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THE INTERACTION OF BRANCHED ELECTRON TRANSPORT
PATHWAYS IN PLANT MITOCHONDRIA.

by

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ABSTRACT

The branched nature of the electron transport pathways of plant mitochondria has been investigated using inhibitors of electron transport and artificial electron acceptors. Activation of succinate dehydrogenase is described, and assays indicated a high level of membrane integrity in the mitochondria used in this study.

The possible pathways for oxidation of endogenous NADH in plant mitochondria were investigated. Evidence was found for compartmentation of malic enzyme and malate dehydrogenase, which was partly removed by piericidin A or the transition from respiratory state 3 to 4. Compartmentation was greatest when respiratory control was highest and the piericidin A-insensitive component of malate oxidase activity lowest. State 3 rates of oxygen consumption were different for each NAD^+ -linked substrate, in the presence or absence of piericidin A, indicating that either the rate of reduction of NAD^+ was different for each substrate, or a different respiratory pathway is used for the oxidation of each substrate.

Citrate inhibited exogenous NADH oxidase activity which appeared to be due to chelation of divalent cations required by this enzyme. The site of inhibition by citrate was located between the ferricyanide and duroquinone oxidation points of exogenous NADH oxidase. The inhibition did not require major uptake of citrate.

Succinate inhibited exogenous NADH oxidase activity by a mechanism requiring activity of succinate dehydrogenase. Exogenous NADH further activated succinate dehydrogenase, and the site of interaction was on the substrate side of cytochrome c.

Interaction required electron transport to be coupled to oxidative phosphorylation. There was no evidence for reverse electron flow from succinate to exogenous NAD^+ or for rapid extra reduction of any of the easily observed redox mediators of the electron transport pathways, with the possible exception of cytochrome c_{549} . A mechanism of interaction involving conformational changes in succinate dehydrogenase and exogenous NADH dehydrogenase is discussed, together with possible cellular roles for interactions between substrates that affect the rates of oxidation of malate, exogenous NADH and succinate.

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ABBREVIATIONS

AMP	adenosine-5'-monophosphate
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumen (fraction V)
CoA	coenzyme A
Duroquinone	2,3,5,6-tetramethyl-1,4-benzoquinone
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(β -amino-ethyl)-N,N' tetraacetic acid
FCCP	carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone
Juglone	5-hydroxy-1,4-naphthoquinone
MOPS	3-(N-morpholino)propane sulphonic acid
mv	millivolt
NAD ⁺	nicotinamide-adenine dinucleotide (oxidized)
NADH	nicotinamide-adenine dinucleotide (reduced)
OAA	oxaloacetate, potassium salt in solution pH 7.2
O.D.	optical density
Pi	inorganic phosphate
S.E.M.	standard error of the mean
Sl3	5-chloro,3-t-butyl-2-nitrosalicylanilide
TES	N-tris(hydroxymethyl)-methyl-2-aminoethane sulphonic acid
TPP	thiamin pyrophosphate hydrochloride
Tris	tris(hydroxymethyl)-methylamine, or 2-amino- 2-(hydroxymethyl)-propane-1:3 diol
Triton X100	octylphenoxypolyethoxyethanol
TCA cycle	tricarboxylic acid cycle

The following trivial names have been used
in this thesis.

<u>Trivial name</u>	<u>Systematic name</u>	<u>Enzyme Commission No.</u>
Alcohol dehydrogenase	Alcohol : NAD ⁺ oxidoreductase	1.1.1.1.
Glutamate transaminase	L-aspartate : 2- oxoglutarate aminotransferase	2.6.1.1.
Glycerol phosphate dehydrogenase	sn-glycerol-3- phosphate: NAD ⁺ oxidoreductase	1.1.1.8.
Isocitrate dehydrogenase (NAD ⁺)	threo-D ₅ -isocitrate : NAD ⁺ oxidoreductase (decarboxylating)	1.1.1.41
Lactate dehydrogenase	L-lactate : NAD ⁺ oxidoreductase	1.1.1.27
Malate dehydrogenase	L-malate : NAD ⁺ oxidoreductase	1.1.1.37
Malic enzyme	L-malate : NAD ⁺ oxidoreductase (decarboxylating)	1.1.1.39
2-Oxoglutarate dehydrogenase	2-oxoglutarate : lipoate oxido- reductase (decarboxyl- ating and acceptor- succinylating)	1.2.4.2.
Pyruvate dehydrogenase	pyruvate : lipoate oxidoreductase decarb- oxylating and acceptor - acetylating	1.2.4.1.
Succinate dehydrogenase	succinate : (acceptor) oxidoreductase	1.3.99.1.
NADH dehydrogenase	reduced-NAD ⁺ : (acceptor) oxidoreductase	1.6.99.3.
Cytochrome oxidase	ferrocytochrome c : oxygen oxidoreductase	1.9.3.1
Catalase	hydrogen-peroxide : hydrogen peroxide oxidoreductase	1.11.1.6.
Pyruvate kinase	ATP : pyruvate phosphotransferase	2.7.1.40

Kinetic Symbols

V	velocity of reaction
$V_{\max}$	maximum velocity of reaction; the value of V when enzyme is saturated with substrate.
K_m	Michaelis constant; the concentration of substrate permitting half $V_{\max}$.
$K_m(\text{app})$	apparent K_m .

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INTRODUCTION

This thesis is concerned with the results of a study of the branched nature of the electron transport pathways of isolated plant mitochondria. It is considered helpful to look first at the arrangement of these pathways, as formulated at present.

1. The electron transport pathways of isolated mitochondria.

The nature of the redox mediators, and their general organisation in the electron transport pathways of plant mitochondria, appear to be similar to those of mammalian and avian mitochondria (Passam, 1971; Ikuma, 1972; Bonner, 1973; Palmer & Coleman, 1974; Palmer, 1976). Since the electron transport pathways of isolated animal mitochondria are better understood than those of plant mitochondria, a brief description of the electron transport pathways of animal mitochondria will be given first.

i) Mammalian mitochondria.

The simplest arrangement of the electron transport pathways of isolated mammalian mitochondria, is illustrated in figure 1, in which it can be seen that NADH dehydrogenase and succinate dehydrogenase both appear to donate reducing equivalents to the same conventional cytochrome chain. The electron transport pathway from succinate to oxygen is shown as meeting the main pathway from NADH, at a point located between the iron-sulphur centres associated with oxidative phosphorylation at site 1, and cytochromes b. Ubiquinone is the only known redox mediator of the respiratory chain which appears to be located there, and so it is shown as being located at the point of junction between the two branches of the respiratory pathways. General accounts of the mammalian respiratory electron transport pathways will be found

in recent reviews (see Slater, 1971; Van Dam & Meyer, 1971; Palmer & Hall, 1972; Baltschevsky & Baltschevsky, 1974; Palmer & Coleman, 1974).

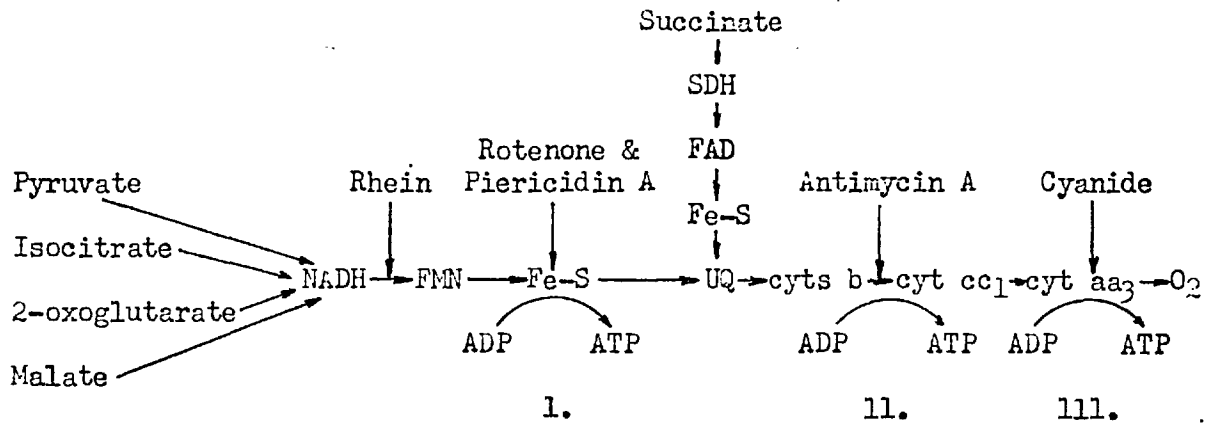


Figure 1. The major components of the electron transport pathways of isolated mammalian mitochondria, showing the sites of action of inhibitors and the 'sites' of ATP synthesis. FMN, flavin mononucleotide; Fe-S, iron-sulphur centres; UQ, ubiquinone; cyt(s), cytochromes; SDH, succinate dehydrogenase; FAD, flavin adenine dinucleotide. (After Palmer & Coleman, 1974).

ii) Plant mitochondria.

A scheme for the possible organisation of the electron transport pathways in isolated plant mitochondria is presented in figure 2, which also illustrates some aspects of the greater complexity of these pathways, compared with those of mammalian mitochondria just presented. This organisation is based upon a number of similar schemes published by Passam (1971), Coleman (1971), Coleman & Palmer (1972), Storey & Bahr (1972), Ikuma (1972), Storey (1973), Bonner (1973), Bahr & Bonner (1973a,b), Brunton & Palmer (1973), Palmer & Coleman (1974), and Palmer (1976), and on data presented by Storey (1970b), for the reduction kinetics of the respiratory chain carriers of mung bean mitochondria, with exogenous NADH as the supplied substrate.

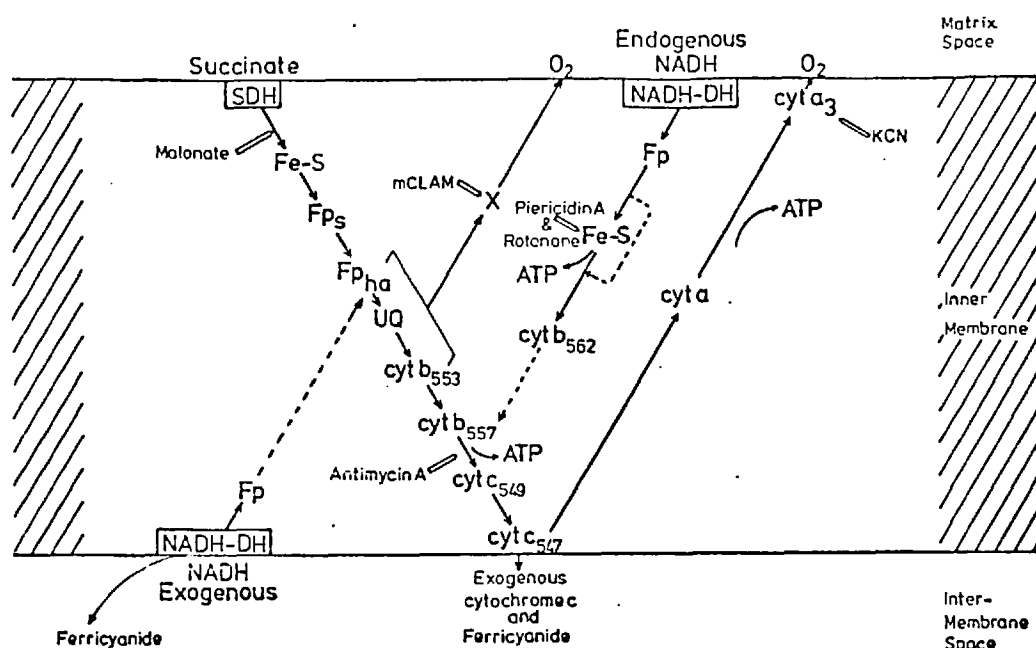


Figure 2. The possible organisation of the electron transport pathways of isolated plant mitochondria, in relation to the faces of the inner membrane. The numerical values shown as subscripts to 'cyt b' and 'cyt c', refer to the wavelength of light at which the α absorbance band of the particular cytochrome has its maximum when measured at 77K. The units of measurement for the wavelengths of light are nanometers (nm.). NADH-DH, NADH dehydrogenase; Fp, flavoprotein; mCLAM, meta-chlorobenz-hydroxamic acid; other abbreviations are as described for figure 1.

The arrangement of the electron transport pathways between succinate and cytochromes aa₃, can be seen from figure 2 to be similar to the pathway that is thought to exist in isolated mammalian mitochondria (figure 1.). Cytochrome c is located near the outer face of the inner membrane, since it appears to be able to interact with exogenously added ferricytochrome c under conditions when the inner mitochondrial membrane is intact (Palmer & Kirk, 1974).

iii) Functional segmentation of the electron transport pathways.

The electron transport pathway in the region of NADH dehydrogenase in animal mitochondria, and the endogenous NADH dehydrogenase in yeast mitochondria, has been further characterized by the use of Electron Paramagnetic Resonance spectroscopy (EPR), and by the use of alternative terminal oxidants of the pathway, in conjunction with artificial reductants of components of the pathway and inhibitors of electron transport. EPR studies have shown the existence of at least four iron-sulphur centres (Fe-S), in the NADH dehydrogenase region of the electron transport pathway, as figure 3 illustrates. This figure also shows that oxidative phosphorylation at 'site 1' is thought to be associated with electron transport through these iron-sulphur centres. Also the site of inhibition by piericidin A and rotenone, is indicated (see Gutman & Singer, 1970, 1971; and Gutman, Singer, Beinert & Casida, 1970).

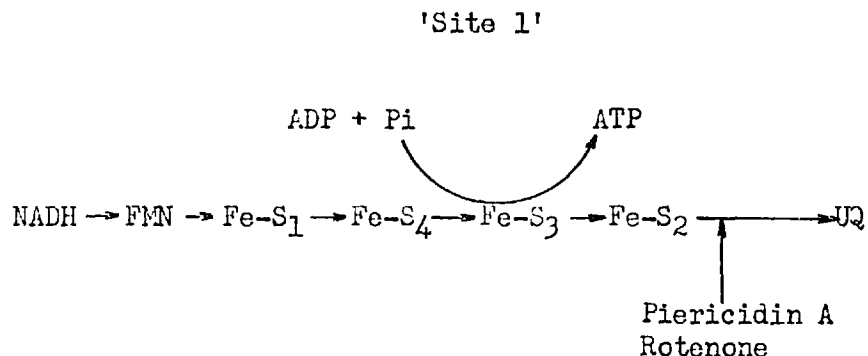


Figure 3. Sequence of electron flow in the rotenone-sensitive NADH dehydrogenase. Fe-S, iron-sulphur centre; FMN, flavin mononucleotide; UQ, ubiquinone. 'Site 1' phosphorylation is associated with electron flow through Fe-S₁ and Fe-S₂ (Ohnishi, 1973). After Palmer & Coleman (1974).

There have been several reviews concerning iron-sulphur proteins in the respiratory pathways of mitochondria, including those of Malkin & Rabinowitz (1967), Hall & Evans (1969), Orme-Johnson (1973), Beinert, Orme-Johnson & Hansen (1973) and Ohnishi (1973). In a recent review of the organization and regulation of electron transport in plant mitochondria, Palmer (1976) concluded that preliminary experiments indicate a similar arrangement of the Fe-S centres of plant mitochondria, to those shown in figure 3 for yeast and animal mitochondria.

Because of the complexity and general insolubility, of NADH dehydrogenase, it has not been possible to study it using the traditional techniques of enzyme purification and characterization. The enzyme is closely associated with phospholipid (Awasthi, Ruzicka & Crane, 1970), and with insoluble protein (Hatefi & Stempel, 1967), constituents of the inner mitochondrial membrane. Attempts to release components of this enzyme complex by rigorous treatment, have resulted in changes in its catalytic properties (Watari, Kearney & Singer, 1963; Salach, Singer & Bader, 1967). Therefore, non-disruptive techniques of assaying NADH dehydrogenase in isolation from as many of the other components of the electron transport pathway as possible, were sought.

One experimental approach employed to investigate the nature of the NADH dehydrogenase further, was to use artificial oxidants and reductants of components of the electron transport pathway, which functionally segmented this pathway, without causing physical disruption of the mitochondrial membranes. Use has been made of artificial oxidants or electron acceptors, such as the ferricyanide anion, which was added to the mitochondria as an aqueous solution of potassium ferricyanide (Copenhaver & Lardy, 1952; Pressman, 1955; Lee, Sottocasa & Ernster, 1967). The ferricyanide anion does not appear to be able to

cross the inner mitochondrial membrane (Mitchell & Moyle, 1969; von Jagow & Klingenberg, 1970; Klingenberg, 1970a; Passam, 1971). The ferricyanide-ferrocyanide redox couple, has a mid-point redox potential at pH 7.0 of +360mV (Palmer & Coleman, 1974), and so the ferricyanide anion is potentially capable of accepting electrons from all known natural redox mediators of the electron transport pathways of isolated mitochondria. The mid-point potentials of redox couples associated with the electron transport pathways, range from NADH-NAD⁺ at -320mV, to cytochrome a oxidized at +290mV, both measured at pH 7.0 (see Palmer & Hall, 1972).

The ferricyanide anion reacts with the terminal part of the electron transport pathways of isolated intact mitochondria, at a point located on the oxygen side of the antimycin A-sensitive step, probably cytochrome c (Pressman, 1955; Jacobs & Sanadi, 1960; Estabrook, 1961; Palmer & Kirk, 1974). It can also interact with flavoprotein dehydrogenases when they are accessible to it (von Jagow & Klingenberg, 1970; Palmer & Passam, 1971; Passam, 1971; Brunton, 1975), which appears to be the case for the oxidation of exogenous NADH by ferricyanide, in isolated intact plant mitochondria, as indicated in figure 2.

Other examples of alternative electron acceptors used to functionally segment the electron transport pathways of isolated mitochondria, are quinones. The natural ubiquinone of the mitochondria can be easily extracted using organic solvents such as petroleum ether (Szarkowska & Klingenberg, 1963; Kröger & Klingenberg, 1966), n-hexane (Storey, 1966) or n-pentane (Szarkowska, 1966). However, as might be expected from its ready solubility in non-polar solvents, ubiquinone (i.e. ubiquinone₁₀), is highly insoluble in aqueous media such as the assay medium used to measure reactions mediated by isolated

intact mitochondria, and so other substituted aromatic quinones were tested for their suitability as artificial terminal oxidants of parts of the mitochondrial electron transport pathways. These quinones were more soluble in aqueous media than ubiquinone₁₀, and among the most useful ones tested were substituted naphthoquinones such as 5-hydroxy-1,4-naphthoquinone, and substituted benzoquinones such as 2,3,5,6-tetramethyl-1,4-benzoquinone. These quinones have trivial names, 5-hydroxy-1,4-naphthoquinone being called 'juglone', because of its natural occurrence as a purple pigment in members of the family of angiosperm plants, the Juglandaceae, for example Juglans nigra L., the black walnut (Aynehchi, Dehpour & Mahmoodian, 1973), and 2,3,5,6-tetramethyl-1,4-benzoquinone, is called 'duroquinone'.

Using ferricyanide, juglone and duroquinone as oxidants of NADH dehydrogenase in sub-mitochondrial particles (SMP) prepared from beef heart mitochondria by ultra-sonic oscillation, followed by centrifugation, Ruzicka & Crane (1970) have investigated the points at which each oxidant appears to accept electrons from the electron transport pathways. In addition, studies by Hare & Crane (1971), which included the use of the reduced form of duroquinone, durohydroquinone, as an electron donor to the electron transport pathways, have enabled these authors to draw up a scheme to illustrate the possible points of oxidation of NADH dehydrogenase by ferricyanide, juglone and duroquinone, in relation to the points at which inhibitors of electron transport have their action. This scheme is reproduced here in figure 4, and also shows the point at which durohydroquinone appears to donate electrons to the electron transport pathways. Subsequent studies using durohydroquinone as a reducing agent of the electron transport pathways of sub-mitochondrial particles (Boveris, Oshino, Erecinska & Chance, 1971; Kröger, 1972; Kröger & Klingenberg, 1973;

von Jagow & Bohrer, 1975), have supported the proposed site of reduction by this agent shown in figure 4, i.e. in the region of the cytochromes b.

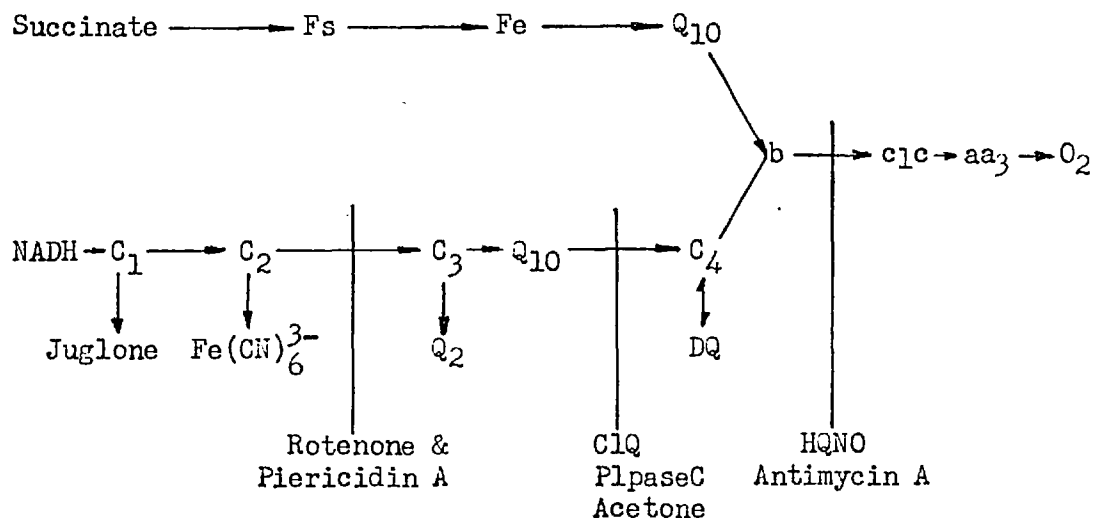


Figure 4. Possible points of oxidation of NADH in beef heart sub-mitochondrial particles, by juglone, ferricyanide, duroquinone and ubiquinone₂, in relation to the points of inhibition by rotenone, piericidin A, chloroquine sulphate (ClQ), phospholipase C (PlpaseC), acetone, 8-hydroxyquinoline n-oxide ($HQNO$), and antimycin A. Fs, the flavoprotein associated with succinate dehydrogenase; Fe, iron-sulphur centre; Q_{10} , ubiquinone₁₀; Q_2 , ubiquinone₂; DQ , duroquinone and durohydroquinone; $Fe(CN)_6^{3-}$, ferricyanide anion; b, c_1c, aa_3 , cytochromes b, c_1c , and aa_3 ; C_1-4 , represent the apparent electron donor sites on the NADH dehydrogenase complex. After Hare & Crane (1971).

Another example of an electron acceptor from the electron transport pathways of isolated mitochondria, is purified ferricytochrome c. This electron acceptor is used to study the part of the electron transport pathway that is located on the oxygen side of the endogenous cytochrome c, from which it can accept electrons directly

(see figure 2.). Unlike the artificial oxidants of the electron transport pathways described so far, ferricytochrome c is a relatively large molecule, having a molecular weight in the region of 12,500. This raises problems concerning its accessibility to the outer face of the inner mitochondrial membrane, and thus to the endogenous cytochrome c (Racker, 1970; Palmer & Hall, 1972), since to do so, the molecules of ferricytochrome c must cross the outer mitochondrial membrane. This problem appears to be overcome by suspending the mitochondria in an assay medium of osmolarity low enough to result in swelling of the inner membrane, which in turn results in the outer membrane becoming stretched, but still sufficiently high to prevent rupture of the inner membrane.

The activity of succinate-cytochrome c reductase has been used as a measure of the degree of intactness of the outer membrane of mung bean mitochondria by Douce, Christensen & Bonner (1972). However, a more recent study by Palmer & Kirk (1974), of this enzyme activity in isolated Jerusalem artichoke mitochondria, has indicated that the rate limiting step for the reduction by succinate of the ferricytochrome c, is not only the rate at which it crosses the outer mitochondrial membrane, but also the ease with which it can react with endogenous cytochrome c. In isolated mitochondria, the enzyme succinate dehydrogenase is often found to be in a deactivated state, and a process of activation is normally carried out before assaying reactions which involve this enzyme. Activation of succinate dehydrogenase can be achieved by preincubating the isolated mitochondria for 2-4 minutes, at a temperature between 25 and 37°C in the presence of molecules which act as activating agents, for example succinate, malonate or ATP, (Kearney, 1957; Kearney, Mayr & Singer, 1972; Singer, Kearney & Kenney, 1972; Oestreicher, Hogue & Singer, 1973). The degree of activation

of succinate dehydrogenase caused by the activating agents, is a function of time, until maximum activation is reached, and Palmer & Kirk (1974) found that, in isolated intact Jerusalem artichoke mitochondria, suspended in assay medium of normal osmolarity (i.e. a medium containing 0.4M sucrose as the main osmoticum), as the succinate dehydrogenase became progressively more active with time, following the addition of ATP and succinate, so the activity of succinate-cytochrome c reductase increased. This indicated that under constant conditions of normal osmolarity, sub-maximal rates of reduction by succinate of exogenously added ferricytochrome c, were not being limited by the permeability of the outer mitochondrial membrane to cytochrome c, but by the activity of the enzyme succinate dehydrogenase. This conclusion was further supported by the results of a similar experiment in which the ferricytochrome c was replaced by the ferricyanide anion. This anion can act as an oxidant of endogenous ferrocytochrome c (see figure 2), but the outer mitochondrial membrane does not appear to act as a permeability barrier to it. Palmer & Kirk (1974), found that the activity of succinate-ferricyanide reductase also increased with time, as succinate dehydrogenase became progressively more active, and this increase was found to closely parallel the increase in the activity of succinate-cytochrome c reductase. Also, the rate of reduction of exogenous ferricytochrome c by succinate or exogenous NADH, was found to be directly related to the maximum rate of electron flow, as determined from the rate of consumption of oxygen.

Lowering the osmolarity of the assay medium, in addition to stimulating the activity of succinate-cytochrome c reductase, also caused simultaneous changes in the permeability of the inner membrane of Jerusalem artichoke mitochondria (Palmer & Kirk, 1974). The first change detected was an increase in the degree of permeability of this

membrane to the ferricyanide anion, then a simultaneous increase in the level of accessibility of exogenous ferricytochrome c to the electron transport pathways, and an increase in the permeability to NADH, followed by rupture, as measured by the release of the enzyme malate dehydrogenase from the mitochondrial matrix. Palmer & Kirk (1974), therefore concluded that the stimulation of succinate-cytochrome c reductase activity in isolated Jerusalem artichoke mitochondria, resulting from decreasing the osmolarity of the assay medium, was associated with conformational changes in the inner mitochondrial membrane, rather than with rupture of the outer membrane. Estimation of the degree of stimulation of succinate-cytochrome c reductase activity as a result of decreasing the osmolarity of the assay medium, is however, still considered to be a sensitive parameter for measuring the integrity of the membranes of isolated plant mitochondria (Palmer, 1976). Whether the outer membrane of such mitochondria acts as a permeability barrier to exogenous ferricytochrome c molecules as proposed by Douce, Christensen & Bonner (1972) and Bonner (1973), has still to be resolved.

The activities of endogenous NADH-ferricyanide reductase and succinate-ferricyanide reductase, in isolated plant mitochondria, have been shown to be mediated by the electron transport pathways, as far as endogenous cytochrome c at least, and hence both activities are found to be almost totally sensitive to inhibition by antimycin A. In contrast, the activity of exogenous NADH-ferricyanide reductase in yeast mitochondria, has been found to be insensitive to inhibition by antimycin A (von Jagow & Klingenberg, 1970), and similarly in isolated plant mitochondria (Palmer & Passam, 1971; Passam, 1971; Coleman, 1971; Coleman & Palmer, 1972). Von Jagow & Klingenberg (1970), concluded that there must be an oxidation site in the electron trans-

port pathway from exogenous NADH, which is accessible to ferricyanide, and located before the antimycin A inhibition point between cytochromes b and c. These authors equated this site with the flavoprotein of the exogenous NADH dehydrogenase.

In addition to the flavoprotein of the exogenous NADH dehydrogenase, ferricyanide is able to react with other flavoproteins, including one located on the outer mitochondrial membrane, and is associated with the enzyme NADH-cytochrome b_5 reductase (Sottocasa, Kylenstierna, Ernster & Bergstrand, 1967a,b). Douce, Mannella & Bonner (1973), have isolated the outer membrane of mung bean mitochondria, and shown that it possesses NADH-ferricyanide reductase activity. It is therefore not possible to be certain that all of the exogenous NADH-ferricyanide reductase activity catalyzed by isolated plant mitochondria, represents activity of the flavoprotein associated with the inner membrane and the electron transport pathways.

There is some evidence however, for each enzyme having different stereospecificity for the hydrogen atom of the NADH molecule involved in the reduction of the flavoprotein. The reaction catalyzed by NADH-cytochrome b_5 reductase has been found to be specific for the 4α hydrogen atom of NADH, while the NADH dehydrogenase associated with the inner membrane of animal mitochondria, has been shown to be specific for the 4β hydrogen atom of NADH (Ohnishi, Kawaguchi & Hagihara, 1966; Sottocasa, Kylenstierna, Ernster & Bergstrand, 1967a,b). The exogenous NADH-ferricyanide reductase activity in yeast mitochondria, has been shown to be insensitive to inhibition by antimycin A, and to be specific for the 4β hydrogen atom of NADH by von Jagow & Klingenberg (1970). From the results obtained with animal mitochondria and just discussed, this finding indicated that the enzyme being assayed in the yeast mitochondria was probably the respiratory chain-linked

exogenous NADH dehydrogenase. Douce, Mannella & Bonner (1973), have questioned this conclusion, since these authors found that exogenous NADH-ferricyanide reductase activity in mung bean mitochondria, was sensitive to inhibition by antimycin A. Since these authors also showed that NADH could not cross the inner mitochondrial membrane, they had to conclude that the antimycin A-sensitive component of exogenous NADH-ferricyanide reductase activity, was a property of the exogenous NADH dehydrogenase which is linked to the respiratory pathways of the mung bean mitochondria used. They suggested that the flavoprotein component of this dehydrogenase was associated with the outer face of the inner mitochondrial membrane, in such a way as to be accessible to NADH but not to the ferricyanide anion. As pointed out by Palmer (1976), " It is impossible to distinguish between this situation, and one in which the mitochondria are permeable to NADH but not to ferricyanide. Oxidation could therefore be catalyzed by an internal enzyme".

Exogenous NADH-cytochrome c reductase activity in intact isolated plant mitochondria, is mediated by the electron transport pathways including the cytochromes b to c step, and hence is largely sensitive to inhibition by antimycin A, unlike exogenous NADH-ferricyanide reductase activity just described (Palmer & Kirk, 1974; see also figure 2, present thesis). The activity of exogenous NADH-cytochrome c reductase was not as sensitive to inhibition by antimycin A as succinate-cytochrome c reductase activity, since in common with the ferricyanide anion, exogenous ferricytochrome c can be reduced by exogenous NADH, in a reaction catalyzed by the enzyme NADH-cytochrome b_5 reductase, located on the outer mitochondrial membrane. This component of the rate of cytochrome c reduction does not involve the electron transport pathways of the inner mitochondrial membrane, and

so is insensitive to inhibition by antimycin A.

The assay of respiratory chain-mediated exogenous NADH-cytochrome c reductase activity in isolated intact plant mitochondria, is normally carried out in the presence of a concentration of potassium cyanide, sufficiently high to cause the maximum level of inhibition of cytochrome c oxidase activity. Any antimycin A-insensitive component of exogenous NADH-cytochrome c reductase activity (which is normally small compared with the total activity) is subtracted from the total rate of reduction of cytochrome c. In the absence of cyanide, the exogenous ferrocytochrome c produced by the activity of NADH-cytochrome b₅ reductase activity, is able to ferry reducing equivalents across the inter-membrane space of the mitochondrion, and be oxidized by oxygen in a reaction catalyzed by cytochrome c oxidase. This results in the consumption of oxygen which is insensitive to inhibition by antimycin A because it does not involve the cytochrome b to c step, but sensitive to inhibition by cyanide, since it involves the activity of cytochrome c oxidase (Hanson & Hodges, 1967; Palmer & Coleman, 1974; Palmer, 1976).

iv) Differences between electron transport pathways of animal and plant mitochondria.

As noted already, comparison of the arrangement of the electron transport pathways of isolated animal mitochondria (figure 1.), with those of isolated plant mitochondria (figure 2.), shows an overall similarity between the two types of mitochondria, particularly between succinate and cytochromes aa₃.

There are however, three major differences between the electron transport pathways of plant mitochondria, and their mammalian

counterpart. These differences are (i) the existence in mitochondria isolated from plant tissues, of an alternative terminal oxidase to the conventional cytochrome c oxidase; the alternative oxidase is insensitive to inhibition by cyanide, but sensitive to inhibition caused by substituted benzhydroxamic acids such as meta-chlorobenzhydroxamic acid (mCLAM), (ii) the presence in mitochondria isolated from plant tissues, of an NADH oxidase capable of catalyzing the oxidation of exogenous NADH by oxygen, in a manner which is coupled to oxidative phosphorylation and characterized by an ADP:O ratio of up to a value of two, and (iii) the ability of isolated plant mitochondria to catalyze the oxidation of malate, in the absence of a system for the rapid removal of oxaloacetate, the product of malate oxidation catalyzed by malate dehydrogenase in mammalian mitochondria.

a) Cyanide-insensitive electron transport pathways.

Mitochondria isolated from vertebrate tissues such as liver, kidney or heart, catalyze the oxidation of respiratory substrates in a manner which is totally sensitive to inhibition by cyanide (see Lehninger, 1964; Palmer & Hall, 1972; Palmer & Coleman, 1974). In contrast, mitochondria isolated from certain other sources catalyze respiration that is insensitive to inhibition by cyanide.

A few invertebrate animals that live in specialized environments, have been found to possess cyanide-insensitive respiration. For example Ascaris lumbricoides, a parasitic worm (Cheah & Chance, 1970), and Crithidia fasciculata, a trypanosomatid haemoflagellate (Kusel & Storey, 1973; Hill & Cross, 1973). However, it is in mitochondria isolated from plant tissues, that cyanide-insensitive respiration is most highly developed (see Henry & Nyns, 1975). Examples have been found in the fungi, particularly mutants as in the yeast Candida utilis (Downie & Garland, 1972; Grimmelikhuijzen & Slater, 1973), and the 'mi'

mutants of Neurospora crassa (Lambowitz, Smith & Slayman, 1972a,b; von Jagow, Weiss & Klingenberg, 1973). In addition, Edwards & Kwiecinski (1973), have investigated cyanide-insensitive respiration in the 'Cni 1' mutant of N.crassa, and Sherald & Sisler (1970), have shown the existence of an antimycin A-insensitive electron transport pathway in Ustilago maydis and Neurospora sitophila.

Culture conditions of fungi, that lead to inhibition of the synthesis of mitochondrial cytochrome c oxidase, can also result in the development of cyanide-insensitive respiration, in their mitochondria. This characteristic makes such fungi valuable experimental tissues for investigations of the nature of cyanide-insensitive respiration. For example, culturing the yeast Candida utilis (Downie & Garland, 1973), and N.crassa (Schwab, 1973), under copper-limited conditions, or culturing wild type hyphae of N.crassa in the presence of the inhibitor of protein synthesis, chloramphenicol, (Lambowitz, Smith & Slayman, 1972b; von Jagow & Klingenberg, 1972; Edwards, Rosenberg & Maroney, 1974). Another example of inducibility of cyanide and antimycin A-insensitive respiration in mitochondria, is that found in the phytoflagellate Euglena gracilis, if it is cultured in the presence of ethanol or succinate plus antimycin A (Sharpless & Butow, 1970).

Mitochondria isolated from angiosperm plant tissues, show a range of sensitivity to cyanide, with white potato (Solanum tuberosum) and Jerusalem artichoke (Helianthus tuberosus) tuber mitochondria catalyzing respiration that is approximately 99% sensitive to inhibition by cyanide, mung bean (Phaseolus aureus) hypocotyl mitochondria being approximately 80-85% sensitive to cyanide, skunk cabbage (Symplocarpus foetidus) spadix mitochondria being approximately 20-30% sensitive, and mitochondria isolated from the spadices of Arum maculatum are almost

totally insensitive to cyanide (see Bahr & Bonner, 1973a). It is possible to induce 30-40% cyanide-insensitive respiration in white potato tuber mitochondria, by suspending slices of the tuber in aerated water for one or two days before isolating the mitochondria from them, (Hackett, Haas, Griffiths & Nierderpruen, 1960; Laties, 1963; Cammack & Palmer, 1973; Solomos & Laties, 1975).

The phenomenon of cyanide-insensitive respiration in angiosperm plants, was first observed by Van Herk (1937). Since then many biochemists have investigated it, including the following: James & Beevers (1950), Bendall & Hill (1956), Hackett (1957), Hackett & Haas (1958), Chance & Hackett (1958), Hackett (1959), Bonner (1962), Chance & Bonner (1965), Bonner, Bendall & Plesnicar (1967), Bonner & Bendall (1968), Chance, Bonner & Storey (1968), Storey & Bahr (1969a,b), Lance & Bonner (1969), Wilson (1970a,b), Erecinska & Storey (1970), Bendall & Bonner (1971), Storey (1971), Bonner, Christensen & Bahr (1972), Passam & Palmer (1972), Palmer (1972), Wilson & Moore (1973), Wedding, McCready & Harley (1973a,b), Bahr & Bonner (1973a,b), Cammack & Palmer (1973), Solomos & Laties (1973,1975), Henry & Nyns (1975) and Meeuse (1975).

Reference to figure 2 shows that the cyanide-insensitive oxidase is thought to branch from the cyanide-sensitive electron transport pathways in the region of the ubiquinone-Fp_{ha}-cytochrome b₅₅₃ complex. The reasons for this position of the point of junction between the two oxidases, were that the cyanide-insensitive component of the rate of respiration, was also found to be insensitive to inhibition by antimycin A in most cases, which placed the point of junction before the point of action of antimycin A, i.e. at or before the cytochromes b. Also, the flavoprotein with the high mid-point redox potential and high value for the fluorescence : absorbance ratio, Fp_{hf}, and the flavoprotein with the high mid-point redox potential and low value for the fluorescence

: absorbance ratio, Fp_{na} , remained oxidized in the presence of antimycin A and succinate, in skunk cabbage mitochondria, and Fp_{na} appears to have a role on the main electron transport pathway to cytochrome c oxidase, while Fp_{nr} appears to be a member of the cyanide-insensitive electron transport pathway (Erecinska & Storey, 1970; Storey, 1970a; Ikuma, 1972; Palmer, 1976). The cyanide-insensitive alternative oxidase can be totally inhibited by substituted benzhydroxamic acids, such as mCLAM (Schonbaum, Bonner, Storey & Bahr, 1971; Lambowitz, Smith & Slayman, 1972a,b; Bahr & Bonner, 1973a,b; Edwards, Rosenberg & Maroney, 1974), a characteristic which has permitted each electron transport pathway to be studied separately in the same mitochondria.

The cyanide-sensitive and cyanide-insensitive electron transport pathways in mitochondria isolated from skunk cabbage spadices and hypocotyls of mung bean and black-eyed pea (Vigna sinensis), appear to be functionally linked to each other, since Bahr & Bonner (1973a,b) found that the electron transport from substrate to oxygen, switched from the cytochrome chain and cytochrome c oxidase to the alternative oxidase, upon the transition from respiratory state 3 to respiratory state 4. The terms 'respiratory state 3' and 'respiratory state 4' refer to the conditions when the isolated mitochondria are in the presence of a respiratory substrate and excess ADP (state 3), and in the presence of a respiratory substrate, a high value for the ratio of the concentrations of ATP : ADP, and the concentration of ADP too low for it to be able to act as phosphate acceptor (state 4), as defined by Chance & Williams (1956). Similar results have been found by Edwards, Rosenberg & Maroney (1974), for mitochondria isolated from hyphae of Neurospora crassa which had been cultured in the presence of chloramphenicol.

b) Electron transport from exogenous NADH.

A major difference between the electron transport pathways of mitochondria isolated from mammalian and other animal tissues, and those isolated from almost all plant tissues, is the presence in the latter of an exogenous NADH oxidase capable of catalyzing the oxidation of NADH which is located externally to the mitochondrial matrix. This oxidation is coupled to oxidative phosphorylation at 'sites' II and III, as shown in figure 2. Animal mitochondria do not possess such an enzyme (Lehninger, 1955; Coleman, 1971; see also Lehninger, 1964), which probably means that the mechanism by which the pool of NAD^+ in the cytosol is maintained at a different level of reduction compared with the level of reduction of the intra-mitochondrial pool of NAD^+ , is different in animal cells compared with plant cells. The ratio of $\text{NADH}:\text{NAD}^+$ has been shown to affect the activity of metabolically important enzymes, and hence it is considered to be of importance for the regulation of cellular metabolism, a point that will be returned to later.

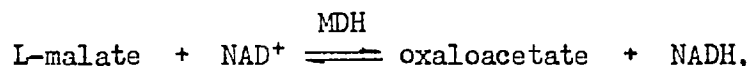
The detailed structural and functional relationship between exogenous NADH oxidase, endogenous NADH oxidase and succinate oxidase, is not yet known, and hence in figure 2 dotted lines connect both exogenous NADH dehydrogenase and endogenous NADH dehydrogenase to the cytochrome chain. However, as figure 2 also shows, there is evidence for the branches of the electron transport pathways from both NADH dehydrogenases and succinate dehydrogenase, meeting a common cytochrome chain at a point located on the substrate side of the antimycin A-sensitive step between cytochromes b and c. The major part of the present thesis is concerned with experiments carried out to investigate the functional relationship between the enzymes exogenous NADH oxidase, endogenous NADH oxidase and succinate oxidase in isolated plant mitochondria.

The scheme illustrated in figure 2, also shows that there is evidence for a bypass of the step in the electron transport pathway from endogenous NADH to oxygen, which is sensitive to inhibition by piericidin A, and also includes oxidative phosphorylation 'site 1'. This evidence comes from the experiments of Brunton & Palmer (1973), Brunton (1975) and Pomeroy (1975); using mitochondria isolated from wheat (Triticum vulgare cv.) coleoptiles, and from barley (Hordeum sp. cv.) scutella, in which it was found that the rate of oxidation of endogenous NAD^+ -linked substrates, was only partially sensitive to inhibition by rotenone or piericidin A, indicating the possible existence of more than one type of electron transport pathway from endogenous NADH to oxygen in these mitochondria, one of which is insensitive to inhibition by piericidin A. That the rotenone and piericidin A-insensitive electron transport pathway also bypassed oxidative phosphorylation at 'site' 1, was indicated by the finding that the value for the ADP:O ratio measured for NAD^+ -linked substrate oxidation, was decreased by an amount which was approximately equal to one, when rotenone was present compared with a similar measurement made in the absence of rotenone. Part of the present thesis is concerned with the results obtained from an investigation of the nature of endogenous NADH oxidase in mitochondria isolated from tubers of Jerusalem artichoke and white potato, and from hypocotyls of mung beans.

c) Malate oxidation.

The rapid oxidation of malate can be catalyzed by isolated plant mitochondria, in the absence of a system for removing oxaloacetate (Wiskich & Bonner, 1963; Ikuma & Bonner, 1967a; Sarkissian & Srivastava, 1968; Macrae & Moorhouse, 1970; Macrae, 1971 a,b,c; Passam, 1971; Coleman, 1971; Coleman & Palmer, 1972; Brunton & Palmer, 1973; Brunton, 1975; Pomeroy, 1975), while malate oxidation in animal mitochondria,

can only be catalyzed if the product, oxaloacetate, is removed by either transamination with glutamate, or by condensation with acetyl-CoA (Banga, Szent-Gyorgyi, 1936; Chappell, 1961; Slater, Tambllyn-Hague & Davis van Thienen, 1965). In this case, the enzyme responsible for catalysis of malate oxidation is malate dehydrogenase, and the reaction may be represented by the equation,



where MDH is malate dehydrogenase. This reaction has an equilibrium constant at pH 7.0 of 1.3×10^{-5} , and thus at equilibrium, malate is favoured. Only when the equilibrium is displaced, by for example removing the oxaloacetate as described above, will malate be converted to oxaloacetate. In the TCA cycle, the condensation reaction between oxaloacetate and acetyl-CoA, is strongly exergonic ($\Delta G^{\circ} = 8.0 \text{ kcal.mol}^{-1}$), in the direction of citrate synthesis, which tends to favour the conversion of malate to oxaloacetate by displacing the equilibrium through the continuous removal of oxaloacetate. Another consequence of the very low equilibrium constant for the reaction catalyzed by malate dehydrogenase, is that low concentrations of oxaloacetate can inhibit the reaction in the forward direction, and hence the subsequent activity of malate oxidase catalyzed by animal mitochondria.

In plant mitochondria, in addition to malate dehydrogenase there is also an NAD^+ -linked 'malic enzyme', which catalyzes the reaction in which malate is converted to pyruvate with simultaneous reduction of NAD^+ . Hence the products of malate oxidation in isolated plant mitochondria, have been shown to be mainly pyruvate, with some citrate and oxaloacetate (Macrae & Moorhouse, 1970; Macrae, 1971 a,b,c; Coleman, 1971; Coleman & Palmer, 1972). The presence of the 'malic enzyme' in plant mitochondria, permits the oxidation of malate in the

absence of a system for the rapid removal of oxaloacetate.

Concerning the location within the mitochondria of the enzymes responsible for the oxidation of malate, Brunton & Palmer (1973) and Brunton (1975), have shown that the activity of malate-ferricyanide reductase measured under state 3 conditions, in isolated wheat mitochondria, was almost totally sensitive to inhibition by antimycin A. This indicated that under the conditions of assay, reducing equivalents from malate were flowing to ferricyanide anions via an electron transport pathway which included the step that is sensitive to antimycin A, i.e. the cytochrome b-c step (see figure 2). In addition, this result indicated that, under these conditions, the initial steps of malate oxidation occurred on the matrix side of the inner mitochondrial membrane, since this membrane appears to be impermeable to the ferricyanide anion (Mitchell & Moyle, 1969; von Jagow & Klingenberg, 1970; Klingenberg, 1970a; Passam, 1971; Brunton, 1975).

The activity of malate-ferricyanide reductase in wheat mitochondria, was however found to be only partially sensitive to inhibition by piericidin A, indicating that both piericidin A-sensitive and piericidin A-insensitive electron transport pathways mediated the flow of electrons from NADH produced in the matrix of these mitochondria (Brunton & Palmer, 1973; Brunton, 1975). This finding was not in agreement with those of Coleman (1971) and Coleman & Palmer (1972), for Jerusalem artichoke mitochondria. These authors found that malate-ferricyanide reductase activity, was almost totally sensitive to inhibition by rotenone, and the addition of NAD^+ following inhibition by rotenone, resulted in the stimulation of malate-ferricyanide reductase activity in a reaction that was insensitive to inhibition by antimycin A. It was concluded that, in the absence of added or exogenous NAD^+ , the initial steps of malate oxidation occurred in the matrix of Jerusalem

artichoke mitochondria, and that the electron transport pathway which mediated the oxidation of the NADH produced during this oxidation, was almost totally sensitive to inhibition by rotenone, as found to be the case for animal and yeast mitochondria (see Palmer & Coleman, 1974). The addition of NAD^+ appeared to allow a pool of malic enzyme located in the inter-membrane space of the mitochondrion, to become active, and the NADH produced by this activity appeared to be oxidized in a reaction catalyzed by the exogenous NADH dehydrogenase. These conclusions were drawn because in the presence of exogenous NAD^+ , malate-ferricyanide reductase activity was largely unaffected by antimycin A, and malate oxidase was characterized by an ADP:O ratio of 1.7 ± 0.1 in the presence of rotenone and exogenous NAD^+ , compared with a value of 2.8 ± 0.2 measured for malate alone, and for exogenous NADH of 1.8 ± 0.1 . A possible explanation was sought for the differences between the pattern of response of malate oxidase activity to piericidin A in wheat mitochondria found by Brunton & Palmer (1973) and Brunton (1975), and in Jerusalem artichoke mitochondria, found by Coleman (1971) and Coleman & Palmer (1972), using rotenone.

Wheat mitochondria were also found to catalyze the rapid oxidation of pyruvate, citrate and 2-oxoglutarate in reactions that were very sensitive to inhibition by added oxaloacetate (Brunton & Palmer, 1973; Brunton, 1975). If a low concentration of oxaloacetate was added to wheat mitochondria that were catalyzing the rapid oxidation of any of the three acids given above, i.e. under state 3 conditions, then the rate of consumption of oxygen in each case, was almost totally inhibited for a period of time, the duration of which was directly related to the amount of oxaloacetate added. In contrast, these authors found that state 3 malate oxidase activity, catalyzed by the same mitochondria, was not affected in the same way as the activities of the

other NAD^+ -linked substrate oxidases. Instead of causing a large but transient inhibition of the rate of oxygen consumption resulting from malate oxidase activity, a low concentration of oxaloacetate (0.2mM), caused a much lower degree of inhibition to a constant rate. Avron & Biale (1957), found similar results with avocado pear mitochondria, and Douce & Bonner (1972) had shown that the inhibition of the rate of oxygen consumption resulting from the oxidation by isolated plant mitochondria, of NAD^+ -linked substrates was a result of the oxaloacetate acting as the oxidant of available NADH, rather than oxygen, mediated by the electron transport pathways. The reduction of oxaloacetate by endogenous NADH required the presence of the enzyme malate dehydrogenase, and confirmed that oxaloacetate could freely enter the matrix of plant mitochondria. Douce & Bonner (1972) found that under state 4 conditions, the rate of oxygen consumption resulting from the oxidation of all NAD^+ -linked substrates, was greatly and temporarily inhibited by a low concentration of oxaloacetate, and concluded that malate dehydrogenase had access to each NAD^+ -linked substrate dehydrogenase, and that the NAD^+ in the matrix of isolated plant mitochondria, existed as a common pool. The findings of Avron & Biale (1957) and Brunton & Palmer (1973) and Brunton (1975), suggested that under state 3 conditions at least, the malic enzyme and/or its associated NAD^+ , was in a separate compartment on the matrix side of the inner membrane of those mitochondria tested, from the NAD^+ associated with the other NAD^+ -linked enzymes of the TCA cycle, including malate dehydrogenase. Brunton & Palmer (1973) and Brunton (1975), also found that the apparent compartmentation between malate dehydrogenase and the malic enzyme, could be removed following the partial inhibition of malate oxidase activity by piericidin A, concluding that the NADH associated with the malic enzyme then had access to oxaloacetate in

the malate dehydrogenase compartment of the mitochondrion. The results of a study of the type carried out by Brunton & Palmer (1973) and Brunton (1975), form a major part of the first half of this thesis. Mitochondria isolated from a range of angiosperm plant tissues were used in this study.

2. Regulation of the rate of mitochondrial electron transport.

The ratio of NADH:NAD^+ in the cytosol, appears to play a significant role in regulating the activity and direction of the glycolytic pathway. In particular, it appears to affect the step that is catalyzed by the enzyme, glycerol-3-phosphate dehydrogenase (see Palmer & Coleman, 1974). It is considered to be of advantage to cell economy therefore, that the rate of cytosolic NADH oxidation be regulated, since a high value for the NADH:NAD^+ ratio, results in inhibition of glycerol-3-phosphate dehydrogenase.

In animal cells, regulation of the rate of oxidation of cytosolic NADH could be effected by the regulation of one of the enzymes associated with the 'shuttle system', by which reducing equivalents are transferred across the inner mitochondrial membrane (Greville, 1966; Klingenberg, 1970b; Quagliariello & Palmieri, 1972; Cederbaum, Lieber, Beattie & Rubin, 1973). In plant cells, however, the existence of an exogenous NADH dehydrogenase in the mitochondria (see figure 2), presents these cells with additional problems not faced by animal cells.

The rate of oxidation of exogenous NADH catalyzed by isolated plant mitochondria, is known to be affected by the magnitude of the 'phosphate potential' i.e. $[\text{ATP}]/[\text{ADP}][\text{Pi}]$, as described by Wilson, Owen, Mela & Weiner (1973). There is also evidence for other possible modes of regulation of exogenous NADH dehydrogenase activity, in isolated plant mitochondria.

Hackett (1961) first reported involvement of divalent cations, such as calcium (Ca^{++}), with exogenous NADH oxidase in isolated plant mitochondria. More recently, other workers have reported similar findings (Miller, Dumford, Koeppe & Hanson, 1970; Miller & Koeppe, 1971; Coleman & Palmer, 1971; Coleman, 1971). In Jerusalem artichoke mitochondria, Coleman & Palmer (1971), reported that the activity of exogenous NADH oxidase was almost totally dependent upon the availability of divalent cations. Thus, the presence of a powerful chelator of such ions, e.g. ethylene glycol bis (2-amino-ethylether) -N,N' tetraacetic acid (EGTA), in the assay medium, caused almost total inhibition of exogenous NADH oxidase activity. Subsequent addition of the divalent cations Ca^{++} or Mn^{++} , but not Mg^{++} , was found to relieve the inhibition caused by the presence of EGTA. The divalent ion requirement was shown to be specific to exogenous NADH oxidase, since neither malate nor succinate oxidases were inhibited by EGTA. In addition, Coleman & Palmer (1971) found that exogenous NADH-ferricyanide reductase activity was not affected by the presence of EGTA, a result that was consistent with the site of interaction between the divalent cation and exogenous NADH oxidase, being located on the oxygen side of the oxidation point of exogenous NADH by ferricyanide in these mitochondria. The presence of EGTA also appeared to prevent the flow of electrons from exogenous NADH, reaching the cytochromes b, and so it was concluded that the site of interaction of the divalent cation requirement was located on the substrate side of the cytochromes b.

The concentration of calcium ions in the cytosol, could be altered by the ratio of mitochondrial influx:efflux of these ions, which would be expected to affect the activity of the mitochondrial exogenous NADH oxidase, which in turn would be expected to affect the

rate of glycolysis.

The major part of this thesis is concerned with experiments in which two respiratory substrates were presented simultaneously to isolated plant mitochondria, in a search for evidence for interaction between them, as measured by a change in the rate of oxidation of each substrate, compared with the rate measured for each substrate alone. One of the substrates in each case was exogenous NADH, and the other was pyruvate or an intermediate of the TCA cycle. Of particular interest were interactions that resulted in the inhibition of exogenous NADH oxidase activity. There have been no reports to date, concerning possible regulation of cellular metabolism resulting from interaction between respiratory substrates in isolated plant mitochondria, although some examples have been reported for isolated animal mitochondria.

Krebs (1935) introduced the concept of competition for oxygen between oxidizable substrates, to explain the failure of more than one substrate presented to respiring kidney slices and yeast cells, to stimulate the rate of consumption of oxygen to the extent expected from the sum of the two rates measured for each substrate presented separately. Haslam (1966) has reviewed much of the work of this type, which has been carried out on animal tissue homogenates and on mitochondria isolated from them. Of particular interest to the studies reported in this thesis, are reports of interactions between succinate and NADH oxidases in animal mitochondria, or sub-mitochondrial particles prepared from them.

Working with sub-mitochondrial particles prepared from pig heart mitochondria by the method of Keilin & Hartree (1947), Wu & Tsou (1955) showed that simultaneous oxidation of succinate and NADH catalyzed by these particles, resulted in a rate of reduction of

either oxygen or cytochrome c, that was less than the sum of the two rates measured for succinate or NADH alone. Thus, there was evidence for interaction occurring between succinate and NADH oxidases in pig heart sub-mitochondrial particles, at a point in the electron transport pathways located on the substrate side of endogenous cytochrome c (see figure 1.).

When the electron transport pathways were bypassed by diverting electron flow to the oxidizing agent, 2,6-dichlorophenol indophenol, the interaction between succinate and NADH oxidases was not found, since the rate of reduction of 2,6-dichlorophenol indophenol resulting from the simultaneous oxidation of succinate and NADH, was equal to the sum of the two rates measured for each substrate separately. Wu & Tsou (1955) also found that the presence of malonate could prevent succinate from having its inhibitory effect upon NADH oxidase activity. Thus it was concluded that interaction could occur between succinate and NADH oxidases in these animal sub-mitochondrial particles, which resulted in a mutual inhibition of each oxidase activity, by a mechanism which required the active participation of succinate dehydrogenase, and electron flow through the electron transport pathways to a point beyond the point(s) of oxidation by 2,6-dichlorophenol indophenol.

In a more recent study of the mutual inhibition of succinate and NADH oxidases in animal sub-mitochondrial particles, Gutman & Silman (1972) have suggested that the mechanism of interaction involves the state of reduction of ubiquinone. It was suggested that ubiquinone could be divided into a kinetic pool associated with NADH dehydrogenase, and another kinetic pool associated with succinate dehydrogenase, and that the mutual inhibition between NADH and succinate oxidases could be the result of a "spill-over" of reducing equiv-

alents over the compartmentation barrier between the two pools of ubiquinone. This, it was stated, might result in NADH dehydrogenase reducing ubiquinone in the pool available to succinate dehydrogenase, to such a level that the concentration of oxidized ubiquinone falls below the saturating level for succinate dehydrogenase, and hence succinate dehydrogenase would become inhibited. If this were the case, it might be expected that NADH oxidase activity would be unaffected and not inhibited, as was found, which possibly indicates that the explanation put forward by Gutman & Silman (1972) for the mechanism by which succinate oxidase and NADH oxidase mutually inhibit each other in animal sub-mitochondrial particles, might bear further investigation. It is of relevance to data to be presented in this thesis, to note that the NADH dehydrogenase which catalyzes the oxidation of added or exogenous NADH in animal sub-mitochondrial particles, is the enzyme that is located on the matrix side of the inner membrane of intact animal mitochondria. During the preparation of sub-mitochondrial particles by sonic-oscillation treatment, the inner membrane appears to form small vesicles, in which the polarity is reversed compared with that in the intact mitochondria. Thus the endogenous NADH dehydrogenase of the mitochondria becomes an exogenous NADH dehydrogenase of the sub-mitochondrial particles, and succinate dehydrogenase also appears on the outside of these particles (see Palmer & Hall, 1972)

Concerning the possibility of a 'pool function' for ubiquinone in animal sub-mitochondrial particles, there are conflicting reports in the literature. For example, Kröger & Klingenberg (1973), have used the finding that the titration curve for the concentration of antimycin A versus the respiratory activity of animal sub-mitochondrial particles, catalyzing the oxidation of either NADH or

succinate, was hyperbolic (sigmoidal) in shape. Thus, at sub-optimal concentrations of antimycin A, no inhibition of NADH oxidase activity occurred, and gradually as the concentration of antimycin A was increased, so inhibition took place. Kröger & Klingenberg (1973), also found that decreasing the electron donor capacity of succinate, by the presence in the reaction cuvette of a low concentration of malonate, to inhibit succinate dehydrogenase, or NADH by the presence of a low concentration of amytal, resulted in the antimycin A titration curve becoming increasingly more sigmoidal in shape. This finding has been interpreted by these authors, to be a result of ubiquinone acting as a hydrogen-transporting pool, capable of ferrying hydrogen atoms from antimycin A-inhibited to non-inhibited electron transport chains, when antimycin A was present at sub-optimal concentrations.

It has been possible to avoid ubiquinone by using the artificial electron donor, durohydroquinone, to donate electrons directly to the cytochromes b (see figure 4), when it was found that the antimycin A versus rate of oxygen consumption titration curve, became linear in shape (Kröger & Klingenberg, 1973). These authors concluded that the linear plot resulted from the lack of involvement of ubiquinone in the oxidation of durohydroquinone, and hence the absence of lateral movement of hydrogen atoms to unblocked electron transport chains at low antimycin A concentrations, when little or no inhibition of NADH or succinate oxidase activity had occurred.

The sigmoidal antimycin A titration curves have been explained by Bryla, Kaniuga & Slater (1969) and later by Bryla, Ksiezak, Rode & Kaniuga (1971) and Berden & Slater (1972), in terms of the allosteric model of Monod, Wyman & Changeux (1965). These authors have proposed that, in sub-mitochondrial particles, the antimycin A-sensitive segment of the electron transport pathways, exists in two enzymatically

active conformation states, R and T, both oligomeric or polymeric, and in equilibrium with each other, $R \rightleftharpoons T$. It was further proposed that in the non-energised particles used, the T state was favoured at equilibrium, and that antimycin A bound more firmly to the R state. Thus R, the antimycin A-sensitive state can be interpreted as an energized state, and Bonner & Slater (1970) have put forward evidence for this hypothesis, since they found that the curve describing the effect of antimycin A on the level of reduction of cytochrome b_{566} in isolated white potato mitochondria, was sigmoidal in shape in the de-energized state (i.e. in the presence of succinate and uncoupler), and more closely approximated to a straight line until saturation was reached, in the energized state (i.e. in the presence of succinate and ATP). On the allosteric model, the linear antimycin A titration curve for duroquinone as the electron donor to the respiratory chain, is explained in terms of the bypassing of ubiquinone by the reducing equivalents from this donor, preventing the formation of the low affinity T state, and therefore not requiring the involvement of ubiquinone as a means of lateral movement of hydrogen atoms between electron transport pathways.

Further possible evidence against ubiquinone existing as separate pool associated with NADH oxidase and succinate oxidase, as suggested by Gutman & Silman (1972), comes from experiments in which the ubiquinone has been extracted from lyophilized sub-mitochondrial particles prepared from beef heart mitochondria. Firstly, Ernster, Lee, Norling & Persson (1969a) found that there was a close relationship between the inactivation of the NADH and succinate oxidases, caused by the extraction of ubiquinone, and the amount of ubiquinone extracted, indicating that all the ubiquinone was necessary for maximal oxidase activities. Also it was found that after only one extraction

of the lyophilized particles by n-pentane, when the remaining amount of ubiquinone was still considerably greater than the amount of cytochromes, on a molar basis, the activities of NADH and succinate oxidases were considerably less than the controls. It was concluded that this finding was in line with the suggestion made by Kröger & Klingenberg (1967), that ubiquinone acts as a "homogeneous hydrogen-collecting pool", operating according to saturation kinetics rather than by a mole-to-mole stoichiometry. The activities of both NADH and succinate oxidases were found to be totally removed following extraction of the ubiquinone, and restored by re-incorporation of the ubiquinone, and so it was concluded that ubiquinone was essential for these activities.

Secondly, Ernster, Lee, Norling & Persson (1969b), reported that normal and lyophilized particles catalyzed a rotenone-sensitive oxidation of NADH by fumarate, indicating that reducing equivalents could flow from NADH dehydrogenase to succinate dehydrogenase. This reaction was completely absent from the ubiquinone depleted particles and was restored upon the re-incorporation of the ubiquinone, indicating that ubiquinone was essential for the functional connection between NADH and succinate dehydrogenases in these particles.

Finally, Norling, Glazek, Nelson & Ernster (1974), have carried out the quantitative incorporation of small amounts of ubiquinone into ubiquinone-depleted lyophilized beef heart sub-mitochondrial particles, and studied its effects on the NADH and succinate oxidase activities. These authors found that both NADH oxidase and succinate oxidase activities could be restored to 100% of that in lyophilized particles, by incorporation of 6-8 nmol ubiquinone per. mg. protein, and concluded that their data did not "support the belief that succinate oxidase and NADH oxidase communicate with separate pools of Q".

It is possible to argue that the lyophilization followed by extraction and re-incorporation of ubiquinone, might result in the destruction of kinetic pools of ubiquinone in sub-mitochondrial particles, Worling, Glazek, Nelson & Ernster (1974), found that 50 to 60% of the incorporated ubiquinone could be reduced by NADH or succinate, independently of the total concentration of ubiquinone in the membrane. Similar reduction values were obtained for lyophilized particles prior to extraction, suggesting that incorporated ubiquinone functioned similarly to that originally present in the particles. Until the detailed arrangement of the electron transport pathways is better understood in the region of ubiquinone, and the mechanism of inhibition of electron transport by antimycin A is better understood (see Slater, 1973), it is not possible to propose a more definite mechanism for the interaction between succinate and NADH oxidases in animal sub-mitochondrial particles, than that suggested by Gutman & Silman (1972).

