REACTIONS OF UBIQUINONE IN HIGHER PLANT MITOCHONDRIA.

BY

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ABSTRACT.

The ubiquinone content of mitochondria from Jerusalem artichoke (*Helianthus tuberosus*) was characterised. Inhibition of exogenous NADH oxidation by succinate was studied, as one example of anomalous behaviour of the plant respiratory chain, observed when two dehydrogenases or two oxidases operate.

Interaction between succinate and exogenous NADH oxidases was very asymmetric, since little inhibition of succinate oxidation was observed in the presence of NADH. This could not be explained as simple competition for ubiquinone. The degree of inhibition of NADH oxidase depended upon the respiratory state, but was not regulated by membrane potential, adenine nucleotides, or by direct interaction between succinate and NADH dehydrogenases. Instead the inhibition reflected the activity of the quinol oxidase. The site of interaction was identified, by the use of specific inhibitors, as ubiquinone.

The suitability of antimycin, myxothiazol, HQNO and BAL as probes of ubiquinone involvement in the separate oxidation of succinate and NADH was examined. Resistance of exogenous NADH oxidase to HQNO was shown to involve superoxide radicals. Oxidation of both substrates proceeded via a highly mobile pool of ubiquinone and no evidence was found for compartmentation of ubiquinone.

Techniques were developed for the production of relatively pure submitochondrial particles of either membrane polarity, and for separation of SMP of different polarity from mixed populations, by variation of ionic conditions and by phase-partition. Using SMP it was shown that organisation of the membrane into cristae was not essential to the asymmetric inhibition of NADH oxidation by succinate.

The kinetics of succinate oxidation by intact mitochondria

differed significantly from those found with NADH as substrate. A simple extension to the original model of ubiquinone function is shown to explain the kinetics of the two oxidases, and the asymmetric interaction between them. This model is discussed in relation to other branches of the plant respiratory chain.

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Abbreviations.

AMP	adenosine-5'-monophosphate
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
BAL	2,3-dimercaptopropanol
BSA	bovine serum albumin (fraction V)
CTC	chlorotetracycline
Duroquinone	2,3,5,6-tetramethyl-1,4-benzoquinone
DQ	duroquinone (oxidised form)
DQH_2	duroquinol (reduced form)
EDTA	ethylenediaminetetraacetic acid
E_m	apparent midpoint potential
E_o'	standard electrode potential at pH 7
FCCP	carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone
HQNO	2-(*n*-heptyl)-4-hydroxyquinoline *N*-oxide
MDH	malate dehydrogenase
MOPS	3-(N-morpholino) propane sulphonic acid
mV	millivolt
NADH	nicotinamide adenine dinucleotide (reduced)
Pi	inorganic phosphate
SDH	succinate dehydrogenase
TES	N-tris(hydroxymethyl)-methyl-2-aminoethane sulphonic acid
Tris	2-amino-2-(hydroxymethyl)-propane-1:3-diol
Triton X-100	octylphenoxypolyethoxyethanol
UHDBT	5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole
UQ-(n)	ubiquinone with a side chain of (n) isoprenoid units

Kinetic symbols

Eo	total concentration of dehydrogenase
E	dehydrogenase without ubiquinone
EQ	dehydrogenase with bound ubiquinone
g(i)	a function linking benzhydroxamate concentration with alternative oxidase activity
Km	Michaelis constant
Ki	inhibition constant
Kd	dissociation constant
Qa	redox active portion of ubiquinone pool
Q	Qa present as ubiquinone
QH_2	Qa present as ubiquinol
V_T or v	rate of electron transfer
Valt	second order rate constant for the oxidation of ubiquinol by the alternative oxidase
Vcyt	second order rate constant for the oxidation of ubiquinol by the cytochrome pathway
Vcyt	(according to context) the maximum rate of oxidation via the cytochrome pathway
V_N	second order rate constant for the reduction of ubiquinone by NADH
Vs	second order rate constant for the reduction of ubiquinone by succinate
Vred	rate constant for the reduction of ubiquinone
Vox	rate constant for the oxidation of ubiquinol
Vm	maximum rate of enzyme catalysis

The following trivial names have been used in this thesis:

Trivial name	Systematic name	Enzyme Commission number
Catalase	hydrogen peroxide: hydrogen peroxide oxidoreductase	1.11.1.6
Cytochrome oxidase	ferrocytochrome c: oxygen oxidoreductase	1.9.3.1
Malate dehydrogenase	L-malate:NAD^+ oxidoreductase	1.1.1.37
Malic enzyme	L-malate:NAD^+ oxidoreductase (decarboxylating)	1.1.1.39
NADH dehydrogenase	reduced-NAD^+:(acceptor) oxidoreductase	1.6.99.3
Succinate dehydrogenase	succinate:(acceptor) oxidoreductase	1.3.99.1
Superoxide dismutase	superoxide: superoxide oxidoreductase	1.15.1.1

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Solubilization of the alternative oxidase of Cuckoo-pint (*Arum maculatum*) mitochondria. (1985) Kay, C.J. & Palmer, J.M. Biochem. J. 228 , 309-318.

Electrostatic screening stimulates rate-limiting steps in mitochondrial electron transport. (1984) Moller, I.M., Kay, C.J. & Palmer, J.M. Biochem. J. 223 , 761-767.

FIGURES

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Chapter 1. Introduction.

1.1 The respiratory activities of isolated higher plant mitochondria.

In many respects the components, organisation and mechanism of operation of the plant and mammalian respiratory chainsare similar. Consequently it is the differences between the two systems which tend to be stressed (eg Palmer, 1979). The principal differences relevant to this project are the additional pathways of substrate oxidation that are present in plant mitochondria. These give flexibility, both in terms of the oxidation routes available, and in the degree of energy conservation attained. These additional pathways of oxidation include the ability to oxidise exogenous NADH and NADPH; the oxidation of glycine by mitochondria from photosynthetic tissue; the oxidation of internal NADH without energy conservation at site I, and the mysterious, non-energy conserving, alternative pathway of ubiquinol oxidation. The only respiratory component thought to be common to all pathways is ubiquinone, which is present in large excess compared to the other components. A deceptively simple model, in which ubiquinone functions as a mobile pool shuttling electrons between relatively immobile protein complexes, has been broadly successful in explaining the way particular ubiquinone-reducing and ubiquinol-oxidising components cooperate in electron transport, but is much less successful when more than one type of dehydrogenase or oxidase is active simultaneously. This is especially the case with the plant respiratory chain, and the experiments presented in this thesis were intended to explore the reasons for this. In this section current understanding of the behaviour and arrangement of the branched respiratory chain is discussed.

