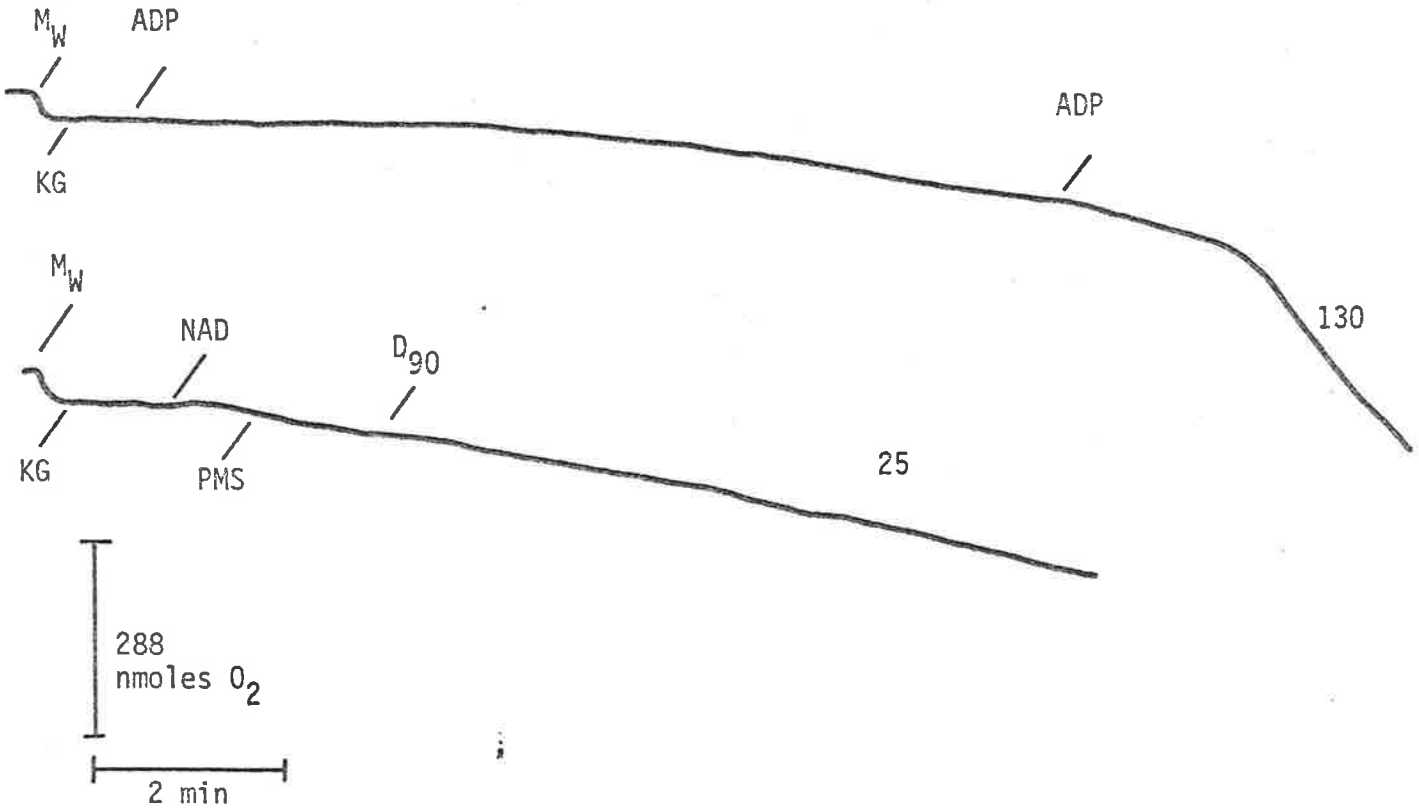
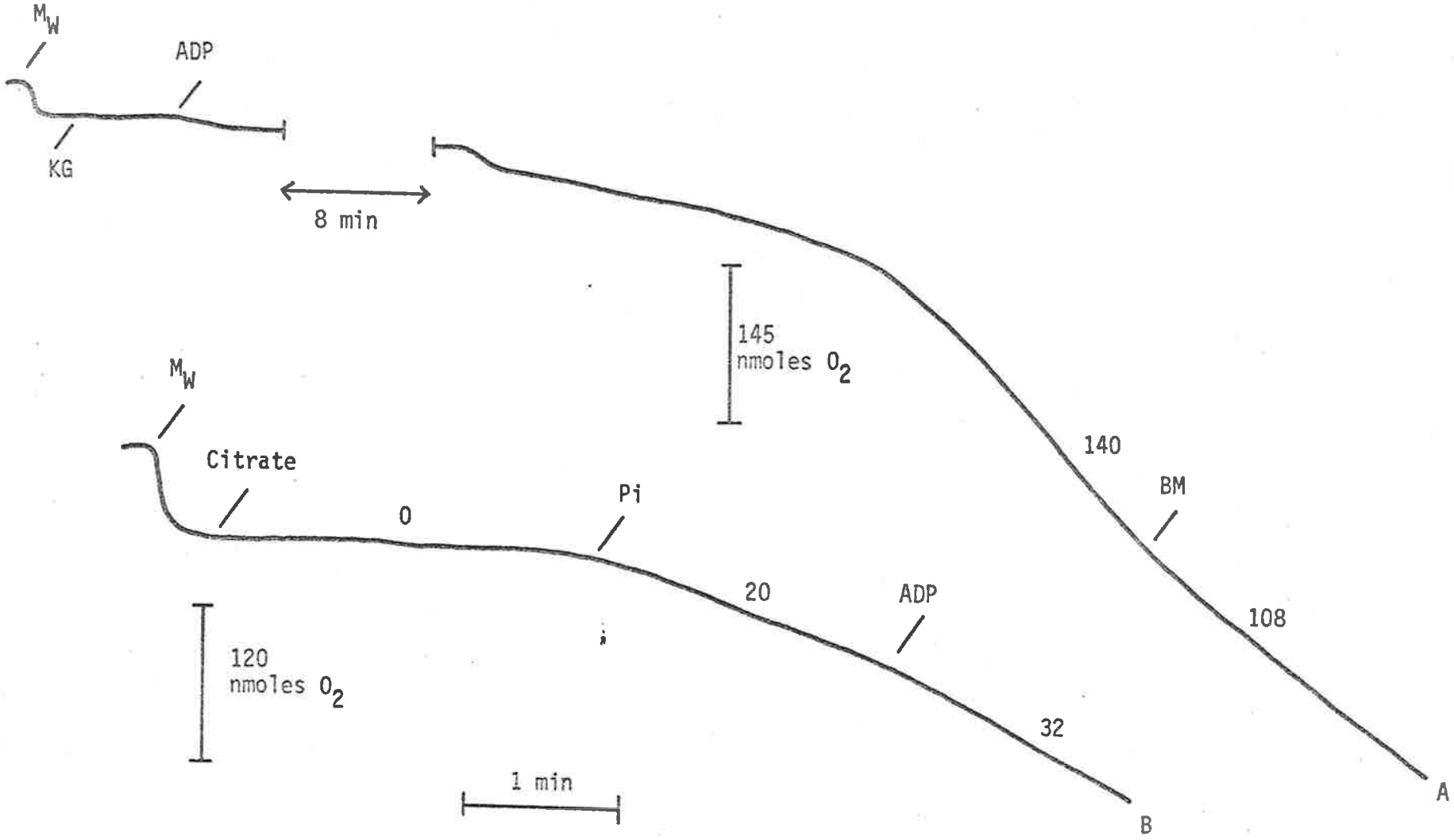


Figure 5.8 α-Ketoglutarate oxidation by beetroot mitochondria.

Oxygen uptake measured as described in Materials and Methods. Additions as indicated were; 1.2 mg mitochondrial protein, 10 mM α-Ketoglutarate (KG), 0.27 mM ADP, 0.5 mM NAD^+, 0.025 mM PMS and 20 µl of 'Decon 90' (D90) detergent. TPP (0.03 mM) and malonate (5 mM) were included in the reaction medium. Oxygen uptake is expressed as nmoles/min. mg protein.

Figure 5.9 Effect of phosphate and butylmalonate on citrate and α-ketoglutarate oxidation.

Oxygen uptake was measured as described in Materials and Methods. Additions as indicated were; A: 1.2 mg beetroot mitochondrial protein, 10 mM α-ketoglutarate, 0.5 mM ADP and 15 mM n-butylmalonate. TPP (0.03 mM) and malonate (5 mM) were included in the reaction medium; B: 1.5 mg cauliflower mitochondrial protein, 20 mM citrate, 10 mM phosphate and 0.23 mM ADP. Malonate (8 mM) was included in the reaction medium which did not contain phosphate. Oxygen uptake is expressed as nmoles/min.mg protein.


M_W
ADP
KG
8 min
145
nmoles O_2
M_W
Citrate
0
Pi
20
ADP
32
B
140
BM
108
A
120
nmoles O_2
1 min

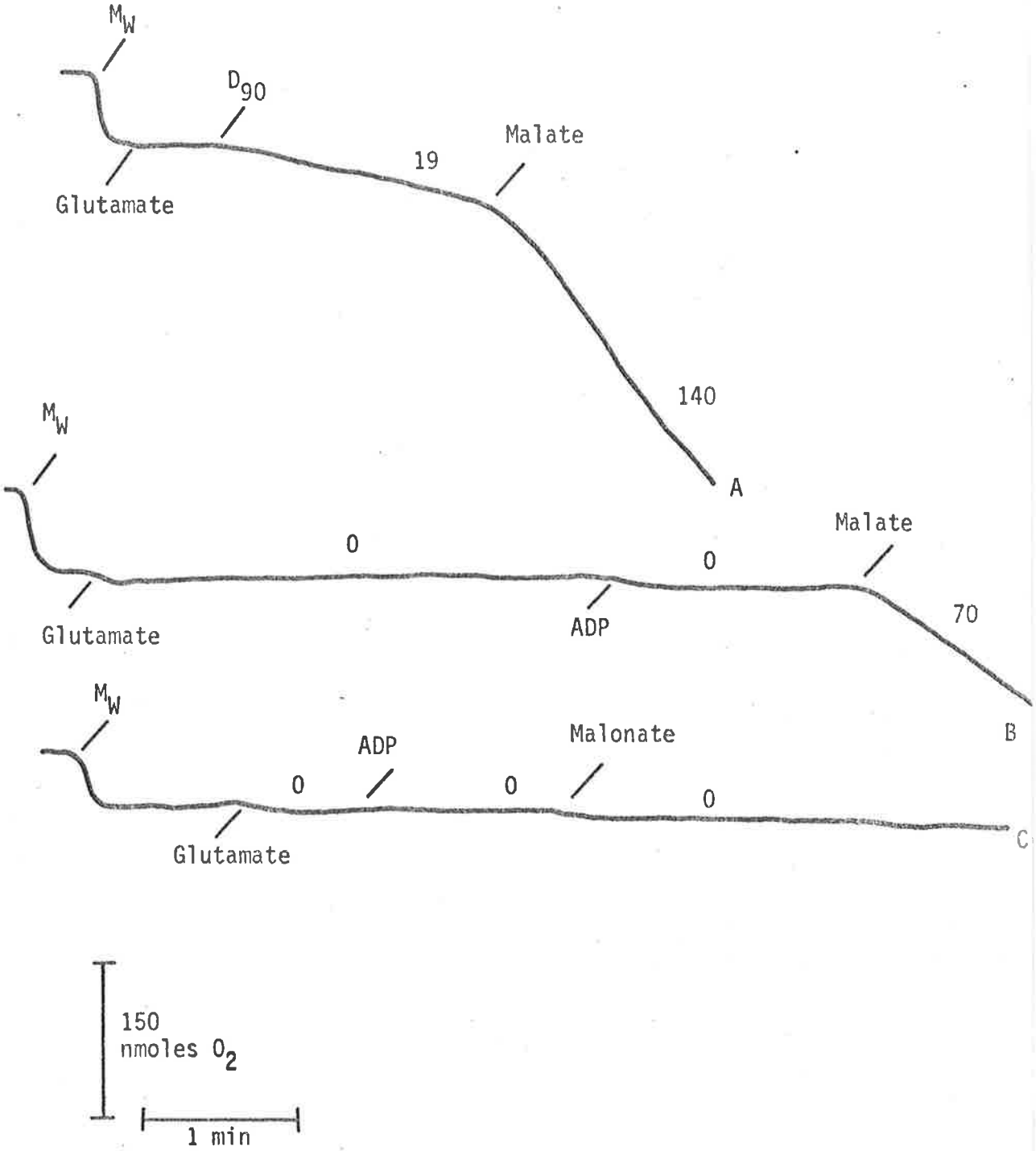


Figure 5.10. Malate and glutamate oxidation by isolated cauliflower bud mitochondria.

Oxygen uptake was measured as described in Materials and Methods. Additions as indicated were; A: 1.6 mg mitochondrial protein, 15 mM glutamate, 30 µl 'Decon 90' (D_{90}) detergent, 15 mM malate. NAD^+ (0.5 mM) and PMS (0.025 mM) were added to the reaction medium; B: 1.6 mg protein, 15 mM glutamate, 0.24 mM ADP, 15 mM malate; C: 2 mg protein, 10 mM glutamate, 0.16 mM ADP, 10 mM malonate. Oxygen consumption is expressed as nmoles/min. mg protein.

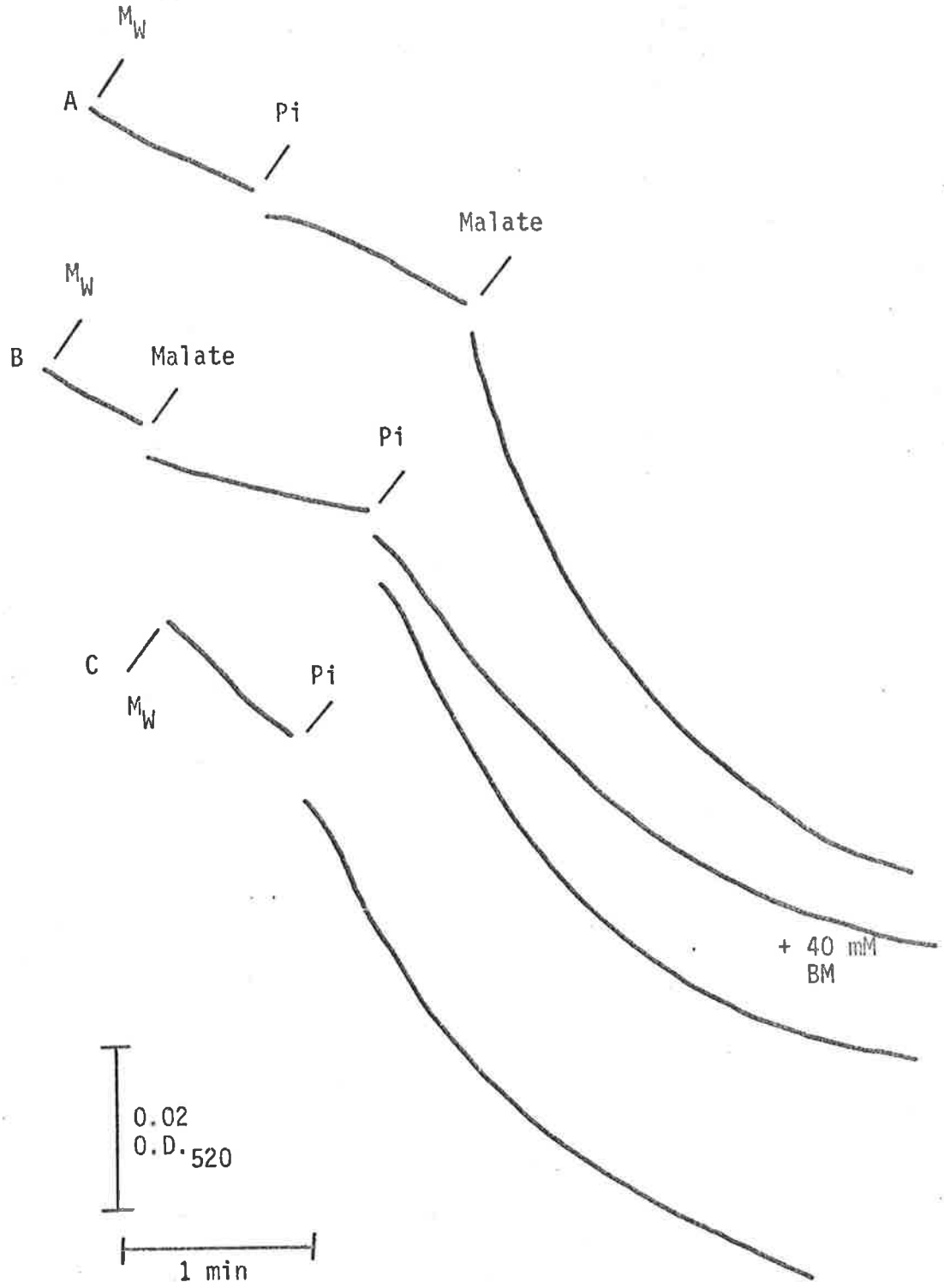


Figure 5.11 Swelling of cauliflower bud mitochondria in ammonium salts

The swelling medium contained 170 mM ammonium glutamate (A and B) or 170 mM ammonium malate (C), 5 mM TES buffer (pH 7.2), 0.11 mM EGTA and 5 µM antimycin A. 10 mM malate and 8 mM phosphate were added as indicated. Swelling was monitored spectrophotometrically as described in Materials and Methods. BM = n-butylmalonate.

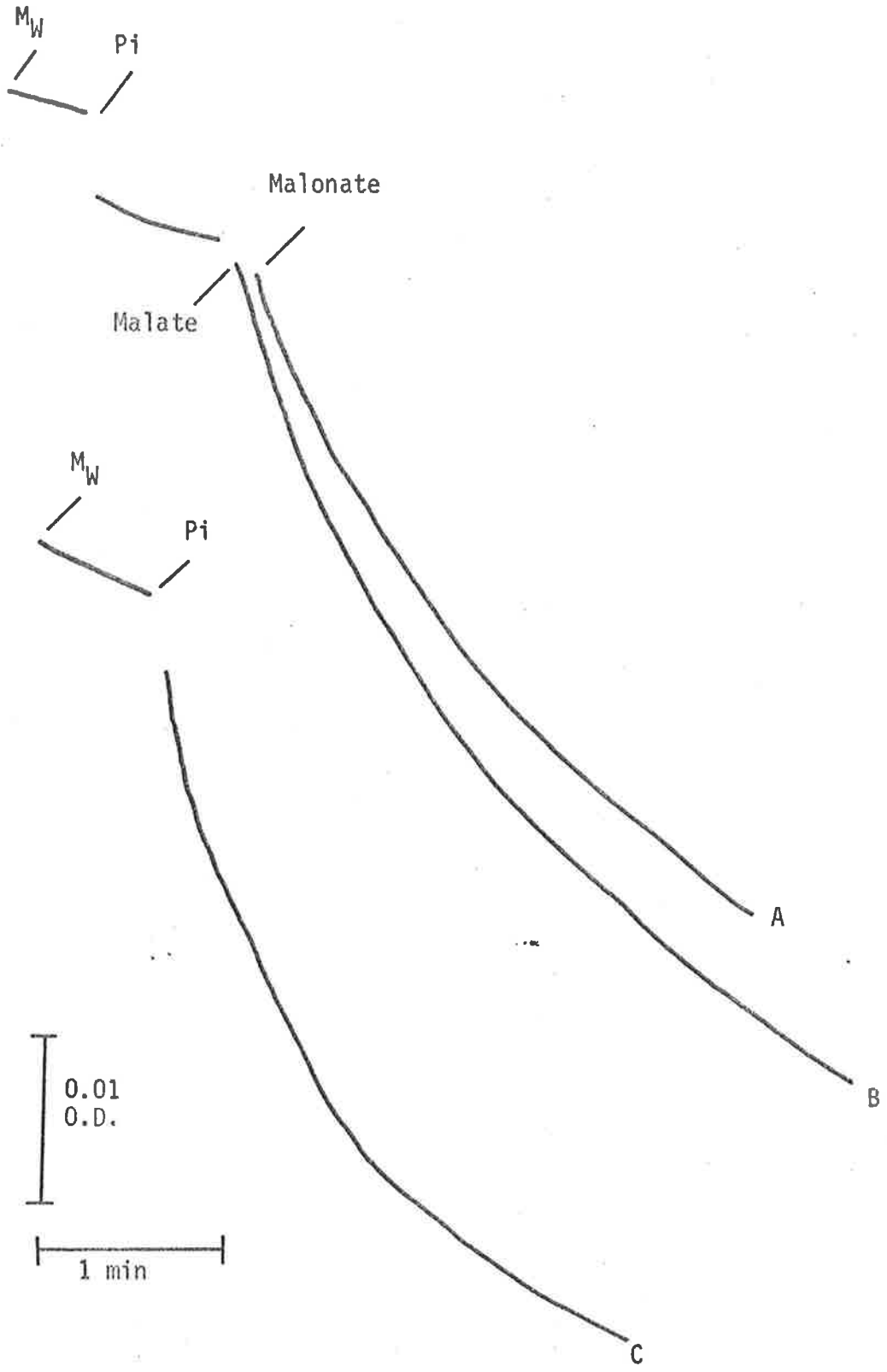
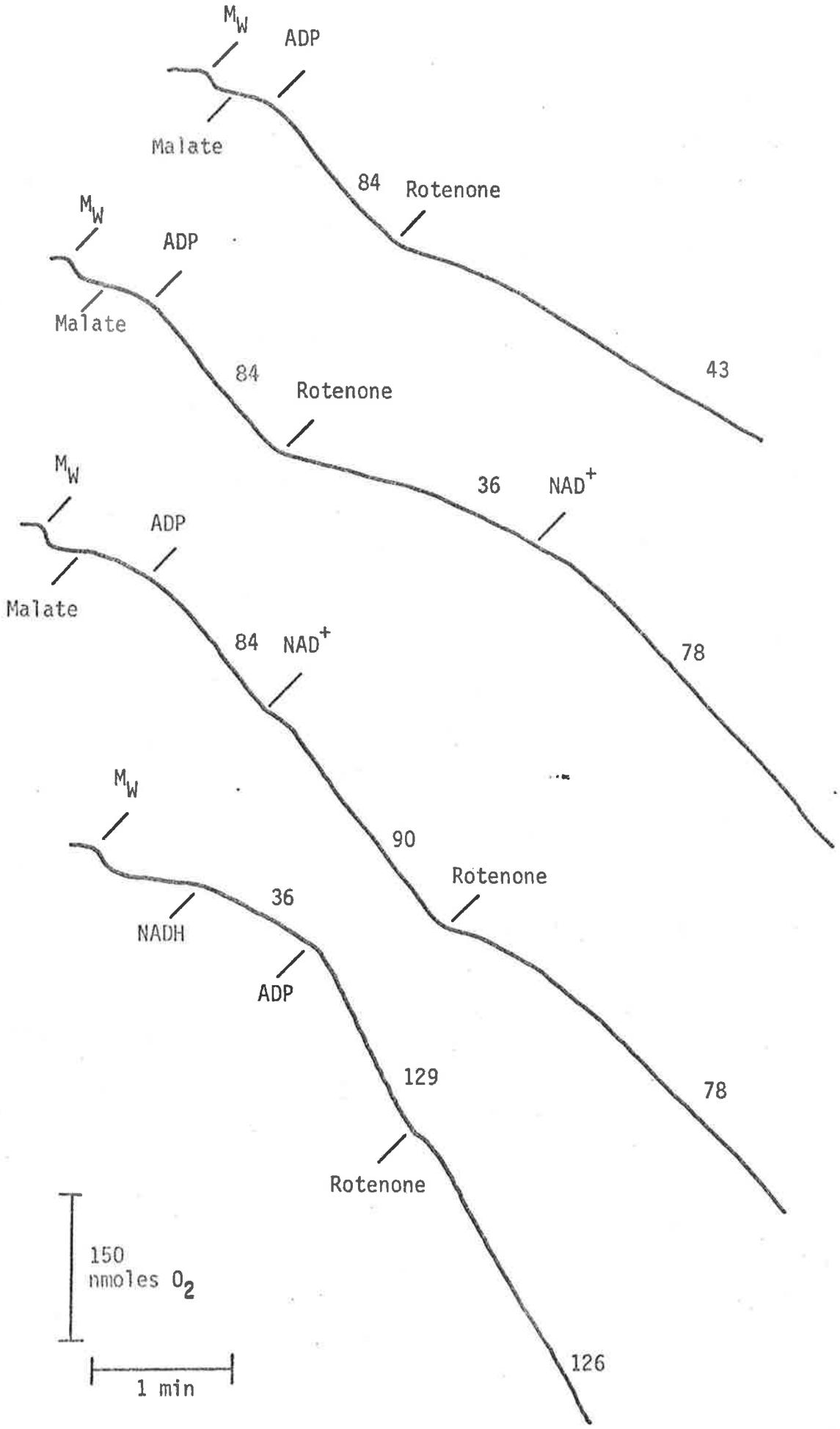


Figure 5.12 Swelling of beetroot mitochondria in ammonium.

Assay conditions were as for Figure 5.11. A and B : ammonium glutamate; C : ammonium malate. 10mM malate, 10 mM malonate and 8 mM phosphate were added as indicated.

Figure 5.13 Effect of exogenous NAD^+ and rotenone on malate oxidation by cauliflower bud mitochondria.

Oxygen uptake was measured as described in Materials and Methods. Additions as indicated were, 2 mg mitochondrial protein, 15 mM malate, 17 µM rotenone, 0.67 mM ADP, 0.5 mM NAD^+ and 1 mM NADH. Oxygen uptake is expressed as nmoles/min. mg protein.