In isolated rat liver mitochondria, Kimura & Singer (1959), found that choline oxidase and succinate oxidase activities could mutually inhibit each other, and Kimura, Singer & Lusty (1960), showed that the interaction required the activity of succinate dehydrogenase because the presence of malonate prevented it, and the activity of NADH dehydrogenase, because amytal also prevented it. The mechanism of interaction between choline oxidase and succinate oxidase also appeared to require electron transport, since diversion of reducing equivalents to phenazine methosulphate, resulted in removal of the interaction. Choline was oxidized at a much slower rate than succinate, and it was thought that the interaction was a result of the electron transport pathway being able to orient itself toward whichever flavo-protein was in the reduced state at a given moment. As a result of this, the electron transport pathway was thought to become unable to

accept electrons from other flavoproteins. This suggestion is consistent with activity of the electron transport pathways causing conformation changes in the inner membrane of the mitochondria, a suggestion that appears to be supported by the results obtained from mitochondrial swelling and contracting experiments (see Hanson & Hodges, 1967; and Palmer & Hall, 1972). Such conformation changes might result in changes occurring in the protein moieties of membrane-bound enzymes, which could affect their activities, for example inhibiting them.

The presence in plant mitochondria, of an exogenous NADH oxidase presents physiological problems to plant cells, that are not faced by animal cells. Particular attention has therefore been paid to interactions between the branched electron transport pathways of plant mitochondria, which involve mediation of electron flow from exogenous NADH, during the course of the present study.

Materials and Methods

1. Chemicals.

The NAD^+ , NADH , ALP , ATP , purified horse heart cytochrome c, chloramphenicol and all of the enzymes used in the present study were purchased from Boehringer Corporation (London) Ltd. Stock solutions of all of the reagents were prepared freshly in distilled water, except for chloramphenicol which was dissolved in 50% aqueous ethanol, before use. The pH value of the stock solutions of NAD^+ , AMP , ADP and ATP was adjusted to approximately 7.2 using aqueous potassium hydroxide. The enzymes were each dissolved in phosphate buffer (0.1M), at a pH value appropriate to the particular assay.

The TCA cycle intermediates and pyruvate were purchased from Sigma (London) Ltd. as the free acid where possible, and stock aqueous solutions were adjusted to a pH value of approximately 7.2, using 10 or 20% aqueous potassium hydroxide, as required. Bovine serum albumen (BSA fraction V), duroquinone, juglone and antimycin A, were also purchased from Sigma (London) Ltd. The BSA was allowed to dissolve in aqueous media by floating it off of the weighing paper onto the surface of the medium, and leaving it until it had passed into solution. Stock solutions of duroquinone, juglone and antimycin A, were prepared in absolute ethanol.

The yeast extract powder was purchased from Oxoid Ltd., London, U.K., and casein hydrolysate from BDH Biochemicals, Poole, U.K. Piericidin A and FCCP were generous gifts from Professor T.P. Singer and Dr. P.G. Heytler, respectively, and were prepared as ethanolic solutions. The mCLAM was synthesized and kindly provided by Mr. R.C. Powell, of the Agricultural Research Council Unit of Developmental Botany, Cambridge. All other reagents used in the present study, were purchased from British Drug Houses (BDH) Poole, U.K., or Hopkin & Williams.

2. Preparation of mitochondria.

i) Jerusalem artichoke mitochondria.

Jerusalem artichoke (Helianthus tuberosus) tubers were obtained from the Botanical Supply Unit, University of London, in the Autumn and stored in polyethylene film bags at a temperature of 5-9°C, until required. Mitochondria were isolated from the tubers using a method which was similar to that described by Palmer (1967). The media that were used for the initial isolation and for subsequent washing and final suspension of the mitochondria, were as follows:

Isolation medium

Sucrose	(0.5M)
MOPS	(10mM)
EDTA	(5 mM)

Immediately before use, 2mM sodium metabisulphite and 1% BSA were added to the isolation medium, and the pH adjusted to a value of 8.0, using 20 and 10% w/v aqueous potassium hydroxide.

Washing and suspending medium

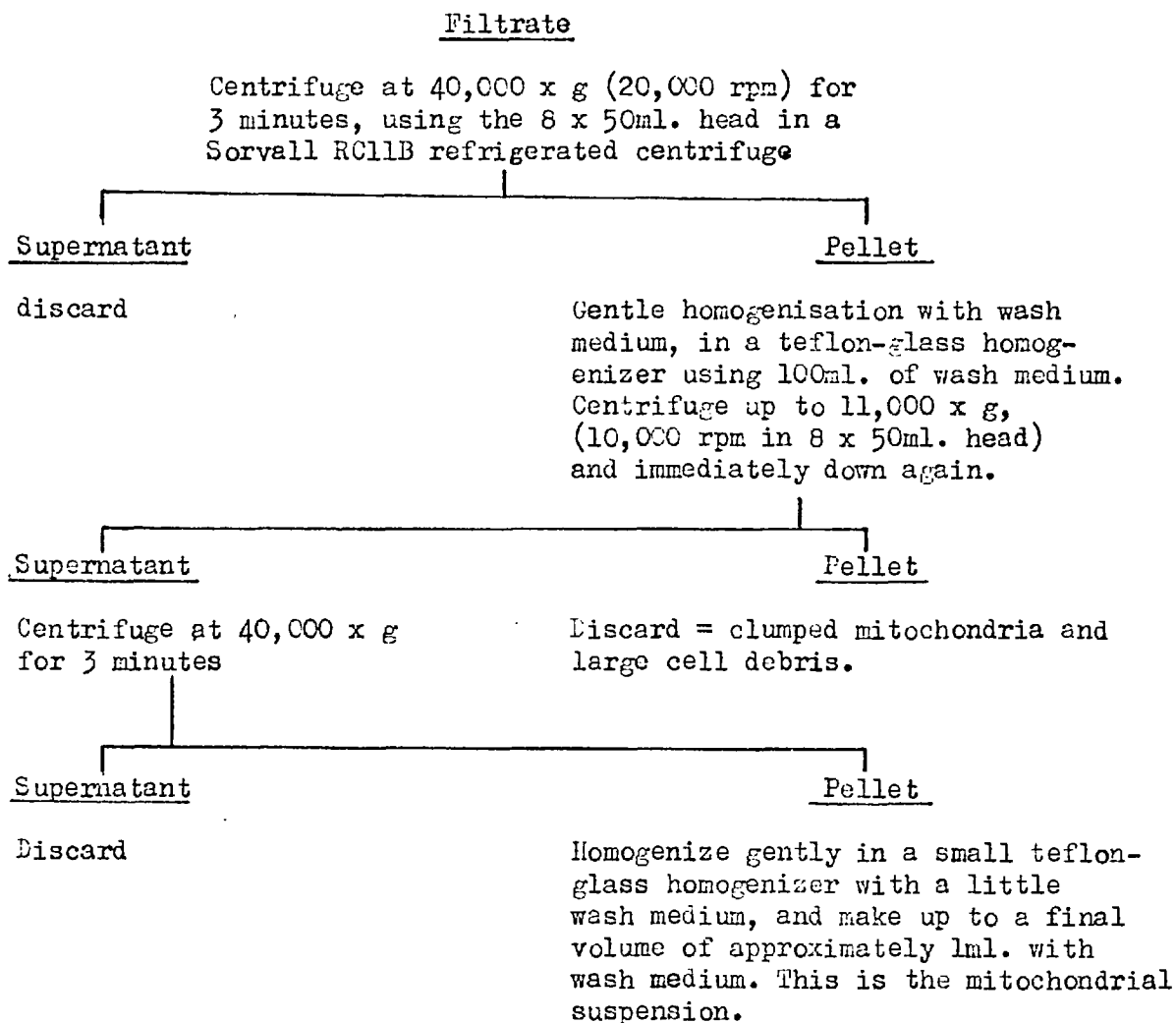
Sucrose	(0.4M)
TES	(5 mM)

Immediately before use, 0.1% BSA was added and the pH of the medium adjusted to a value of 7.2 using 10% w/v aqueous potassium hydroxide. All solutions were kept at 0-4°C.

Sodium metabisulphite was included in the initial isolation medium because it is a mild reducing agent in aqueous solution, and helps to protect the mitochondria from damage by phenolic compounds released during tissue disruption (Stokes, Anderson & Rowan, 1968).

BSA was included in the media to protect the mitochondria from the deleterious effects of free fatty acids released during the isolation procedure (see Schewe, Ludwig & Rapoport, 1974).

Using a stainless steel household vegetable grater with 2mm diameter holes, 250g of peeled and washed artichoke tubers were grated in 300ml. of isolation medium, at a temperature of 0-4°C. The ground tissue slurry was then filtered through two layers of fine mesh muslin, and the filtrate centrifuged as indicated below:



All solutions and apparatus used during the isolation were maintained at a temperature of 1-4°C, and a 250g batch of peeled and washed Jerusalem artichoke tuber tissue, yielded approximately 15-20 mg.

protein ml.⁻¹, as determined by the method of Lowry, Rosebrough, Farr & Randal (1951).

ii) White potato mitochondria.

White potato (Solanum tuberosum) tubers were purchased from local markets, and stored at 5-9°C until required. Mitochondria were isolated from these tubers, by a method similar to that described above for Jerusalem artichokes, except that an additional centrifugation of the filtrate was carried out in order to deposit the majority of the starch grains which are released upon disruption of the potato tissue. The centrifugation was identical to that used to deposit large cell debris in the flow diagram for isolation of Jerusalem artichoke mitochondria, just described i.e. up to 11,000 x g and down again. Once this initial step had been completed, then the resultant supernatant was treated as if it was the filtrate from the Jerusalem artichoke tubers according to the flow diagram presented above. The reason for the absence of the extra initial centrifugation step when isolating mitochondria from Jerusalem artichoke tubers compared with potato tubers, is that the former tissue contains inulin as its carbohydrate reserve and not starch as is the case with potatoes, and inulin is soluble in aqueous media, while starch is not. The yield of protein from white potato tubers was approximately 12-15 mg. from 250g of peeled and washed tubers.

iii) Arum maculatum spadix mitochondria.

Inflorescences of Arum maculatum were collected at stages just prior to the opening of the spathe (stages β - γ of James & Beevers, 1950), from wild colonies in North Surrey and North Kent. The sterile heads of the spadices were used in experiments after the removal of papillae and the superficial purple pigment, by wiping with cotton wool, moistened with distilled water.

The media used for the isolation, washing and final suspension of mitochondria from Arum spadices, were similar to those described above for Jerusalem artichoke and white potato, and differed from them only by the pH of the isolation medium being adjusted to a value of 7.4 for the Arum spadices, instead of 8.0, as in the other cases.

Washed spadices were cut with a razor blade in ice-cold isolation medium, contained in a precooled mortar of appropriate size for the quantity of spadices. A tissue : isolating medium ratio of up to 1 : 3 w/v was used. When all of the pieces of spadix were approximately 5mm cubes, a pre-cooled pestel was used to grind the tissue into a creamy homogenate. The homogenate was then filtered through two layers of fine muslin, and centrifuged as described above for the isolation of white potato mitochondria. The yield of protein was approximately 5 mg. from 10g of spadix tissue, and the mitochondria were finally suspended at a concentration of 20-30 mg. protein ml.⁻¹

iv) Mung bean mitochondria.

Mung bean (Phaseolus aureus) seeds were obtained from a local domestic supplier. Before sowing the seeds, they were surface sterilized with a 10% v/v aqueous solution of sodium hypochlorite, followed by eight rinses with distilled water. The seeds were then sown thinly over the surface of 2mm mesh nylon gauze, which was stretched over a circular wire frame made to fit the walls of a round plastic domestic bowl (30cm. diameter), at a position approximately two inches above its base. Tap water was then added to the bowl, to a level corresponding to the upper surface of the gauze, such that the lower part of each seed was submerged beneath the surface of the water, i.e. the lower 1mm only. The bowl with seeds and water was then placed in a dark incubator (Gallenkamp, Model IH-150), at a temperature of 27°C

for 3 to 4 days, to allow the seeds to germinate and the hypocotyls to elongate to a length of 10-15cm. At this stage, the etiolated hypocotyls were harvested using a sharp scissors, under conditions of low light intensity to minimize any light-induced reactions in the tissue. The conditions of low light intensity were achieved by placing venetian type blinds at any windows, and turning off any artificial sources of illumination.

Following the separation from the roots and cotyledons, the hypocotyls were washed in ice-cold distilled water, then transferred to a pre-cooled mortar of appropriate size, and finally chopped under isolation medium into sections up to 1cm. long, using sharp scissors. The isolation and washing media were similar to those described above for the isolation of mitochondria from Arum spadices, except for sodium metabisulphite, which was not included in the isolation medium for Mung bean mitochondria. The 1cm. lengths of hypocotyl were then disrupted by mild homogenisation with a pre-cooled pestel, until most of them were crushed, when the homogenate was filtered through two layers of fine mesh muslin. The resultant filtrate was then centrifuged as described for Jerusalem artichoke. Mung bean hypocotyls contain some starch which is precipitated during the first centrifugation, which can be left in the base of the centrifuge tube after removing the mitochondrial pellet from the upper surface of the starch, by careful pipetting with a Pasteur pipette. The yield of protein from Mung bean hypocotyls was between 7.5 and 10 mg. from 100g of hypocotyls, and the final mitochondrial pellet was suspended in wash/suspending medium at a protein concentration of 10-15 mg. ml.⁻¹

v) Neurospora crassa mitochondria.

Hyphae of wild type Neurospora crassa (Y74A), were cultured on 2% agar slopes in McCartney bottles, at a temperature of approximately 25°C and in daylight, until well conidiated whereupon they were transferred to a refrigerator for storage at a temperature of 0-4°C, until required. The agar slopes were prepared as follows, after the method described by Wainwright (1959).

Medium for culture slopes (Wainwright's conidiation).

To 2 litres of distilled water were added the following:

40g Potassium sodium tartrate
24g Sodium nitrate
24g Potassium dihydrogen phosphate
4g Magnesium sulphate (heptahydrate)
2.4g Potassium sulphate
0.8g Sodium chloride
0.8g Calcium chloride (anhydrous)
4ug Biotin
160g Sucrose
0.8ml. Vogel's trace element solution, see below for details.

500ml. of Wainwright's conidiation medium was added to 40g agar and 1.5 litres of distilled water, contained in a large pyrex beaker. The mixture was heated with stirring, until all of the agar had dissolved, when 10ml. aliquots were poured into clean McCartney bottles. The lids of these bottles were loosely screwed down, and the bottles plus culture medium, were then sterilized in an autoclave set to a temperature of 120°C for 20 minutes (15p.s.i.). Upon removal from the autoclave, the bottles were rested at an angle of approximately 30° to permit slopes to form as the agar set upon cooling. Once cooled, the lids

were screwed down tightly, and the slopes stored until required. Slopes were inoculated with conidia taken from stock cultures kept at 0-4°C until required, using sterile technique in an inoculation room. Once inoculated, the agar slopes were incubated in daylight at a temperature of approximately 25°C. The presence of daylight induced the synthesis of an orange pigment in the conidia, which permitted visual estimation of the 'conidial harvest'. The conidia from these cultures were used to inoculate liquid culture medium, used for the production of a hyphal mat, from which the mitochondria were isolated. The liquid culture medium will now be described.

Medium for the liquid culture of *N.crassa* hyphae.

This medium was based upon that described by Vogel (1956). A stock solution of Vogel's 'N' Medium was prepared by adding in sequence to 750ml. of distilled water, the following:

- 130g Sodium citrate
- 250g Potassium dihydrogen phosphate
- 100g Ammonium nitrate
- 10g Magnesium sulphate (heptahydrate)
- 5g Calcium chloride (dihydrate)
- 5ml. Vogel's trace element solution, see below for details.
- 2.5ml. Biotin (5mg. per 50ml. of distilled water)

This stock solution was stored in a deep freeze at a temperature of -15 to -20°C until required, alternatively, it is understood that a drop of chloroform added to the solution acts as a preservative of it. The stock solution was diluted 1:50 with distilled water, and to every 500ml. of the diluted solution, was added 2.5g of yeast extract powder and 2.5g of casein hydrolysate powder. The pH of

the resultant solution was then adjusted to a value of 5.8 if necessary, using aqueous potassium hydroxide or hydrochloric acid. Two litre Erlenmeyer flasks were used to culture the hyphae, and into each was poured 500ml. of the diluted medium described above. The mouths of the flasks were then covered with a 1cm. thick layer of non-absorbent cotton wool, sandwiched between two layers of muslin. A strong rubber band held this cap on, and a generous cap, made out of aluminium foil, was then placed over the muslin to prevent excessive wetting during sterilization, and to help to maintain the medium in a sterile condition during storage. The medium in the two litre flasks was then sterilized by autoclave at 120°C for 20 minutes (15p.s.i.), and the sterilized flasks were stored in a laboratory cupboard, until required.

The constituents of Vogel's trace element solution will now be given. To 95ml. of distilled water, the following were added in sequence:

- 5g Citric acid
- 5g Zinc sulphate (heptahydrate)
- 1g Ferrous ammonium sulphate (hexahydrate)
- 0.25g Cupric sulphate (pentahydrate)
- 0.05g Manganese sulphate
- 0.05g Borax (H_2BO_3 , anhydrous)
- 0.05g Sodium molybdate (dihydrate)
- 1ml. Chloroform, as preservative if stored at room temperature.

Inoculation of the liquid culture medium.

For the inoculation of the liquid culture medium with conidia of N.crassa, 10ml. of sterile water and 10ml. of a solution of sucrose containing 1g sucrose per ml., and also sterile, were required

for each Erlenmeyer flask. These were sterilized separately in cotton wool stoppered test tubes, with metal caps, purchased from Oxoid Ltd., London U.K., as described above for the liquid culture medium. The sterilized water and sucrose solution, were stored in a laboratory cupboard, until required.

All additions made to the liquid culture flasks, were carried out in an inoculation room with the minimum of air movement, to prevent cross-contamination of other cultures by N.crassa conidia. The cotton wool and muslin covers were kept over the mouths of the culture flasks at all times, with additions to the flasks being made by lifting one side of the cover only, to minimize any contamination from the air immediately above each flask. Using sterile technique, the orange coloured conidia were transferred from the McCartney bottles to the Erlenmeyer flasks containing the sterilized culture medium, by suspending them in 10ml. of sterile water, and pouring the suspension from one bottle, into each flask. The contents of one of the sucrose test tubes sterilized as described above, were then poured into each culture flasks, and the caps replaced, before placing the flasks on an orbital shaker within a Gallenkamp incubator. The shaker was set to 120 rpm, and the temperature of incubation was approximately 30°C for a period of 21-24 hours.

In some experiments, chloramphenicol was added to the liquid culture flasks before placing them on the orbital shaker. When this was done, the chloramphenicol was first dissolved in 50% aqueous ethanol (10ml.), and then transferred to the liquid culture flask containing the culture liquid, conidial suspension and sucrose.

Harvesting the hyphae and isolation of the mitochondria.

After the prescribed period of incubation, the flasks were removed from the shaker and their contents were filtered through two layers of fine mesh muslin, followed by squeezing to remove most of the liquid medium. This procedure resulted in the production of a wad of damp hyphae, which was then weighed, and the weight recorded. The filtrate was then returned to the flask, which was then covered ready for sterilization. The wad of hyphae was cut with scissors into pieces approximately 5mm. cubes, and washed three times with ice cold distilled water. The hyphae were then separated from the water by a second filtration through two layers of muslin, and pressed into a wad again. This hyphal wad was then cut with scissors into approximately 5mm. cubes and into a beaker which contained mitochondrial isolation medium, at a tissue:isolating medium ratio of approximately 1:10 w/v. The isolation medium used was that described by Weiss, von Jagow, Klingenberg & Bucher (1970), and it's constituents are given below. This medium was also used for washing the mitochondria, and for the suspension of the final mitochondrial pellet.

Isolation, wash and suspending medium.

Sucrose	(0.44M)
Tris-acetate	(10 mM), pH 7.3
EDTA	(2 mM)
BSA	(0.2%, w/v)

All solutions and apparatus used for isolation of the mitochondria, were maintained at a temperature between 0-4°C, by an ice bath.

Mitochondria were released from the hyphae by hand homogenisation, using a ground glass homogenizer with a 3cm. diameter, and made by Wiklund, Stockholm. Five firm passes of the ground glass

male component of the homogenizer, with a little twisting, was found to be sufficient to release the mitochondria from the hyphae. The resultant homogenate was filtered by squeezing through two layers of muslin, and this filtrate was then differentially centrifuged, as described above for the isolation of white potato tuber mitochondria. The yield of hyphae for wild type N.crassa (Y74A), was approximately 10g of press-dried hyphae per culture flask, and each gramme of hyphae yielded approximately 2 mg. of mitochondrial protein. When chloramphenicol, at a concentration of 3g per litre, was present in the culture medium, the yield of hyphae was approximately 3g per flask which in turn yielded approximately 2 mg. of mitochondrial protein per gramme of hyphae.

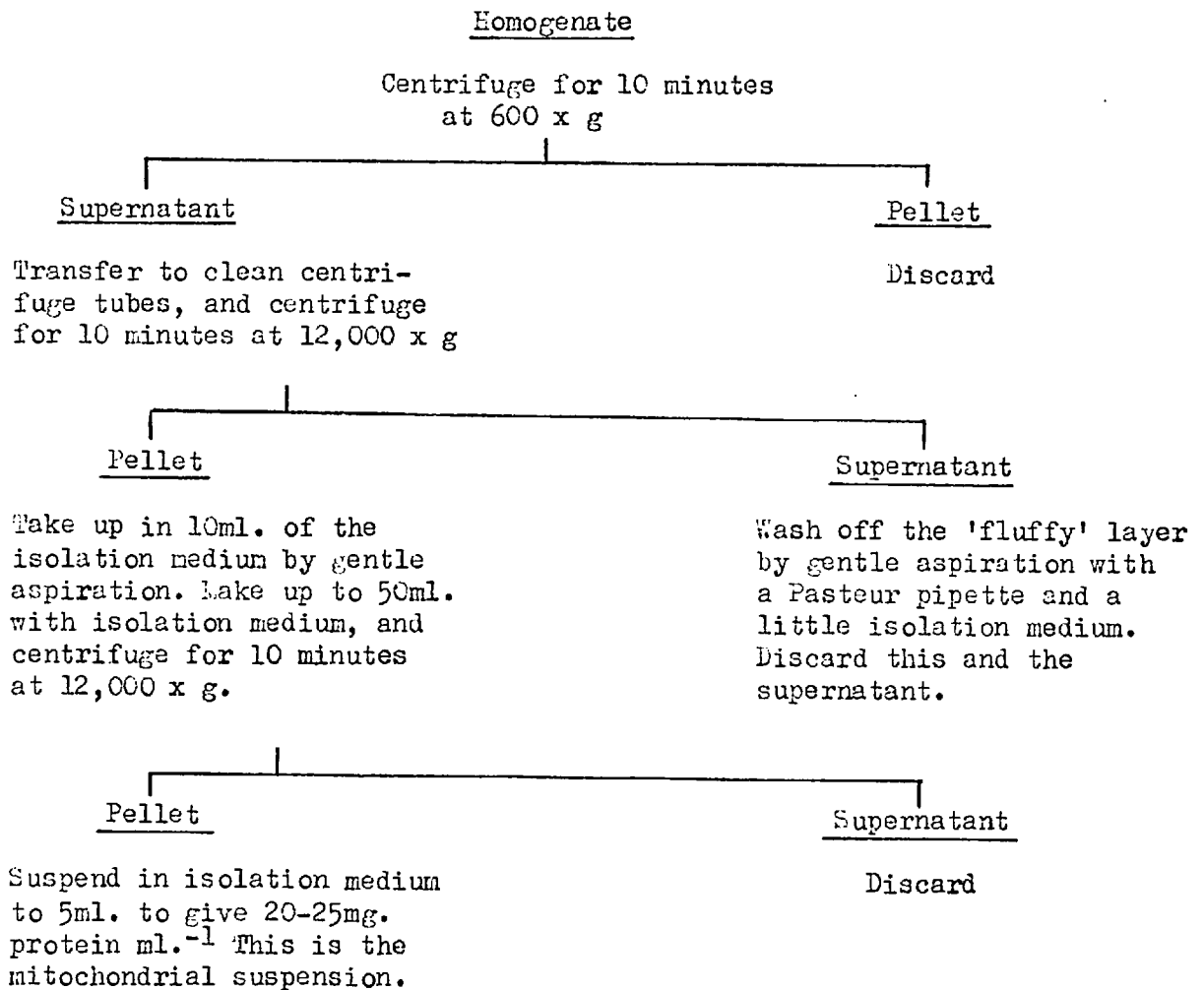
vi) Rat liver mitochondria.

Mitochondria were isolated from the liver of Sprague-Dawley adult male rats (200-250g), which had been killed by a sharp blow to their cervical region. The liver was rapidly excised and transferred to a beaker containing 100ml. of isolation medium held at a temperature of 0-4°C on ice. The liver was washed in this medium and then it was transferred to a second beaker which contained 50ml. of ice cold isolation medium, into which the liver was cut using a sharp scissors, to result in thin strips of tissue suspended in the liquid medium. This mixture was then transferred, in suitable portions, to a glass homogenizer having an internal diameter of 2.5cm., and purchased from Wesley-Coe (Cambridge) Limited. The same medium was used for the initial isolation, washing and final suspension of the mitochondria, and its constituents are given below.

Isolation, wash and suspending medium.

Sucrose	(0.25M)
EDTA	(0.1mM)
Tris-HCl	(10mM), pH 7.6
BSA	0.1% w/v.

The slices of liver were homogenized by hand, using 6 twisting passes of the male component of the glass homogenizer, and the resultant homogenate was then transferred to centrifuge tubes held on ice. The mitochondria were isolated from the homogenate by differential centrifugation, in a refrigerated centrifuge, as outlined in the flow diagram presented below, and carried out with all solutions being kept at 0-4°C.



3. Purification of isolated plant mitochondria.

Isolated and washed plant mitochondria were purified by passage through a discontinuous sucrose density gradient, under the influence of a centrifugal force, as described by Douce, Christensen & Bonner (1972). The discontinuous sucrose density gradients were prepared by layering fixed volumes of aqueous sucrose solutions, which contained 0.1% BSA (w/v), and 10mM orthophosphate buffer, pH 7.2, into 3 inch x 1 inch cellulose nitrate centrifuge tubes (Beckman (California), Type No. 302236). The sequence in which the sucrose solutions were added to the tubes was as follows:

1.8M sucrose (6ml.), 1.45M sucrose (6ml.), 1.2M sucrose (6ml.),
0.9M sucrose (3ml.), and 0.6M sucrose (3ml.).

Washed plant mitochondria (20-40mg. protein), were layered onto the top of each sucrose density gradient, which was kept at a temperature of 1-4°C on ice, and the whole placed in the swinging bucket rotor of a Beckman Model L preparative centrifuge (SW 25:1 rotor). The gradients were centrifuged at 20,000 rpm. (40,692 x g), and at a temperature of 1-4°C, for 45 minutes, and this resulted in the purified mitochondria moving to the boundary between the two layers of sucrose solution which corresponded to the concentrations 1.2 and 1.45M sucrose, respectively.

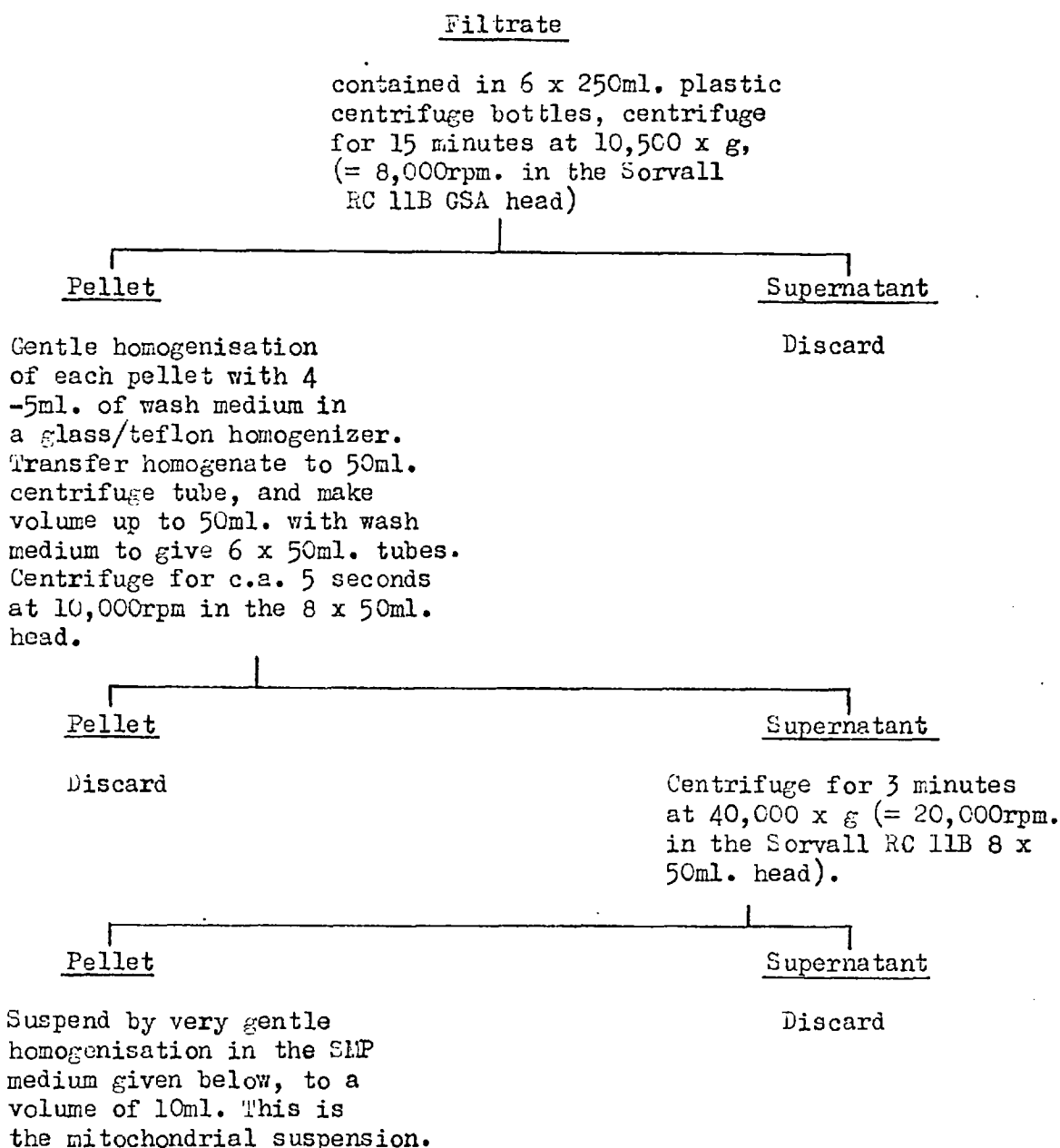
Removal of the purified mitochondria from the sucrose density gradient was achieved by injecting a 2.0M sucrose solution, prepared in 10mM orthophosphate buffer, into the base of the centrifuge tube, and collecting the mitochondrial fraction as it was pushed out of the top of the tube. The purified mitochondria were now contained in a 1.35M sucrose solution, and a process of slow dilution to a sucrose concentration of 0.3M was carried out using 10mM orthophosphate buffer pH 7.2 at 1-4°C which also contained 0.1% BSA. The time taken to complete

the dilution of the mitochondria, was approximately 20 minutes, and a peristaltic pump (H.R.Flow Inducer, Type HHE 22, made by Watson & Marlow Ltd., Falmouth, Cornwall, U.K.), was used to deliver the dilutant to the vessel in which the purified mitochondria were contained, and at the desired rate. All of the apparatus for the dilution procedure was arranged in a cold-room at a temperature of 5-9°C, and the diluted mitochondria were transferred to 10ml. capacity centrifuge tubes, and were then centrifuged at 9,000 x g for 15 minutes, and at 1-4°C, in a Sorvall RC 11B refrigerated centrifuge, using the 8 x 50ml. capacity head fitted with a 10ml. tube adapter for each tube. The resultant pellets of purified mitochondria, were suspended in a small volume of 0.3M sucrose solution at 1-4°C, which also contained 10mM orthophosphate at pH 7.2 and 0.1% BSA, the final volume of the mitochondrial suspension being in the region of 0.5ml. The final mitochondrial suspension contained up to 100mg. of protein per ml.

4. Preparation of sub-mitochondrial particles.

Sub-mitochondrial particles (SMP), were prepared from isolated Jerusalem artichoke mitochondria by subjecting them to ultrasonic oscillations, using a method similar to that described for the preparation of SMP from isolated mammalian mitochondria, by Low & Vallin (1963). Firstly, washed mitochondria were prepared from 2Kg. of washed and peeled Jerusalem artichoke tubers, using the same isolation and wash media, as described for the preparation of mitochondria from a 250g batch of tubers, (see above section 2.i.). The tissue was processed in two equal portions of 1Kg. each, which was cut into 1cm. cubes using a sharp knife, which were then suspended in 1 litre of isolation medium held at 1-4°C on ice. The tissue was then ground using a Moulinex Mixer 66 (Alenson, France), for 30-40 seconds, and the resultant slurry was then filtered through two layers of fine mesh muslin, into a beaker

which was standing in an ice bath. The filtrate was centrifuged, in order to precipitate the mitochondria, as outlined in the flow diagram outlined below:



The medium used for the final suspension of the mitochondria, was as follows:

Sucrose	(0.4M)
TES	(5 mM), pH 7.2
MgCl ₂	(10mM)
ATP	(5 mM)

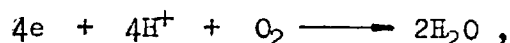
The SMP were prepared by subjecting the mitochondria to ultra-sonic oscillations generated by the probe of an ultra-sonicator (Soniprobe, Type 75 30A, made by Dawe Instruments Ltd., U.K.). The probe was positioned such that its tip was approximately $\frac{1}{4}$ inch above the base of the special sonication tube containing the mitochondrial suspension, of 10ml. at least. The sonication tube was embedded in ice and the tip of the sonicator probe was pre-cooled in ice, before immersing it into the mitochondrial suspension. Sonication was carried out for four periods of 10 seconds each, at a setting of 5 and tuned to between 4 and 5 amps. The use of 10 second periods of sonication, with a pause of approximately 30 seconds between each period, helped to prevent excessive rises in temperature of the suspension being sonicated. Following sonication, the resultant sonicate was centrifuged at 40,000 x g (20,000 rpm. in the 8x50ml. head of the Sorvall RC 11B centrifuge), for 10 minutes, in 10ml. capacity centrifuge tubes, using the 10ml. tube adapters. The centrifuge head was maintained at 1-4°C. This centrifugation precipitated larger membrane fragments, leaving the SMP in the supernatant, which was then subjected to centrifugation for 1 hour at 100,000 x g (40,000 rpm. using the Type 50 head in a Beckman Model L, preparative centrifuge), and at temperature setting 22, which resulted in the SMP suspending medium maintaining a temperature of approximately 4°C. Following this high-speed centrifugation, the supernatant was discarded, and the reddish translucent pellet was suspended in normal

mitochondrial suspending medium, to a volume of 0.5-1.0 ml., to form the SLP suspension, which was called a suspension of 'ATP/Mg⁺⁺ particles', in order to distinguish it from other types of SLP which can be made, see Beechey & Cattell (1973).

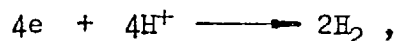
5. Polarographic assay of oxygen consumption.

i) General principle of operation of the oxygen electrode.

The oxygen electrode is a device which is used to measure the concentration of oxygen in a solution in contact with it. It consists of a platinum cathode (-), which is separated from a silver anode (+), by a saturated solution of aqueous potassium chloride. When a potentiating voltage of 600-700 mv. is applied, a small current will flow. The size of this current is proportional to the oxygen concentration in the vicinity of the platinum cathode. The reasons for this are not clear, but it may be that, under these conditions the reaction,



proceeds more readily than the reaction,



which normally occurs at the cathode during the electrolysis of water. When all of the oxygen is reduced, no further current flows.

If a solution in which oxygen is dissolved, is separated from the platinum cathode by a membrane which is permeable to oxygen, then the rate of diffusion of oxygen to the cathode, will control the current flowing. The current will therefore be directly proportional to the concentration of oxygen which is dissolved in the solution. A suitable membrane can be made from a thin sheet of teflon or polyethylene. The current generated is very small and for convenience, it is measured as the voltage across a variable resistor. This is amplified

and can be recorded continuously on a chart recorder, which was the method used in the present study. Clark, Wolf, Granger & Taylor (1953), have described the practicability of a membrane-covered oxygen electrode.

In the present study, two makes of oxygen electrodes were used, a Clark electrode (Yellow Springs Instrument Co., Ohio, U.S.A.), connected to an Oxygraph recorder (Gilson Medical Electronics, Wisconsin, U.S.A.), and a specially made Rank electrode, with a $\frac{3}{8}$ inch internal diameter reaction chamber (Rank Bros., Bottisham, Cambridge, U.K.), which was connected to a Servoscribe potentiometric recorder (Type RE 520.20). Both of the electrodes employed a membrane between the surface of cathode and anode, and the reaction medium, of which the oxygen concentration was to be determined. This prevented contamination of the electrode surfaces by reagents added to the reaction chamber of the electrode, which in turn might affect its response to changes in the concentration of oxygen. This was the modification employed by Clark, Wolf, Granger & Taylor (1953). In the experiments described here, a teflon membrane was used to cover the electrode surface, which was held in place by a rubber O-ring. Compared with an uncovered electrode vibrating within the reaction mixture, the presence of the membrane increased the response time of the electrode to changes in the concentration of oxygen. However, avoidance of contamination by reagents used in many of the experiments which are described here, made the membrane essential. The use of a small magnetic stirrer (Cole-Parmer Co. I 11, USA., Micro-V stirrer), achieved a rapid mixing of reactants, and decreased the electrode response time.

The reaction chamber, or cuvette, of both electrodes used in the present study, had a 1ml. capacity, although that of the Rank electrode was continuously variable, and could be used with a reaction volume of 0.5ml. Each reaction cuvette was surrounded by a water jacket, which contained water at a temperature of 25°C, which was heated and

circulated by the same thermostatic heater and pump (Julabo Paratherm, Scienco Western, Wiltshire, U.K.). Tightly fitting stoppers, with an unstirred capillary through which reactants were introduced from hypodermic syringes, prevented oxygen from dissolving into the reaction medium, from the air.

In experiments where very low concentrations of respiratory substrates were used, it was necessary to increase the sensitivity of the oxygen electrode, such that full-scale deflection on the chart recorder corresponded to a change in concentration of oxygen from 100% air-saturated, to a value which was greater than the 0% air-saturated level, used normally. This was achieved by alternate adjustment of the zero control of the chart recorder and the sensitivity control of the electrode voltage supply, and the degree of increase of the sensitivity of the electrode was determined by the magnitude of the deflection of the recorder pen following the addition of a given aliquot of exogenous NADH to isolated plant mitochondria suspended in the reaction cuvette of the electrode, before and after increasing its sensitivity. This allowed the calculation of rates of oxygen consumption by mitochondria, in units of nmoles oxygen per unit time.

ii) Mitochondrial assay media.

The standard assay medium used to measure reactions catalyzed by isolated mitochondria, including the rate of oxygen consumption in the presence of respiratory substrates, during the present study were as outlined below.

a) Assay medium for mitochondria isolated from Jerusalem artichoke and white potato tubers, Mung bean hypocotyls and *Arum maculatum* spadices.

Sucrose	(0.4M)
KH_2PO_4	(5 mM)
TES	(5 mM), pH 7.2
MgCl_2	(2.5mM)

b) Assay medium for *Neurospora crassa* mitochondria.

Sucrose	(0.44M)
Tris-acetate	(10 mM), pH 7.3
KH_2PO_4	(10 mM)
MgSO_4	(10 mM)
EDTA	(2 mM)
BSA	0.2% w/v.

c) Assay medium for rat liver mitochondria.

Sucrose	(25mM)
KCl	(100mM)
MgCl_2	(10 mM)
EDTA	(1 mM)
TES	(5 mM), pH 7.2
KH_2PO_4	(10 mM).

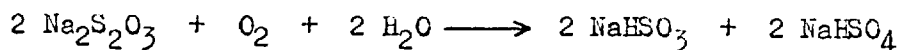
iii) Calibration of the oxygen electrode.

The oxygen electrode was calibrated using two different methods. The first method involved the evolution of oxygen from hydrogen peroxide, catalyzed by the enzyme, catalase, and the second method involved the consumption of oxygen during the oxidation of exogenous NADH, catalyzed by isolated plant mitochondria.

a) The hydrogen peroxide technique.

Calibration of the oxygen electrode by the hydrogen peroxide technique, followed the method described by Dixon & Kleppe (1965). A fixed volume of distilled water (1ml.), was added to the reaction cuvette of the oxygen electrode, and the initial reading of air saturated distilled water, at a temperature of 25°C, was set at 90% of full scale deflection on the chart recorder, making necessary adjustments using the sensitivity control. At standard barometric pressure, this reading corresponded to a concentration of oxygen of 257 nmoles per ml. of distilled water (oxygen standard after Hodgman 1957-8).

The zero current reading was checked by the addition to the water contained in the reaction cuvette, of a crystal of sodium dithionite. This rapidly brought about anaerobiosis upon interaction with the dissolved oxygen, by a reaction which can be expressed as,



The solubility of oxygen in the assay media used in the present study, was then calibrated by comparing the magnitude of the deflection of the chart recorder pen, following the addition of a given amount of hydrogen peroxide to 1ml. of distilled water, containing excess catalase (20ul. catalase, at 260,000 U per ml. 25°C), and contained

in the reaction cuvette of the oxygen electrode. To measure the evolution of oxygen from the hydrogen peroxide, the oxygen electrode was first calibrated as described above, so that full scale deflection of the chart recorder pen corresponded to an oxygen concentration range of from 0 to 100% air saturated distilled water in the presence of the catalase. The electrode sensitivity was then decreased by 45%, which resulted in the pen moving from 90 to 50, on the chart recorder for 100% air saturated oxygen. Then a small volume of an aqueous solution of hydrogen peroxide (20ul. of a 1:2000 v/v dilution of 100 volume H_2O_2), was added to the oxygen electrode via the unstirred capillary in the stopper, using a 25ul. micro-syringe, and the upward deflection of the recorder pen recorded. This procedure was carried out for distilled water and assay medium, and the numerical values obtained for the magnitude of deflection of the pen with each, were entered into the following equation:

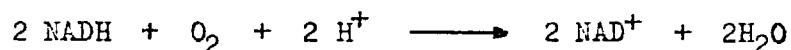
$$257 \times \frac{x}{y} = S$$

where 257 = the solubility of oxygen in distilled water at 25°C and normal barometric pressure (760mm. mercury), x = the magnitude of the pen deflection for the distilled water, and y = the magnitude of the pen deflection for the assay medium, and S = the solubility of oxygen in the assay medium. The numerical value of S was found to be 240uM oxygen, for the standard assay media used here.

b) The exogenous NADH oxidation technique.

Calibration of the oxygen electrode by this technique, was carried out by a method which was similar to that described by Chappell (1964), and later used by Brunton (1975), and it involved the measurement of the magnitude of the deflection of the recorder pen resulting from the oxidation of a known amount of exogenous NADH and catalyzed by isolated Jerusalem artichoke mitochondria, suspended in standard assay medium contained in the reaction cuvette of the oxygen

electrode, at 25°C. The full scale deflection was then calibrated in units of oxygen, using a value of 2.0, for the molar ratio between NADH and oxygen according to the following equation for the oxidation of exogenous NADH by oxygen, catalyzed by the enzyme, exogenous NADH oxidase in isolated and intact Jerusalem artichoke mitochondria:



This method also gave a value of approximately 240uM, for the concentration of oxygen in air saturated assay medium, and so this value was used throughout the present study. This value was the same as that used by other workers (e.g. Chance, 1952; Chance & Williams, 1955a,b; Chappell, 1964; Ikuma & Bonner, 1967a; Douce, Christensen & Bonner, 1972; Brunton & Palmer, 1973).

6. Absorbance and fluorescence spectroscopy.

The spectroscopy used in the present study was confined to wavelengths within the ultra-violet and visible parts of the spectrum of electromagnetic radiation. The spectrophotometer used for absorbance measurements was an Aminco DW2 dual-wavelength model (American Instrument Company, Silver Spring, Maryland 20910, U.S.A.), with wavelength alignment and calibration carried out using holmium and didymium filters and horse-heart cytochrome c in aqueous solution. The spectrophotometer used for fluorescence measurements was a Perkin-Elmer (Model MPF-3).

i) Measurement of the kinetics of reduction of mitochondrial endogenous pyridine nucleotide.

The redox state of endogenous pyridine nucleotide in isolated mitochondria was determined by observations made with the dual-wavelength absorbance spectrophotometer (Aminco DW2), using a method similar to that described by Haslam & Krebs (1968) and later used by Brunton & Palmer (1973) and Brunton (1975), to follow the kinetics of reduction of pyridine nucleotide in isolated wheat mitochondria.

The wavelength pair used was 340 nm. measuring and 374 nm. reference. The band-pass was 1.0 nm., and the pen carriage velocity was set to 100 seconds inch⁻¹. 2.5 ml. of assay medium were pipetted into a 3 ml. capacity silica cuvette, with a 1 cm. light path length. Mitochondria were added to the cuvette (5-6 mg. protein), and the whole contents of the cuvette was then mixed. The cuvette was then placed in the cuvette carriage of the spectrophotometer, and the two light beams balanced on the cuvette, with the zero set near the lower margin of the chart recorder paper. The appropriate substrate was then added to the cuvette with mixing, the pen set in motion, and the changes in absorbance recorded until no further change occurred.

ii) Measurement of the kinetics of oxidation and reduction of endogenous mitochondrial flavoproteins.

The technique used to measure changes in the level of reduction of light absorbing flavoproteins, in isolated plant mitochondria, was similar to the method described above for endogenous pyridine nucleotide. The wavelength pair used was 468 nm. measuring and 493 nm. reference, as used by Storey (1970a). A decrease in absorbance at 468 nm. relative to that at 493 nm. corresponded to an increase in the degree of reduction of the light absorbing flavoproteins.

iii) Measurement of the kinetics of oxidation and reduction of endogenous mitochondrial cytochromes b and c.

The technique used to measure changes in the level of reduction of cytochromes b and c, in isolated plant mitochondria, was similar to those described above for pyridine nucleotide and flavoproteins, but using different wavelength pairs.

a) Cytochromes b.

The electron transport pathways of plant mitochondria, contain at least three species of cytochromes b, which possess absorbance maxima for their α bands corresponding approximately to the wavelengths of light 557, 560 and 566 nm. respectively, at a temperature of 25°C (see Palmer, 1976). In the present study measuring wavelengths of 560 nm. and 566 nm. were used, with 575 nm. as the reference wavelength, which corresponds to a wavelength at which no change in absorbance occurs upon the change from oxidized to reduced, in these cytochromes, i.e. an 'isosbestic point', as used by Lance & Bonner (1968). Increases in absorbance at 560 nm. or 566 nm. relative to 575 nm. corresponded to increases in reduction of mainly cytochromes b_{560} and b_{566} respectively, and vice versa for oxidation.

b) Cytochromes c.

Although the electron transport pathways of plant mitochondria appear to contain two species of cytochrome c, their absorbance spectra overlap so much, that only a single absorbance maximum can be seen in difference spectra, which is centred at 550 nm. This absorbance maximum is due to absorbance by the α bands of cytochromes c, at 25°C. The wavelengths used to measure the kinetics of changes in reduction of cytochromes c in isolated plant mitochondria, were those used by Lance & Bonner (1968), and were 550 nm. measuring, and 540 nm. reference.

iv) Measurement of the kinetics of oxidation and reduction of endogenous mitochondrial ubiquinone.

The kinetics of oxidation and reduction of endogenous mitochondrial ubiquinone was similar to that used by Storey & Bahr (1969a), and employed a dual-wavelength technique using the wavelength pair 282-295 nm. The deuterium lamp was used in the Aminco DW2 spectrophotometer, and silica cuvettes with a light path of length 2mm. were used to minimize interference from turbidity of the mitochondrial suspension, and so enhance the sensitivity of the technique (see Szarkowska & Klingenberg, 1963).

Jerusalem artichoke mitochondria were preincubated with ATP/Pi/MgCl₂ to activate succinate dehydrogenase (see Results figure 9), and suspended in standard assay medium to a final volume of 0.5 ml. in the silica cuvette, and absorbance changes in response to additions to the cuvette were measured at 282-295 nm. Decreases in absorbance corresponded to increases in the degree of reduction of ubiquinone, and vice versa for increases in absorbance at 282-295 nm.

v) Measurement of the kinetics of oxidation of exogenous NADH.

Dual-wavelength or split-beam modes of operation of the Aminco DW2 spectrophotometer can be used to measure rates of oxidation of exogenous NADH catalyzed by isolated plant mitochondria, or sub-mitochondrial particles made from them.

a) Dual-wavelength technique.

Measuring and reference wavelengths used were 340 nm. and 374 nm. respectively, with a band-pass of 3 nm., full-scale deflection was set to 0.2 O.D. units, and the pen carriage velocity was set to 50 seconds inch⁻¹. Three ml. of the appropriate assay medium was pipetted into a 1 cm. light path silica spectrophotometer cuvette, and the light beams balanced on it, with the pen set to zero near the lower margin of the recorder chart. Approximately 100μM NADH was added to the cuvette, and stirred in, followed by setting the pen to the upper margin of the recorder chart, with the baseline set control. The reaction was triggered by adding the appropriate quantity of mitochondria or sub-mitochondrial particles, (c.a. 0.25 mg. protein). Additions could be made during the experiment, and an 'extinction' coefficient of 3.73 mM cm.⁻¹ was used to convert changes in absorbance at 340-374 nm. into units of NADH, as used by Haslam & Krebs (1968).

b) Split-beam technique.

The Aminco DW2 spectrophotometer was set to the split-beam mode, 340 nm. wavelength and a band pass of 3 nm.. The pen carriage velocity was set to 50 seconds inch⁻¹ and full-scale deflection was set to 0.5 O.D. units for 100μM NADH as the initial concentration in the reaction cuvette. Assay medium and mitochondria (c.a. 1 mg. protein) to a total volume of 6 ml. were mixed thoroughly in a clean 25 ml. beaker, followed by pipetting of 3 ml. of the

mixture into a 1 cm. light path silica spectrophotometer cuvette, the sample cuvette, and pouring of the remaining volume of the mixture into a similar cuvette, the reference cuvette.

The sample and reference cuvettes were placed in the spectrophotometer, and the pen was set to zero near the lower margin of the recorder chart, with the split light beam balanced on the two cuvettes. NADH (c.a. 100uM) was then added to the sample cuvette by microsyringe, and stirred in, followed by rapid re-setting of the pen to the upper margin of the recorder chart, and setting in motion of the pen carriage. Additions to the sample cuvette were made as indicated, and stirred in using a plastic stirrer.

A millimolar extinction coefficient of 6.22 cm.^{-1} was used to convert absorbance at 340 nm. into a concentration of NADH (Horecker & Kornberg, 1948). The following formula was used to convert absorbance at 340 nm. into units of nmoles NADH oxidized $\text{min.}^{-1} \text{ mg. protein}^{-1}$:

$$\frac{\Delta \text{ O.D.}_{340} \text{ min.}^{-1}}{6.22} \times \frac{V}{m} = \text{nmoles NADH min.}^{-1} \text{ mg. protein}^{-1},$$

where V = the reaction volume in uL, m = the quantity of mitochondrial protein used in the assay, in mg., and as estimated by the method outlined in Materials & Methods 8, and 6.22 = the millimolar extinction coefficient for NADH at 340 nm. cm.^{-1} .

vi) Absorbance spectra.

Reduced minus oxidized absorbance difference spectra of the α bands of cytochromes b and c, in isolated plant mitochondria, were determined at room temperature (c.a. 25°C) and at low temperature (c.a. 77K), using the Aminco DW2 spectrophotometer operating in the split beam mode.

a) Room temperature spectra.

Mitochondria (3-4 mg. protein) were thoroughly mixed with assay medium to a volume of 6 ml., in a clean 25 ml. beaker at room temperature (c.a. 25°C), and 3 ml. of the mixture pipetted into the sample cuvette, and the remainder poured into the reference cuvette. The cuvettes used were 1 cm. light path glass cuvettes of 3 ml. capacity, and a matched pair (Aminco visible). Both cuvettes were wiped with a tissue and placed in the cuvette carriage of the spectrophotometer. The beam was then balanced at a wavelength of 540 nm., and the pen set to zero near the centre of the y axis of the chart recorder paper, with a full-scale deflection setting of 0.01 O.D. units, and a band pass of 1.0 nm. The appropriate wavelength range was then scanned, and adjustments made until a baseline was as close to a horizontal line as possible. This represented the oxidized-minus-oxidized absorbance spectrum of the cytochromes b and c. Once a horizontal baseline had been achieved, the full-scale deflection was reset to 0.02 O.D. units, and the appropriate reducing agent was added to the sample cuvette with stirring, and the reduced-minus-oxidized absorbance difference spectrum drawn

b) Low temperature spectra.

For the measurement of reduced-minus-oxidized absorbance difference spectra at 77K, the low temperature attachment was placed

in position on the Aminco DW2 spectrophotometer. The low temperature cuvettes were of capacity 1 ml., had plastic windows and a light path length equal to 2 mm.

Mitochondria (2-5 mg. protein) and assay medium, were mixed thoroughly to a volume of 2.0 ml. in a clean 10 ml. beaker, and 1 ml. of the mixture pipetted into the sample cuvette, and the remainder pipetted into the reference cuvette. The cuvettes were then immersed in liquid nitrogen contained in a Dewar, and cooled until the cuvette contents produced cracked ice (c.a. 5 minutes). The cuvettes were then rapidly transferred to the Dewar on the spectrophotometer, which also contained liquid nitrogen maintained at a level just beneath the cuvette windows to avoid absorbance interference due to surface boiling.

A slow scan through the wavelength span under study, was then made, with corrections for baseline deviation from a horizontal line, at a full-scale deflection setting of 0.01 O.D. units, and a band-pass of 0.9 nm. Once a reasonably horizontal oxidized-minus-oxidized baseline had been achieved, the cuvette contents were thawed out, and a crystal of potassium ferricyanide added to the contents of the sample cuvette. The contents of both cuvettes were then mixed by withdrawal into a plastic disposable hypodermic syringe (2.5 ml.), fitted with a soft polyethylene extension to the needle, a device added to prevent the needle from scratching the plastic cuvette windows. The oxidized mixture was then returned to the sample and reference cuvettes, and frozen down by immersion in liquid nitrogen. The baseline was then redrawn, to check that the presence of potassium ferricyanide had not caused irregularities in it.

The steps outlined above were followed after the addition to the mitochondrial suspensions contained in the cuvettes, of the

appropriate reducing agent, before the initial freezing down. For substrate-mediated reduction of the cytochromes under anaerobic conditions, the cuvettes were kept at room temperature for sufficient time to allow full onset of anaerobiosis (5-10 minutes).

Aerobic steady-state 4 levels of reduction of cytochromes b and c were measured by freezing the mitochondrial suspensions very shortly after adding the appropriate respiratory substrate, in the presence of ATP, i.e. after 20-30 seconds, using a method similar to that used by Chance & Spencer (1959). Also the concentration of the mitochondria was adjusted to prevent onset of anaerobiosis before freezing down. The absorbance difference spectra were scanned at a pen carriage velocity of 2-5 nm. second⁻¹ at the medium response setting, to check that absorbance maxima did not exceed the recorder chart, followed by recording the spectra at a pen carriage velocity of 0.1 nm. second⁻¹, and set to the slow response to reduce the noise : signal ratio to a minimum.

vii) Measurement of electron transport to potassium ferricyanide.

The rate of electron transport from exogenous NADH and intermediates of the TCA cycle to the ferricyanide anion, and catalyzed by isolated plant mitochondria, was followed by measuring either the rate of oxidation of NADH, in the case of exogenous NADH, and the rate of reduction of ferricyanide, in all cases. This was carried out using the Aminco DW2 spectrophotometer, operating in the dual-wavelength mode and set to a measuring wavelength of 420 nm. and reference wavelength of 490 nm. to follow ferricyanide reduction to ferrocyanide. Full-scale deflection was set to 0.2 O.D. units, with a band-pass of 1.5 nm. and a pen carriage velocity of 50 seconds inch⁻¹.

Mitochondria (0.1 - 1.0 mg. protein) were pipetted into a 3 ml. capacity 1 cm. light path glass spectrophotometer cuvette and mixed with assay medium to a volume of 3 ml., and 3 umoles of KCN added and stirred in. The KCN prevented the flow of electrons to oxygen catalyzed by cytochrome c oxidase. The cuvette was then placed in the spectrophotometer, and the light beams balanced on it, with the pen set to the lower margin of the recorder chart. Potassium ferricyanide (0.6 umol) was added to the cuvette and stirred in, as 12 uL of aqueous potassium ferricyanide at a concentration of 50 ug. ions ml.⁻¹, added by microsyringe. The pen was reset with the baseline locate control to the upper margin of the recorder chart, and any background rate of reduction of ferricyanide, recorded as a decrease in absorbance at 420-490 nm. The appropriate respiratory substrate was then added to the cuvette, with stirring, and the rate of reduction of ferricyanide recorded. Other additions to the cuvette were made as indicated.

Absorbance changes at 420-490 nm. were calibrated in units of ferricyanide, by measuring the absorbance by known concentrations of potassium ferricyanide, made from distilled water and aliquots of a stock solution prepared gravimetrically from Analar grade potassium ferricyanide solid and distilled water.

The wide range of quantities of protein indicated above for the assay of ferricyanide reduction, reflects different rates of reduction according to the substrate used. For exogenous NADH-ferricyanide reductase, only approximately 0.1 mg. protein was required, while reduction of ferricyanide by the TCA cycle intermediates required approximately 1.0 mg. protein to produce an easily measured rate of reduction of ferricyanide, catalyzed by Jerusalem artichoke mitochondria.

viii) Measurement of electron transport from exogenous NADH to juglone and duroquinone.

Ethanollic solutions of juglone and duroquinone were prepared at a concentration of 50mM. Exogenous NADH-juglone reductase, and exogenous NADH-duroquinone reductase activities catalyzed by isolated plant mitochondria, were measured by following the rate of oxidation of exogenous NADH spectrophotometrically as described in Materials & Methods 6.v.a, in the presence of 1.0mM KCN and either juglone or duroquinone at a concentration of 150uM initially.

ix) Measurement of exogenous NADH-cytochrome c reductase, and succinate-cytochrome c reductase activities.

The activities of exogenous NADH-cytochrome c reductase, and succinate-cytochrome c reductase activities, catalyzed by isolated plant mitochondria, were measured by following the reduction of exogenous ferricytochrome c spectrophotometrically. The Aminco DW2 spectrophotometer was used in the dual-wavelength mode, with a measuring wavelength of 550 nm. and reference wavelength of 540 nm., as used by Palmer & Kirk (1974). The assay medium used was as follows:

KH_2PO_4	(5mM)
TES	(5mM), pH 7.2
MgCl_2	(2.5mM)

A 3 ml. capacity, 1 cm. light path length glass spectrophotometer cuvette was set-up containing the above assay medium, 1mM KCN, horse heart ferricytochrome c, at 33-36 nmoles ml.^{-1} , and isolated mitochondria (0.15-0.2 mg. protein), to a final volume of 3.0 ml. The cuvette was then placed in the spectrophotometer and the beams balanced on it, with a full-scale deflection setting of 0.5 O.D. units, and the pen carriage velocity set to 20 seconds inch^{-1} .

The pen was set to zero near the lower margin of the recorder chart and set in motion. The substrate was added to the cuvette, and stirred-in, and the rate of reduction of the ferricytochrome c, recorded. Absorbance changes at 550-540 nm. were converted into units of ferricytochrome c reduced min.⁻¹ mg. protein⁻¹ by use of the following formula:

$$\frac{\Delta \text{O.D.}_{550-540} \text{ min.}^{-1}}{19} \times \frac{V}{m} = \text{nmoles ferricytochrome c reduced min.}^{-1} \text{ mg. protein}^{-1}$$

where, 19 is the millimolar extinction coefficient for cytochrome c at 550-540 nm. cm.⁻¹ (Lance & Bonner, 1968), V is the reaction volume in uL., and m is the quantity of mitochondrial protein used in the assay, in mg. and estimated by the method outlined in Materials & Methods 8.

For measurement of the activity of succinate-cytochrome c reductase, the enzyme succinate dehydrogenase was activated by preincubating the mitochondria with ATP, Pi and MgCl₂, as described in the legend to figure 9, Results 1.ii.

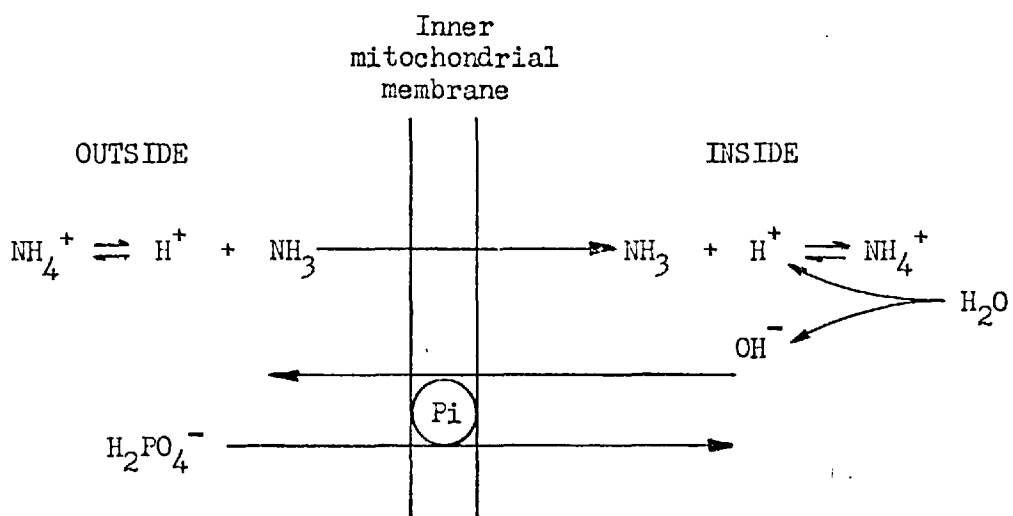
x. Measurement of citrate entering the mitochondrial matrix.

The entrance of the mitochondrial matrix by citrate, in isolated plant mitochondria, was measured indirectly by a method which involved swelling of the mitochondria suspended in a solution of ammonium citrate, and following the swelling spectrophotometrically. Ammonium citrate and water enter the mitochondrial matrix which results in swelling, and the method was first described by Chappell & Haarhof (1966), and later used in studies with isolated plant mitochondria by Phillips & Williams (1973a), and Wiskich (1974).