1.1.1 The oxidation of exogenous NADH and NADPH.

Mitochondria from Neurospora crassa (Weiss *et al*, 1970), yeast von Jagow and Klingenberg, 1970) and all higher plant species and tissues tested to date are capable of oxidising added NADH via the respiratory chain, with the exception of red beetroot (*Beta vulgaris* L.), and even in this case the activity can be induced by prolonged washing of slices in a dilute aerobic solution of $CaSO_4$ (Day *et al*, 1976).

When the ability of mitochondria to oxidise exogenous NADH in the absence of added cytochrome c was first noted (Humphreys and Conn, 1956) it was proposed that an externally facing dehydrogenase was involved, on the basis that NADH could not penetrate the inner membrane. The resistance of exogenous NADH oxidation to rotenone (Wilson and Hanson, 1969) and coupling of oxidation to only two sites of phosphorylation (Ikuma and Bonner, 1967a, Wilson and Hanson, 1969, Palmer and Passam, 1971, Tomlinson and Moreland, 1975) seemed to distinguish further this pathway from endogenous NADH oxidation. However this view was weakened by the discovery of a pathway of endogenous NADH oxidation also rotenone resistant and bypassing one site of phosphorylation (Ikuma and Bonner, 1967b, Brunton and Palmer, 1973. See also section 1.1.2). In order to determine on which face of the inner membrane added NADH was oxidised, von Jagow and Klingenberg (1970) used radiolabelling to show that NADH oxidation by yeast mitochondria occurred without NADH crossing the inner membrane. Similar experiments have not been carried out on higher plant mitochondria, but indirect evidence suggests that oxidation of NADH by higher plant mitochondria does indeed occur via a dehydrogenase with the site of oxidation for NADH located on the outer face of the inner membrane (Douce *et al*, 1973, Palmer and Coleman, 1974).

The typical rates and P:O ratios reported for oxidation of exogenous NADH through the cytochrome pathway are similar to those for succinate oxidation, and the P:O ratios are approximately two thirds of those reported for malate oxidation, suggesting that, like succinate oxidation, no energy conservation occurs prior to complex III, as indicated in Figure 1 (Ikuma and Bonner, 1967a, Wilson and Hanson, 1969, Palmer and Passam, 1971, Tomlinson and Moreland, 1975).

The dehydrogenase can reduce artificial quinones such as duroquinone and juglone (5-hydroxy-1,4-naphthoquinone), both in intact Jerusalem artichoke (*Helianthus tuberosus*) mitochondria (Cowley and Palmer, 1978) and in the isolated form (see below) as well as ubiquinone-1. Extraction of endogenous ubiquinone prevented oxidation of exogenous NADH by Arum mitochondria and activity could be restored by reincorporation of the extracted lipid, ubiquinone-10 or duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone) (Huq and Palmer, 1978a). Molecular mobility between this dehydrogenase and complex III is suggested by the sigmoidal inhibition of oxygen uptake by antimycin (Cottingham and Moore, 1983). This mobility is usually attributed to free ubiquinone (section 1.2.1) and so the most reasonable interpretation of this data is that the immediate natural redox acceptor of the dehydrogenase is ubiquinone.

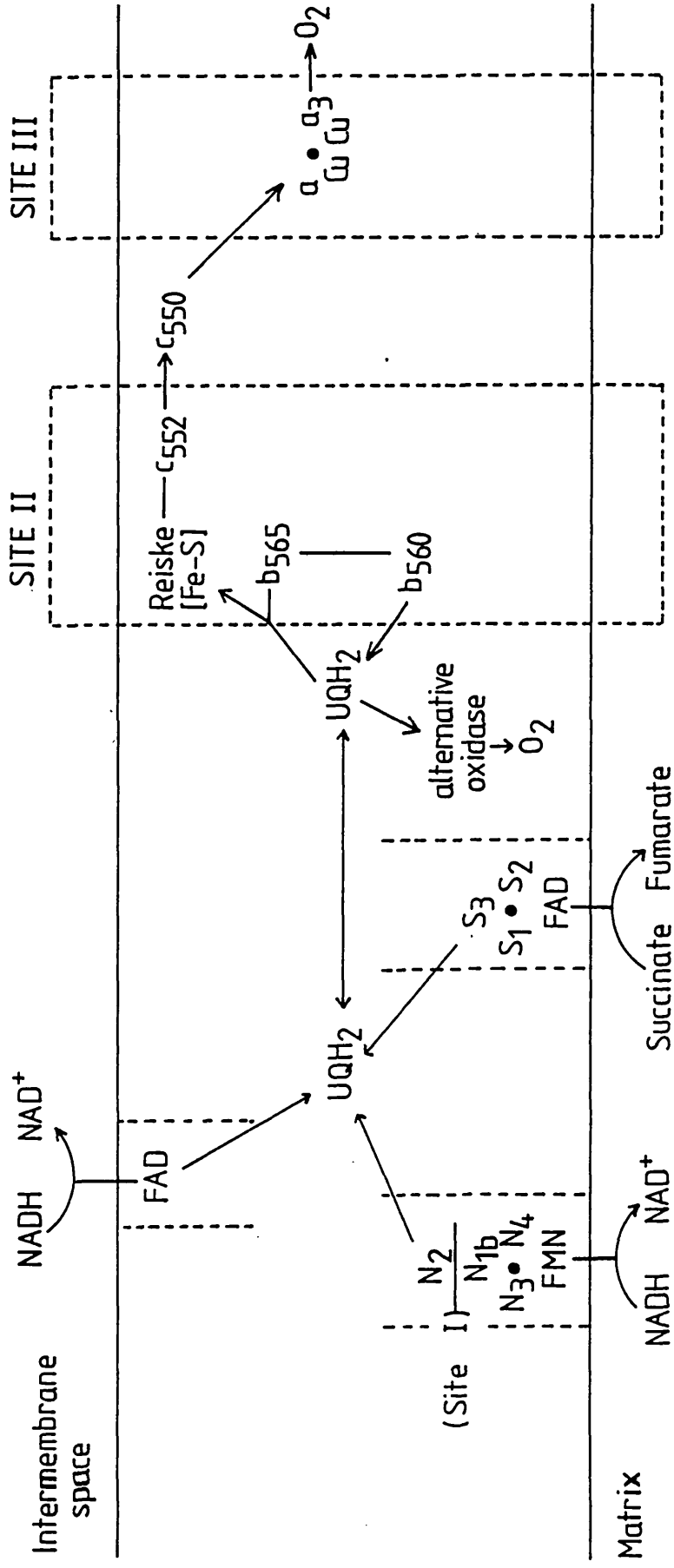
There have been three recent reports of isolation of rotenone insensitive NADH dehydrogenases from plant mitochondria: two from *Arum maculatum* spadix (Cook and Cammack, 1984, Cottingham and Moore, 1984) and one from cauliflower (*Brassica oleracea* L.) (Klein and Burke, 1984). Cook and Cammack (1984) used the detergent lauryl dimethylamine *N*-oxide (LDAO) followed by 5'-ADP affinity chromatography to separate rotenone sensitive and insensitive NADH dependent ubiquinone-1 reductase activities.Three major polypeptides were associated with the rotenone insensitive activity, with

Figure 1. The respiratory chain of non-photosynthetic plant mitochondria.