M_W
ADP
Malate
84
Rotenone
43
36
NAD^+
78
NADH
90
129
126
150
nmoles O_2
1 min

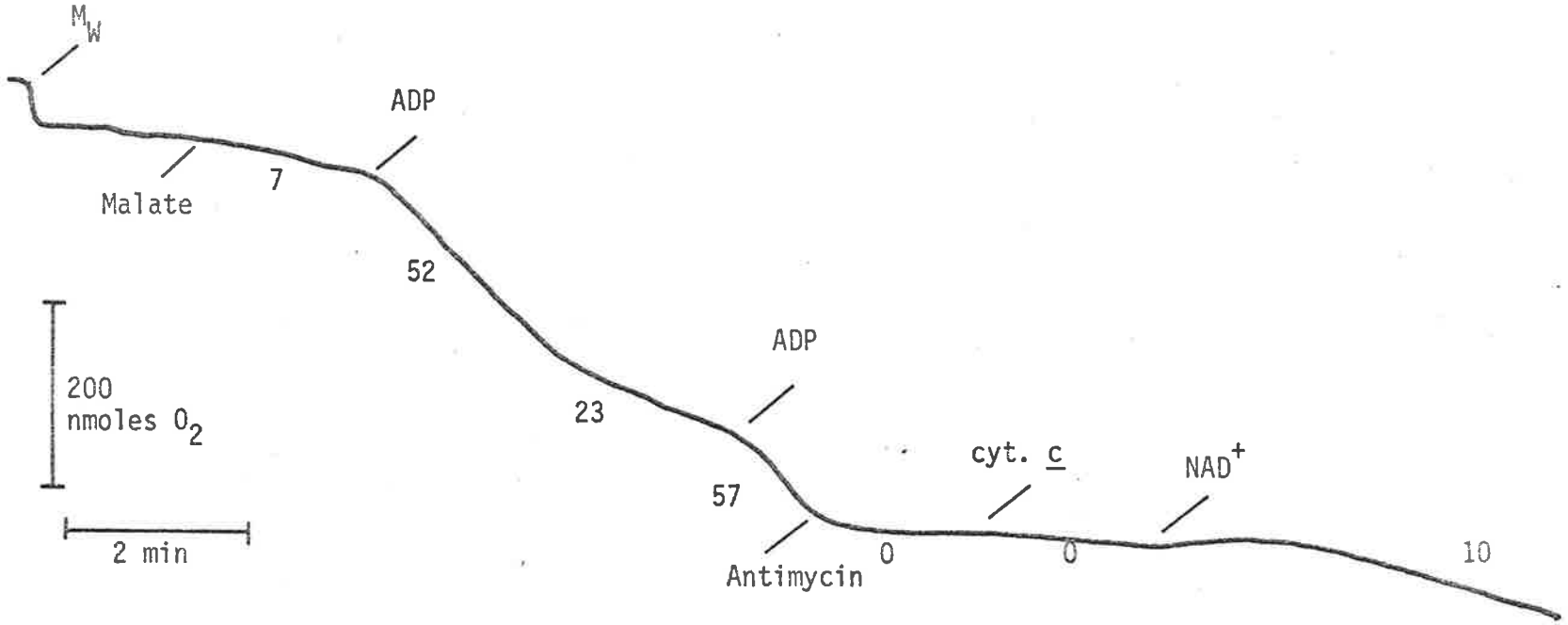


Figure 5.14 Effect of exogenous NAD^+, antimycin A and cytochrome c on malate oxidation by cauliflower mitochondria.

Oxygen consumption measured as in Materials and Methods. Additions as indicated were; 1.4 mg mitochondrial protein, 20 mM malate, 0.26 mM ADP, 5 μM antimycin A, 0.05 mM cyt. c and 0.5 mM NAD^+. Rates expressed as nmoles O_2/min.mg protein. Glutamate (20 mM) was added to the reaction medium.

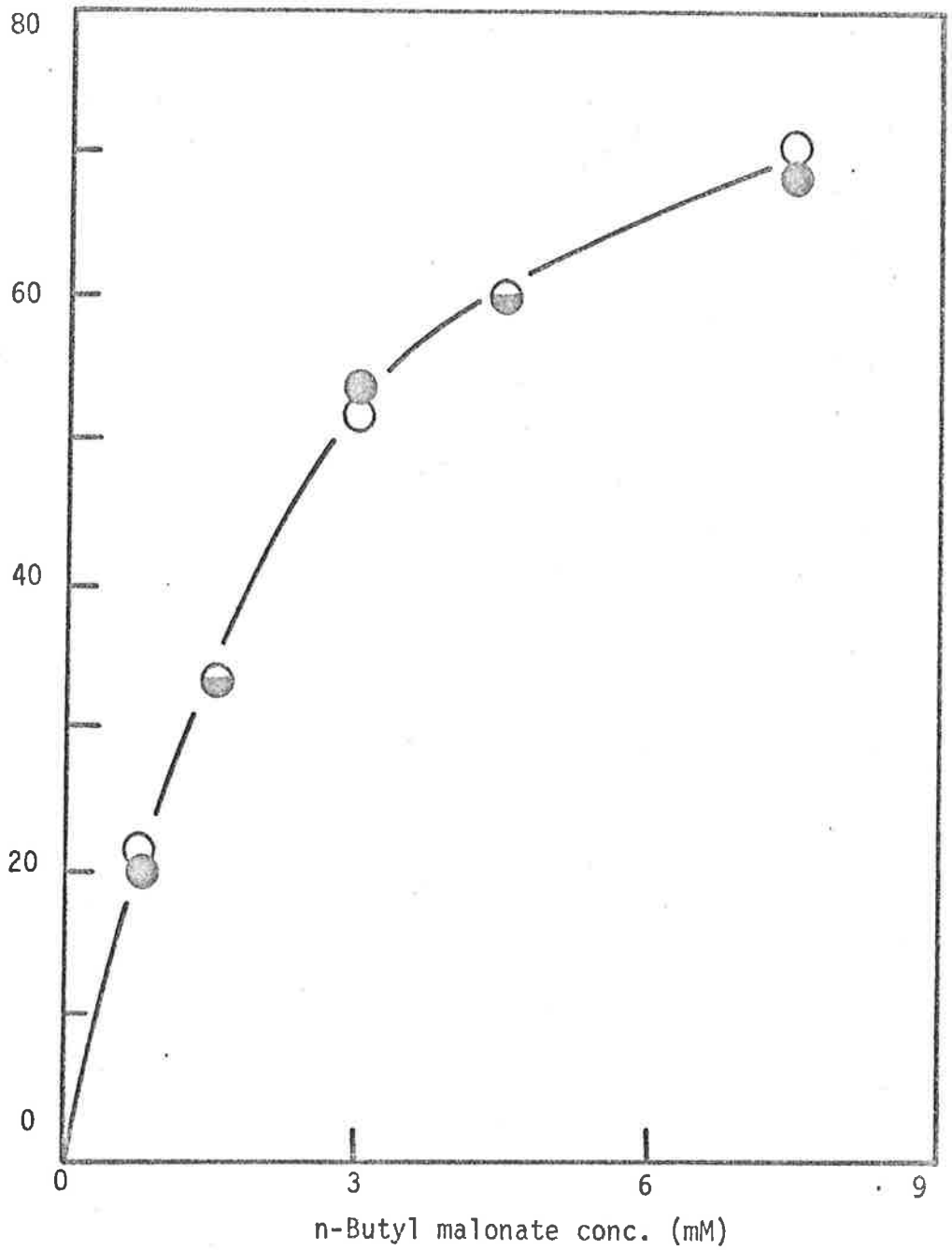


Figure 5.15 Effect of n-butylmalonate concentration on malate oxidation by cauliflower bud mitochondria.

Oxygen consumption was measured upon addition of mitochondria (1.6 mg protein) to 3 ml standard reaction medium which included 0.5 mM ADP and up to 7.5 mM n-butylmalonate. O — O; 10 mM malate was substrate; ● — ● 10 mM malate, plus 0.5 mM NAD^+ and 12 μM rotenone. In all experiments, all reagents were added to the reaction vessel prior to addition of mitochondria. The initial rate of oxygen consumption was 48 nmoles/min. mg protein. The values shown are averages of three separate experiments.

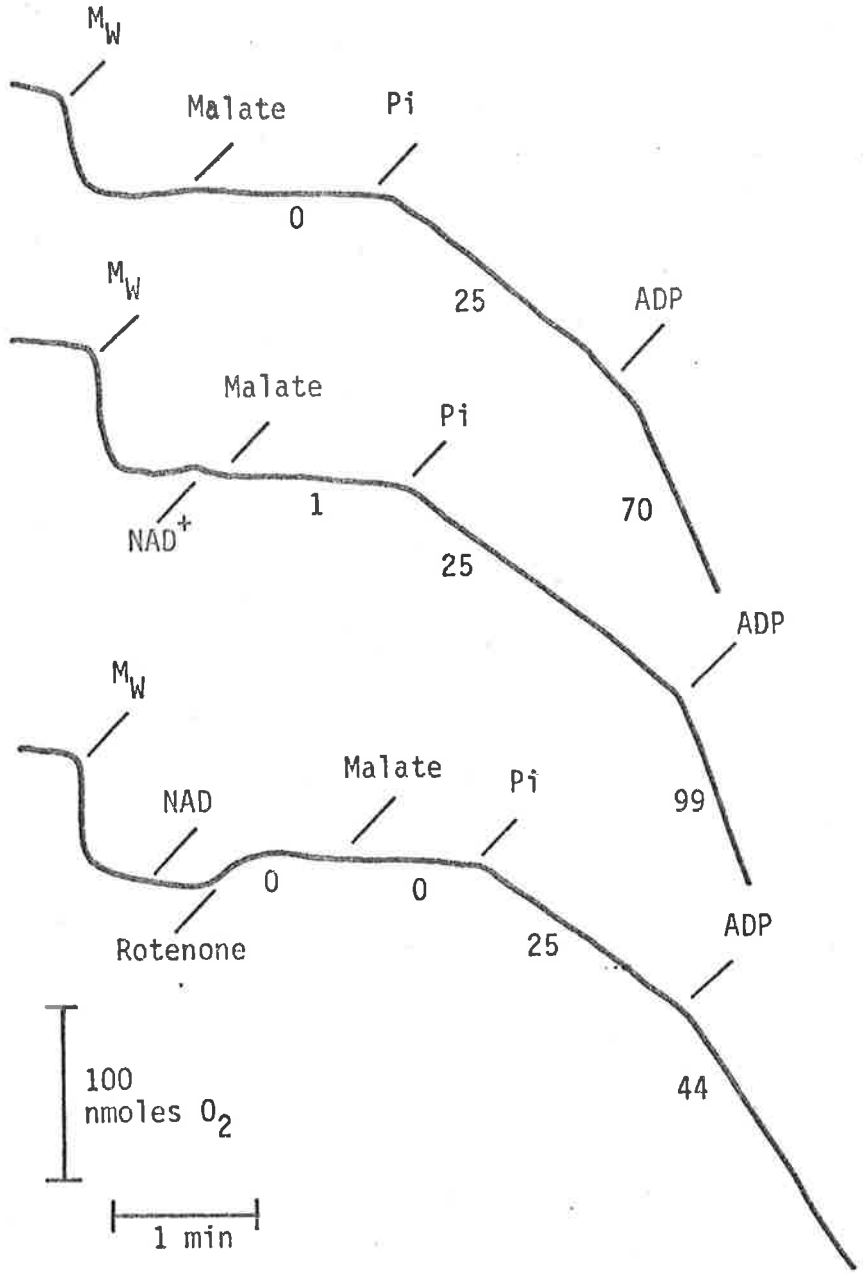


Figure 5.16 Dependence of malate oxidation on inorganic phosphate, and the effect of rotenone and exogenous NAD+.

Oxygen uptake measured as in Materials and Methods. Additions as indicated were; 2.4 mg protein, 10 mM malate, 10 mM phosphate, 0.26 mM ADP, 0.25 mM NAD^+ and 7 µM rotenone. Rates expressed as nmoles O_2/min. mg protein.

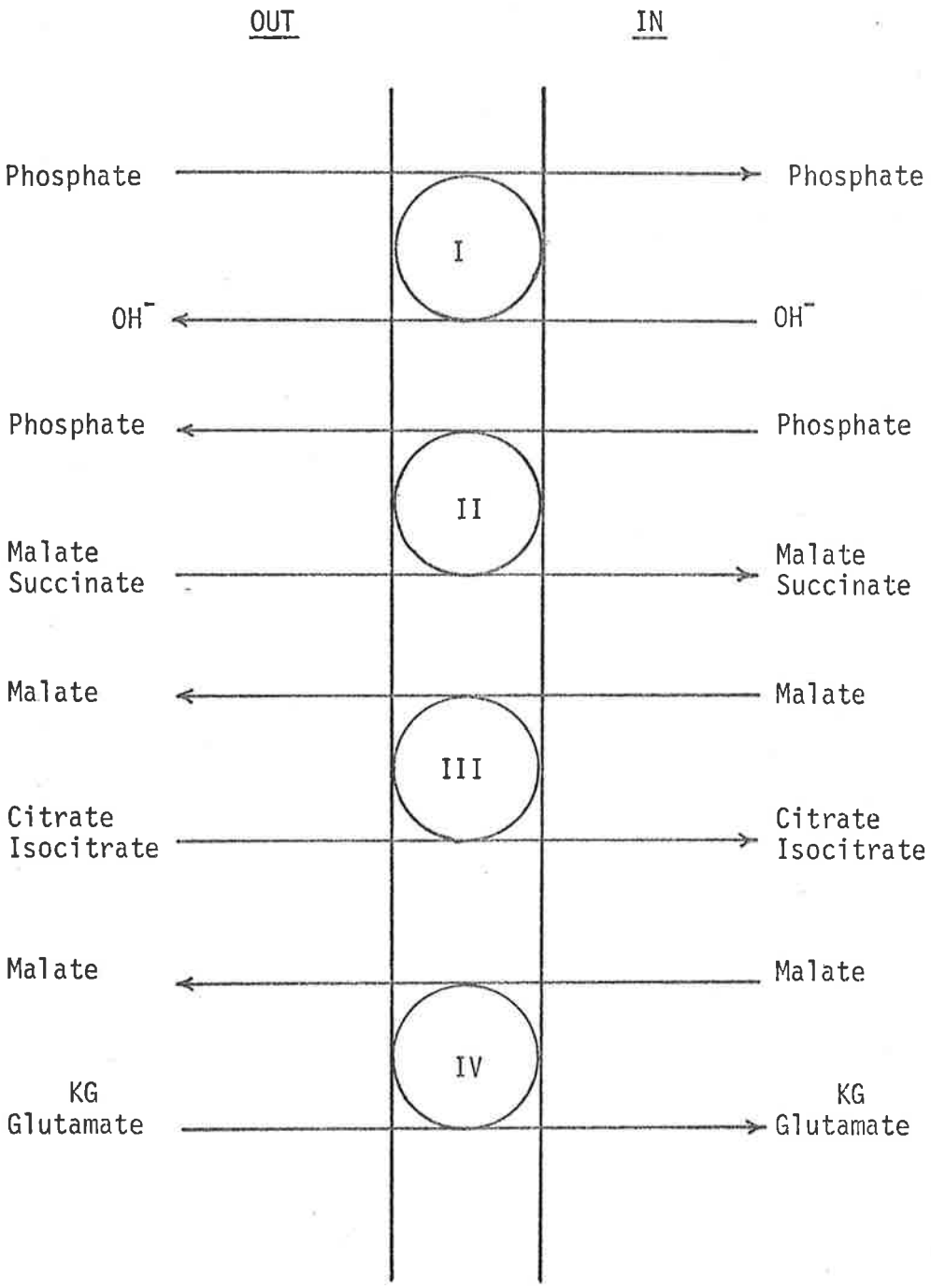
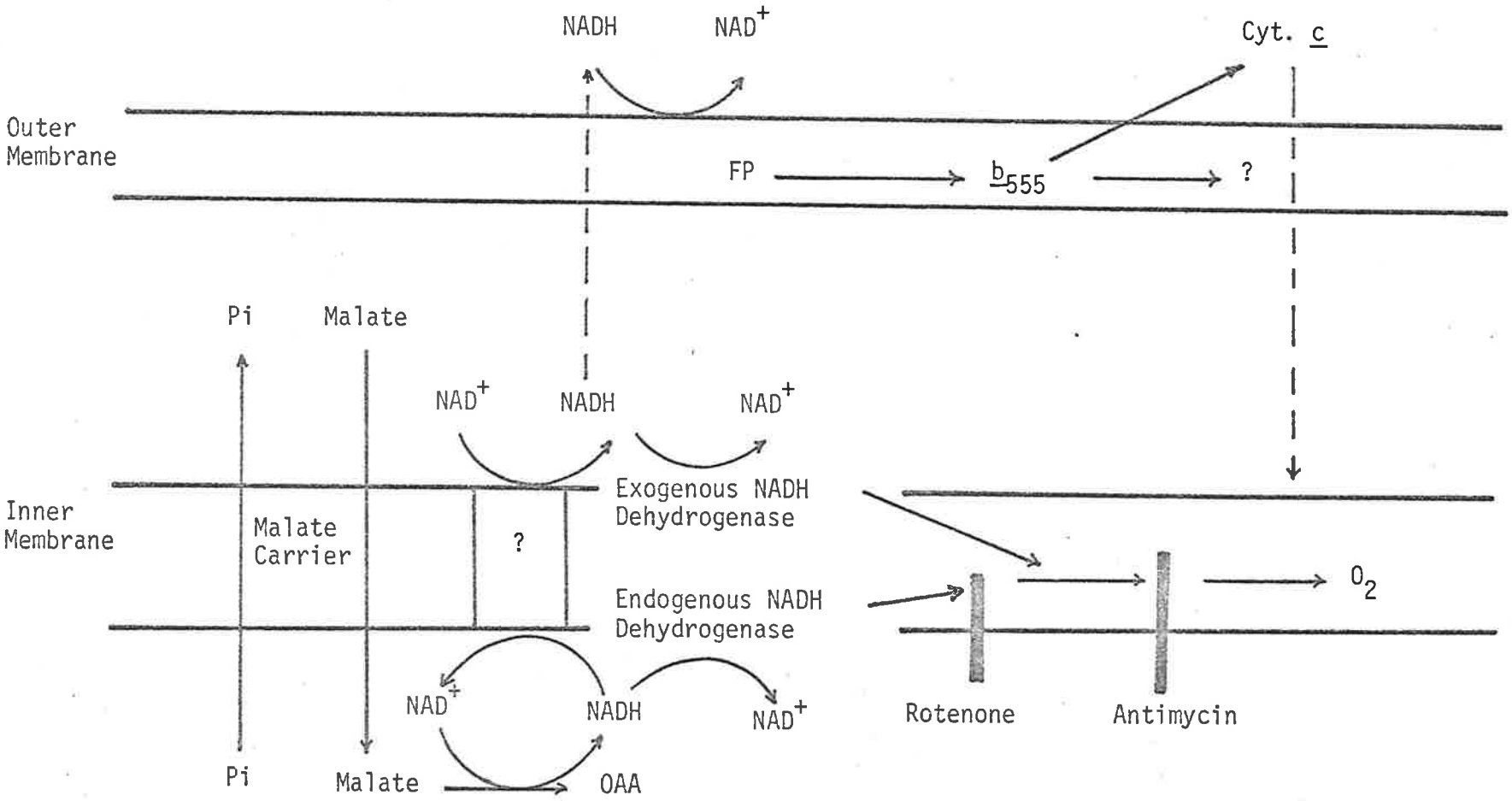


Figure 5.17 Anion transporters of plant mitochondria.

I	=	phosphate transporter
II	=	dicarboxylate transporter
III	=	tricarboxylate transporter
IV	=	glutamate and/or α-ketoglutarate transporter.

N.B. Carriers III and IV may be identical.

Fig. 5.18 Malate oxidation by cauliflower mitochondria in the presence of exogenous NAD^+.



CHAPTER VI

TRANSFER OF REDUCING EQUIVALENTS ACROSS THE INNER MEMBRANE

In an attempt to elucidate the mechanism by which reducing equivalents are transferred from intromitochondrial NADH to extramitochondrial NAD^+, the reduction of exogenous NAD^+ during the oxidation of NAD-linked substrates was investigated. Particular attention was paid to the intactness of the mitochondria employed, and integrity assays are described. Measurements were made with mitochondria from several different plant tissues and an important difference was found with *Beta vulgaris* (beetroot) tuber mitochondria, which appear unable to oxidize exogenous NADH directly via the respiratory chain.

RESULTS AND DISCUSSION

A. *Cauliflower mitochondria*

1. *NAD^+ reduction*

When the mitochondria were disrupted (by detergent), exogenous NAD^+ was reduced rapidly and at a steady rate in the presence of malate and antimycin A (Fig. 6.1A). Under these conditions NAD^+ is reduced directly, upon oxidation of malate, by malate dehydrogenase (or malic enzyme) released from the matrix.

N.B. [H] = reducing equivalent

Since the reaction occurred in a relatively large volume (3 ml), product inhibition by NADH or oxaloacetate was not observed over the period of measurement (Fig. 6.1A).

The addition of malate to intact mitochondria resulted in a brief but rapid reduction of exogenous NAD^+; this reduction decreased to a very low steady rate within one minute (Fig. 6.1B and 6.2). The rate and extent of this reduction could be increased by increasing the concentration of exogenous NAD^+ and/or malate (Fig. 6.2). When glutamate was omitted from the reaction medium, less NAD^+ was reduced and the reaction quickly slowed to zero. (Fig. 6.3B); a subsequent addition of glutamate to the cuvette restored the final steady rate (Figure 6.3B *c.f.* 6.3A). If phosphate was not present, very little exogenous NAD^+ was reduced, the reaction coming to a halt within 0.3 minutes; when phosphate was added to the medium a slow rate of reduction occurred (Fig. 6.3C).

The results suggest that the reduction of added NAD^+ by intact mitochondria is governed by the rate of malate oxidation within the matrix. The rapid decrease in the rate of NAD^+ reduction (Figs. 6.1, 6.2 and 6.3) is probably due to the equilibrium of the malate dehydrogenase reaction. Since antimycin A was present, NADH in the matrix was not oxidized via the respiratory chain and therefore would have accumulated; although either (H) or NADH moves out of the matrix, only a small quantity would need to remain to cause inhibition of malate oxidation (since the malate dehydrogenase equilibrium does not favour this reaction). When glutamate was omitted from the reaction medium, oxaloacetate also accumulated and malate oxidation was more severely inhibited (as was NAD^+ reduc-

tion, Fig. 6.3). If phosphate was not present, malate entry was restricted (See Chapter V) and very little NAD^+ reduction occurred (Fig. 6.3). Malate entry may not be very great in the presence of antimycin A anyway, since a pH gradient cannot be generated across the inner membrane when electron transport does not occur (Mitchell, 1966). Addition of ATP (to generate a membrane potential via the ATPase ; Skulachev, 1972) led to a small increase in the amount of exogenous NAD^+ reduced but did not stimulate the initial rate of reduction (Fig. 6.4A and B).

It also appears that a large concentration gradient of NADH (high in matrix, low in the medium) is needed for a rapid efflux of (H) or NADH to be observed. The addition of relatively small amounts (10 % of NAD^+ conc.) of NADH to the external medium prevented the initial rapid reduction of exogenous NAD^+, and subsequent reduction was very slow (Fig. 6.4 C and D). This may simply reflect a restriction of NADH or NAD^+ diffusion across the inner membrane. However if (H) transfer involves a trans-membrane transhydrogenase, then the inhibition of NAD^+ reduction by external NADH may also involve competition between the reduced and oxidized nucleotides for binding sites on the outer surface of the inner membrane.

The results presented above suggest that external NAD^+ is in equilibrium, across the inner membrane, with internal NADH, in isolated cauliflower bud mitochondria oxidizing malate. The rate of external NAD^+ reduction is governed by: (i) the rate of malate penetration of the inner membrane (i.e. the malate concentration inside the matrix, (ii) the removal of oxaloacetate

(by transamination with glutamate), and (iii) the NAD^+/NADH ratio in the medium. Glutamate did not contribute directly to NAD^+ reduction, since no reduction was observed until malate was added (Figs. 6.1, 6.2 and 6.3). Antimycin A could be replaced by a combination of 50 μM dicoumarol (which inhibited exogenous NADH oxidation, but not malate oxidation, completely; see Fig. 3.10) and rotenone (a specific inhibitor of the internal NADH dehydrogenase), as shown in Fig. 6.5 (A and B). However, when dicoumarol alone replaced antimycin A as a respiratory inhibitor, less NAD^+ reduction occurred and the initial rate was slower (Fig. 6.5C). This was probably due to oxidation of some intramitochondrial NADH by the internal NADH dehydrogenase. Nevertheless, significant NAD^+ reduction occurred when the external NADH dehydrogenase was inhibited, implying that this flavoprotein was not involved in the transfer of (H) out of the matrix.

The results presented in Fig. 6.6 suggest that very little external NAD^+ reduction was attributable to malic enzyme activity. In the absence of glutamate, coenzyme A (an activator of malic enzyme - Macrae, 1971a; Hatch and Kagawa, 1974) did not affect NAD^+ reduction (Figure 6.6.B), although glutamate had a marked effect (Fig. 6.6A). When phosphate was absent, $MnCl_2$ (a cofactor of malic enzyme - Macrae, 1971a; Hatch and Kagawa, 1974) stimulated NAD^+ reduction only slightly, although subsequent addition of phosphate caused a marked stimulation (Fig. 6.6.C). This result confirms those presented in the previous chapter, which showed that extramitochondrial malic enzyme was not involved.

Palmitoyl CoA (an inhibitor of the NAD(P) energy-linked transhydrogenase of rat liver mitochondria; Rydstrom, 1972) had little effect on the reduction of exogenous NAD^+, and added $NADP^+$ was not reduced when malate was added (Fig. 6.7), suggesting that the energy-linked pyridine nucleotide transhydrogenase of these mitochondria (Wilson and Bonner, 1970) was not involved.

Unlike malate, isocitrate did not induce external NAD^+ reduction in the presence of antimycin A, by intact cauliflower mitochondria, (Fig. 6.8A), although a rapid rate of reduction was observed upon disruption of the mitochondria (Fig. 6.8D). NAD^+ reduction with isocitrate as substrate was only observed when dicoumarol (50 μM) replaced antimycin A as an inhibitor of electron transport, and this reduction was very small compared to that with malate as substrate (Fig. 6.8). Von Jagow and Klingenberg (1970) also failed to observe exogenous NAD^+ reduction with citrate and α-ketoglutarate as substrates.

One of the problems with isocitrate may be its poor penetration of the inner membrane in the presence of antimycin A (as discussed above for malate). Citrate and isocitrate penetration of cauliflower bud mitochondria is slower and more dependent on added phosphate, than is malate penetration (Wiskich, 1975), and hence is likely to be more restricted by lack of a pH gradient across the inner membrane. However, preincubation of the mitochondria with citrate and ATP did not lead to any exogenous NAD^+ reduction. Isocitrate dehydrogenase is also susceptible to inhibition by NADH (Cox and Davies, 1967) and therefore restriction of NADH oxidation by antimycin A would tend to inhibit isocitrate

oxidation by this enzyme. When dicoumarol, instead of antimycin A, was used, some intramitochondrial NADH oxidation would have occurred.

Studies of cytochrome *c* reduction supported the NAD^+ reduction data. Addition of NAD^+ stimulated the low malate cytochrome *c* reductase activity observed with intact mitochondria, and this reduction in the presence of NAD^+ was largely insensitive to antimycin A (Table 6.1A). Cytochrome *c* reduction with citrate and ∝-ketoglutarate as substrates was also very slow, but adding NAD^+ produced only a slight increase in rate (Table 6.1A). Cytochrome *c* reduction in the absence of external NAD^+ presumably occurred only in the small percentage of broken mitochondria present (see chapter III), but addition of NAD^+ allowed malate oxidation by intact mitochondria to proceed, by transferring reducing equivalents to the outermembrane NADH-cytochrome *c* reductase. Cytochrome *c* reduction with citrate and ∝-ketoglutarate as substrates did not occur even in the presence of exogenous NAD^+, probably for the same reasons that isocitrate-induced NAD^+ reduction was not observed when electron transport was severely inhibited (see above).