Mitochondrial swelling was measured optically as a

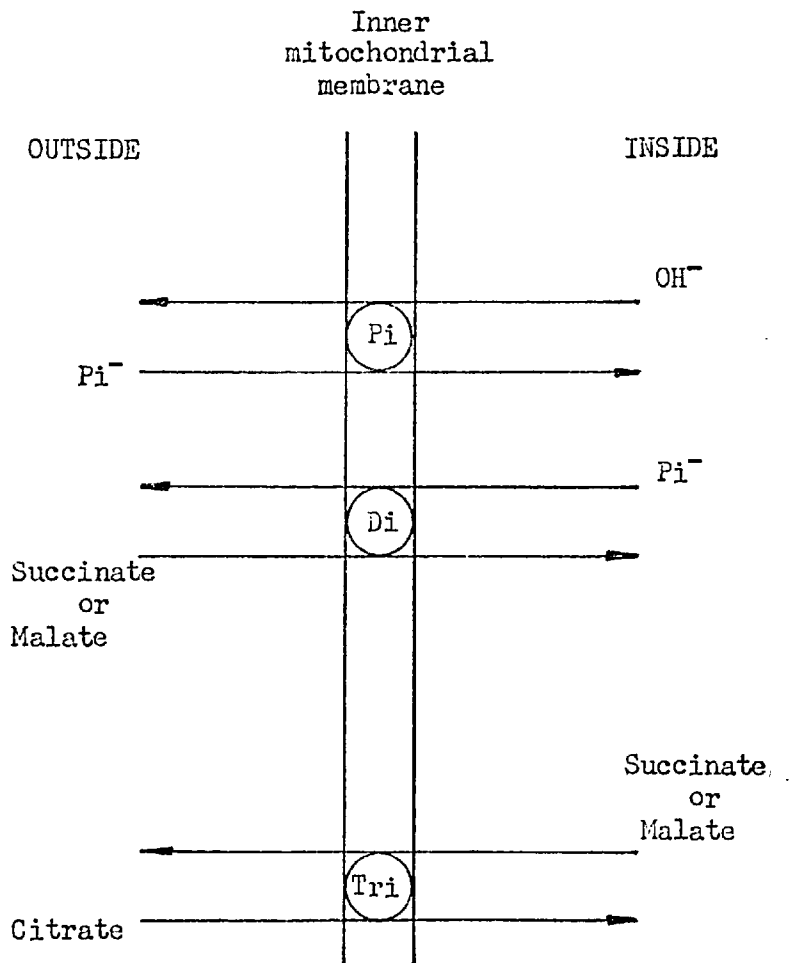
decrease in light scattering, using the Aminco DW2 spectrophotometer, in the split-beam mode and set to a wavelength of 520 nm., a full-scale deflection of 0.1 O.D. units, 3 nm. band-pass, and a pen carriage velocity of 50 seconds inch⁻¹. Isolated Jerusalem artichoke mitochondria (2 mg. protein) were suspended in 100mM ammonium citrate, (i.e. citric acid adjusted to pH 7.2 with 880 ammonia), to a volume of 2 ml. and mixed well in a 10 ml. beaker. One ml. of this mixture was pipetted into the sample cuvette, and the remainder pipetted into the reference cuvette. The cuvettes were a matching pair of 1 ml. capacity, made of glass and having a light path of length 1 cm. Antimycin A (200 ng.) was stirred into each cuvette contents, and the light beam balanced between the two cuvettes, with the pen set to zero near the upper margin of the recorder chart. Additions were made to the sample cuvette using a microsyringe, followed by stirring and recording of changes in the degree of swelling of the mitochondria.

The mechanism by which the swelling just described is thought to occur, has been outlined by Phillips & Williams (1973a), in a scheme which has been reproduced below:



Pi = the phosphate carrier.

Ammonia appears to diffuse across the inner mitochondrial membrane, down a concentration gradient. Once inside the mitochondria, NH_3 probably associates with a proton to form NH_4^+ , leaving an excess of OH^- which can exchange for inorganic phosphate (H_2PO_4^-) on the phosphate-hydroxyl antiporter, if the mitochondria are suspended in ammonium phosphate. In the present study the mitochondria were suspended in ammonium citrate, and only when citrate entered the matrix did swelling occur due to the increase in osmotic pressure inside the mitochondria thus created. In white potato mitochondria, Phillips & Williams (1973a), found that citrate entry of the matrix required inorganic phosphate and malate indicating that the scheme reproduced below, which is based on that thought to occur in animal mitochondria, was operating.



where Pi^- = inorganic phosphate, Pi = phosphate carrier, Di = dicarboxylate carrier and Tri = tricarboxylate carrier.

xi) Measurement of fluorescence changes of exogenous NADH.

A Perkin-Elmer fluorescence spectrophotometer, model MPF - 3, was used to follow the decrease in fluorescence of NADH as it was oxidized by oxaloacetate in the present study.

The nicotinamide adenine dinucleotide fluorescence was excited by light of wavelength 340 nm., and measured at a wavelength of 460 nm. as described by Palmer & Kirk (1974). The percentage fluorescence was calibrated with NADH of known concentration, it having been calibrated as described in Materials & Methods 9.ii. The mitochondria were suspended in the standard assay medium for potato mitochondria, in a 3 ml. capacity fluorescence spectrophotometer cuvette, and additions made as indicated, while measuring changes in the level of fluorescence of NADH.

7. Extraction of mitochondrial ubiquinone, and measurement of its absorbance spectrum.

i) Extraction of ubiquinone from mitochondria in aqueous medium.

Ubiquinone was extracted from Jerusalem artichoke mitochondria suspended in the suspending medium described in Materials & Methods 2.i., by the method described by Storey (1966). Up to 2 mg. of mitochondrial protein contained in 0.5 ml. of suspending medium, was added to a glass-stoppered glass 50 ml. capacity tube which contained the following:

n- Hexane (4.5 ml.)

Methanol (2.0 ml.)

and a number of small glass beads (Sigma (London)Ltd.).

The mixture was then shaken vigorously for 45 seconds using an electric vortex mixer, followed by addition to the tube of 1.5 ml. of a 40% (v/v) solution of triethanolamine in methanol, and shaking for a further 15 seconds. The aqueous and non-aqueous layers were separated by centrifugation at 1500 r.p.m. for 3 minutes, using an MSE bench centrifuge. This produced an aqueous lower layer, a precipitate at the interface and an upper hexane layer containing the ubiquinone. The upper layer was transferred to another clean glass stoppered tube by careful Pasteur pipetting, and the precipitate and aqueous layer, extracted again with 4.5 ml. of n-hexane. The hexane fractions were combined and evaporated to dryness using a rotary evaporator at room temperature. The n-hexane extract was dissolved in spectroscopic grade ethanol, and stored in a ground glass- stoppered bottle at 0-4°C for spectroscopic analysis.

ii) Extraction of ubiquinone from lyophilized mitochondria.

Mitochondria isolated from Arum maculatum spadices, were washed and suspended in 0.15M KCl solution, and lyophilized for 24 hours at -40°C. The lyophylate was stored in a dessicator at -15°C until required.

Ubiquinone was extracted from the lyophylate by the method described by Szarkowska (1966). Quantities (50 mg.) of the lyophylate were shaken with 5 ml. of n-pentane at 0°C, contained in a 10 ml. glass centrifuge tube, followed by precipitation of the extracted lyophylate by centrifugation at 10,000 r.p.m. for 3 minutes in a Sorvall RC 11B centrifuge, using the 8 x 50 ml. head fitted with 10 ml. adapters. The n-pentane containing the ubiquinone, was carefully poured into a clean 50 ml. ground glass-stoppered tube (Quickfit), and the solid precipitate re-extracted four times with 5 ml. aliquots of n-pentane. The n-pentane fractions were combined and evaporated

to dryness using a rotary evaporater at room temperature. The extract was dissolved in 2-5 ml. of spectroscopic grade ethanol, and stored at 0-4°C in a ground glass stoppered tube until required.

iii) Absorbance spectra of mitochondrial organic solvent extracts and ubiquinone 10.

The absorbance spectra of the organic solvent extracts of mitochondria and of a sample of ubiquinone₁₀ purchased from a chemical supplier (Sigma(London)Ltd.), which were all dissolved in spectroscopic grade ethanol, were measured with the Aminco DW2 spectrophotometer. The split-beam mode was selected, together with the deuterium lamp, and the sodium borohydride reduced minus air oxidized absorbance difference spectra drawn in each case, using a method which was similar to that described by Szarkowska & Klingenberg (1963) and Storey (1966).

Three ml. of an ethanolic solution of ubiquinone₁₀ were pipetted into each of a matching pair of 1 cm. light path silica spectrophotometer cuvettes, which were then placed in the Aminco DW2 spectrophotometer. The beam was balanced on the two cuvettes at a wavelength of 295 nm., an isosbestic point (Storey, 1972), and the oxidized-minus-oxidized baseline drawn, after adjustments for deviation from a horizontal line had been made. Full-scale deflection was set to 0.02 O.D. units when the solution contained approximately 2 ug. ml.⁻¹ of ubiquinone₁₀. A small crystal of sodium borohydride was transferred from a bottle kept in a dessicator, to the sample cuvette, and once bubbling had ceased, the reduced-minus-oxidized spectrum was drawn. The wavelength limits were set to 250 nm. and 350 nm.

8. Determination of mitochondrial protein concentration.

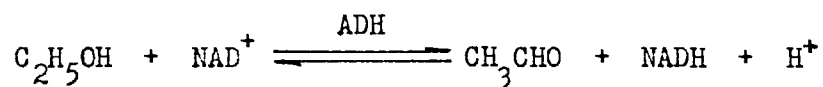
Mitochondrial protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), after solubilization by 10% (w/v) sodium deoxycholate. A standard aqueous solution of Bovine Serum Albumen was prepared gravimetrically from fraction V solid purchased from Sigma(London)Ltd., and containing approximately 5 mg. ml.⁻¹ of the solid, such that each μ L taken from it would contain of the order of 5 μ g. of the BSA.

Mitochondrial protein in the range 0-150 μ g. of protein was added to 0.4 ml. of 10% (w/v) sodium deoxycholate contained in a clean test tube, using a microsyringe. The volume was then made up to 0.5 ml. with distilled water added by microsyringe, followed by the addition of 5.0 ml. of 2% sodium carbonate (w/v, anhydrous) in 0.1N sodium hydroxide solution. A fresh mixture of 1.0% cupric sulphate pentahydrate and 2% potassium sodium tartrate (both w/v), was prepared in the ratio of 1:1 by volume, and 0.1 ml. of this mixture was added to the reaction tube, with stirring on a vortex mixer. The mixture was left at room temperature for 10 minutes, after which time, 0.5 ml. of a diluted solution of Folin's phenol reagent, containing 1 part by volume of the stock solution, and 2 parts by volume of distilled water, was added with stirring to the reaction tube. The resultant mixture was allowed to stand for 30 minutes to allow the colour to develop, when its absorbance at a wavelength of 600 nm. was measured against an appropriate blank, in the Aminco DW2 spectrophotometer operating in the split-beam mode, with a band pass of 1 nm. The blank was prepared by substituting an equal volume of suspending medium for the mitochondrial volume, and the calibration curve was checked against the BSA standard from time to time. (See Materials & Methods 1. for details of BSA solution).

9. Determination of NAD⁺ and NADH concentration.

i) NAD⁺ concentration.

The concentration of an unknown solution of NAD⁺ was determined by measuring the increase in absorbance of light by the solution at a wavelength of 340 nm., upon reduction to NADH by ethanol, in a reaction catalyzed by the enzyme, alcohol dehydrogenase, using the method of Klingenberg (1962). The reaction may be summarized as follows:



An alkaline assay medium which contained semi-carbazide, was used in order to obtain a quantitative reduction of NAD⁺ to NADH. In addition, pyrophosphate was used as the buffer, as it can chelate heavy metals which might have inhibited alcohol dehydrogenase, shown in the equation above, as ADH. The assay medium consisted of pyrophosphate buffer pH 8.8 (0.1M) and semi-carbazide hydrochloride (5 mg. ml.⁻¹).

The Aminco DW2 spectrophotometer was set to a wavelength of 340 nm. in the split-beam mode, with a band-pass of 3 nm. and full-scale deflection set to 0.5 -1.0 O.D. units. The beam was balanced on a matching pair of 3 ml. capacity 1 cm. light path length, silica spectrophotometer cuvettes, each containing 2 ml. of the assay medium. To the sample cuvette was added 20 uL of absolute ethanol plus a known volume of the unknown solution of NAD⁺, to result in approximately 50-100 uM in the cuvette, for example 5-10 uL of a solution prepared gravimetrically as 25mM. Beam balance was checked, and the pen set to zero near the lower margin of the recorder chart, and a record of the zero made. The reaction was started by adding 20 uL of

yeast alcohol dehydrogenase, containing approximately 1-2 mg. of the enzyme solid, as purchased from Sigma(London)Ltd. (308 U mg.⁻¹ at 25°C), which was then stirred in with a plastic stirrer. After 3-4 minutes when no further change in absorbance at 340 nm. took place, the total absorbance change was substituted in the equation given below, to calculate the concentration of the original solution of NAD⁺ of unknown concentration.

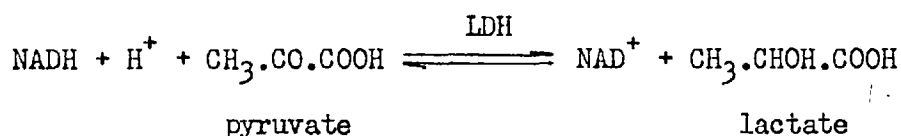
$$\frac{\Delta \text{O.D.}_{340}}{6.22} \times \frac{V}{L} = N \text{ nmoles NAD}^+ \text{ uL}^{-1} \text{ added to the assay}$$

cuvette = NAD^+ uM concentration of unknown solution.

where $\Delta \text{O.D.}_{340}$ = total change in absorbance at 340 nm. in O.D. units;
6.22 = millimolar absorbance extinction coefficient for NADH at 340 nm.
 cm.^{-1} , after Horecker & Kornberg (1948); V = the assay volume in μL ,
and L = the number of μL of the unknown NAD^+ solution added to the
assay cuvette.

ii) NADH concentration.

The concentration of aqueous solutions of NADH, was determined by measuring the decrease in absorbance at a wavelength of 340 nm., upon the total oxidation of a known volume of each solution, by pyruvate, in a reaction catalyzed by lactate dehydrogenase. This method was similar to that described by Klingenberg (1962), and the reaction involved may be summarized as follows:



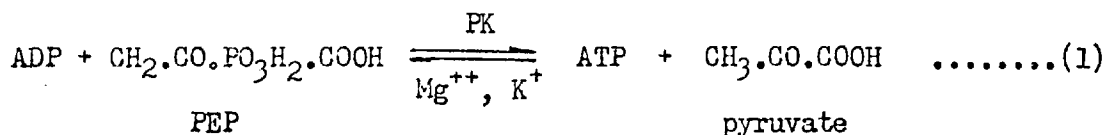
where LDH = lactate dehydrogenase.

The assay medium used was phosphate buffer (0.1M), pH 7.4.

The spectrophotometer and cuvette details, were as described above for the determination of NAD^+ concentration. To 2 ml. of the assay medium contained in the sample cuvette, were added with mixing, 5 umoles of sodium pyruvate, as 5 uL of a 1.0M solution, and up to 200 nmoles of NADH taken from the solution whose concentration is to be determined. The beam was balanced on the cuvettes and the pen set to the upper margin of the recorder chart with full-scale deflection set to 1.0 O.D. unit, with a band pass of 3 nm. The reaction was started by adding lactate dehydrogenase to the sample cuvette and stirring it in, (20 uL of a solution containing 5 mg. ml^{-1} , which contained 500 U mg^{-1} at 25°C). The total absorbance change at 340 nm. was then substituted in the equation given for NAD^+ in part (i) above, which gave a value for the concentration of the unknown solution of NADH as μM .

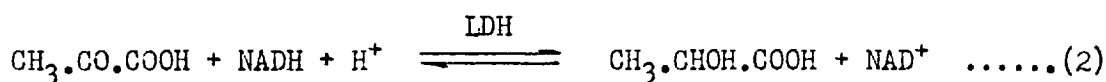
10. Determination of ADP concentration.

The concentration of a solution of ADP was determined by measuring the decrease in absorbance at 340 nm. wavelength, which resulted from the oxidation of NADH in a 'coupled' reaction in which pyruvate was reduced to lactate, as described by Adam (1962). The reactions may be summarized as shown below:



where PK = pyruvate kinase, and PEP = phosphoenol pyruvate.

The pyruvate formed as shown in equation (1), was then assayed for in the reaction summarized in equation (2) below:



in which pyruvate is reduced to lactate by NADH , catalyzed by the enzyme lactate dehydrogenase (LDH).

The assay medium used was Tris/HCl buffer (0.1M), pH 7.4. To 2 ml. of this medium contained in a 3 ml. capacity 1 cm. light path silica spectrophotometer cuvette, were added 0.15 ml. of a fresh solution which contained the following:

Sodium phosphoenolpyruvate (PEP)	(10mM)
KCl	(1.3M)
MgSO ₄	(0.4M)

The details of spectrophotometry were the same as described for the determination of the concentration of NADH solutions, given in Materials & Methods 9.ii., using the Tris/HCl buffer as the blank.

Microsyringes were used to pipette the following into the sample cuvette, after balancing the beam:

Pyruvate kinase, 20 uL of a solution containing 2 mg. ml.⁻¹ and 200 U per. mg. at 25°C.

NADH to c.a. 125uM, as 5 uL of a 50uM solution

ADP to c.a. 100uM

The pen was set to the upper margin of the recorder chart, and lactate dehydrogenase added to the sample cuvette to start the reactions, as 20 uL of a 5 mg. ml.⁻¹ solution; 500 U mg.⁻¹ at 25°C, with stirring. The total absorbance decrease at 340 nm. was recorded and the value substituted in the equation given below, to find the concentration of the unknown ADP solution:

$$\frac{\Delta \text{O.D.}_{340}}{6.22} \times \frac{V}{L} = \text{concentration of the unknown ADP solution as uM.}$$

where 6.22 = extinction coefficient of NADH at 340 nm. cm.⁻¹,

V = the total assay volume in uL, and L = the volume of ADP solution added to the assay cuvette.

The above relationship follows from the 1:1 stoichiometry between NADH oxidized by pyruvate, and ADP phosphorylated to ATP, shown by equations (1) and (2) above, which describe the reactions of this assay of ADP concentration.

RESULTS

1. Some respiratory characteristics of isolated mitochondria.

The objective of the experiments described in this section of the results, is to show that mitochondria isolated from Jerusalem artichoke tubers can catalyze active oxidation of intermediates of the TCA cycle, pyruvate and exogenous NADH. Data for the magnitude of the ADP:O ratios and the sensitivity of the rates of substrate oxidation, to inhibitors of electron transport, are also presented to indicate the apparent branched nature of the electron transport pathways. Finally, data which illustrate the complexity of the endogenous NADH dehydrogenases in plant mitochondria, are presented.

i) Substrate utilization and oxidative phosphorylation.

Mitochondria isolated from plant tissues, by the technique described in Materials & Methods 2, were shown to catalyze the rapid oxidation of intermediates of the TCA cycle, pyruvate and exogenous NADH. Oxygen electrode traces for isolated Jerusalem artichoke mitochondria, catalyzing the oxidation of pyruvate and malate, are shown in figure 5. As described in the introduction to this thesis, the initial step of oxidation of malate in plant mitochondria, appears to be catalyzed mainly by an NAD^+ -linked malic enzyme, and hence no oxaloacetate removing system was required .

In the presence of a respirable substrate alone (i.e. the 'substrate state'), the addition of ADP caused a stimulation of the rate of consumption of oxygen by the mitochondria, until the ADP had been phosphorylated to ATP, when the rate returned to a lower magnitude. Following the nomenclature of Chance & Williams (1956), the ADP stimulated rate is referred to as the 'state 3' rate, and the slower post state 3 rate, is referred to as the 'state 4' rate. The rate of

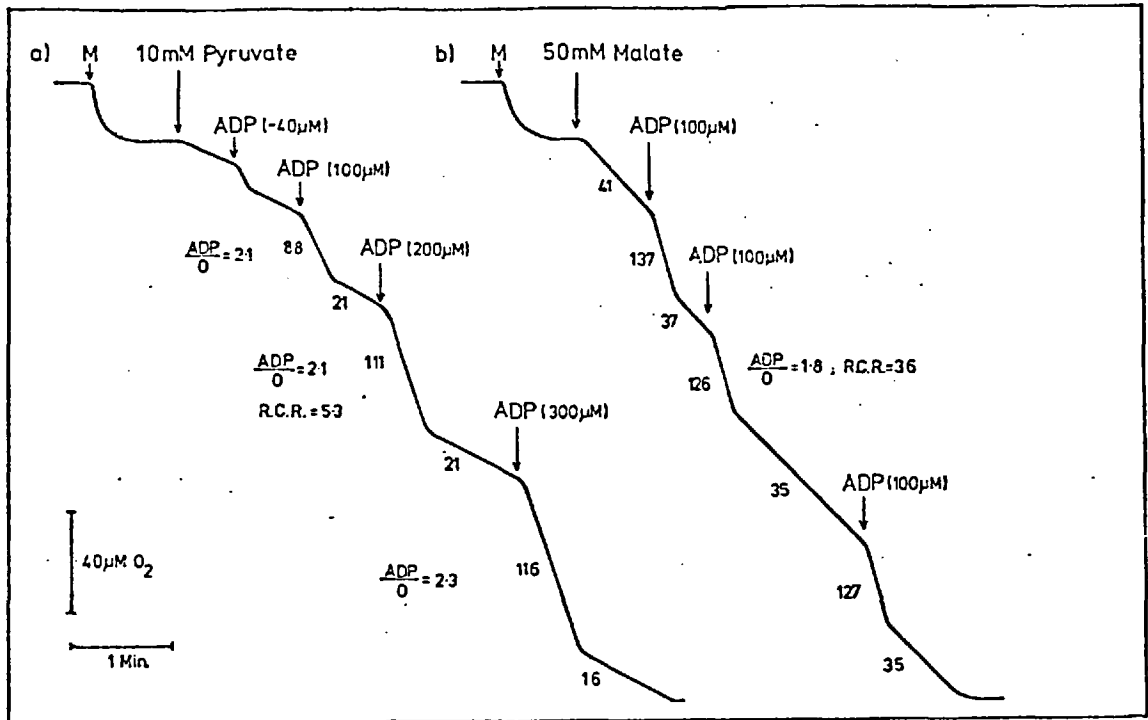


Figure 5. The nature of respiratory control in isolated Jerusalem artichoke mitochondria, oxidizing pyruvate and malate. Oxygen electrode traces showing the pattern of oxygen consumption. Mitochondria, M, 1 mg. protein ml^{-1} , were suspended in standard assay medium, to a final volume of 0.5 ml. For trace (a) the assay medium also contained 1mM malate and 0.35mM TPP. Numerals under the traces represent oxygen consumption as $\text{nmoles min}^{-1} \text{mg. protein}^{-1}$. R.C.R. , respiratory control ratio. Oxygen consumption was measured as described in Materials & Methods 5.

respiration catalyzed by suspensions of mammalian mitochondria, in the presence of excess oxygen and respiratory substrate, has been shown to be dependent upon the ratio of the activity of ATP to that of the product of the activities of ADP and Pi, which is designated as the 'phosphate potential' i.e. $[ATP]/[ADP][Pi]$ (Wilson, Owen, Mela & Weiner, 1973). The activities of ATP, ADP and Pi appear to be very similar to the concentrations of each as measured, and similar results have been obtained with avian mitochondria (Erecinska, Veech & Wilson, 1974), and for suspensions of liver cells (Wilson, Stubbs, Veech, Erecinska & Krebs, 1974). The respiratory states 3 and 4, are characterized by low and high values for the phosphate potential, respectively.

A parameter used to measure the degree of control that is imposed by the oxidative phosphorylation system, upon the rate of electron transport, is called the 'respiratory control ratio' after Chance & Williams (1956). It has been abbreviated to R.C.R. in this thesis, and has a numerical value greater than one, and is obtained by dividing the numerical value for the rate of respiration measured under state 3 conditions, by that measured under state 4 conditions. The greater the value for the respiratory control ratio, the greater the degree of respiratory control, i.e. the greater the degree of coupling between electron transport and oxidative phosphorylation.

The respiratory control ratio is sensitive to damage to the mitochondria, which can be introduced at the stage of their isolation from the parent tissue, and hence it can vary between mitochondrial preparations, as comparison of trace (a) in figure 5, with trace (a) figure 6, shows, as each trace was obtained with a different preparation of mitochondria. The state 3 rate appeared to be much less variable than the state 4 rate. Figure 6 (b) and (c) show that citrate

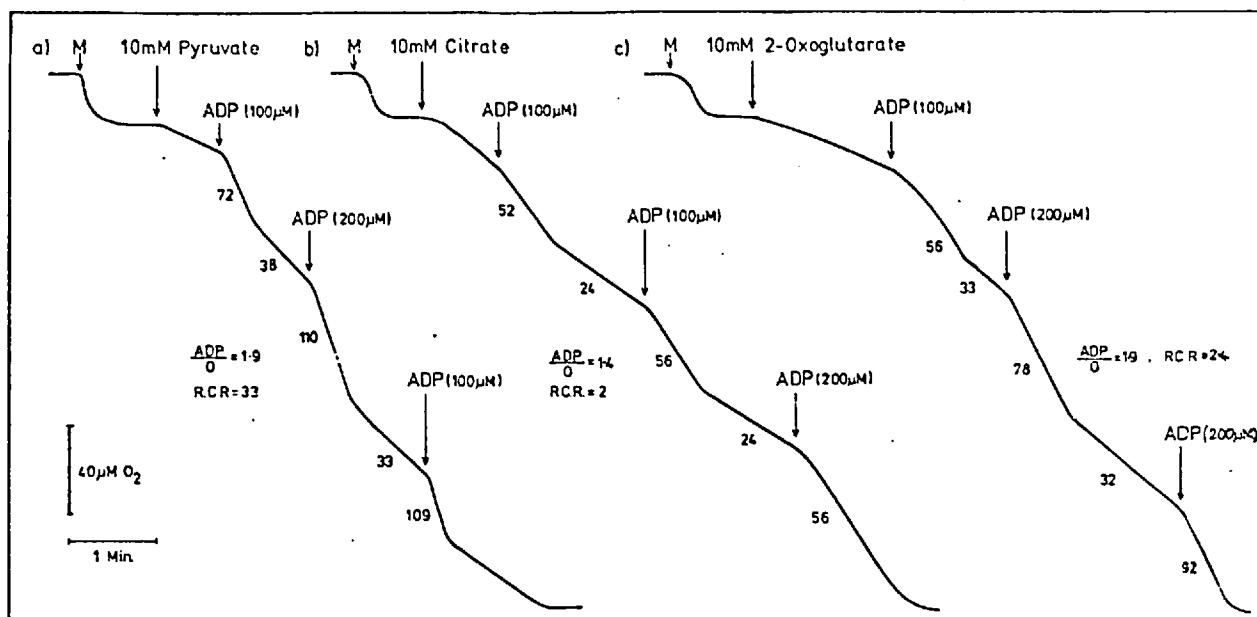


Figure 6. Oxidative phosphorylation in isolated Jerusalem artichoke mitochondria, oxidizing pyruvate, citrate and 2-oxoglutarate. Oxygen electrode traces showing the pattern of oxygen consumption. For traces (a) and (c), 0.35mM TPP was present in the assay medium. Other details were as described for figure 5.

and 2-oxoglutarate oxidation was also catalyzed by isolated Jerusalem artichoke mitochondria, but at rates lower than those measured for pyruvate and malate (figure 5 and figure 6a). The oxidation of these substrates was also stimulated by ADP, and hence was shown to be coupled to phosphorylation, although the values measured for the ADP:O ratio can be seen to be lower than the expected maximum values of 3 for malate, pyruvate and citrate, and 4 for 2-oxoglutarate. This suggested that electron transport was bypassing phosphorylation 'sites' (see figure 2), and/or the efficiency of coupling of electron transport to oxidative phosphorylation was low.

A similar pattern of oxygen consumption was observed when exogenous NADH or succinate were supplied to the mitochondria as the respiratory substrate, which can be seen from figure 7. The expected maximum value for the ADP:O ratio measured for these substrates is 2. Of particular relevance to the data to be presented in this thesis, is the pattern of oxidation of succinate shown in figure 7 (b) and (c); trace (c) shows the usual pattern of oxygen consumption catalyzed by Jerusalem artichoke mitochondria, in which the enzyme succinate dehydrogenase was initially greatly deactivated, and became progressively activated by the presence of succinate and probably ADP and ATP, as time elapsed, in agreement with Wiskich & Bonner (1963). Trace (b) however, shows that it was also possible to prepare mitochondria from Jerusalem artichoke tubers, in which the succinate dehydrogenase was not greatly deactivated, as measured by the activity of succinate oxidase. Such mitochondrial preparations were infrequently made, and it may be of significance that only Jerusalem artichoke tubers that had not been stored at low temperature, yielded mitochondria in which succinate dehydrogenase was not greatly deactivated. In relation to this phenomenon, it should perhaps be pointed out that Raison, Lyons

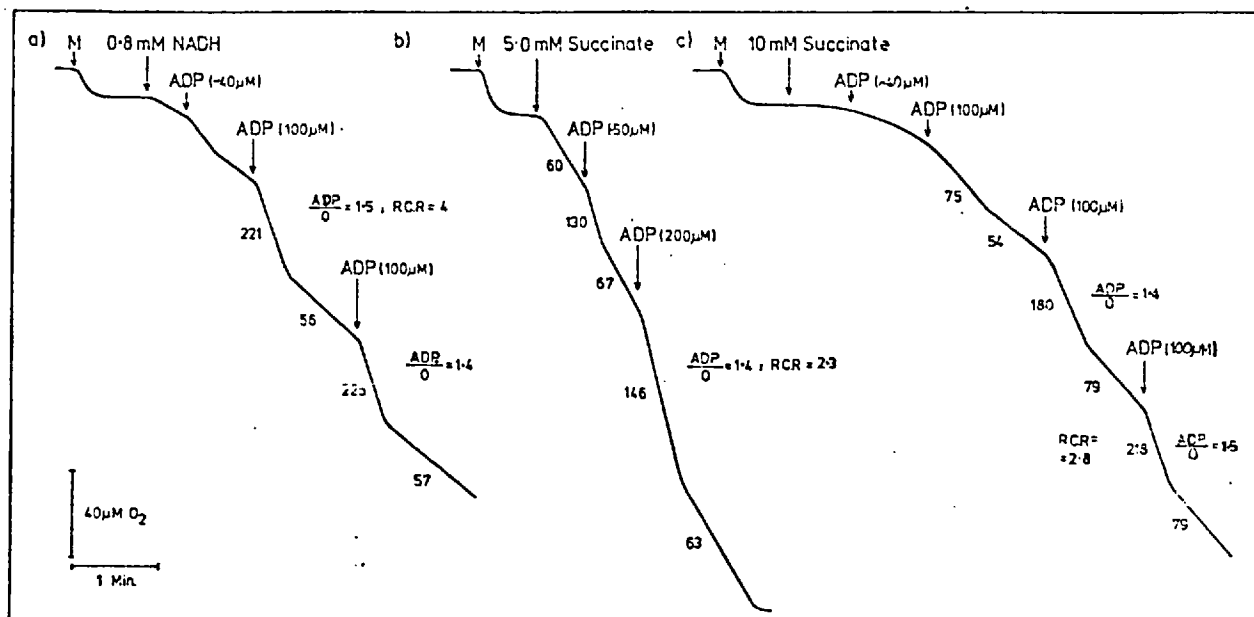


Figure 7. Oxidative phosphorylation in isolated Jerusalem artichoke mitochondria, oxidizing exogenous NADH and succinate. Oxygen electrode traces showing the pattern of oxygen consumption. Traces (a) and (c), were obtained using 0.6 mg. mitochondrial protein ml.^{-1} of assay medium, the mitochondria having been isolated from one sample of tubers, and trace (b) was obtained using mitochondria isolated from a second sample of tubers, and suspended in assay medium at a protein concentration of 1 mg. ml.^{-1} assay medium. For other details, see figure 5.

& Campbell (1973) have reported that it was possible to prepare mitochondria from white potato tissue, in which the state of activation of succinate dehydrogenase appeared to be dependent upon who carried out the steps of mitochondrial isolation. This suggested that the enzyme succinate dehydrogenase was sensitive to physical changes occurring during mitochondrial isolation, which could affect its state of activation. The mechanism of the deactivation of succinate dehydrogenase was not investigated further in the course of the present study, although a method of activation of this enzyme will be given shortly.

It has already been indicated that the values for the ADP:O ratios obtained with Jerusalem artichoke mitochondria, were lower than the expected maximum values. Coleman & Palmer (1972) have reported values of 2 and 3 for the ADP:O ratios measured for exogenous NADH and malate as the supplied respiratory substrate, respectively, which closely approached the expected maximum values. Thus it appeared to be possible to achieve the expected ADP:O ratios with Jerusalem artichoke mitochondria, and in order to check the validity of the ADP:O ratios found here, a direct comparison was made with mitochondria isolated from rat liver, as described under Materials & Methods 2.vi, and figure 8 shows results obtained. Although values for the ADP:O ratios measured for rat liver mitochondria closely approached the expected maximum values of 3 for β -hydroxybutyrate and 2 for succinate, (figure 8 a,b), it can be seen from trace (c) in figure 8 that the same solution of ADP resulted in the measurement of an ADP:O ratio of 1.4 for exogenous NADH oxidase activity in Jerusalem artichoke mitochondria. Thus there was evidence that mitochondria isolated from plant tissue, can have lower phosphorylative efficiency than their mammalian counterparts, a conclusion that is supported by most reports in the literature for such data, some of which are included in table 1.

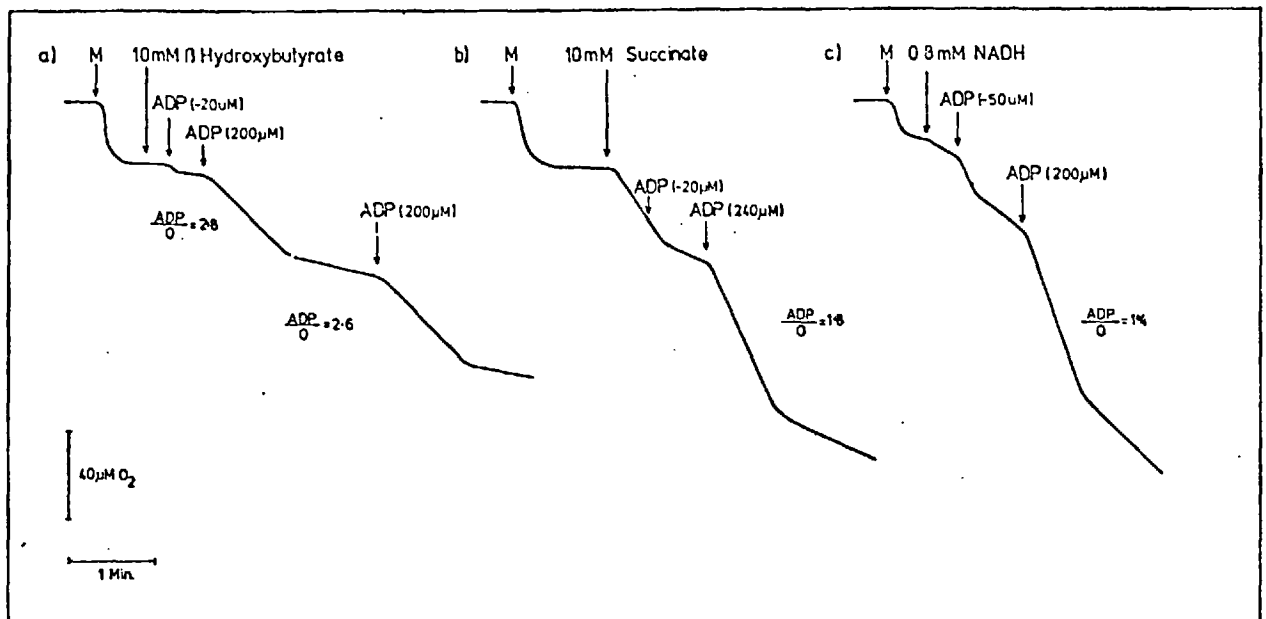


Figure 8. Oxidative phosphorylation in isolated rat liver and Jerusalem artichoke mitochondria. Oxygen electrode traces showing the pattern of oxygen consumption. Traces (a) and (b) were obtained using rat liver mitochondria at 1 mg. and 2 mg. mitochondrial protein ml^{-1} assay medium respectively, and trace (c) was obtained using Jerusalem artichoke mitochondria (0.5 mg. mitochondrial protein ml^{-1} assay medium). The assay medium used for each type of mitochondria are described in Materials & Methods 5.ii. For trace (b) the rat liver mitochondria were preincubated with 0.1 μmol each of ATP and Pi mg. protein^{-1} and 5mM MgCl_2 at 25°C for 4 minutes to activate succinate dehydrogenase. Other details were as described for figure 5.

TABLE 1.

Oxidative Phosphorylation in Plant Mitochondria. ADP:O Ratios with a Range of Substrates.

<u>Source of Mitochondria</u>	<u>Substrate Presented to Mitochondria</u>						<u>References</u>
	<u>NADH</u>	<u>Succinate</u>	<u>Pyruvate</u>	<u>Citrate</u>	<u>2-oxoglut.</u>	<u>Malate</u>	
White Potato Tuber	1.0	-	-	-	-	1.8	Wiskich & Bonner (1963)
"	-	-	-	1.6	-	-	Hawker & Laties (1963)
"	1.29 $\pm$ 0.1	1.57 $\pm$ 0.12	-	-	-	2.4 $\pm$ 0.22	Douce <u>et al</u> (1972)
"	1.0	1.0	1.7	1.8	2.2	1.8	Present thesis (1976)
Jerusalem Artichoke	1.8	1.7	-	-	-	2.8	Coleman & Palmer (1972)
"	1.2 $\pm$ 0.07	1.3 $\pm$ 0.05	2.1 $\pm$ 0.15	1.5	2.1	1.83 $\pm$ 0.2	Present thesis (1976)
Mung Bean Hypocotyl	1.31 $\pm$ 0.11	1.54 $\pm$ 0.1	-	-	-	2.38	Douce <u>et al</u> (1972)
"	-	1.6	-	-	-	2.0	Ikuma (1970)
"	1.4	1.4	-	-	-	2.1 to 2.6	Present thesis (1976)
Barley Coleoptiles	1.0	1.0	1.6	-	2.2	1.5	Brunton & Palmer (1973)
Wheat Coleoptiles	1.48	1.64	2.39	-	2.62	2.4	Brunton (p.92) (1975)
"	1.9	1.7	-	-	3.3	2.5	Pomeroy (1975)

(For Douce et al. values = Mean $\pm$ S.D. ; For present thesis values = Mean $\pm$ SEM , 19 assays NADH, 8 assays Succinate & Malate)

ii) Activation of succinate oxidase.

Deactivation of succinate oxidase in isolated mitochondria, has been shown to be the result of deactivation of the enzyme that catalyzes the initial step of succinate oxidation, succinate dehydrogenase (Kearney, Singer & Zastrow, 1955; Kearney, 1957; Singer, Kearney & Kenney, 1972; Singer, Oestreicher, Hogue, Contreiras & Brandao, 1973; Oestreicher, Hogue & Singer, 1973). The deactivation can be reversed by incubation of the mitochondria at a temperature between 25 and 37°C, in the presence of activating agents, including ATP, Pi and Mg^{++} .

The data presented in table 2, permit comparison to be made between the uncoupled activity of succinate oxidase catalyzed by Jerusalem artichoke mitochondria containing succinate dehydrogenase in its 'normal' deactivated state, and following activation of succinate dehydrogenase resulting from preincubation with combinations of ATP, Pi and Mg^{++} . It can be seen that all the combinations of ATP, Pi and Mg^{++} resulted in considerable degrees of activation of succinate dehydrogenase, with the presence of all three agents, resulting in slightly greater activation than that obtained with ATP plus Pi or ATP plus Mg^{++} . Also, increasing the concentration of $MgCl_2$ above 5mM, failed to result in further activation, and so this concentration was employed routinely throughout the present study, whenever activation of succinate dehydrogenase was required, together with ATP and Pi, each at a final concentration of 1 μ mol per mg. protein, in the bulk activation vessel.

The activation of succinate dehydrogenase was carried out by adding the ATP and Pi, prepared as aqueous solutions containing 0.4M sucrose to avoid osmotic changes to the mitochondria, which might cause damage, and at a concentration of 50mM in each case, to a suspension of the mitochondria as prepared i.e. at a protein concentration of

TABLE 2.

Activation of Mitochondrial Succinate Dehydrogenase. The effect of ATP, Pi, and MgCl₂ on the degree of activation.

<u>Activating Agents</u>	<u>Succinate oxidase activity (Uncoupled).</u>
<u>Present</u>	(nmoles oxygen min. ⁻¹ mg. protein ⁻¹)
None	22.2 ± 2.8
ATP + Pi	206.5 ± 8.5
ATP + MgCl ₂ (5mM)	207.5 ± 2.5
ATP + Pi + MgCl ₂ (5mM)	236.3 ± 3.5
ATP + Pi + MgCl ₂ (10mM)	240.0 ± 10.0

'Activated' mitochondria (0.4 mg. protein ml.⁻¹) and 'non-activated' mitochondria (0.62 mg. protein ml.⁻¹), were suspended in standard assay medium. Succinate (30mM) was added and succinate oxidase activity measured polarographically, as described in Materials & Methods 5. Uncoupler (Sl3, 10⁻⁶M) was added to the reaction cuvette one minute after the succinate, and the initial rate of oxygen consumption recorded. The degree of activation of succinate dehydrogenase was measured by the activity of succinate oxidase. The values for succinate oxidase activity, are the means of 3 assays ± S.E.M. Activation of succinate dehydrogenase was carried out by preincubating the mitochondria for 4 minutes at 26°C in the presence of the appropriate activating agents. ATP and Pi were used at a concentration of 1μmole mg. protein⁻¹ each, and each agent was prepared as stock solutions at pH 7.2 in 0.4M sucrose solution.

approximately 20 mg. protein ml.^{-1} , to result in final concentrations of ATP and Pi of 1 $\mu\text{mol mg. protein}^{-1}$, and MgCl_2 at 5mM. The mixture was then incubated at a temperature of 26°C for 4 minutes, and then returned to an ice bath. (The ATP and Pi stock solutions were adjusted to a pH value of 7.2, using 10% w/v aqueous potassium hydroxide solution). Subsequent dilution of the activated mitochondria for the appropriate enzyme assay, resulted in concentrations of ATP ranging from approximately 50 to 500 μM , according to the assay. These concentrations were similar to those used by other workers (Douce, Christensen & Bonner, 1972; Bonner, 1973; Palmer & Kirk, 1974).

Characteristic patterns of succinate oxidase activity, catalyzed by isolated Jerusalem artichoke mitochondria, are shown in figure 9. Trace (a) shows that, without activation of succinate dehydrogenase, uncoupler had little or no effect on the activity of succinate oxidase, as expected if the rate limiting step of the reaction was the conversion of succinate to fumarate. Figure 9 (b) shows how the pattern of oxygen consumption changed after activation of succinate dehydrogenase, and traces (c) and (d) show similar effects but using a much higher concentration of succinate, to emphasise how greatly succinate dehydrogenase was deactivated in these mitochondria. Trace (e) indicates that the process of activation of succinate dehydrogenase did not result in electron transport becoming uncoupled from oxidative phosphorylation, since respiratory control was easily measurable after activation. Activation did result in a greatly increased affinity of succinate dehydrogenase for succinate, however, as demonstrated by the much lower value for the apparent K_m ($K_{m_{\text{app.}}}$) for succinate, measured in the presence of the uncoupler, S13 (figure 10).

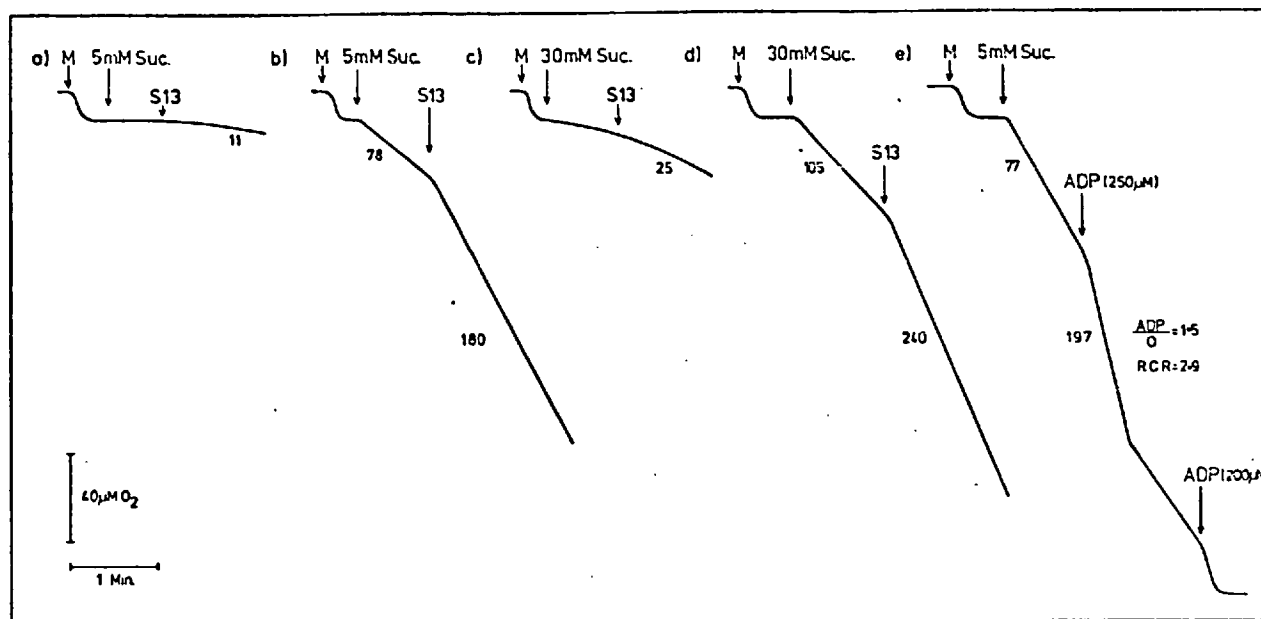


Figure 9. The effect of activation of succinate dehydrogenase on the activity of succinate oxidase in isolated Jerusalem artichoke mitochondria. Oxygen electrode traces showing the pattern of oxygen consumption. Traces (a) and (c) were obtained using mitochondria ($0.62 \text{ mg. protein ml.}^{-1}$) in which succinate dehydrogenase had not been activated. Traces (b), (d) and (e) were obtained using mitochondria ($0.42 \text{ mg. protein ml.}^{-1}$, for (b) and (d), and $0.83 \text{ mg. protein ml.}^{-1}$, for (e)), in which succinate dehydrogenase had been activated by preincubation for 4 minutes at 26°C in the presence of ATP and Pi at $1 \mu\text{mole mg. protein}^{-1}$, and MgCl_2 , 5 mM . Numerals under the traces represent the rate of oxygen consumption as $\text{nmoles oxygen min.}^{-1} \text{ mg. protein}^{-1}$. R.C.R., respiratory control ratio; Suc, succinate; S13, 10^{-6} M . Final reaction volume was 0.5 ml. , using standard assay medium.

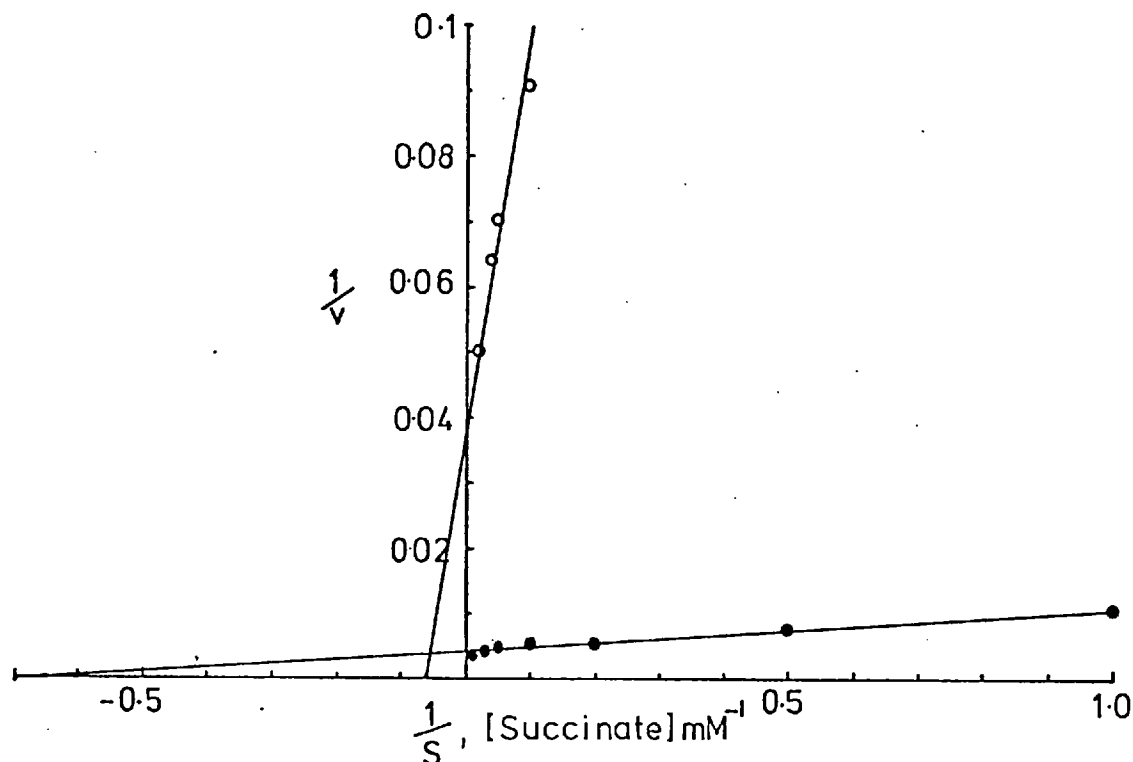


Figure 10. The effect of activation of succinate dehydrogenase on the uncoupled activity of succinate oxidase in isolated Jerusalem artichoke mitochondria. A double reciprocal plot of $1/V$ versus $1/S$ where V = velocity of succinate oxidase activity as nmoles oxygen $\text{min.}^{-1} \text{mg. protein}^{-1}$, and S = concentration of substrate, succinate, mM. Mitochondria ($0.5 \text{ mg. protein ml.}^{-1}$) were suspended in standard assay medium, and succinate of the appropriate concentration, was then added, followed by measurement of the rate of oxygen consumption polarographically, as described in Materials & Methods 5. After one minute, uncoupler (S13, 10^{-6}M) was added and the subsequent rate of oxygen consumption recorded, V . ●, activated mitochondria; O, non-activated mitochondria. For details of activation, see figure 9.

iii) Assessment of mitochondrial membrane integrity.

The integrity of mitochondrial membranes has been assessed indirectly using methods that depend upon their apparent permeability to various agents. For example, NADH and oxaloacetate added to intact isolated mitochondria, in which electron transport has been inhibited, can only react with each other to a major extent following the disruption of the inner mitochondrial membrane. This is because the enzyme responsible for catalyzing the reduction of oxaloacetate by NADH, malate dehydrogenase, is located on the matrix side of this membrane, and the inner membrane is impermeable to NADH. Thus, comparison of the activity of NADH-oxaloacetate oxido-reductase, catalyzed by isolated mitochondria, before and after disruption of their membranes by for example, a detergent, indicates the level of integrity of the inner membranes before disruption (Palmer & Kirk, 1974; see also table 7, present thesis).

Secondly, it is possible to measure the permeability of the inner mitochondrial membrane to the ferricyanide anion, by estimating the sensitivity of succinate-ferricyanide reductase activity to inhibition by antimycin A. A high level of sensitivity to antimycin A reflects a high level of integrity of the inner mitochondrial membrane, since it shows that ferricyanide is acting as an oxidant of the electron transport pathway from succinate, at a point beyond the step that is sensitive to antimycin A, probably cytochrome c (see figure 2). A low level of integrity of the inner mitochondrial membrane, allows ferricyanide anions to penetrate it and so reach the flavoprotein of succinate dehydrogenase, with which it can interact in a reaction that is insensitive to inhibition by antimycin A (Palmer & Kirk, 1974).

A third indirect test for the integrity of the membranes of isolated mitochondria, which is used with plant mitochondria in

particular, is to determine the rate of reduction of exogenous ferri-cytochrome c by succinate catalyzed by them, when suspended in normal assay medium (i.e. containing 0.4M sucrose), and when suspended in an assay medium of low osmolarity (i.e. one based upon 5mM phosphate buffer). This method is probably the most sensitive, since it depends upon the penetration of the outer mitochondrial membrane, by molecules of ferricytochrome c, the molecular weight of which is approximately 12,500 and so is very close to the accepted limit of permeability of this membrane, of 10,000 molecular weight (Douce, Christensen & Bonner, 1972; Bonner, 1973; Palmer & Kirk, 1974; see also Palmer, 1976).

The data presented in table 3 were obtained with mitochondria as used throughout the present study, and it can be seen from them that lowering the osmolarity of the assay medium, resulted in a large increase in the activity of succinate-cytochrome c reductase, equivalent to a twenty-fold stimulation, when compared with that measured in the assay medium containing 0.4M sucrose. A similar level of stimulation has been reported by Bonner (1973), and indicates a high degree of membrane integrity in the mitochondria used in the present study.

iv) Inhibitor sensitivity of electron transport.

Compounds which inhibit the rate of electron transport in isolated mitochondria have been useful in investigations of the arrangement of the electron transport pathways, particularly with regard to branching of the pathways, and to the possible ordering of the redox mediators making up the pathways. The site of action of the inhibitor potassium cyanide is cytochrome c oxidase (see Lehninger, 1964; Wilson & Hanson, 1969; and figures 1 and 2, present thesis), while the site of action of antimycin A, is located between the cytochromes b and c (Ikuma & Bonner, 1967b; Slater, 1973). The site of

TABLE 3.

The Effect of Osmolarity of the Assay Medium on the Activity of Succinate-cytochrome c Reductase in Isolated Jerusalem Artichoke Mitochondria.

<u>Osmolarity of</u> <u>Assay Medium</u>	<u>Succinate-cytochrome c</u> <u>Reductase Activity</u>	Ratio $\frac{A}{B}$
High	19.8 (B)	
Low	407.0 (A)	20.5

Assay medium: High Osmolarity = Standard Assay Medium,

Low Osmolarity = Cytochrome c Reductase Assay Medium.

ATP/Pi/MgCl₂ activated mitochondria (0.05 mg. protein ml.⁻¹), were suspended in the appropriate assay medium containing KCN (1.0mM), and ferricytochrome c (36μM). Succinate (20mM) was added to the mitochondria, stirred in and the rate of reduction of ferricytochrome c measured spectrophotometrically, using the wavelength pair 550-540 nm. as described in Materials & Methods 6.ix. The units of succinate-cytochrome c reductase activity are nmoles cytochrome c reduced min.⁻¹ mg. protein⁻¹. For details of standard assay medium and cytochrome c reductase assay medium, see Materials & Methods 5.ii.a. and 6.ix., respectively.

action of low concentrations of the inhibitors rotenone and piericidin A, which are too low to affect succinate oxidase activity, is close to the iron-sulphur centres associated with endogenous NADH dehydrogenase and oxidative phosphorylation at 'site 1' in animal and yeast mitochondria (Gutman, Singer Beinert & Casida, 1970; Gutman & Singer, 1970, 1971; see also figures 1 to 4, present thesis). Results obtained with mitochondria isolated from plant tissues, are also consistent with this location for the site of action by rotenone and piericidin A in these mitochondria (Coleman & Palmer, 1972; Brunton & Palmer, 1973; Brunton, 1975; Pomeroy, 1975).

The data presented in table 4, were obtained using Jerusalem artichoke mitochondria, and are consistent with the sites of action of each of the inhibitors just described. They are also consistent with branches of the electron transport pathways from the enzymes succinate dehydrogenase and exogenous NADH dehydrogenase, donating electrons to the oxygen side of phosphorylation 'site 1', but on the substrate side of cytochromes b, since the rates of oxidation of both succinate and exogenous NADH were found to be insensitive to inhibition by piericidin A, but sensitive to antimycin A.

The data in table 4 also show that the rate of oxidation of endogenous NADH produced as a result of the NAD^+ -linked oxidation of pyruvate, citrate, 2-oxoglutarate or malate, was not totally sensitive to inhibition by piericidin A, which was in agreement with the findings of Brunton & Palmer (1973) and Brunton (1975), for mitochondria isolated from wheat coleoptiles. The different activities of each of the named carboxylic acid oxidases, in the presence of a concentration of piericidin A that caused maximum inhibition of each, suggested that each acid may be oxidized via a different branch of the electron transport pathways to oxygen, with each branch having a different sensitivity to piericidin A. A detailed investigation of this aspect of

TABLE 4.

The Effects of Inhibitors of Respiratory Electron Transport on the Rate of Consumption of Oxygen by Isolated Jerusalem Artichoke Mitochondria, Oxidizing a Range of Substrates.

Rates of oxygen uptake in the presence of each inhibitor, as nmoles min.⁻¹ mg. protein⁻¹

<u>Substrate</u>	<u>Control</u>	<u>Pyrrolnitrin</u>	<u>Piericidin A</u>	<u>Antimycin A</u>	<u>Cyanide</u>
Exog. NADH	190	<5	190	<5	<5
Pyruvate	94	<5	23	<5	<5
Citrate	51	-	29	<5	<5
2-Oxoglutarate	65	-	36	<5	<5
Succinate	192	<5	192	<5	<5
Malate	110	<5	47	<5	<5

Mitochondria (0.5 - 1.0 mg. protein ml.⁻¹), were suspended in standard assay medium containing 1mM ADP, and the appropriate substrate added. The rate of oxygen consumption was measured polarographically, as described in Materials & Methods 5., and the appropriate inhibitor was added c.a. 30 seconds after attainment of a constant rate. For pyruvate, citrate and 2-oxoglutarate, 0.35mM TPP was also present in the assay medium. Substrate concentrations initially were, exogenous NADH, 1.0mM; Pyruvate, citrate, 2-oxoglutarate and succinate, 10mM; Malate, 60mM. Inhibitor concentrations were, pyrrolnitrin, 200 ug., piericidin A, 50 ng., and antimycin A, 200 ng. mg. protein⁻¹, and potassium cyanide, 1.0mM.

endogenous NADH oxidation in isolated plant mitochondria, forms a later part of the present study.

Pyrrolnitrin is an antibiotic which inhibits fungal respiration (Arima, Imanaka, Kousaka, Fukata & Tamura, 1964; Tripathi & Gottlieb, 1969; Wong & Airall, 1970; Wong, Horng & Gordee, 1971). It has been shown to inhibit electron transport in isolated Neurospora crassa mitochondria at site(s) located on the substrate side of cytochromes b, since it appeared to prevent electrons from reaching these components, when donated by succinate or exogenous NADH (Lambowitz & Slayman, 1972). This result was consistent with pyrrolnitrin inhibiting the rate of electron transport at a site or sites close to the flavoprotein components of the electron transport pathways. If this is so then the oxidation of all the respiratory substrates tested here would be expected to be sensitive to inhibition by pyrrolnitrin, since they all involve flavoproteins and cytochromes b in the electron transport pathways by which they appear to be oxidized (see figure 2). The data presented in table 4 support this suggestion, since the oxidation of all substrates tested was found to be totally sensitive to inhibition by pyrrolnitrin.

The enzyme exogenous NADH dehydrogenase, appears to be located on the outer face of the inner membrane, of isolated plant mitochondria, and to be able to catalyze the reduction of ferricyanide in a reaction that bypasses the cytochrome b to c step in the electron transport pathways. Hence exogenous NADH-ferricyanide reductase activity is insensitive to antimycin A, while succinate-ferricyanide reductase activity is largely sensitive to it in intact mitochondria, since it involves the antimycin A sensitive step between cytochromes b and c, (see figure 2). These conclusions were confirmed in the present study, as the data presented in table 5 indicate.

TABLE 5.

The Effect of Antimycin A on the Activity of Exogenous NADH-ferri-
cyanide Reductase and Succinate-ferricyanide Reductase Activities,
in Isolated Jerusalem Artichoke Mitochondria.

<u>Additions to the</u> <u>reaction cuvette</u>	<u>Ferricyanide Reductase Activity (ng. ions</u> <u>ferricyanide reduced min.⁻¹ mg. protein⁻¹)</u>	
	<u>Experiment 1.</u>	<u>Experiment 2.</u>
Succinate	525	495
Antimycin A	18	-
NADH	1650	2070

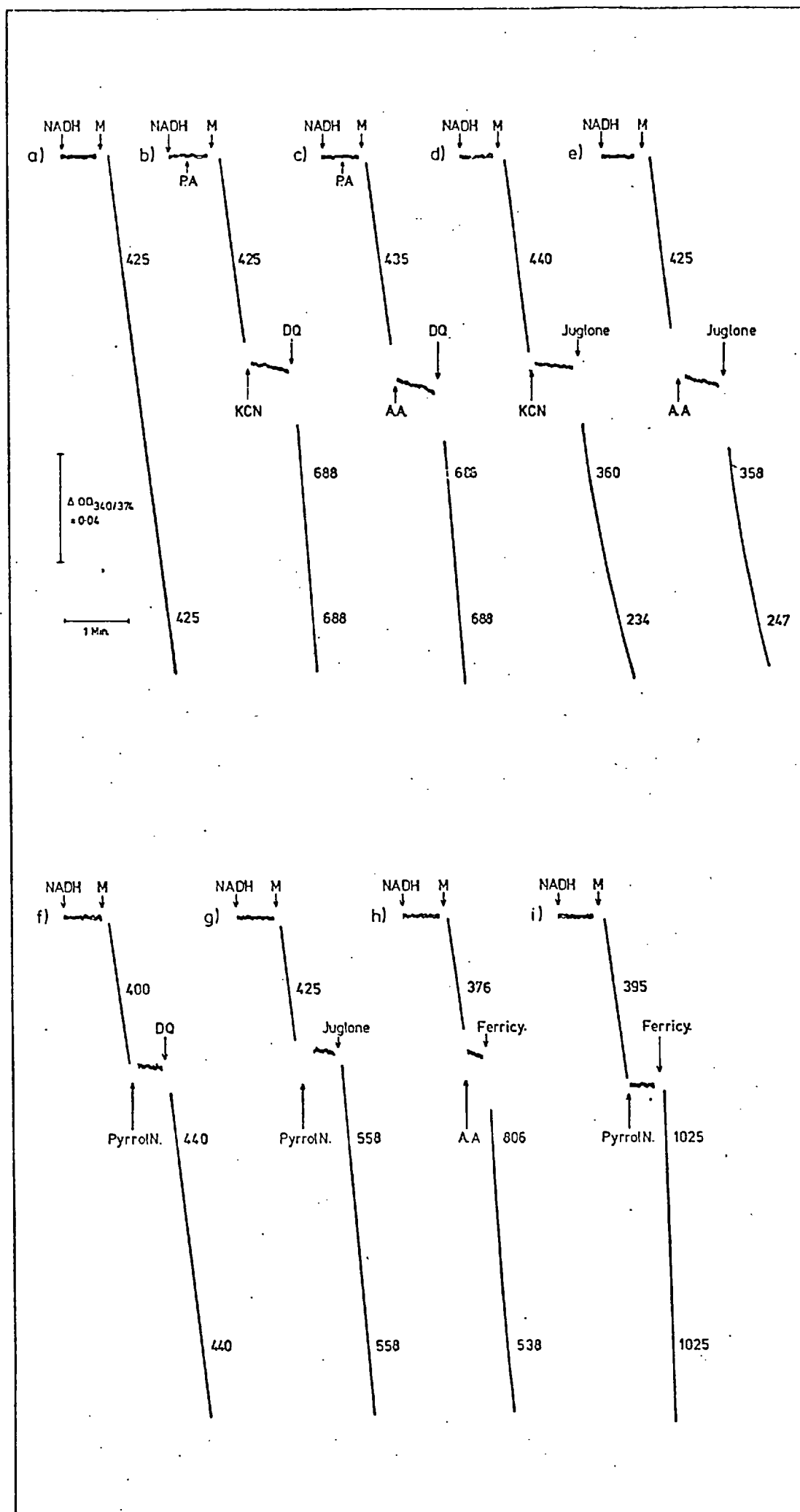
ATP/Pi/MgCl₂ Activated mitochondria (0.2 mg. protein ml.⁻¹), were suspended in standard assay medium containing 1mM KCN, and 200 ng. ions ml.⁻¹ of potassium ferricyanide, and 0.5mM ADP. Additions of succinate (20mM), antimycin A (200 ng. mg. protein⁻¹) and NADH (0.4mM), were added to the reaction cuvette sequentially as indicated, and the constant rates of reduction of ferricyanide were measured spectrophotometrically using the wavelength pair 420-490 nm., as described in Materials & Methods 6.vii. For details of ATP/Pi/MgCl₂ activation of succinate dehydrogenase in plant mitochondria, see Results 1.ii.