The components of the separate respiratory complexes are bounded by broken lines, but the relative positions of the components are not meant to signify their physical arrangement within the membrane. Sites I-III represent the points at which energy is conserved. Site I is shown in parentheses to indicates the ability of endogenous NADH oxidation to bypass this site. The cytochrome spectral peaks are those measured at room temperature, and values are taken from the reports of Ducet and Diano (1978) and Lambowitz and Bonner (1974a,b). The organisation of the components of the bc_1 complex is adapted from Rich and Moore (1976).

FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
N1b-4	Iron-sulphur centres associated with complex I
S1-3	Iron-sulphur centres associated with complex II
UQH_2	ubiquinol
a, b, c	cytochrome components of type a, b or c
Cu	Copper prosthetic groups of cytochrome oxidase.

Figure 1. The respiratory chain of non-photosynthetic plant mitochondria.



molecular weights estimated by SDS-polyacrylamide gel electrophoresis to be 55 kDa, 39.2 kDa and 33.9 kDa. These values are different to those reported by Cottingham and Moore (1984) who used the detergent deoxycholate, ion-exchange and gel-chromatography to obtain a preparation containing polypeptides of molecular weights 78 kDa, 76 kDa and 65 kDa. In other respects the two preparations were similar. Both had a pH optimum for NADH-UQ-1 reductase activity of approximately 7.3, in close agreement with that found for NADH oxidase activity in intact Arum mitochondria of pH 7.2 (Moller and Palmer, 1981a). The apparent Km values for NADH dependent, rotenone insensitive, UQ-1 reduction were identical at 28μM. Reported values for the Km for NADH oxidation by intact mitochondria are variable and dependent on the ionic composition of the assay medium used, probably due to screening of negative charges on the inner membrane, facilitating approach of negatively charged NADH (Moller and Palmer, 1981b, Moller *et al.*, 1984). Reported values include 19-31μM for Jerusalem artichoke mitochondria (Moller and Palmer, 1981a), 70μM for mung bean mitochondria (Ikuma and Bonner, 1967a) and 25μM for white potato mitochondria (Arron and Edwards, 1979). Screening of the membrane surface negative charges decreases the Km for NADH and also increases the apparent Vmax (Moller and Palmer, 1981b, Moller *et al.*, 1984). The change in Vmax shows that screening has other effects than just allowing NADH to approach the membrane surface.

Both rotenone insensitive preparations from Arum mitochondria were unable to oxidise NADPH at pH 7.4, but between pH 7 and pH 6 Cook and Cammack (1984) found increasing NADPH dependent UQ-1 reductase activity with decreasing pH. The pH optimum for NADPH oxidation by intact mitochondria is between pH 6.0 and pH 6.5, depending on the source of the mitochondria. It has been suggested that the difference in the pH optimum of NADPH oxidation from that

found for NADH oxidation is due to the state of protonation of the 2'-phosphate group of NADPH. This group has a pKa of 6.2-6.3 (Theorell, 1935). As the pH is lowered this group will become increasingly protonated and, in terms of charge, NADPH will resemble NADH. Consequently the approach of NADPH to the negatively charged membrane surface is easier at low pH (Moller and Palmer, 1981b).

This explanation for the different pH optima of NADH and NADPH oxidation raises the question of whether the same dehydrogenase is involved in the oxidation of both NADH and NADPH. At present evidence on this point is inconclusive. Because NADH and NADPH oxidation differ in their sensitivity to chelators and mersalyl (Arron and Edwards, 1980) it seems that the available evidence favours the idea of separate dehydrogenases (Moller and Palmer, 1981a, Nash and Wiskich, 1983). The report of Cottingham and Moore (1984) that in intact Arum mitochondria NADH could donate electrons about eight times faster to UQ-1 than to oxygen, while with NADPH as substrate the rate to UQ-1 was only a quarter of that to oxygen, is difficult to reconcile with a common site of quinone reduction. However, the oxidation of NADPH by the isolate of Cook and Cammack (1984) suggests either that a single dehydrogenase can catalyse the oxidation of both substrates, or that more than one type of dehydrogenase was still present.

Both Cook and Cammack (1984) and Cottingham and Moore (1984) attempted to identify the redox centres associated with the rotenone insensitive NADH dehydrogenase activity. Acid extractable flavin was present in both cases, mostly as FAD but with some FMN as well. Cook and Cammack (1984) also detected riboflavin and, since acid treatment of FAD can result in breakdown of FAD to FMN and riboflavin, these authors concluded that FAD was probably the active flavin. In an earlier investigation of EPR signals arising during oxidation of NADH

by Arum submitochondrial particles, Cammack and Palmer (1977a) found a signal at g=1.93 (77K) with an estimated midpoint potential of -20mV, which was possibly an iron-sulphur centre associated with the external NADH dehydrogenase. This signal was not found to be present in the preparation of Cook and Cammack (1984) and it was concluded that no iron-sulphur centres were involved in the operation of the enzyme.

The oxidation of exogenous NADH by intact mitochondria is inhibited by a variety of compounds. Low concentrations of dicoumarol (50μM) caused full inhibition of NADH oxidation, with an apparent Ki of 5μM (Day and Wiskich, 1975). A range of sulphydryl group reagents were tested by Arron and Edwards (1980) and *p*-chloromercuribenzoate, N-ethyl maleimide and mersalyl were found to be powerful inhibitors of NADPH oxidation, but were less effective against NADH oxidation. The potency of inhibition by mersalyl is dependent upon pH. The oxidation of NADH by Jerusalem artichoke mitochondria is inhibited at pH 7.2 but not at pH 5.2. Conversely, the oxidation of NADPH, which is not oxidised at pH 7.2, is inhibited at pH 5.2 (Moller and Palmer, 1981a). More recently Tissut *et al.* (1984) have shown that several flavenoids, including rotenone derivatives, were potent inhibitors of exogenous NADH oxidation. A strong correlation was found between the lipophilicity of the compounds and the effectiveness of inhibition, but whether the inhibitory effects are due to alterations in membrane properties, such as fluidity, is not yet clear.

Metal ion chelators of such as citrate (Cowley and Palmer, 1978), EDTA and EGTA (Coleman and Palmer, 1971) also inhibit exogenous NADH oxidation. Inhibition can be reversed by the addition of Ca^{2+} (Palmer and Coleman, 1974). Stimulation of oxidation by Ca^{2+} is not due soley to charge screening effects, but to a specific calcium

requirement (Moller *et al.*, 1981a). It has been suggested that Ca^{2+} may play a role in the binding of the flavin of the dehydrogenase to the membrane (Coleman and Palmer, 1971, Storey, 1980), and also that Ca^{2+} may change the conformation of the dehydrogenase such that it interacts more efficiently with NADH (Palmer and Moller, 1982).