Rupture of the outer membrane by incubating the mitochondria with a low concentration of digitonin resulted in faster rates of cytochrome *c* reduction with all three substrates, and this reduction was completely inhibited by antimycin A (Table 6.1B). However, only malate-cytochrome *c* reductase recovered activity when NAD^+ was added to the medium (Table 6.1B). These results suggest that inhibition of citrate entry, in the presence of antimycin A, is not the only factor involved, since the mitochondria oxidized citrate prior to the addition of antimycin A. Therefore, some

citrate should have been present in the matrix, and a pmf generated across the membrane. Obviously the interpretation of these results is more complicated than at first apparent; it seems that inhibition of electron transport has a direct effect on isocitrate (and ∝-ketoglutarate) dehydrogenase activity. NADH and ATP levels within the matrix may be important in this respect.

2. *Oxygen electrode studies*

Transfer of reducing equivalents across the inner membrane was also measured indirectly by following oxygen uptake by cauliflower mitochondria in the presence of NAD^+, PMS and antimycin A.

Antimycin A inhibited malate oxidation severely and PMS had very little effect on the inhibited rate (Fig. 6.9A), suggesting that PMS did not penetrate the inner membrane to react with intramitochondrial NADH. However subsequent addition of NAD^+ stimulated oxygen uptake several-fold (Fig. 6.9A), although not nearly as much as did the addition of detergent (Fig. 6.9A), indicating that exogenous NAD^+ did not penetrate the inner membrane rapidly. Inorganic phosphate was required to elicit fast rates of malate oxidation, even in the presence of NAD^+ and PMS (Fig. 6.9B) showing that internal malate oxidation was the main contributor to oxygen uptake. That is, reducing equivalents were transferred out of the matrix upon oxidation of malate, to reduce extramitochondrial NAD^+ and lead to oxygen uptake via PMS (see Chapter V).

The rate of (H) transfer measured by the above method was

almost identical to the initial rate of NAD^+ reduction measured spectrophotometrically (Table 6.2). However oxygen uptake in the presence of NAD^+ and PMS was linear, whereas NAD^+ reduction slowed almost to a halt, generally within one or two minutes (compare Figs. 6.1 and 6.9). This difference is probably attributable to the reoxidation of NADH, formed in the intermembrane space, by PMS. NAD^+ stimulated rotenone-inhibited malate oxidation much more than NAD^+ and PMS stimulated antimycin - inhibited malate oxidation (Table 6.2). Once again this may reflect faster penetration of the inner membrane by malate when electron transport was operating. Alternatively, if release of NADH from the outer surface of the inner membrane was rate limiting, then reoxidation of NADH may have been faster via the external NADH dehydrogenase (see Chapter III) than by PMS. This may have been possible if a transmembrane transhydrogenase was involved but not if the inner membrane was permeable (or leaky) to pyridine nucleotides, since added NADH was oxidized far more rapidly by PMS alone than by the electron transport chain.

Exogenous $NADP^+$ had little effect on rotenone-inhibited oxidation of NAD-linked substrates although external NADPH was oxidized by these mitochondria (Table 6.3). This supports the NAD^+ reduction data and indicates that the energy-linked NAD(P)-transhydrogenase was not involved. Further support for this idea came from studies with uncoupled mitochondria. DNP uncoupled malate and citrate oxidation both in the presence and absence of exogenous NAD^+ and rotenone, but did not prevent the relief of rotenone inhibition by NAD^+ (Table 6.4), suggesting that this process is not dependent on a supply of energy.

Citrate oxidation inhibited by antimycin A was only slightly stimulated by PMS and NAD^+, although exogenous NAD^+ restored rotenone-inhibited citrate oxidation to the state 3 rate (Fig. 6.10). These results agree with those obtained with malate (Fig. 6.9). The fact that some oxygen uptake was observed when NAD^+ and PMS were added in the presence of antimycin A (Fig. 6.10A) suggests that NADH accumulation prevented NAD^+ reduction during citrate oxidation (Fig. 6.8A).

3. Integrity assays

The possibility that the inner membranes of the mitochondria used in this study had been damaged, thereby allowing passage of exogen-NAD^+ into the matrix, is extremely remote as the following observations demonstrate the intactness of their membranes. (i) Exogenous NAD^+ was reduced by mitochondria which only oxidized malate when Pi was present to allow operation of the dicarboxylate carrier (Fig. 6.11). (ii) NAD^+ relieved rotenone inhibition of citrate and malate oxidation although neither substrate was oxidized until phosphate was added (Fig. 6.12 and 6.13 A and B). (iii) Exogenous NADH oxidation by the same mitochondrial preparation was insensitive to rotenone (Fig. 6.13C). (iv) Malate and succinate-cytochrome *c* reductase activities were very low unless NAD^+ was added (with malate) or the mitochondria were disrupted (Table 6.5). Also, the NADH-cytochrome *c* reductase was largely insensitive to antimycin A (Table 6.5). These results suggest that the outer membranes were intact (Douce et al, 1973), although if the mitochondria were swollen and damaged one would expect their outer membranes to be ruptured.

(v) ADP/O ratios were high for all substrates, indicative of tight coupling and intact membranes.

It should be noted that observations (ii) to (v) above were made with the same batch of mitochondria. Although some of these results have been presented previously in this study, they were obtained with a number of different mitochondrial preparations over a relatively long period of time. Since mitochondrial properties (particularly those which are closely associated with membrane function and structure) may vary with season and the length of time a particular tissue has been stored, as well as with unintentional differences in the isolating procedure, it was considered important to demonstrate that mitochondria from a single preparation were intact and displayed the whole spectrum of results obtained previously with a number of different preparations.

Points (i) and (ii) above show that the inner membrane is intact with respect to organic acid entry. Point (iii) indicates that NADH does not penetrate into the matrix, while point (v) shows that the inner membrane was intact with respect to maintenance of a high energy state. Any damage to the inner membrane would increase the passive diffusion of organic acids and decrease ADP/O ratios, as well as allowing exogenous NADH to be oxidized via the rotenone-sensitive internal NADH dehydrogenase. (NADH can be oxidized simultaneously by the external and internal dehydrogenases, since malate *plus* NADH oxidation was sensitive to rotenone - Table 5.6).

Point (v) demonstrates that very little damage due to swelling had occurred, since swelling results in breakage of the less flexible outermembrane. In fact, this forms the rationale behind some methods used to isolate the outer mitochondrial membrane (Douce et al, 1973). Douce et al (1972) have used the ratio of succinate-cytochrome *c* reductase activity in purified mitochondria to that in disrupted mitochondria as an indication of the percentage of mitochondria with intact outer membranes (since the outer membrane is impermeable to cytochrome *c* - see Chapter III). For the mitochondria used here this ratio was 11.5 (Table 6.5), suggesting that approximately 87% 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-cytochrome *c* reductase activity in the untreated mitochondria, providing that the penetration of the outer membrane by the added cytochrome *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 sould display complete dependence on the inner membrane substrate transport systems.

The above integrity assays, together with the observed kinetics of malate-induced NAD^+ reduction (particularly in the absence of glutamate and phosphate), and the lack of citrate-induced NAD^+ reduction, strongly suggest that leakage of pyridine

nucleotides across the inner membrane of these mitochondria did not take place. Hence, any pyridine nucleotide passage across the membrane would have to be via a transporter, for which there is no precedence. Whichever is transported, either reducing equivalents or nucleotide, the transfer appears to be unidirectional ({H} or NADH out; nothing or NAD^+ in), since exogenous NADH oxidation is insensitive to rotenone.

Perhaps the best explanation of the results presented in this study is the existence of a transhydrogenase across the inner membrane of plant mitochondria, which is capable of transferring reducing equivalents from within the matrix to exogenous NAD^+ in the intermembrane space, upon oxidation of NAD-linked substrates. The NADH thus formed in the outer compartment is oxidized via the external NADH-dehydrogenase (s), or, if a respiratory inhibitor is present, accumulates and prevents further reduction of exogenous NAD^+.

In contrast to the results presented here, Davis et al (1972) found that exogenous NAD^+ was not reduced by addition of malate to heart mitochondria, unless they were disrupted. In fact, it appears that most animal mitochondria are impermeable to pyridine nucleotides and extra-and intra-mitochondrial NAD(H) cannot communicate across the inner membrane. (Pfaff and Schwalbach, 1967; Lehninger, 1951).

B. Turnip and Maize mitochondria

Rotenone (10 μM) inhibited malate oxidation by both maize root and turnip mitochondria, but this inhibition was relieved

upon addition of NAD^+ (Table 6.6). In general exogenous NAD^+ stimulated the rotenone-inhibited rate of oxygen consumption two-fold (Table 6.6). Exogenous NAD^+ was reduced by isolated turnip mitochondria upon addition of malate (Fig. 6.14), in much the same manner as by cauliflower bud mitochondria.

These results suggest that transfer of reducing equivalents across the inner membrane can occur in all isolated plant mitochondria.

C. *Beetroot Mitochondria*

1. *Mitochondria from fresh tissue*

Unlike most other plant mitochondria (see Chapter I and III), mitochondria isolated from fresh beetroot tubers did not oxidize exogenous NADH readily (Fig. 6.15A) unless they were disrupted (e.g. by addition of deoxycholate; Fig. 6.15B). If external NADH was oxidized at all, it was inhibited by rotenone, as was NADH oxidation by disrupted beetroot mitochondria (Fig. 6.15A). However, malate was oxidized readily when glutamate was present (Fig. 6.15C). That is, all NADH oxidation by beetroot mitochondria occurred via the internal NADH dehydrogenase.

To ensure that the different isolation procedure used with beetroot (see Materials and Methods) did not produce this anomalous result, turnip mitochondria were prepared by essentially the same method.

Exogenous NADH was oxidized readily by turnip mitochondria, was insensitive to rotenone and yielded ADP/O values between 1 and 2 (Fig. 6.15D). Mitochondria isolated from beetroots which were sliced into disks and homogenized with a 'Polytron', did not oxidize added NADH (Fig. 6.23A). It thus seems that lack of external NADH oxidation by beetroot mitochondria is not attributable to the isolation procedure employed.

Nor was it due to lack of a Ca^{++} requirement of the external NADH dehydrogenase (Coleman and Palmer, 1971). Addition of 0.5 mM $CaCl_2$ did not induce exogenous NADH oxidation (Fig. 6.16A). However addition of cytochrome *c* or Fe CN did stimulate NADH oxidation, indicating that the outer membrane reductase was active in isolated beetroot mitochondria (Fig. 6.16B and D). Some of this cytochrome *c*-stimulated oxygen consumption was sensitive to rotenone and antimycin A (Fig. 6.16B and C). This was probably due to the presence of some broken mitochondria in the preparations used. Oxygen uptake by these disrupted mitochondria would not have been evident in the absence of added cytochrome *c*, because endogenous cytochrome *c* is readily removed from the inner membrane upon disruption (see Chapter III).

Studies on cytochrome *c* and Fe CN reduction support the oxygen uptake data. Succinate-cytochrome *c* reductase activity increased almost ten-fold upon rupture of the outer membrane with digitonin, but NADH-cytochrome *c* reductase hardly increased at all (Table 6.7).

Some of the NADH-cytochrome *c* reduction was sensitive to rotenone (Table 6.7), indicating that some inner membranes were broken, both before and after digitonin treatment. In the presence of rotenone, NADH-cytochrome *c* reductase activity was not increased by digitonin (Table 6.7), and this activity was not inhibited further by antimycin A (Table 6.7). In other words, in intact mitochondria only the outer membrane reductase contributed to cytochrome *c* reduction when exogenous NADH was the substrate.

NADH-Fe CN reductase activity of turnip mitochondria was only partially inhibited by antimycin A (Table 6.8), indicating that two pathways were operating (see Chapter III). However, antimycin did not inhibit NADH-Fe CN reductase activity of isolated beetroot mitochondria (Table 6.8). That is, only one pathway (insensitive to antimycin) operated in beet mitochondria. Succinate-Fe CN reductase activity was completely inhibited by antimycin A, showing that in undamaged mitochondria Fe CN accepted electrons from the respiratory chain at the cyt. *c* site.

From these results it appears that mitochondria from *Beta vulgaris* lack the external NADH-dehydrogenase associated with the outer surface of the inner membrane of most other plant mitochondria (see Chapter III). That is, beetroot mitochondria do not oxidize exogenous NADH directly via the respiratory chain, and in this respect resemble animal mitochondria (Lehninger, 1951). However, their outer membrane NADH-cytochrome *c* reductase appears to be identical to that of cauliflower bud and turnip mitochondria,

at least with respect to pCMB inhibition (Table 6.9). When the mitochondria were preincubated with pCMB cytochrome *c* reduction was inhibited almost completely, but NADH, if added before pCMB, protected the dehydrogenase to some extent from attack by the mercurial (Table 6.9). Cauliflower bud mitochondria displayed a similar pattern of inhibition (Table 6.9; see also Chapter III). Obviously, the outer membrane NADH-dehydrogenase contains similar-SH groups and binds NADH in the same manner in beetroot and other plant mitochondria.

Because beet mitochondria do not oxidize exogenous NADH via the respiratory chain, external NAD^+ did not stimulate malate - induced oxygen uptake in the presence of rotenone (Fig. 6.17A), although antimycin A inhibition was slightly relieved upon addition of NAD^+ and cytochrome *c* (Fig. 6.17B), as it was in other plant mitochondria (Fig. 6.17C). This suggests that reducing equivalents can be transported across the inner membrane of beetroot mitochondria, as well as other plant mitochondria. This is more clearly illustrated by the effect of exogenous NAD^+ on malate-cytochrome *c* reduction. Cytochrome *c* was reduced upon addition of malate to beet mitochondria (Fig. 6.18), indicating that some of the outer membranes were damaged; this cytochrome *c* reduction was completely inhibited by antimycin A but addition of NAD^+ relieved this inhibition (Fig. 6.18). That cytochrome *c* reduction in the presence of NAD^+ was not due to mitochondria with broken inner membranes, or attributable to extra-mitochondrial

malate dehydrogenase (or malic enzyme) activity, is shown by the n-butylmalonate inhibition of the NAD^+-stimulated rate (Fig. 6.18). Butylmalonate does not inhibit malate dehydrogenase of these mitochondria (Wiskich, 1975), and oxidized cytochrome *c* was not limiting since NADH stimulated reduction (Fig. 6.18).

Rotenone inhibited malate-Fe CN reductase activity by approximately 80%, but this inhibition was partly relieved by adding NAD^+ (Fig 6.19). This NAD^+-induced Fe CN reduction was via the outer membrane NADH dehydrogenase; antimycin A inhibited Fe CN reduction in the presence of exogenous NAD^+ by eliminating the contribution by malate alone (Fig. 6.19). That is, in the presence of antimycin A only NADH-Fe CN reductase activity was measured, again implying a transmembrane transfer of reducing equivalents.

Malate-induced reduction of exogenous NAD^+ by beetroot mitochondria was also measured, and displayed similar kinetics, both in intact and disrupted mitochondria, to that by cauliflower bud mitochondria (Fig. 6.20; see Fig. 6.1). Exogenous NAD^+ reduction by beet mitochondria was inhibited by the omission of glutamate or phosphate from the reaction medium (Figs. 6.21 and 6.22 respectively). Subsequent addition of phosphate or glutamate to the medium stimulated NAD^+ reduction (Figs. 6.21B and 6.22B). These results show that the NAD^+ reduction observed was not due to extramitochondrial enzyme activity, and that exogenous NAD^+ reduction by beetroot mitochondria occurred in a similar fashion to that by cauliflower mitochondria.

The most important feature of these studies with beetroot mitochondria (at least with respect to the present study) is that they show that although exogenous NADH is not oxidized by the respiratory chain and therefore does not penetrate the inner membrane, exogenous NAD^+ reduction still occurs. That is, despite the fact that added NADH cannot penetrate the intact inner membrane, reducing equivalents can be transferred across the membrane upon oxidation of NAD-linked substrate. This strongly suggests that such a transfer occurs in a controlled manner, either *via* a transmembrane transhydrogenase or some other transporting system. The results also indicate that (H) can only be transferred in one direction, *viz.* out of the matrix.

2. *Mitochondria from 'aged' tissue*

Mitochondria isolated from beetroot which had been sliced into discs and 'aged', by bubbling in $CaSO_4$, for 48 hours, oxidized exogenous NADH in a rotenone insensitive manner (Fig. 6.23B). However, malate oxidation by these mitochondria was inhibited by the same concentration of rotenone (Fig. 6.23C). Neither the slicing process alone, nor use of a different isolation method, were responsible for the induction of exogenous NADH oxidation (Fig. 6.23A).

ADP/O ratios associated with exogenous NADH oxidation by mitochondria from aged beetroot were always less than two, unlike those found with malate as substrate which were between two and three (Table 6.10). External NADH oxidation was

completely inhibited by antimycin A, but this inhibition was partly relieved by adding cytochrome *c* (Fig. 6.24B). That is, these mitochondria oxidized added NADH in much the same manner as those isolated from other plant tissues (see Chapter III), such as cauliflower buds. NADH oxidation was not due to penetration of the inner membrane by this compound, since it was insensitive to rotenone and coupled to only two phosphorylation sites. The small amount of oxygen uptake in the presence of antimycin A and cytochrome *c* also suggests that most of the mitochondria were intact (Fig. 6.24B). Dicoumarol inhibited NADH oxidation, but not to the same extent as that by other plant mitochondria (NADH oxidation by cauliflower and turnip mitochondria was inhibited completely by 50 μM dicoumarol - see Chapter III). This implies that the external NADH dehydrogenases of the different mitochondria are not identical.

Two other minor differences between fresh and 'aged' beetroot were observed. Fresh beetroot contained very little detectable starch; no starch was found in either the low speed or high speed (12000g) pellets during isolation of mitochondria. However, considerable starch deposits were found in the 1500g pellet during isolation of mitochondria from 'aged' beetroot discs, suggesting that starch was produced during the aging process. This may represent an osmotic adjustment of the tissue to its incubation in hypotonic $CaSO_4$ solution; the significance of starch accumulation to the induction of external NADH oxidation is not immediately apparent.

It was also noticeable that rotenone inhibited malate oxidation by mitochondria from fresh beetroot, more strongly than it inhibited malate oxidation by mitochondria that oxidized exogenous NADH (such as aged beetroot, cauliflower and maize mitochondria - Table 6.11). In general, malate oxidation by mitochondria from fresh beet was inhibited 10 - 20% more, by 15 μM rotenone, than that by mitochondria from the other tissues (Table 6.11). This suggests that the insensitivity of malate oxidation to rotenone, generally observed with most plant mitochondria, is associated with the presence of external NADH dehydrogenase activity. Perhaps some reducing equivalents are transferred directly to this enzyme during malate oxidation, although this would be expected to give lower ADP/O values than those observed.

Whether the induction of exogenous NADH oxidation by beetroot mitochondria (upon slicing and aging) is due to *de novo* synthesis of an enzyme or, more simply, to an activation (or 'derepression') of the dehydrogenase involved, has yet to be determined. The role (if any) this phenomenom plays in the respiratory rise observed upon aging plant storage tissues (e.g. see Laties, 1968) should also be determined. Nonetheless, the results presented here do show that plant tissues can modify NADH oxidation by their mitochondria. Future studies using protein synthesis inhibitors (particularly on whole tissue or cells) should shed some light on the development of NADH oxidation by plant mitochondria, and its importance to cellular metabolism.

3. *NADH oxidation via substrate shuttles*

Although exogenous NADH is not oxidized directly by fresh beetroot mitochondria, it can be oxidized extra-mitochondrially via a malate-oxaloacetate shuttle. NADH added to beetroot mitochondria was not oxidized, even in the presence of exogenous malate dehydrogenase and ADP, until malate was added to the medium (Fig. 6.25). This oxidation took several minutes to reach its maximum rate, and displayed respiratory control; rotenone inhibited NADH oxidation very severely (Fig. 6.25), as did antimycin A. Glutamate also inhibited NADH oxidation, but subsequent addition of glutamate - oxaloacetate transaminase did not stimulate much (Fig. 6.26). When the mitochondria were preincubated for a few minutes with malate and ADP, prior to the addition of NADH, the initial lag in NADH oxidation was abolished (Fig. 6.27B). Under these conditions, a rapid burst of oxidation was observed initially, followed by a considerable slowing of the rate which then gradually increased again (Fig. 6.27B). If glutamate was also present during preincubation with malate, NADH oxidation was dramatically inhibited (Fig. 6.27A). However, glutamate did not always completely inhibit NADH oxidation (Fig. 6.27A; *c.f.* Fig. 6.26).

These results are interpreted as follows (Fig. 6.29); malate enters the matrix and is oxidized to form oxaloacetate which, in the absence of glutamate, leaves the matrix and is reconverted to malate by the exogenous malate dehydrogenase.

A concommittant oxidation of added NADH is seen. Since continuing malate oxidation relies on oxidation of intramitochondrial NADH by the respiratory chain, external NADH oxidation is inhibited by rotenone and antimycin A. Likewise, when ADP is limiting, NADH oxidation is restricted. Electron transport inhibitors did not affect exogenous NADH oxidation directly, because added NADH is not oxidized via the respiratory chain of beetroot mitochondria(Fig.6.15). That is, exogenous NADH oxidation by these mitochondria is dependent on added MDH and internal malate oxidation.

Preincubation of the mitochondria with malate allowed oxaloacetate to accumulate in the medium and hence a rapid burst of NADH oxidation was observed, until oxaloacetate efflux again became limiting. Presumably the lag observed when malate was first added to the mitochondria (Fig. 6.25) was due to the time taken for oxaloacetate to build up in the medium; a steady state was reached when oxaloacetate efflux equalled its removal (externally) by MDH.

In the presence of glutamate, oxaloacetate is transaminated (in the matrix) to ∝-ketoglutarate and aspartate and therefore NADH oxidation was inhibited. The different degrees of inhibition by glutamate probably reflect varying permeability of the inner membrane to OAA, or varying aspartate aminotransferase activity, in different mitochondrial preparations. Since addition of GOT, in the presence of glutamate, did not stimulate NADH oxidation significantly (Fig. 6.26), it appears that the malate-

aspartate cycle does not operate in these mitochondria. This also implies that ∝-ketoglutarate, and/or aspartate, transport out of the matrix is very slow compared to malate and oxaloacetate movement across the inner membrane.

The operation of the malate-oxaloacetate shuttle could also be demonstrated using the oxygen electrode. Oxygen uptake with malate as substrate was inhibited within a few minutes unless glutamate was also present (Fig. 6.28B). Addition of NADH and MDH during the inhibited state stimulated oxygen uptake to the same extent as glutamate (Fig. 6.28A). Addition of NADH and glutamate together stimulated malate oxidation more than did addition of either substance alone (Fig. 6.28B), indicating that not all oxaloacetate formed in the matrix was removed by transamination or OAA efflux across the inner membrane.

Such a malate-oxaloacetate cycle may also operate under certain conditions in mitochondria isolated from other tissues, but its demonstration is more difficult because such mitochondria oxidize exogenous NADH directly via their respiratory chain.

Table 6.1. Effect of antimycin A and NAD^+ on cytochrome c reduction by cauliflower mitochondria

Cytochrome *c* reduction was measured as described in Materials and Methods. 1 mg mitochondrial protein, 5 μM antimycin A and 0.5 mM NAD^+ were used. Substances added to the cuvette are shown in order of addition.

Additions to cuvette	Malate	Isocitrate	KG
A. *Intact Mitochondria*	*nmoles/min. mg. protein*		
substrate (+ADP)	4	3	0
NAD^+	29	8	3
antimycin A	18	4	3
B. *Mitochondria preincubated with digitonin**			
substrate (+ADP)	36	17	28
antimycin A	0	0	0
NAD^+	21	4	0

* 0.1 mg/mg protein, for 30 min at 0C.

Table 6.2 Transfer of reducing equivalents out of cauliflower mitochondria

Oxygen uptake and NAD^+ reduction were measured as described in Materials and Methods. 10 mM malate (+10 mM glutamate) was the substrate in each case. Concentrations of other reagents were; 0.5 mM NAD^+, 25 μM PMS, 5 μM antimycin A and 10 μM rotenone.

Rate measured		Oxidized or reduced NAD (H)
		nmoles/ min. mg protein
NAD^+ - induced increase of rotenone inhibited malate oxidation.	(1)*	163
	(2)	164
NAD^+ - induced increase of malate oxidation in the presence of PMS and antimycin A.	(1)	56
	(2)	60
Initial rate of malate -induced NAD^+ reduction.	(1)	55
	(2)	50

* Numbers in parentheses indicate separate experiments.

Table 6.3 The effect of exogenous NAD(P)H On malate, citrate and ∝-ketoglutarate oxidation by cauliflower mitochondria

Oxygen uptake was measured as described in Materials and Methods. Concentrations of reagents used were: 10 mM malate, 10 mM citrate, 10 mM ∝-ketoglutarate, 15 µM rotenone, 0.5 mM $NAD(P)^+$, 0.28 mM ADP and 1 mM NADPH. Rotenone and $NAD(P)^+$ were added during state 3 in each case.

Substrate	state 3	Oxygen uptake + rotenone	+rotenone & NADP	+rotenone & NAD
		nmoles/min. mg protein		
malate	113	40	52	110
citrate	58	14	20	54
∝-KG	48	12	15	36
NADPH	33	33	-	-

Table 6.4 The effect of DNP on the recovery of malate and citrate oxidation by NAD^+

Oxygen uptake was measured as described in Materials and Methods. Final concentrations were ; malate and citrate 20 mM, NAD^+ 1 mM, DNP 30 µM and ADP 0.3 mM.

Addition to reaction vessel	Rate of O_2 uptake			
	Malate		Citrate	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
	nmoles/ min. mg protein			
1. Substrate	36	29	23	18
2. ADP	68	104	30	45
3. Rotenone	32	42	15	24
4. NAD^+	62	79	29	45
1. Substrate	38	29	28	21
2. DNP	68	104	35	46
3. Rotenone	20	36	18	22
4. NAD^+	46	72	35	52

Table 6.5 Cytochrome c reductase activity in cauliflower bud mitochondria

Details of assays are described in Materials and Methods. NAD^+ (0.5 mM) and 5 µM Antimycin A were used.

Substrate	Rate cyt. *c* reduction
	nmoles/min. mg. protein
malate	3.4
malate + NAD^+	23.5
Succinate	9.7
Succinate[1]	114
NADH	68.2
NADH + Antimycin A	51.4

[1] mitochondria were incubated in distilled water for five minutes prior to assaying.

Table 6.6 The effect of exogenous NAD^+ on malate oxidation by maize and turnip root mitochondria

Oxygen uptake was measured as described in Materials and Methods. Final concentrations were: 10 mM malate, 0.5 mM NAD^+, 10 μM rotenone, and 0.5 mM ADP.

Mitochondrial source	Oxygen uptake		
	State 3	+ Rotenone	+ Rotenone & NAD^+
	nmoles / min. mg protein		
Maize roots	128	48	85
Turnip roots	112	35	60

Table 6.7 Cytochrome c reduction by beetroot mitochondria

Details of assays are given in Materials and Methods. Final concentrations were; 0.3 mg mitochondrial protein, 5 µM antimycin A, 25 µM cytochrome *c*, 10 µM rotenone, 0.14 mM ADP, 0.5 mM NADH and 10 mM succinate.