The data presented in table 5 also support the location of the enzyme, succinate dehydrogenase, on the inside of the permeability barrier to ferricyanide anions, which is equated with the inner mitochondrial membrane, and the location of exogenous NADH dehydrogenase outside this barrier. The establishment of the location of these two enzymes in relation to the faces of the inner membrane of isolated mitochondria is of importance to a later section of this thesis, in which interaction between exogenous NADH oxidase and succinate oxidase, is investigated.

In addition to exogenous cytochrome c and ferricyanide, juglone and duroquinone were employed as alternative terminal oxidants of the electron transport pathways of isolated plant mitochondria. The data presented in figure 11, show the pattern of oxidation of exogenous NADH catalyzed by isolated Jerusalem artichoke mitochondria using oxygen, duroquinone, juglone and ferricyanide as terminal oxidants of the electron transport pathways. In addition the data indicate the response of the rate of electron transport from exogenous NADH to each oxidant, to the inhibitors piericidin A, potassium cyanide, antimycin A and pyrrolnitrin.

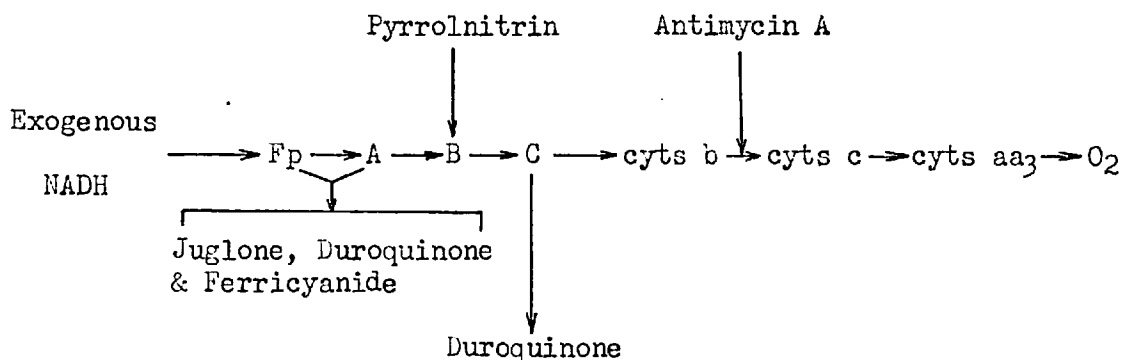
It can be seen from figure 11 that all of the inhibitors, except piericidin A, caused almost total inhibition of electron transport to oxygen, as expected from data already presented (table 4). When duroquinone or juglone were used as the terminal oxidants, the rates of oxidation of exogenous NADH were found to be very similar in the presence of either potassium cyanide or antimycin A, which indicated that acceptance of electrons by these oxidants, was occurring on the substrate side of the antimycin A-sensitive step in the electron transport pathway, as found for beef heart sub-mitochondrial particles (see figure 4).

Figure 11. Inhibitor sensitivity of exogenous NADH-oxygen reductase, exogenous NADH-duroquinone reductase, exogenous NADH-juglone reductase and exogenous NADH-ferricyanide reductase activities, in isolated Jerusalem artichoke mitochondria. Dual wavelength spectrophotometer traces, measured at 340-374 nm., showing the pattern of oxidation of exogenous NADH by oxygen, duroquinone, juglone and ferricyanide. Mitochondria (M, 0.12 mg. protein ml.⁻¹), were suspended in standard assay medium containing 0.5mM ADP. Exogenous NADH (106uM) was added, and the rate of oxidation of NADH was measured by following the rate of decrease in absorbance at 340-374 nm. as described in Materials & Methods 6.v. Inhibitors and alternative terminal oxidants of the electron transport pathways were added as indicated. Numerals under the traces represent the rate of oxidation of exogenous NADH, as nmoles NADH min.⁻¹ mg. protein⁻¹. Inhibitor concentrations were, piericidin A, 100 ng. = P.A; antimycin A, 200 ng. = A.A; pyrrolnitrin, 417 ug. = PyrrolN; all mg. protein⁻¹, and potassium cyanide, 1.0mM = KCN. The concentrations of the alternative oxidants were initially 150 uM for duroquinone and juglone, and 200 ng. ions ml.⁻¹ for ferricyanide, added as potassium ferricyanide.



Duroquinone appeared to be a more efficient oxidant of exogenous NADH than juglone, as comparison of traces (b) and (c), with traces (d) and (e), in figure 11, suggest. This difference might have been a result of duroquinone having greater access to the electron transport pathways of the mitochondria used for these experiments, than did juglone. This was not investigated further as part of the present study.

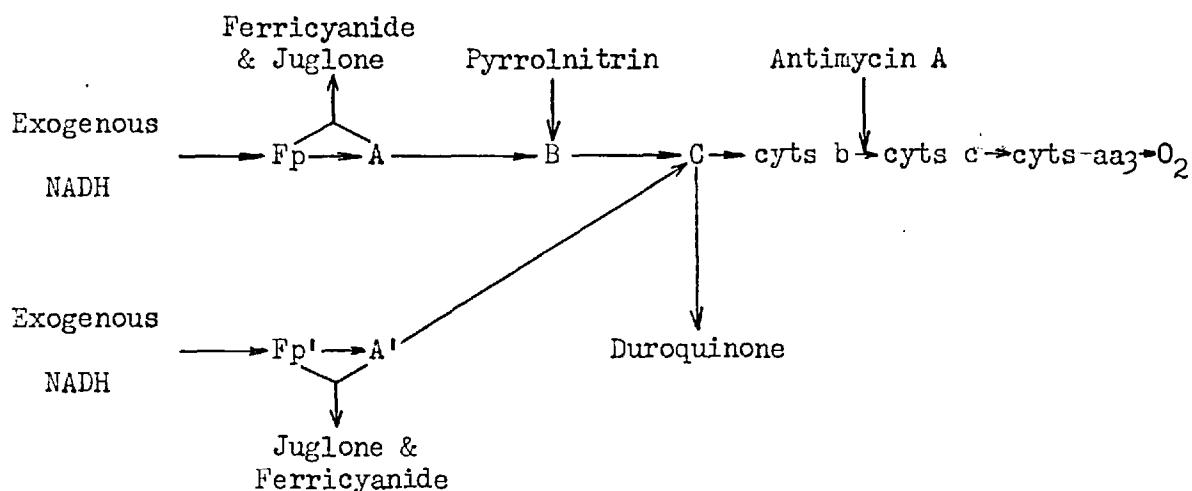
It was of interest to find that pyrrolnitrin caused approximately 36% inhibition of the activity of exogenous NADH-duroquinone reductase (figure 11,b,c & f), while causing a major stimulation to the activities of exogenous NADH-juglone reductase (figure 11, d,e, & g), and exogenous NADH-ferricyanide reductase (figure 11,h & i), respectively. These responses to pyrrolnitrin are not understood yet, and require further investigation. The data in figure 11 are considered to be consistent with the schemes outlined below, for the possible pathways of electron transport from exogenous NADH to the oxidants used, and in relation to the site(s) of inhibition by pyrrolnitrin and antimycin A.



Fp, the flavoprotein associated with exogenous NADH dehydrogenase, and A,B, & C represent hypothetical components of the electron transport pathway, which are able to reduce juglone, ferricyanide and/or duroquinone as indicated.

In this scheme, pyrrolnitrin is seen as inhibiting only electron flow from component C to duroquinone, leaving a maximum of 64% of the total to flow from component A, and/or the flavoprotein associated with exogenous NADH dehydrogenase, to duroquinone.

Alternatively, the partial inhibition of exogenous NADH-duroquinone reductase activity, caused by pyrrolnitrin, might be reflecting the existence of two types of exogenous NADH dehydrogenases in isolated Jerusalem artichoke mitochondria. One type of dehydrogenase could catalyze the reduction of duroquinone via a pathway that is totally inhibited by pyrrolnitrin, while the other type of exogenous NADH dehydrogenase could catalyze the reduction of duroquinone, via a pathway that is totally insensitive to inhibition by pyrrolnitrin. Such a possibility formed the basis of the alternative scheme outlined below, for the possible pathways of electron transport from exogenous NADH, to the oxidants used here.



In this scheme, the flow of electrons from NADH oxidized via the pathway associated with Fp' and A', to duroquinone, would be insensitive to inhibition by pyrrolnitrin, and would be expected to represent a maximum of approximately 64% of the total flow.

v) The effect of chloramphenicol on the respiration and cytochrome content of *Neurospora crassa* mitochondria.

Culturing hyphae of wild type *Neurospora crassa* (Y74A), in a liquid medium containing chloramphenicol, resulted in the appearance of an active cyanide-insensitive oxidase, which was found to be sensitive to inhibition by mCLAM, as can be seen from the data presented in table 6.

In addition to the appearance of an active alternative oxidase, chloramphenicol caused changes in the quantities of certain redox mediators of the electron transport pathways of the mitochondria, which included a very large decrease in light absorbance due to cytochromes b, and an increase in that due to cytochrome c, as the room temperature reduced-minus-oxidized absorbance difference spectra in figure 12 illustrate.

A more detailed study of the effects of chloramphenicol on the absorbance spectra of the cytochromes in *N. crassa* mitochondria, has been carried out by von Jagow & Klingenberg (1972), in which it was found that the cytochrome b that was decreased to the greatest extent, was the one which has an absorbance maximum due to the α band, at 562 nm. wavelength, at a temperature of 77K. This cytochrome is designated cytochrome b_{562} , and corresponds with b_{563} in isolated Jerusalem artichoke mitochondria.

It is not possible to distinguish between individual cytochromes b from the spectra drawn in figure 12, since they were measured at room temperature, when the resolution is much lower than at 77K, but the spectra do indicate a general decrease in absorbance due to cytochromes b, and an increase due to absorbance by cytochromes c, following growth of the hyphae in the presence of chloramphenicol, see trace (c) compared with trace (a).

TABLE 6.

The Effect of KCN and mCLAM on the Activity of Exogenous NADH Oxidase in Mitochondria Isolated from Hyphae of *Neurospora crassa* (Y74A) grown in the Presence and Absence of Chloramphenicol.

<u>Exogenous NADH Oxidase Activity (nmoles NADH min.⁻¹ mg. protein⁻¹).</u>				
<u>Chloramphenicol</u>	<u>Control</u>	<u>+ KCN</u>	<u>+ mCLAM</u>	<u>+ KCN, + mCLAM</u>
-	259.0 ± 2.5	31.9 ± 0.34	214.5 ± 4.8	< 5
+	325.3 ± 2.5	255.1 ± 7.1	160.2 ± 3.7	< 5

Mitochondria (0.1 mg. when KCN was used, or 0.04 mg. protein ml.⁻¹ minus chloramphenicol, and 0.06 mg. when mCLAM was used, or 0.02 mg. protein ml.⁻¹ plus chloramphenicol), were suspended in *N. crassa* assay medium, as described in Materials & Methods 5.ii.b. NADH (50μM) was added, and exogenous NADH oxidase activity measured spectrophotometrically, as described in Materials & Methods 6.v. The inhibitors KCN (1mM) and mCLAM (1.67mM), were added to the reaction cuvette, as indicated. The values for exogenous NADH oxidase activity are the mean of 14 assays ± S.E.M. in each case. The hyphae of *N. crassa* (Y74A), were cultured in the presence and absence of 3g. L⁻¹ chloramphenicol for 24 hours at 30°C and mitochondria isolated as described in Materials & Methods 2.v.

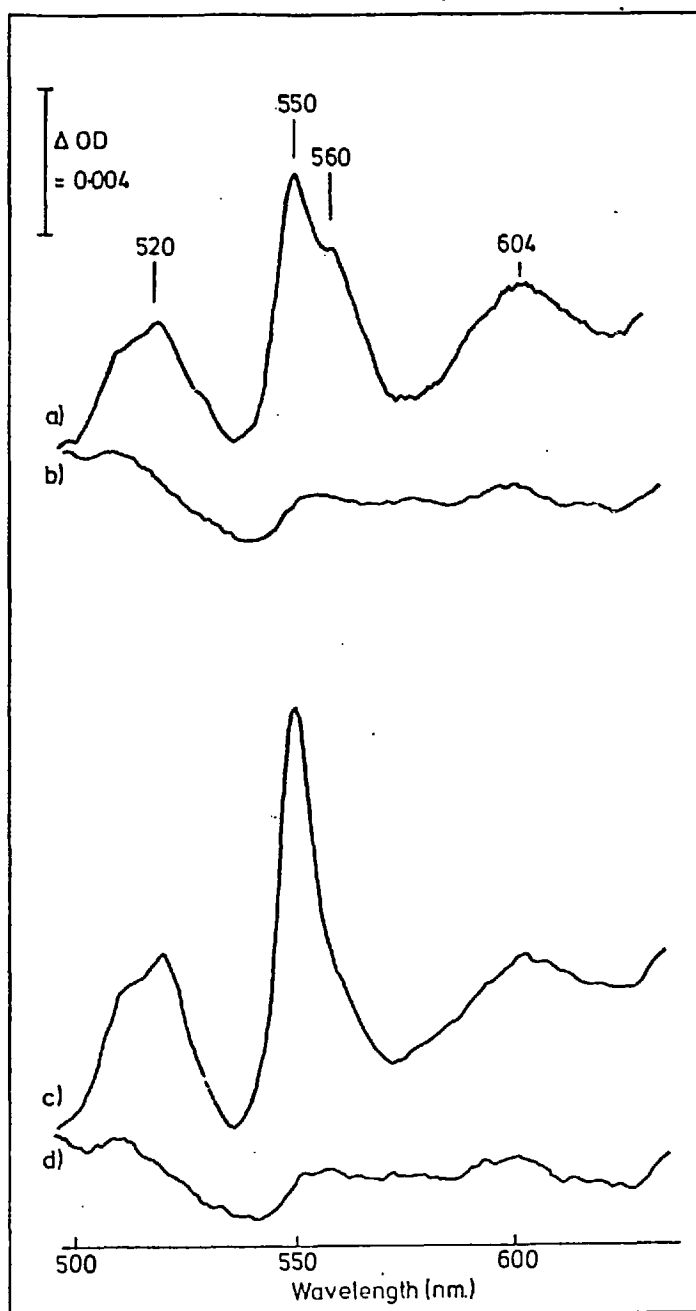


Figure 12. The effect of chloramphenicol on the cytochrome content of *Neurospora crassa* mitochondria. Split-beam spectrophotometer traces of *N. crassa* (Y74A) mitochondria from hyphae grown in the absence and presence of chloramphenicol (CAP), drawn as described in Materials & Methods 6.vi.a. Traces (a) and (c) are dithionite reduced minus air oxidized spectra, and traces (b) and (d) are air oxidized minus air oxidized spectra. Traces (a) & (b), control mitochondria (0.4 mg. protein ml.⁻¹); traces (c) & (d), +CAP mitochondria (0.3 mg. protein ml.⁻¹).

vi) Possible pathways for the oxidation of endogenous NADH.

The oxidation of malate in mitochondria isolated from plant tissue, appears to be catalyzed by two enzymes, malate dehydrogenase and an NAD^+ -linked malic enzyme. Also, there is evidence obtained with wheat mitochondria, for the NADH produced as the result of malic enzyme activity, being located in a functionally separate compartment of the mitochondrial matrix, from that in which the enzyme malate dehydrogenase exists, at least under respiratory state 3 conditions. This was concluded because added oxaloacetate was unable to cause a large but temporary inhibition of the rate of oxygen consumption by the mitochondria when catalyzing the oxidation of malate under state 3 conditions, which was observed with pyruvate as the supplied substrate (Brunton & Palmer, 1973; Brunton, 1975). In the present study, evidence was found for a similar functional separation of malate dehydrogenase and malic enzyme in mitochondria isolated from Jerusalem artichoke and white potato tubers, and from mung bean hypocotyls, as the data presented in this section of the results illustrate.

a) Location of the enzymes catalyzing the oxidation of malate.

In wheat coleoptile mitochondria, Brunton & Palmer (1973), found that the activity of malate-ferricyanide reductase was only partially sensitive to inhibition by piericidin A, and almost totally sensitive to inhibition by antimycin A, indicating that all of the initial steps of malate oxidation, took place on the matrix side of the inner membrane of these mitochondria. The partial sensitivity of malate-ferricyanide reductase activity to piericidin A, indicated that NADH arising from malate oxidation could be oxidized via more than one pathway, with one pathway lacking the piericidin A-sensitive step.

Similar results to those just described for wheat

mitochondria, have been observed with Jerusalem artichoke mitochondria, as the data in figure 13 show. Thus, the conclusions reached above concerning malate oxidation in isolated wheat mitochondria, also apply to Jerusalem artichoke mitochondria. (The stimulation of malate-ferri-cyanide reductase activity, following the addition of NAD^+ which is also shown in this figure, will be investigated further in a later part of this section of the results).

The enzyme malate dehydrogenase can be shown to be located mainly in the matrix of isolated intact plant mitochondria, by measuring its latency in catalyzing the reduction of oxaloacetate by NADH, as the data presented in table 7 illustrate. It can be seen that the activity of malate dehydrogenase increased greatly following the disruption of the mitochondrial membranes by the detergent, TX 100. Oxaloacetate can freely enter the matrix of intact plant mitochondria (Avron & Biale, 1957; Hulme, Rhodes & Woollorton, 1967; Douce & Bonner, 1972; Brunton & Palmer, 1973; Brunton, 1975), and so these results confirm the impermeability of the inner mitochondrial membrane, to NADH (see Lehninger, 1951; Hogeboom & Schneider, 1953; Chance & Williams, 1956; Pullman & Racker, 1956; and Devlin & Bedell, 1960), and that most of the malate dehydrogenase of isolated potato mitochondria, is located in the matrix space, which is in agreement with the findings of Brunton (1975), who found similar results with wheat mitochondria.

b) The functional relationship between malic enzyme and malate dehydrogenase.

The ability of oxaloacetate to inhibit the rate of oxidation of NAD^+ -linked substrates catalyzed by isolated plant mitochondria, was tested in order to investigate the possibility that the various

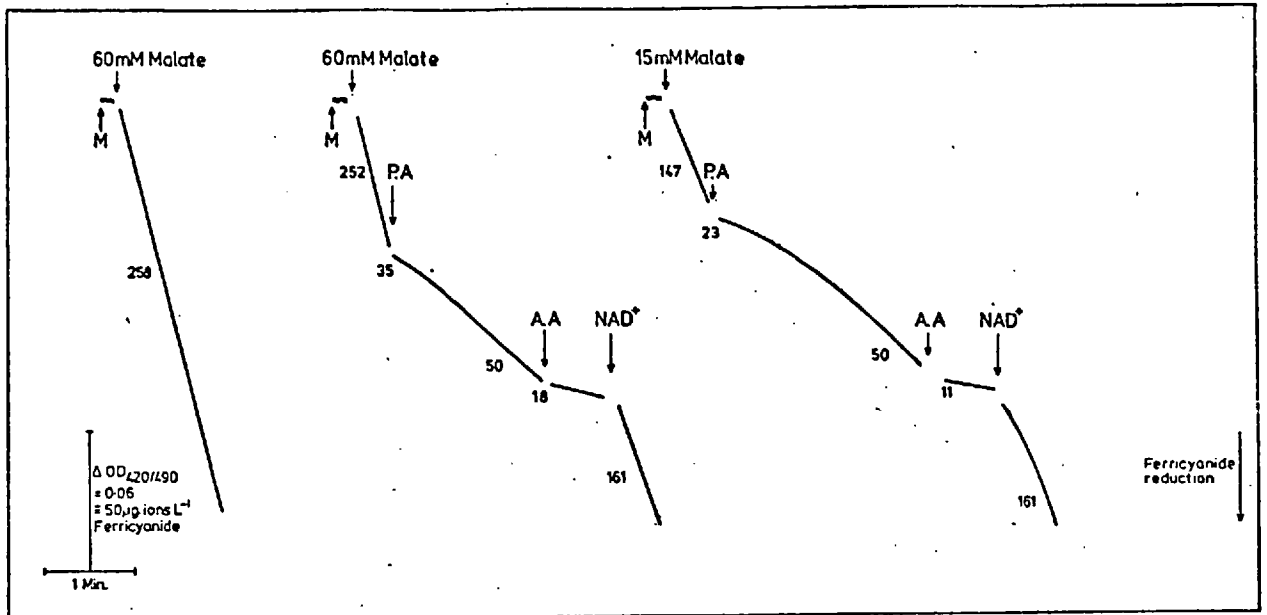


Figure 13. The effect of piericidin A, antimycin A and NAD^+ , on the activity of malate-ferricyanide reductase in Jerusalem artichoke mitochondria. Dual wavelength spectrophotometer traces showing the pattern of reduction of potassium ferricyanide, as a decrease in absorbance at 420-490 nm. as described in Materials & Methods 6.vii. Mitochondria (M, 0.83 mg. protein⁻¹), were suspended in 3ml. standard assay medium containing 1mM ADP, 1mM potassium cyanide and 200 ng. ions potassium ferricyanide ml.⁻¹. P.A, piericidin A (100 ng.), A.A, antimycin A (300 ng.) NAD^+ , nicotinamide adenine dinucleotide (1mM). Numerals below the traces represent the rate of reduction of potassium ferricyanide as ng. ions ferricyanide reduced min.⁻¹ mg. protein⁻¹.

TABLE 7.

Estimation of the Activity of Malate Dehydrogenase in Intact and Disrupted Mitochondria Isolated from White Potato Tubers.

<u>Exogenous NADH-Oxaloacetate</u>		<u>Control Rate as a</u>
<u>Oxido - Reductase Activity</u>		<u>percentage of the</u>
<u>Control</u>	<u>+ TX100</u>	<u>rate plus TX100</u>
0.32	7.85	4.1

Mitochondria (0.15 mg. protein ml.⁻¹), were suspended in standard assay medium containing 1mM KCN, and 2mM OAA, pH 7.2. NADH (200uM), was then added to the reaction cuvette, and the rate of oxidation of NADH by OAA was measured fluorimetrically as described in Materials & Methods 6.xi. One minute after the attainment of a constant rate, TX100 (5 ul. of a 20% aqueous solution 2.5 ml.⁻¹ reaction volume), was added to the reaction cuvette, and the resultant rate of oxidation of NADH recorded. Units of NADH oxidation are umoles NADH min.⁻¹ mg. protein⁻¹.

dehydrogenases responsible for catalysis of the initial steps of oxidation of each substrate, existed in different compartments of the mitochondrion with respect to malate dehydrogenase. Figure 14 shows data obtained with Jerusalem artichoke mitochondria, where 0.2mM oxaloacetate caused a large but temporary inhibition of the rate of oxygen consumption due to oxidation of pyruvate or citrate, under state 3 conditions, but malate oxidase activity was only inhibited to a small extent, and permanently. These results were similar to those obtained by Brunton & Palmer (1973) with wheat mitochondria.

Douce & Bonner (1972) have shown the almost total inhibition of the rate of oxygen consumption resulting from the oxidation of NAD^+ -linked substrates, caused by oxaloacetate, to be a result of oxidation of available NADH by the oxaloacetate rather than via the electron transport pathways to oxygen. Once the oxaloacetate had been completely reduced by the NADH, in a reaction catalyzed by malate dehydrogenase, oxidation of the NADH by oxygen resumed, as shown by the recovery of the rate of oxygen consumption following the period of inhibition. As expected, the duration of the period of inhibition caused by oxaloacetate, was found to be directly proportional to the amount of oxaloacetate added to the mitochondria. Thus it was found that, in the presence of malate dehydrogenase and oxaloacetate, the reaction in which endogenous NADH is oxidized by oxaloacetate, competed totally for available NADH, with the NADH dehydrogenases associated with the electron transport pathways to oxygen.

Similar results to those shown in figure 14, have been obtained with avocado pear mitochondria by Avron & Biale (1957), and with mitochondria isolated from white potato tubers and mung bean hypocotyls, in the present study, which is in agreement with the results

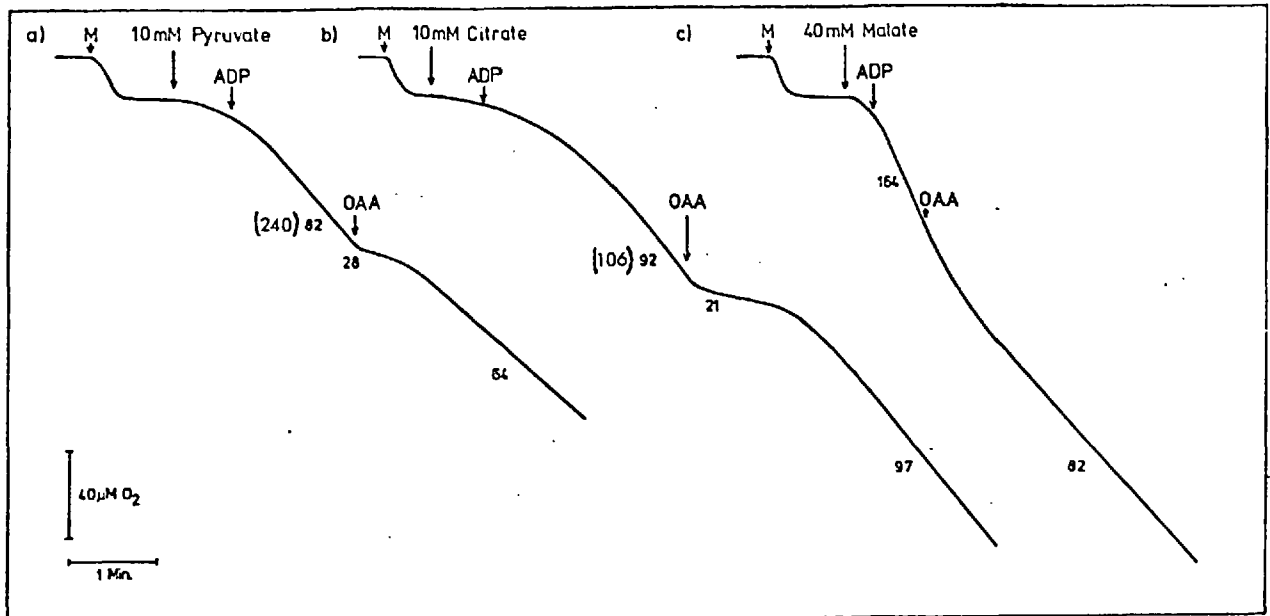
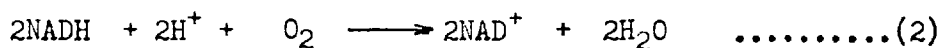


Figure 14. The effect of oxaloacetate on pyruvate, citrate and malate oxidation in Jerusalem artichoke mitochondria, under state 3 conditions. Oxygen electrode traces showing the pattern of oxygen consumption. Mitochondria (M, 0.52 mg. protein ml.⁻¹), were suspended in standard assay medium containing 1mM malate for traces (a) and (b), and 0.35mM TPP, for trace (a). Concentrations of additions to reaction cuvette were ADP, 1.5mM, OAA, 0.2mM (pH 7.2). Numerals below traces represent the rate of oxygen consumption as nmoles oxygen min.⁻¹ mg. protein⁻¹ as measured while the values given in parentheses were estimated from the time taken for the removal of approximately 100 nmoles OAA. The final reaction volume was 0.5 ml.

of preliminary experiments carried out by Brunton (1975). It is therefore concluded that malic enzyme and/or its associated NAD^+ , can be separate from malate dehydrogenase in all isolated plant mitochondria tested, (see also figures 15 & 16).

It is possible to estimate the rate of reduction of the added oxaloacetate, by NADH resulting from the activity of the NAD^+ -linked dehydrogenases which are located in the malate dehydrogenase compartment of isolated plant mitochondria. This is done by estimating the duration of the period of inhibition of the rate of oxygen consumption caused by a known amount of oxaloacetate, in this case approximately 100 nmol added to a final reaction volume of 0.5ml., and converting the value obtained into the units nmoles of oxaloacetate reduced $\text{min.}^{-1} \text{ mg. protein}^{-1}$.

The reactions for the reduction of oxaloacetate and of oxygen, by NADH which are catalyzed by the enzyme malate dehydrogenase or the enzymes associated with the electron transport pathways, respectively, may be represented by the following equations:



It can be seen from equation (1), that the molar ratio between NADH and oxaloacetate is one, while the molar ratio between NADH and oxygen, would be expected to be two, by reference to equation (2), a value which was confirmed experimentally for isolated Jerusalem artichoke mitochondria (table 13). Thus it has been possible to estimate the potential maximum rate of NAD^+ reduction resulting from the oxidation of each NAD^+ -linked substrate which occurs in the malate

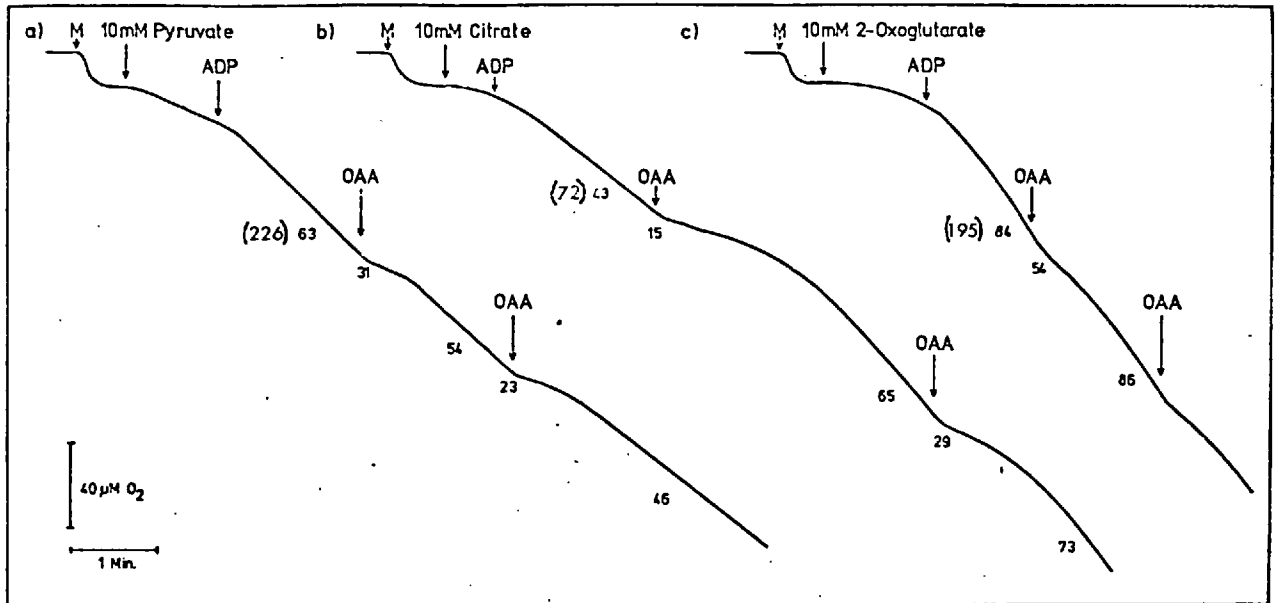


Figure 15. The effect of oxaloacetate on pyruvate, citrate and 2-oxo-glutarate oxidation in white potato mitochondria, under state 3 conditions. Oxygen electrode traces showing the pattern of oxygen consumption. Mitochondria (M, 0.7 mg. protein ml^{-1}), were suspended in standard assay medium containing 1.5mM ADP and 0.35mM TPP. Other details were as described for figure 14.

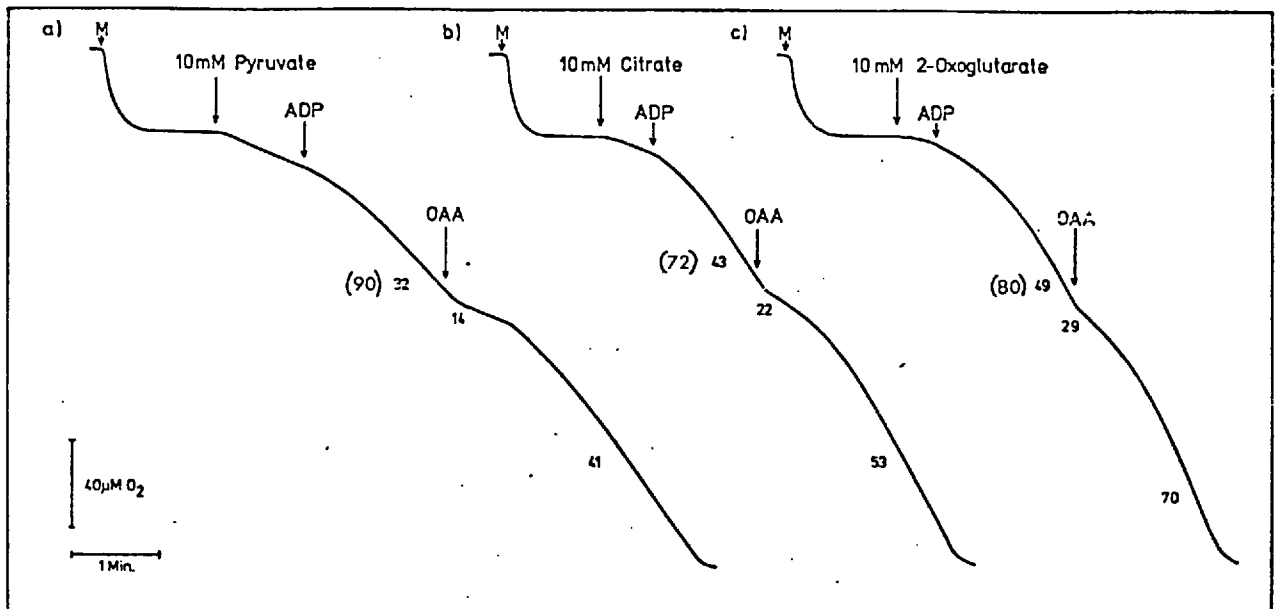


Figure 16. The effect of oxaloacetate on pyruvate, citrate and 2-oxo-glutarate oxidation in mung bean mitochondria, under state 3 conditions. Oxygen electrode traces showing the pattern of oxygen consumption. Mitochondria (M, 1.4 mg. protein ml^{-1}), were suspended in standard assay medium, containing 1.5mM ADP and 0.35mM TPP. Other details were as described for figure 14.

dehydrogenase compartment of the mitochondrion, since it would be expected to be equal to the rate at which oxaloacetate is reduced by NADH because of the molar ratio of one for the reaction, shown in equation (1) above. The estimated potential maximum rate of NAD^+ reduction has been compared with the measured rate of oxygen consumption under state 3 conditions, for those NAD^+ -linked substrates oxidized in the malate dehydrogenase compartment of the mitochondrion, and in each case, the estimated rate of NAD^+ reduction exceeded the equivalent measured rate of oxygen consumption. This was found to be true for mitochondria isolated from Jerusalem artichoke (figure 14), and white potato (figure 15) tubers, and mung bean hypocotyls (figure 16), when pyruvate, citrate or 2-oxoglutarate were the supplied respiratory substrates. The potential maximum equivalent rate of oxygen consumption is shown in parentheses alongside the measured value, in figures 14, 15, and 16, and was obtained by dividing the estimated rate of NAD^+ reduction by two, the molar ratio between NADH and oxygen.

The finding that the estimated potential maximum rate of reduction of NAD^+ by pyruvate, citrate or 2-oxoglutarate appeared to exceed the measured rate of oxygen consumption in all the mitochondria tested, indicates that the rate of production of NADH in the matrix of these mitochondria, may exceed the rate at which it is oxidized by oxygen, even under state 3 conditions. If this is so, then the finding that the maximum measured rate of oxygen consumption under state 3 conditions, differed according to which NAD^+ -linked substrate was being oxidized by the mitochondria, might be indicating that a different step is limiting the rate of electron transport to oxygen from the NADH produced during the initial step of oxidation of each substrate (see figures 14, 15, and 16). This could be evidence favouring the involvement of a different set of electron transport pathways

in the oxidation of each of the NAD^+ -linked substrates, pyruvate, citrate and 2-oxoglutarate. Alternatively, the rate of reduction of NAD^+ by each NAD^+ -linked substrate, in the absence of oxaloacetate, might be limiting the rate of consumption of oxygen. For example, under these conditions each NAD^+ -linked substrate dehydrogenase might be allosterically controlled by the redox poise of endogenous NAD^+/NADH . There is some evidence that pyruvate dehydrogenase (Crompton & Laties, 1971), and isocitrate dehydrogenase (Cox & Davies, 1967), isolated from plant mitochondria, are sensitive to the $\text{NADH}:\text{NAD}^+$ ratio, with a high value for this ratio resulting in each enzyme becoming inhibited, as required to explain the data presented here.

The inhibition of malate oxidase activity by 0.2mM oxaloacetate shown in figure 14, could be due to competitive inhibition of the malic enzyme by the oxaloacetate, as reported by Macrae (1971a, b, c) and Brunton & Palmer (1973), and Brunton (1975). The data presented in figure 17 support this suggestion, and thus it appears that oxaloacetate has access to the active site of the malic enzyme, under conditions when it does not appear to have access to the NADH associated with this enzyme. (There was possible evidence for inhibition of pyruvate dehydrogenase caused by oxaloacetate in figures 14a and 15a; observations which were not investigated further in the course of the present study).

Douce & Bonner (1972) reported that malate dehydrogenase in mung bean mitochondria, could catalyze the oxidation of NADH produced by the activity of the malic enzyme and the other NAD^+ -linked substrate dehydrogenases, and concluded that the NAD^+ in the matrix of the mitochondria, was acting as a common pool to all the enzymes. There were however, two differences at least between the experiments reported

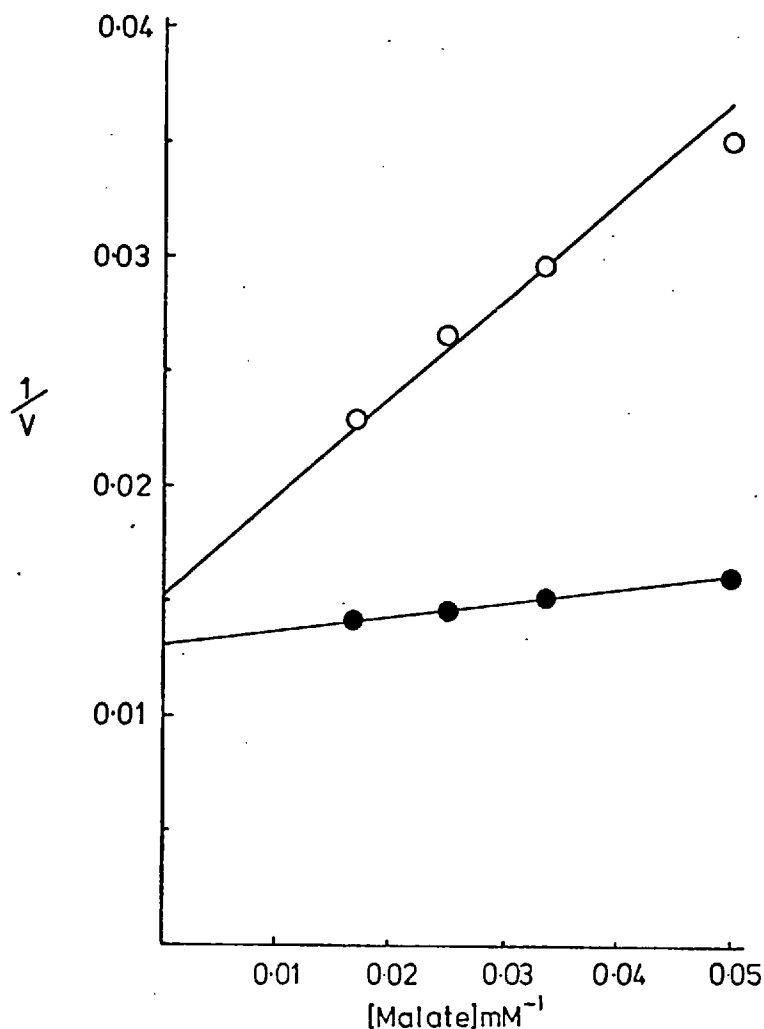


Figure 17. Inhibition of malate oxidation by oxaloacetate in white potato mitochondria. A double-reciprocal plot of $1/V$ versus $1/S$, where V = the velocity of malate oxidase activity (state 3), as nmoles oxygen min.^{-1} mg. protein, in the absence (●) and presence (O), of 0.2mM OAA, and S = substrate concentration as mM malate. Mitochondria ($0.77 \text{ mg. protein ml.}^{-1}$) were suspended in standard assay medium containing 1mM ADP. Malate was then added to give the appropriate initial concentration, and the resultant constant rate of oxygen consumption measured in the absence or presence of OAA, as described in Materials & Methods 5.

by these authors, and those reported by Avron & Biale (1957), Brunton & Palmer (1973), Brunton (1975) and in the present thesis, figures 14, 15 and 16.

Firstly, Douce & Bonner (1972) purified their mitochondria, by passing them through a discontinuous sucrose density gradient under the influence of a centrifugal force, using the method described by Douce, Christensen & Bonner (1972). In order to test whether this type of treatment could cause the NADH associated with the malic enzyme, to appear in the presence of malate dehydrogenase, as required to explain the data of Douce & Bonner (1972), Jerusalem artichoke mitochondria were purified by sucrose density gradient centrifugation, as described by Douce, Christensen & Bonner (1972), see also Materials & Methods 3. Experiments of the type illustrated in figures 14, 15 and 16, were then repeated using the purified mitochondria, and figure 18 shows results obtained. It can be seen from this figure, that the purification procedure did not affect the pattern of inhibition of inhibition caused by oxaloacetate, to the rate of oxygen consumption resulting from the oxidation of pyruvate, citrate or malate. Thus, it was considered unlikely that it was the purification step which resulted in the difference between the data of Douce & Bonner (1972), and those of the other authors.

The second difference between the experiments reported by Douce & Bonner (1972), and those reported by the other authors given above, was the metabolic state of the mitochondria at the time of adding the oxaloacetate to them. Douce & Bonner (1972) added the oxaloacetate under state 4 conditions, while Avron & Biale (1957), Brunton & Palmer (1973), Brunton (1975) and the present author, all added the oxaloacetate under state 3 conditions. An experiment of the type illustrated in figure 14c, was therefore carried out under

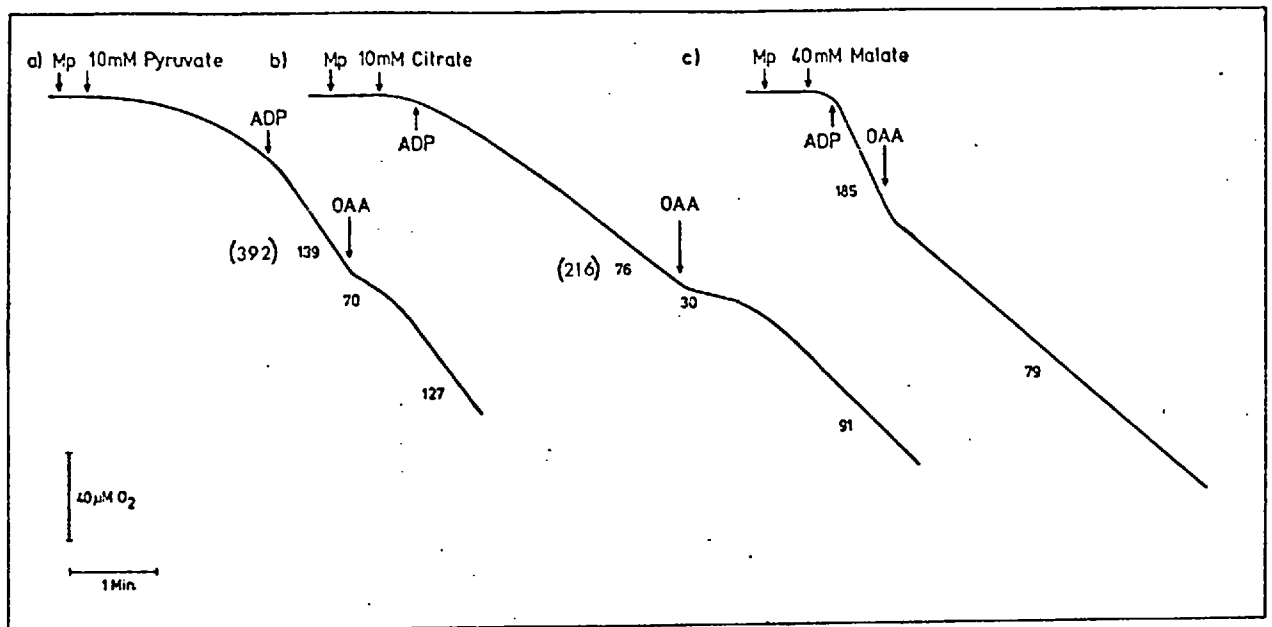


Figure 18. The effect of oxaloacetate on pyruvate, citrate and malate oxidation in 'purified' Jerusalem artichoke mitochondria, under state 3 conditions. Oxygen electrode traces showing the pattern of oxygen consumption. Mitochondria (Mp, $0.44 \text{ mg. protein ml.}^{-1}$), which had been 'purified' by passage through a discontinuous sucrose concentration gradient, as described in Materials & Methods 3., were suspended in standard assay medium containing 1.5 mM ADP and for traces (a) and (b), 0.35 mM TPP . Concentration of OAA was 0.2 mM initially and the reaction volume was 0.5 ml. Other details were as described for figure 14.

state 4 conditions, in both washed and purified Jerusalem artichoke mitochondria, and figure 19 (a) and (b), show that there was evidence for oxaloacetate being able to cause a temporary inhibition of the rate of oxygen consumption, resulting from malate oxidation under state 4 conditions, with purification having little or no effect upon the pattern of oxygen consumption catalyzed by these mitochondria. Thus it was found that the transition from state 3 to state 4, caused the pattern of response of malate oxidase activity in Jerusalem artichoke mitochondria to approach that found by Douce & Bonner (1972). These authors however, found that the degree of inhibition by oxaloacetate, was almost total indicating that in their mitochondria, all of the NADH associated with the malic enzyme appeared in the malate dehydrogenase compartment. In the experiments reported here (figure 19), oxaloacetate still only caused a partial, although temporary, inhibition of state 4 malate oxidase activity, indicating that only a proportion of the NADH associated with the malic enzyme was appearing in the malate dehydrogenase compartment of the mitochondria.

The data presented by Avron & Diale (1957), Brunton & Palmer (1973), Brunton (1975), and by the present author (figures 14, 15, 16, 18 and 19), indicate that endogenous NAD^+ can function as at least two distinct pools in the mitochondria tested, one pool associated with the malic enzyme, and the other pool associated with malate dehydrogenase. Brunton & Palmer (1973) and Brunton (1975), concluded that their data were consistent with a kinetic compartmentation of the malic enzyme and malate dehydrogenase, together with all of the other NAD^+ -linked dehydrogenases, as outlined in their scheme, which has been reproduced here in figure 20.

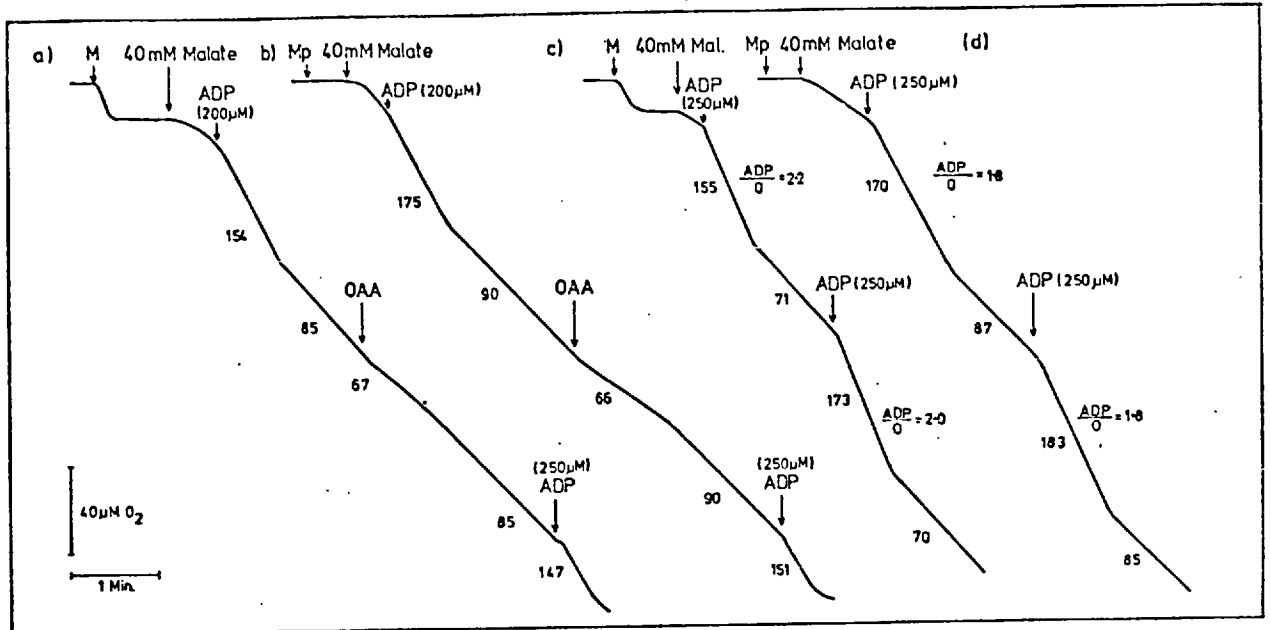


Figure 19. The effect of discontinuous sucrose density gradient purification, on oxidative phosphorylation and inhibition of state 4 malate oxidase activity by oxaloacetate, in Jerusalem artichoke mitochondria. Oxygen electrode traces showing the pattern of oxygen consumption. Mitochondria (M, 0.52 mg. protein ml.⁻¹ = washed; 0.44 mg. protein ml.⁻¹ Mp = 'purified'), were suspended in standard assay medium, and the rate of oxygen consumption was measured using an oxygen electrode, as described in Materials & Methods 5. Mitochondria were 'purified' as described in Materials & Methods 3, and the numerals under the traces represent the rate of oxygen consumption as nmoles min.⁻¹ mg. protein⁻¹. Oxaloacetate (OAA) concentration = 0.2mM, pH 7.2.

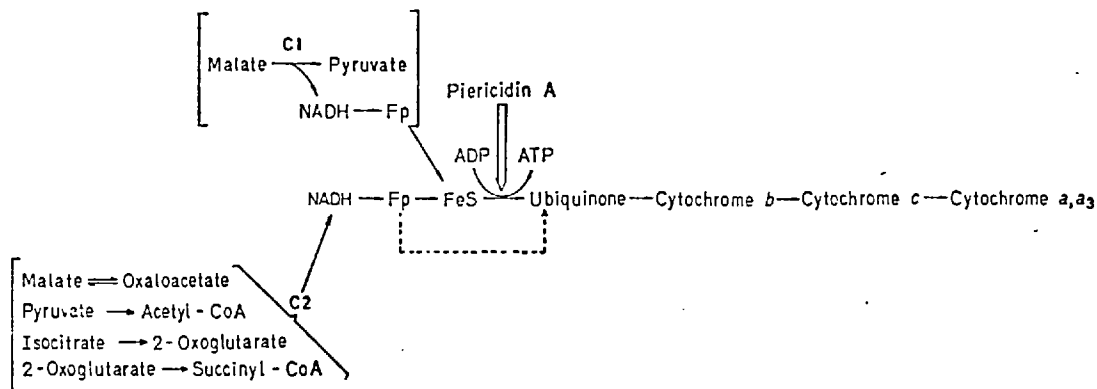


Figure 20. "Possible pathways of malate oxidation. This scheme illustrates the spatial separation of malate dehydrogenase and malic enzyme, together with a flavoprotein component. It also includes a possible pathway to by-pass the piericidin A-sensitive step, which will only oxidise NADH from the malate dehydrogenase compartment. Fp, flavoprotein; FeS, iron-sulphur centre." After Brunton & Palmer (1973).

c) Estimation of the extent of reduction of endogenous pyridine nucleotide.

The failure of oxaloacetate to cause a large and transient inhibition of malate oxidase activity under state 3 conditions in wheat mitochondria, suggested the existence of at least two pools of endogenous NAD^+ , one associated with the malic enzyme, and the other with the other NAD^+ -linked mitochondrial carboxylic acid dehydrogenases. This prompted Brunton & Palmer (1973) to investigate the extent of reduction of the endogenous pyridine nucleotide caused by malate or citrate as the supplied respiratory substrate, to wheat mitochondria.

With malate as substrate, Brunton & Palmer (1973) found that the onset of anaerobiosis was accompanied by a biphasic increase in absorbance of light, measured at the wavelength 340-374 nm., and using a dual-wavelength spectrophotometer. The corresponding absorbance increase with citrate as substrate however, was monophasic. The two results represented evidence for two pools of NAD^+ associated with the oxidation of malate, and one pool associated with the oxidation of pyruvate, citrate and 2-oxoglutarate, together with the enzyme, malate dehydrogenase, as shown in figure 20. If the absorbance increase at anaerobiosis represents the reduction by malate of both pools of NAD^+ shown in figure 20, while that observed with citrate as the supplied substrate, represents the reduction of the pool of NAD^+ located in the malate dehydrogenase compartment, as suggested by Brunton & Palmer (1973) and Brunton (1975), then it seems reasonable to expect one of the phases of reduction of NAD^+ by malate to be equal in magnitude, to the single phase of reduction of NAD^+ by citrate. That this was not found to be the case, can be seen by reference to figure 5 in Brunton & Palmer (1973), in which it can be seen that neither the first nor the second phase of absorbance increase at 340-374 nm., was as great as the single phase of absorbance measured for citrate. This suggested that the arrangement of endogenous NAD^+ , and the NAD^+ -linked dehydrogenases in isolated wheat mitochondria, are probably different from the arrangement indicated by Brunton & Palmer (1973) and Brunton (1975), and shown here in figure 20.

That the scheme proposed for wheat mitochondria was less likely to apply to isolated Jerusalem artichoke mitochondria, than to isolated wheat mitochondria, can be seen from the data presented in figure 21. Thus, upon the transition from aerobic to anaerobic with citrate as supplied substrate, (trace b), the absorbance increase was

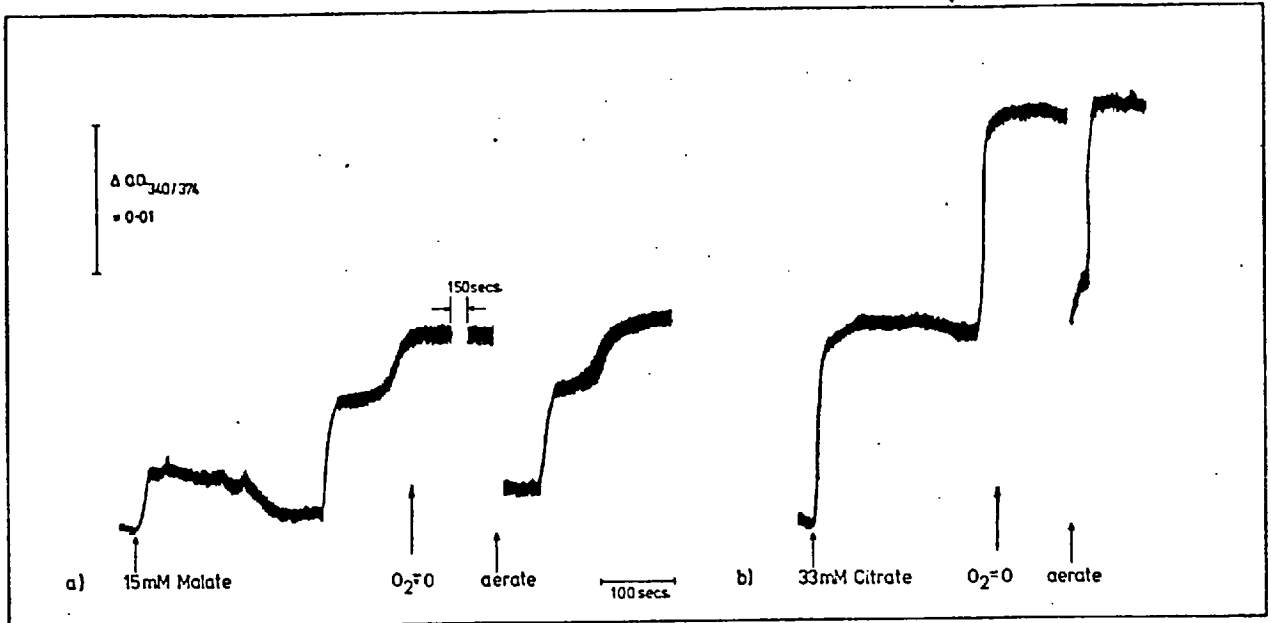


Figure 21. Reduction and oxidation changes of endogenous pyridine nucleotide in Jerusalem artichoke mitochondria. Dual wavelength spectrophotometer traces measured at the wavelength pair 340-374 nm. showing the pattern of reduction and oxidation of endogenous pyridine nucleotide. Mitochondria ($2.6 \text{ mg. protein ml.}^{-1}$) were suspended in standard assay medium, substrate additions made to the reaction cuvette as indicated, and absorbance changes recorded as shown, and as described in Materials & Methods 6.i.

monophasic, and the total absorbance change from the addition of citrate to anaerobiosis was approximately twice that measured for malate as the supplied substrate (trace a). It could be therefore, that the biphasic increase in absorbance at 340-374 nm. seen with malate, is reflecting the existence of two kinetically separate pools of NAD^+ associated specifically with malate oxidation, and probably with the malic enzyme.

It is assumed that absorbance changes occurring in isolated plant mitochondria at 340-374 nm., are mainly a result of reduction or oxidation of endogenous NAD^+ , since no evidence has been found for the presence in these mitochondria, of significant amounts of NADP^+ (Hawker & Laties, 1963; Harmey, Ikuma & Bonner, 1966; Ikuma, 1967).

Further support for the suggestion made above, that the arrangement of endogenous NAD^+ and NAD^+ -linked substrate dehydrogenases, in isolated plant mitochondria is probably different from that proposed by Brunton & Palmer (1973) and Brunton (1975) for wheat mitochondria, came from the results of experiments using the inhibitor of electron transport, piericidin A. Experiments will now be described, in which piericidin A has been used to further test the hypothesis that NAD^+ is unlikely to exist as a simple soluble pool common to all of the NAD^+ -linked substrate dehydrogenases, as proposed by Douce & Bonner (1972), and to test the hypothesis that the NAD^+ associated with the NAD^+ -linked dehydrogenases, excluding malic enzyme, is unlikely to exist as a common pool in the malate dehydrogenase compartment of the isolated plant mitochondria tested, as suggested by Brunton & Palmer (1973) and Brunton (1975).

d) The effect of piericidin A on the rate of oxidation of NAD⁺-linked respiratory substrates.

A concentration of piericidin A, which was too low to cause inhibition of succinate oxidase activity (see table 4), was found to have two effects on the activity of malate oxidase, measured under state 3 conditions. Firstly it caused the NADH associated with the malic enzyme, to appear in the malate dehydrogenase compartment of the mitochondrion, and secondly it caused a partial inhibition of the rate of malate oxidase activity.

These effects were seen as a biphasic pattern of oxygen consumption following the addition of the piericidin A to the mitochondria, as shown in figures 22 and 23. Proof that the initial slower phase of oxygen consumption, was a result of reduction by NADH of accumulated oxaloacetate, came from the observation that a subsequent addition of oxaloacetate caused a similar effect, i.e. an initial high level of inhibition followed by recovery of the rate of oxygen consumption. Thus NADH associated with malic enzyme activity, had appeared in the malate dehydrogenase compartment. In addition, the pattern of oxygen consumption following the addition of piericidin A, was similar to that already seen for the effect of oxaloacetate on the state 3 activity of pyruvate, and citrate oxidases (figures 14, 15 and 16). Also, Palmer & Arron (1976) have shown that the rate of consumption of oxygen due to state 3 malate oxidase activity in isolated Jerusalem artichoke mitochondria, was inhibited immediately to a constant rate, by piericidin A when a system was present in the assay medium for the removal of oxaloacetate, in this case glutamate and glutamate transaminase, were used. Thus, when no oxaloacetate could accumulate in the mitochondria, following malate dehydrogenase activity, then no initial phase of almost total inhibition of malate

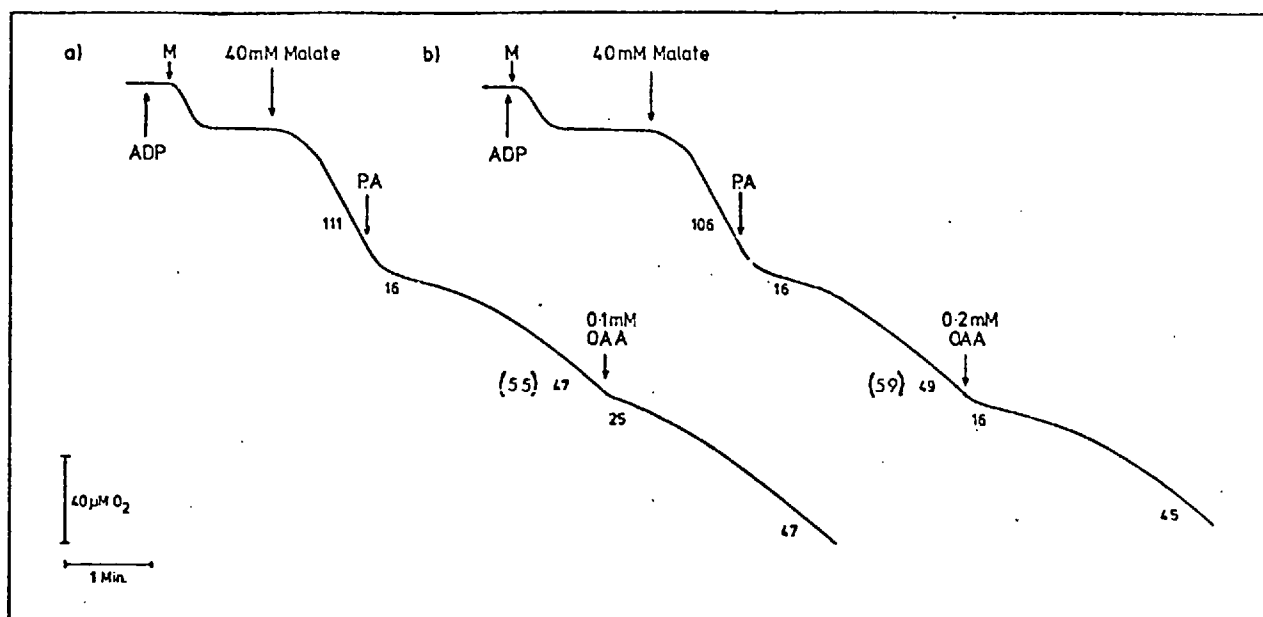


Figure 22. The effect of the sequential addition of piericidin A and oxaloacetate, on state 3 malate oxidase activity in Jerusalem artichoke mitochondria. Oxygen electrode traces showing the pattern of oxygen consumption. Mitochondria (M, 0.7 mg. protein ml.⁻¹), were suspended in standard assay medium, and additions made to the reaction cuvette, as indicated. The initial concentrations of additions were ADP, 1.5mM, piericidin A (P.A), 50 ng., OAA, pH 7.2, as shown. Numerals under the traces represent the rate of oxygen consumption as nmoles oxygen min.⁻¹ mg. protein.⁻¹ as measured, while the values given in parentheses were estimated from the time taken for the removal of the oxaloacetate; the final reaction volume was 0.5 ml., as described for figure 14.