The effectiveness of inhibition of NADH oxidation by EDTA and EGTA is much less at pH 6.5 than at pH 7.5, an effect attributed to changes in the surface charge properties of the membrane at low pH (Moller and Palmer, 1981a). The kinetics of inhibition by chelators are complicated as maximum inhibition is obtained more quickly if chelator is added before, rather than after, NADH, and this has been interpreted as meaning that Ca^{2+} becomes more firmly attached to the dehydrogenase once substrate is added (Cowley and Palmer, 1978). It is clear that the role of Ca^{2+} in NADH oxidation is not fully understood, and it is of interest that no calcium requirement or chelator sensitivity has been found in isolated rotenone insensitive NADH dehydrogenase preparations from plant mitochondria (Cook and Cammack, 1984, Cottingham and Moore, 1984, Klein and Burke, 1984).

1.1.2 The oxidation of endogenous NADH.

Electron transfer to oxygen via the internal NADH dehydrogenase of the animal respiratory chain is completely inhibited by rotenone or piericidin (Jeng *et al.*, 1968, Hatefi and Stiggall, 1976) and is coupled to three sites of phosphorylation. Endogenous NADH oxidation by plant mitochondria is often only partially inhibited by rotenone (Ikuma and Bonner, 1967b, Brunton and Palmer, 1973, Day and Wiskich, 1974) and, in the presence of rotenone or piericidin, is coupled to the synthesis of a maximum of two moles of ATP (Palmer and Coleman, 1974, Brunton and Palmer, 1973, Marx and Brinkman, 1978). The

rotenone sensitive component of respiration is thought to involve a similar dehydrogenase to mammalian complex I, while in the presence of rotenone NADH is oxidised by a different pathway that also donates electrons to ubiquinone, but does not conserve energy at site I (see Figure 1).

Most of the current understanding of the internal NADH dehydrogenases present in plant mitochondria has come from attempts to understand the complex kinetics of malate oxidation. The oxidation of malate by plant mitochondria is carried out by malate dehydrogenase and an NAD^+-linked malic enzyme (Macrae and Moorhouse, 1970), which catalyse the following reactions:

Malate dehydrogenase: Malate + NAD^+ = Oxaloacetate + NADH + H^+

Malic enzyme: Malate + NAD^+ = Pyruvate + CO_2 + NADH + H^+

Malate dehydrogenase is a soluble enzyme which is present in the matrix at an activity that can, in the presence of detergent to rupture the mitochondria, reduce NAD^+ at 2000 nmol NADH/min/mg protein, which is ten times faster than intact mitochondria can oxidise malate (Palmer, 1980). It has therefore been assumed that this enzyme is at all times at equilibrium. The equilibrium position at pH 7 strongly favours formation of malate and NAD^+ and so concentrations of oxaloacetate and NADH will be low (Keq, pH 7 = 2.2×10^{-5}) (Palmer, 1984).

Malic enzyme is also located in the matrix but is much less active than malate dehydrogenase, reducing NAD^+ at only 100 nmol/min/mg protein (Coleman and Palmer, 1972). The equilibrium position of malate dehydrogenase is pH dependent since hydrogen ions are produced by the reaction. The activity of the malic enzyme is markedly affected by pH with a rapid decline, with increasing pH, from the optimum at pH 7.2 (Coleman and Palmer, 1972). Consequently increasing pH values of higher than pH 7.2 will shift the equilibrium

for malate dehydrogenase to favour product accumulation and also decrease NADH production by the malic enzyme.

Many of the reported properties of malate oxidation can be explained on the assumption that the rotenone-sensitive NADH dehydrogenase has a greater affinity for NADH than does the rotenone-insensitive activity (see Palmer and Moller, 1982). Moller and Palmer (1982) studied the affinities of the two activities for NADH using submitochondrial particles of mixed polarity. Calcium chelator was present in order to minimise interference by NADH oxidation via the external NADH dehydrogenase (see section 1.1.1) by particles which had retained their original polarity during sonication. The Km for NADH for the rotenone-sensitive activity was 8μM while for the rotenone-insensitive activity it was 80μM.

Addition of piericidin to mitochondria oxidising malate under state 3 conditions can cause a transient inhibition of oxygen uptake with partial recovery occuring within minutes (Brunton and Palmer, 1973, Palmer and Arron, 1976). This effect was attributed to the inverse relation between oxaloacetate concentration and the concentration of NADH. During state 3 oxidation oxaloacetate accumulates and NADH concentration is lowered as a consequence of the perpetual equilibrium of malate dehydrogenase. On addition of piericidin or rotenone the concentration of NADH is low and so the rotenone-insensitive dehydrogenase is only partially saturated, and so the rate of oxygen uptake is slow. After the addition of rotenone, malic enzyme continues to produce NADH which is oxidised by malate dehydrogenase, progressively decreasing the oxaloacetate concentration allowing NADH concentration to rise. As NADH levels rise the rotenone-insensitive dehydrogenase becomes more saturated and the rate of oxygen consumption increases. This explanation has been supported by measurement and manipulation of oxaloacetate levels

(Palmer *et al.*, 1978, Tobin *et al.*, 1980) and is consistent with reported biphasic rates of oxidation of malate under state 4 conditions (Lance *et al.*, 1967), since oxaloacetate accumulated during state 3 oxidation must be reduced by the NADH produced by malic enzyme before the non-energy conserving, rotenone-insensitive dehydrogenase can engage. Stimulation of rates of piericidin-insensitive malate oxidation by added NAD^+ provides further evidence of regulation of the pathway of NADH oxidation according to NADH concentration, since it is now clear that NAD^+ can cross the inner mitochondrial membrane raising the concentration in the matrix (Neuberger and Douce, 1978).

Another factor which can regulate the oxidation of endogenous NADH is AMP, which is required to achieve maximum rates of oxidation of NAD^+-linked substrates by uncoupled mitochondria (Sotthibandhu and Palmer, 1975). The stimulation of rate was unaffected by the inhibitors of adenine nucleotide translocation bongkrekic acid and atractyloside, and so it seems that AMP stimulates respiration from outside the matrix. Addition of AMP caused simultaneous oxidation of NADH and reduction of cytochrome b, but only in the absence of rotenone, suggesting that only the rotenone-sensitive dehydrogenase was stimulated.

Most of our knowledge of the rotenone-sensitive NADH dehydrogenase (complex I) has come from studies on the mammalian, fungal or avian mitochondrial enzyme. A summary of the properties of these enzymes is given here because, from the little that is so far known, it is thought that the plant mitochondrial enzyme is similar. Isolated complex I catalyses NADH dependent ubiquinone reduction that is rotenone sensitive, but will also reduce other acceptors such as ferricyanide, though these activities are not inhibited by rotenone.