Substrate	Control	Digitonin* - treated mitochondria
	nmoles cyt. c reduced/min.mg protein	
Succinate	28	213
Succinate + antimycin	0	0
NADH	113	137
NADH + rotenone	85	83
NADH - antimycin	85	80

* 0.1 mg / mg mitochondrial protein, for 30 min at 0C.

Table 6.8 Ferricyanide reduction by beetroot and turnip mitochondria

Ferricyanide reduction was measured as described in Materials and Methods. Final concentrations were; 0.9 mM Fe CN, 15 mM succinate, 0.25 mM NADH, 0.13 mM ADP, 5 µM antimycin A.

Substrate	Source of mitochondria	
	Beetroot	Turnip
	µmoles Fe CN reduced/min.mg protein	
Succinate	0.514	0.823
Succinate + antimycin	0.0	0.0
NADH	1.714	3.528
NADH + antimycin	1.714	2.508

Table 6.9 Effect of pCMB on antimycin-insensitive NADH-cytochrome c reductase activity of isolated plant mitochondria

Cytochrome *c* reduction was assayed as described in Materials and Methods. 0.1 mg outer membrane protein 0.5 mM NADH, 25 µM pCMB, 5 µM antimycin A and 0.025 mM cytochrome *c* were used. Initial rates of cytochrome *c* are shown.

	Beetroot mitochondria	Cauliflower bud mitochondria
	nmoles / min. mg protein	
NADH	50	50
NADH + pCMB (NADH added first)	35	24
NADH + pCMB (pCMB added first)	5	0

Table 6.10 Respiratory control and ADP/O ratios of aged beetroot mitochondria

Oxygen uptake and ADP/O values were measured as described in Materials and Methods. 10 mM malate and 0.5 mM NADH were used as substrates.

Substrate	ADP/O ratio	R.C.R.
MALATE	2.6	3.0
	2.3	2.5
NADH	1.3	2.0
	1.1	1.7

Table 6.11 The effect of rotenone on malate oxidation by various plant mitochondria

Oxygen uptake was measured as described in Materials and Methods. Rates are expressed as nmoles O_2/ min. mg protein. Final concentrations were; 10 mM malate, 1 mM NADH, 0.33 mM ADP and 15 µM rotenone.

Mitochondrial Source	Malate oxidation State 3	Malate oxidation + Rotenone	NADH oxidation State 3
Fresh beetroot	101	21 (79%)	0
	150	31 (80%)	3
Aged beetroot	92	45 (52%)	62
	47	18 (62%)	44
Cauliflower	82	36 (57%)	129
	76	24 (69%)	132
Maize roots	128	48 (63%)	220

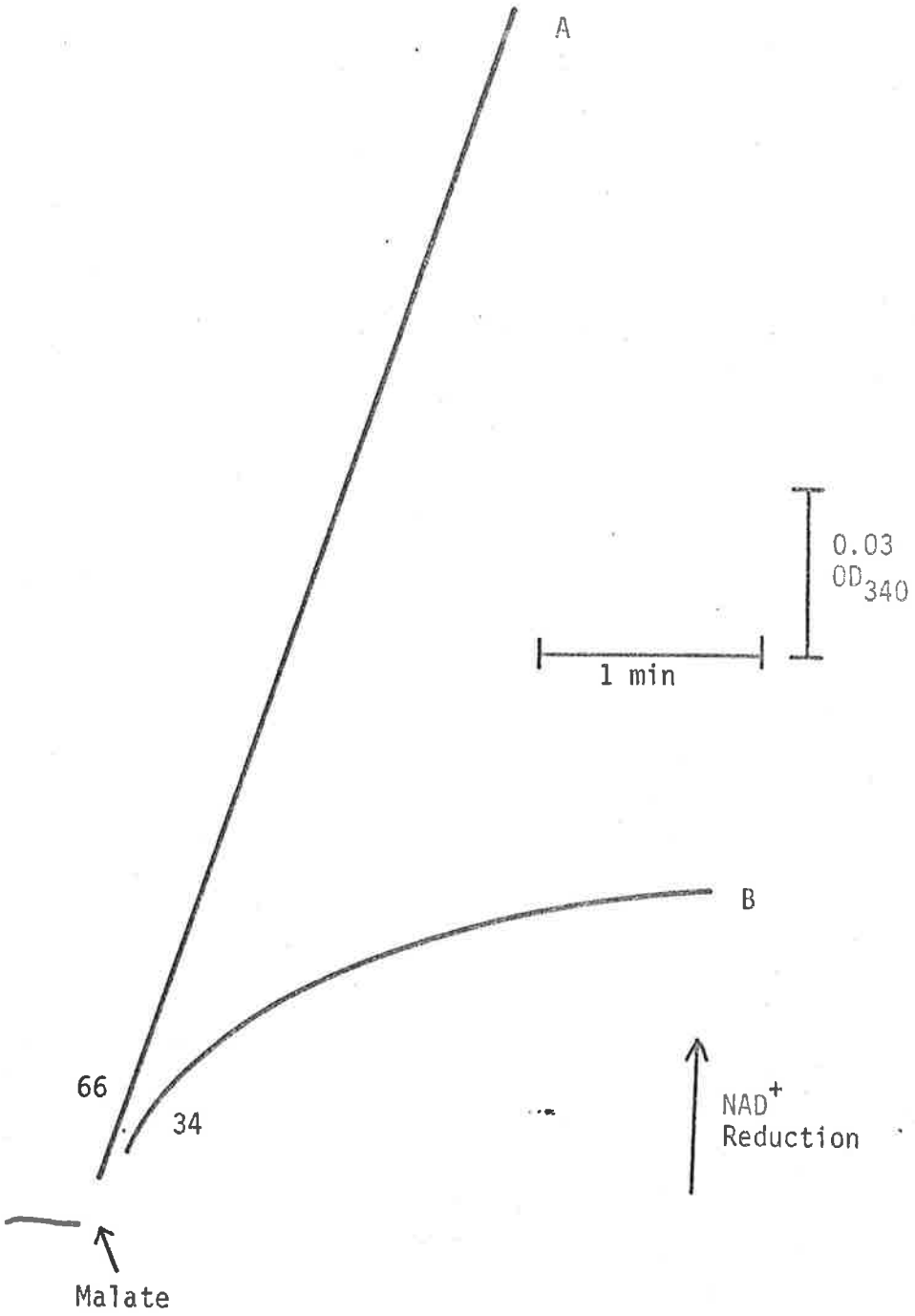


Figure 6.1 Exogenous NAD^+ reduction by cauliflower bud mitochondria.

NAD^+ reduction was measured spectrophotometrically as described in Materials and Methods. The reaction medium also contained 1.1 mg mitochondrial protein, 10 mM glutamate, 0.3 mM NAD^+ and 5 µM antimycin A. 10 mM malate was added as indicated. Rates are expressed as nmoles NAD^+ reduced/min. mg protein.

A : mitochondria pre-treated with 'Decon 90' detergent.
B : untreated mitochondria.

(N.B. Separate traces were superimposed for comparison)

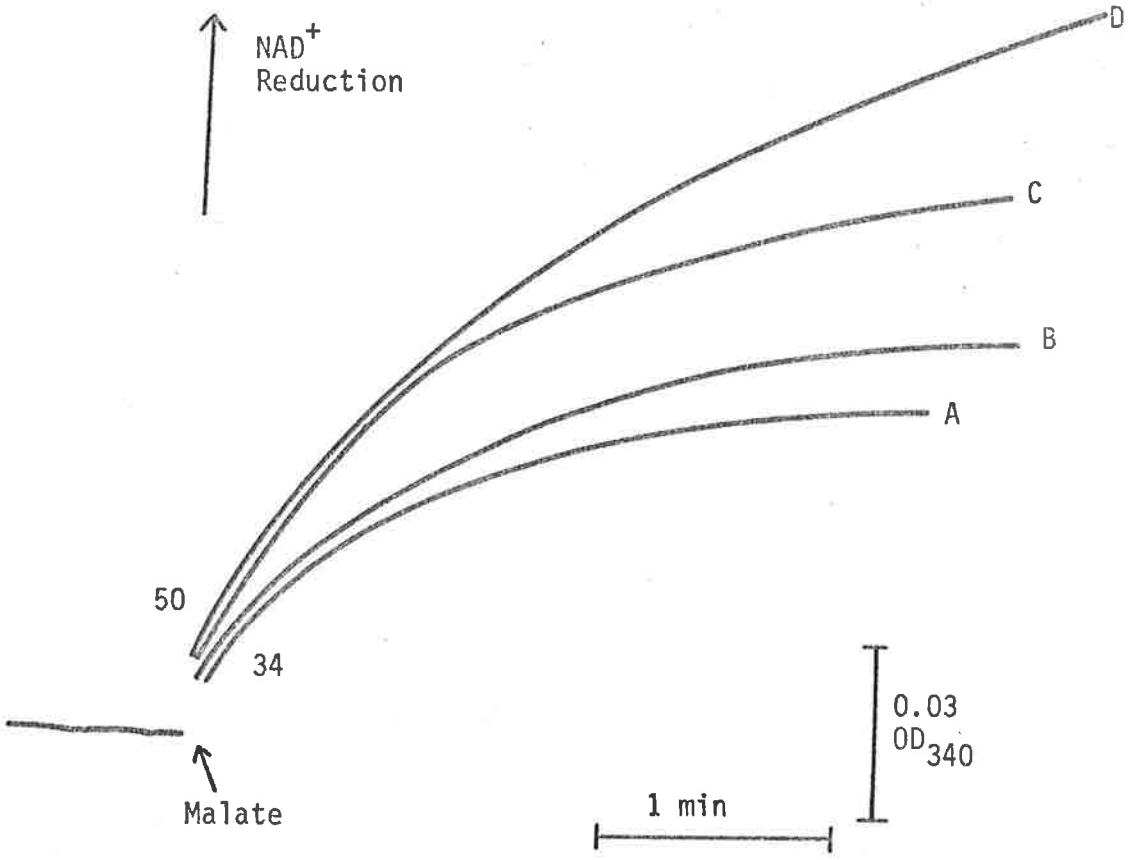


Figure 6.2 Effect of varying NAD^+ and malate concentrations on exogenous NAD^+ reduction by cauliflower mitochondria.

Assay conditions are described in Figure 6.1, except that malate and NAD^+ concentrations were varied.

A : 0.3 mM NAD^+ and 8 mM malate

B : 0.6 mM NAD^+ and 8 mM malate

C : 0.6 mM NAD^+ and 30 mM malate

D : 1.2 mM NAD^+ and 30 mM malate.

Rates are expressed as nmoles NAD^+ reduced per min.mg protein.

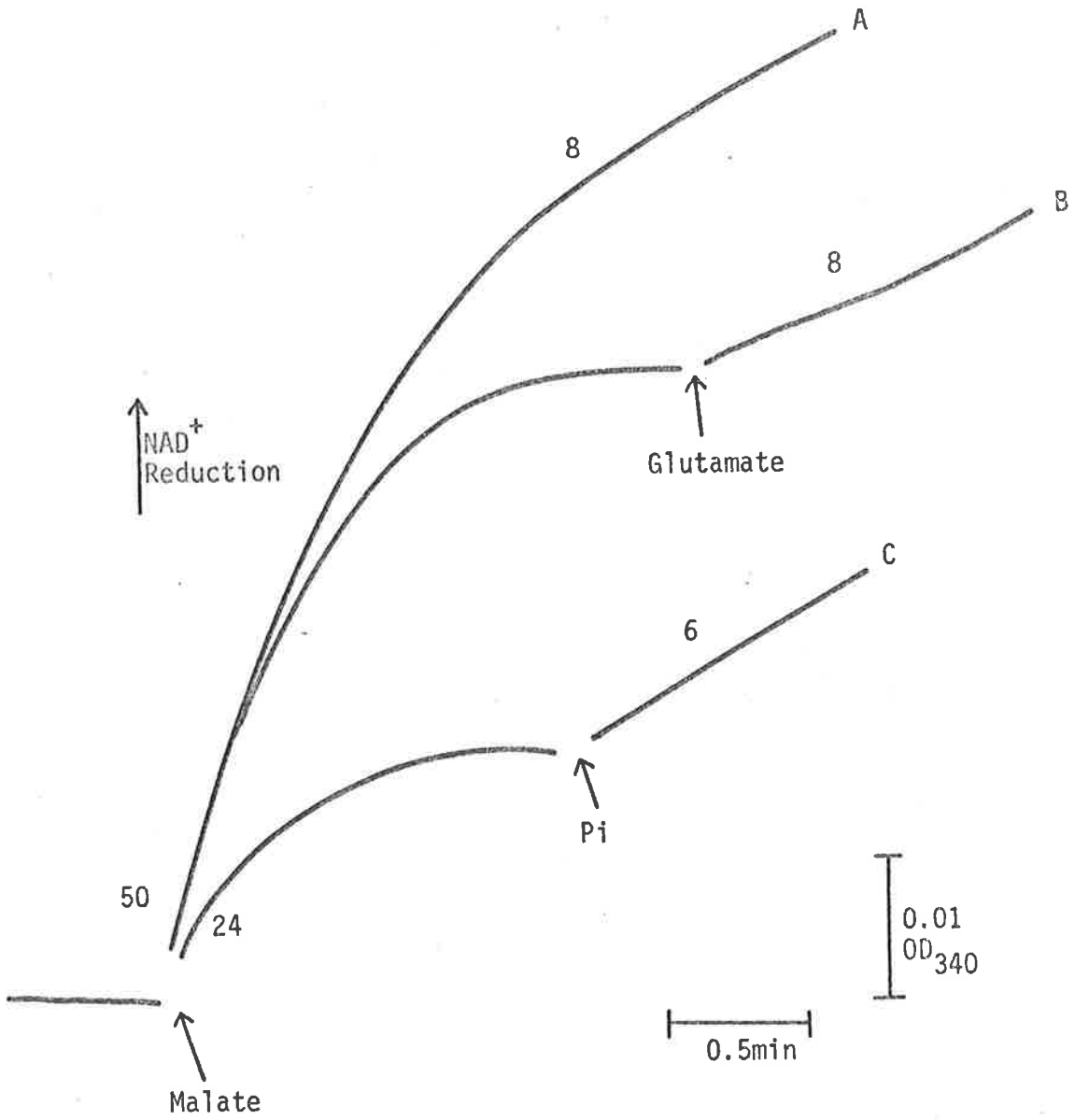


Figure 6.3 Effect of glutamate and phosphate on exogenous NAD^+ reduction by cauliflower mitochondria.

Assay conditions were the same as those for Figure 6.1, except that: B; glutamate (10mM) was omitted from the medium and added as indicated, and C; phosphate (10 mM) was omitted from the medium and added as indicated. Rates are expressed as nmoles/min. mg protein.

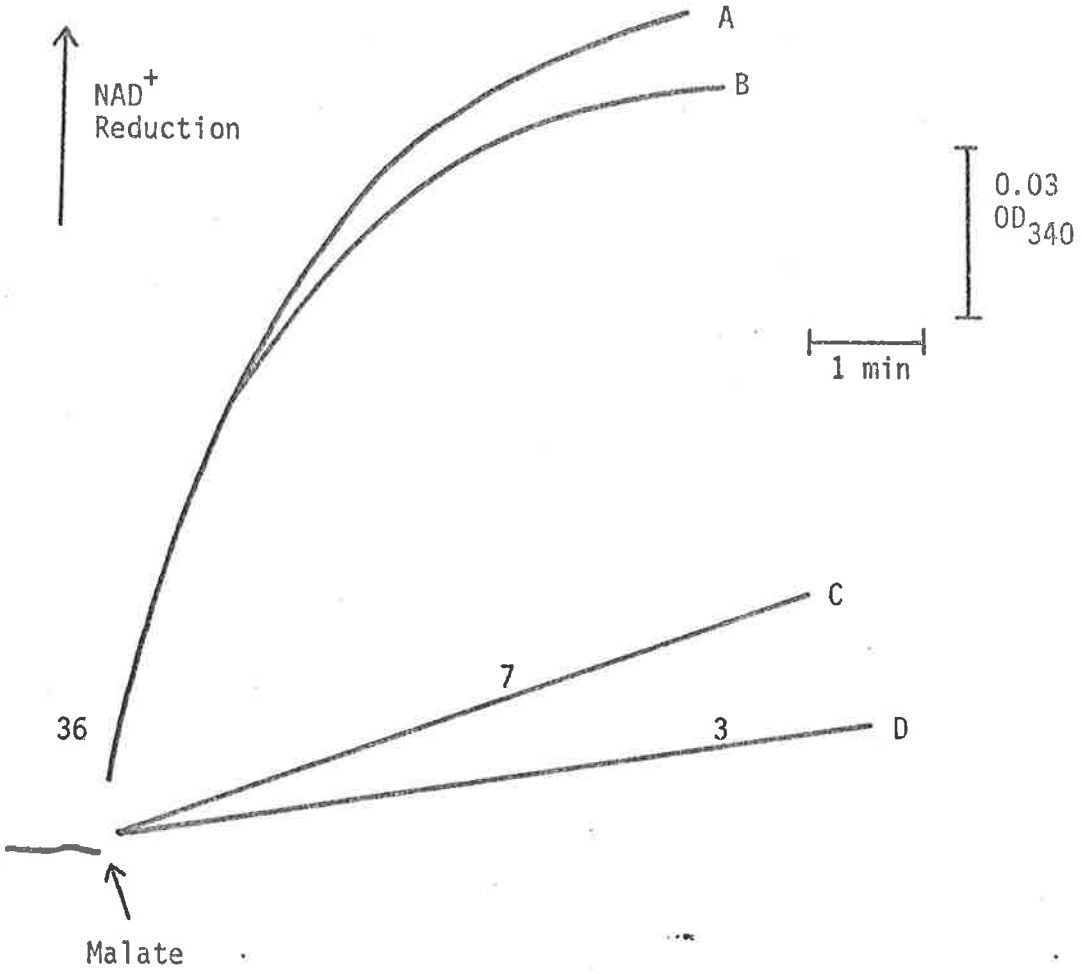


Figure 6.4 Effect of ATP and NADH on exogenous NAD^+ reduction by cauliflower mitochondria.

NAD^+ reduction was measured as described in Materials and Methods. 10 mM glutamate, 5 µM antimycin A, 0.5 mM NAD^+ and 2.4 mg mitochondrial protein were included in the reaction medium, and 10 mM malate was added as indicated.

A : 0.6 mM ATP also added to reacton medium.

B : control

C : 0.1 mM NADH added to medium

D : 0.05 mM NADH added to medium

Rates are expressed as nmoles/min. mg protein.

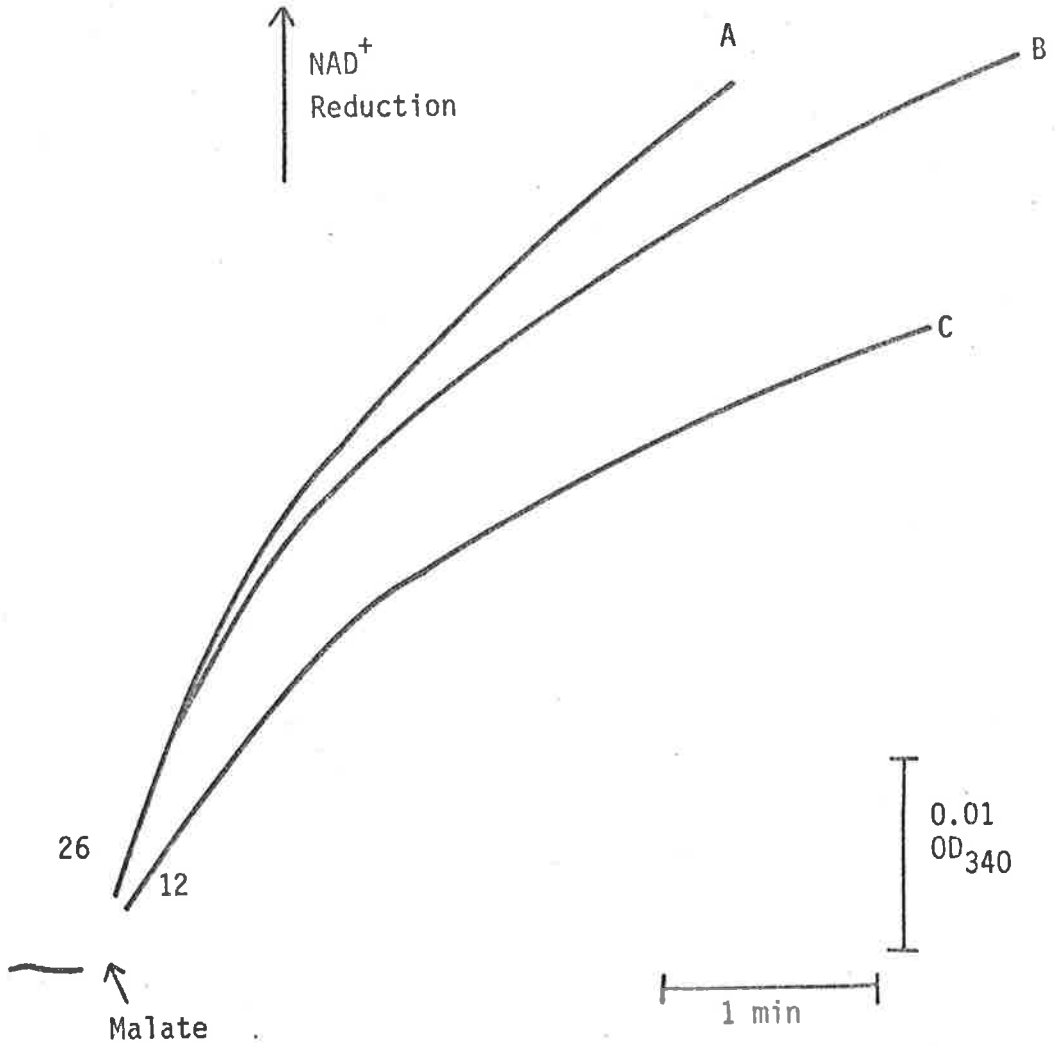


Figure 6.5 Effect of rotenone and dicoumarol on NAD^+ reduction by cauliflower mitochondria.

NAD^+ reduction was measured as described in Materials and Methods. In each case 0.5 mM NAD^+, 10 mM glutamate and 0.7 mg mitochondrial protein were included in the reaction medium, and 10 mM malate added as shown.

A : + 50 µM dicoumarol and 25 µM rotenone

B : + 5 µM antimycin A

C : + 50 µM dicoumarol.

Rates are expressed as nmoles/min. mg protein.

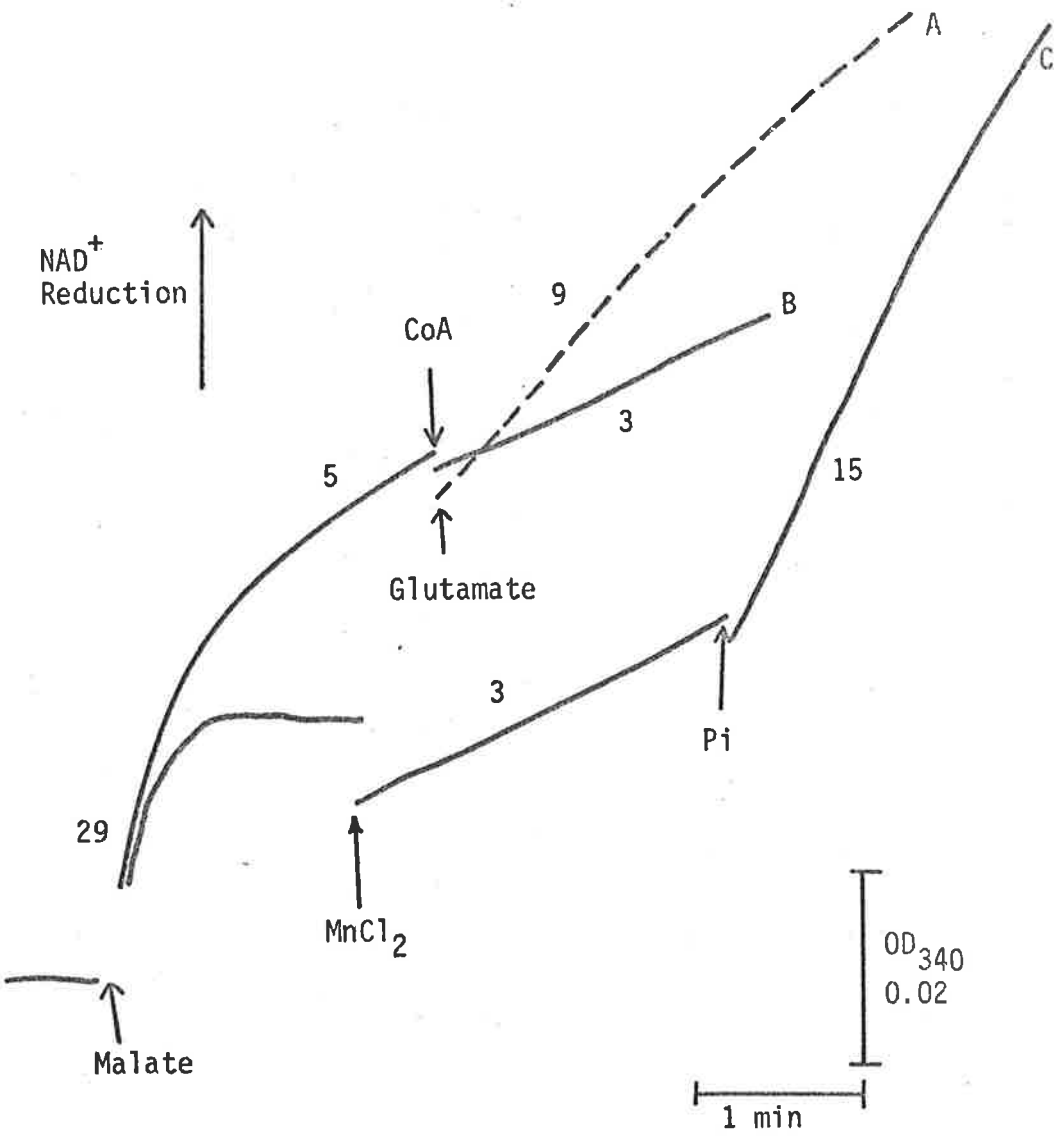


Figure 6.6 Effect of $MnCl_2$ and CoA on exogenous NAD^+ reduction by cauliflower mitochondria.

NAD^+ reduction was measured as described in Materials and Methods. 5 µM antimycin A, 1 mg mitochondrial protein and 0.5 mM NAD^+ were included in the reaction medium.

A and B : Glutamate (10 mM) and CoA (0.1 mM) were added as shown.

C : Phosphate was omitted from the medium and added (10 mM) as shown, as was $MnCl_2$ (6 mM).