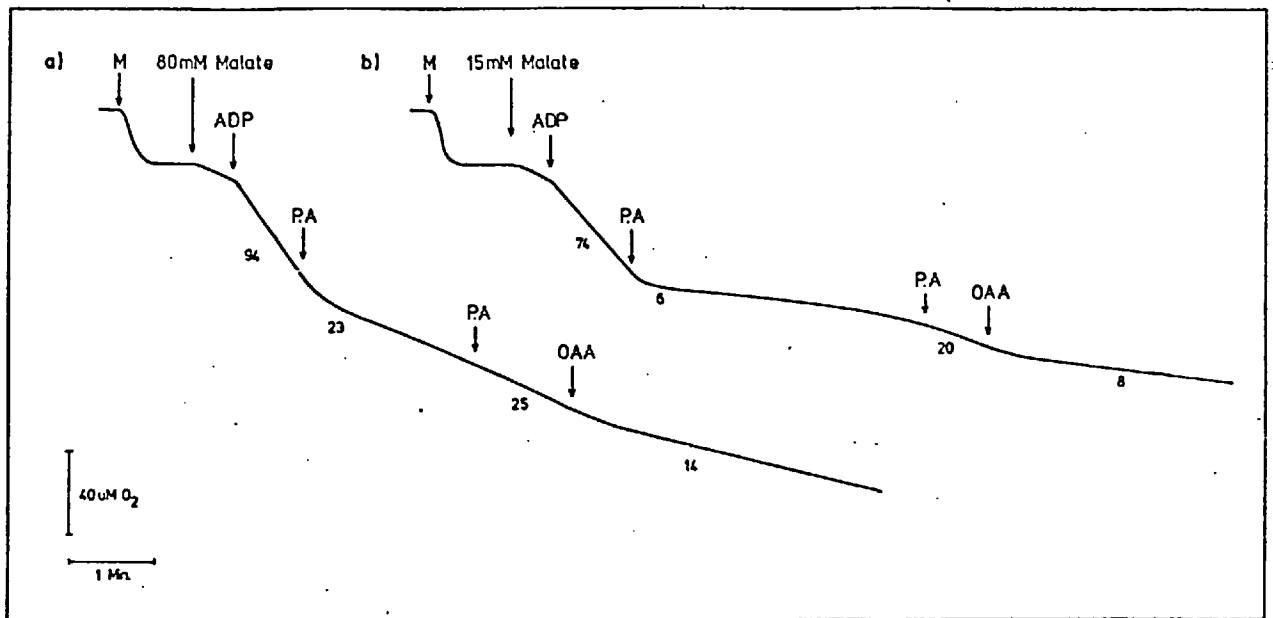


Figure 23. The effect of the sequential addition of piericidin A and oxaloacetate, on state 3 malate oxidase activity in mung bean mitochondria. Oxygen electrode traces showing the pattern of oxygen consumption. Mitochondria (M , $1.4 \text{ mg. protein ml.}^{-1}$), were suspended in standard assay medium, and additions made to the reaction cuvette as indicated. Other details were as described for figure 22.

oxidase activity, occurred, following the addition of piericidin A.

Comparison of the measured rate of oxygen consumption, with the rate estimated from the time taken to reduce approximately 100 nmoles of oxaloacetate (given in parentheses in figures 22 and 23), following the partial recovery of the rate of oxygen consumption, after piericidin A addition, indicated that the estimated rate was less than the control rate measured before piericidin A was added. The estimated rate of oxygen consumption, calculated as described already (Results 1.vi.b), was found to approximate quite closely, the piericidin A-recovered maximum rate. One explanation of this finding could be that only a proportion of the malic enzyme molecules are induced by the presence of piericidin A, to expose the NADH produced by their activity, to the malate dehydrogenase. In addition, only these molecules would be required to remain active in the catalysis of NAD^+ reduction, to explain the low estimated rate of oxygen consumption following partial recovery from piericidin A, which in turn reflects an equally low potential maximum rate of NAD^+ reduction by malate, under these conditions.

The possible explanation just given, requires the existence of two types of malic enzymes, each associated with a pool of NAD^+ . One type of malic enzyme would not be able to expose its associated NADH to the malate dehydrogenase compartment of the mitochondrion, and would be associated with electron transport to oxygen via pathways which are totally sensitive to inhibition by piericidin A. The other type of malic enzyme would be capable of being induced by the presence of piericidin A (or by the transition from state 3 to state 4), to expose its associated NADH to the malate dehydrogenase compartment. Once in the malate dehydrogenase compartment, the NADH would reduce any accumulated oxaloacetate, and then proceed to be oxidized by oxygen,

via electron transport pathways which are insensitive to inhibition by piericidin A.

This explanation of the data was supported by the finding that the duration of the initial period of inhibition of state 3 malate oxidase activity, caused by piericidin A, appeared to be inversely related to the magnitude of the maximum piericidin A-insensitive component of this activity, measured for a given preparation of mung bean mitochondria (figure 24), and Jerusalem artichoke mitochondria (table 8). In addition, the magnitude of the respiratory control ratio was also shown to be directly related to the duration of the initial phase of piericidin A inhibition, of state 3 malate oxidase activity, as figure 24 and table 8, show. Thus, as the value for the respiratory control ratio increased for a preparation of mitochondria, so the magnitude of the piericidin A-insensitive component of malate oxidase activity decreased, and the duration of the initial phase of inhibition of state 3 malate oxidase activity, increased.

Thus, there is evidence for the existence of two types of malic enzymes in isolated plant mitochondria, in contrast with the one type suggested by Brunton & Palmer (1973) and Brunton (1975), (see figure 20), which would be expected to be associated with two pools of NAD^+ , in addition to any pool(s) associated with the other NAD^+ -linked substrate dehydrogenases in the mitochondria.

Concerning the oxidation of pyruvate, citrate and 2-oxo-glutarate in isolated plant mitochondria, Brunton & Palmer (1973) and Brunton (1975) have proposed a common pool of NAD^+ associated with all of them in wheat mitochondria. If this were the case, and the resultant NADH from the oxidation of any of these acids had access to the same NADH dehydrogenase and electron transport pathway, as also indicated by these authors, then it would be expected that the piericidin A-

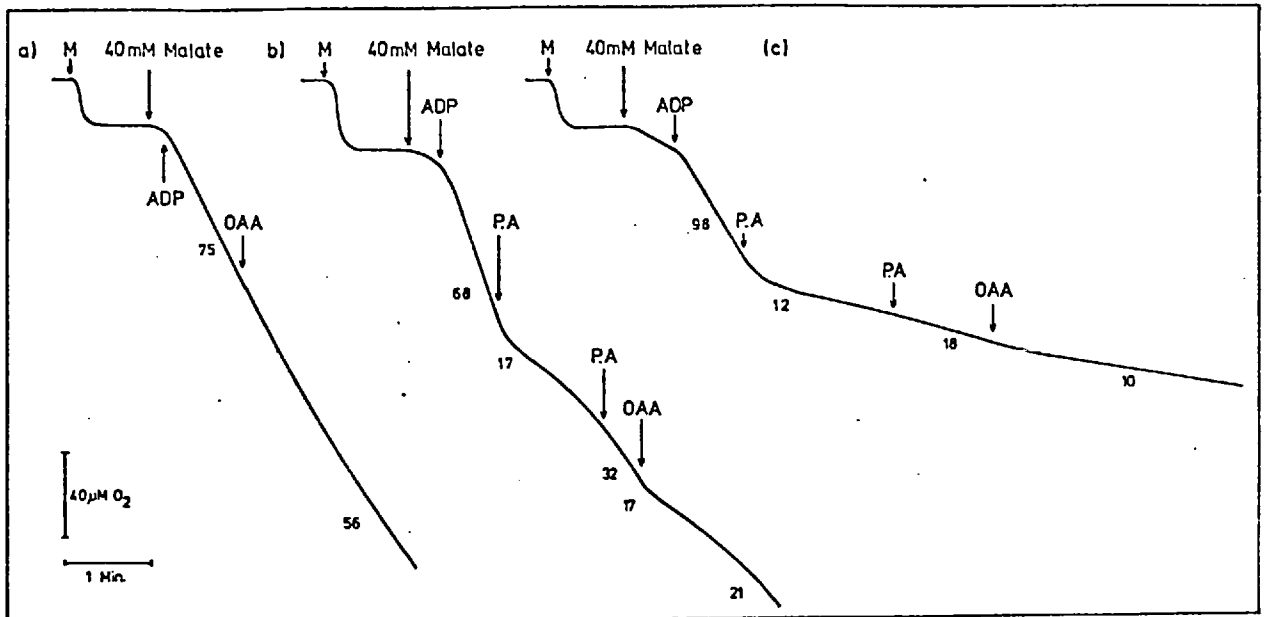


Figure 24. Piericidin A inhibition and the effect of oxaloacetate on state 3 malate oxidase activity in mung bean mitochondria, characterized by high and low levels of respiratory control. Oxygen electrode traces showing the pattern of oxygen consumption. Mitochondria (M, 0.5, 1.0 and 0.7 mg. protein ml.^{-1} for traces (a), (b) and (c), respectively), were suspended in standard assay medium and additions made to the reaction cuvette as indicated, and the rate of consumption of oxygen measured using an oxygen electrode, as described in Materials & Methods 5. The mitochondria used to produce traces (a) and (b) were characterized by a value of 3.1-3.25 for the R.C.R. measured with 40mM malate as substrate, and those used to produce trace (c) had a value of 5.0-5.2 for the R.C.R. with the same substrate. Other details were as for figure 22.

TABLE 8.

The Effect of Piericidin A and Oxaloacetate on the State 3 malate oxidase Activity in
Jerusalem Artichoke Mitochondria Characterised by Different Degrees of Respiratory Control.

<u>Experiment Number</u>	<u>State 3 malate oxidase activity</u> (nmoles O ₂ min. ⁻¹ mg. protein ⁻¹)			Duration of the Initial phase of Inhibition by Piericidin A (secs.)	Duration of the OAA Inhibited phase of Piericidin A insen- sitive malate oxidase activity (secs.)	<u>Respiratory Control Ratio</u>
	Control	Plus Piericidin A (Initially)	Plus Piericidin A (Maximum)			
193	111	48.5	69	40	45	2.0,2.2,2.3
204	102	3.9	43	50	90	3.0,3.1
	106	15.7	49	65	85	
207	125	12.5	42.5	110	130	4.1,4.8
	* 130	10.0	35	110	120	

The experiments were carried out as indicated in figure 20. The oxaloacetate (OAA) concentration used was 0.2mM, and the substrate used for determining the Respiratory Control Ratio, was malate (40mM), and in all experiments the concentration of malate used as respiratory substrate, was also 40mM, except experiment 207*, when 100mM malate was used.

insensitive component of the rates of oxidation of each of these substrates, would be of the same magnitude. This is because the rate of oxidation of each substrate under these conditions, would be limited by the electron transport capacity of the piericidin A-insensitive bypass shown in figure 20, assuming that no other step became rate limiting to the rate of oxygen consumption.

This was not found to be the case in wheat mitochondria, as figure 6 in Brunton & Palmer (1973) shows. The magnitude of the piericidin A-insensitive component of pyruvate oxidase activity was only approximately $9.5 \text{ nmoles oxygen min.}^{-1} \text{ mg. protein}^{-1}$, while those of 2-oxoglutarate and malate oxidases, were approximately 23 and 27 $\text{nmoles oxygen min.}^{-1} \text{ mg. protein}^{-1}$, respectively.

It has already been noted that although the rate of NADH production by the activity of pyruvate, isocitrate and 2-oxoglutarate dehydrogenases in isolated plant mitochondria, appears to exceed the equivalent measured rate of oxygen consumption, in each case, the rates of oxygen consumption differed under state 3 conditions, (see Results 1.vi.b). This indicated that different electron transport pathways might be mediating the reduction of oxygen by each substrate, and so it is perhaps reasonable to find that such pathways possess different degrees of sensitivity to piericidin A, as was found to be the case for Jerusalem artichoke mitochondria (figure 25), white potato mitochondria (figure 26) and mung bean mitochondria (figure 27).

These observations suggest that isolated plant mitochondria contain two types of NADH dehydrogenases associated with the malate dehydrogenase compartment. As proposed for malate oxidation, one type of NADH dehydrogenase would be associated with piericidin A - sensitive electron transport pathways, while the other type would be associated with piericidin A-insensitive pathways. The different

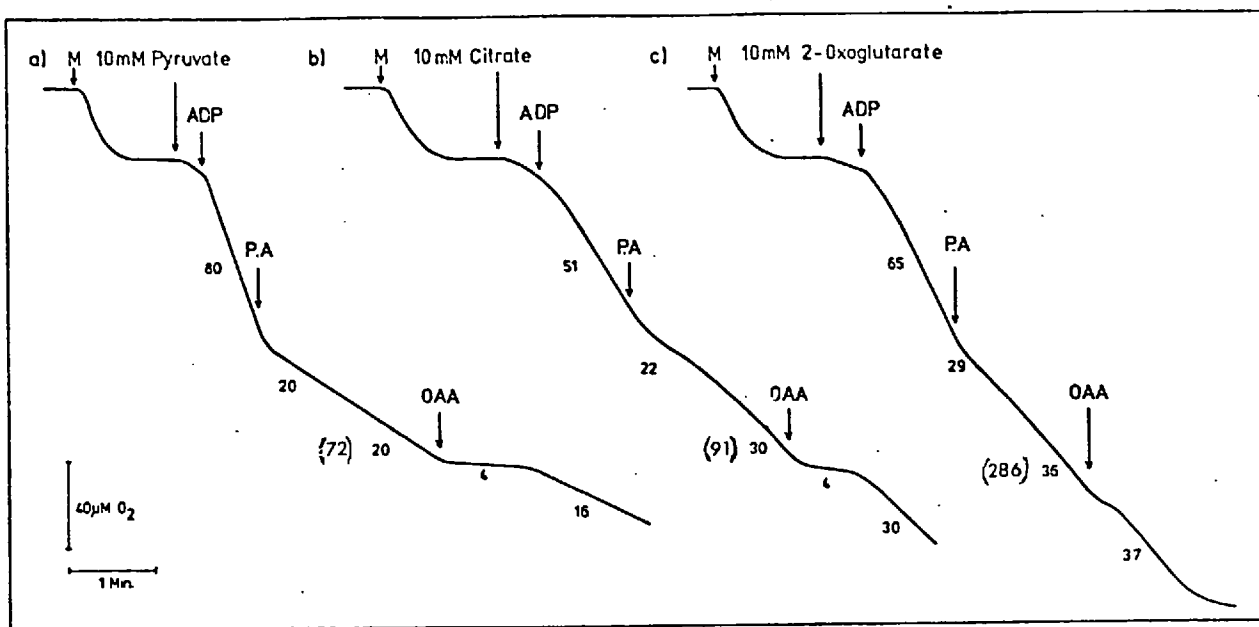


Figure 25. The effect of the sequential addition of piericidin A and oxaloacetate, on the oxidation of pyruvate, citrate and 2-oxoglutarate in Jerusalem artichoke mitochondria. Oxygen electrode traces showing the pattern of consumption of oxygen. Mitochondria (M, 1.4 mg. protein ml.^{-1}), were suspended in standard assay medium containing 1mM malate, and 0.35mM TPP. Other details were as described for figure 22.

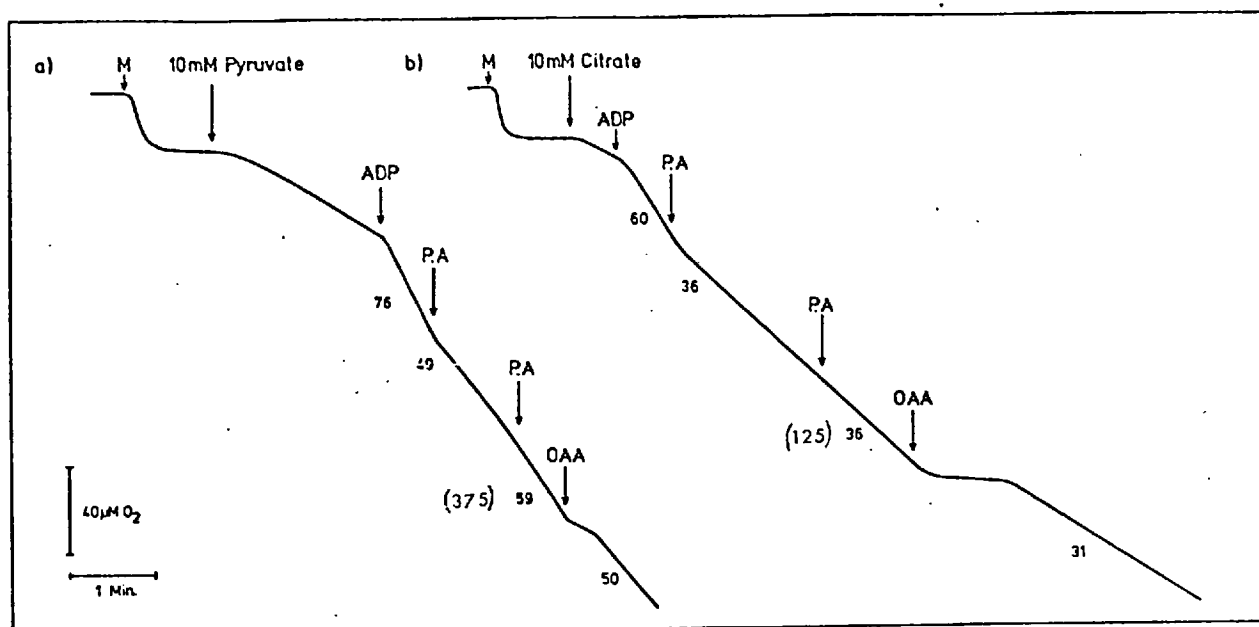


Figure 26. The effect of the sequential addition of piericidin A and oxaloacetate, on the oxidation of pyruvate and citrate in white potato mitochondria. Oxygen electrode traces showing the pattern of oxygen consumption. Mitochondria (M, 0.8 mg. protein ml.^{-1}), were suspended in standard assay medium containing 1mM malate and 0.35mM TPP. Other details were as described for figure 22.

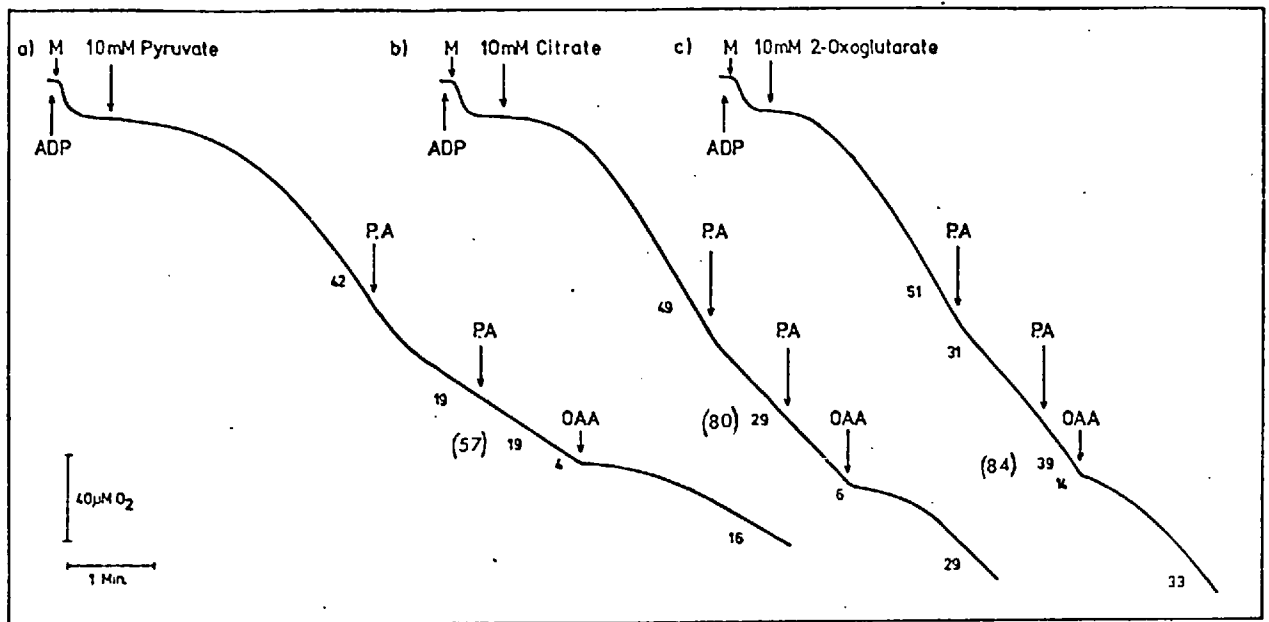


Figure 27. The effect of the sequential addition of piericidin A and oxaloacetate on the oxidation of pyruvate, citrate and 2-oxoglutarate in mung bean mitochondria. Mitochondria (M, 1.4 mg..protein ml.⁻¹). Other details were as for figures 25 and 26.

degrees of sensitivity to piericidin A observed for each NAD^+ -linked substrate, could be reflecting that each NAD^+ -linked substrate dehydrogenase was associated with specific NADH dehydrogenases, with no possibility of NADH moving from one dehydrogenase to another, a situation that would lead to equal rates of oxygen consumption for each NAD^+ -linked substrate, particularly following partial inhibition of oxygen consumption by piericidin A.

The expected rates of oxygen consumption resulting from the oxidation of pyruvate, citrate and 2-oxoglutarate, and estimated from the time taken for the reduction of a given amount of oxaloacetate, (shown as the numerical values in parentheses in figures 25, 26, and 27) appeared to exceed the rates measured in the presence of piericidin A, but comparison with the estimated values shown in figures 14, 15 and 16, in the absence of piericidin A, suggested that piericidin A might have been causing inhibition of NAD^+ reduction, as well as inhibition of the rate of oxygen consumption. Alternatively, the presence of piericidin A might decrease the accessibility of oxaloacetate, NADH and malate dehydrogenase, to each other. This aspect of the effect of piericidin A on the oxidation of NAD^+ -linked substrates, requires further study to distinguish between these possibilities. Thus it appears that the number of piericidin A-sensitive NADH dehydrogenases, associated with the oxidation of a particular substrate, probably governs the magnitude of the maximum piericidin A-insensitive rate of oxygen consumption with each NAD^+ -linked substrate.

As outlined above, from the scheme of Brunton & Palmer (1973), equal rates of oxygen consumption would be expected in the presence of piericidin A, irrespective of the NAD^+ -linked substrate presented to the plant mitochondria, in the presence of ADP, and assuming that no other step becomes limiting to the rate of oxygen

consumption as a result of the presence of piericidin A. For example, it might be considered that, in addition to inhibiting the rate of electron transport, the presence of piericidin A inhibits the rate of production of NADH, to such an extent that it becomes rate limiting to oxygen consumption. While it cannot be certain that this does not happen, the data in figure 28 suggest that it is unlikely to be the rate limiting step for pyruvate oxidase activity in the presence of piericidin A, since under state 4 conditions (trace b), ADP could not stimulate the piericidin A-insensitive component of this activity. Crompton & Laties (1971) found that pyruvate dehydrogenase isolated from white potato mitochondria, was sensitive to the ratio of NADH:NAD⁺ in the assay medium, with a high value for this ratio resulting in the enzyme becoming inhibited. In the presence of piericidin A, the value for the NADH:NAD⁺ ratio in the mitochondria might be expected to rise due to partial inhibition of the rate of electron transport, when an NAD⁺-linked substrate is present, particularly under state 4 conditions perhaps, but under these conditions, the addition of ADP would be expected to release respiratory control, and so lower the NADH:NAD⁺ ratio. This would be expected to release at least some of the inhibition of pyruvate dehydrogenase caused by NADH, which was not found to be the case here, suggesting that in the case of pyruvate at least, the piericidin A-insensitive component of the rate of oxygen consumption, was limited by the maximum rate of electron transport in the presence of piericidin A.

Following on from the pyruvate oxidase activity under state 4 conditions, and its response to piericidin A and ADP, an interesting observation was made concerning malate oxidase activity in isolated Jerusalem artichoke mitochondria. In some preparations of these mitochondria, malate oxidase activity under state 4 conditions

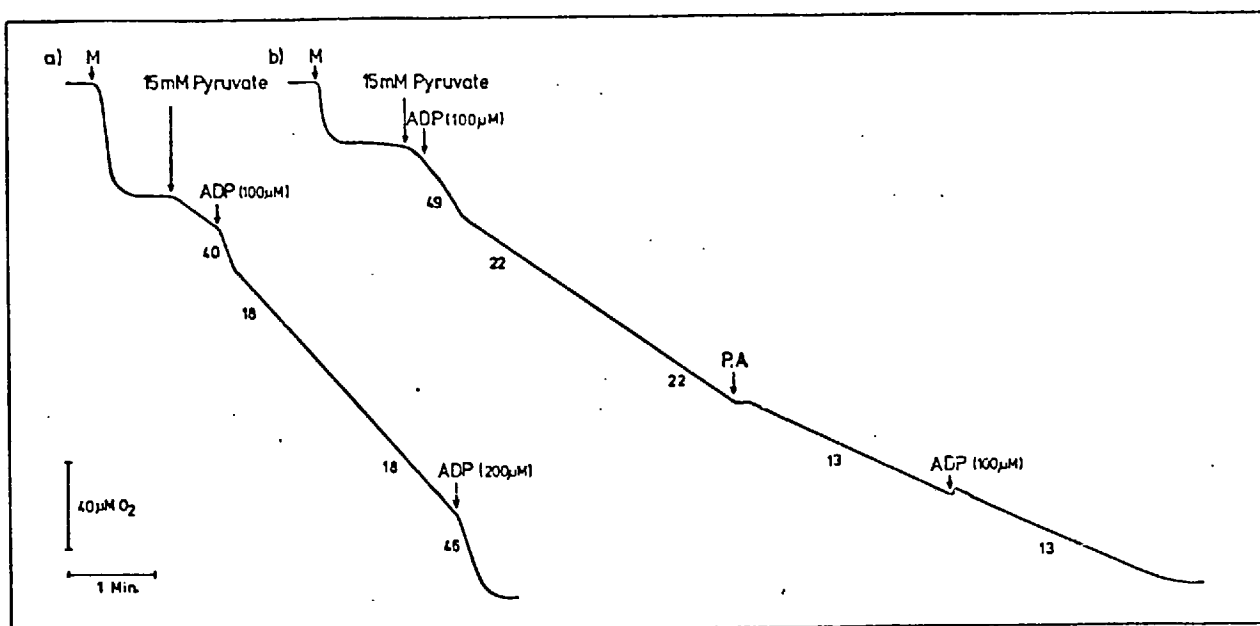


Figure 28. The effect of piericidin A on the oxidation of pyruvate under state 4 conditions, in Jerusalem artichoke mitochondria. Oxygen electrode traces showing the pattern of consumption of oxygen. Mitochondria (M, 1.2 mg. protein ml.⁻¹, for (a), and 0.6 mg. protein ml.⁻¹ for (b)), were suspended in standard assay medium containing 1mM malate and 0.35mM TPP. P.A = piericidin A, 50 ng. Numerals under the traces represent the rate of consumption of oxygen as nmol min.⁻¹ mg. protein⁻¹.

was found to be biphasic. Upon entering state 4, a lower rate of oxygen consumption occurred, compared with the rate observed about one minute later. The second phase of activity, was constant, and the cycle could be repeated if the aliquots of ADP were small, as can be seen from the data presented in figure 29 illustrate. Biphasic state 4 malate oxidase activity has been seen before in isolated plant mitochondria by Lance, Hobson, Young & Biale (1965, 1967), Hobson (1970), and Brunton (1975); see also Palmer (1976), however in the present study, the second phase of activity was found to be totally insensitive to inhibition by piericidin A, as can also be seen from figure 29a.

Thus, it appears that in the mitochondria used to produce the data presented in figure 29, the transition from state 3 to state 4, induced a change in the site of NADH associated with the malic enzyme, from the malic enzyme compartment to the malate dehydrogenase compartment, where it reduced accumulated oxaloacetate, hence the initial slower phase of state 4 malate oxidase activity observed, followed by its oxidation catalyzed by NADH dehydrogenases terminating piericidin A-insensitive electron transport pathways. This was quite different from the response of pyruvate oxidase activity under state 4 conditions (figure 28), and is considered to represent further evidence in support of the proposal made earlier that the possible pathway for the oxidation of endogenous NADH in isolated plant mitochondria, is different from that described in figure 20, and proposed by Brunton & Palmer (1973) and Brunton (1975) for wheat mitochondria. Indeed the arrangement appears to be considerably more complex than suggested by these authors.

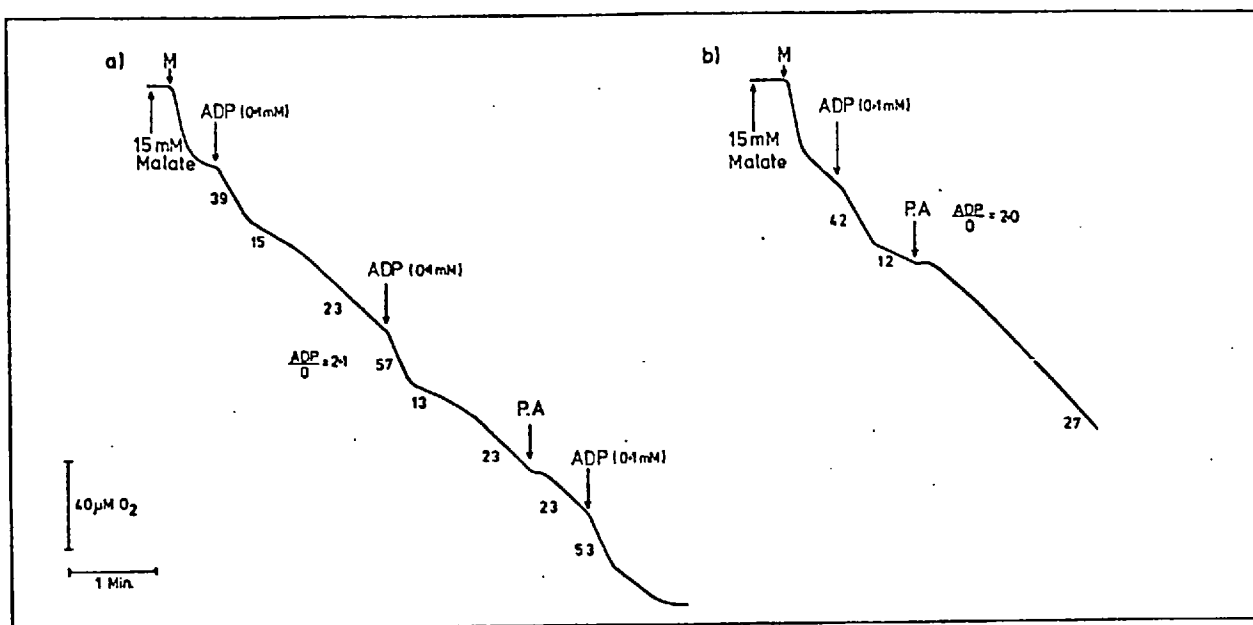


Figure 29. The pattern of malate oxidase activity under state 4 conditions in Jerusalem artichoke mitochondria characterized by non-linear kinetics, and its sensitivity to piericidin A. Oxygen electrode traces showing the pattern of consumption of oxygen. Mitochondria (M, 1.6 mg. protein ml.⁻¹), were suspended in standard assay medium, and additions to the reaction cuvette made as indicated. P.A = piericidin A (100 ng. ml.⁻¹). Numerals under the traces represent the rate of consumption of oxygen as nmoles min.⁻¹ mg. protein⁻¹.

e) The effect of exogenous NAD^+ on malate, citrate and 2-oxoglutarate oxidation.

The stimulation of malate-ferricyanide reductase activity by exogenous NAD^+ in the presence of antimycin A (see figure 13), indicated that the reduction of ferricyanide by NADH under these conditions, was being catalyzed by the exogenous NADH dehydrogenase, in agreement with Coleman & Palmer (1972), Brunton & Palmer (1973), Day & Wiskich (1974a,b) and Brunton (1975). The site of reduction of the exogenous NAD^+ was probably located externally to the inner membrane of the Jerusalem artichoke mitochondria used, since all the evidence available indicates that this membrane is impermeable to NAD^+ , see results l.vi.a.

The activity of malate-ferricyanide reductase catalyzed by isolated Jerusalem artichoke mitochondria in the absence of exogenously added NAD^+ , piericidin A and antimycin A, was found to be increased by increasing the concentration of malate from 15mM to 60mM, as can be seen from figure 13. Following the almost total inhibition of malate-ferricyanide reductase activity by antimycin A however, the NAD^+ -stimulated activity of this enzyme was the same whether 15mM or 60mM malate was used. This indicated that the rate-limiting step for ferricyanide reduction by malate was different in the absence of exogenously added NAD^+ , from that in its presence.

Coleman & Palmer (1972) and Palmer & Arron (1976), have proposed the existence of an NAD^+ -linked malic enzyme located outside the inner membrane of isolated Jerusalem artichoke mitochondria, and saturating at a much lower concentration of malate than the internally located enzymes. From experiments carried out using mitochondria isolated from cauliflower (Brassica oleracea L.) bud mitochondria, Day & Wiskich (1974a,b), have proposed the existence of a 'trans-

membrane transhydrogenase' capable of catalyzing the transfer of reducing equivalents from NADH located in the matrix of the mitochondria, to NAD^+ located outside the inner membrane. The subsequent oxidation of the NADH produced, was then considered to take place outside the inner membrane, catalyzed by the exogenous NADH dehydrogenase.

Day & Wiskich (1974a,b) found that malate, citrate and 2-oxoglutarate oxidase activities were all considerably stimulated by added NAD^+ , a result which supported the existence of a general transmembrane transhydrogenase in isolated cauliflower mitochondria, since these authors argued that it is unlikely that all of the NAD^+ -linked dehydrogenase are located outside the inner mitochondrial membrane.

In the present study, the activities of malate, citrate and 2-oxoglutarate-ferricyanide reductases were tested for their sensitivity to piericidin A, NAD^+ and antimycin A in isolated white potato mitochondria, and the data presented in table 9, show that, only malate-ferricyanide reductase activity was significantly stimulated by NAD^+ , and the stimulated component was almost insensitive to antimycin A. These results are considered to be more consistent with malic enzyme being located in the inter-membrane space of white potato mitochondria, as well as on the matrix side of the inner membrane, as suggested by Coleman & Palmer (1972) and Palmer & Arron (1976) for Jerusalem artichoke mitochondria, and Brunton & Palmer (1973) for wheat mitochondria, than with the existence of a transmembrane transhydrogenase, as proposed by Day & Wiskich (1974a,b), for cauliflower mitochondria.

TABLE 9.

The Effect of Piericidin A, NAD^+ and Antimycin A on the Activities of Malate-ferricyanide Reductase, Citrate-ferricyanide Reductase and 2-Oxoglutarate-ferricyanide Reductase in White Potato Mitochondria.

<u>Substrate</u>	<u>Ferricyanide Reductase Activity</u>			
	<u>Control</u>	<u>+ Piericidin A</u>	<u>+ NAD^+</u>	<u>+ Antimycin A</u>
Malate	122	23	71	45
Citrate	74	43	45	5
2-oxoglutarate	63	63	70	15

Mitochondria ($0.2 \text{ mg. protein ml.}^{-1}$), were suspended in the standard assay medium, containing 0.3 mM ADP , 1 mM KCN , and $100 \text{ ng. ions ml.}^{-1}$ potassium ferricyanide. The appropriate substrate was then added to the reaction cuvette, followed, after the attainment of a constant rate of reduction of ferricyanide, by the addition of piericidin A (50 ng.). One minute later NAD^+ was added ($350 \mu\text{M}$), and the subsequent rate of ferricyanide reduction recorded, followed by addition of the antimycin A (100 ng.). The reaction volume was 3 ml. , and the rate of reduction of ferricyanide was measured spectrophotometrically using the wavelength pair $420\text{--}490 \text{ nm.}$, as described in Materials & Methods 6.vii. The initial concentrations of substrates used were, malate, 60 mM ; Citrate, 20 mM and 2-oxoglutarate 20 mM . For citrate and 2-oxoglutarate, 1 mM malate was also present in the assay medium. The units of ferricyanide reductase activity are $\text{ng. ions ferricyanide reduced min.}^{-1} \text{ mg. protein}^{-1}$.

2. Oxidation of exogenous NADH.

In the preceding section, some of the complexities of endogenous NADH oxidation catalyzed by isolated plant mitochondria, were investigated. It is the purpose of this section of the results, to present data obtained from experiments which formed the main part of the present study. An investigation has been made of the relationship between exogenous NADH oxidase activity, and the oxidation of pyruvate and intermediates of the TCA cycle, with the aim of learning a little more about the branched nature of the electron transport pathways in plant mitochondria, from interactions between them.

i) The effect of NADH concentration on the activity of exogenous NADH oxidase.

An aqueous solution of NADH, has an absorbance maximum at a wavelength of 340 nm., while NAD^+ has no absorbance at this wavelength (see Lehninger, 1970). The millimolar extinction coefficient at 340 nm. for NADH used in the present study, was 6.22 cm.^{-1} , as defined by Horecker & Kornberg (1948). In addition to absorbance measurements made at 340 nm., concentrations of NADH were also measured at the wavelength pair 340-374 nm., using the dual wavelength spectrophotometer as used by Haslam & Krebs (1968), and described in Materials & Methods 6.v., and using a millimolar extinction coefficient of 3.73 cm.^{-1} . Figure 30 shows the relationship found between the concentration of NADH in the measuring cuvette of the spectrophotometer, and the absorbance of light measured at wavelengths of 340 nm. and 340-374 nm. It can be seen that at concentrations of NADH above approximately $100 \mu\text{M}$, absorbance as a function of concentration, deviated from a linear relationship, which was expected from the Beer-Lambert law relating the concentration of a light absorbing solute

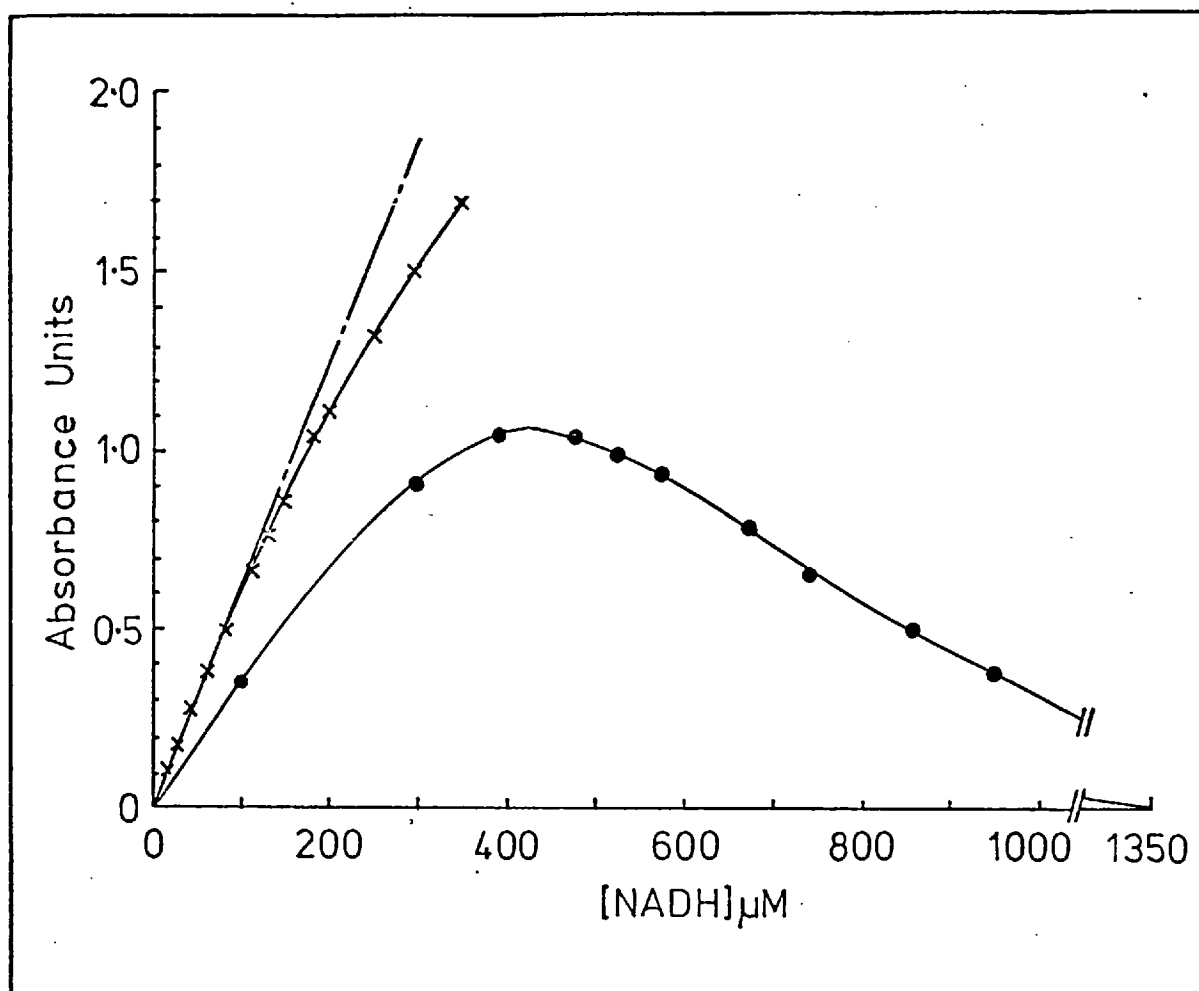


Figure 30. The relationship between the concentration of NADH in aqueous solution, and the absorbance of light at 340 nm. and 340-374 nm. The measurement of absorbance of light by NADH was carried out as described in Materials & Methods 6.v. X = absorbance at 340 nm. measured using the split-beam mode, with distilled water as a reference; ● = absorbance at the wavelength pair 340-374 nm., using the dual-wavelength mode. The concentration of NADH was determined as described in Materials & Methods 9.ii., and the broken line represents the expected plot of absorbance at 340 nm. versus concentration of NADH, using 6.22 cm^{-1} as the millimolar extinction coefficient of NADH at this wavelength.

species, to the absorbance by the species.

The plot for absorbance at 340-374 nm. in figure 30, shows that at concentrations above approximately 500 μ M NADH, the spectrophotometer probably became limiting, with the greater effect occurring at 340 nm., and hence ever increasing magnitudes of absorbance at the reference wavelength were being subtracted from ever decreasing absorbance 'visible' to the spectrophotometer at the measuring wavelength of 340 nm. Thus, the recorded absorbance at 340-374 nm., progressively decreased as the concentration of NADH was increased above 500 μ M. The initial concentration of NADH for use in subsequent experiments was therefore most suitably selected to fall between 100 and 200 μ M upon spectrophotometric grounds.

Assays of exogenous NADH oxidase activity in isolated Jerusalem artichoke mitochondria, indicated that 100 μ M NADH saturated this enzyme activity under state 4 conditions, and resulted in an activity of near saturation, under state 3 conditions (figure 31). Thus, the use of a concentration of NADH of the order of 100 μ M, for assay of the activity of exogenous NADH oxidase in isolated Jerusalem artichoke mitochondria, was considered to be satisfactory on spectrophotometric grounds, and reasonable on enzyme kinetic grounds. The use of such a low concentration of NADH did however mean that the sensitivity of the oxygen electrode had to be increased, since the normal range of oxygen concentration used in mitochondrial studies, from 100% air saturated down to 0% air saturated at 25°C, required at least 480 μ M NADH to be present in the reaction cuvette of the oxygen electrode initially (i.e. 240×2 , where 240 = the concentration of oxygen in the standard assay medium at 25°C as μ M, and 2 = the molar ratio between NADH and oxygen). The sensitivity of the oxygen electrode was increased as described in Materials & Methods 5.

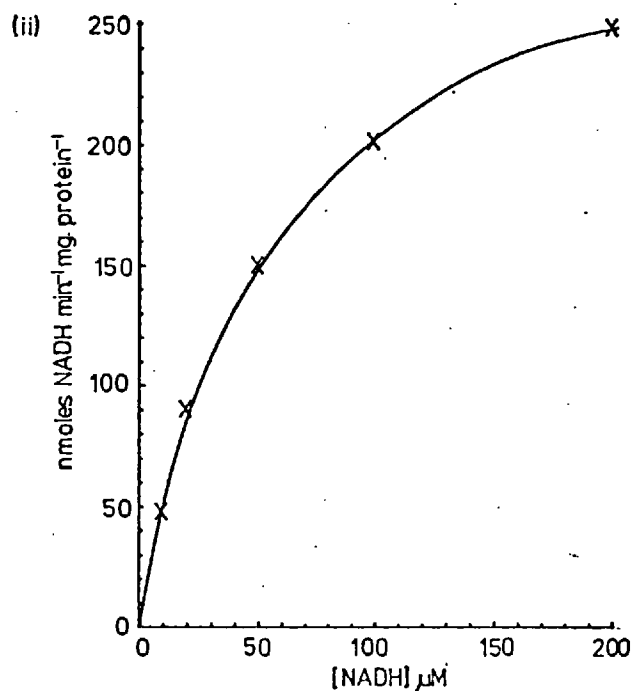
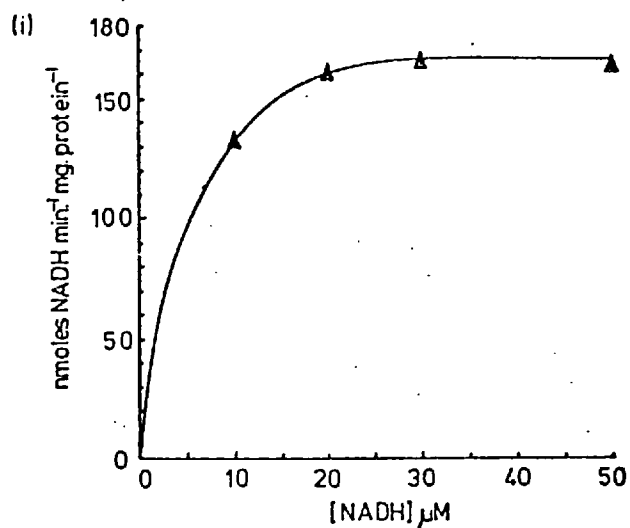


Figure 31. The effect of the initial concentration of NADH on the activity of exogenous NADH oxidase, in Jerusalem artichoke mitochondria. Mitochondria ($0.2 \text{ mg. protein ml.}^{-1}$), were suspended in standard assay medium in both sample and reference cuvettes. For graph (ii), 0.5 mM ADP was also present in the sample cuvette. Exogenous NADH oxidase was then activated by the addition of the appropriate concentration of NADH to the sample cell. Exogenous NADH oxidation was measured spectrophotometrically, as described in Materials & Methods 6.v.

(i) Substrate state, (ii) State 3.

ii) The effect of carboxylic acids on the activity of exogenous NADH oxidase.

Experiments were carried out to see if there was any evidence, in isolated Jerusalem artichoke mitochondria, for interaction between exogenous NADH oxidase and a range of carboxylic acids, all of which have been shown in section 1 of the results, to be readily oxidized by these mitochondria. The data presented in table 10 indicate possible interaction between exogenous NADH oxidase and citrate and succinate, since both of these acids caused high levels of inhibition of exogenous NADH oxidase activity.

Citrate was found to inhibit a greater proportion of the state 3 activity compared with the substrate state, while the reverse was true for succinate. It is the aim of the remainder of the results section to present data obtained from experiments designed to investigate the interactions between citrate and exogenous NADH oxidase and succinate and exogenous NADH oxidase, in isolated plant mitochondria.

TABLE 10.

The Effect of a Range of Respiratory Substrate Carboxylic Acids on the Activity of Exogenous NADH Oxidase in Jerusalem Artichoke Mitochondria, under State 3 and under Substrate State Conditions.

Carboxylic Acid	State 3				Substrate State		
	Activity of each carboxylic acid oxidase. (nmoles O ₂ min. ⁻¹ mg. protein ⁻¹)	Activity of Exogenous NADH Oxidase. (Control)	Activity of Exogenous NADH Oxidase in the presence of each carboxylic acid.	%Inhibition of Exogenous NADH oxidase by each carboxylic acid.	Activity of Exogenous NADH Oxidase (Control)	Activity of Exogenous NADH Oxidase in the presence of each carboxylic acid.	%Inhibition of Exogenous NADH oxidase by each carboxylic acid.
Pyruvate	110	270	310	0	165	169	0
Citrate	45	266	82	70	163	119	28
2-oxoglutarate	65	266	315	0	164	197	0
Succinate	125	266	162	39	164	60	64
Malate	110	278	290	0	158	135	14

Mitochondria (0.43 mg. protein ml.⁻¹, for NADH oxidation and 0.87 mg. protein ml.⁻¹, for oxygen uptake), were suspended in standard assay medium. The rate of oxygen uptake was measured polarographically, as described in Materials & Methods 4, in the presence of each carboxylic acid at the following concentrations: pyruvate, 16.7mM; citrate, 16.7mM; 2-oxoglutarate, 16.7mM; succinate, 5.0mM; malate, 5.0mM; each in the presence of 0.3mM ADP. Exogenous NADH oxidase activity was measured spectrophotometrically at 340nm. as described in Materials & Methods 5, using an initial concentration of 50μM NADH, and 0.3mM ADP for state 3 measurements. Each carboxylic acid was added to the reaction cuvette approximately 30 seconds before adding the NADH, and to an initial concentration equal to that used for oxygen uptake for each acid. The units of exogenous NADH oxidase are nmoles NADH min.⁻¹ mg. protein⁻¹.

iii) Interaction between citrate and exogenous NADH oxidase.

Citrate is an anion which is known to chelate divalent cations, such as calcium (see Jones, 1964; Stecher, 1968), and it has been suggested that exogenous NADH oxidase in plant mitochondria has a requirement for such cations (Hackett, 1961; Miller, Dumford, Koeppe & Hanson, 1970; Miller & Koeppe, 1971; Coleman, 1971; Coleman & Palmer, 1971). It was therefore possible that the inhibition of exogenous NADH oxidase activity caused by citrate, involved the chelation of Ca^{++} ions, rather than interaction between citrate oxidase and exogenous NADH oxidase. It was therefore considered reasonable to test for the chelation possibility first.

a) The effect of citrate on the activity of exogenous NADH oxidase.

The pattern of inhibition of exogenous NADH oxidase by citrate, was found to be dependent upon the sequence of addition of citrate and NADH, to the mitochondria. Citrate added after NADH, was found to cause a lower level of inhibition of exogenous NADH oxidase activity than citrate added before the NADH, at least over the time course of the experiments carried out here, data for which are presented in figure 32 (b) and (c). Figure 32 also shows that the chelator of divalent cations, EGTA, caused high levels of inhibition of exogenous NADH oxidase activity, whether added before or after NADH, (see traces d,e,f). Calcium ions were found to relieve the inhibition caused by both citrate and EGTA, indicating that both inhibitors were acting in a similar way upon exogenous NADH oxidase, probably as chelators of divalent cations such as Ca^{++} , which appear to be necessary for this enzyme activity.

The finding that inhibition by citrate was less when it was added after NADH than when it was added before, suggested that under conditions when exogenous NADH oxidase is active, Ca^{++} ions might be more tightly associated with the enzyme, than under conditions when

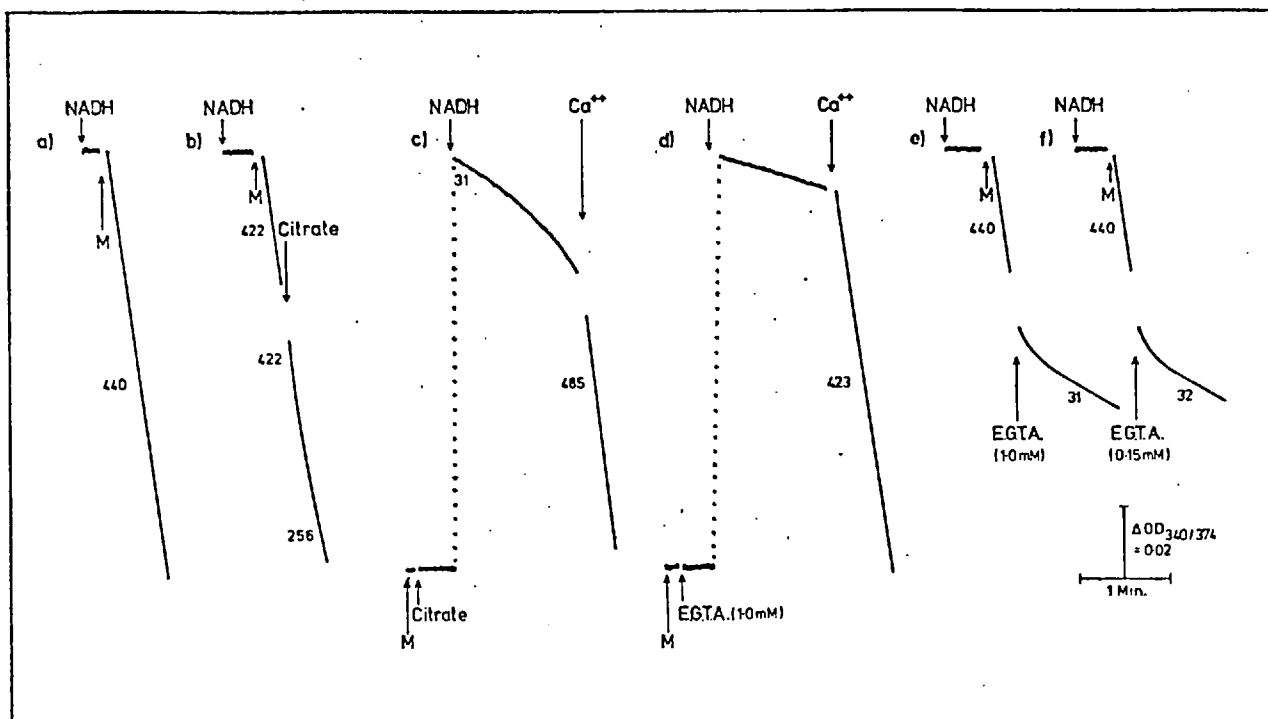


Figure 32. The effect of citrate and EGTA on the activity of exogenous NADH oxidase, in Jerusalem artichoke mitochondria. Dual-wavelength spectrophotometer traces, measured at 340-374nm. and showing the pattern of oxidation of exogenous NADH. Mitochondria (M, 0.12 mg. protein ml.⁻¹) were suspended in standard assay medium, and additions made to the cuvette as indicated. For details of the spectrophotometer measurements, see Materials & Methods 6.v.a. The assay medium contained 0.3mM ADP, and the concentrations of additions were, NADH, 100μM; citrate, 20mM; Ca⁺⁺ as aqueous calcium chloride solution, 83 ug. ions mg. protein⁻¹. The numerals below the traces represent the rate of oxidation of exogenous NADH, as nmol NADH min.⁻¹ mg. protein⁻¹.

it is inactive. The finding that EGTA caused a high level of inhibition of exogenous NADH oxidase activity, whether it was added before or after NADH, might be reflecting a greater chelating power of EGTA, compared with citrate. Testing of this possibility, was beyond the scope of the present study.

The data presented in figure 33, show that the degree of inhibition of the activity of exogenous NADH oxidase, was a function of the concentration of citrate added, as might be expected if it was chelating Ca^{++} ions. Also, the inhibition was found to be of a non-competitive type (figure 34), a result which was similar to that reported by Coleman & Palmer (1971), for the inhibition of exogenous NADH oxidase activity by EGTA, in Jerusalem artichoke mitochondria. These authors found that the inhibition could be relieved by Mn^{++} ions as well as Ca^{++} , which was also found to be the case for citrate inhibition. The effect of increasing the concentration of Ca^{++} ions added to Jerusalem artichoke mitochondria following inhibition of exogenous NADH oxidase activity by citrate, can be seen in figure 35, and it is consistent with the added Ca^{++} ions activating the exogenous NADH oxidase.

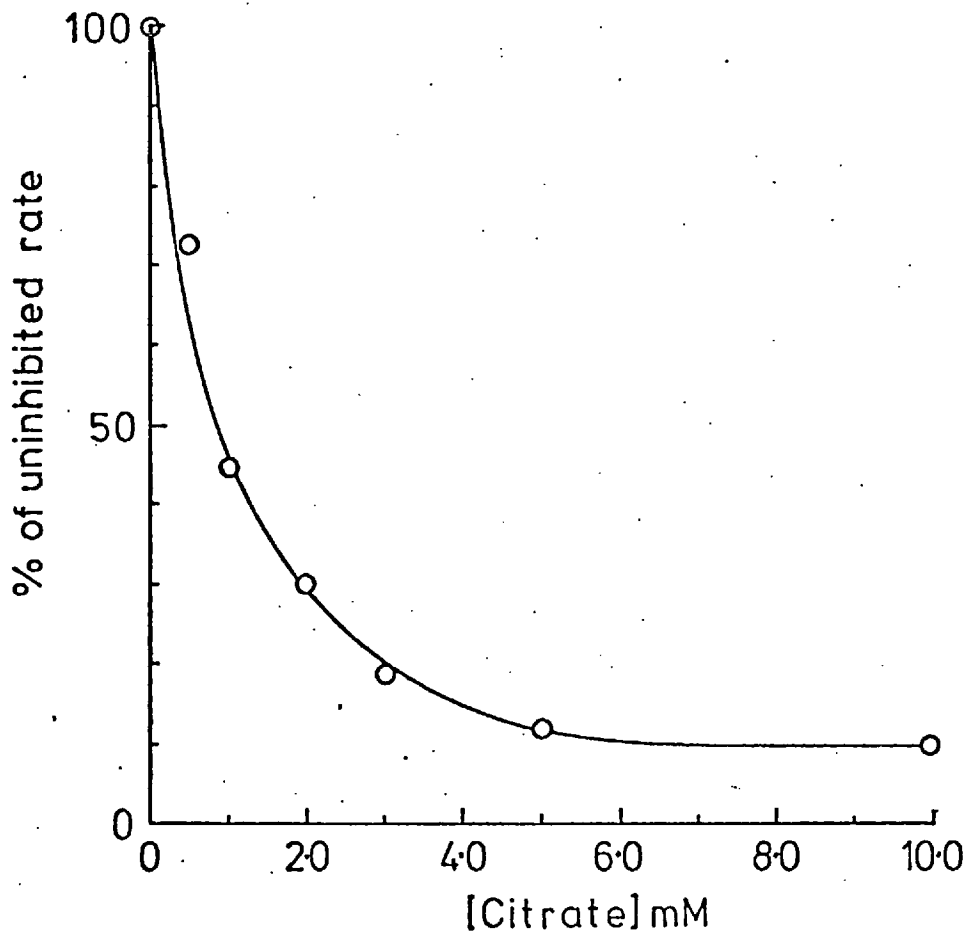


Figure 33. The effect of citrate on the activity of exogenous NADH oxidase in Jerusalem artichoke mitochondria. Mitochondria ($0.14 \text{ mg. protein ml.}^{-1}$), were suspended in the standard assay medium containing 0.3 mM ADP , and incubated for 30 seconds with the appropriate concentration of potassium citrate (pH 7.2). Exogenous NADH ($100 \mu\text{M}$) was added to the reaction cuvette, and stirred in followed by measuring the rate of oxidation of the NADH spectrophotometrically, as described in Materials & Methods 6.v. using the split-beam mode of operation. The control 100% value was $248 \text{ nmoles NADH min.}^{-1} \text{ mg. protein}^{-1}$.

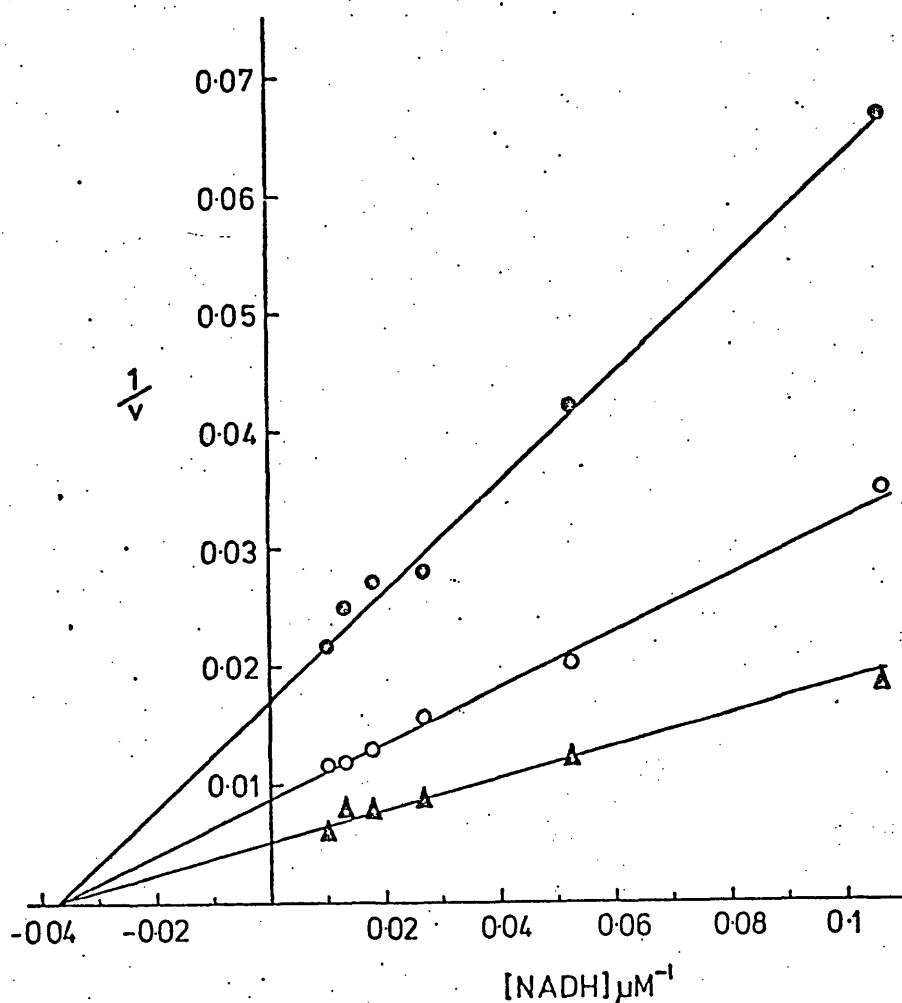


Figure 34. The nature of the inhibition of exogenous NADH oxidase caused by citrate, in Jerusalem artichoke mitochondria.

A double-reciprocal plot of $1/V$ versus $1/S$, where V = the velocity of oxidation of exogenous NADH measured alone (Δ), and in the presence of 1.0mM citrate ($\circ$) and 2.0mM citrate ($\bullet$). The units of V are nmoles NADH min.^{-1} mg. protein^{-1} . S = the initial concentration of substrate added to the mitochondria as μM NADH. Mitochondria ($0.14 \text{ mg. protein ml.}^{-1}$), were suspended in standard assay medium containing 0.3mM ADP, and the appropriate concentration of potassium citrate. After 30 seconds, the appropriate concentration of NADH was added to the sample cuvette; and the rate of oxidation of NADH measured spectrophotometrically, using the split-beam mode of operation as described in Materials & Methods 6.v.