The striking feature of active preparations of complex I is the

large number of associated polypeptides not containing redox active groups. The complex is thought to have a molecular weight of between 600 and 900 kDa, though precise measurement is difficult as the complex is heterogenous when subjected to ultracentrifugation. This heterogeneity, together with the observation that only 0.1-0.2 mole of rotenone per mole of FMN can cause 50% inhibition of activity (Ragan and Heron, 1978), has prompted suggestions that several complexes may associate to form the functional unit in the membrane (Ragan, 1980). Individual complexes are comprised of approximately 26 different polypeptides, although with so many present detection of contaminants is difficult (Heron *et al.*, 1979b). Chaotropic agents such as urea, sodium perchlorate or trichloroacetic acid solubilize about 30% of the protein of complex I, leaving a very hydrophobic residue. Antisera raised to either complex I or an iron-protein fragment co-ordinately precipitated all the polypeptides of complex I except one of 42 kDa (Smith and Ragan, 1978) and loss of the 42kDa polypeptide was accompanied by loss of ubiquinone reductase activity (Heron *et al.*, 1979b), supporting the view that all 26 polypeptides are part of complex I.

It is generally accepted that phospholipid is necessary for rotenone sensitive ubiquinone reduction (Ragan, 1976,1980). Submitochondrial particles depleted of phospholipid by added phospholipase show decreased ubiquinone reductase activity that can be partially restored by added phospholipid (Machinist and Singer, 1965). Phospholipid removal also promotes denaturation of centre N2 (Ohnishi *et al.*, 1974). Heron *et al.* (1977) concluded that complex I had a specific lipid requirement for cardiolipin and either one of phosphatidylcholine or phosphatidylethanolamine.

Various protein labelling agents have been used to study the arrangement of the polypeptides in the membrane. As diazobenzene sulphonate (DABS) cannot penetrate the membrane it has been used to label subunits not deeply buried within the membrane. [^{32}S] DABS labelled most of the polypeptides of complex I (Smith and Ragan, 1978), but subsequent fractionation of complex I with perchlorate showed that the soluble fraction was only lightly labelled. This was not due to inherent unreactivity of these polypeptides since they could be labelled once isolated. Similar results were obtained following labelling of the polypeptides of the complex with radioactive iodine (Ragan, 1976, Smith and Ragan, 1978). Ragan (1980) concluded that the hydrophilic iron and flavin containing regions of the complex were probably shielded in the membrane by the hydrophobic small molecular weight polypeptides.

Complex I preparations have been recombined with other respiratory enzymes to reconstitute NADH oxidase activity (Hatefi *et al.*, 1962a) and when reincorporated into phospholipid vesicles can catalyse proton translocation linked to electron transport (Ragan and Hinkle, 1975).

Complex I contains acid extractable FMN that is not covalently attached to the protein (Hatefi and Reiske, 1967). Because the flavin absorbtion maximum of 420nm (oxidised form) is little affected by the protein environment, while the fluorescence of the flavin is, the ratio of fluorescence to absorbance can be used to characterise different flavins. This technique has been applied to animal mitochondria (Chance *et al.*, 1967) and supported by redox potential measurements (Erecinska *et al.*, 1970). Storey (1970, 1971) distinguished 5 flavins in plant mitochondria, only one of which responded to the endogenous pyridine nucleotide pool. This was designated Fp_{LF} (meaning "low potential, fluorescent") and had a

midpoint potential at pH 7.2 of -155mV, but this flavin is probably associated with lipoate dehydrogenase rather than complex I. Identification of these components as flavins is complicated by the fact that the wavelengths used by Storey (464-492nm) will also detect iron-sulphur centres (Hall *et al.*, 1974) but these can usually be distinguished from flavin by redox titration as single electron carriers. The problems of flavin identification were discussed by Storey (1980).

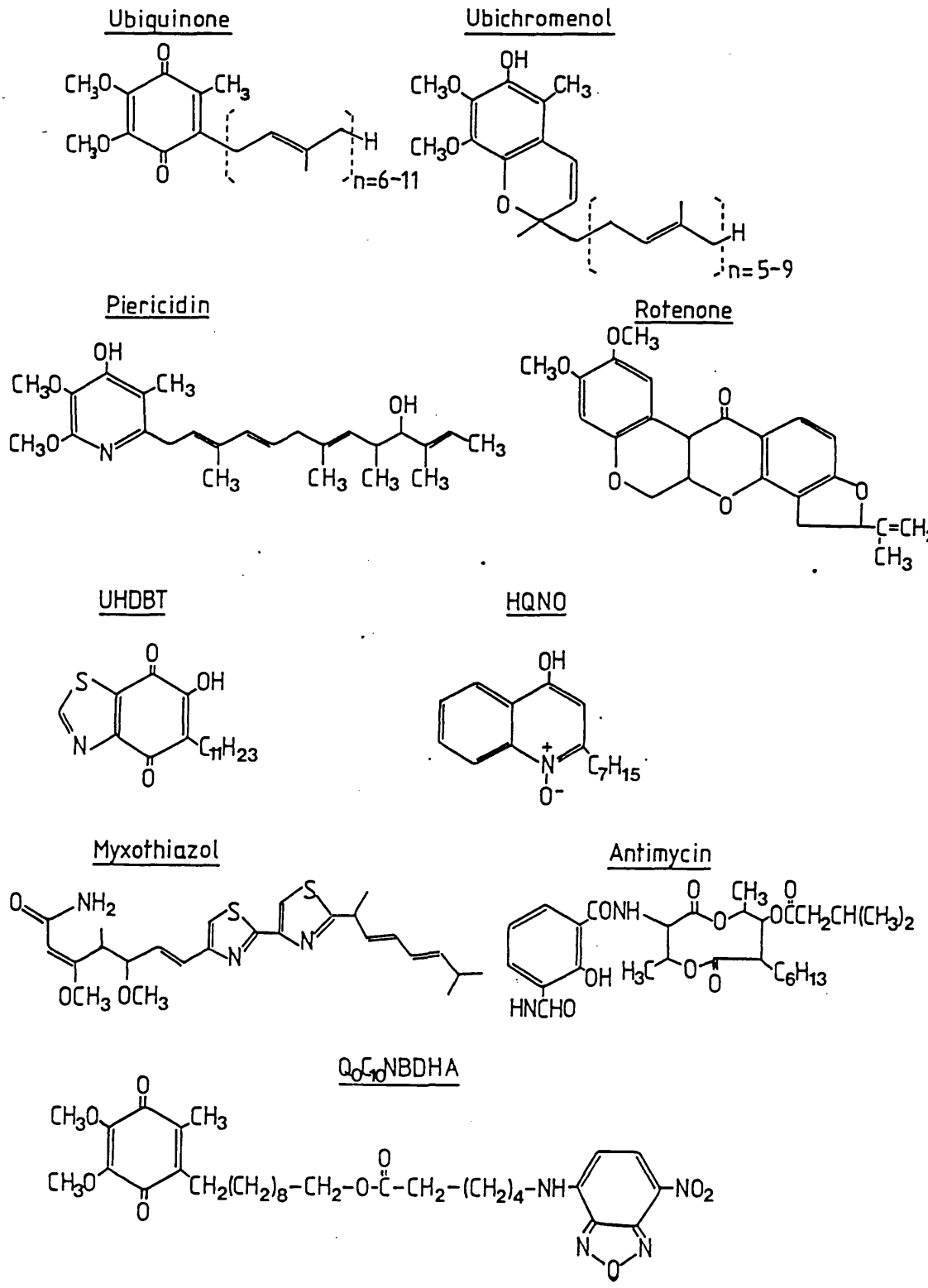
In addition to flavin there are at least five different EPR-detectable species of iron-sulphur (Fe-S) clusters: centres 1a, 1b, 2, 3, 4 (Orme-Johnson *et al.*, 1974, Albracht *et al.*, 1977). Centres 2, 3 and 4 are present in equal amounts (Orme-Johnson *et al.*, 1974), but the stoichiometry of centres 1a and 1b is disputed. Orme-Johnson *et al.* (1974) found that the concentration of centre 1 (ie.1a + 1b) was equal to that of the other centres, while Albracht *et al.* (1977,1979) have reported centres 1a and 1b to be present at only one-quarter the concentration of the other centres, both in isolated complex I and in submitochondrial particles.