In each case 10 mM malate was added where indicated. Rates are expressed as nmoles/min. mg protein.

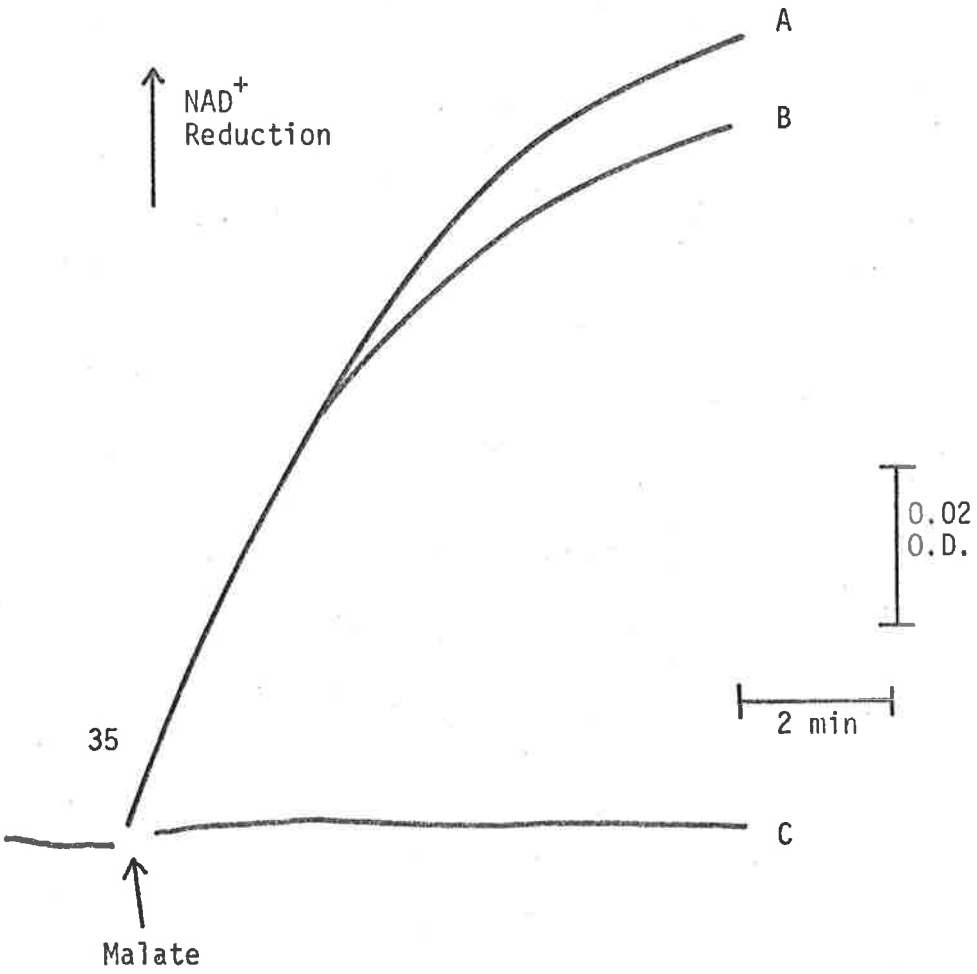


Figure 6.7 Effect of palmitoyl CoA on $NAD(P)^+$ reduction by cauliflower mitochondria.

$NAD(P)^+$ reduction was measured as described in Materials and Methods. 1.2 mg mitochondrial protein, 10 mM glutamate, 5 µM antimycin A and 0.3 mM $NAD(P)^+$ were included in the reaction medium, and 10 mM malate was added as shown. Rates are expressed as nmoles/min. mg protein.

A : +50 µg palmitoyl CoA.

B : control.

C : 0.3 mM $NADP^+$ instead of NAD^+.

Figure 6.8 NAD^+ reduction by cauliflower mitochondria with isocitrate and malate as substrates.

NAD^+ reduction was measured as described in Materials and Methods. In each case, 0.3 mM NAD^+ was used.

A; + 10 mM isocitrate and 5 µM antimycin A.

B; + 10 mM isocitrate + 50 µM dicoumarol.

C; + 10 mM malate + 50 µM dicoumarol.

D; + 10 mM isocitrate, 5 µM antimycin A and 20 µl 'Decon 90' detergent.

Isocitrate and malate were added as indicated by the arrow. Rates are expressed as nmoles/min.mg protein. (In C, 0.7 mg mitochondrial protein was used, while 1.4 mg was used in A, B and D).

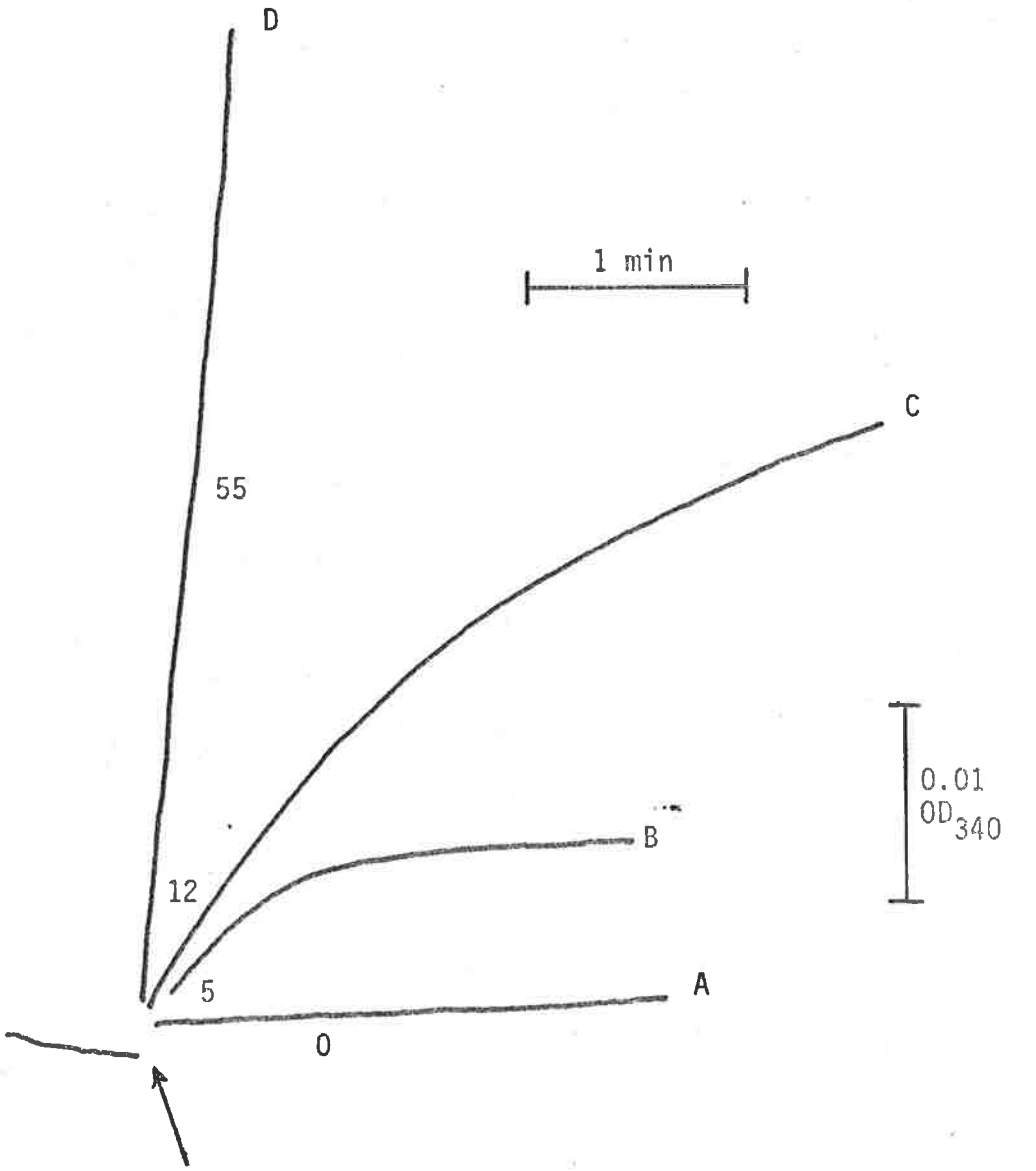

D
1 min
C
55
12
B
5
A
0
0.01
OD340

Figure 6.9 Effect of phosphate, NAD^+ and PMS on malate oxidation by cauliflower mitochondria.

Oxygen uptake was measured as described in Materials and Methods except Pi was omitted from the medium. Additions as indicated were; 3 mg mitochondrial protein, 10 mM malate, 10 mM phosphate, 0.17 mM ADP, 10 µM antimycin A, 0.3 mM NAD^+ and 30 µl 'Decon 90' detergent. In (B) NAD^+ and PMS were included in the reaction medium. Glutamate (10 mM) was present in both cases. Rates are expressed as nmoles O_2/min. mg protein.

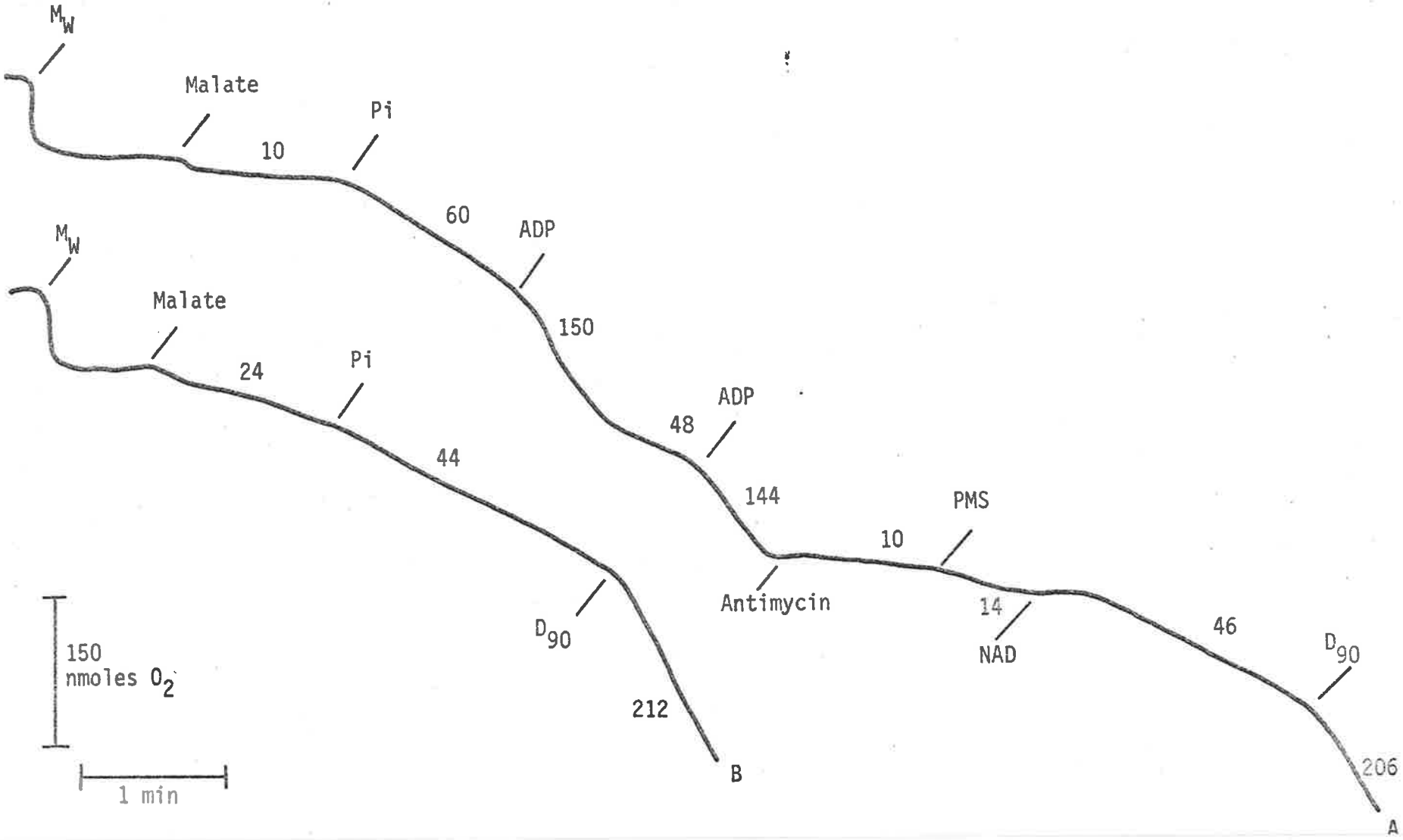

M_W
Malate
Pi
10
60
ADP
150
ADP
48
144
PMS
10
Antimycin
14
NAD
46
D_{90}
206
A
M_W
Malate
24
Pi
44
D_{90}
212
B
150
nmoles O_2
1 min

Figure 6.10 The effect of NAD^+, PMS, rotenone and antimycin A on citrate oxidation by cauliflower mitochondria.

Oxygen uptake was measured as described in Materials and Methods. Additions as indicated were; 2.6 mg mitochondrial protein, 0.175 mM ADP, 5 µM antimycin, 15 µM rotenone, 25 µM PMS, 0.5 mM NAD^+ and 15 mM citrate. Oxygen uptake is expressed as nmoles/min. mg protein.

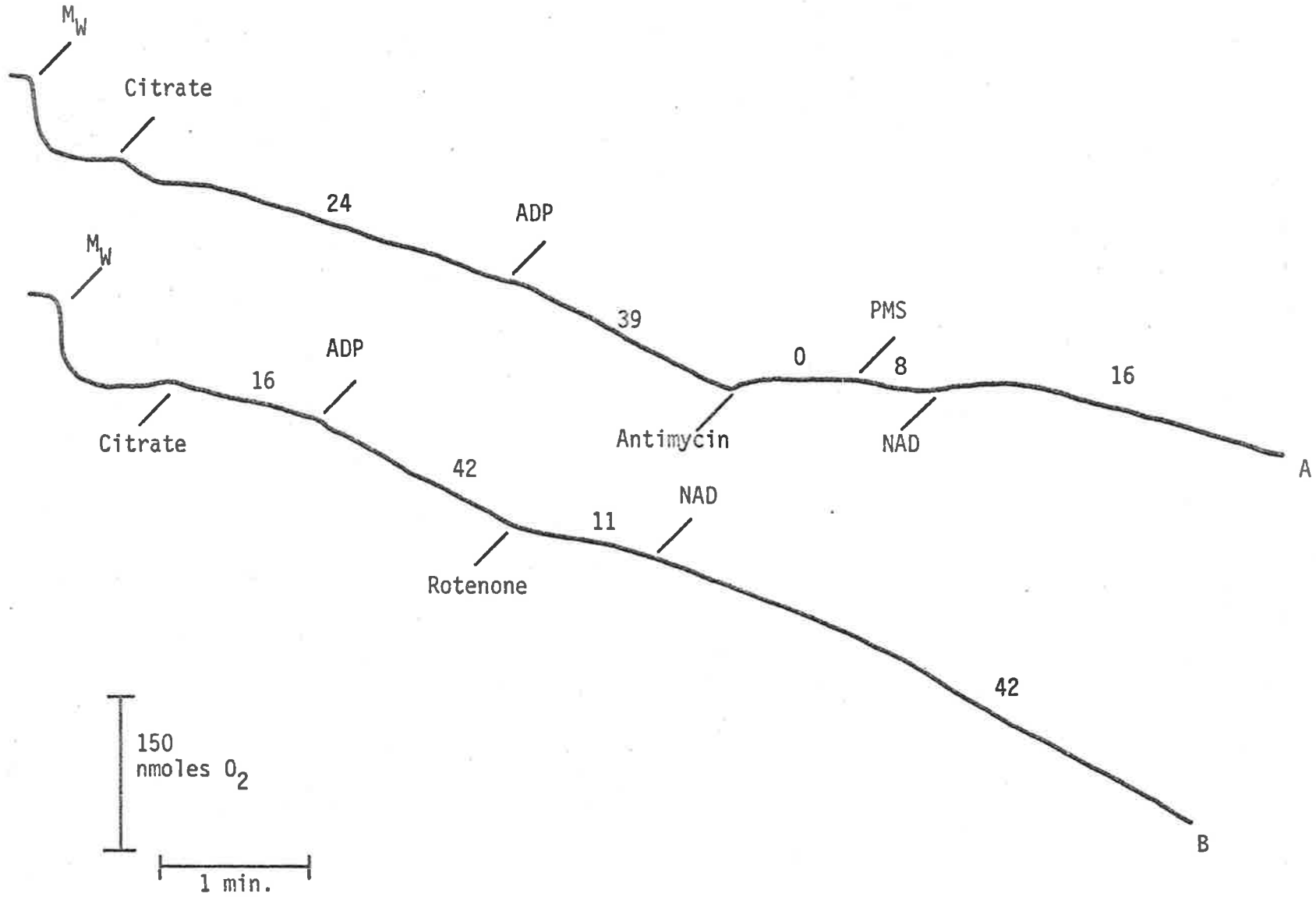
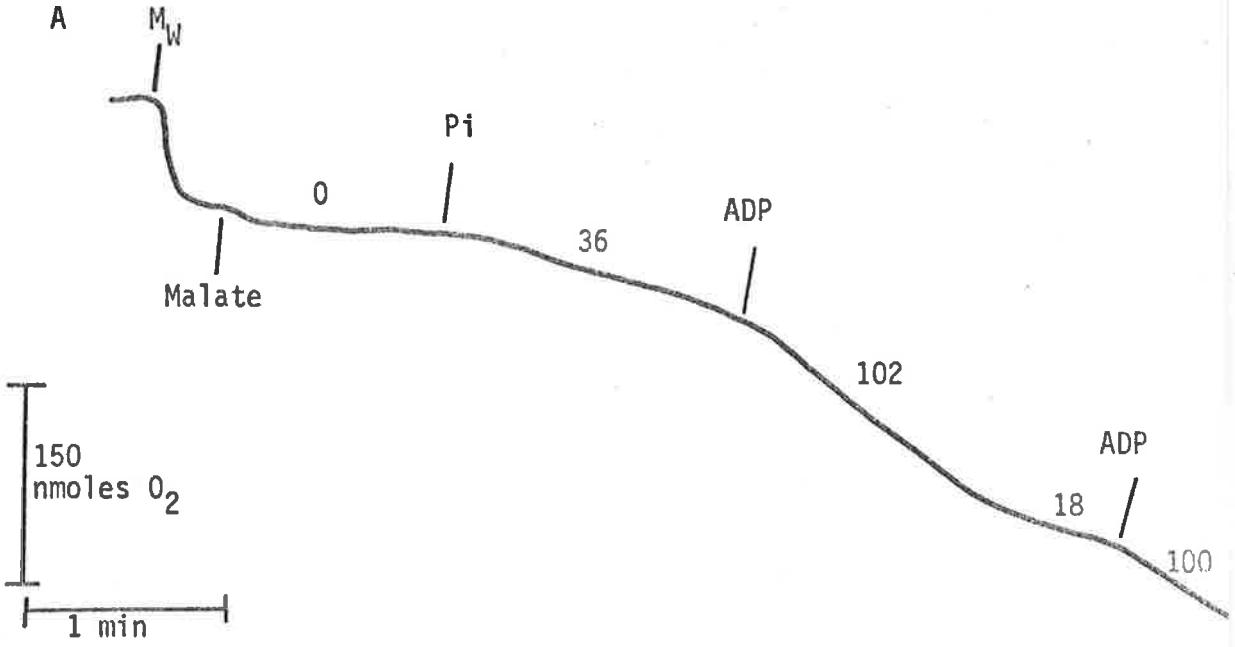

M_W
Citrate
24
ADP
39
PMS
0
8
16
A
M_W
ADP
16
Citrate
Antimycin
NAD
42
NAD
11
Rotenone
42
B
150
nmoles O_2
1 min.

Figure 6.11 Malate oxidation and NAD^+ reduction by cauliflower mitochondria.

Oxygen consumption and NAD^+ reduction were measured as described in Materials and Methods. A. Additions as indicated were; 2.6 mg mitochondrial protein, 10 mM malate, 10 mM phosphate and 0.175 mM ADP. B. The reaction medium included 0.3 mM NAD^+, 5 µM antimycin A and 10 mM glutamate. 10 mM malate was added as shown. Oxygen uptake and NAD^+ reduction are expressed as nmoles/min. mg protein.


A
M_W
Pi
0
ADP
36
Malate
102
ADP
18
100
150
nmoles O_2
1 min

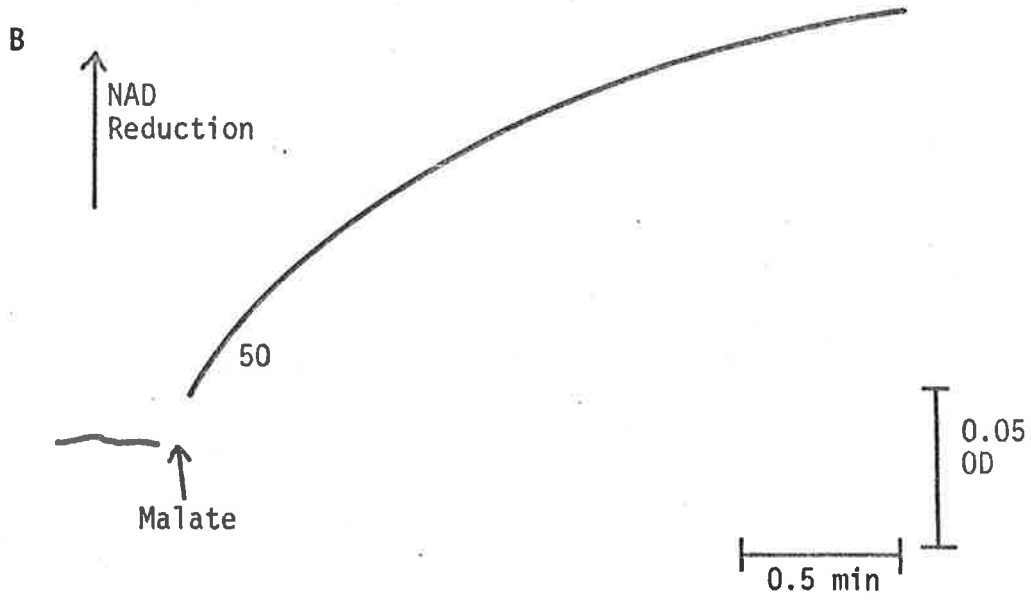

B
NAD
Reduction
50
Malate
0.05
OD
0.5 min

Figure 6.12 The effect of phosphate, rotenone and NAD^+ on isocitrate oxidation by cauliflower mitochondria.

Oxygen uptake was measured as described in Materials and Methods. Additions as indicated were; 1.5 mg mitochondrial protein, 15 mM isocitrate, 10 mM Pi, 0.235 mM ADP, 10 µM rotenone and 0.5 mM NAD^+. Malonate (10 mM) was included in the reaction medium, but in trace A phosphate was omitted and added as indicated. Rates are expressed as nmoles O_2/min. mg protein.

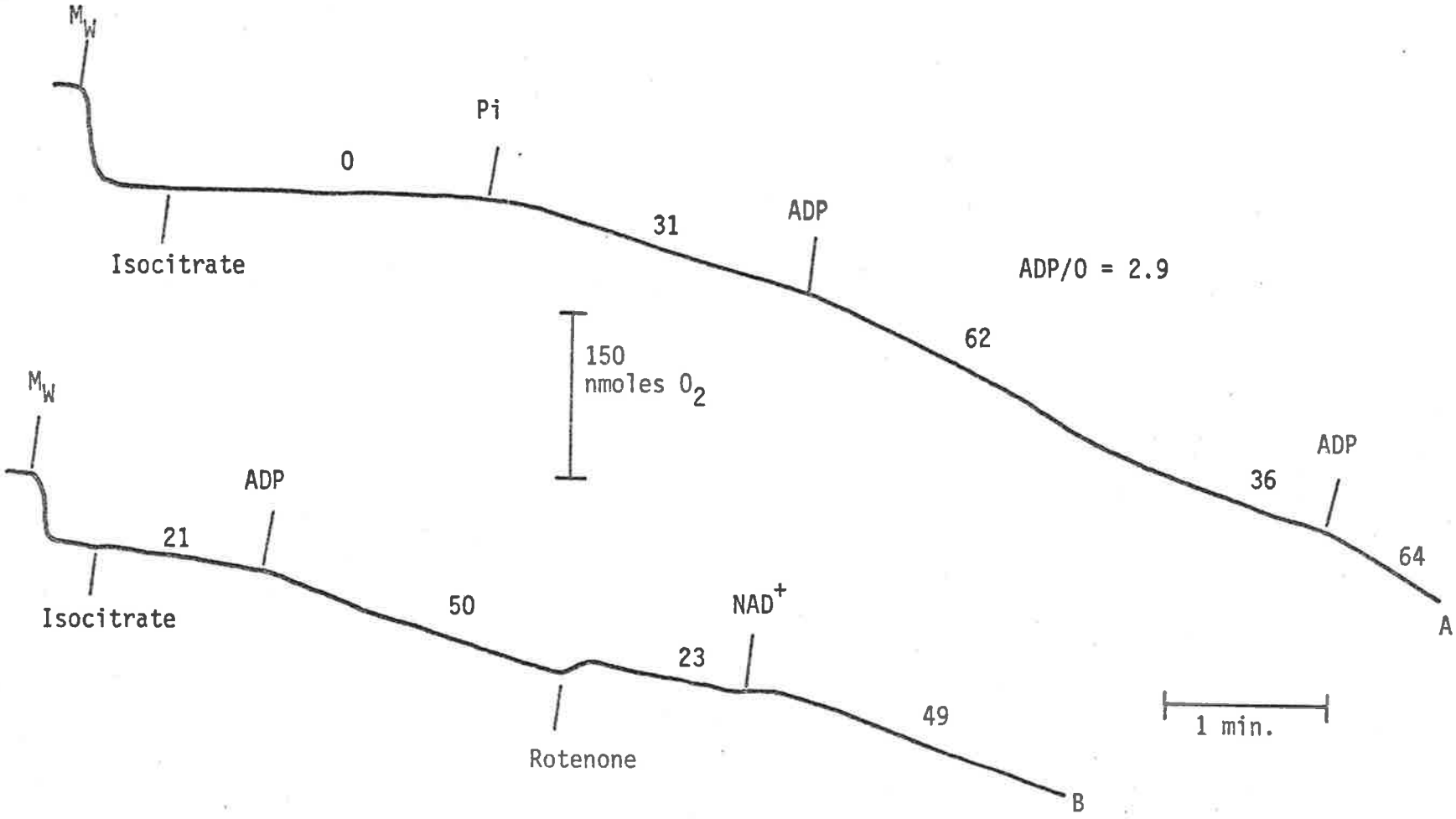
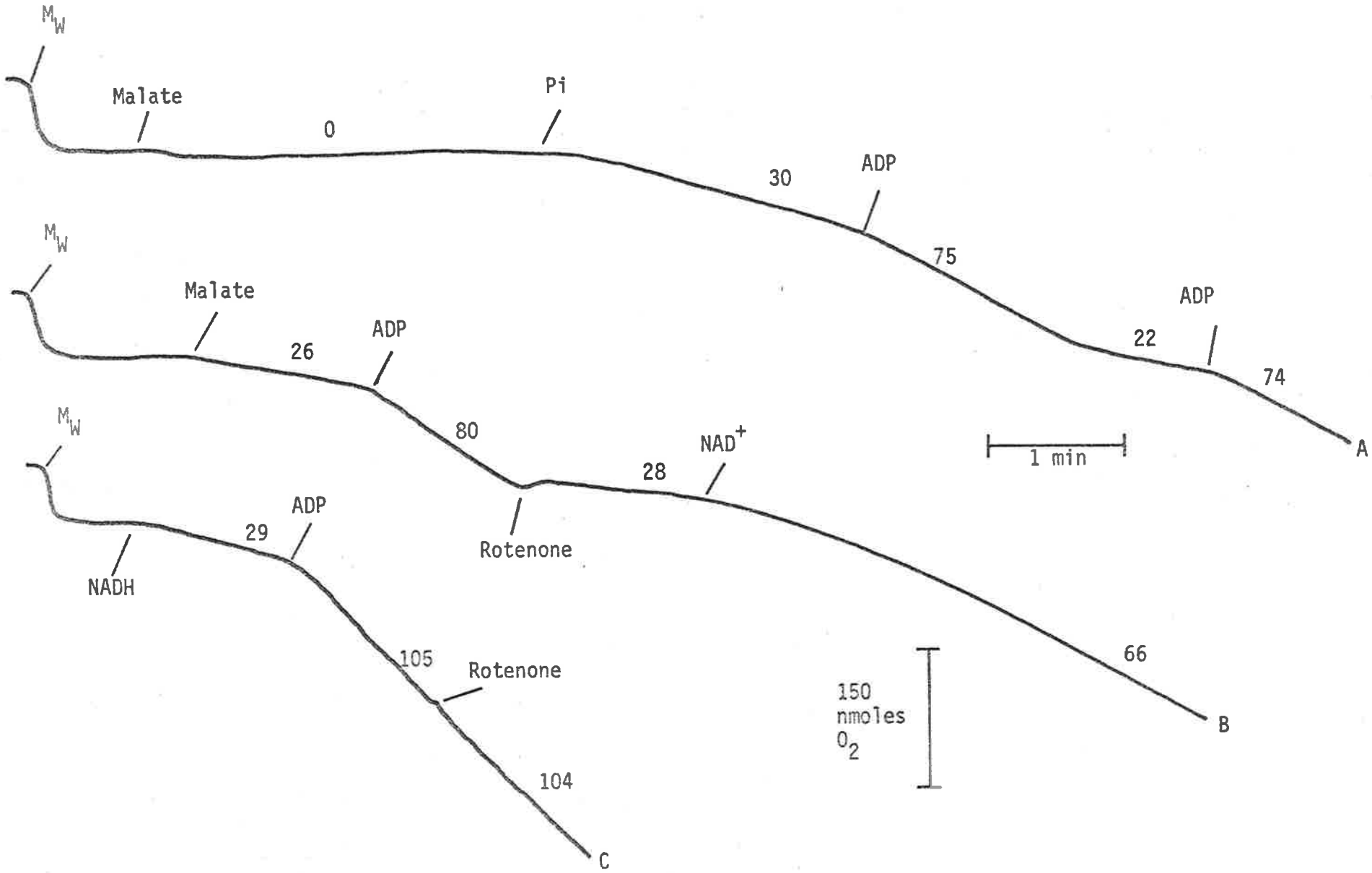

M_W
Pi
0
Isocitrate
31
ADP
ADP/O = 2.9
62
150
nmoles O_2
M_W
ADP
36
ADP
64
21
Isocitrate
50
NAD^+
23
49
Rotenone
A
B
1 min.