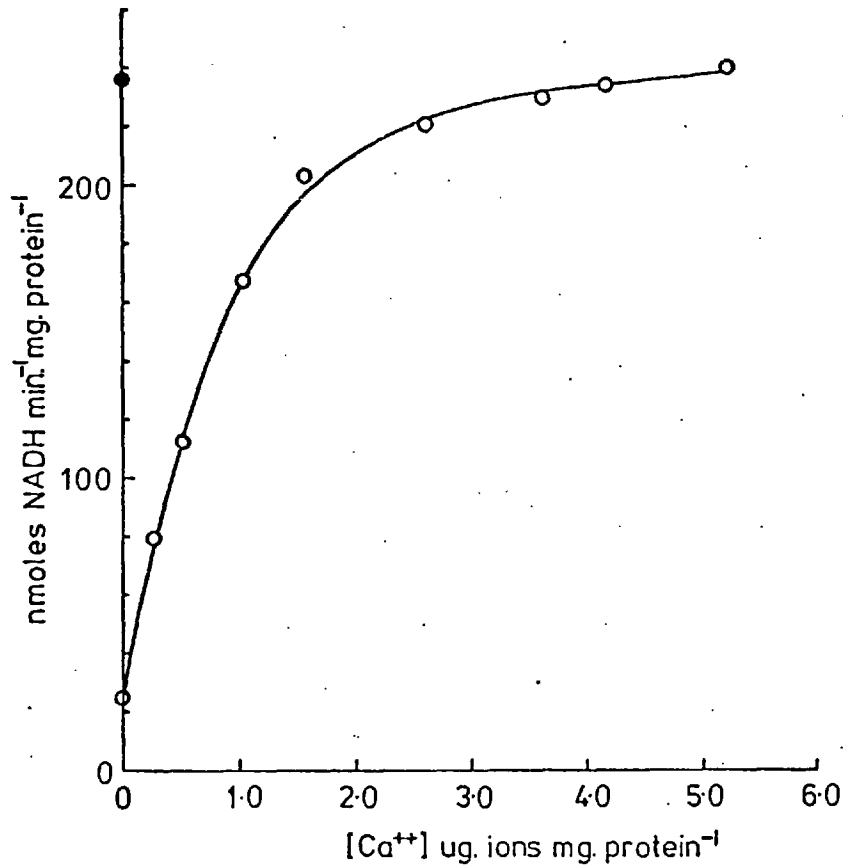


Figure 35. The effect of calcium chloride concentration on the activity of exogenous NADH oxidase in the presence of citrate, in Jerusalem artichoke mitochondria. Mitochondria ($0.2 \text{ mg. protein ml.}^{-1}$), were suspended in standard assay medium containing 0.3 mM ADP and 20 mM citrate . After 30 seconds, NADH ($100 \mu\text{M}$) was added to the sample cuvette, stirred in, and the activity of exogenous NADH oxidase measured as for figure 34, for 1 minute, when the appropriate concentration of calcium chloride solution was added to the sample cuvette, and the subsequent rate of NADH oxidation recorded (○).
(●) = the control activity of exogenous NADH oxidase.

Citrate has been shown to be able to exert a very powerful inhibitory effect upon exogenous NADH oxidase activity in isolated Jerusalem artichoke mitochondria, and it was therefore of considerable interest to see how general this type of inhibition is, in isolated plant mitochondria. Data presented in table 11 were obtained using mitochondria isolated from mung bean hypocotyls, which showed a very high level of sensitivity to citrate, white potato tubers, which showed intermediate sensitivity, and Neurospora crassa hyphae, which showed the lowest degree of sensitivity of exogenous NADH oxidase to citrate. Thus, inhibition of exogenous NADH oxidase activity by citrate, was found to be of general occurrence in mitochondria isolated from a range of angiosperm tissues and a fungus.

b) Location of the site of interaction between citrate and exogenous NADH oxidase.

A brief preliminary investigation of the possible site of interaction between citrate and exogenous NADH oxidase, in isolated Jerusalem artichoke mitochondria, has been carried out using artificial oxidants of the electron transport pathways mediating the oxidation of exogenous NADH (see Results 1.iv.). The data in table 12, were obtained from this type of study, and indicate that the site of citrate interaction is located on the oxygen side of the ferricyanide acceptance point, of electrons arising from exogenous NADH, as found for EGTA by Coleman & Palmer (1971), and on the substrate side of the site(s) of electron acceptance by duroquinone. It is suggested that these results indicate a site of interaction between citrate, and therefore probably Ca^{++} , and exogenous NADH oxidase in Jerusalem artichoke mitochondria, which was close to the exogenous NADH dehydrogenase itself. This could mean that, in order to inhibit exogenous NADH

TABLE 11.

The Effect of Citrate on the Activity of Exogenous NADH Oxidase in Mitochondria Isolated from a Range of Plant Tissues.

<u>Source of</u> <u>Mitochondria.</u>	<u>Exogenous NADH Oxidase Activity</u> (nmoles NADH min. ⁻¹ mg. protein ⁻¹)		<u>% Inhibition</u> <u>by citrate</u>
	<u>- Citrate</u>	<u>+ Citrate</u>	
Mung bean hypocotyl	160	16.5	87
White potato tuber	56	28	50
<u>Neurospora crassa</u> hyphae	460	320	31

Mung bean and potato mitochondria were suspended in standard assay medium containing 0.3mM ADP and 20mM citrate, and 50uM NADH was added to the sample cuvette, after 30 seconds. The rate of oxidation of exogenous NADH was then measured spectrophotometrically, as described in Materials & Methods 6.v. The experiments were then repeated in the absence of citrate, and the percent inhibition of exogenous NADH oxidase activity by citrate calculated. The whole procedure was repeated using N.crassa mitochondria suspended in N.crassa assay medium, as in Materials & Methods 5.ii.b. The mitochondrial protein concentrations used were, mung bean, 0.25 mg. ml.⁻¹; potato, 0.22 mg. ml.⁻¹ and N.crassa (Y74A), 0.43 mg. ml.⁻¹.

TABLE 12.

The Effect of Citrate on the Activities of Exogenous NADH-Oxygen Reductase, Exogenous NADH-Duroquinone Reductase, Exogenous NADH-Juglone Reductase and Exogenous NADH-Ferricyanide Reductase, in Jerusalem Artichoke Mitochondria.

<u>Rate of Electron Transport</u>			
<u>Terminal Electron</u>	<u>(nmoles NADH min.⁻¹mg. protein⁻¹)</u>		<u>% Inhibition</u>
<u>Acceptor.</u>	<u>- Citrate</u>	<u>+ Citrate</u>	<u>by Citrate.</u>
Oxygen	200	10	95
Duroquinone	265	57.5	78
Juglone	122.5	70	43
Ferricyanide	640	612.5	5

The activity of exogenous NADH-ferricyanide reductase was calculated from the rate of reduction of ferricyanide by NADH catalyzed by the mitochondria (0.06 mg. protein ml.⁻¹), as ng. ions ferricyanide reduced min.⁻¹ mg. protein⁻¹, by dividing by a factor of two. For details of the method see Materials & Methods 6.vii. Mitochondria (0.16 mg. protein ml.⁻¹) were suspended in standard assay medium containing 0.5mM ADP and 1.67mM KCN for all except when oxygen was the terminal electron acceptor, when KCN was omitted, and 20mM citrate. After 30 seconds, 80uM NADH was added to the reaction cuvette, and the rate of NADH oxidation measured as described in Materials & Methods 6.v. The experiments were repeated in the absence of citrate. Electron acceptor concentrations were, duroquinone and juglone, 150uM; ferricyanide 200 ng. ions ml.⁻¹.

oxidase, citrate did not need to cross the inner mitochondrial membrane, and so enter the matrix space.

Support for this possibility came from the similarity found between the pattern of inhibition caused by citrate and EGTA, to exogenous NADH oxidase activity (see figure 32), since EGTA is thought to be unable to cross the inner membrane of intact mitochondria (Reed & Bygrave, 1974). Also, it was found that both citrate and EGTA, could cause a high level of inhibition of exogenous NADH oxidase activity, in the absence of inorganic phosphate, as the data in figure 36 show. That inorganic phosphate was required for major uptake of citrate by isolated Jerusalem artichoke mitochondria, can be seen from the data presented in figure 37, which were obtained from mitochondrial swelling studies. Swelling of the mitochondria, causes a decrease in the degree of light scattering by them, which was measured spectrophotometrically, as described in Materials & Methods 6.x. This result was in agreement with that found for beet-root (Beta vulgaris cv.) mitochondria, by Wiskich (1974).

Thus, citrate, a carboxylic acid which can be present as a major constituent of plant cells (Walker, 1962), has been found to be capable of exerting a powerful inhibitory effect on exogenous NADH oxidase, particularly in mitochondria isolated from angiosperm plant tissues. The inhibition appears to involve the chelation of Ca^{++} ions, required for the activity of exogenous NADH oxidase, and the site of interaction between citrate/calcium and exogenous NADH oxidase, appears to be close to the NADH dehydrogenase. There was no evidence for citrate needing to enter the mitochondrial matrix, for it to exert its inhibitory effect on exogenous NADH oxidase, in Jerusalem artichoke mitochondria.

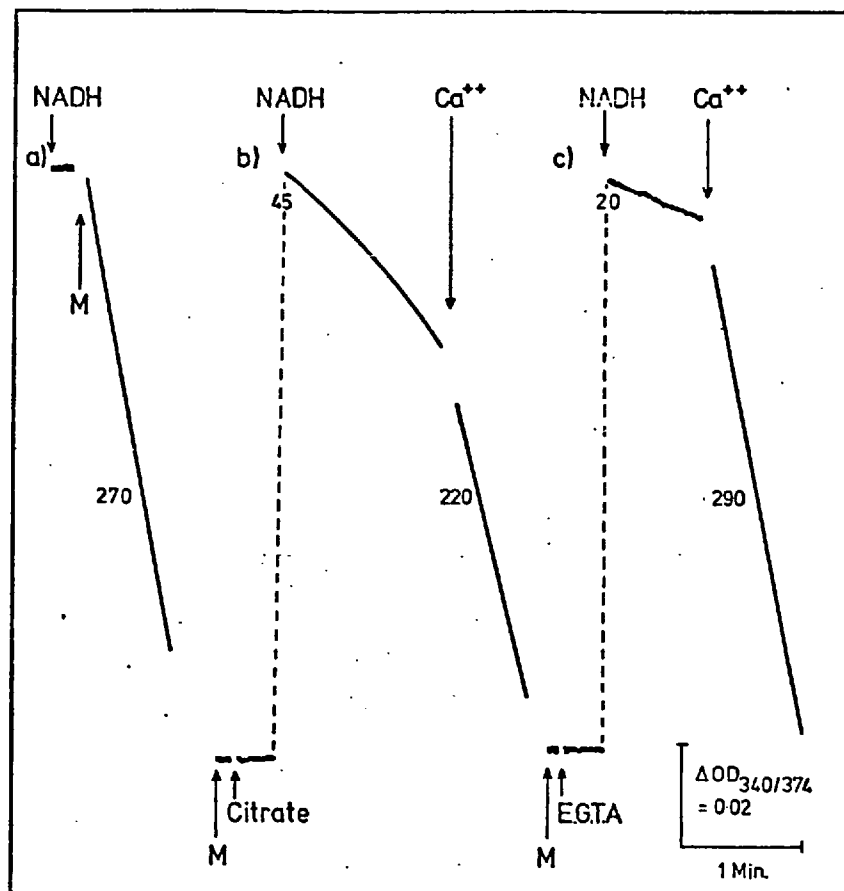


Figure 36. The effect of citrate and EGTA on the activity of exogenous NADH oxidase in Jerusalem artichoke mitochondria, in the absence of inorganic phosphate. For these experiments, the assay medium used was the standard medium minus inorganic phosphate. Other experimental details were as described for figure 32. EGTA, 1mM.

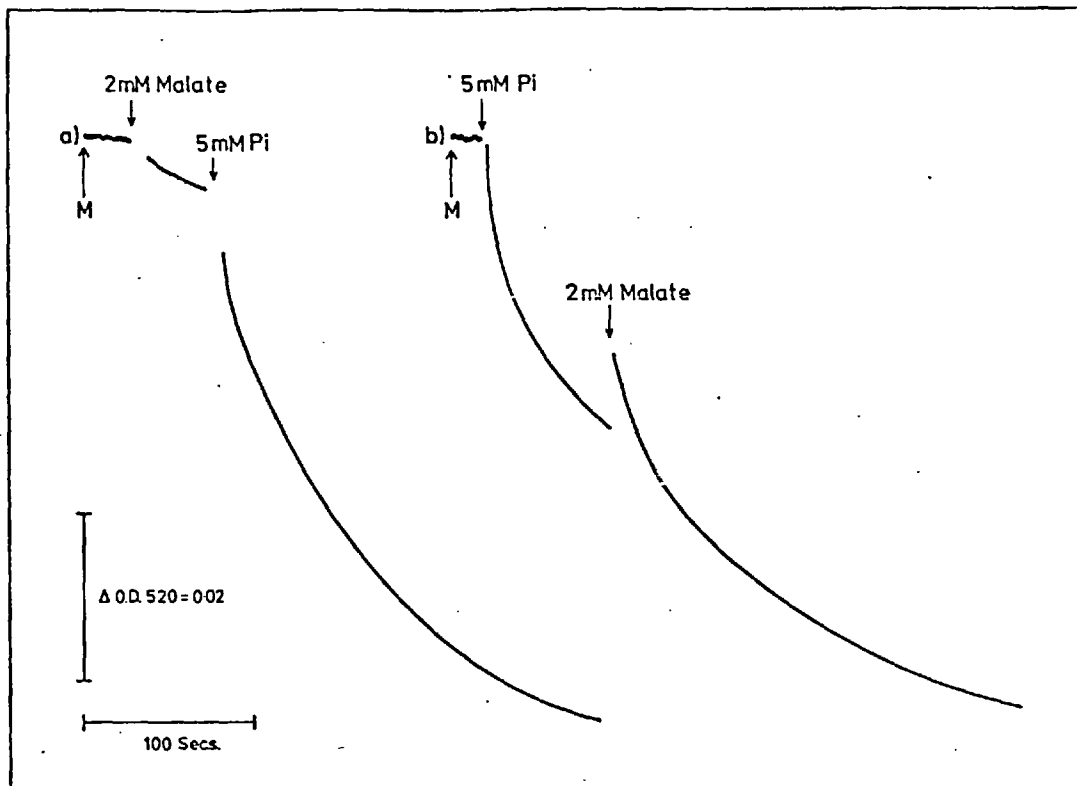


Figure 37. The effect of malate and inorganic phosphate on the state of contraction of the membranes of Jerusalem artichoke mitochondria suspended in ammonium citrate solution. Split-beam spectrophotometer traces measured at a wavelength of 520 nm., as described in Materials & Methods 6.x., and showing the pattern of swelling of the mitochondria, as a decrease in absorbance at 520 nm. The mitochondria were suspended in 100mM ammonium citrate (pH 7.2), at a protein concentration of 1 mg. ml.⁻¹. Additions were made to the sample cuvette as indicated.

iv) Interaction between succinate and exogenous NADH oxidase.

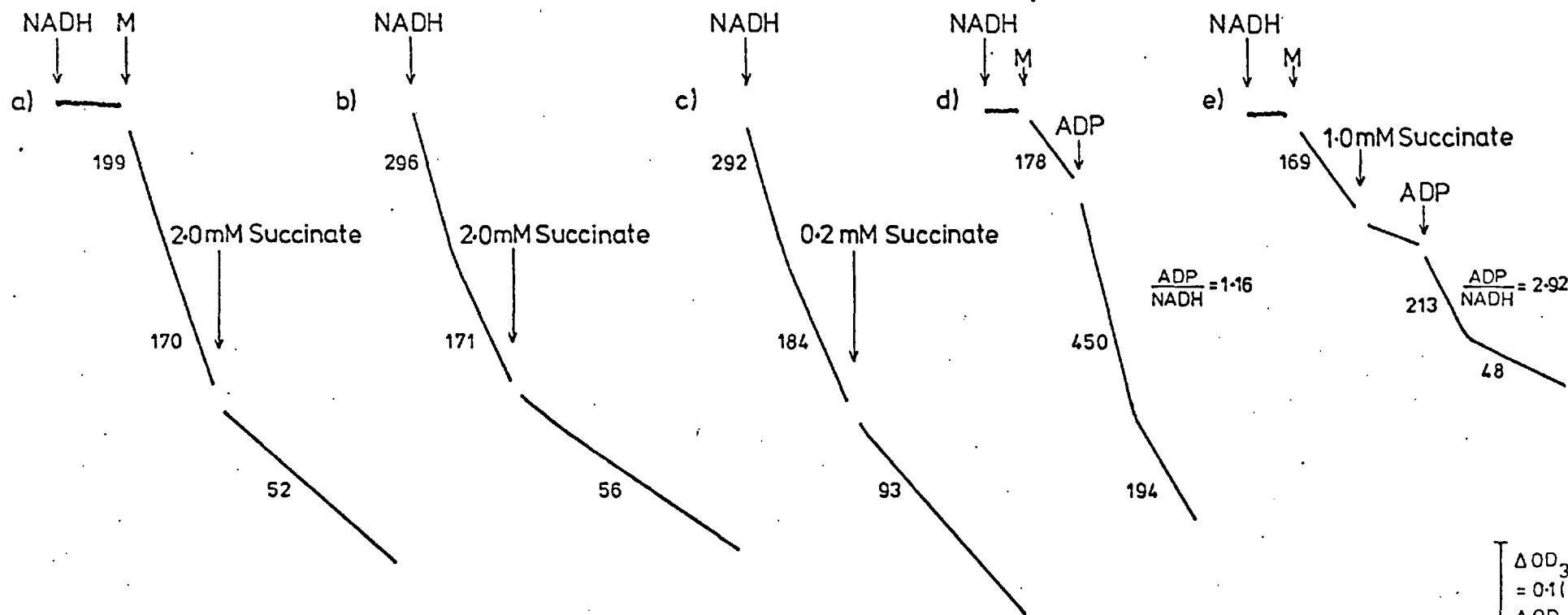
The inhibition of exogenous NADH oxidase caused by succinate (see table 10), was found to involve the interaction between the enzymes exogenous NADH oxidase and succinate oxidase, and thus it appeared to represent a possible example of interaction between branches of the electron transport pathways of isolated plant mitochondria. The aim of this section of the results is to present data obtained from experiments designed to further characterise this interaction.

a) The effect of succinate on the activity of exogenous NADH oxidase.

In most preparations of mitochondria isolated from tubers of Jerusalem artichoke and white potato, the enzyme succinate dehydrogenase, was found to be in a highly deactivated state, and was therefore activated by preincubation of the mitochondria with ATP, Pi and $MgCl_2$, as described in Results 1.ii.

Using Jerusalem artichoke mitochondria in which succinate dehydrogenase had been activated, the data presented in figure 38 were obtained. It can be seen from this figure that the degree of inhibition of exogenous NADH oxidase caused by succinate, was a function of the concentration of succinate used. The dual wavelength spectrophotometric technique was found to give similar results (trace a), to those obtained using the split beam technique (trace b). The split beam technique was however, more sensitive to changes in concentration of NADH, as indicated by the greater resolution of the initial phase of NADH oxidation seen in trace (a) in figure 38, compared with trace (b). The split-beam technique was therefore used in all experiments requiring the maximum degree of precision of measurement of the rate of oxidation of exogenous NADH. (The initial faster phase of exogenous NADH oxidase activity seen in figure 38, is thought to be due to the

Figure 38. The effect of succinate on the activity of exogenous NADH oxidase under state 4 conditions in Jerusalem artichoke mitochondria. Dual-wavelength and split-beam spectrophotometer traces, showing the pattern of exogenous NADH oxidation. Mitochondria ($0.12 \text{ mg. protein ml.}^{-1}$), in which the enzyme succinate dehydrogenase had been activated by preincubation with ATP, Pi and MgCl_2 , as described in the legend to figure 9, see Results 1.ii., were suspended in standard assay medium. Additions were made to the reaction cuvette as indicated, and the rate of oxidation of exogenous NADH measured as described in Materials & Methods 6.v. The concentration of NADH was initially $100 \mu\text{M}$ for traces (a), (b) and (c), and $200 \mu\text{M}$ for traces (d) and (e). The initial concentration of ADP was $60 \mu\text{M}$. The numerals below the traces, represent the rate of oxidation of exogenous NADH as $\text{nmoles NADH min.}^{-1} \text{ mg. protein}^{-1}$. Dual-wavelength traces are shown in (a), (d) and (e); Split-beam traces are shown in (b) and (c).



ΔOD_{340}
 $= 0.1$ (for b,c)
 $\Delta OD_{340/374}$
 $= 0.04$ (for d,e)
 $= 0.1$ (for a)

1Min.

presence of a little ADP in the assay medium, resulting from hydrolysis of some of the ATP used in the procedure of activating succinate dehydrogenase).

The amount of NADH oxidized under state 3 conditions, by isolated plant mitochondria, and following addition of a given aliquot of ADP of known concentration, can be expressed as the ADP:NADH ratio. The ADP:NADH ratio is analogous to the ADP:O ratio, and is numerically equal to this ratio, when the molar ratio between NADH and oxygen, is two, as appears to be the case for exogenous NADH oxidation, catalyzed by isolated Jerusalem artichoke mitochondria (table 13).

Comparison of the data shown in trace (d) in figure 38, with those in trace (e) in the same figure, shows that the ADP:NADH ratio was greater when measured in the presence of succinate, than in the absence of succinate. This indicated that, under the condition when succinate was present, it was being oxidized simultaneously with the NADH, and in a manner that was coupled to oxidative phosphorylation. That oxidation of succinate was taking place, can be seen from the data presented in figure 39. Increasing the concentration of succinate from 0 to 3.0mM, would be expected to result in the rate of oxidation of succinate increasing, and so to increase the ADP:NADH ratio measured in the presence of the increasing concentrations of succinate, as was found to be the case. In addition figure 39 shows that the value measured for the ADP:O ratio for the simultaneous oxidation of exogenous NADH and succinate, remain constant irrespective of the concentration of succinate initially added to the mitochondria, thus confirming that succinate and exogenous NADH were being oxidized simultaneously.

TABLE 13.

Estimation of the Molar Ratio between Exogenous NADH Oxidized and
Oxygen Reduced by Jerusalem Artichoke Mitochondria.

<u>NADH Oxidation</u>	<u>Oxygen Reduction</u>	Ratio $\frac{\text{NADH}}{\text{Oxygen}}$
(nmoles NADH min. ⁻¹ mg. protein ⁻¹)	(nmoles oxygen min. ⁻¹ mg. protein ⁻¹)	
131.2 ± 1.64	91.0 ± 3.45	2.0

ATP/Pi/MgCl₂ 'Activated' mitochondria (0.07 mg. protein ml.⁻¹ for NADH oxidation, and 0.32 mg. protein ml.⁻¹ for oxygen reduction), were suspended in the standard assay medium, and 90μM NADH was added. Exogenous NADH oxidation was measured spectrophotometrically using the split-beam mode of operation as described in Materials & Methods 6.v., and oxygen reduction was measured polarographically, as described in Materials & Methods 5. The numerical values represent the mean of 20 assays ± S.E.M. For details of ATP/Pi/MgCl₂ activation of succinate dehydrogenase see figure 9, Results 1.ii.

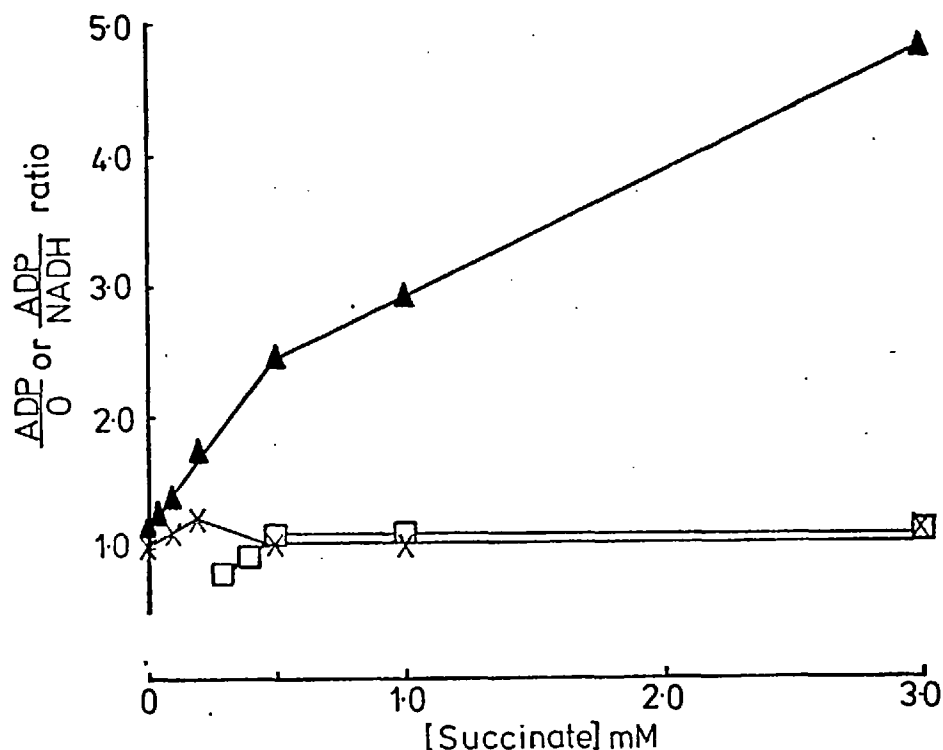


Figure 39. The effect of succinate concentration on the ADP:O ratio and ADP:NADH ratio, in Jerusalem artichoke mitochondria oxidizing exogenous NADH and succinate simultaneously. ATP/Pi/MgCl₂ 'activated' mitochondria (0.13 mg. protein ml.⁻¹ for measurement of ADP:NADH ratios and 0.45 mg. protein ml.⁻¹ for measurement of ADP:O ratios), were suspended in standard assay medium. The ADP:NADH ratios were measured as shown in figure 38d,e, using an initial concentration of NADH equal to 200μM, and succinate additions were made as indicated and ADP pulses of 60μM were used. The ADP:O ratios were measured polarographically as described by Chance & Williams (1956), see also figures 5 to 8, using identical concentrations of reagents as used for measuring ADP:NADH ratios. The sensitivity of the oxygen electrode was increased as described in Materials & Methods 5.

▲ = ADP:NADH ratio; X = ADP:O ratio (NADH + Succinate);

□ = ADP:O ratio (Succinate).

Evidence for the active and direct involvement of succinate dehydrogenase activity in the mechanism by which succinate caused the inhibition of exogenous NADH oxidase activity catalyzed by isolated Jerusalem artichoke mitochondria, came from experiments in which malonate was employed, in order to inhibit succinate dehydrogenase. The presence of malonate was found to prevent succinate from causing inhibition of exogenous NADH oxidase activity, as the data in table 14 show. Figure 40 confirms that malonate acts as a competitive inhibitor of succinate dehydrogenase, in Jerusalem artichoke mitochondria.

The inhibition of exogenous NADH oxidase activity, by succinate was found to be non-competitive in nature, as figure 41 indicates. Also, there was no difference in the pattern of inhibition by succinate of exogenous NADH oxidase, irrespective of whether the succinate was added before or after the NADH.

The possibility that the oxidation of exogenous NADH by isolated Jerusalem artichoke mitochondria, might result in modification of succinate oxidase activity, has also been investigated. This was a possibility since the enzyme succinate dehydrogenase, is known to be capable of undergoing allosteric modification. Such modification is thought to accompany the activation and deactivation of this enzyme, since these changes have a high energy requirement, a large component of which is associated with an entropy change, a finding which is consistent with them involving changes in the conformation of the protein moiety of succinate dehydrogenase (Davis & Hatefi, 1971; Hanstein, Davis, Ghalambor & Hatefi, 1971; Singer, Kearney & Gutman, 1972; Singer, Kearney & Kenney, 1972; Singer, Oestreicher, Hogue, Contreiras & Brandao, 1973; Oestreicher, Hogue & Singer, 1973).

TABLE 14.

The Effect of Succinate on the Activity of Exogenous NADH Oxidase,
in the Presence and Absence of Malonate, in Jerusalem Artichoke
Mitochondria.

<u>Malonate</u>	<u>Exogenous NADH Oxidase Activity</u> (nmoles NADH min. ⁻¹ mg. protein ⁻¹)		<u>% Inhibition</u> <u>by Succinate</u>
	<u>Control</u>	<u>+ Succinate</u>	
-	167	46	72.4
+	150	150	0

ATP/Pi/MgCl₂ 'Activated' mitochondria (0.1 mg. protein ml.⁻¹), were suspended in standard assay medium plus or minus 5mM malonate. NADH (84μM) was added to the reaction cuvette, and the activity of exogenous NADH oxidase measured spectrophotometrically, as described in Materials & Methods 6.v. Succinate (1.67mM) was added to the reaction cuvette 1 minute after attainment of a constant rate of oxidation of NADH, and the subsequent constant rate recorded. For details of ATP/Pi/MgCl₂ activation of succinate dehydrogenase, see figure 9, Results 1.ii.

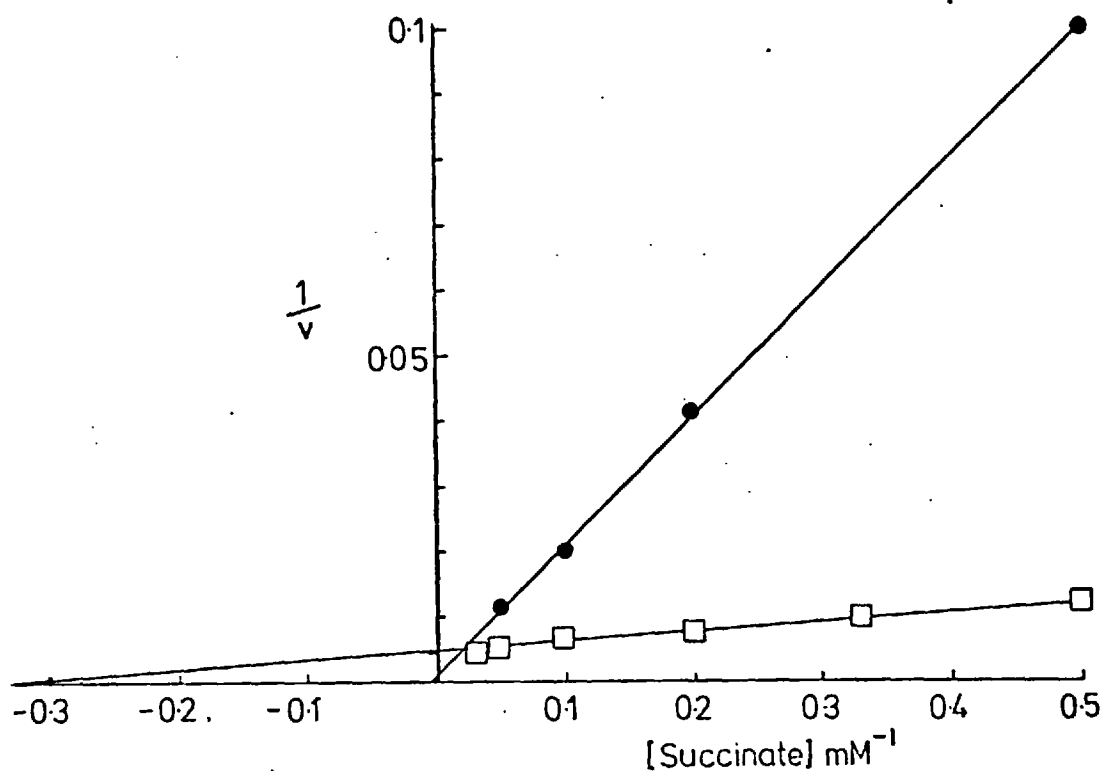


Figure 40. The inhibition of the activity of succinate oxidase by malonate in Jerusalem artichoke mitochondria. A double-reciprocal plot of $1/V$ versus $1/S$, where V = the velocity of succinate oxidase activity measured alone ($\square$) and in the presence of 1mM malonate ($\bullet$), as $\text{nmoles oxygen min.}^{-1} \text{mg. protein}^{-1}$. S = substrate concentration as mM succinate . ATP/Pi/MgCl_2 'activated' mitochondria ($1\text{mg. protein ml.}^{-1}$), were suspended in standard assay medium containing 1mM ADP, and 1mM malonate when appropriate, and the appropriate concentration of succinate added to the reaction cuvette. The rate of consumption of oxygen was measured polarographically, as described in Materials & Methods 5. For details of activation of succinate dehydrogenase by ATP/Pi/MgCl_2 , see figure 9, Results 1.ii.

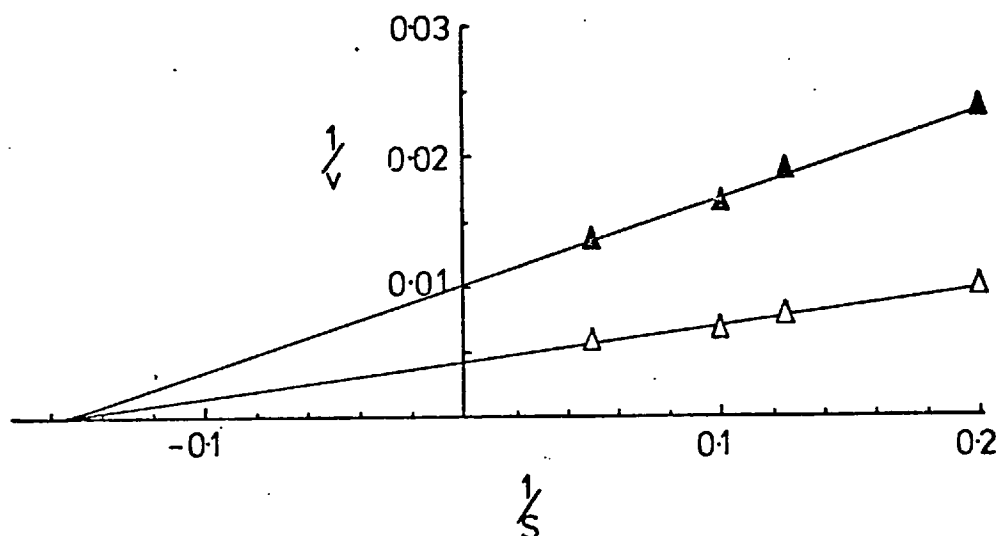


Figure 41. The effect of the initial concentration of NADH on the activity of exogenous NADH oxidase under state 4 conditions in Jerusalem artichoke mitochondria, and in the presence and absence of succinate. A double-reciprocal plot of $1/V$ versus $1/S$, where V = the velocity of exogenous NADH oxidase activity measured in the absence (Δ) and presence ($\blacktriangle$) of 0.17mM succinate, as nmoles NADH oxidized $\text{min.}^{-1} \text{ mg. protein}^{-1}$. S = substrate concentration as μM NADH. ATP/Pi/MgCl₂ 'activated' mitochondria (0.14 mg. protein ml.^{-1}), were suspended in standard assay medium in sample and reference cuvettes. Succinate to 0.17mM was then added to the sample cuvette followed by the appropriate concentration of NADH, and the rate of oxidation of exogenous NADH measured as described in Materials & Methods 6.v. This procedure was repeated in the absence of succinate. For details of activation of succinate dehydrogenase by ATP/Pi/MgCl₂, see figure 9.

b) The effect of exogenous NADH on succinate dehydrogenase activity.

In order to investigate the effect of exogenous NADH on the activity of succinate dehydrogenase, in isolated Jerusalem artichoke mitochondria, it was necessary to measure the activity of succinate dehydrogenase indirectly, as the activity of succinate oxidase i.e. the rate of oxidation of succinate by oxygen, catalyzed by succinate dehydrogenase and the enzymes associated with the electron transport pathways of the mitochondria. This was because it was not possible to assay succinate dehydrogenase directly in the presence of NADH, since the oxidant employed, phenazine methosulphate, was found to react directly with the NADH.

The state 4 activity of succinate oxidase, which occurred in the presence of exogenous NADH oxidase activity, was estimated by first measuring the rate of oxidation of exogenous NADH spectrophotometrically, for an initially constant concentration of NADH of the order of 100 μ M, and in the presence of a given concentration of succinate from 0 to 1.0 mM. This was carried out using the Aminco DW2 spectrophotometer in the split-beam mode and set to a wavelength of 340 nm. The rate of consumption of oxygen was then measured in the presence of identical concentrations of NADH and succinate, to those used for the spectrophotometric assays, and using mitochondria from the same preparation, which had been preincubated to activate the succinate dehydrogenase. The rates of oxygen consumption were measured with an oxygen electrode connected to a chart recorder, with its sensitivity to changes in the concentration of oxygen in the assay medium being increased, as described in Materials & Methods 5.

The rates of oxidation of exogenous NADH were then converted from the units of nmoles NADH min^{-1} mg. protein $^{-1}$ to nmoles oxygen min^{-1} mg. protein $^{-1}$, by dividing by the value for the molar

ratio between NADH and oxygen in Jerusalem artichoke mitochondria, i.e. two (see table 13). The resultant value for the measured rate of oxidation of exogenous NADH, in units of $\text{nmoles oxygen min.}^{-1} \text{ mg. protein}^{-1}$, and in the presence of a given concentration of succinate, was then subtracted from the value for the rate of oxygen consumption under the same conditions, measured directly using an oxygen electrode. Thus, by subtracting the rate of oxygen consumption due to exogenous NADH oxidase activity in the presence of a given concentration of succinate, from the rate of oxygen consumption measured for both substrates simultaneously, a value for the rate of succinate oxidation taking place in the presence of exogenous NADH oxidation, was arrived at. The final step was to plot the data obtained for exogenous NADH oxidase, and succinate oxidase activities, as a function of the concentration of succinate initially added to the mitochondria (figure 42).

It can be seen from figure 42 that exogenous NADH appeared to cause stimulation of succinate oxidase activity, compared with the activity measured in the absence of exogenous NADH. Also the relationship between the activity of exogenous NADH oxidase following inhibition by a given concentration of succinate, and the activity of succinate oxidase stimulated by exogenous NADH, were both described by hyperbolic functions. This suggested that the two effects were closely related to each other.

A double reciprocal plot of the data for succinate oxidase activity measured as a function of the initial concentration of succinate added to the mitochondria, in the presence and absence of exogenous NADH oxidase activity, is shown in figure 43. As can be seen from this figure, the presence of exogenous NADH was found to cause a large decrease in the value for the apparent K_m for succinate, from a value of 0.33mM succinate to a value of 0.06mM succinate.

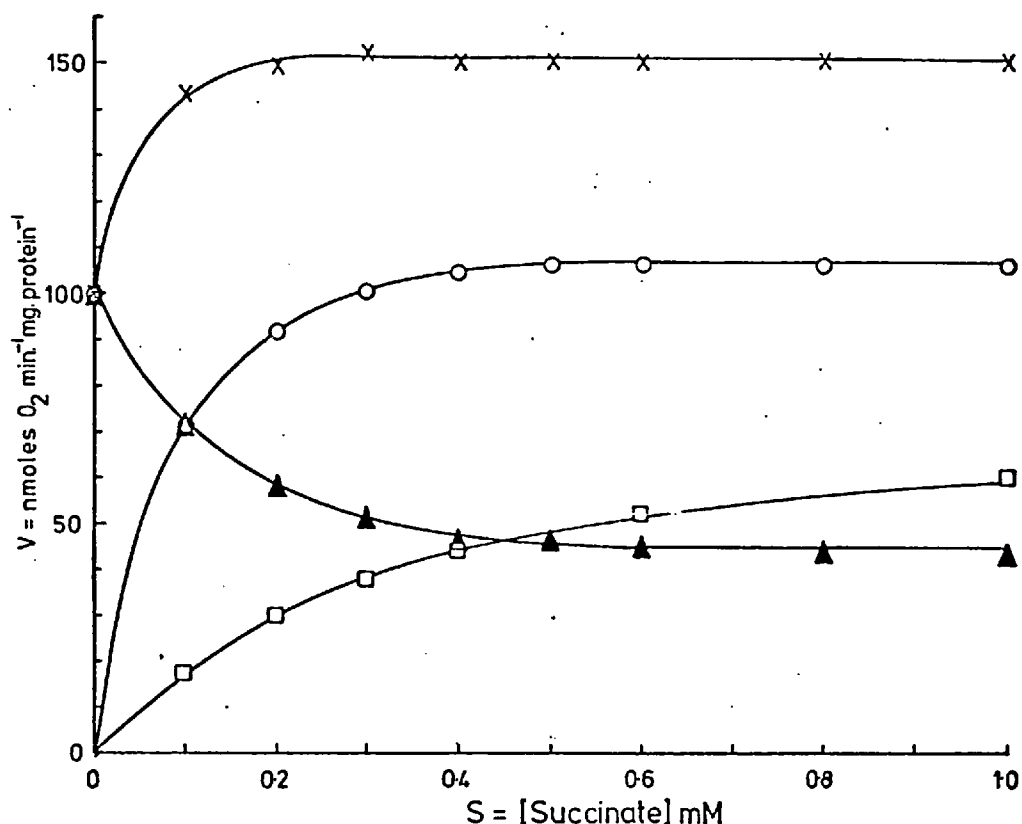


Figure 42. The effect of succinate on the simultaneous activities of exogenous NADH and succinate oxidases under state 4 conditions in Jerusalem artichoke mitochondria. ATP/Pi/MgCl₂ 'activated' mitochondria (0.1 mg. protein ml.⁻¹), were suspended in standard assay medium in either the cuvettes of a split-beam spectrophotometer, or the cuvette of an oxygen electrode. NADH (90μM) was then added to the reaction cuvettes, followed 1 minute later, by the appropriate concentration of succinate. The rate of oxidation of exogenous NADH was measured as described in Materials & Methods 6.v., and the rate of oxygen consumption for exogenous NADH and succinate oxidases simultaneously, was measured polarographically as described in Materials & Methods 5. The activity of succinate oxidase alone was measured polarographically, and its activity in the presence of exogenous NADH, was computed as described in the text.

X = exogenous NADH oxidase plus succinate oxidase;

Δ = exogenous NADH oxidase; □ = succinate oxidase alone;

O = succinate oxidase in the presence of exogenous NADH oxidase.

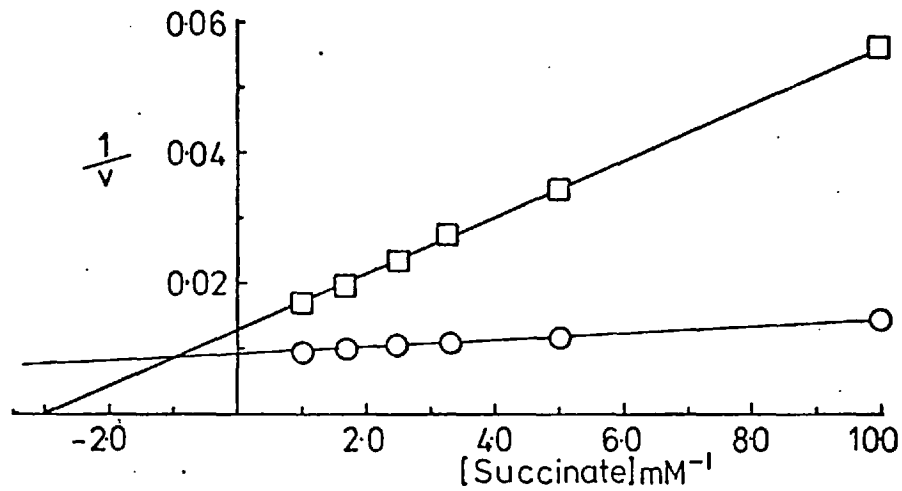


Figure 43. The effect of exogenous NADH on the activity of succinate oxidase in Jerusalem artichoke mitochondria.

A double-reciprocal plot of $1/V$ versus $1/S$, where V = the velocity of succinate oxidase activity taken from figure 42, measured in the absence of exogenous NADH (□) and in the presence of exogenous NADH (○).

This result suggested that exogenous NADH was causing extra activation of succinate dehydrogenase, above the level of activation already achieved by preincubation of the mitochondria with ATP, Pi and $MgCl_2$. Thus there was possible evidence in favour of reciprocal interaction between succinate dehydrogenase and exogenous NADH dehydrogenase, in Jerusalem artichoke mitochondria. It was not possible to test whether the further activation of succinate dehydrogenase by exogenous NADH, involved the active participation of exogenous NADH dehydrogenase, since there is no inhibitor known to the author, which specifically inhibits exogenous NADH oxidase without causing undesirable effects on succinate oxidase as well, (c.f. malonate inhibition of succinate dehydrogenase, table 14 and figure 40)

The hyperbolic relationships found between the initial concentration of succinate added to the mitochondria, and the inhibited activity of exogenous NADH oxidase, or the stimulated activity of succinate oxidase, shown in figure 42, suggested a close functional relationship between the two enzymes. This suggestion was further supported by the finding that data for succinate oxidase activity in the presence of exogenous NADH, and for different concentrations of succinate, produced a straight line when plotted against the inhibited rates of exogenous NADH oxidation which were measured under similar conditions, as can be seen from figure 44.

Data for succinate inhibition of exogenous NADH oxidase activity presented earlier (table 10), indicated that the degree of inhibition was less under state 3 conditions, than under the more controlled substrate state. However, the magnitude of the inhibited component was the same in both cases, being equivalent to $104 \text{ nmoles NADH min.}^{-1} \text{ mg. protein}^{-1}$ or $52 \text{ nmoles oxygen min.}^{-1} \text{ mg. protein}^{-1}$. This suggested that succinate could be inhibiting specific exogenous

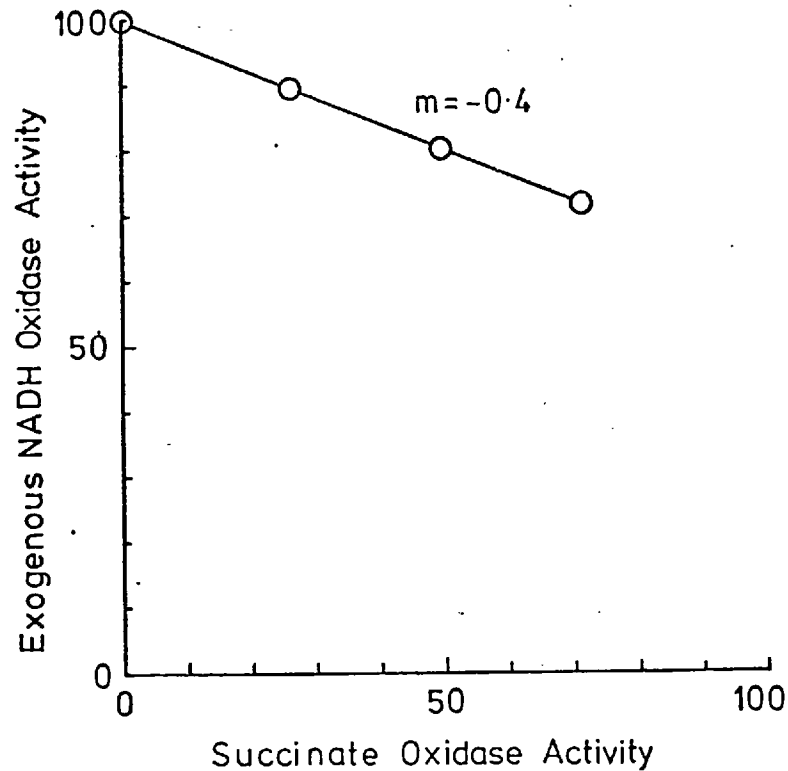


Figure 44. The relationship between the activities of exogenous NADH oxidase and succinate oxidase, measured simultaneously in Jerusalem artichoke mitochondria. This graph was obtained by plotting the data from figure 42 for exogenous NADH oxidase activity (i.e. Δ), on the ordinate, and for succinate oxidase activity (i.e. $\circ$), on the abscissa. 'm' = the slope of the plot. For other details, see figure 42.

NADH dehydrogenases, under both respiratory states, with the greater magnitude of the control activity under state 3 conditions, resulting in the inhibited component representing a smaller fraction of the control, than under substrate state conditions. This possibility was supported by data obtained from experiments designed to test the effect of the concentration of succinate, on the activities of exogenous NADH oxidase and succinate oxidase, under state 3 conditions, in Jerusalem artichoke mitochondria, as the data presented in figures 45, 46, and 47, show. Thus, the degree of stimulation of succinate oxidase activity caused by the presence of exogenous NADH, and measured by the decrease in the value for the apparent K_m for succinate, was similar to that found under state 4 conditions i.e. approximately a five fold decrease, as can be seen from a comparison of figures 43 & 46. Also, the slope of the plot shown in figure 47 was similar to that measured under state 4 conditions, and shown in figure 44.

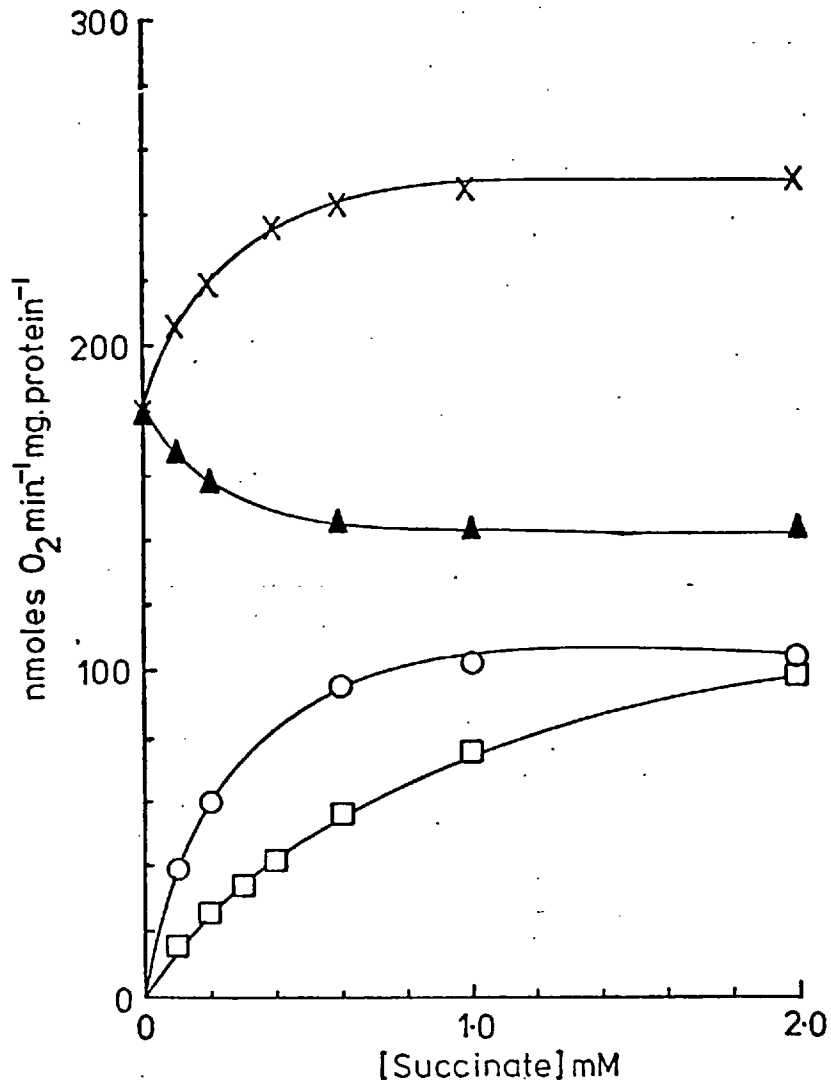


Figure 45. The effect of succinate on the activities of exogenous NADH oxidase and succinate oxidase, measured simultaneously under state 3 conditions, in Jerusalem artichoke mitochondria. The experimental details were similar to those described for figure 42, except that the assay medium contained 0.3mM ADP.

X = exogenous NADH oxidase plus succinate oxidase;
▲ = exogenous NADH oxidase; □ = succinate oxidase alone;
O = succinate oxidase in the presence of exogenous NADH.

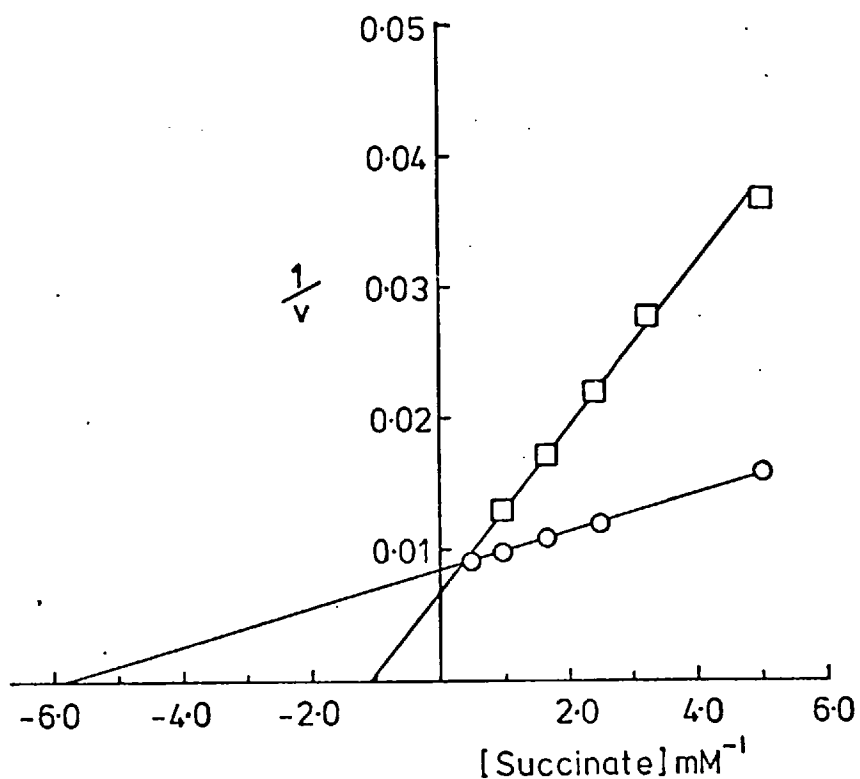


Figure 46. The effect of exogenous NADH on the activity of succinate oxidase under state 3 conditions, in Jerusalem artichoke mitochondria. The details concerning the plotting of this graph are similar to those described for figure 43, except that here, the data used for V and S were taken from figure 45.

□ = succinate oxidase alone; ○ = succinate oxidase in the presence of exogenous NADH.

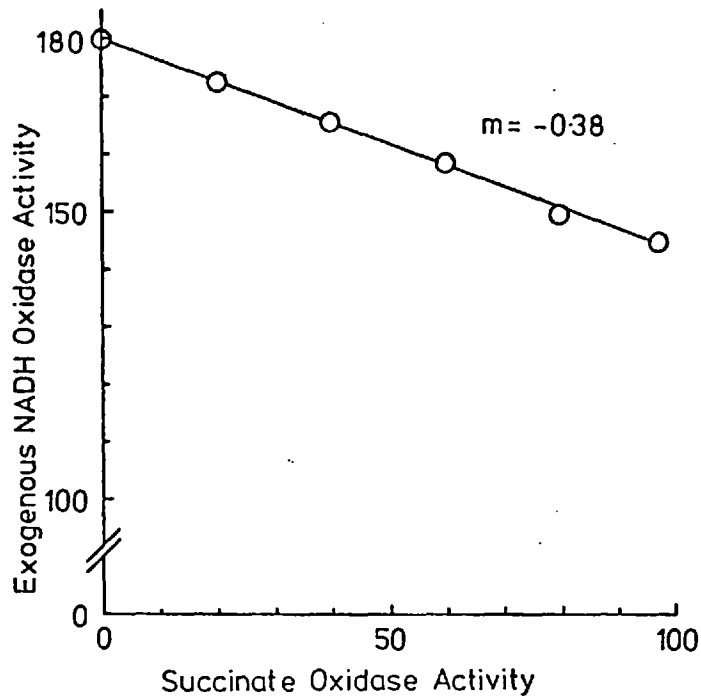


Figure 47. The relationship between the activities of exogenous NADH oxidase and succinate oxidase measured simultaneously under state 3 conditions, in Jerusalem artichoke mitochondria. This graph was obtained by plotting the data from figure 45 for exogenous NADH oxidase, ($\blacktriangle$), on the ordinate against the data for succinate oxidase ($\circ$), on the abscissa. 'm' = the slope of the plot. For other details see figure 45.

c) Interaction between succinate and exogenous NADH oxidases, in mung bean and Neurospora crassa mitochondria.

In the light of the potentially interesting nature of the interaction between succinate and exogenous NADH oxidases found in Jerusalem artichoke mitochondria, it was of interest to see if there was evidence for a similar interaction taking place in other mitochondria, also known to possess an active exogenous NADH oxidase.

Firstly mitochondria isolated from mung bean hypocotyls, were tested, and shown to behave in a manner similar to Jerusalem artichoke mitochondria, as the data presented in figures 48, 49 and 50 show. Secondly, there was evidence for interaction between succinate and exogenous NADH oxidases, in mitochondria isolated from wild type (Y74A) hyphae of Neurospora crassa, as the data in figure 51, show. Thus evidence was found for interaction between succinate and exogenous NADH oxidases, occurring in mitochondria isolated from tubers of Jerusalem artichokes, an example of an angiosperm plant storage tissue, mung bean hypocotyls, an example of a rapidly growing angiosperm plant tissue, and N. crassa hyphae, an example of a fungal tissue.

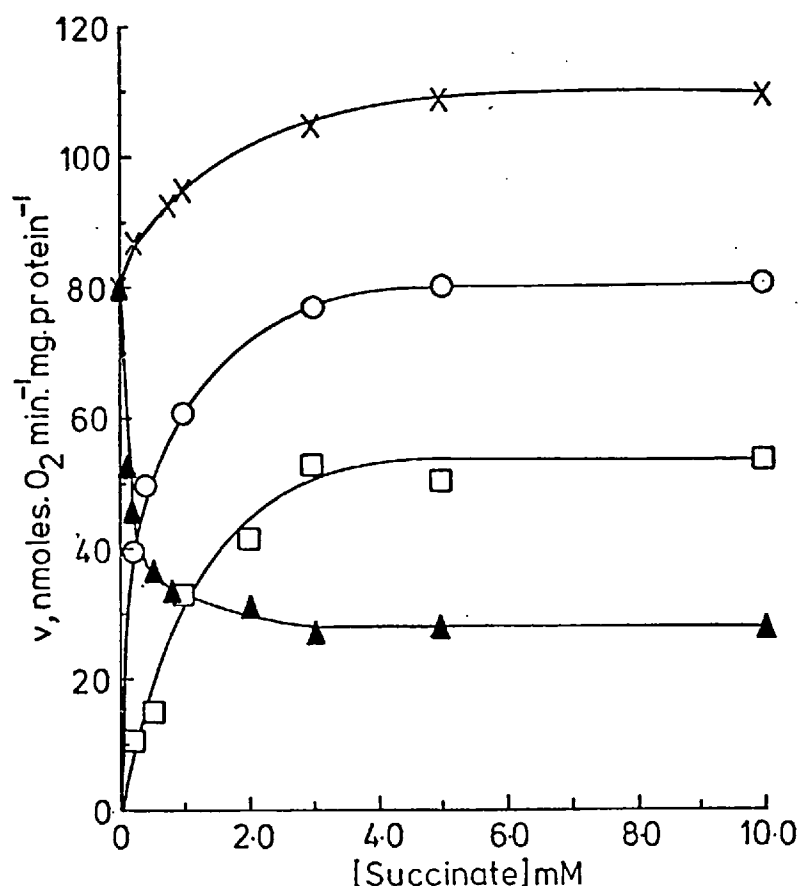


Figure 48. The effect of succinate on the activities of exogenous NADH oxidase and succinate oxidase, measured simultaneously under state 4 conditions, in mung bean mitochondria. The experimental details were similar to those described for Jerusalem artichoke mitochondria in figure 42. ATP/Pi/MgCl₂ 'activated' mitochondria (0.22 mg. protein ml.⁻¹, for X and ▲ ; and 0.67 mg. protein ml.⁻¹ for □.), were suspended in standard assay medium. The initial concentration of exogenous NADH was 114μM.

X = exogenous NADH oxidase plus succinate oxidase;

▲ = exogenous NADH oxidase; □ = succinate oxidase alone;

○ = succinate oxidase in the presence of exogenous NADH.

For details of ATP/Pi/MgCl₂ activation of succinate dehydrogenase, see figure 9, Results 1.ii.

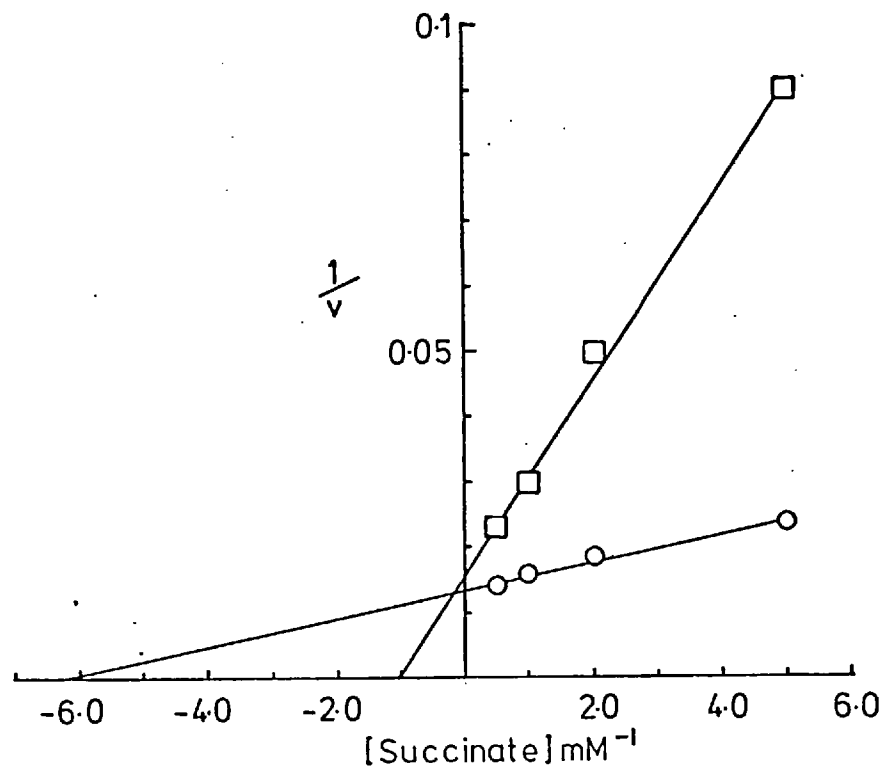


Figure 49. The effect of exogenous NADH on the activity of succinate oxidase under state 4 conditions in mung bean mitochondria. The details concerning the plotting of this graph were similar to those described for figure 43, except that the data used as V and S , were taken from figure 48. $\square$ = succinate oxidase alone; $\circ$ = succinate oxidase measured in the presence of exogenous NADH. For other details see figure 48.