All the Fe-S centres are on the substrate side of the rotenone and piericidin binding site (Ohnishi, 1973) which might be expected on the basis of the close structural similarity of piericidin to ubiquinone (see Fig. 2). Centre 2, since it is the centre with the highest redox potential, may be involved in ubiquinone reduction. The site of energy conservation (site I) is not known with certainty but the difference in redox potential between centre 2 and centres 1, 3 and 4 suggest it is located in this span. Only centres 1a and 2 have pH dependent redox potentials (60 mV per unit pH) and ATP induces a midpoint potential shift in centres 1 and 2 (Singer and Gutman, 1974, Gutman *et al.*, 1970). The properties of site I have been reviewed by Ohnishi (1973).

Cammack and Palmer (1977a,b) resolved centres 1b, 2, 3 and 4 in plant mitochondria. Centre 1b was detected at 77K (g=2.03 and 1.93) and had a midpoint potential of -240mV. A signal at g= 1.928 (65K), which was readily reducable by NADH was suggested to be associated with rotenone-insensitive NADH oxidation, but this signal was not reported in a subsequent study (Rich and Bonner, 1978b).

Rotenone sensitive NADH dehydrogenase has been purified from Arum spadix mitochondria by Cook and Cammack (1984) using the detergent LDAO and 5'ADP affinity chromatography. The Km for NADH was 8.3μM, in agreement with the measurement of Moller and Palmer (1982) using Jerusalem artichoke submitochondrial particles. No information was presented concerning the polypeptide composition of this fraction, presumably because of the complexity of complex I. Klein and Burke (1984) separated a NADH dehydrogenase fraction from cauliflower mitochondria that was specific for NADH and was inhibited by both sulphydryl reagents and low pH. The fraction contained major polypeptides of molecular weights 57.6kDa and 32.6kDa. The presence of EPR signals characteristic of a reduced (ferrodoxin-type) iron-sulphur centre suggested it may oxidise endogenous NADH, but unfortunately the fraction was not tested for rotenone sensitivity.

Figure 2. Chemical structures of ubiquinone, quinone analogues and inhibitors of the bc_1 complex.



1.1.3 The oxidation of Succinate.

Succinate is oxidised to fumarate during operation of the tricarboxylic acid cycle by succinate dehydrogenase, an enzyme present in all plant mitochondria. Succinate dehydrogenase is firmly bound to the inner membrane and donates electrons to ubiquinone. The similarity between the midpoint potentials for the succinate/fumarate couple ($E^{o\prime}$ (pH 7)= 31mV, Metzler 1977) and the pool of ubiquinone (+70mV Storey, 1970) precludes energy conservation before ubiquinone, and P:O ratios of approximately 2 have been reported, corresponding to energy conservation at sites II and III.

The lipoprotein complex (complex II) which catalyses ubiquinone reduction by succinate has been isolated and characterised from animal tissues (King 1963, Davies and Hatefi, 1971, Ackrell *et al.*, 1977a). Complex II includes the enzyme succinate dehydrogenase, which cannot reduce ubiquinone but only artificial acceptors such as dichloroindophenol (Ackrell *et al.*, 1980) and is insensitive to the inhibitors of ubiquinone reductase activity of complex II, thenoyl trifluoroacatone (TTFA) and carboxin. The complex contains five polypeptides: the two larger ones of molecular weights 73 and 25 kDa (Davis and Hatefi, 1971) comprise succinate dehydrogenase and contain the recognised redox components. The largest subunit (SD_1) contains one molecule of flavin (FAD), covalently attached to a histidine residue of the polypeptide (Hemmerich *et al.*, 1969), and four of the iron and acid-labile sulphides. The iron is generally thought to be organised into two binuclear 2 iron-2 sulphur clusters termed S1 and S2 (Ohnishi and Salerno, 1982), although the existence of S2 *in vivo* has been questioned (see Beinert and Albracht, 1982 for a discussion). The 25 kDa subunit (SD_2) contains the remaining four iron and acid-labile sulphides as a 4Fe-4S cluster known as the S3 or HiPIP centre. This centre is extremely labile towards oxidants such

as oxygen or ferricyanide in the isolated enzyme and destruction of this centre prevents ubiquinone reductase activity. Centre S3 is thought to be directly involved in the reduction of ubiquinone (see section 1.2.3).

In addition to the flavin and the three iron-sulphur centres small amounts of a b-type cytochrome (b_{560}) are found in complex II preparations (Davis and Hatefi, 1971). This cytochrome co-purifies with polypeptides of apparent molecular weights 15.5 and 13.5 kDa (Hatefi and Galante, 1980), and is spectrally distinct from the b-type cytochromes associated with complex III (Davis *et al.*, 1973). It has a high redox potential and is not thought to have a function in electron transport from succinate to ubiquinone (Hatefi and Galante, 1980). The small polypeptides of complex II have molecular weights 13.5, 8 and 6.5 kDa (Davis and Hatefi, 1971, Capaldi, 1982) and, on the basis of amino acid analysis, are very hydrophobic (Capaldi *et al.*, 1977, Hatefi and Galante, 1980). These polypeptides can reconstitute ubiquinone reductase activity to succinate dehydrogenase enzyme.

In intact mitochondria and submitochondrial particles the flavin is accessable to oxidants and reductants only from the matrix side (Klingenberg, 1970). Similarly, antibody-binding and protein-labelling experiments using non-membrane permeating agents locate the two larger subunits (SD_1, SD_2) exclusively on the matrix side of the membrane (Merli *et al.*, 1979).