Figure 6.13 The effect of exogenous NAD^+ and rotenone on malate and NADH by cauliflower mitochondria.

Oxygen consumption was measured as described in Materials and Methods. Additions as indicated were; 1.5 mg mitochondrial protein, 10 mM malate, 10 mM Pi, 0.235 mM ADP, 15 μM rotenone, 0.5 mM NAD^+ and 1 mM NADH. Rates are expressed as nmoles O_2/min. mg protein.


M_W
Malate
0
Pi
30
ADP
75
ADP
22
74
A
M_W
Malate
26
ADP
80
NAD^+
28
Rotenone
66
B
M_W
NADH
29
ADP
105
Rotenone
104
C
1 min
150
nmoles
O_2

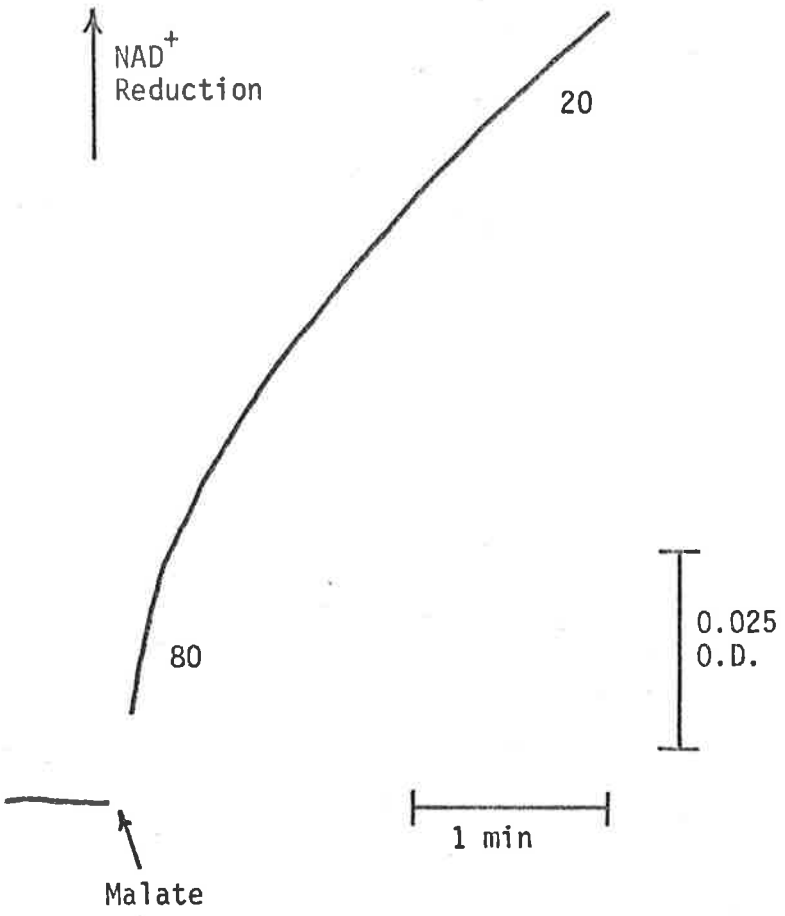


Figure 6.14 Exogenous NAD^+ reduction by turnip mitochondria.

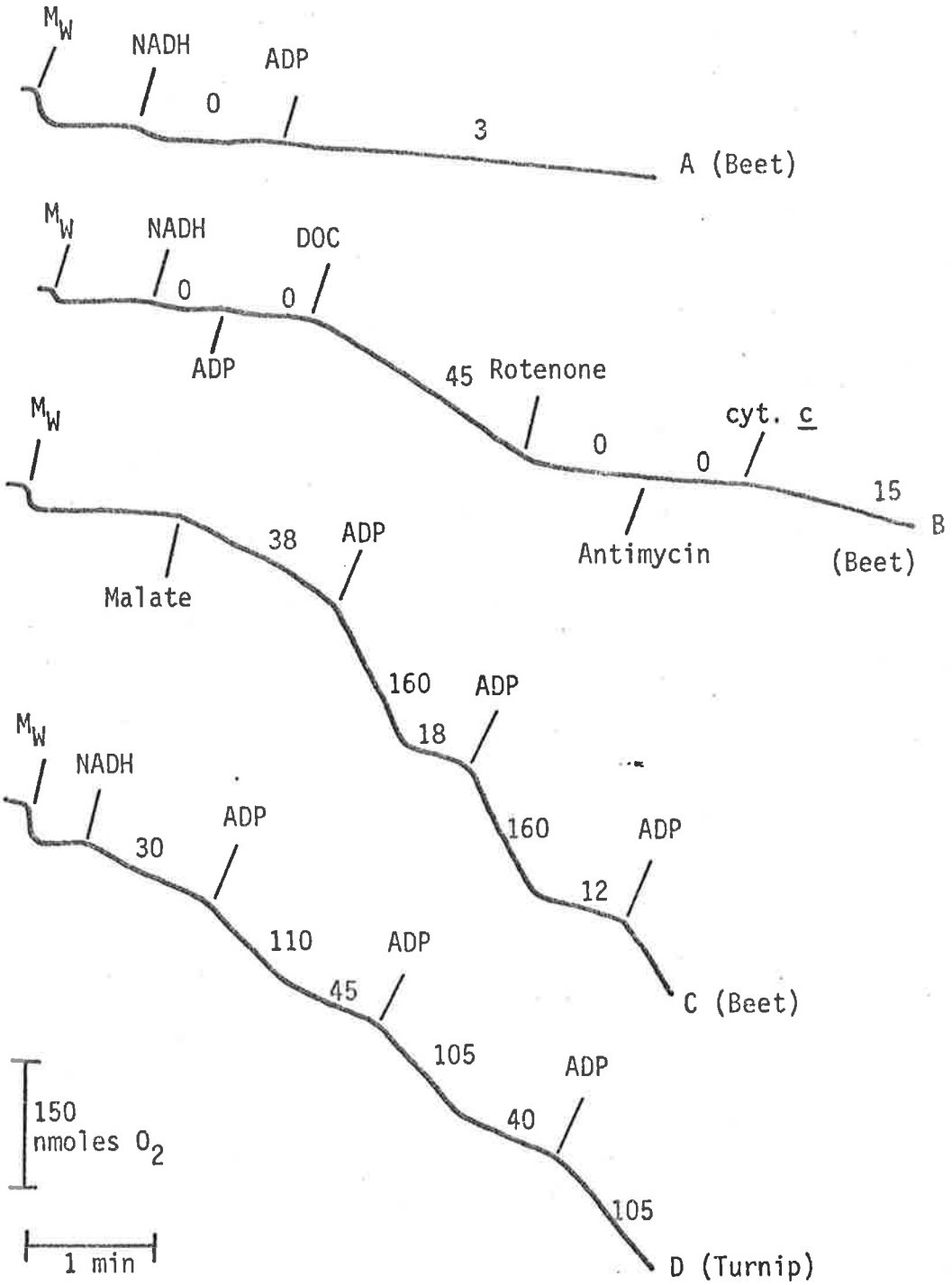
NAD^+ reduction was measured as described in Materials and Mathods. The reaction medium included, 0.75 mg mitochondrial protein, 10mM glutamate, 5 µM antimycin A, and 0.5 mM NAD^+; 10 mM malate was added as shown. Rates are expressed as nmoles/min. mg protein.

Figure 6.15 Malate and NADH oxidation by beetroot and turnip mitochondria.

Oxygen consumption was measured as described in Materials and Methods. Additions as indicated were; 1 mM NADH, 0.3 mM (A, B and C) or 0.1 mM (D) ADP, 10 µM rotenone, 5 µM antimycin A, 0.025 mM cytochrome c, 10 mM glutamate, 10 mM malate and 0.05% deoxycholate (DOC). Rates are expressed as nmoles O_2/min. mg protein.

A, B and C : Beetroot mitochondria

D : Turnip mitochondria.


M_W
NADH
ADP
0
3
A (Beet)
M_W
NADH
DOC
0
0
ADP
45 Rotenone
cyt. c
0
0
15
B
(Beet)
Antimycin
M_W
Malate
38
ADP
160
ADP
18
160
ADP
12
C (Beet)
M_W
NADH
30
ADP
110
ADP
45
105
ADP
40
105
D (Turnip)
150
nmoles O2
1 min

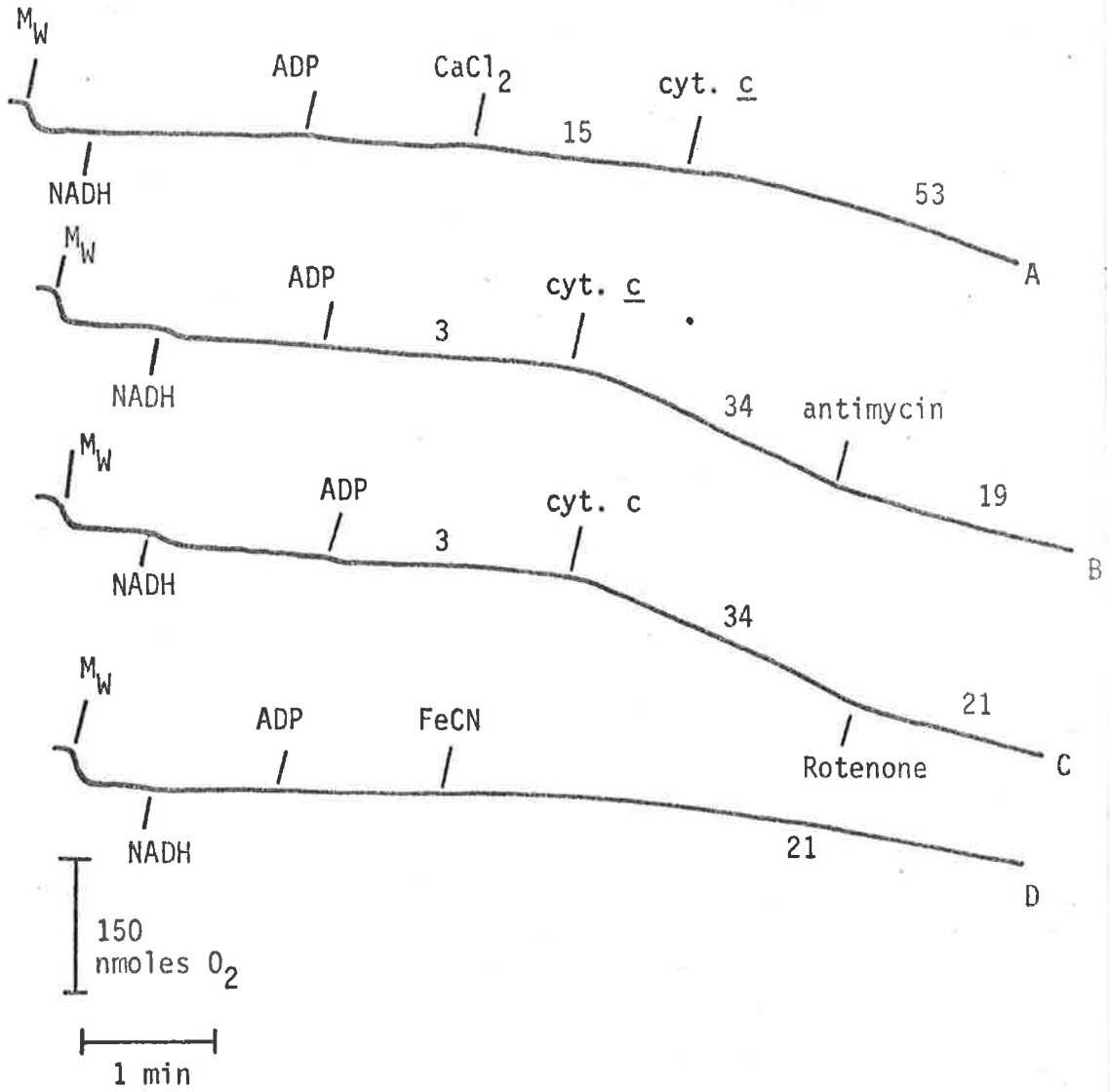


Figure 6.16 The effect of cytochrome c, FeCN and $CaCl_2$ on exogenous NADH oxidation by beetroot mitochondria.

Oxygen consumption was measured as described in Materials and Methods. Additions as indicated were; 1 mM NADH, 0.3 mM ADP, 25 µM cyt. c, 5 µM antimycin A, 10 µM rotenone, 0.9 mM FeCN and 0.6 mM $CaCl_2$. Rates are expressed as nmoles O_2/min.mg protein.

Figure 6.17. The effect of exogenous NAD on malate oxidation by beetroot and maize root mitochondria.

Oxygen uptake was measured as described in Materials and Methods. Additions as indicated were; A and B: 2.4 mg mitochondrial protein, 15 mM malate, 0.32 mM ADP, 10 µM rotenone, 0.5 mM NAD^+, 5 µM antimycin A, 0.05 mM cytochrome c. Glutamate (16 mM) was present in each experiment. Rates are expressed as nmoles/min. mg protein.

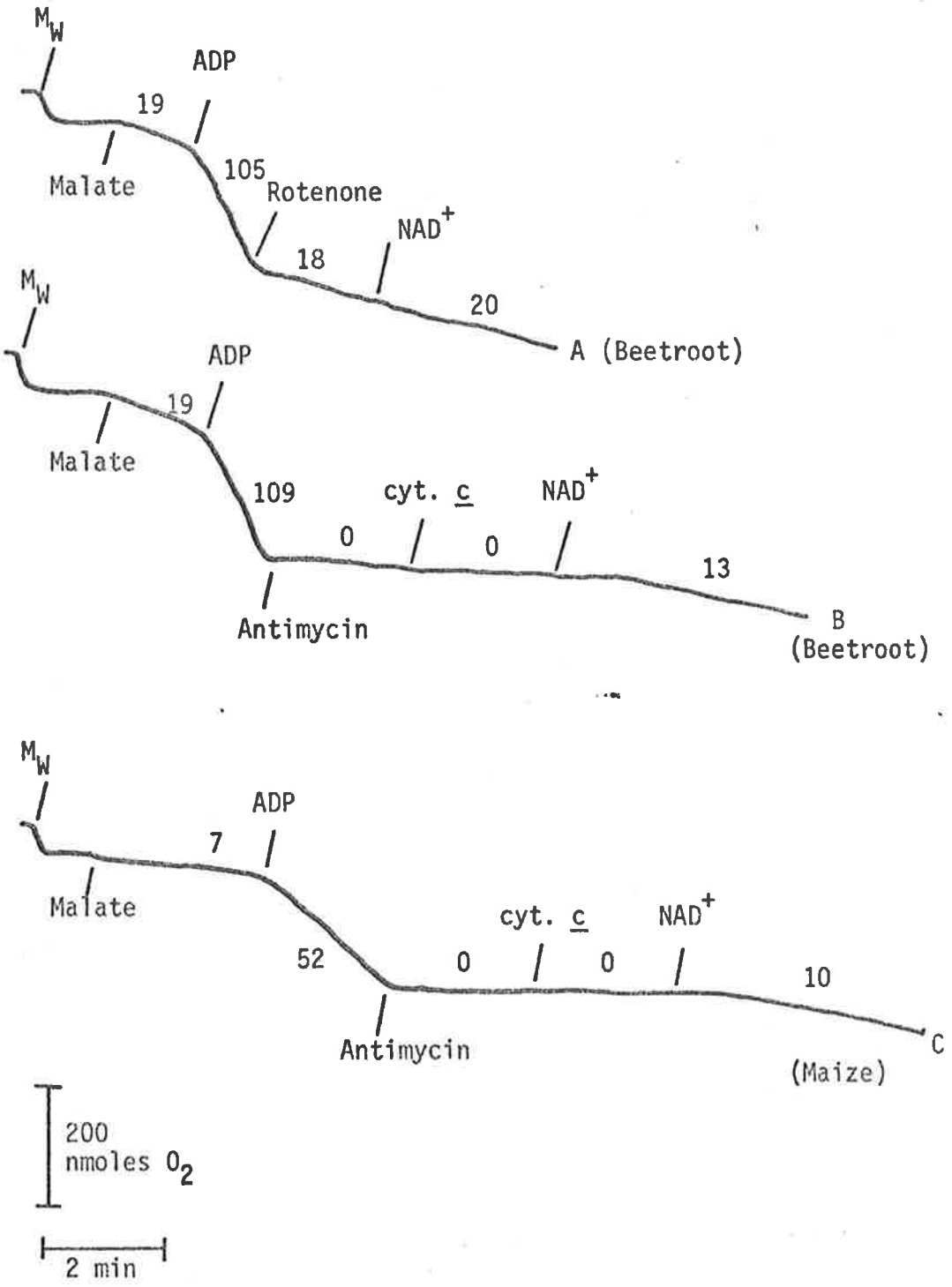
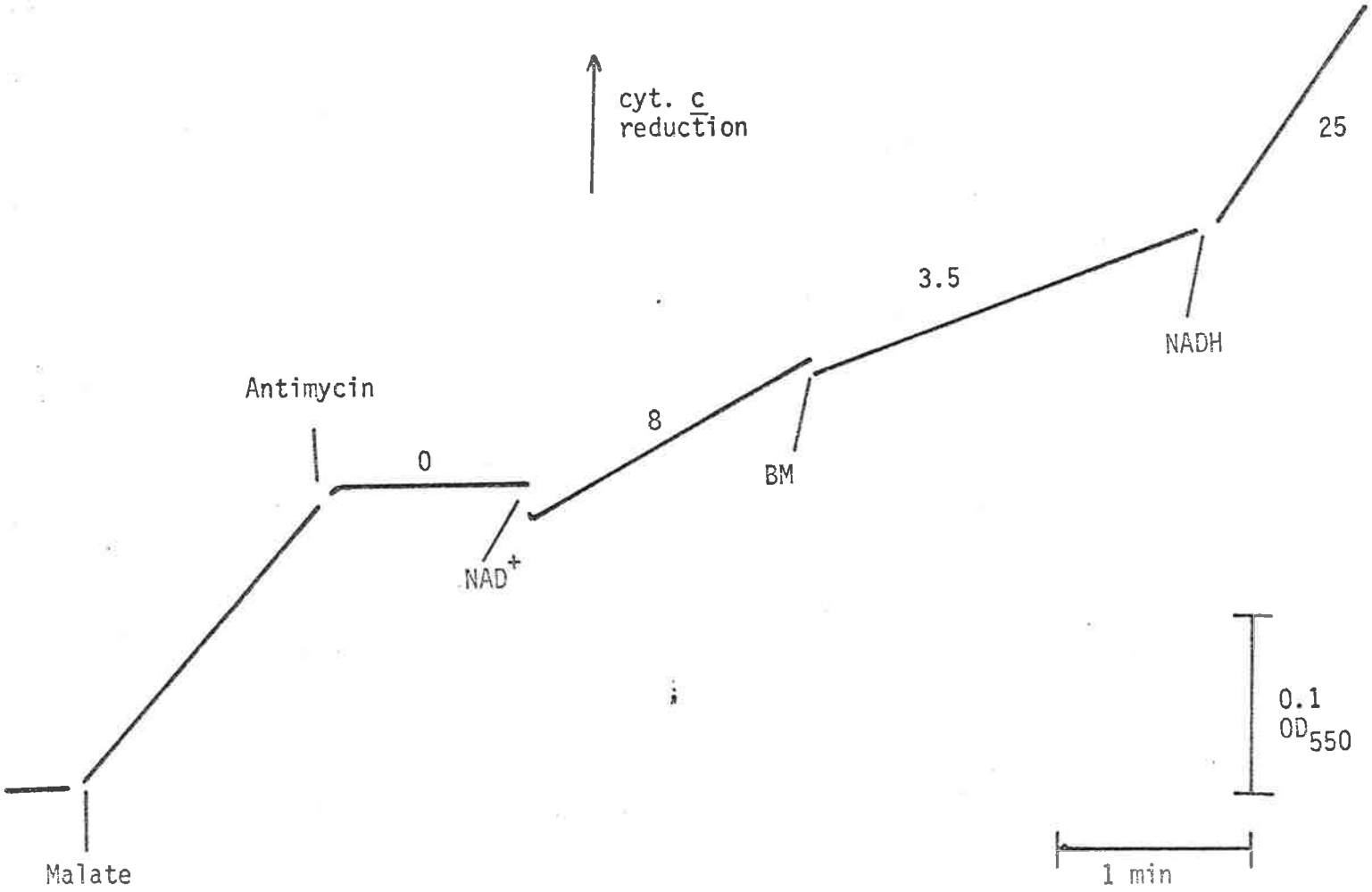

M_W
ADP
19
Malate
105
Rotenone
NAD+
18
20
A (Beetroot)
M_W
ADP
19
Malate
109
cyt. c
NAD+
0
0
13
Antimycin
B
(Beetroot)
M_W
ADP
7
Malate
cyt. c
NAD+
52
0
0
10
Antimycin
C
(Maize)
200
nmoles O2
2 min

Figure 6.18. Effect of exogenous NAD^+ on malate-cytochrome c reductase activity of beetroot mitochondria.

Cyt. c reduction was measured as described in Materials and Methods. Additions as shown were; 10 mM malate, 5 µM antimycin A, 0.5 mM NAD^+, 8 mM butylmalonate and 0.5 mM NADH. Rates are expressed as nmoles/min. mg protein.


cyt. c
reduction
25
3.5
NADH
Antimycin
8
0
BM
NAD+
0.1
OD550
Malate
1 min

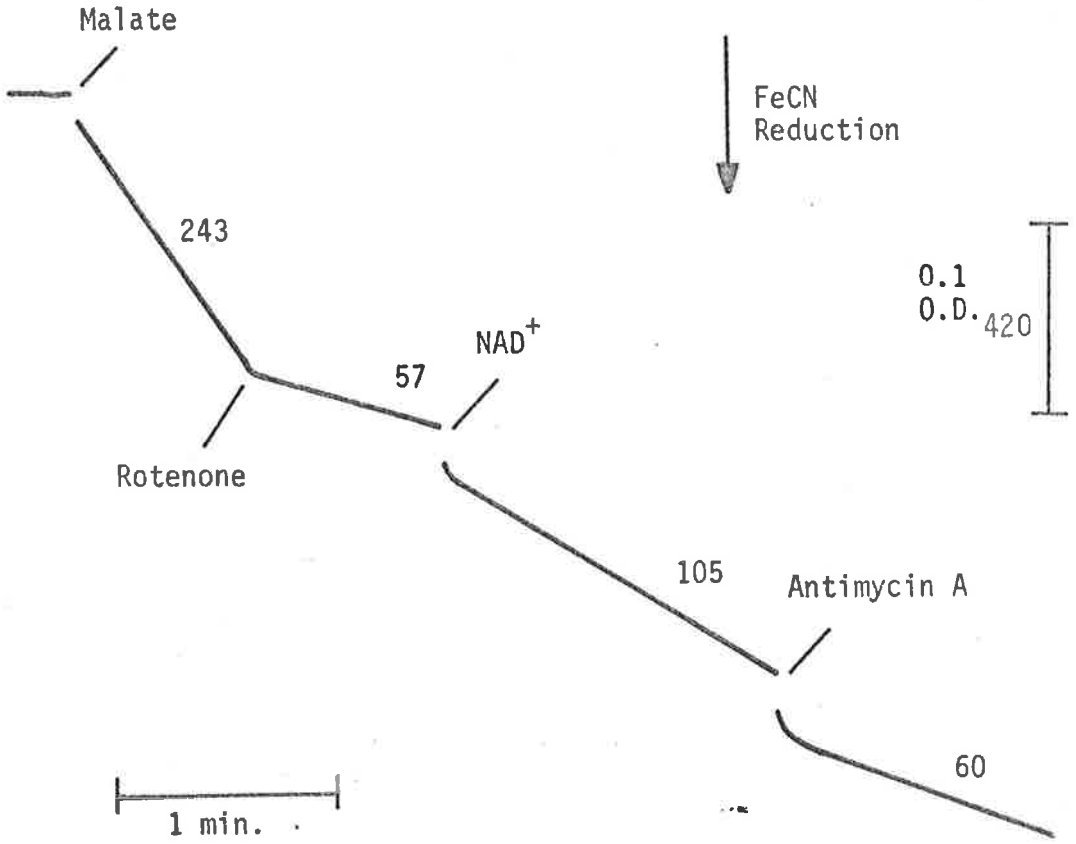


Fig. 6.19 The effect of NAD^+ and rotenone on malate-FeCN reductase activity of beetroot mitochondria.