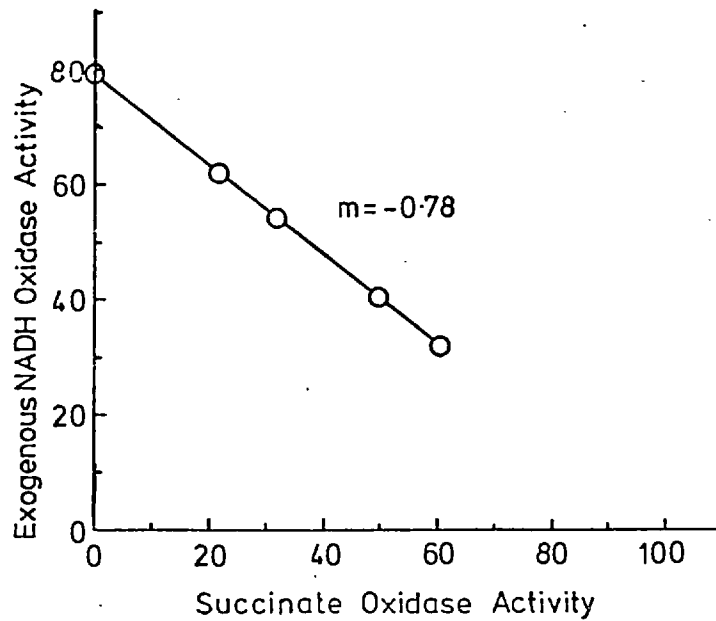


Figure 50. The relationship between the activities of exogenous NADH oxidase and succinate oxidase, measured simultaneously under state 4 conditions in mung bean mitochondria. This graph was obtained by plotting the data from figure 48 for exogenous NADH oxidase activity ($\blacktriangle$), on the ordinate, against data for succinate oxidase activity ($\circ$), on the abscissa. 'm' = the slope of the plot. Other details are as described for figure 48.

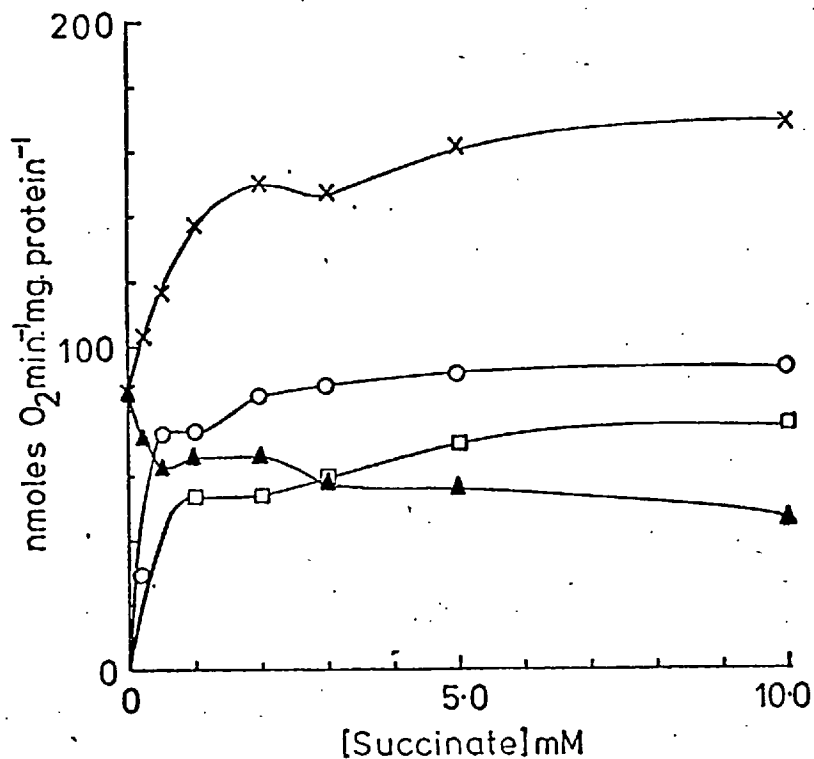


Figure 51. The effect of succinate on the activities of exogenous NADH oxidase and succinate oxidase, under substrate state conditions in *Neurospora crassa* (Y74A) mitochondria. Hyphae of *N.crassa* were cultured, and mitochondria isolated as described in Materials & Methods 2.v. Mitochondria ($0.2 \text{ mg. protein ml.}^{-1}$), were suspended in *N.crassa* assay medium, and a similar experiment carried out to that described for Jerusalem artichoke mitochondria in figure 42.

The initial concentration of exogenous NADH was $100\mu\text{M}$.

X = exogenous NADH oxidase plus succinate oxidase;

▲ = exogenous NADH oxidase; □ = succinate oxidase alone;

○ = succinate oxidase in the presence of exogenous NADH.

d) The effect of the respiratory state on the interaction between succinate and exogenous NADH oxidases.

Under substrate state conditions of exogenous NADH oxidation, succinate was found to inhibit the same amount of the rate of this oxidation, compared with that inhibited under state 3 conditions, (see table 10). There was however, evidence that the component of exogenous NADH oxidase activity that was inhibited by succinate, could be greater under state 4 conditions, than under state 3 conditions. In addition, preliminary experiments indicated that even under state 3 conditions, the activity of exogenous NADH oxidase in isolated Jerusalem artichoke mitochondria, was probably still limited by a reaction involved in the coupling of electron transport to phosphorylation, since substances which are known to uncouple electron transport to oxidative phosphorylation, such as FCCP and S13, were able to result in exogenous NADH oxidase activity becoming stimulated to a greater extent than occurred following the addition of ADP. It was therefore of interest to see if the degree of inhibition of exogenous NADH oxidase activity, caused by succinate, was less under uncoupled conditions than under state 3 and state 4 conditions.

In Jerusalem artichoke mitochondria, figure 52 shows that no inhibition of exogenous NADH oxidase activity by succinate occurred in the presence of uncoupler, compared with a maximum of 24% inhibition under state 3 conditions, and 60% under state 4 conditions. This indicated that coupling of electron transport to oxidative phosphorylation was required, for succinate to be capable of inhibiting the activity of exogenous NADH oxidase, in Jerusalem artichoke mitochondria. Also the magnitude of the component of exogenous NADH oxidase activity, that was inhibited by 2.0mM succinate, in the experiment described in figure 52, was less under state 3 conditions than under state 4

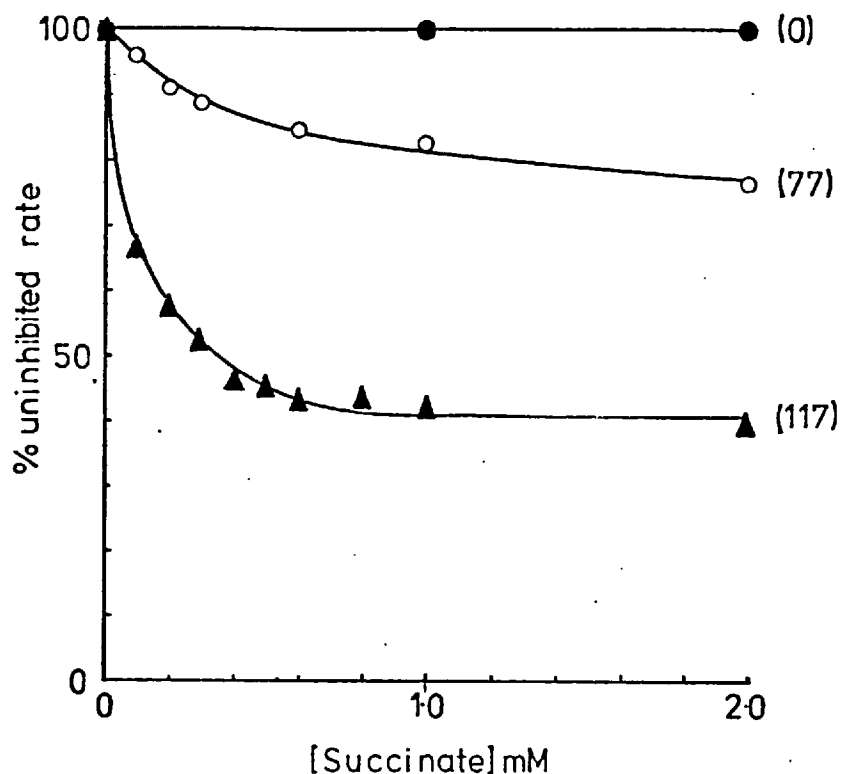


Figure 52. The effect of succinate on the activity of exogenous NADH oxidase, under different respiratory states, in Jerusalem artichoke mitochondria. ATP/Pi/MgCl₂ 'activated' mitochondria (0.15 mg. protein ml.⁻¹), were suspended in standard assay medium for state 4 (▲), standard assay medium plus 0.3mM ADP for state 3 (○), and standard assay medium plus 10⁻⁶M S13, for the uncoupled state (●). Exogenous NADH oxidase activity was measured as described in Materials & Methods 6.v., using an initial concentration of NADH of 106μM, and in the presence of initial concentrations of succinate as indicated in the graphs. Control rates of NADH oxidation were, 194.0 ± 1.6 state 4; 359.0 ± 1.3 state 3; 478.2 ± 0.16 uncoupled, which are the mean of 10 assays ± S.E.M. in each case. The numbers in parentheses represent the maximum succinate-sensitive component of exogenous NADH oxidase activity, as nmoles NADH min.⁻¹ mg. protein⁻¹.

conditions, as the numerical values for these components show (see numbers in parentheses against each curve plotted in figure 52). Reference to the control rates of exogenous NADH oxidation under each respiratory state, which are given in the legend to figure 52, confirms that uncoupler could cause a greater degree of stimulation of this rate, than ADP. A similar pattern of behaviour of exogenous NADH oxidase activity in response to succinate under similar conditions to those just described for Jerusalem artichoke mitochondria, was also found with mung bean mitochondria, as the data in figure 53 show.

Similar effects to those just described for exogenous NADH oxidation, were reflected in oxygen consumption studies, where non-additivity of the rates of oxygen consumption measured for each of succinate or exogenous NADH, added to the mitochondria separately, compared with the rate measured for both substrates supplied together, and oxidized simultaneously, indicated interaction between succinate and exogenous NADH oxidases (table 15). A coefficient of interaction of 1.0, probably indicated absence of interaction, while values greater than 1.0, indicated an interaction in which the rate of consumption of oxygen measured for both substrates oxidized simultaneously, was less than that expected from values obtained for the oxidation of each substrate alone. As can be seen from table 15, interaction between succinate and exogenous NADH oxidases, appeared to be greatest under state 4 conditions, less under state 3 and absent under uncoupled conditions, as expected from the inhibition of exogenous NADH oxidase activity by succinate, shown in figure 52.

It is possible to restrict the rate of electron transport in isolated mitochondria, by inhibiting the energy conservation reactions coupled to it. The inhibitor oligomycin is an 'energy-transfer' inhibitor, which appears to inhibit phosphorylation of

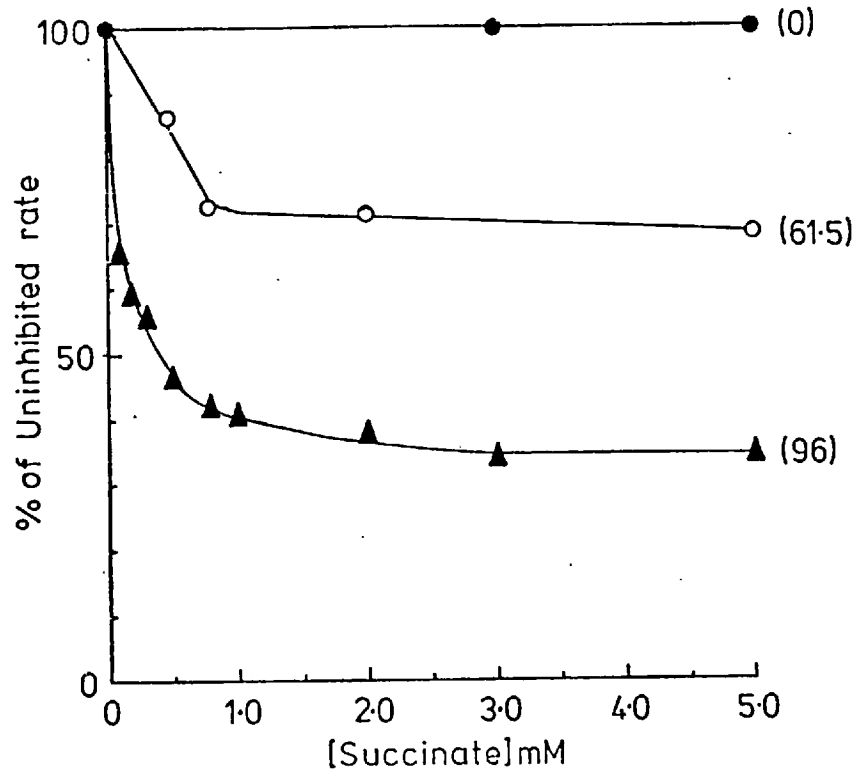


Figure 53. The effect of succinate on the activity of exogenous NADH oxidase, under different respiratory states, in mung bean mitochondria. The experimental details for this figure were similar to those for figure 52, except that mung bean mitochondria ($0.22 \text{ mg. protein ml.}^{-1}$), were used instead of Jerusalem artichoke mitochondria. The mitochondria were preincubated with ATP/Pi/MgCl_2 , to activate succinate dehydrogenase, as described for figure 9. The control rates of NADH oxidation were, 146.1 ± 2.1 state 4 (10 assays); 198.8 ± 1.8 state 3 (5 assays), and 229.8 ± 0.14 (6 assays). These values represent the mean of the assay values $\pm$ S.E.M. The numbers in the parentheses represent the maximum succinate-sensitive component of exogenous NADH oxidase activity, as $\text{nmoles NADH min.}^{-1} \text{ mg. protein}^{-1}$. $\blacktriangle$ = state 4; $\circ$ = state 3; $\bullet$ = uncoupled state.

TABLE 15.

The Effect on the Rate of Consumption of Oxygen of the Simultaneous Oxidation of Exogenous NADH and Succinate in Jerusalem Artichoke Mitochondria, under states 4, 3 and under uncoupled conditions.

<u>Substrate</u>	<u>Rate of Consumption of Oxygen</u> (nmoles O ₂ min. ⁻¹ mg. protein ⁻¹).		
	<u>State 4</u>	<u>State 3</u>	<u>Uncoupled</u>
NADH (0.85mM)	77.4	172.1	244
Succinate (15mM)	84.6	172.3	176.3
NADH (0.85mM) + Succinate (15mM) = y	99.3	236.7	381.8
NADH + Succinate Expected if Additive = x	162	344.4	420.3
Coefficient of Interaction = $\frac{x}{y}$	1.64	1.46	1.1

ATP/Pi/MgCl₂ 'activated' mitochondria (0.4 mg. protein ml.⁻¹), were suspended in standard assay medium, which also contained 0.5mM ADP for state 3 measurements, and 10⁻⁶ M Sl3 for uncoupled state measurements. NADH (0.85mM) or succinate (15mM), or both substrates simultaneously, were added to the mitochondria and the rate of consumption of oxygen measured polarographically, as described in Materials & Methods 5. For details of ATP/Pi/MgCl₂ activation of succinate dehydrogenase, see figure 9, Results 1.ii.

a non-phosphorylated 'high energy' intermediate of the reaction between electron transport and the synthesis of ATP (see Palmer & Hall, 1972). Hence, oligomycin causes partial inhibition of the state 3 rate of electron transport, to a rate similar to the 'natural' state 4 rate, but in the presence of excess ADP. When the sensitivity of the oligomycin inhibited state 3 rate of exogenous NADH oxidation, in Jerusalem artichoke mitochondria, to inhibition by succinate, was compared with the sensitivity under state 4 and state 3 conditions, it was found that the oligomycin inhibited activity was much more sensitive to succinate than the state 3 activity, although not quite as sensitive as the state 4 activity of exogenous NADH oxidase, as the data in table 16 show. Thus, whether the rate of electron transport from exogenous NADH to oxygen was restricted by the absence of phosphate acceptor, as under state 4 conditions, or by the presence of an energy transfer inhibitor such as oligomycin, the restricted rate of electron transport was more sensitive to inhibition by succinate, than the less restricted state 3 rate.

It has been established in the preceding sub-sections of the results, that mitochondria isolated from plant tissues possess the capacity for interaction between the enzymes, exogenous NADH oxidase and succinate oxidase. In order to study the mechanism of this interaction further, it was considered desirable to simplify the system. An initial step in such simplification was to prepare sub-mitochondrial particles from the mitochondria, which are membrane vesicles which possess both exogenous NADH oxidase and succinate oxidase activity, but unfortunately lack respiratory control.

TABLE 16.

The Effect of Succinate on the Activity of Exogenous NADH Oxidase under State 4, State 3 and State 3
in the Presence of Oligomycin, Conditions, in Jerusalem Artichoke Mitochondria.

<u>Experiment</u>	<u>Exogenous NADH Oxidase Activity (nmoles NADH min.⁻¹ mg. protein)</u>				<u>% Inhibition</u>
	<u>State 3</u>	<u>State 4</u>	<u>+ Oligomycin</u>	<u>+ Succinate</u>	<u>by Succinate</u>
A	-	171	-	84.5	51
B	-	170	170	-	-
C	277	-	173	104.5	39.5
D	282	-	-	238	15.5

ATP/Pi/MgCl₂ 'activated' mitochondria (0.1 mg. protein ml.⁻¹), were suspended in standard assay medium in the presence of 0.2mM ADP for state 3 measurements, in a spectrophotometer cuvette. NADH (133uM) was added, and the rate of oxidation of NADH was measured at 340-374 nm. as described in Materials & Methods 6.v. Oligomycin (13.6 ug. mg. protein⁻¹) and succinate (0.8mM) were added as indicated, and the rate of oxidation of NADH recorded before and after each addition. For details of ATP/Pi/MgCl₂ activation of succinate dehydrogenase see figure 9, Results 1.ii.

e) Estimation of interaction between exogenous NADH and succinate oxidases in sub-mitochondrial particles.

The apparent dependence of the inhibition of exogenous NADH oxidase by succinate, upon functional respiratory control, indicated that preparation of sub-mitochondrial particles, would result in the loss of the capacity for this interaction between exogenous NADH and succinate oxidases. However sub-mitochondrial particles were prepared from Jerusalem artichoke mitochondria, and their succinate dehydrogenases were activated as described for intact mitochondria in Results 1.ii.

The data presented in table 17, show that succinate dehydrogenase was proportionately less deactivated in the sub-mitochondrial particles, compared with the degree of deactivation found earlier for intact mitochondria (see table 2 and figures 9 and 10), although the absolute maximum value for succinate oxidase activity measured following activation, was greater for whole mitochondria than for sub-mitochondrial particles.

Sub-mitochondrial particles in which the enzyme succinate dehydrogenase had been activated, catalyzed the oxidation of exogenous NADH, in a manner that was only slightly inhibited by succinate, as the data presented in table 18 indicate. This table also shows data obtained from an experiment in which oligomycin was present, in an attempt to maximize the degree of restriction imposed upon the rate of electron transport, by reactions associated with the transfer of energy to ATP. As can be seen, oligomycin caused a low level of inhibition of exogenous NADH oxidase activity in its own right, but it did not result in an increase in the level of inhibition of the rate of NADH oxidation caused by succinate.

TABLE 17.

The Effect of Preincubation with ATP/Pi/MgCl₂ on the Activity of Succinate Oxidase in Sub-mitochondrial Particles Prepared from Jerusalem Artichoke Mitochondria.

<u>Preincubation</u>	<u>Succinate Oxidase Activity</u> (nmoles oxygen min. ⁻¹ mg. protein ⁻¹)
-	44
+	75

Sub-mitochondrial particles were prepared from Jerusalem artichoke mitochondria by sonic disruption, as described in Materials & Methods 4., and suspended in standard assay medium at a protein concentration of 0.5 mg. ml.⁻¹ before activation of succinate dehydrogenase with ATP/Pi/MgCl₂ (see figure 9 for details of procedure), and 0.4 mg. ml.⁻¹ after such activation. Succinate (10mM) was added to the particles, and the resultant rate of consumption of oxygen measured polarographically, as described in Materials & Methods 5. The same concentrations of ATP, Pi and MgCl₂ were used to activate succinate dehydrogenase as were used for whole mitochondria (figure 9).

TABLE 18.

The Effect of Succinate on the Activity of Exogenous NADH Oxidase in Jerusalem Artichoke Sub-mitochondrial Particles, in the Presence and Absence of Oligomycin.

<u>Oligomycin</u>	<u>NADH Oxidase Activity</u> (nmoles NADH min. ⁻¹ mg. protein ⁻¹)			<u>% Inhibition by Succinate</u>
	<u>Control</u>	<u>+ Succinate</u>		
-	385	314		18.3
+	326	326		0.0

ATP/Pi/MgCl₂ 'activated' sub-mitochondrial particles (0.1 mg. protein ml.⁻¹), were suspended in standard assay medium plus or minus oligomycin (50 ug. mg. protein⁻¹). NADH (117uM) was added to the reaction cuvette, and NADH oxidase activity measured spectrophotometrically, using the wavelength pair 340-374 nm., as described in Materials & Methods 6.v. One minute after a constant rate was obtained, 8mM succinate was added, and the following constant rate of NADH oxidation recorded. Details of preparation and 'activation' of the sub-mitochondrial particles, were as for table 17.

As might be expected from the results just presented, oxygen uptake studies with sub-mitochondrial particles also failed to show evidence for any major interaction between exogenous NADH and succinate oxidases, as the data in table 19 indicate. It was therefore concluded that the preparation of sub-mitochondrial particles from Jerusalem artichoke mitochondria, resulted in the loss of the capacity for interaction between exogenous NADH and succinate oxidases, as expected from the loss of respiratory control which accompanies this preparation. Thus, further investigations into the nature of the mechanism of the interaction between succinate and exogenous NADH oxidases, were carried out using intact mitochondria.

Interaction between exogenous NADH and succinate oxidases in intact plant mitochondria, has been shown to result in the activity of exogenous NADH oxidase becoming partially inhibited by succinate, while the activity of succinate oxidase was stimulated, but only under conditions when electron transport from exogenous NADH was limited by the energy transfer reactions. It was therefore considered that both effects might be explained if succinate was able to donate reducing equivalents to exogenous NAD^+ , the product of oxidation of exogenous NADH, and so effectively recycle the exogenous NADH. Such behaviour would be expected to result in an apparent inhibition of exogenous NADH oxidase activity, while simultaneously producing an apparent stimulation of succinate oxidase activity, as found here (see Results 2.iv.b.) This possibility was therefore tested experimentally.

TABLE 19.

The Effect on the Rate of Consumption of Oxygen of the Simultaneous
Oxidation of NADH and Succinate in Jerusalem Artichoke Sub-mito-
chondrial Particles

<u>Substrate</u>	<u>Rate of Consumption of Oxygen</u> (nmoles min. ⁻¹ mg. protein ⁻¹).
NADH (1.0mM)	190
Succinate (15mM)	70
NADH (1.0mM) + Succinate (15mM) = y	274
NADH + Succinate Expected if additive = x	260
Coefficient of Interaction = $\frac{x}{y}$	0.95

ATP/Pi/MgCl₂ 'activated' sub-mitochondrial particles (0.4 mg. protein ml.⁻¹), were suspended in standard assay medium. NADH (1.0mM) or succinate (15mM) or both substrates together, were added to the reaction cuvette, and the rate of consumption of oxygen measured polarographically as described in Materials & Methods 5. The expected rate of consumption of oxygen assumes additivity of the rates measured separately for NADH and succinate = x, and y = the rate actually measured). For details of ATP/Pi/MgCl₂ activation of succinate dehydrogenase, see table 17.

f) Estimation of the rate of reduction of exogenous NAD^+ by succinate.

In order to see whether mitochondria isolated from Jerusalem artichoke tubers, could catalyze the reduction of exogenous NAD^+ by succinate, an experiment was carried out in which these mitochondria were supplied with NAD^+ and succinate, in the presence of sufficient potassium cyanide to totally inhibit the oxidation of any NADH produced catalyzed by the mitochondrial exogenous NADH oxidase. No evidence was found for the existence of an enzyme capable of catalyzing the reduction of exogenous NAD^+ by succinate in isolated Jerusalem artichoke mitochondria, as the data presented in table 20 show. It can also be seen from this data, that exogenous NADH oxidation catalyzed by mitochondria from the same preparation, was found to be very sensitive to inhibition by succinate, as expected.

This result is in accord with the lack of any evidence to date, for energy conservation taking place between ubiquinone and exogenous NADH dehydrogenase in isolated plant mitochondria. Such a reaction would be expected to be required for the catalysis of the reverse electron flow necessary for succinate to reduce NAD^+ , since the succinate/fumarate redox couple has a mid-point potential at pH 7.0 of 0mV, while the equivalent value for NADH/NAD^+ is -320mV. Also, there have been no reports of the existence of a transhydrogenase capable of catalyzing the transfer of reducing equivalents from succinate to exogenous NAD^+ in isolated plant mitochondria, a reaction that would require reducing equivalents to be transferred across the inner mitochondrial membrane. It was therefore considered that the inhibition of exogenous NADH oxidase activity by succinate, and the stimulation of succinate oxidase activity by exogenous NADH, was probably real and not apparent, as first thought.

TABLE 20

Estimation of the Rate of Reduction of Exogenous NAD^+ by Succinate catalyzed by Jerusalem Artichoke Mitochondria.

Rate of reverse electron flow from succinate to exogenous NAD^+ as nmoles NAD^+ reduced $\text{min.}^{-1} \text{ mg. protein}^{-1}$.		Rate of forward electron flow from exogenous NADH to oxygen as nmoles NADH oxidized $\text{min.}^{-1} \text{ mg. protein}^{-1}$.	
Reagents present in the reaction cuvette.		Reagents present in the reaction cuvette.	
NAD^+ (100uM)	NAD^+ (100uM) plus succinate (10mM)	NADH (100uM)	NADH (100uM) plus succinate (10mM)
0	0	187.0	44.4

ATP/Pi/ MgCl_2 'activated' mitochondria ($0.1 \text{ mg. protein ml.}^{-1}$), were suspended in standard assay medium. For reverse electron flow only, 3mM KCN was also present, to prevent forward electron flow to oxygen. Other reagents were added to the reaction cuvette as indicated, and the rate of change in NADH concentration, was measured spectrophotometrically at 340 nm. as described in Materials & Methods 6.v. and 9. For details of ATP/Pi/ MgCl_2 activation of succinate dehydrogenase see figure 9 Results 1.ii.

The next possibility considered for the mechanism of interaction between succinate and exogenous NADH oxidases, was based on the conclusion drawn by Gutman & Silman (1972), from a study of the interaction between succinate and NADH oxidases in electron transport particles prepared from beef heart mitochondria. These authors found evidence for a mutual inhibition between succinate and NADH oxidases, to the maximum extent of approximately 10% in each case, which they concluded could be explained by assuming that ubiquinone is compartmentalized with respect to succinate dehydrogenase and NADH dehydrogenase, as discussed in the Introduction 2.

Although the results found in the present study of the interaction between exogenous NADH and succinate oxidases, were different from those reported by Gutman & Silman (1972) for electron transport particles prepared from beef heart mitochondria, it was not possible at this stage to rule out the involvement of changes in the state of reduction of a component of the electron transport pathways of isolated plant mitochondria, in the interaction between these two enzymes. A study was therefore made of the oxidation-reduction states of those components of the electron transport pathways of Jerusalem artichoke mitochondria, which are visible in a light absorbance spectrophotometer, and undergo changes in absorbance as a result of changes in their redox state. Firstly however, an attempt was made to locate the site of interaction between succinate oxidase and exogenous NADH oxidase, by functional segmentation of the electron transport pathways, using alternative artificial terminal oxidants of these pathways.

g) Location of the site of interaction between succinate and exogenous NADH oxidases.

It is possible to functionally separate that part of the electron transport pathway which lies on the substrate side of cytochrome c, from that which lies on the oxygen side of cytochrome c, by diverting the flow of reducing equivalents from oxygen, to added, or exogenous, ferricytochrome c. The inhibitor of cytochrome c oxidase, potassium cyanide, was present in the assay medium to prevent the flow of reducing equivalents to oxygen, and a low osmolarity assay medium was used, to permit ferricytochrome c to interact to the maximum extent with the endogenous cytochrome c molecules; see Results 1.iii. and Materials & Methods 6.ix., for details.

The activities of succinate and exogenous NADH -cytochrome c reductases were first measured as a function of the initial concentration of succinate or exogenous NADH, in isolated Jerusalem artichoke mitochondria, under state 4 conditions i.e. following the activation of succinate dehydrogenase by preincubation with ATP, Pi and $MgCl_2$, and the data presented in figure 54, show results obtained. It can be seen from this figure, that succinate-cytochrome c reductase activity saturated at approximately 0.4mM succinate, and the activity of exogenous NADH-cytochrome c reductase saturated at approximately 100uM NADH, under the conditions of assay.

Interaction between succinate-cytochrome c reductase and exogenous NADH-cytochrome c reductase, was estimated by measuring the rate of reduction of added ferricytochrome c, by succinate or exogenous NADH and comparing the sum of the two rates, with that measured for both succinate and exogenous NADH present simultaneously. This method was similar to that used earlier for estimating the degree of interaction between succinate and exogenous NADH oxidases, by

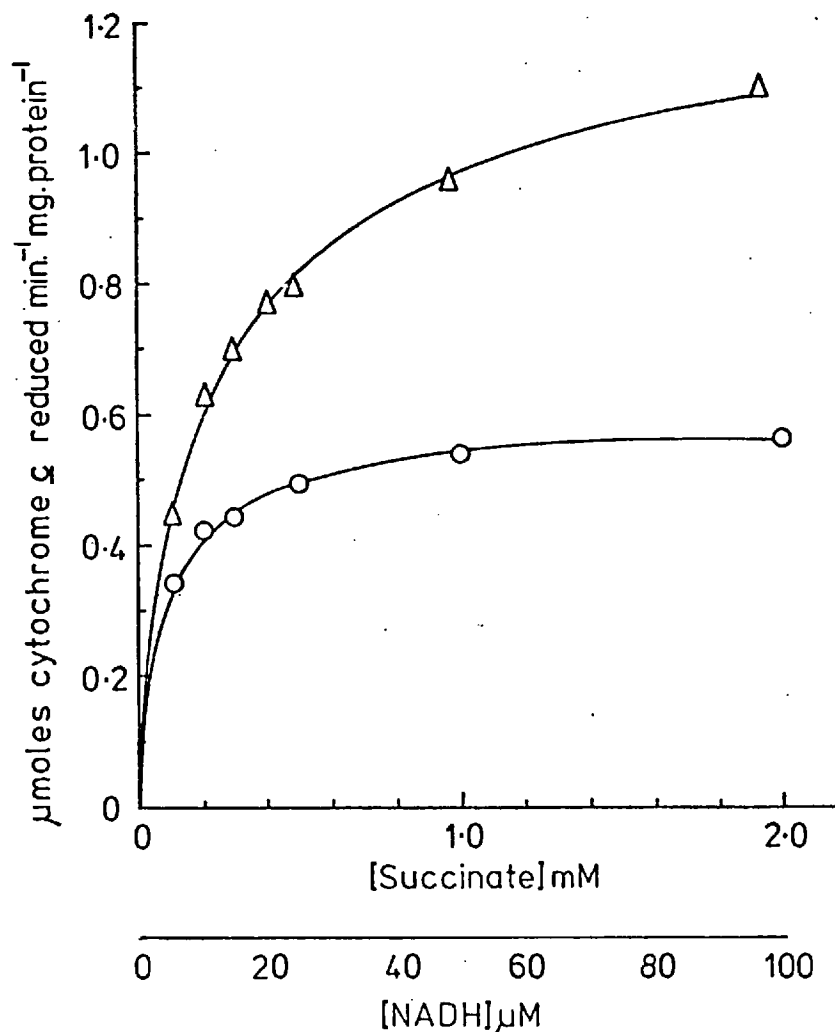


Figure 54. The effect of the concentration of NADH on the activity of exogenous NADH-cytochrome c reductase, and the concentration of succinate on the activity of succinate-cytochrome c reductase, in Jerusalem artichoke mitochondria. ATP/Pi/MgCl₂ 'activated' mitochondria (0.012 mg. protein ml.⁻¹), were suspended in cytochrome c reductase assay medium as described in Materials & Methods 6.ix., which contained 1mM KCN, and 36μM ferricytochrome c (horse heart). The reaction was started by the addition of the appropriate concentration of NADH or succinate, and the rate of reduction of ferricytochrome c was measured spectrophotometrically at 550-540 nm., as described in Materials & Methods 6.ix. Δ = exogenous NADH-cytochrome reductase; O = succinate-cytochrome c reductase.

measurement of the rates of oxygen consumption (see tables 15 and 19). The data presented in table 21, show that a numerical value of 1.63 was found, for the coefficient of interaction between succinate-cytochrome c reductase and exogenous NADH-cytochrome c reductase, in isolated Jerusalem artichoke mitochondria, under state 4 conditions. This value was similar to that found for oxygen consumption (see table 15), and indicated that the site of interaction between succinate and exogenous NADH oxidases is located on the substrate side of cytochrome c, in Jerusalem artichoke mitochondria.

The artificial electron acceptors, juglone and duroquinone, were then used as oxidants of exogenous NADH and succinate in isolated Jerusalem artichoke mitochondria, under conditions when interaction between succinate and exogenous NADH oxidases would be expected to be maximal, i.e. state 4. The data already presented (figure 7), has shown that both juglone and duroquinone, accept electrons from exogenous NADH on the substrate side of the antimycin A-sensitive site in the electron transport pathways of isolated Jerusalem artichoke mitochondria. The data presented in table 22, show that exogenous NADH-juglone and exogenous NADH-duroquinone reductase activities, were both insensitive to succinate, in contrast with exogenous NADH-oxygen reductase activity, i.e. exogenous NADH oxidase activity, which was found to be very sensitive to inhibition by succinate, as expected from data already presented (see figures 42 and 52). The data in table 22 are consistent with juglone and duroquinone accepting electrons from exogenous NADH, at a point in the electron transport pathways, located before the point of interaction between succinate and exogenous NADH oxidases in isolated Jerusalem artichoke mitochondria.

TABLE 21.

The Effect on the Rate of Reduction of Ferricytochrome c by Exogenous NADH and Succinate Simultaneously Compared with each Substrate added Separately to Jerusalem Artichoke Mitochondria.

<u>Substrate</u>	<u>Rate of Reduction of Ferricytochrome c,</u> <u>(Antimycin A-Sensitive Component) as</u> nmoles ferricytochrome c reduced min. ⁻¹ mg. protein ⁻¹ .
NADH (473uM)	752.8 ± 1.3
Succinate (10mM)	432.1 ± 3.6
NADH (473uM) + Succinate (10mM) = y	729.2 ± 3.2
NADH + Succinate	
Expected if Additive = x	1184.9
Coefficient of	
Interaction = $\frac{x}{y}$	1.63

ATP/Pi/MgCl₂ 'activated' mitochondria (0.15 mg. protein ml.⁻¹), were suspended in cytochrome c reductase assay medium, and the rate of reduction of ferricytochrome c, was measured as described for table 54. The initial concentration of ferricytochrome c was 33uM. For details of ATP/Pi/MgCl₂ activation of succinate dehydrogenase, see figure 9. The values represent the mean of 3 assays ± S.E.M. in each case.

TABLE 22.

The Effect of Succinate on the Activity of Exogenous NADH-Oxygen Reductase, Exogenous NADH-Duroquinone Reductase, and Exogenous NADH-Juglone Reductase, in Jerusalem Artichoke Mitochondria.

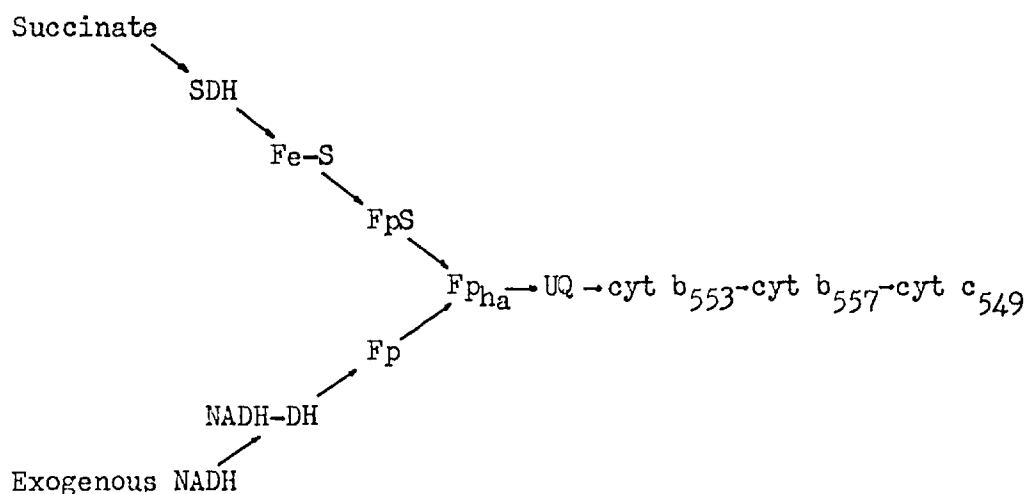
<u>Reductase</u>	<u>Rate of oxidation of</u>		
	<u>Exogenous NADH (nmoles</u>		<u>% Inhibition</u>
	<u>min.⁻¹</u>	<u>mg. protein⁻¹)</u>	
	<u>Control</u>	<u>+ Succinate</u>	<u>by Succinate</u>
NADH - oxygen	123	26.2	79.0
NADH - duroquinone	692	685	< 5
NADH - juglone	1000	950	5.0

ATP/Pi/MgCl₂ 'activated' mitochondria (0.1 mg. protein ml.⁻¹), were suspended in standard assay medium containing (a) no additions, (b) duroquinone (150uM) and KCN (1.0mM), and (c) juglone (150uM) and KCN (1.0mM). NADH (100uM) was added to the reaction cuvette, and the rate of oxidation of exogenous NADH by each of the three terminal electron acceptors measured spectrophotometrically at 340-374 nm. as described in Materials & Methods 6.v. (see also 6.viii.) One minute after attainment of a constant rate of NADH oxidation, 2.0mM succinate was added to the reaction cuvette, and the following rate recorded. For details of ATP/Pi/MgCl₂ activation of succinate dehydrogenase see figure 9, Results 1.ii.

h) The effects of exogenous NADH and succinate oxidation on the level of reduction of components mediating electron transport.

An attempt was made to identify any component(s) mediating electron transport to oxygen from exogenous NADH and/or succinate in isolated Jerusalem artichoke mitochondria, under state 4 conditions, which might be located between exogenous NADH dehydrogenase and the point of interaction between succinate oxidase and exogenous NADH oxidase. This was carried out by measuring the steady-state levels of reduction of those components of the electron transport pathways, which undergo measurable changes in absorbance of light, upon changing their state of reduction, and are thought to be located on the substrate side of cytochrome c.

The present state of knowledge concerning this part of the electron transport pathways of plant mitochondria, is outlined below:



SDH, succinate dehydrogenase; Fe-S, iron sulphur centres; FpS, flavo-protein associated with SDH; Fp_{ha}, flavoprotein with high absorbance: fluorescence ratio; NADH-DH, exogenous NADH dehydrogenase; UQ, ubiquinone; cyt, cytochrome, and the subscript numbers, refer to the

wavelength in nm., at which each cytochrome has maximum absorbance by its α band, at 77K. (see also figure 4).

This scheme indicates that the components of particular interest to the present investigation, were the flavoproteins, and especially Fp_{ha} , since it was thought to be located at the point of junction between exogenous NADH oxidase and succinate oxidase. Also, of interest were ubiquinone, and the cytochromes b and c. Thus, evidence was sought for absorbance changes corresponding to changes in the level of reduction of these components, as a result of adding succinate following exogenous NADH, to Jerusalem artichoke mitochondria, under state 4 conditions. In particular, evidence was sought for very rapid extra reduction of each component following the addition of the succinate, in the presence of exogenous NADH. These experiments were attempted using the dual-wavelength spectrophotometer, as described in Materials & Methods 6.

Looking first at ubiquinone, because of its suggested role in the mechanism of interaction between succinate and NADH oxidases in animal electron transport particles (Gutman & Silman, 1972). Changes in the level of reduction of endogenous mitochondrial ubiquinone, were estimated by the method used by Storey (1972), described here in Materials & Methods 6.iv., in which an increase in the level of reduction of ubiquinone was seen as a decrease in absorbance measured at 282-295 nm. Figure 55 shows results obtained when succinate was the substrate for Jerusalem artichoke mitochondria. It can be seen that the signal:noise ratio was low, which made this type of measurement difficult. In addition to this, it was found that the absorbance by NADH at 282-295 nm. wavelength, was extremely large, so it was not possible to carry out the type of experiment outlined above, for ubiquinone.

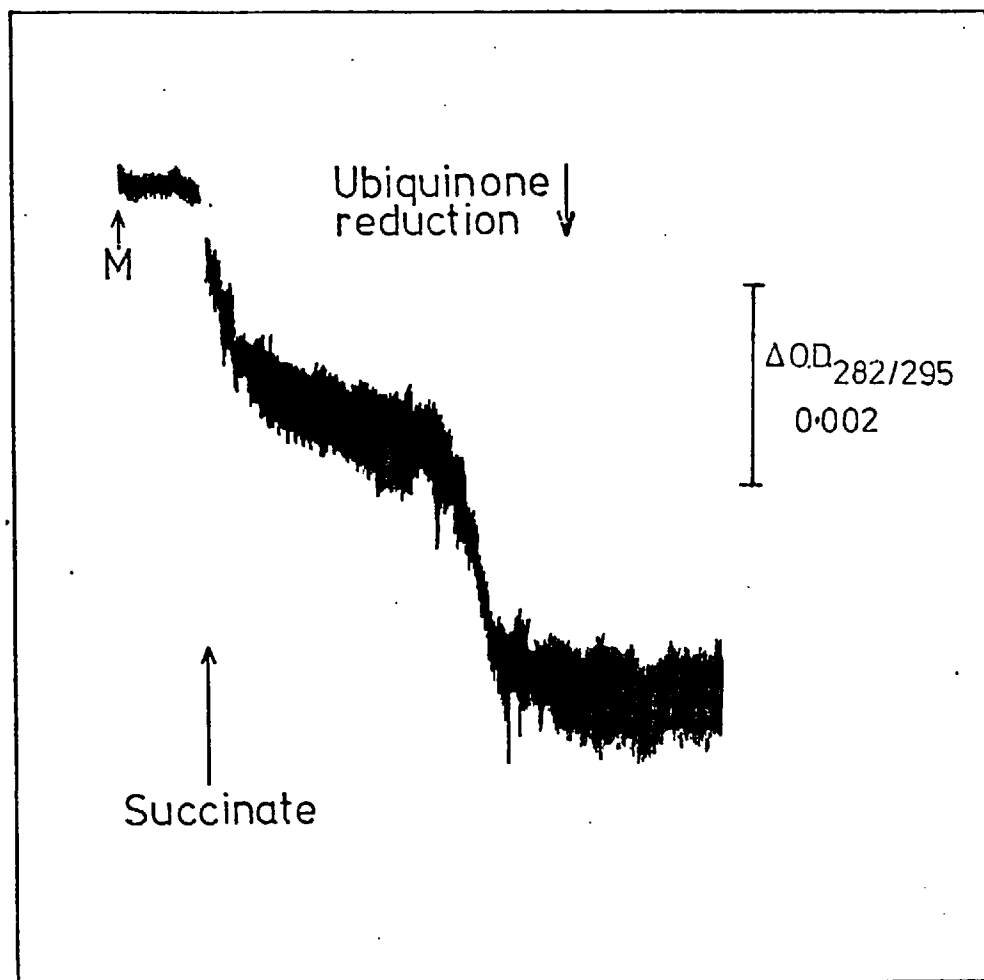


Figure 55. The kinetics of reduction of endogenous ubiquinone by succinate, in Jerusalem artichoke mitochondria. Dual-wavelength spectrophotometer traces showing the pattern of reduction of endogenous ubiquinone by succinate. ATP/Pi/MgCl₂ 'activated' mitochondria (1.2 mg. protein ml.⁻¹) were suspended in standard assay medium in a 2mm. light path length silica cuvette, and endogenous ubiquinone reduction followed using a dual-wavelength spectrophotometer at 282-295 nm., as described in Materials & Methods 6.iv. For details of ATP/Pi/MgCl₂ activation of succinate dehydrogenase see figure 9, Results 1.ii. The concentration of succinate added to the mitochondria (M), was 10mM.

There are reports in the literature that mitochondrial ubiquinone can be extracted in the state of reduction which existed at the moment of extraction. Szarkowska & Klingenberg (1963), and Kroger & Klingenberg (1966), have used an extraction mixture consisting of methanol and petroleum spirit, and Storey (1966), has used a mixture of methanol and n-hexane. All of the extractions were carried out under anaerobic conditions, to prevent auto-oxidation of reduced ubiquinone by the oxygen of the laboratory atmosphere. Both methods of extraction of ubiquinone from isolated Jerusalem artichoke mitochondria, were attempted in the present study, but it proved impossible within the time available, to obtain sufficiently consistent values for the ratio of reduced:oxidized ubiquinone, for their serious consideration as part of the present thesis.

The extraction of ubiquinone from isolated plant mitochondria was relatively simple however, it was the trapping of the redox state that was difficult to reproduce. Absorbance spectra of reduced minus oxidized ubiquinone, have been included to show that the spectra obtained with organic solvent extracts from Jerusalem artichoke and Arum maculatum spadix mitochondria, were very similar to the spectrum measured for ubiquinone₁₀ purchased from a chemical supplier (Sigma(London) Chemical Co. U.K.), indicating a low level of absorbance due to contaminants, even in these crude extracts (see figure 56). The similarity between the spectra, might be indicating that the ubiquinone extracted from both mitochondrial sources, was ubiquinone₁₀, as reported to be the case in mung bean mitochondria by Beyer, Peters & Ikuma (1968). The verification of this requires chromatographic characterization of the extracted ubiquinone, which was considered to be beyond the scope of the present study.

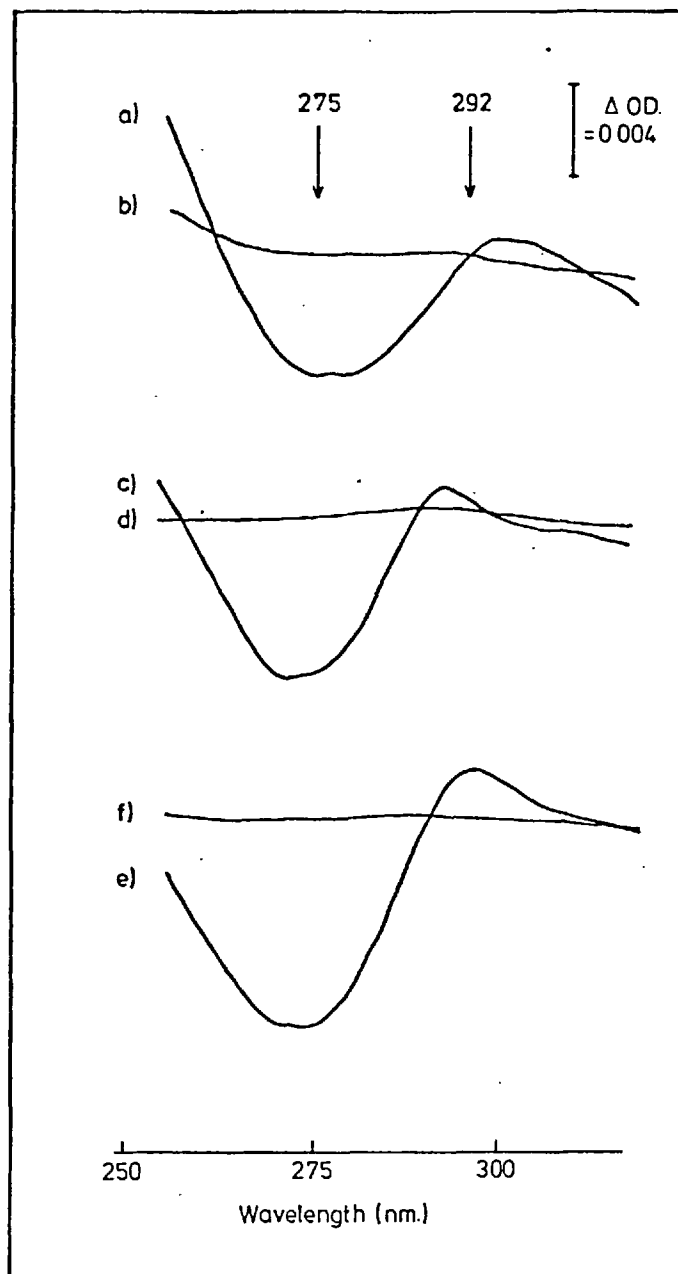


Figure 56. Reduced-minus-oxidized difference absorbance spectra of ubiquinone. Traces (b), (d) and (f) are oxidized minus oxidized spectra, and traces (a), (c) and (e) are sodium borohydride (crystal) reduced minus oxidized spectra, obtained as described in Materials & Methods 7.iii. Traces (a) and (b) were obtained with a methanol-hexane extract of Jerusalem artichoke mitochondria, traces (c) and (d) were obtained with a pentane extract of *Arum maculatum* spadix mitochondrial lyophylate, and traces (e) and (f) were obtained with ubiquinone₁₀ purchased from Sigma(London)Chemical Co. U.K., at a concentration of approximately 2 ug. ml.⁻¹ ethanol. See Materials & Methods 7.i.&ii. for details of extracting mitochondrial ubiquinone.

Turning now to the absorbing flavoproteins in isolated Jerusalem artichoke mitochondria. These were studied using the method described by Storey (1970a), which is reproduced in the Materials & Methods section 6.ii., in which a decrease in absorbance at the wavelength pair 468-493 nm., indicated an increase in the level of reduction of absorbing flavoproteins. Figure 57 shows traces obtained from an experiment designed to see if the absorbing flavoproteins responded to the addition of succinate, following that of exogenous NADH, in a rapid way, indicating the possible involvement of such a component at the junction point between exogenous NADH and succinate oxidases. It can be seen from trace (c) in figure 57, that there was no evidence for such a rapid increase in reduction of the absorbing flavoproteins. The reduction of absorbing flavoproteins as measured by absorbance decreases at 468-493 nm., and resulting from the addition of succinate following exogenous NADH, was considered to be too slow for the to be closely involved in the mechanism by which succinate caused the inhibition of exogenous NADH oxidase activity, and hence also too slow for their possible existence at the point of junction between succinate and exogenous NADH oxidases.

The effect of exogenous NADH and succinate, on the level of reduction of cytochromes b and c, in isolated Jerusalem artichoke mitochondria was also investigated at some length, using the dual-wavelength spectrophotometer, and a technique similar to those used for ubiquinone and flavoproteins, which is described in Materials & Methods 6.iii. In addition, a low temperature spectral analysis technique was employed, in which the cytochromes were freeze-trapped in the state of reduction characteristic of the moment of freezing the mitochondria. A similar technique has been described by Chance & Spencer (1959).

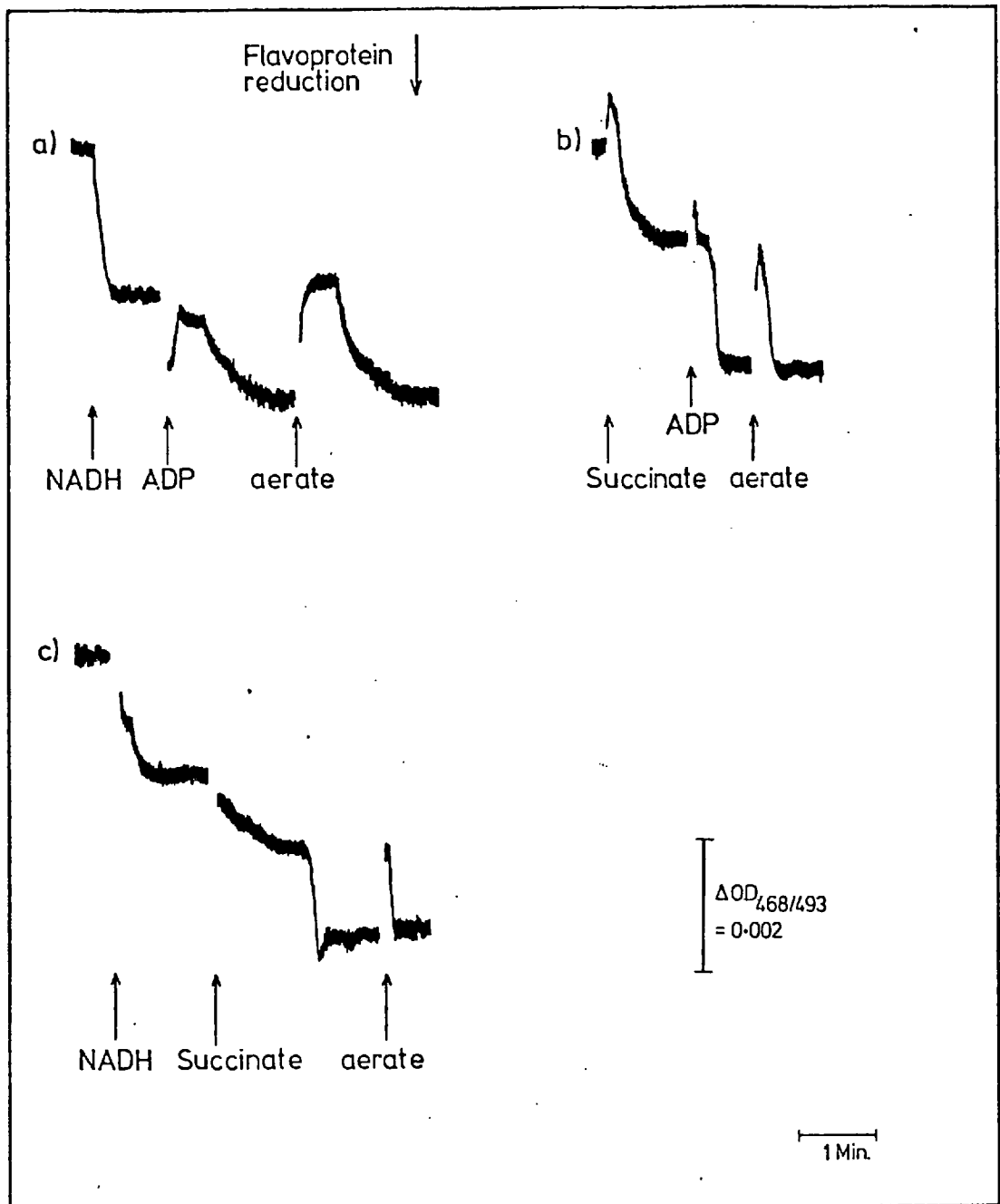


Figure 57. The kinetics of reduction of light absorbing flavoproteins in Jerusalem artichoke mitochondria, under state 4 conditions with exogenous NADH and succinate as substrates. Dual-wavelength spectrophotometer traces showing the pattern of reduction of light absorbing flavoproteins. For details of the method see Materials & Methods 5.ii. ATP/Pi/MgCl₂ 'activated' mitochondria (1.2 mg. protein ml.⁻¹), were suspended in standard assay medium, and additions made to the cuvette as indicated. Concentrations of reagents were, NADH 1.5mM, succinate 5mM, and ADP 50μM aliquots. For details of ATP/Pi/MgCl₂ activation of succinate dehydrogenase see figure 9.

The wavelength-pairs used here for measuring changes in absorbance due to cytochromes b and c, were the same as those used by Lance & Bonner (1968), and were 550-540 nm. for cytochromes c, and 560-575 nm. for cytochromes b. This technique produced no evidence of consistent absorbance changes from one preparation of mitochondria, to another and resulting from the addition to the mitochondria, of exogenous NADH followed by succinate, even though all preparations were characterized by a very similar and high level of, sensitivity of exogenous NADH oxidase to inhibition by succinate. In some preparations of Jerusalem artichoke mitochondria, there was evidence for small increases in the level of reduction of cytochromes b and/or c, while other preparations showed no increases. Thus it was concluded that these results were consistent with the junction point between succinate and exogenous NADH oxidases in Jerusalem artichoke mitochondria, being located on the substrate side of the cytochromes b.

In order to check this suggestion, the low temperature spectral analysis technique, was employed to see if absorbance spectra characteristic of the aerobic steady state 4 conditions, were similar whether exogenous NADH or succinate were the supplied substrate to isolated Jerusalem artichoke mitochondria, or when both substrates were supplied simultaneously. This low temperature technique overcomes the problem of rapid onset of anaerobiosis, which often accompanies room temperature spectral analysis of mitochondrial suspensions supplied with respiratory substrate, due to the use of high concentrations of mitochondria required to avoid low signal:noise ratios. The low temperature technique achieves this by plunging the mitochondria, suspended in assay medium and contained in special low temperature spectrophotometer cuvettes, into a dewar containing liquid nitrogen. This was done after a short lapse of time following the

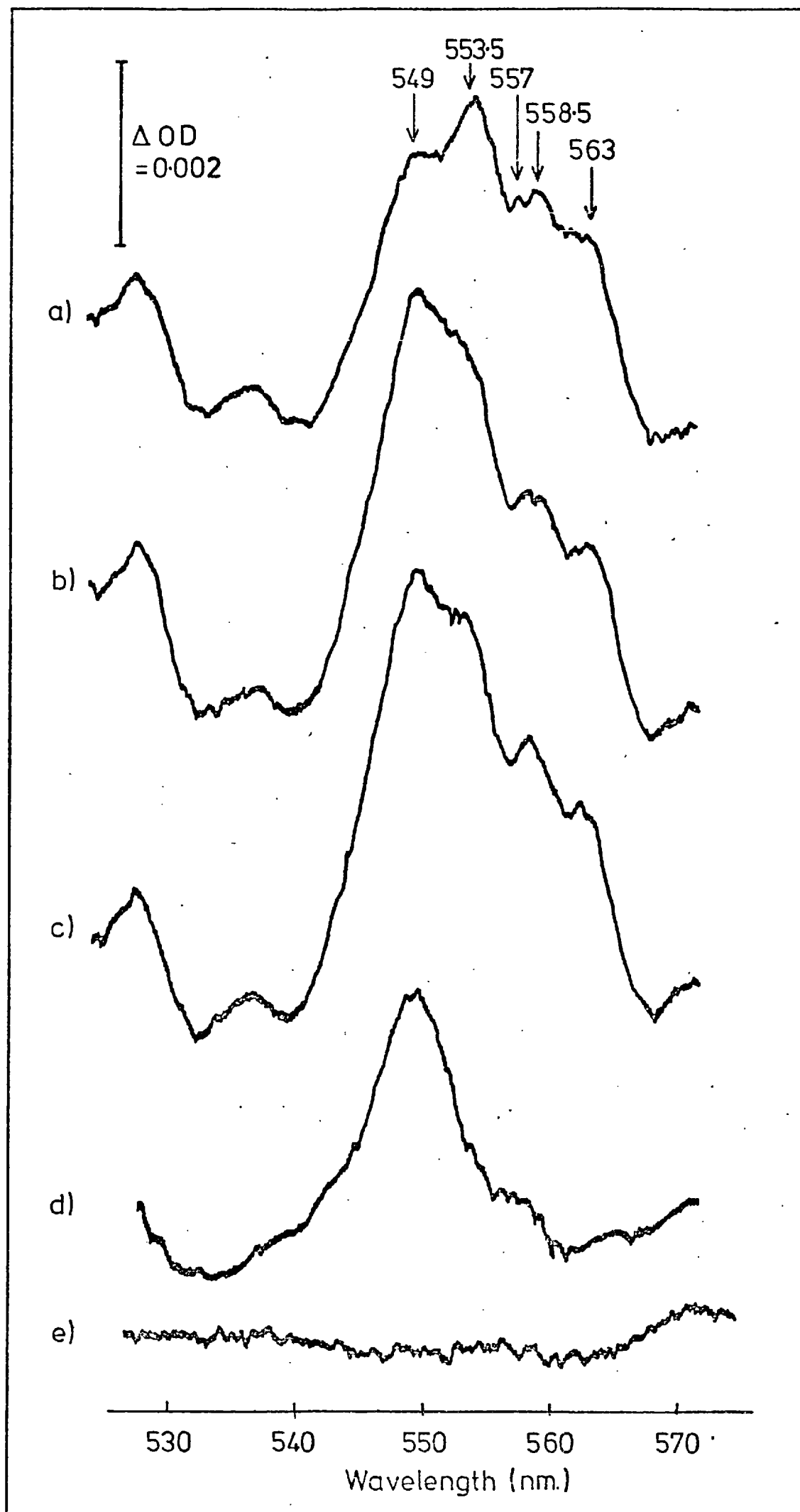
addition to the appropriate cuvette, of the required substance such as NADH or succinate or both, followed by stirring, as described in Materials & Methods 6.vi.b. The short lapse of time following the addition of substrate, allowed the establishment of the steady state under aerobic conditions, before freeze-trapping it. Another advantage of this technique is that the resolution and intensity of the absorbance of light by the cytochromes, is greatly increased compared with those measured at room temperature. This is probably due to the crystalline structure of the ice between the mitochondria, greatly increasing the length of the light path through the mitochondrial suspension, and to the low temperature retarding the velocity of molecular movements, compared with their velocity at room temperature.

Succinate and/or exogenous NADH reduced minus oxidized spectra of isolated Jerusalem artichoke mitochondria, under aerobic steady state 4 conditions, at 77K are shown in figure 58. It can be seen from this figure, that NADH and succinate reduced cytochromes c, which have absorbance maxima at 547 and 549 nm., and cytochromes b, which have absorbance maxima at 553.5 nm., 557 nm., 558.5 nm. and 563 nm., which was in agreement with the findings of Lance & Bonner (1968) and Passam, Berden & Slater (1973).

Plant mitochondria possess two cytochromes c, one of which has been found to be easily solubilized from the mitochondria by treatment with phosphate buffer, and has an absorbance maximum at 77K of 547 nm. The other cytochrome c remained attached to the inner mitochondrial membrane following this treatment, and was found to have an absorbance maximum at 77K of 549 nm. (Lance & Bonner, 1968). It can be seen from figure 58 (a) and (b), that succinate was able to reduce more cytochrome c under state 4 conditions, than was reduced by exogenous NADH. In addition, trace (c) in figure 58 shows that

Figure 58. Low temperature (77K) absorbance difference spectra of Jerusalem artichoke mitochondria, under aerobic steady-state 4 conditions. Split-beam spectrophotometer traces showing the pattern of absorbance of light by the α bands of cytochromes b and c reduced by the substrates shown below, in the sample cuvette, minus their absorbance in the reference cuvette, measured at 77K as described in Materials & Methods 6.vi.b, under aerobic conditions, by freeze-trapping the steady-state 4. ATP/Pi/MgCl₂ 'activated' mitochondria (1.7 mg. protein ml.⁻¹), were used in all cases, suspended in standard assay medium. For details of ATP/Pi/MgCl₂ activation of succinate dehydrogenase, see figure 9, Results 1.ii.

<u>Trace</u>	<u>Additions to cuvettes</u>	
	<u>Sample cuvette</u>	<u>Reference cuvette</u>
(a)	Exogenous NADH (2mM)	none = air oxidized
(b)	Succinate (10mM)	none = air oxidized
(c)	Exogenous NADH (2mM) plus succinate (10mM)	none = air oxidized
(d)	Exogenous NADH (2mM) plus succinate (10mM)	Exogenous NADH (2mM)
(e)	potassium ferricyanide	potassium ferricyanide.



the presence of NADH and succinate in the sample cuvette, did not result in reduction of any more cytochrome than succinate alone, trace (b). When NADH was added to the reference cuvette, and NADH plus succinate was added to the sample cuvette, and the absorbance by the cytochromes in the reference cuvette automatically subtracted from that in the sample cuvette, trace (d) shows results obtained. The almost symmetrical peak of absorbance centred at 549 nm. shown in trace (d), possibly indicates absorbance by cytochrome c_{549} . This difference spectrum is equivalent to subtracting the spectrum drawn in trace (a) from that drawn in trace (c) in figure 58.