While almost all of our knowledge of complex II comes from studies using animal tissue it seems, from the limited evidence available, that the plant complex is similar, particularly since succinate dehydrogenase from several microbial and animal sources are essentially similar (Hederstedt and Rutberg, 1981). Succinate dehydrogenase has been purified from a number of plant mitochondria:

from mung bean (*Vigna radiata*) hypocotyls and soybean (*Glycine max*) cotyledons (Burke *et al.*, 1982), and from sweet potato tuber (Hattori and Asahi, 1982). In each case two major polypeptides were present of mw 67kDa and 30kDa (Burke *et al.*, 1982) and of 65kDa and 26kDa (Hattori and Asahi, 1982). Very similar EPR signals to those found in mammalian complex II corresponded to centres SI, SII, SIII (Burke *et al.*, 1982). These centres have also been detected by EPR studies of intact mitochondria (Cammack and Palmer, 1977a, Moore *et al.*, 1976). The larger subunit was found to contain the flavin (Hattori and Asahi, 1982). Cytochrome b with an alpha peak at 562nm was also present (Burke *et al.*, 1982) : the b cytochrome of the complex from beef heart has an alpha peak at 560nm (Hatefi and Galante, 1980). However, despite the overall similarity of these preparations, minor differences were found in the kinetic parameters of the preparations from the two sources. The Ki values for inhibition by oxaloacetate were 0.34µM for mung bean and 1.15µM for soybean, and the Ki values for inhibition by malonate, a classic competitive inhibitor of complex II (Dervartanian and Veeger, 1964), were 21.8µM for mung bean and 24.6µM for soybean (Burke *et al.*, 1982). The Km for succinate was 0.29mM in the preparation from sweet potato (Hattori and Asahi, 1982).

1.1.4 The cytochrome pathway of ubiquinol oxidation.

The cytochrome pathway consists of two large lipoprotein complexes, termed the bc_1 complex and cytochrome oxidase, which together oxidise endogenous ubiquinol and reduce oxygen. This process is coupled to energy conservation at sites II and III, as shown in Figure 1. Electrons are passed from the bc_1 complex to cytochrome oxidase by cytochrome c.

Cytochrome oxidase has been isolated from a variety of plant mitochondria, including sweet potato tuber (Maeshima and Asahi, 1979) and pea (Matsuoka *et al.*, 1981). The active complex only contained five polypeptides, in contrast to the 8 present in the mammalian enzyme (Capaldi, 1982), but contains similar prosthetic groups in cytochromes a and a_3 and two associated copper ions. This section will concentrate on the complex that directly oxidises ubiquinol: the bc_1 complex, since it is the site of action of most of the inhibitors used in this study.

The bc_1 complex has been isolated from mammalian mitochondria and generally contains, for each mol of complex, 2 mol cytochrome b, 1 mol cytochrome c_1 and 1 mol (when Triton is not the detergent used) of a 2Fe-2S centre known as the Rieske centre, together with a variable amount (up to 1 mol) of bound ubiquinone (Hauska *et al.*, 1983, Rich, 1984). The bound ubiquinone can difuse freely if lipid is restored to isolated complex (Heron *et al.*, 1978). The two cytochromes b differ in their spectral and redox properties: there is a low potential form (b-566, E_m -34mV) and a high potential form (b-562, E_m +93mV, pH 7.2, Nelson and Gellerfors, 1974). Both redox potentials are pH dependent at physiological values. The bc_1 complex of plant mitochondria is much less well characterised than the mammalian enzyme and the cytochrome b content of intact plant mitochondria is complicated by the presence of at least four distinct

species. At 77K, and by using antimycin to remove spectral interference from other types of cytochrome, Lambowitz and Bonner (1974a,b) resolved 3 b cytochromes, with alpha peaks (77K) at 553, 557 and 562nm. At room temperature, which is the temperature at which cytochrome peak values are assigned, these values were shifted 3nm towards the longer wavelengths, and so were named, respectively, cytochromes b-556, 560 and 565. An additional b type cytochrome was also detected at 554nm, but could only be reduced by dithionite and not by natural substrates. On the basis of the low midpoint potential (-100mV, pH 7) it is probably not directly involved in ubiquinol oxidation. The redox potentials (pH 7) of all three of the substrate reducible b cytochromes have been measured by Dutton and Storey (1971) and by Lambowitz and Bonner (1974b), and the values obtained in the two studies are quoted here in that order: Cytochrome b-556 (+75,+88mV), b-560 (+42,+79) and b-565 (-77,-76mV). The reduction of all three cytochromes by succinate was enhanced by antimycin, and the binding of antimycin causes a bathochromic peak shift of 1-2nm in the alpha peak of cytochrome b-560, suggesting that antimycin binds close to it (Passam *et al.*, 1973, Lambowitz and Bonner, 1974a). The reduction of the low potential b-565 was also promoted in energised mitochondria, and so could be selectively monitored in succinate energised mitochondria, using uncoupled, succinate reduced mitochondria as the reference. These results have led to the view (Rich and Moore, 1976) that the plant b-560 is analogous to b-562 of mammalian mitochondria, and that b-565 corresponds to b-566. Three b cytochromes could be distinguished in bc_1 complex isolated from potato mitochondria by Ducet and Diano (1978). These had alpha peaks (77K) at 553, 557 and 563nm. In addition, cytochrome c_1 was also present. The other redox carrier always present in the bc_1 complex, the Rieske centre, has also been detected in mung bean and potato

mitochondria, although it is usually not visible in plant mitochondria using EPR, unless UHDBT is present. The binding of UHDBT alters the EPR spectrum, enabling it to be seen (Prince *et al.*, 1981, Bonner and Prince, 1984). The midpoint potential in potato mitochondria was +300mV, similar to the typical values of 280-290mV reported for the mammalian centre (Hauska *et al.*, 1983), and close to the reported value for cytochrome c_1 (225-250mV, Nelson and Gellerfors, 1974).