FeCN reductase was measured as described in Materials and Methods. Additions as indicated were; 10 mM malate, 10 µM rotenone, 0.5 mM NAD^+ and 5 µM antimycin A. Rates are expressed as nmoles/min. mg protein.

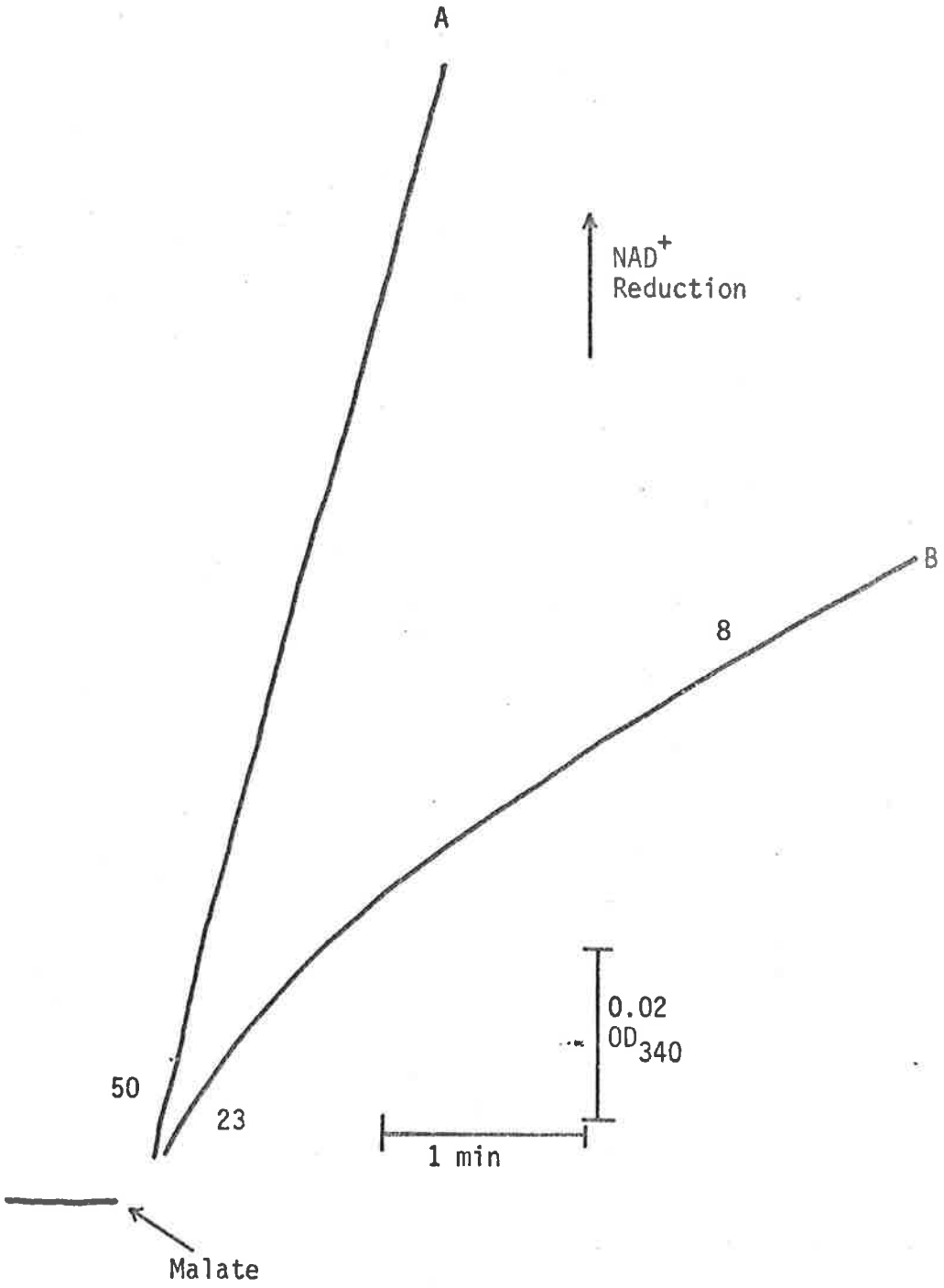


Fig. 6.20 Exogenous NAD^+ reduction by beetroot mitochondria.

NAD^+ reduction was measured as described in Materials and Methods. The reaction medium included 10 mM glutamate, 5 µM antimycin A, 0.5 mM NAD^+ and 1 mg mitochondrial protein. Rates are expressed as nmoles/min. mg protein.

A : + 20 µl Decon 90 detergent.

B : control.

10 mM malate was added where shown.

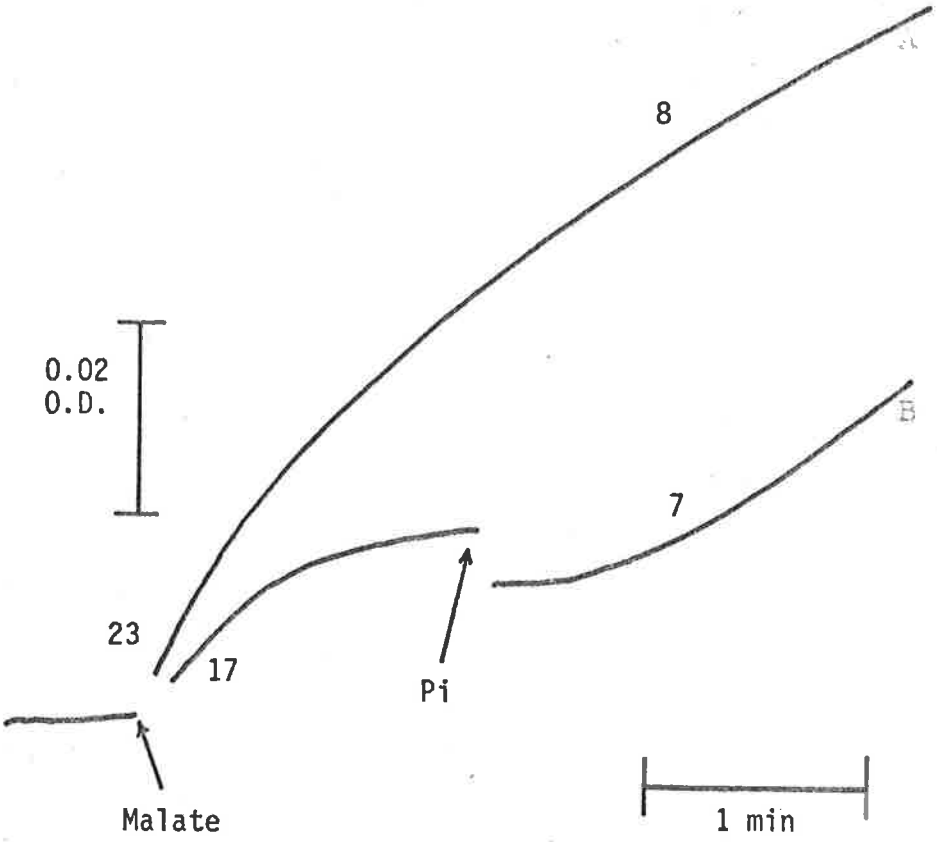


Fig. 6.21 The effect of phosphate on NAD^+ reduction by beetroot mitochondria.

Conditions of assay were the same as those described in Figure 6.20, except that in (B), Pi was omitted from the reaction medium and added as shown (10 mM).

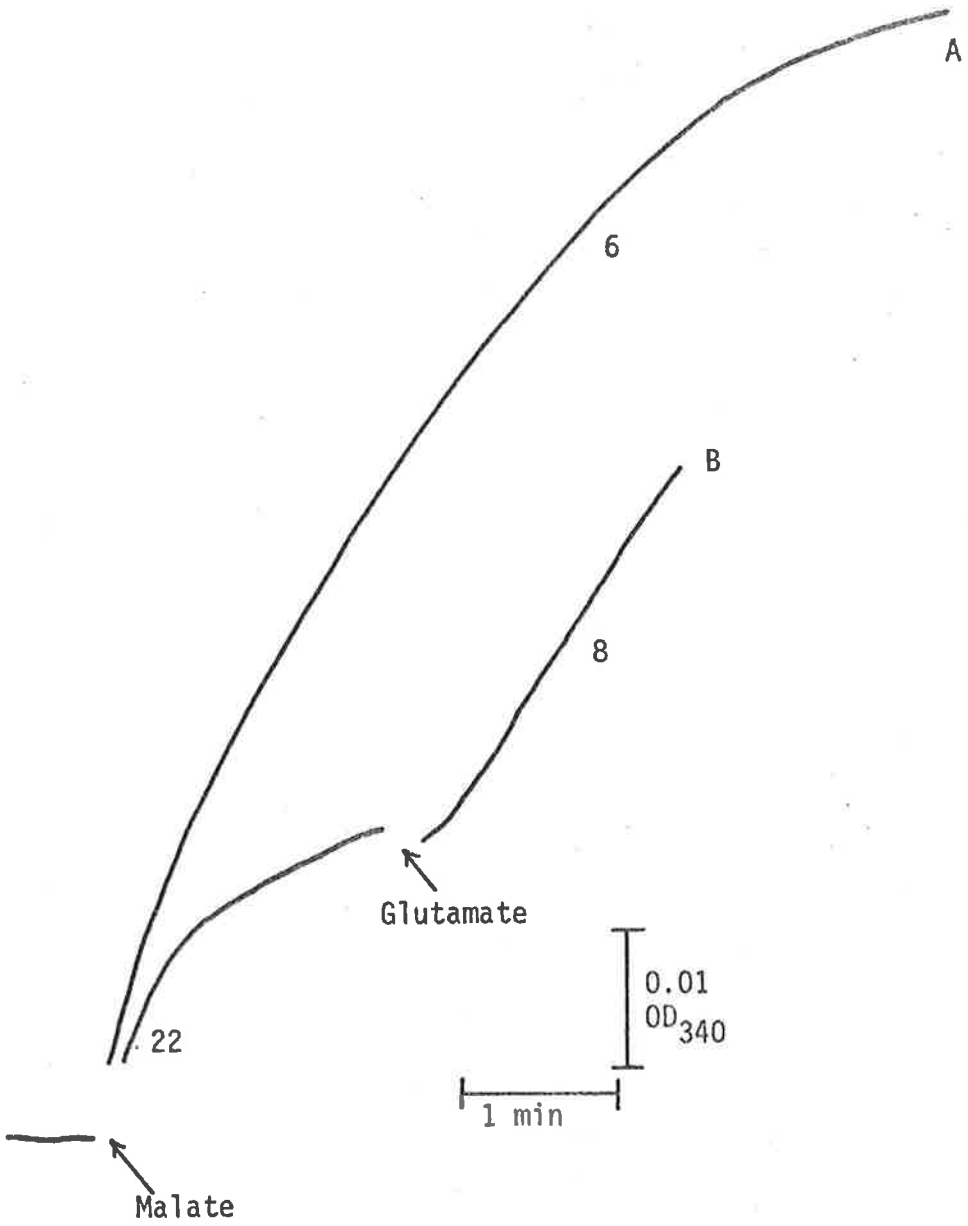


Figure 6.22 Effect of glutamate on exogenous NAD^+ reduction by beetroot mitochondria.

NAD^+ reduction was measured as described in Materials and Methods. The reaction medium contained; A: 0.3 mM NAD^+, 5 µM antimycin A, 10 mM glutamate and 1.2 mg mitochondrial protein; B: as above except that glutamate was omitted from the medium and added as shown. Rates are expressed as nmoles/min. mg protein.

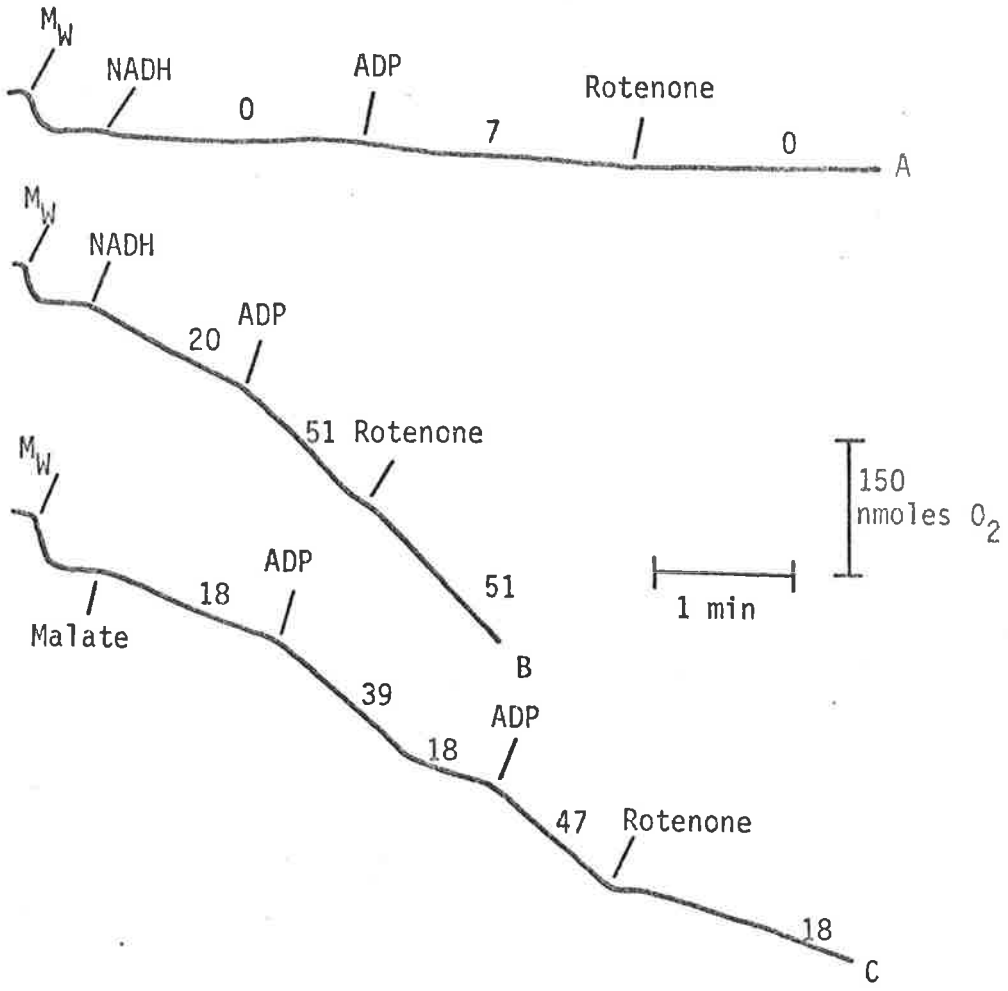


Figure 6.23 Malate and exogenous NADH oxidation by mitochondria from aged beetroot.

Oxygen uptake was measured as described in Materials and Methods. Additions as indicated were; 1 mM NADH; 0.21 mM ADP, 10 µM rotenone and 10 mM malate. Rates are expressed as nmoles/min. mg protein.

A : mitochondria from 'fresh' beetroot discs.

B and C : mitochondria from 'aged' beetroot discs.

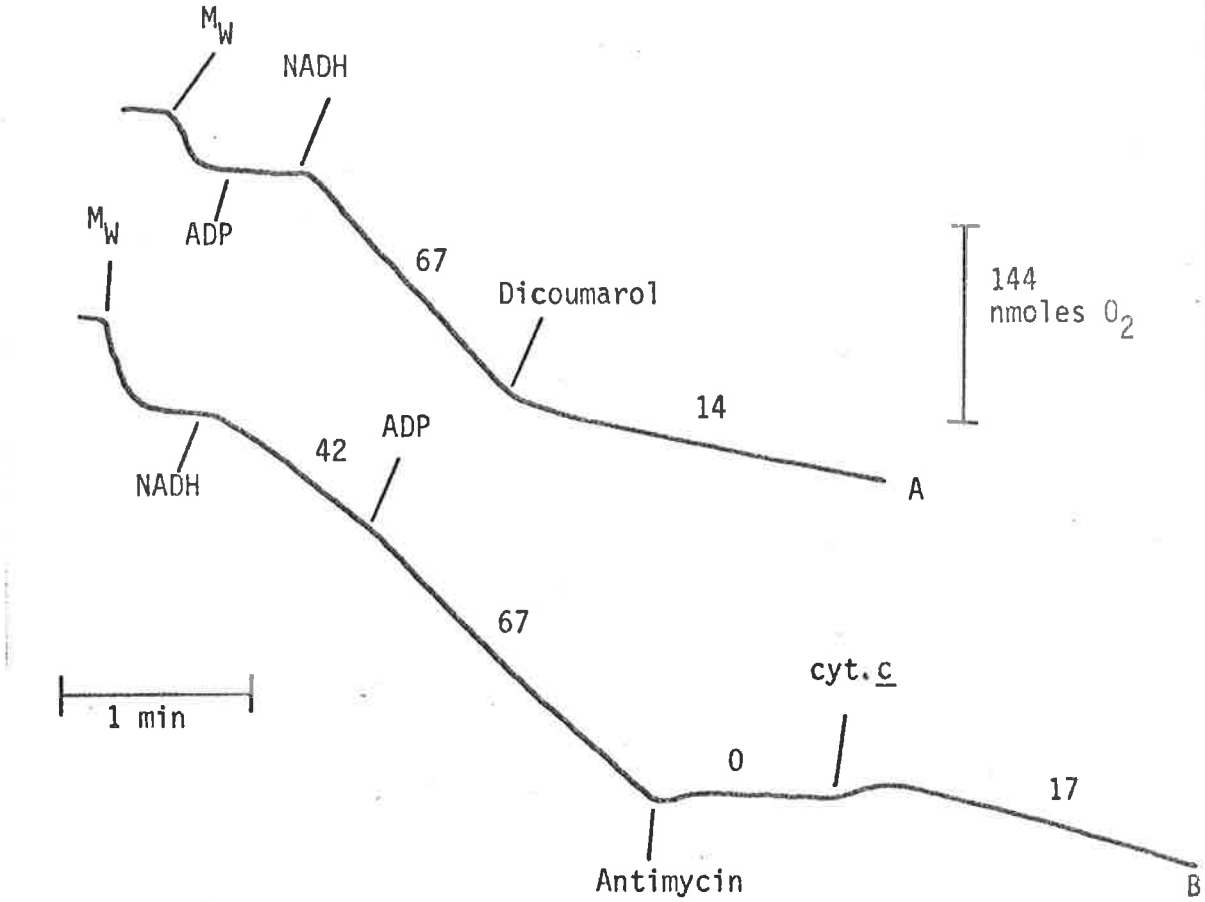
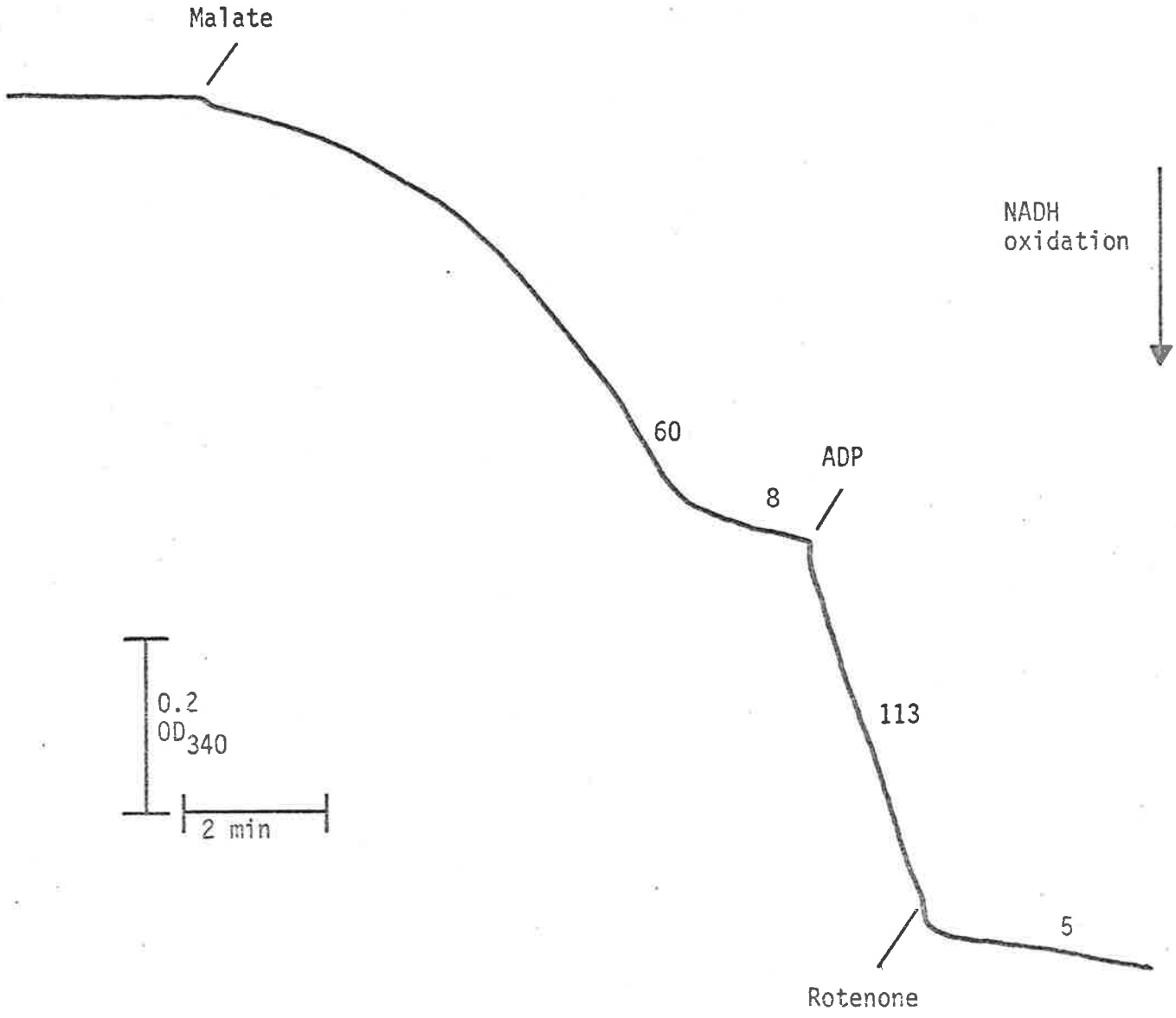


Fig. 6.24 The effect of dicoumarol and antimycin A on exogenous NADH oxidation by mitochondria from aged beetroot discs.

Oxygen consumption was measured as described in Materials and Methods. Additions as indicated were; 2.8 mg mitochondrial protein, 1 mM NADH, 0.21 mM ADP, 5 µM antimycin A, 0.05 mM cytochrome c and 50 µM dicoumarol. Rates are expressed as nmoles/min. mg protein.

Figure 6.25 NADH Oxidation by beetroot mitochondria.

NADH oxidation was measured spectrophotometrically as described in Materials and Methods. 0.5 mM NADH, 0.75 mM ADP and 50 units of malate dehydrogenase were included in the reaction medium. 1 mM ADP, 10 mM malate and 15 μM rotenone were added as shown. Rates are expressed as nmoles/min. mg protein.


Malate
NADH
oxidation
60
ADP
8
113
5
Rotenone
0.2
OD340
2 min

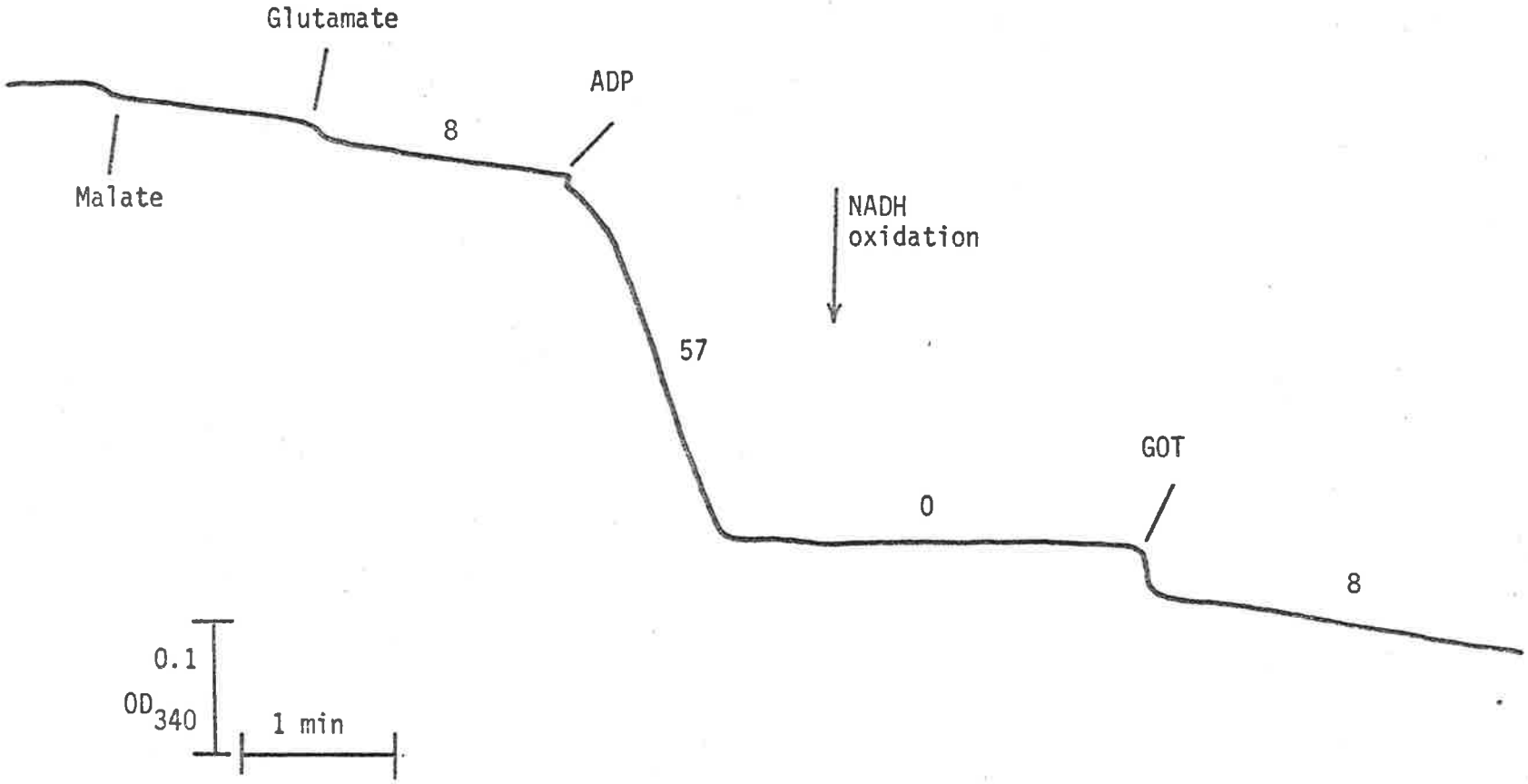


Figure 6.26 The effect of glutamate on NADH oxidation by beetroot mitochondria.
The conditions of assay were the same as those described in the previous figure (6.25), except that ADP was omitted from the reaction mixture. 10 mM malate, 10 mM glutamate, 1 mM ADP and 25 units of glutamate-oxaloacetate transaminase were added as shown. Rates are expressed as nmoles/min. mg protein.