Thus, reducing equivalents from succinate could apparently enter cytochrome c more rapidly than they could leave it, when compared with those from exogenous NADH, under state 4 conditions, which might be reflecting the presence of a rate-limiting step for exogenous NADH oxidation located on the substrate side of cytochromes c, which does not act as a rate-limiting step for succinate oxidation (see figures 52 and 53, and table 15, for the differential response of succinate oxidase and exogenous NADH oxidase activities to uncoupler). It was concluded that cytochrome c_{549} was probably not located at the point of interaction between succinate and exogenous NADH oxidases in Jerusalem artichoke mitochondria, since it appears to be located on the oxygen side of oxidative phosphorylation 'site 11', which appears to be involved in the reaction which limits the rate of electron flow from exogenous NADH to oxygen under state 4 conditions, and so enhances the degree of inhibition caused by succinate to the rate of oxidation of exogenous NADH, under these conditions. Also, there was no evidence that exogenous NADH plus succinate could reduce more cytochrome c_{549} than succinate alone, under state 4 conditions.

The data in figure 58 also show that the steady state 4

level of reduction of cytochrome b_{563} , was approximately the same whether reduced by exogenous NADH, succinate or both of these substrates together. Under anaerobic conditions, however, this cytochrome was not found to be reduced by exogenous NADH, as figure 59c shows. When succinate was present, cytochrome b_{563} was reduced under anaerobic conditions, as traces (d) and (e) in figure 59 show. The spectra measured under anaerobic conditions with exogenous NADH, succinate, and citrate were otherwise found to be similar to each other, as expected if the oxidation of all of these substrates, involved the same cytochrome section of the electron transport pathways to oxygen, as suggested by the scheme in figure 4.

The finding that cytochrome b_{563} could change from the reduced state to an oxidized state during the transition from the aerobic state 4 to the anaerobic state, was in agreement with Storey (1974), who also found that, at least part of the oxidation of cytochrome b_{563} seen at anaerobiosis, was synchronous with extra reduction of cytochrome c_{549} . The magnitude of the absorbance due to cytochromes c reduced under anaerobic conditions, when subtracted from their absorbance under aerobic conditions, was too great to enable detection of a little extra reduction of cytochrome c_{549} , when succinate was present, compared with that measured in the absence of succinate (see figure 59c,d,e). This problem was overcome by using twice as much mitochondrial protein in the cuvettes, and measuring the difference spectrum of the α absorbance bands of cytochromes b and c as reduced by succinate plus exogenous NADH minus those reduced by exogenous NADH alone and under anaerobic conditions. Figure 60 shows that there was evidence for electron flow out of cytochrome b_{563} and into cytochrome c, possibly cytochrome c_{549} , when exogenous NADH was the substrate supplied to the mitochondria.

Figure 59. Low temperature (77K) absorbance difference spectra of Jerusalem artichoke mitochondria, under anaerobic conditions. Split-beam spectrophotometer traces showing the pattern of absorbance of light by the α bands of cytochromes b and c reduced by the substrates shown below, in the sample cuvette, minus their absorbance in the reference cuvette, measured at 77K as described in the Materials & Methods 6.vi.b, under anaerobic conditions. ATP/Pi/MgCl₂ 'activated' mitochondria (1.7 mg. protein ml.⁻¹), were used for all traces except trace (b), for which 1.9 mg. protein ml.⁻¹ of non-activated mitochondria were used. All mitochondria were suspended in standard assay medium.

Trace	<u>Additions to cuvettes</u>	
	<u>Sample cuvette</u>	<u>Reference cuvette</u>
(a)	Sodium dithionite (one crystal)	none = air oxidized
(b)	Citrate (10mM)	none = air oxidized
(c)	Exogenous NADH (2mM)	none = air oxidized
(d)	Succinate (10mM)	none = air oxidized
(e)	Exogenous NADH (2mM) plus succinate (10mM)	none = air oxidized
(f)	Potassium ferricyanide	potassium ferricyanide

ADP at an initial concentration of 1.5mM was present in the sample cuvette for traces (b), (c), (d) and (e).

For details of ATP/Pi/MgCl₂ activation of succinate dehydrogenase, see figure 9, Results 1.ii.

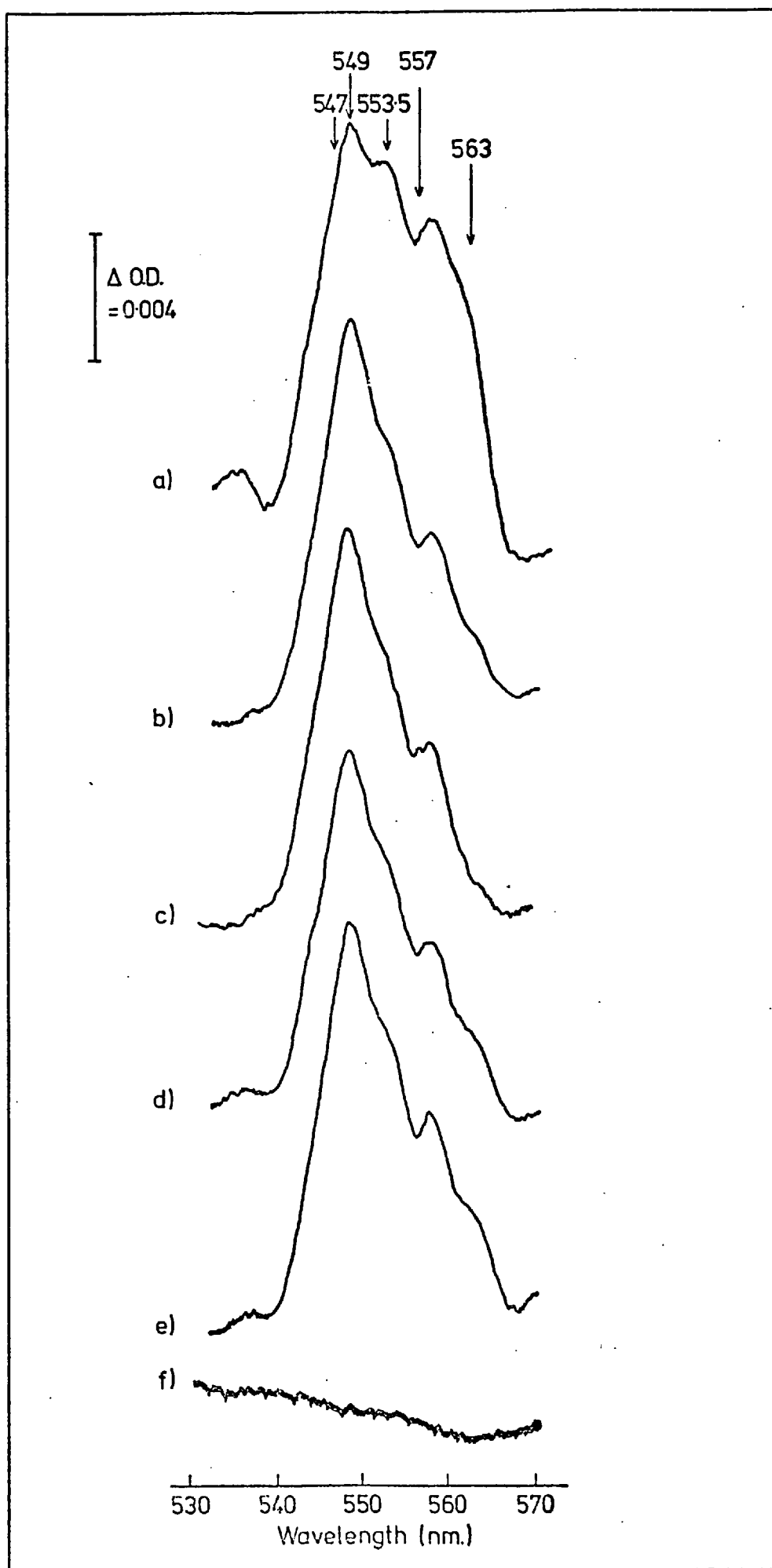
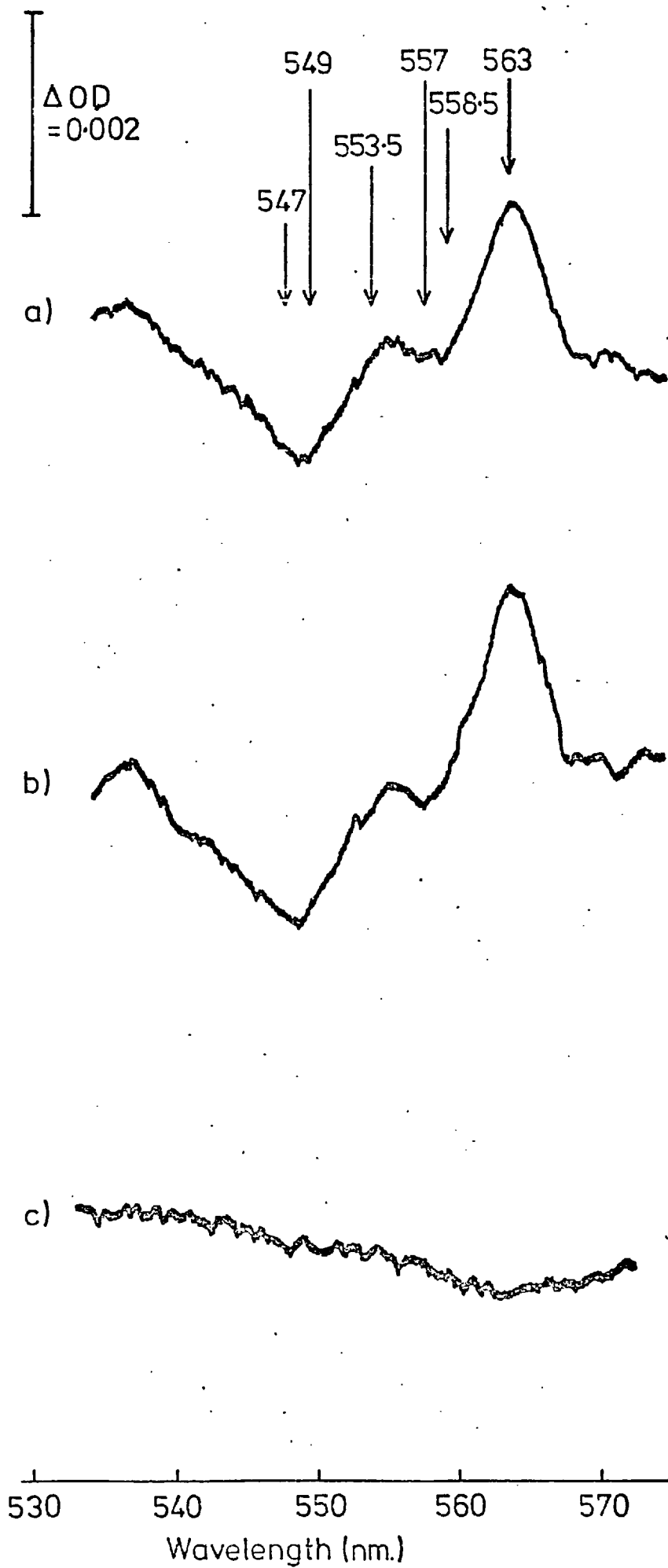


Figure 60. Low temperature (77K) absorbance difference spectra of Jerusalem artichoke mitochondria, under anaerobic conditions. Experimental details were similar to those described for figure 59. For trace (a) ATP/Pi/MgCl₂ 'activated' mitochondria (2.6 mg. protein ml.⁻¹) were used, and for trace (b) and trace (c), non-activated mitochondria (2.3 mg. protein ml.⁻¹) were used. For details of activation of succinate dehydrogenase by ATP/Pi/MgCl₂ see figure 9, Results 1.ii.

<u>Trace</u>	<u>Additions to cuvettes</u>	
	<u>Sample cuvette</u>	<u>Reference cuvette</u>
(a)	Exogenous NADH (2mM) plus succinate (10mM) and ADP (1.5mM)	Exogenous NADH (2mM) plus ADP (1.5mM)
(b)	Exogenous NADH (2mM) plus succinate (10mM) and S13 (10 ⁻⁶ M)	Exogenous NADH (2mM) plus S13 (10 ⁻⁶ M)
(c)	Potassium ferricyanide	Potassium ferricyanide.



Thus, upon the transition from the aerobic to the anaerobic state, electrons appear to flow in a forward direction from cytochrome b_{563} to cytochrome c, when exogenous NADH was substrate but not with succinate present. As expected, this forward electron flow did not require the oxidative phosphorylation reaction to be coupled to electron transport, since a similar difference spectrum was measured in the absence (figure 60 a) and presence (figure 60 b) of uncoupler. A similar pattern of reduction and oxidation of cytochromes b and c in Jerusalem artichoke mitochondria in the presence of succinate and induced by the presence of uncoupler in the reference cell, by Passam, Berden & Slater (1973), who interpreted their results in terms of reverse electron flow from cytochrome c to b in the sample cell, upon transition from aerobic to anaerobic conditions. In the light of the findings made here, the data of these authors could also be explained in terms of uncoupler inducing forward electron flow from cytochrome b_{563} to cytochrome c. The general similarity between the data presented here and that of Passam, Berden & Slater (1973), suggests that exogenous NADH somehow acts as an uncoupler in permitting forward electron flow from cytochrome b_{563} to cytochrome c, at anaerobiosis. A similar pattern of redox behaviour of cytochromes b and c at anaerobiosis, has been reported by Trumpower & Katki (1975), to occur in bovine heart mitochondria.

- j) The effect of succinate on the activity of exogenous NADH oxidase in mitochondria from hyphae of *Neurospora crassa* grown in the presence of chloramphenicol.

There is no evidence to suggest that the unusual redox behaviour described above for cytochrome b_{563} is directly involved in the mechanism of interaction between succinate and exogenous NADH oxidases in isolated plant mitochondria. This suggestion was further supported by the finding that succinate was able to cause a powerful inhibition of exogenous NADH oxidase activity, in mitochondria isolated from *Neurospora crassa* hyphae, which had been cultured in a medium containing chloramphenicol, which results in a major decrease in the absorbance due to cytochromes b in these mitochondria, and particularly b_{562} , see Results l.v. Data obtained from this type of experiment are presented in figure 6l, from which it can be seen that succinate caused over 60% inhibition of the cyanide-sensitive component of exogenous NADH oxidase activity in these mitochondria, i.e. the component measured in the presence of mCLAM. When the alternative cyanide-insensitive oxidase was functional i.e. in the absence of mCLAM, the degree of inhibition was found to be much less than conditions when it was not functional.

Thus, the results of the present search for evidence of involvement of absorbing flavoproteins, cytochromes b or c, at or close to the point at which exogenous NADH oxidase and succinate oxidase interact with each other in isolated plant mitochondria, were negative in each case, which was consistent with the point of interaction between these two enzymes being located on the substrate side of the flavoprotein Fp_{ha} . As reference to figure 4 will show, this part of the electron transport pathway from exogenous NADH to oxygen is poorly understood at present, with no evidence of easily

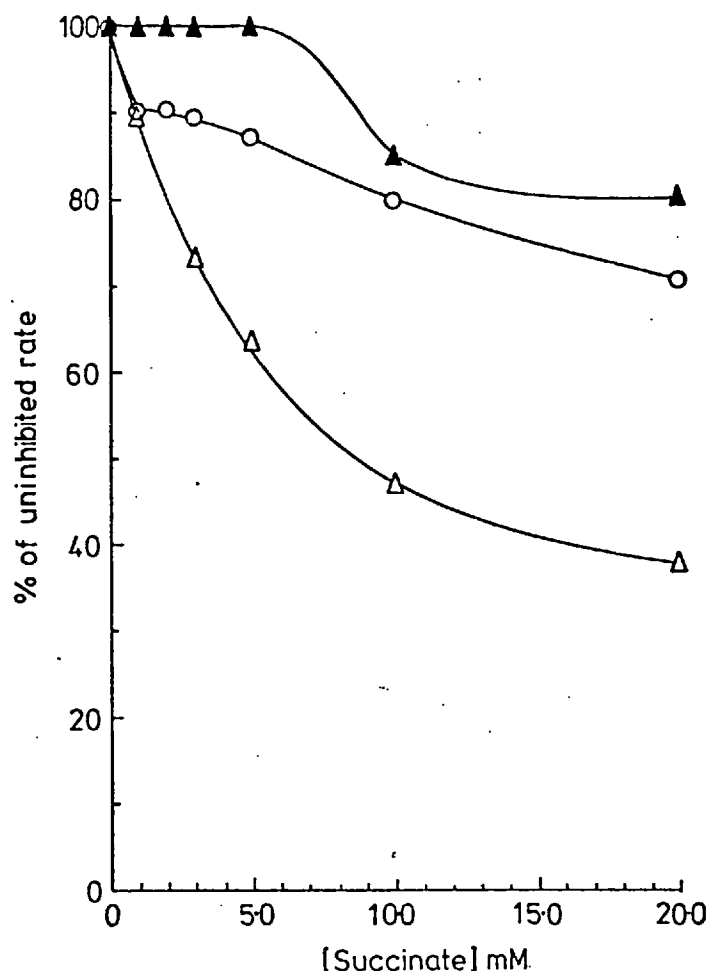


Figure 61. The effect of succinate on the activity of exogenous NADH oxidase in Neurospora crassa mitochondria, isolated from hyphae grown in the presence of chloramphenicol. Hyphae of wild type (Y74A) N. crassa, were grown in liquid culture in the presence of 3 g. L^{-1} chloramphenicol, and mitochondria isolated from the hyphae, as described in Materials & Methods 2.v. Mitochondria ($0.1 \text{ mg. protein ml.}^{-1}$ for Δ data, and $0.03 \text{ mg. protein ml.}^{-1}$ for $\blacktriangle$ and $\circ$ data), were suspended in N. crassa mitochondrial assay medium (see Materials & Methods 5.ii.b), containing 1.67 mM mCLAM for Δ data, and 1.67 mM KCN for $\blacktriangle$ data. NADH ($50 \mu\text{M}$) was added to the reaction cuvette, and one minute after attaining a constant rate of exogenous NADH oxidation, succinate of the appropriate concentration was added, and the rates before and after succinate recorded as described in Materials & Methods 5. The control (100%) rates as the mean of 14 assays $\pm$ S.E.M. in each case, were as follows:

$\circ = 325.3 \pm 2.5$ (no inhibitor); $\blacktriangle = 255.1 \pm 7.1$ (+ KCN);
 $\Delta = 160.2 \pm 3.7$ (+ mCLAM), as nmoles NADH $\text{min.}^{-1} \text{ mg. protein}^{-1}$.

detectable redox mediators of the known types being located there, except for a flavoprotein associated with the NADH dehydrogenase itself. The arrangement of the electron transport pathway from succinate to oxygen is a little better understood, and appears to include a flavoprotein FpS, and iron-sulphur centres Fe-S, and it could be that succinate oxidase and exogenous NADH oxidase meet at one of these components. The flavoprotein FpS is as yet spectroscopically undetectable in the mitochondria (Storey, 1970a), but it may be possible to find evidence for exogenous NADH reducing the iron-sulphur centres associated with succinate dehydrogenase, by using Electron Paramagnetic Resonance spectroscopy (EPR), although experiments carried out under aerobic conditions would probably prove very difficult or impossible, since it is understood that a very high concentration of mitochondrial protein is required to be present in the sample cuvette to produce measurable signals with this technique, which would become anaerobic very shortly after the addition of NADH or succinate. This type of investigation was not carried out during the course of the present study.

An alternative possibility for the 'point' of interaction between succinate and exogenous NADH oxidases, is at the level of the protein moieties of succinate dehydrogenase and exogenous NADH dehydrogenase, with the inner mitochondrial membrane acting as a functional connection between the two enzymes, such that they can behave like sub-units of an allosteric protein. This alternative possibility will be developed further in the discussion.

DISCUSSION

Some detailed aspects of the data have already been discussed in the preceeding section. Thus it is proposed to discuss the results in more general terms, starting with possible pathways for the oxidation of endogenous NADH followed by a discussion of the interaction between exogenous NADH and succinate oxidases. Finally, the possible metabolic significance of the regulatory functions of the reactions described will be discussed.

1. Possible pathways for the oxidation of endogenous NADH.

The data confirmed the established view that isolated plant mitochondria can oxidize malate in the absence of a system for the removal of oxaloacetate (see Results 1. figure 5, table 4, and section vi.). This ability appears to be conferred by the operation of an NAD^+ -linked malic enzyme associated with these mitochondria (Macrae & Moorhouse, 1970; Macrae, 1971a,c; Coleman, 1971; Coleman & Palmer, 1972; Brunton & Palmer, 1973; Brunton, 1975).

The reduction of ferricyanide by all NAD^+ -linked substrates in the absence of exogenous NAD^+ has been found to be almost totally sensitive to inhibition by antimycin A. If it is accepted that ferricyanide cannot cross the inner mitochondrial membrane, it follows that the oxidation occurred on the matrix side of this membrane; this is in accord with previous published data (Coleman, 1971; Coleman & Palmer, 1972; Brunton & Palmer, 1973; Brunton, 1975; Palmer & Arron, 1976), and with data presented here in figure 13 and table 9. The reduction of ferricyanide by all NAD^+ -linked substrates was found to be only partially sensitive to inhibition by piericidin A (Brunton & Palmer, 1973; Brunton, 1975; Palmer & Arron, 1976; present thesis, figure 13, table 9), which

indicated the existence of at least two types of endogenous NADH dehydrogenases located on the matrix side of the inner mitochondrial membrane. One type of NADH dehydrogenase appears to initiate a branch of the electron transport which is sensitive to piericidin A, while the other type appears to initiate a system that lacks the piericidin A-sensitive site. If both types of NADH dehydrogenases catalyze the oxidation of endogenous NADH under state 3 conditions it might explain the low values obtained for the ADP:O ratios when using NAD^+ -linked substrates, in isolated plant mitochondria (table 1), since Brunton & Palmer (1973), Brunton (1975) and Pomeroy (1975), have reported that the rotenone (and hence piericidin A) insensitive pathway in isolated wheat mitochondria is probably not coupled to oxidative phosphorylation at 'site 1'.

The location of malate dehydrogenase in the matrix of intact plant mitochondria, has been supported by experiments which show the latency of this enzyme in catalyzing the reduction of added oxaloacetate, by added (exogenous) NADH (Brunton, 1975; present thesis, table 7). Only after disruption of the inner mitochondrial membrane by the detergent TX100, was malate dehydrogenase able to catalyze this reaction to a significant extent. Thus it was concluded that the enzymes which catalyze the initial steps of oxidation of all of the NAD^+ -linked substrates, in the absence of added NAD^+ , are probably located on the matrix side of the inner membrane of intact plant mitochondria.

Coleman (1971) and Coleman & Palmer (1972), found no evidence for a major rotenone-insensitive component of state 3 malate oxidation in Jerusalem artichoke mitochondria, in the absence of added NAD^+ . Since the pattern of oxidation of NAD^+ -linked substrates by isolated plant mitochondria following the addition of

rotenone, appears to be the same as that following the addition of piericidin A (Brunton & Palmer, 1973; Brunton, 1975; present author, unpublished data), this finding appeared to contrast with data presented in this thesis (figures 22, 23 and 24b,c) and those published by Brunton & Palmer (1973) and Brunton (1975). A possible explanation for this apparent difference will be discussed later.

Douce & Bonner (1972) used oxaloacetate to investigate the behaviour of the pool of endogenous NAD^+ under state 4 conditions. They observed that exogenous oxaloacetate was able to oxidize all the endogenous NADH and cause severe inhibition of the rate of oxygen uptake until all of the oxaloacetate had been converted to malate, catalyzed by malate dehydrogenase. Since oxaloacetate was able to cause a transient inhibition with all NAD^+ -linked substrates, including malate, Douce & Bonner (1972) concluded that the endogenous NAD^+ acted as if it was an homogeneous pool.

When oxaloacetate was tested for its ability to cause inhibition of oxygen uptake resulting from the oxidation of NAD^+ -linked substrates under state 3 conditions, in the present study, it was found to cause a large and transient inhibition of oxygen uptake when using pyruvate, citrate and 2-oxoglutarate; however malate oxidase activity was only slightly and permanently inhibited (figures 14, 15 and 16). Similar results have been reported by Avron & Biale (1957), Brunton & Palmer (1973) and Brunton (1975).

Under state 4 conditions in the present study, there was evidence for malate oxidase activity becoming sensitive to oxaloacetate in a manner similar to that of pyruvate and citrate oxidase activities under state 3 conditions (figure 19). It is therefore concluded that under state 3 conditions, a proportion of the NAD^+ reduced by the malic enzyme is more likely to be

oxidized by the respiratory chain than by oxaloacetate, and thus it appears to occur in a pool which is functionally separate from the NAD^+ reduced by the other NAD^+ -linked TCA cycle intermediate dehydrogenases. This heterogeneity of the endogenous pool of NAD^+ was observed in Jerusalem artichoke, potato and mung bean mitochondria. It seems that when the phosphate potential rises under state 4 conditions, and NADH is not so rapidly oxidized by the respiratory chain, NADH produced by the activity of the malic enzyme is capable of being used to reduce oxaloacetate.

Another way in which the NADH resulting from malic enzyme activity can be made available to malate dehydrogenase, is by the presence of piericidin A or rotenone. This has been shown to occur in the presence of concentrations of these inhibitors that were too low to affect the activity of succinate oxidase, while sufficiently high to cause maximum, albeit partial, inhibition of malate oxidase activity under state 3 conditions (Brunton & Palmer, 1973; Brunton, 1975; present thesis, figures 22, 23 and 24).

Since both the partial inhibition of state 3 malate oxidase activity by rotenone or piericidin A, and the transition from respiratory state 3 to 4, appeared to result in the same type of change in the relationship between malate dehydrogenase, and the NADH produced by the malic enzyme activity, it is considered likely that a common characteristic of both situations, induced the change. Such a common characteristic is the restriction imposed upon the rate of electron transport from malate to oxygen, under both sets of conditions; a restriction that occurs in the region of the iron-sulphur centres associated with the coupling of electron transport to oxidative phosphorylation at 'site 1' (figure 4). Hanson & Hodges (1967) have observed that the degree of contraction of the

inner membrane of isolated plant mitochondria, is a function of the rate of electron transport. It could be therefore, that changes in the conformation of the inner membrane occur on the transition from state 3 to state 4. or on the addition of rotenone or piericidin A, to plant mitochondria oxidizing malate under state 3 conditions, which result in malate dehydrogenase and the NADH associated with the malic enzyme, appearing in the same compartment of the mitochondrion. This point will be discussed further.

In an attempt to explain the effects of piericidin A and oxaloacetate, on the pattern of consumption of oxygen in wheat mitochondria catalyzing the oxidation of NAD^+ -linked substrates under state 3 conditions, Brunton & Palmer (1973) put forward a scheme for the possible pathways for the oxidation of endogenous NADH in these mitochondria (figure 20). These authors have proposed the existence of kinetic compartmentation between malate dehydrogenase and malic enzyme in the matrix of wheat mitochondria. This kinetic compartmentation involved the spatial separation of the malic enzyme and its associated pool of NAD^+ , from the other NAD^+ -linked substrate dehydrogenases, and their pool of NAD^+ . A similar situation was suggested to exist in mitochondria isolated from other plant tissues by Brunton (1975), from results of preliminary experiments carried out with mitochondria isolated from mung bean hypocotyls and Jerusalem artichoke tubers. From the scheme proposed by Brunton & Palmer (1973), it is required that NADH associated with the malic enzyme is oxidized by oxygen, via the electron transport pathways, under state 3 conditions rather than diffusing to the malate dehydrogenase compartment of the matrix, where it would be used to reduce added oxaloacetate, as was the case for NADH associated with the other NAD^+ -linked substrate

dehydrogenase activities under state 3 conditions. Such an arrangement would explain the failure of 0.2 mM oxaloacetate to cause a large, but temporary, inhibition of the rate of consumption of oxygen, resulting from state 3 malate oxidation. Instead it was found to cause only a low degree of inhibition, which appeared to be more permanent in nature and could be the result of oxaloacetate activity as a competitive inhibitor of malic enzyme itself (Brunton & Palmer, 1973; Brunton, 1975; present thesis, figure 17).

It is significant to observe that in the results presented in this thesis (figures 22 and 23), the rate of NADH produced by the oxidation of malate in the presence of piericidin A, as determined by the time taken to reduce a pulse of oxaloacetate, was in the same order as that predicted from the rate of oxygen consumption in the absence of added oxaloacetate. Thus it would seem that the rate of oxygen uptake or oxaloacetate reduction in the presence of piericidin A, is limited by the capacity of the malic enzyme to reduce NAD^+ , rather than the activity of the piericidin A-insensitive oxidation chain or the malate dehydrogenase, respectively. It is not felt that the low rate of NADH production from the malic enzyme is due to a low level of that enzyme. It appears from the data that the rate at which the malic enzyme produces NADH for the removal of oxaloacetate or oxidation via the piericidin A-insensitive electron transport pathway, is inversely proportional to the magnitude of the respiratory control ratio (see figure 24 and table 8). Thus there is evidence that there may be two types of malic enzyme and only one type can be induced to supply NADH for the reduction of oxaloacetate and oxidation via the piericidin A-insensitive electron transport pathway to oxygen. The activity of the other type of malic enzyme is inhibited in the

presence of piericidin A, presumably because the NADH it produces can no longer be oxidized by the piericidin A-sensitive NADH dehydrogenase. The proportion of the total malic enzyme that is capable of supplying NADH to malate dehydrogenase apparently increases as the respiratory control decreases. Both of these parameters appear to be affected by the integrity of the inner membrane of the organelle. Thus it is tempting to speculate that the compartmentation between the pool of NAD^+ reduced by the malic enzyme and the pool of NAD^+ reduced by malate dehydrogenase, may be much more rigidly separate than Brunton & Palmer (1973) suggested, and that the transfer of reducing equivalents between the two pools of NAD^+ is an artifact resulting from damage to the membrane occurring during isolation. Such a proposal provides an explanation of why Coleman (1971) and Coleman & Palmer (1972) failed to observe a rotenone-insensitive component for the oxidation of malate in the matrix of Jerusalem artichoke mitochondria, since these authors report that the respiratory control ratio of their preparation was 7.5, which is very high.

It is possible to speculate from the data presented in this thesis, that the other NAD^+ -linked substrate dehydrogenases may exist in separate functional compartments. In all cases, the maximum rate at which the TCA cycle dehydrogenases could supply NADH for the removal of oxaloacetate greatly exceeded that expected from the rates of oxygen uptake (Results 1.vi.b). Thus one might conclude that the rate limiting step of oxygen uptake lies in the capacity of the electron transport pathway to supply electrons to oxygen, assuming that all the NADH produced by the NAD^+ -linked substrate dehydrogenases, except the malic enzyme, is oxidized by the same NADH dehydrogenase. If this was the case, then the rate

of oxidation of all NAD^+ -linked substrates other than malate, would be expected to be identical. However, this was not the case; there was a considerable variation between the state 3 rates of oxygen consumption for the various substrates tested, both in the absence and presence of piericidin A. Maintaining the assumption that the rate of reduction of NAD^+ was far in excess of the rate of NADH oxidation, it follows that the NADH produced by each type of NAD^+ -linked substrate dehydrogenase was oxidized by a separate NADH dehydrogenase which had a different capacity to transfer electrons to oxygen and different accessibility to the by-pass of the piericidin A sensitive site. Such a possibility represents a very complex system for the compartmentation of enzymes in the matrix, and it is difficult to visualize how it could occur. It could be considered that the inner membrane is composed of structural units which may be best compared with the pieces of a jig-saw puzzle; each piece would contain a given type of NADH dehydrogenase together with the appropriate NAD^+ -linked dehydrogenase associated with it. Such a structural relationship between soluble enzymes and the membrane surface, has been suggested by the studies of Matlib & O'Brien (1975). To explain the data in this thesis, it would also be necessary to propose that the malic enzyme occupies one area alone with no presence of malate dehydrogenase, whilst pyruvate, isocitrate and 2-oxo-glutarate dehydrogenase, although separate from each other, are closely associated with malate dehydrogenase. This is necessary to explain how the NADH produced by these enzymes is so readily available for the reduction of oxaloacetate.

There is another alternative explanation which is that the rate at which the NAD^+ -linked dehydrogenases reduce NAD^+ in the presence of oxaloacetate is very much faster than the rate in the

absence of oxaloacetate, when oxygen uptake occurs. In the presence of oxaloacetate the redox poise of the NAD^+/NADH would be very oxidized, whilst under normal conditions if the activity of the respiratory system is limiting, one would expect the redox poise to become more reduced; as the level of NADH increases it is possible that an allosteric inhibition of the NAD^+ -linked substrate dehydrogenases may occur. There is some experimental evidence to show that both the pyruvate dehydrogenase (Crompton & Laties, 1971) and isocitrate dehydrogenase (Cox & Davies, 1967) activities are affected by the NAD^+/NADH ratio, with a low value causing the inhibition of each enzyme. Enzymes most sensitive to this type of inhibition would catalyze the production of NADH at a slower rate than enzymes that are less sensitive. No work has been carried out to resolve which of these two alternatives is correct, since the major experimental interest of this research was the interaction of the branched electron transport pathways of plant mitochondria.

2. Oxidation of endogenous NADH in the presence of exogenous NAD^+ .

The rate of reduction of oxygen or ferricyanide by isolated plant mitochondria, has been shown to be stimulated by exogenous NAD^+ when malate was the supplied substrate (Coleman, 1971; Coleman & Palmer, 1972; Brunton & Palmer, 1973; Day & Wiskich, 1974a,b; Brunton, 1975; Pomeroy, 1975; Palmer & Arron, 1976; present thesis, Results 1.vi.f). The apparent K_m for malate of the NAD^+ -dependent malate oxidation in Jerusalem artichoke mitochondria has been found to be 2mM, which is much lower than that obtained for the same reaction in the absence of NAD^+ which was found to be 20mM (Palmer & Arron, 1976). A similar trend was observed in the present study (figure 13), where it was found that increasing the concentration

of malate from 15 to 60mM increased the rate in the absence of NAD^+ however, the NAD^+ -dependent rate was fully saturated at 15mM malate.

Pyruvate was found to be the major product of the NAD^+ -dependent malate oxidation (Brunton & Palmer, 1973; Brunton, 1975; Palmer & Arron, 1976), therefore it is concluded that the addition of NAD^+ stimulates the malic enzyme. Since the NAD^+ stimulated malate-ferricyanide reductase activity was insensitive to inhibition by antimycin A (figure 13) it has been concluded that the NADH produced by the malic enzyme under these conditions is located in the inter-membrane space of the mitochondrion. These results are consistent with malic enzyme being located in the inter-membrane space of the mitochondrion, as proposed by Coleman (1971) and Coleman & Palmer (1972). However, the observation that malate oxidation by ferricyanide in the presence of oxaloacetate was sensitive to antimycin A, indicates that some malic enzyme is also located in the matrix, as proposed by Brunton & Palmer (1973), Brunton (1975) and Palmer & Arron (1976). The apparent decrease in the K_m for malate observed in the presence of exogenous NAD^+ , would seem to be due to the activation of a malic enzyme in the inter-membrane space of the mitochondrion.

Day & Wiskich (1974a,b) using cauliflower bud mitochondria, observed that the rate of oxidation of malate, citrate and 2-oxoglutarate could be stimulated by the addition of NAD^+ in the presence of rotenone. These authors argued that it was unlikely that all of the NAD^+ -linked dehydrogenases responsible for the initial step of oxidation of each of these substrates, were located in the inter-membrane space. They also found that either 2-n-butylmalonate or the absence of inorganic phosphate, both of which would prevent the uptake of dicarboxylic acids into the mitochondrial matrix,

inhibited the rate of oxidation of malate in the presence or absence of exogenous NAD^+ . They therefore concluded that the initial steps of malate oxidation in isolated cauliflower bud mitochondria, took place in the matrix together with the other NAD^+ -linked substrates, and that in the presence of exogenous NAD^+ and rotenone, reducing equivalents were transferred from endogenous NADH to exogenous NAD^+ , catalyzed by a transmembrane transhydrogenase. This enzyme was not reversible and could not therefore catalyze the transfer of reducing equivalents from exogenous NADH to endogenous NAD^+ .

It is concluded that the data in this thesis do not support the existence of a transmembrane transhydrogenase in isolated white potato, of the type proposed by Day & Wiskich (1974a,b) for cauliflower bud mitochondria, since only malate-ferricyanide reductase activity was found to be markedly insensitive to inhibition by antimycin A, in the presence of exogenous NAD^+ (table 9). Similar evidence against the existence of a general transmembrane transhydrogenase existing in isolated Jerusalem artichoke mitochondria, has been reported by Palmer & Arron (1976). Thus, if there is transfer of reducing equivalents from endogenous NADH to exogenous NAD^+ in isolated white potato and Jerusalem artichoke mitochondria, it appears to be specific for the substrate malate.

It is not yet possible to test whether malate needs to enter the matrix of plant mitochondria in order that NADH can be generated there and then transhydrogenated to exogenous NAD^+ , since the inhibitors of malate uptake such as 2-n-butylnalonnate and phenyl succinate, have also been found to be inhibitors of the enzymes that can catalyze the oxidation of malate in isolated plant mitochondria (Phillips & Williams, 1973b; Palmer & Arron, 1976). Inorganic phosphate has been shown to be required for the uptake of

substrate anions (Phillips & Williams, 1973a; Wiskich, 1974; present thesis, figure 37). Palmer & Arron (1976) found that the absence of inorganic phosphate totally prevented the oxidation of malate in the absence of exogenous NAD^+ , whilst it only resulted in a partial inhibition of the NAD^+ -stimulated malate oxidation. Thus it seems that in Jerusalem artichoke mitochondria, malate does not need to enter the matrix to be oxidized in the presence of exogenous NAD^+ .

3. Interaction between exogenous NADH oxidase and succinate oxidase.

The interaction between exogenous NADH oxidase and succinate oxidase, in isolated plant mitochondria described in this thesis, represents the first report of such an interaction.

Succinate was found to cause a powerful, although not total, inhibition of exogenous NADH oxidase activity under conditions when the phosphate potential was limiting the rate of respiration (table 10 and figure 38). The inhibition was shown to involve the activity of succinate dehydrogenase since malonate, an inhibitor of this enzyme, prevented succinate from inhibiting the exogenous NADH oxidase activity (table 14). The inhibition of exogenous NADH oxidase activity by succinate under state 4 conditions was found to be an hyperbolic function of the concentration of succinate, up to 0.6mM (figure 42). This was not unexpected since the activity of succinate oxidase followed a similar saturation curve.

Evidence was found for a reciprocal effect of exogenous NADH upon the activity of succinate oxidase in Jerusalem artichoke mitochondria, since the presence of exogenous NADH stimulated the succinate oxidase activity. The stimulation was greatest at very low concentrations of succinate, and diminished as the concentration of succinate approached 0.3mM. Thus the effect of

exogenous NADH was mainly to lower the apparent K_m for succinate. Thus it seems that exogenous NADH was causing a positive modification of succinate dehydrogenase; it was not possible to test whether electron flux through exogenous NADH dehydrogenase was necessary for this activation, in the same way as succinate dehydrogenase activity had been shown to be involved in the inhibition of exogenous NADH oxidase. This was due to the lack of an appropriate inhibitor of exogenous NADH oxidase, which has no effect on other systems. For example, pyrrolnitrin also inhibits succinate oxidase (table 4), and dicoumarol, which can inhibit the oxidation of exogenous NADH (J.M. Palmer, personal communication), also uncouples oxidative phosphorylation from electron transport and so would lead to respiratory conditions under which no interaction would be expected to occur between succinate and exogenous NADH oxidases (figures 52 and 53, table 15).

The hyperbolic relationship between the concentration of succinate added to Jerusalem artichoke mitochondria which were oxidizing exogenous NADH under state 4 conditions, and (i) the subsequent rate of exogenous NADH oxidation, and (ii) the rate of succinate oxidation under the same conditions, suggested that there may be a direct relationship between the two events. This was supported by the finding that a plot of succinate-inhibited NADH oxidase activity versus exogenous NADH-stimulated succinate oxidase activity, gave a straight line (figure 44). Similar effects were found for mitochondria isolated from mung bean hypocotyls (figures 48, 49 and 50), and probably Neurospora crassa (Y74A) hyphae as well (figure 51).

Under respiratory state 3 conditions, the percentage inhibition of exogenous NADH oxidase activity caused by succinate,

was found to be less than that which occurred under state 4 conditions (figures 45, 52 and 53). In contrast, the degree of stimulation of succinate oxidase activity caused by exogenous NADH, appeared to be the same under both respiratory states, as measured by the five-fold decrease in the value for the apparent K_m for succinate (figures 43 and 46) and the similar value obtained for the slope of the plots of exogenous NADH oxidase activity versus succinate oxidase activity (figures 44 and 47). In addition, the double reciprocal plots of $1/V$ versus $1/S$ for succinate oxidase activity and succinate concentration, were linear (figures 43 and 46). This probably indicates that under both respiratory states 3 and 4, the apparent stimulatory effects of exogenous NADH were changing the K_m of all the succinate dehydrogenase molecules, while the partial inhibition of exogenous NADH oxidase activity caused by succinate, probably indicates that in isolated plant mitochondria, not all of the exogenous NADH dehydrogenase molecules were capable of being inhibited by succinate.

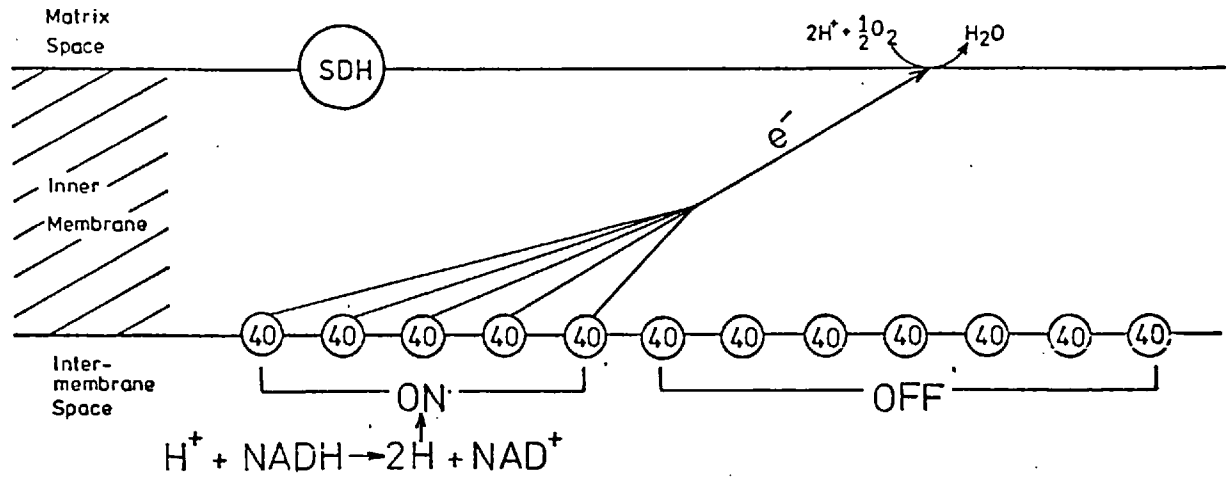
A scheme which attempts to represent diagrammatically, the possible way in which succinate inhibits exogenous NADH oxidase activity in isolated Jerusalem artichoke mitochondria, is given in figure 62. The numerals which appear in circles on the line representing the outer face of the inner mitochondrial membrane, in this figure, represent convenient and approximate units of exogenous NADH oxidase activity, for Jerusalem artichoke mitochondria, as nmoles NADH oxidized $\text{min.}^{-1} \text{ mg. protein}^{-1}$. It can be seen from figure 62(i) that under state 4 conditions, 5 of the units of exogenous NADH dehydrogenase are considered to be functional, with the remaining 7 units 'switched off' by the energy conservation mechanism which is coupled to electron transport. When succinate was added to the mitochondria, figure 62(ii) shows that it is considered that 3 units

Figure 62. The possible effect of succinate on the activity of exogenous NADH oxidase in Jerusalem artichoke mitochondria. This figure illustrates the spatial arrangement of succinate dehydrogenase and exogenous NADH dehydrogenase, with respect to the faces of the inner mitochondrial membrane, and the control imposed upon exogenous NADH oxidase by coupling of electron transport to oxidative phosphorylation and by succinate. The circles on the line representing the outer face of the membrane, represent convenient units of exogenous NADH dehydrogenase activity, each capable of oxidizing $40 \text{ nmoles NADH min.}^{-1} \text{ mg. protein}^{-1}$. The arrows leading from SDH, succinate dehydrogenase, to units of exogenous NADH dehydrogenase, indicate total inhibition of the units touched by their heads.

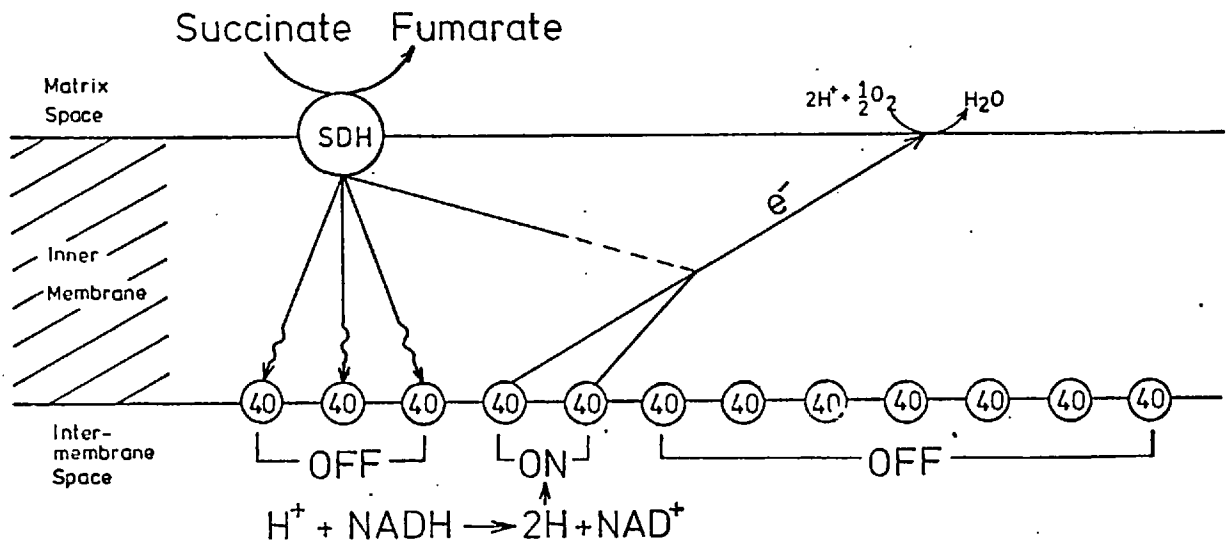
KEY:

- ON = units of exogenous NADH dehydrogenase
which are active
- OFF = units of exogenous NADH dehydrogenase
which are not active.

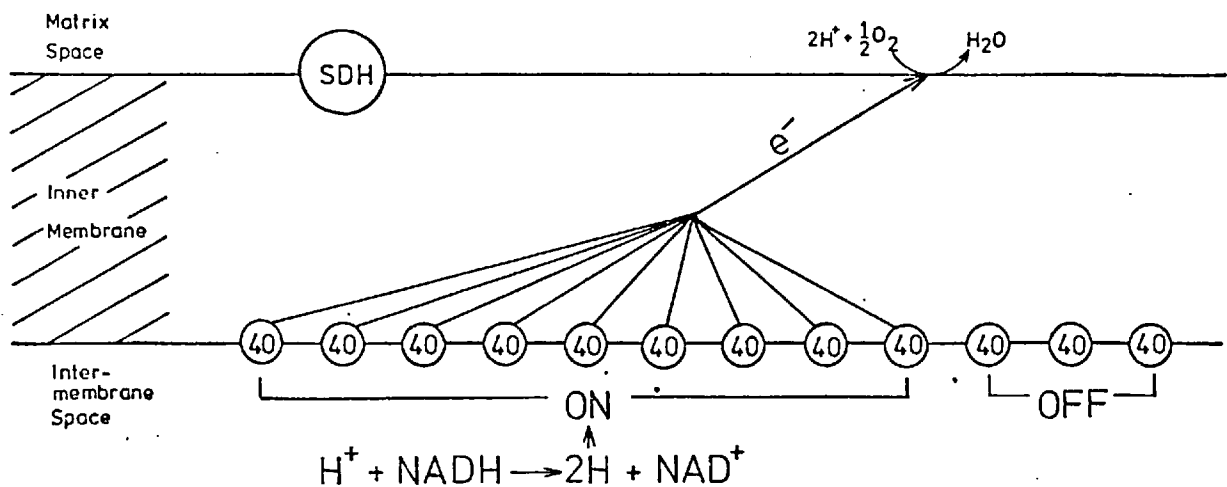
i) State 4.



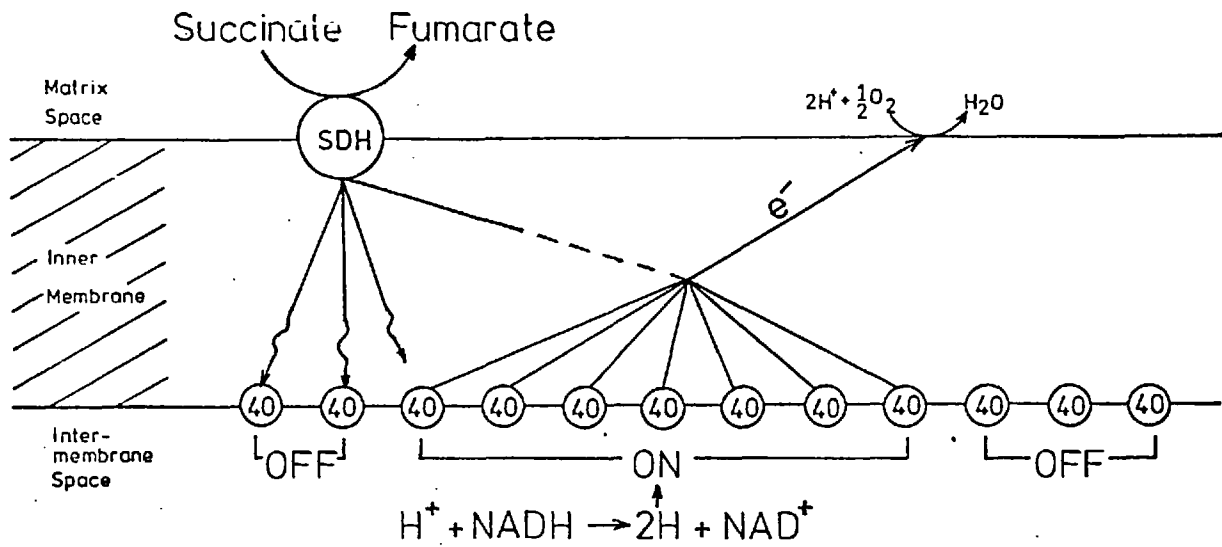
ii) State 4 plus Succinate (> 0.5mM).



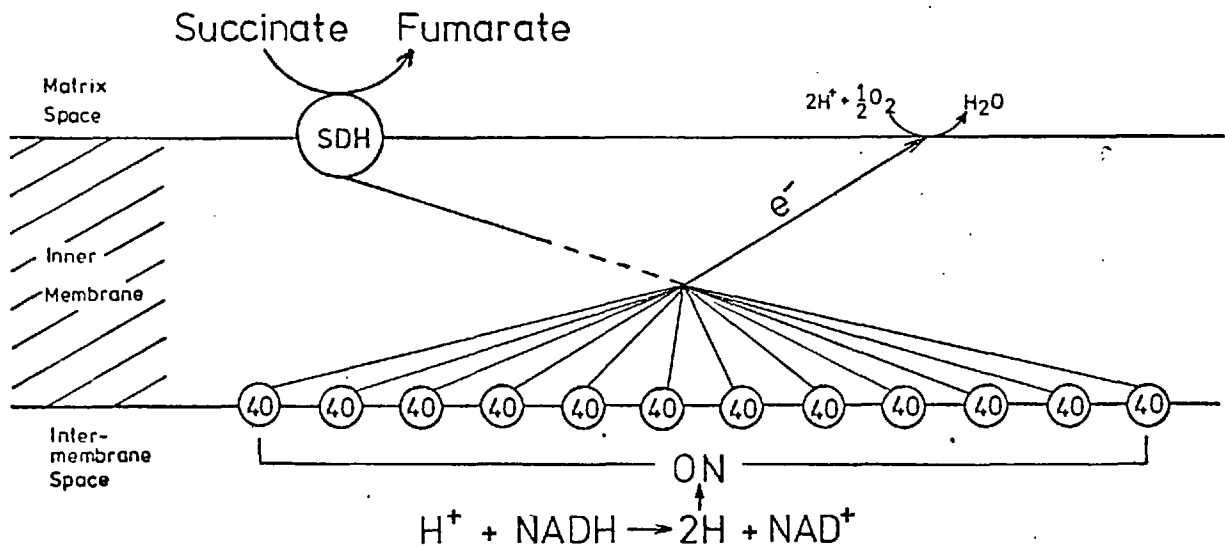
iii) State 3.



iv) State 3 plus Succinate (>1.0mM).



v) Uncoupled State.



are inhibited by succinate, which is equivalent to 60% inhibition of exogenous NADH oxidase activity, under state 4 conditions i.e. in mitochondria in which the enzyme succinate dehydrogenase had been activated by preincubation with ATP, Pi and $MgCl_2$.

Under state 3 conditions, 9 units of exogenous NADH oxidase are considered to be active, before succinate is added, leaving 3 units still controlled by a reaction which appears to be a function of the coupled or energised state (figure 62.iii.). Upon the addition of succinate to the mitochondria under these conditions, is considered to result in maximum inhibition of exogenous NADH oxidase activity, equivalent to 2 units, with the possibility of a third unit being inhibited in some preparations of mitochondria (table 10), as found under state 4 conditions. This leaves a much greater activity of exogenous NADH oxidase unaffected by succinate, compared with that remaining unaffected under state 4 conditions, and so the degree of inhibition was found to be less under state 3 conditions than under state 4 or substrate state conditions (see figures 42, 45 & 52, & table 10).

Only when uncoupler was present in the reaction cuvette, did the extra 3 units of exogenous NADH oxidase activity become apparent, as can be seen from figure 62 (v), indicating that the rate limiting step for consumption of oxygen resulting from the oxidation of exogenous NADH, in Jerusalem artichoke mitochondria, under state 3 conditions, is probably still a function of the mechanism of coupling between oxidative phosphorylation and electron transport. Under the uncoupled state, succinate was unable to inhibit exogenous NADH oxidase activity, and hence the arrows from succinate dehydrogenase to exogenous NADH dehydrogenase, are shown as not reaching the latter enzyme i.e. the inhibitory influence from succinate dehydrogenase, could not reach exogenous NADH dehydrogenase.

Concerning the mechanism of interaction between succinate and exogenous NADH oxidases in plant mitochondria, only a preliminary investigation was carried out during the course of the present study, however, it does appear that the mechanism which seems most simple at first sight, that is one in which reversed electron flow from succinate to exogenous NAD^+ occurs, was not the answer. Such a mechanism would involve only apparent inhibition of the rate of oxidation of exogenous NADH by succinate, since recycling of the NADH by reduction of its oxidation product, NAD^+ , would be expected to give the impression of the activity of exogenous NADH oxidase being inhibited, whilst succinate oxidase will simultaneously appear to be stimulated.

A mechanism involving the direct flow of electrons from succinate to exogenous NAD^+ , is considered to be unlikely on thermodynamic grounds, since there is no evidence for a 'site' of energy conservation between exogenous NADH dehydrogenase and ubiquinone, in isolated plant mitochondria, which would be required to drive reverse electron flow from succinate to exogenous NAD^+ . Also, an experiment designed to see if succinate could cause a measurable rate of reduction of exogenous NAD^+ in Jerusalem artichoke mitochondria, failed to show any evidence for such a reverse flow of electrons (table 20).

The alternative view of how such an interaction between succinate oxidase and exogenous NADH oxidase could occur would be to propose that there was competition between the two systems for a common component of the electron transport pathways. Experiments designed to investigate this possibility met with little success. No clear evidence was found for the involvement of a change in the level of reduction of any of the more easily observed components

of the electron transport pathways in the mechanism of interaction in Jerusalem artichoke mitochondria. In the absence of any clear information concerning interaction between the two oxidase systems at a common point along the electron transport pathways, it is reasonable to consider that the two dehydrogenase systems are functionally linked within the inner mitochondrial membrane, in a way that allows them to behave like sub-units of an allosteric protein. This would require that changes occurring in one enzyme, induced by its substrate, could be transmitted directly to the other enzyme in a manner that affects the catalytic activity of each enzyme. The close relationship between two enzymes which would be required for such a mechanism of interaction to be able to operate, probably exists since the addition of exogenous NADH lowered the apparent K_m for succinate, presumably by changing the conformation of succinate dehydrogenase.

The interaction between succinate and exogenous NADH oxidases in isolated plant mitochondria, represents a way in which the oxidation of a respiratory substrate, succinate, the initial steps of which take place on the matrix side of the inner membrane, can cause inhibition of the rate of oxidation of another substrate, exogenous NADH, the initial steps of which take place outside this membrane. In the cytosol, NADH appears to be a very important molecule in determining the rate and direction of the glycolytic pathway (see Palmer & Coleman, 1974), and so any reaction which alters its concentration, such as the rate of its oxidation catalyzed by the mitochondrial exogenous NADH oxidase, is of interest, assuming that it takes place in vivo. Possible cellular aspects of intra-mitochondrial substrate interactions which affect the rates of oxidation of malate, exogenous NADH and succinate in isolated

plant mitochondria, will now be discussed.

4. Possible cellular aspects of intra-mitochondrial substrate interactions that affect the oxidation of malate, exogenous NADH and succinate.

i) Interactions affecting the rate of oxidation of malate.

Under state 3 conditions, malate oxidase activity in isolated plant mitochondria was not inhibited greatly by low concentrations of oxaloacetate, while the oxidation of other NAD^+ -linked substrates was greatly, although temporarily inhibited by such additions (Avron & Biale, 1957; Brunton & Palmer, 1973; Brunton, 1975; present thesis, figures 14c and 18c). Thus it seems likely that under state 3 conditions in the cell, the NADH associated with malic enzyme cannot be oxidized by oxaloacetate.

Under state 4 conditions, there was some evidence for malate oxidase activity becoming more sensitive to inhibition by oxaloacetate (figure 19a,b). This is consistent with the possibility that NADH associated with the malic enzyme had changed its location to a site accessible to malate dehydrogenase. Whether the ability to switch the site of appearance of the NADH associated with a proportion of their malic enzyme molecules is also a characteristic of the mitochondria in vivo is not known. The observation that this ability in vitro decreased as the value for the respiratory control ratio of preparations of mung bean mitochondria (figure 23) and Jerusalem artichoke mitochondria (table 8), increased, might mean that in vivo none of the NADH associated with the malic enzyme, can change its location in response to a change from a less controlled to a more controlled state of malate oxidation. Thus it could be that in vivo, the compartmentation between malic enzyme and malate

dehydrogenase could be total. If the NADH associated with the malic enzyme and malate dehydrogenase were not functionally separate from each other in the matrix of plant mitochondria, their combined reactions would be expected to result in the depletion of the TCA cycle intermediates as oxaloacetate is reduced to malate by malic enzyme produced NADH, which would have serious consequences for cellular economy.

ii) Interactions affecting the rates of oxidation of exogenous NADH and succinate.

The rate of oxidation of exogenous NADH, catalyzed by isolated plant mitochondria, has been shown in the course of the present study to be inhibited by the respiratory substrates citrate and succinate.

Citrate appears to act as an inhibitor of exogenous NADH oxidase activity by chelating divalent cations, probably calcium, which are required for exogenous NADH oxidase to function. The inhibition caused by citrate was found to be relieved by the subsequent addition of calcium or manganese ions to the mitochondria. A similar result has been found for inhibition of exogenous NADH oxidase activity in Jerusalem artichoke mitochondria, which had been inhibited by the presence of EGTA in the assay medium (Coleman, 1971; Coleman & Palmer, 1971; present thesis, figures 32 and 36). The degree of inhibition caused by citrate, was found to be high, particularly in mitochondria isolated from Jerusalem artichoke tubers and mung bean hypocotyls (table 11), and when the citrate was added before the NADH (figure 32). The inhibition was markedly less if the citrate was added once oxygen uptake had been initiated by the addition of NADH. The last finding could mean that calcium is bound

more tightly to exogenous NADH oxidase, when it is actively catalyzing electron transport.

If citrate causes inhibition of exogenous NADH oxidase activity in vivo, to the same extent as that in vitro, it would alter the redox poise of the NAD^+/NADH in the cytosol which could then affect the rate of glycolysis. This is because the step in the glycolytic pathway catalyzed by glyceraldehyde 3-phosphate dehydrogenase appears to be sensitive to the value for the $\text{NADH}:\text{NAD}^+$ ratio, and is inhibited in the forward direction when the value of this ratio is high (see Palmer & Coleman, 1974). Walker (1962), has found evidence for the accumulation of citrate by plant cells, and so it is possible that it could be involved in causing this type of inhibition of exogenous NADH oxidase activity in the intact cell.

Succinate was the other respiratory substrate carboxylic acid that has been shown to be capable of imposing an inhibitory effect upon the activity of exogenous NADH oxidase in isolated plant mitochondria, which might also be of significance in vivo. The inhibition depended on the activity of succinate dehydrogenase, and was found to be maximal under respiratory state 4.

If the respiratory control ratio for exogenous NADH oxidase has a value of 3, as often found to be the case with mitochondria isolated from mung bean hypocotyls and Jerusalem artichoke tubers in the present study, and the rate of oxidation of exogenous NADH is inhibited by succinate to the extent of 60% under state 4 conditions, as also found here, then succinate would have the ability to cause the rate of oxidation of exogenous NADH to be inhibited to a level equal to only 13% compared with the rate measured under state 3 conditions. It is possible that in vivo, succinate can

inhibit exogenous NADH oxidase activity to a greater extent than occurred in vitro, due to possible damage to the mechanism of interaction between succinate and exogenous NADH oxidases introduced as a result of isolation of the mitochondria. If this is so, then succinate inhibition of exogenous NADH oxidase represents a potentially powerful mechanism for the control of the rate of oxidation of cytosolic NADH in plant cells.

The simultaneous inhibition of external NADH oxidation by succinate in plant mitochondria, and the stimulation of succinate oxidase activity by the NADH oxidation could result in the stimulated production of carbon skeleton for synthetic reactions, and the conservation of cytosolic NADH required for such reactions. Thus it is considered that the inhibition of exogenous NADH oxidase activity caused by citrate and succinate, represent possible ways by which cytosolic NADH in plant cells is conserved. The stimulation of succinate oxidase activity by exogenous NADH, could be of importance for synthetic reactions in the cell.

Finally in conclusion, it can be said that, of the respiratory substrates tested for their ability to interact with exogenous NADH oxidase in plant mitochondria, only succinate involved the active participation of oxidation, although the mechanism of interaction did not appear to involve the competition by reducing equivalents, for a common intermediate in the electron transport pathways. The data presented in this thesis are consistent with the mechanism of interaction being one of interaction between two enzymes on the same membrane, which might be mediated by changes in the conformation of this membrane induced by the activity of either exogenous NADH dehydrogenase or succinate dehydrogenase. Malic enzyme molecules in isolated plant mitochondria have also

been shown to be able to change the location of NADH associated with their activity, to a site which is accessible to malate dehydrogenase upon the transition from state 3 to state 4, and in the presence of rotenone or piericidin A. This change could also involve changes in the conformation of the inner mitochondrial membrane, since the decrease in the rate of electron transport that accompanies both conditions, might be expected to result in a change in the degree of contraction of this membrane (see Hanson & Hodges, 1967). Thus, it could be that conformation changes in the inner membranes of plant mitochondria, represent an important mode of control of the activity of enzymes that are closely associated with it, and so might represent an example of a mechanism for mediating the regulation of mitochondrial enzyme activity.

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