Figure 6.27. NADH oxidation by beetroot mitochondria.

NADH oxidation was measured spectrophotometrically as described in Materials and Methods. The mitochondria were preincubated with 10 mM malate and 1 mM ADP for 2.5 min. prior to the addition of NADH (1mM). In (A), 10 mM glutamate was also included in the reaction medium. Rates are expressed as nmoles/min. mg protein.

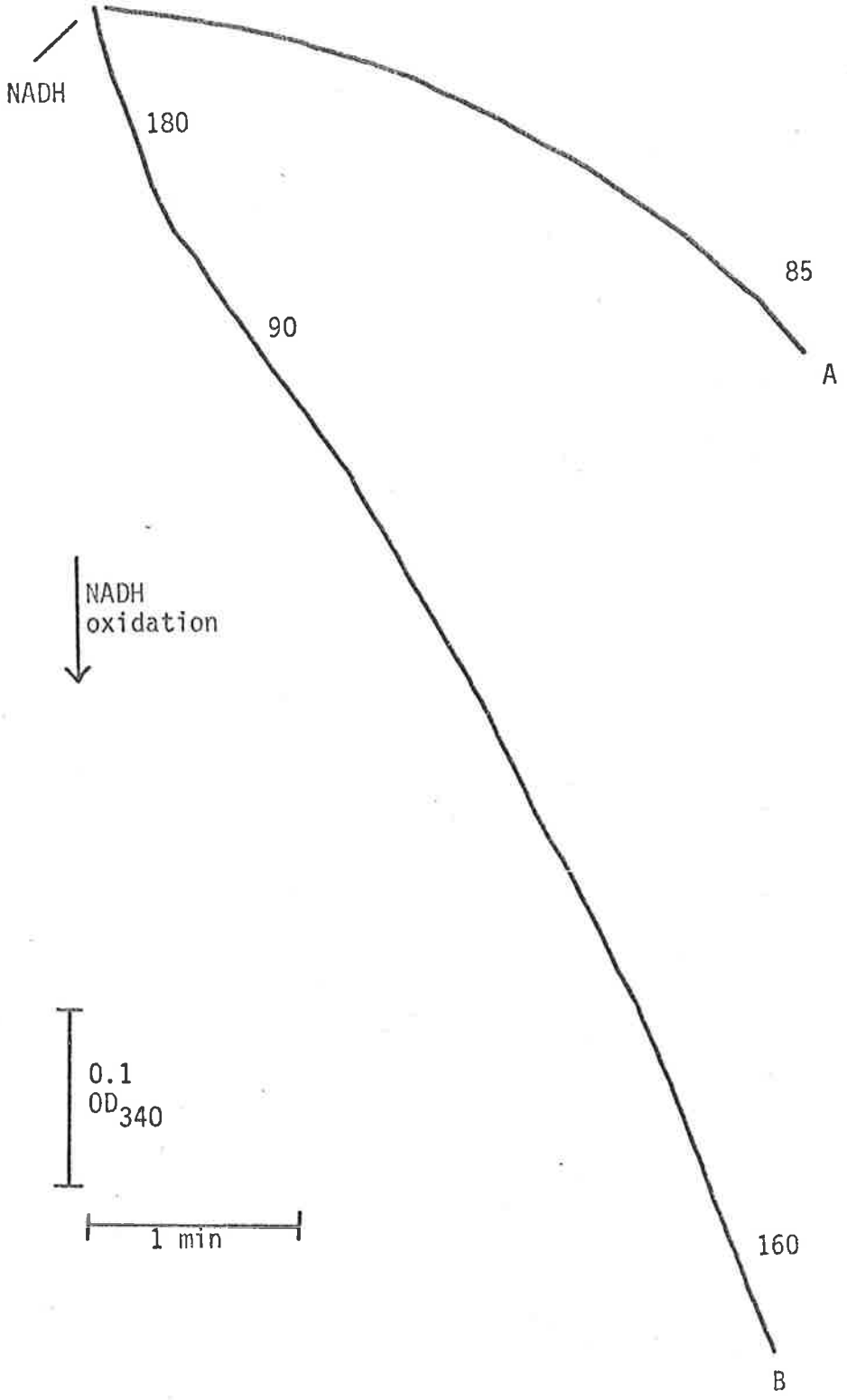
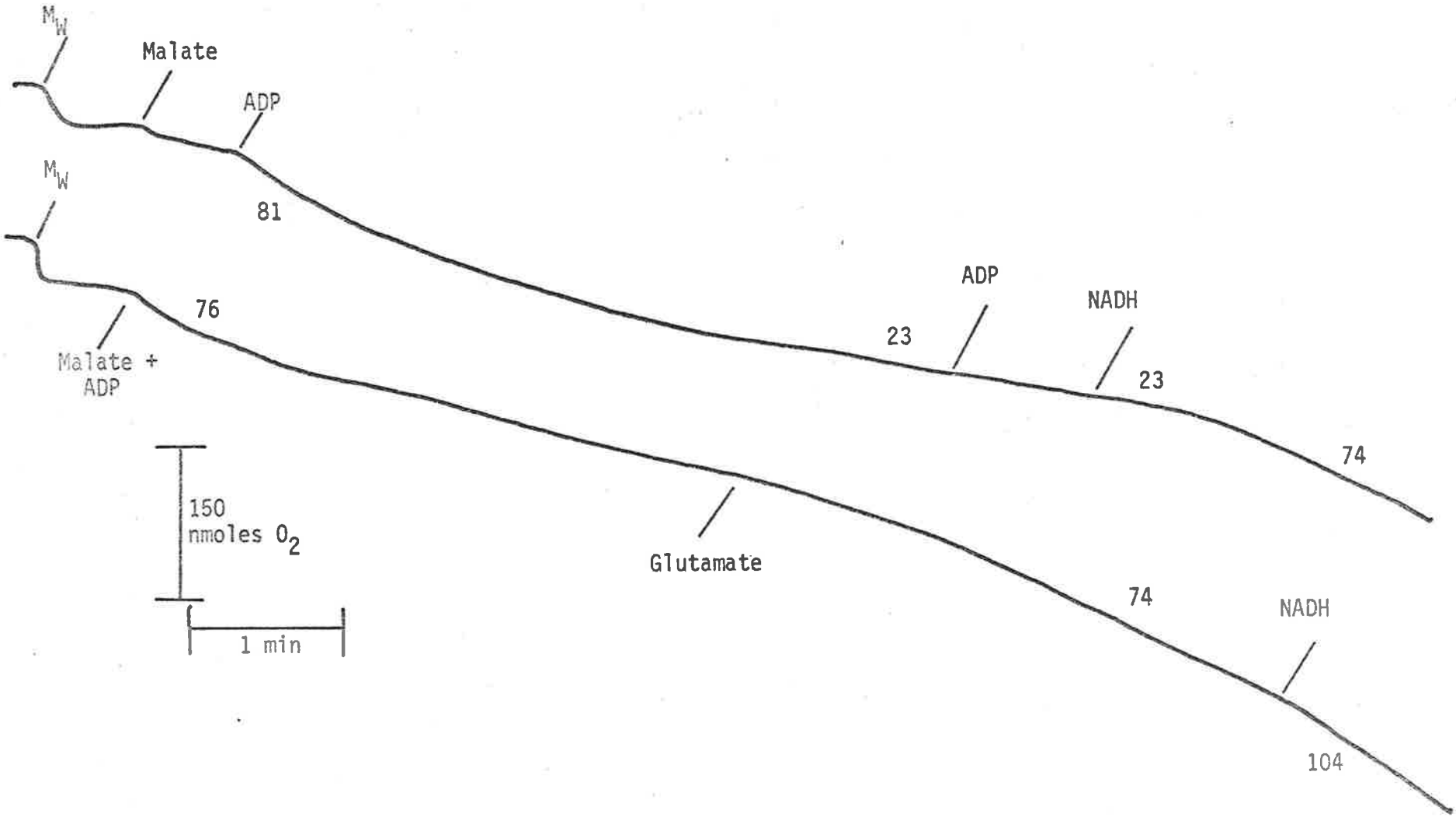

NADH
180
85
90
A
NADH
oxidation
0.1
OD340
1 min
160
B

Figure 6.28 The effect of glutamate and NADH on malate oxidation by beetroot mitochondria.

Oxygen consumption was measured as described in Materials and Methods. 50 units of malate dehydrogenase were included in the reaction mixture. Additions as indicated were; 10 mM malate, 0.7 mM ADP, 0.5 mM NADH and 10 mM glutamate. Rates are expressed as nmoles/min. mg protein.


M_W
Malate
ADP
81
23
ADP
NADH
23
74
M_W
76
Malate + ADP
150 nmoles O_2
1 min
Glutamate
74
NADH
104

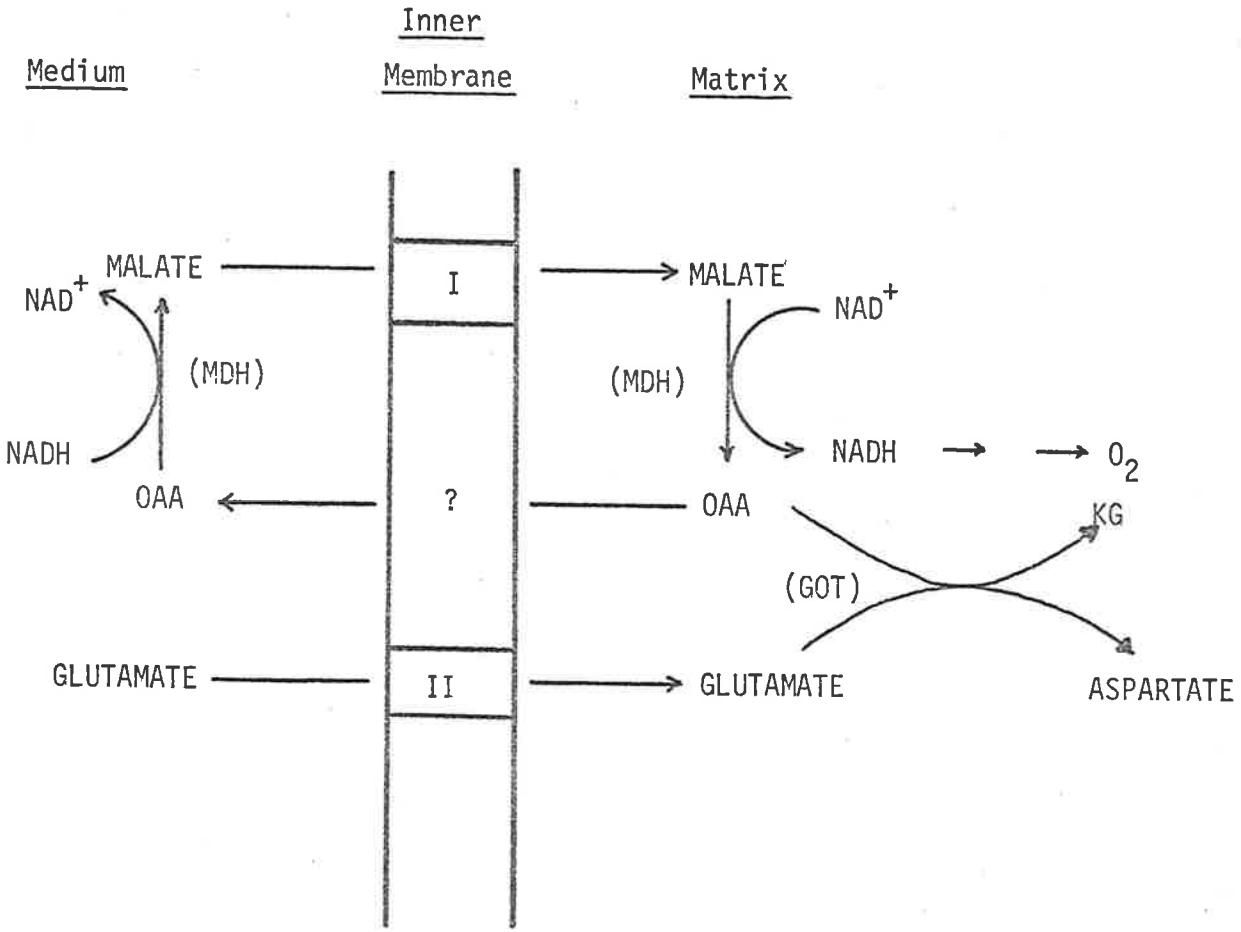


Figure 6.29. The malate-oxaloacetate shuttle of isolated beetroot mitochondria.
I : dicarboxylate carrier; II : glutamate carrier; MDH : malate dehydrogenase
GOT: glutamate- oxaloacetate transaminase.

CHAPTER VII

FINAL DISCUSSION AND FUTURE WORK

Although plant mitochondria are generally considered to be similar to animal mitochondria (see Chapter I), this study shows that pyridine nucleotide interactions of the two types of mitochondria are vastly different. Not only do isolated plant mitochondria oxidize extramitochondrial NADH directly via their respiratory chain but exogenous NAD^+ appears to be in equilibrium with the intramitochondrial pyridine nucleotide pool (mainly via MDH). Minor differences between the outer membrane NADH - reductase systems of plant and animal mitochondria were also demonstrated.

It appears that plant mitochondria possess an additional dehydrogenase, on the outer surface of the inner membrane, for the oxidation of external NADH. NADH oxidation by this flavoprotein is insensitive to rotenone and coupled to two phosphorylation sites. Animal mitochondria do not contain such an enzyme (Lehninger, 1951).

When oxygen uptake associated with the oxidation of NAD-linked substrates by isolated plant mitochondria is inhibited, reducing equivalents are transferred from the matrix to external NAD^+. Two possible mechanisms for this process are shown in Figure 7.1. If actual transport of nucleotides occurred, one would expect to observe some antimycin-insensitive malate-cytochrome *c* reductase activity; no such activity was detected.

Hence, it seems that a transmembrane transhydrogenase is responsible for transport of reducing equivalents across the inner membrane of isolated plant mitochondria.

Transhydrogenase activity was not assayed for directly in whole mitochondria since a number of NADH dehydrogenases exist which could catalyse a hydrogen transfer between NADH and NAD^+, without being involved in the transmembrane transfer. However, Hackett (1963) reported NADH-NAD^+ transhydrogenase activity in mitochondria isolated from a number of plant tissues. The rates of NAD^+ reduction measured by Hackett (1963) were of the same order of magnitude as those reported here. Either, or both, of the inner membrane NADH dehydrogenases could be involved, although the results with beetroot mitochondria would tend to eliminate the external dehydrogenase. If the internal NADH dehydrogenase is responsible for the (H) transfer, the rotenone-sensitive site appears not to be involved.

Purvis and Greenspan (1965) obtained evidence for a pyridine nucleotide translocator which mediated the incorporation of NAD^+ into isolated rat liver mitochondria. However, this incorporation was very slow (e.g. when compared to adenine nucleotide transport) and was dependent on a supply of energy. Electron transport inhibitors abolished NAD^+ incorporation, and no NADH transport out of the mitochondria was observed, implying that the translocator (if real) did not catalyse transfer of reducing equivalents across the inner membrane. Pfaff and Schwalbach (1967) have subsequently shown that leakage of pyridine nucleotides from rat liver mitochondria does not occur over a period of several hours.

Anion movement across the inner membrane of isolated plant mitochondria also differs from that of animal mitochondria. In particular, oxaloacetate appears to move readily across the membrane. It seems unlikely that this movement is passive, since the transport of other anions is controlled via carriers. Oxaloacetate may also be transported by a carrier (perhaps the dicarboxylate carrier, since some OAA is transported in this manner across the rat liver mitochondrial membrane {Gimpel et al, 1973 }), or simply by facilitated diffusion in exchange for hydroxyl ions (or via an H^+ symport) as for acetate in animal mitochondria (Quagliariello and Palmieri, 1971), without a specific carrier involved. Obviously, a more detailed investigation of oxaloacetate transport by plant mitochondria is warranted.

As discussed in chapter I, one of the most important functions of the anion transporters in animal cells is the regulation of the malate-aspartate cycle. This cycle is responsible for the transfer of reducing equivalents between the cytosol and mitochondria during glycolysis and gluconeogenesis, and was first proposed by Borst (1962) who, since animal mitochondria are relatively impermeable to OAA, postulated a double transamination of OAA and glutamate, one on either side of the inner mitochondrial membrane (see Fig. 1.5). The ability of the specific anion carriers to maintain an asymmetric concentration of their substrates across the inner membrane, and the membrane's impermeability to OAA, is important (together with the midpoint potentials and activities of the dehydrogenase systems duplicated in the cytoplasm and mito-

chondria) in preserving the observed difference in pyridine nucleotide redox potential (more negative in the mitochondria) between the cytosol and mitochondria of animal cells (Chappell, 1969; Krebs and Veech, 1969; Williamson et al, 1967). Such a regulation of the NAD^+/NADH ratio in the two compartments is only possible because the nucleotides do not interact directly across the mitochondrial membrane.

It has not been determined whether a similar difference in NAD^+/NADH ratios exists between the cytosol and mitochondria of plant cells. Assuming that such a disequilibrium does exist, the factors that regulate and maintain it are likely to be different than those that operate in animal cells. Not only must oxaloacetate movement be regulated, but also exogenous NADH oxidation and the transmembrane transhydrogenase.

If the concentrations of malate and oxaloacetate are the same on both sides of the inner mitochondrial membrane, then so would the NAD^+/NADH ratios be since the nucleotides are in equilibrium with these substrates of malate dehydrogenase. This is particularly important in beetroot mitochondria which rely on the malate-oxaloacetate shuttle to oxidize extramitochondrial NADH. To keep cytoplasmic NAD oxidized (and mitochondrial NAD reduced), these mitochondria need to accumulate malate and expel oxaloacetate. If the malate dehydrogenase system of the mitochondria has a more negative redox potential than the cytoplasmic system, then this would also help to maintain intramitochondrial NAD in the reduced form.

In contrast to rat liver mitochondria which did not oxidize exogenous NADH, even in the presence of malate and added malate dehydrogenase, until glutamate, aspartate and aspartate aminotransferase were added (Chappell, 1969), oxidation of external NADH by beetroot mitochondria was inhibited by glutamate, even when transaminase was added (Fig. 6.27). This apparent lack of malate-aspartate cycle activity in plant mitochondria fits in well with the non-specificity of some anion transporters of plant mitochondria. Thus glutamate (see Chapter V) and α-ketoglutarate (Wiskich unpublished results) appear to be transported via the tricarboxylate carrier. Swelling of beetroot and cauliflower bud mitochondria in NH_4^+ salts of glutamate and α-ketoglutarate was activated by malate and phosphate and inhibited by n-butylmalonate. However, a more thorough investigation of these transporters, using a variety of substrate analogues and potential activators and inhibitors, may reveal subtle differences.

On the basis of studies with animal tissues (see Chappell, 1968), differences between anion transporters of mitochondria from different plant tissues can be anticipated. In fact Wiskich (1974 and 1975) has found that citrate transport by isolated beetroot mitochondria has similar properties to dicarboxylate-ion transport, although different carriers operate in cauliflower (Wiskich, 1974) and mung bean mitochondria (Phillips and Williams, 1973).

The apparent ease with which OAA penetrates the inner membrane of cauliflower mitochondria suggests that the malate-OAA shuttle may operate in other plant tissues.

However the oxidation of extra mitochondrial NADH by the respiratory chain of these mitochondria would seem to obviate the need for such a shuttle to oxidize NADH produced glycolytically in the cytoplasm. Undoubtedly, the anion transporters of plant mitochondria perform other functions, such as maintenance of membrane potentials (this is particularly important to the chemiosmotic hypothesis of energy conservation) and the intramitochondrial balance of carbon skeletons (Wiskich, 1974). Mitochondrial transport may also be involved in other metabolic pathways, depending on the tissue in question. For example mitochondria of bundle sheath cells in the leaves of plants such as *Atriplex* may be involved in the C_4-pathway of photosynthesis. According to the scheme proposed by Hatch and his co-workers (Hatch and Mau, 1973; Hatch and Kagawa, 1975), bundle sheath mitochondria should contain transporters for aspartate, glutamate, malate (or oxaloacetate) and α-ketoglutarate. If present, these transporting systems may play a role in regulation of the C_4-pathway.

Obviously the pyridine nucleotide interactions with plant mitochondria reported in this study will exert a strong influence on cytoplasmic metabolism. While respiratory activity is high, that is when cellular ADP concentrations are high and there is a supply of carbon to the Kreb's cycle, oxidation of cytoplasmic NADH by the mitochondria will proceed, keeping the NAD^+/NADH ratio in the outer compartment of the cell high. Since pyridine nucleotides are cofactors for several reversible glycolytic enzymes, a high NAD^+/NADH ratio will tend to favour breakdown of glucose via glycolysis.

On the other hand, when the cellular ATP concentration is high and electron transport inhibited, cytoplasmic NAD^+ can be reduced via the transmembrane transhydrogenase, thus allowing gluconeogenesis (and hence starch production) to occur. Such a transfer of reducing equivalents out of the mitochondria is strictly controlled by the extramitochondrial NAD^+/NADH ratio (Fig. 6.4). Unless the cytoplasmic NADH is reoxidized, the transhydrogenase will be inhibited.

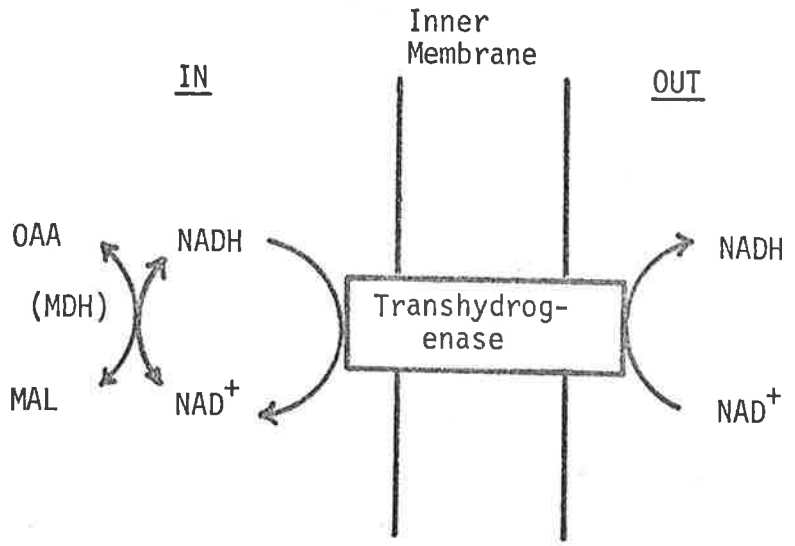
Exogenous NADH oxidation by plant mitochondria may also be important during fatty acid oxidation by glyoxisomes. β-oxidation of fatty acids in these organelles may lead to generation of extramitochondrial NADH, which can be directly reoxidized by the mitochondria. On the other hand, β-oxidation of fatty acids by animal cells occurs in the mitochondria (Lehninger, 1971), which cannot oxidize cytoplasmic NADH directly. Here, reducing equivalents are transferred to the electron transport by $FP._{ETF}$, upon fatty acid oxidation.

Control of extramitochondrial NADH oxidation, and NAD^+ reduction, *in vivo*, is probably more complex than that outlined above. Some light may be shed on this, and on the importance of exogenous NADH oxidation to the cell, by studies with aged beetroot tissue. However these problems may not be solved until measurements of the NAD^+/NADH ratios in the different compartments of plant cells are made. Such estimations are difficult to make, since a detailed knowledge of at least two enzymes in each cell compartment, for one particular tissue, is required if conventional techniques are to be employed (Williamson, 1969).

Measurement of metabolite concentrations are also difficult to interpret when made on heterogenous cell populations (which occur in most plant tissues), but cell or protoplast cultures may help overcome this problem.

The results presented in Chapter III, and those reported by Koeppe and Miller (1972), suggest that at least some plant mitochondria can oxidize extramitochondrial NADPH. This may be of particular importance in photosynthetic cells. However, it appears that external $NADP^+$ is not reduced by the transmembrane transhydrogenase. This, together with the low rates of the energy-linked NADH-NADP transhydrogenase (Hackett, 1963; Wilson and Bonner, 1970) and the general lack of NADP-linked enzymes (see Chapter V) in plant mitochondria, suggest that the mitochondria do not play a prominent role in generation NADPH for biosynthetic purposes, in plant cells.

A.



B.

OR

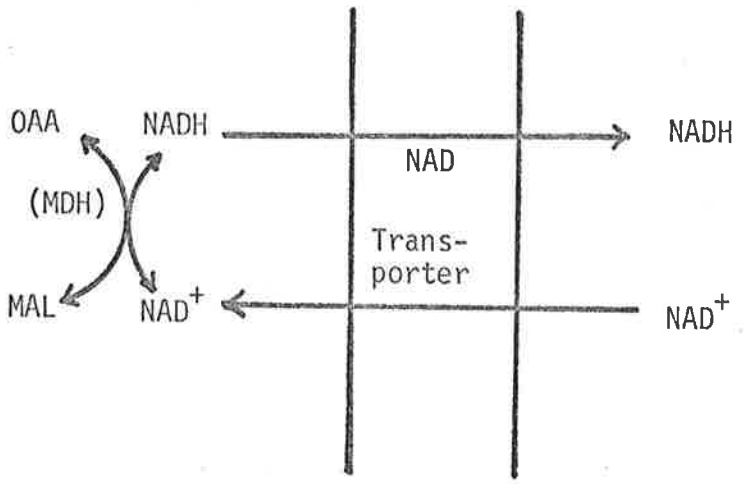


Figure 7.1 Transfer of reducing equivalents across the inner membrane of isolated plant mitochondria.

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