The mechanism of the mammalian bc_1 complex has been studied extensively, with a view to explaining the observed stoichiometry of proton translocation of 4 protons per 2 electrons, and to explain the phenomenon of oxidant-induced reduction, whereby addition of oxidant to partially reduced bc_1 complex results in oxidation of the c cytochromes but, paradoxically, in reduction of the b cytochromes (Baum *et al.*, 1967). The reduction of ubiquinone on the inner face (the "p" surface) of the membrane, followed by oxidation on the outer face (the "n" surface) results in only half the required proton ejection. Two main reaction mechanisms have been proposed to account for this. These are the Q-cycle (Mitchell, 1976) and the b-cycle (Wikstrom *et al.*, 1981, Wikstrom and Krab, 1980). The suggested pathways of electron and proton transfer are shown in Figure 3. Both schemes rely upon the concerted oxidation of ubiquinol to explain oxidant induced reduction. Ubiquinol is oxidised to the ubisemiquinone by the Rieske centre, and this electron passes to cytochrome c_1 and to cytochrome c. The Rieske centre is a powerful oxidant, and generates a powerful reductant in ubisemiquinone, which reduces the low potential b cytochrome. Hence oxidant induced reduction is explained by the generation of a strong reductant (ubisemiquinone) upon addition of an oxidant (Wikstrom and Berden, 1972). The electron on the low potential b cytochrome is rapidly

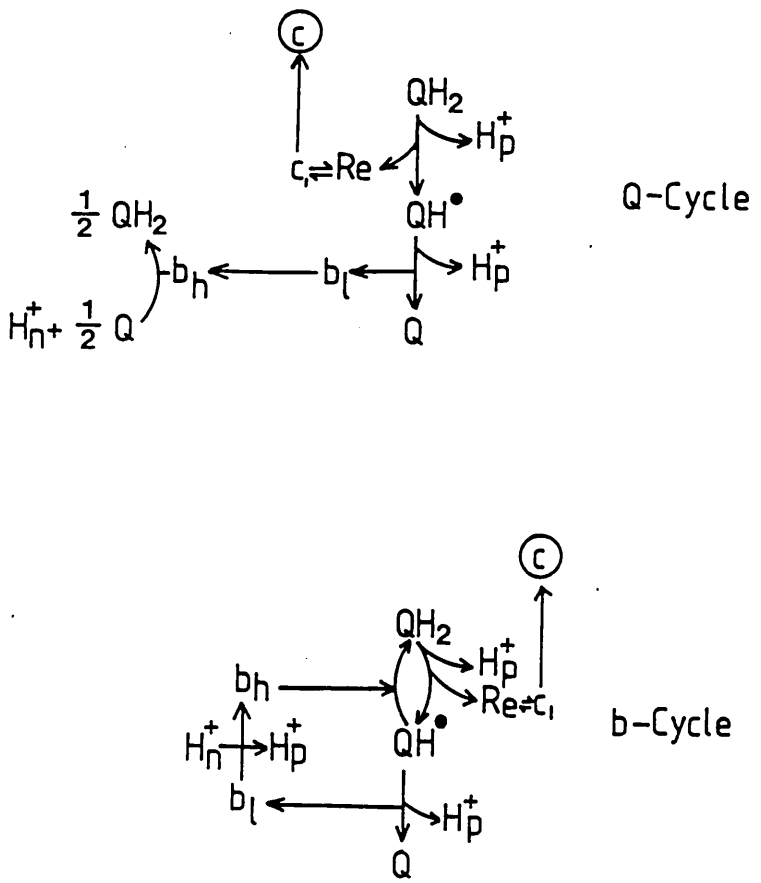
Figure 3. Comparison of the Q-cycle and b-cycle mechanisms of the bc_1 complex.

The proposed pathways of electron and proton transfer during the oxidation of ubiquinol and reduction of cytochrome c by the bc_1 complex. The schemes for the Q-cycle and the b-cycle are adapted from Rich (1984) and Wikstrom (1980) reapectively. These schemes are explained more fully in section 1.1.4.

Abbreviations:

Q	ubiquinone
$QH^{\bullet}$	ubisemiquinone
QH_2	ubiquinol
b_h	cytochrome b, high potential
b_l	cytochrome b, low potential
H^+n	proton present on the inner surface of the inner mitochondrial membrane.
H^+p	proton present on the outer surface of the inner mitochondrial membrane.
Re	Rieske iron-sulphur centre
c_1	cytochrome c_1
c	cytochrome c

Figure 3. Comparison of Q-cycle and b-cycle mechanisms of the bc_1 complex.



passed to the high potential b, but the two models differ as to the subsequent fate of this electron. According to the Q-cycle ubiquinone is reduced on the p side of the membrane, picking up the additional protons ($2H^+:2e^-$) which are then translocated when the ubiquinol is reoxidised. But in the b-cycle mechanism an extra proton is translocated by a proteinaceous pump as each electron is passed from the low potential to the high potential b cytochrome. The high potential b can then reduce ubisemiquinone, generated by the oxidation of ubiquinol by the Rieske centre. Thus the site of oxidation of ubiquinol functions in two different modes, depending on the redox state of the b cytochromes.

The models differ in that two sites of interation of ubiquinol are required by the Q-cycle, but only one according to the b-cycle. Also, the Q-cycle explains the observed $H^+:e^-$ stoichiometry of 2 by the reduction and oxidation of ubiquinone on opposite sides of the membrane, but with the b-cycle it is necessary to propose a proton pump. Two forms of bound ubisemiquinone have been detected by EPR, in accord with the prediction of the Q-cycle. These differ in that one is sensitive to antimycin (termed Qi) while the other is enhanced by oxidant induced reduction of the b cytochromes and is destroyed by BAL (called Qo) (Ohnishi and Trumpower, 1980, De Vries *et al*, 1980, 1981, 1982). The finding of independent spectral and inhibitory effects of myxothiazol and antimycin is further evidence in favour of a Q-cycle (Von Jagow and Engel, 1981, Meinhardt and Crofts, 1982, Thierbach and Riechenbach, 1981, Rich, 1983).

Both these models assume that the functional unit of the bc_1 complex is a monomer. There is some evidence that the complex may associate into heterodimers in the membrane. This arises from the detection of 2 forms of the Rieske centre, and four forms of b cytochrome (De Vries *et al.*, 1981, 1982) and led these authors to

propose a double Q-cycle model, the key point of which is that the order of reduction of the Rieske centre and cytochrome b, by the two one electron steps of oxidation of ubiquinol, is reversed in the second monomer. This enables one monomer to provide the ubisemiquinone (Qi) that acts as the oxidant for the cytochrome b of the other monomer.

Oxidant induced reduction has helped to define the site of action of inhibitors of the bc_1 complex. Hauska *et al* (1983) distinguished three classes of inhibitor. UHDBT binds at, or close to the Rieske centre, changing the EPR spectrum (see above) and drastically slowing the rate of cytochrome b reduction during oxidant induced reduction (Papa *et al.*, 1982). BAL also acts at the Rieske centre, but chemically destroys it by an undefined mechanism, rather than reversably binding and inhibiting (Slater and De Vries, 1980). A second group of inhibitors includes myxothiazol and also act upon ubiquinol oxidation, though not by interaction with the Reiske centre. Myxothiazol slows cytochrome b reduction during oxidant induced reduction, but although the presence of the Rieske centre is not essential to binding (von Jagow and Engel, 1981), binding of myxothiazol can displace UHDBT bound to the Rieske centre (Meinhardt and Crofts, 1982). While most reports of myxothiazol binding have shown spectral shifts in cytochrome b-566, some authors report a blue shift (Meinhardt and Crofts, 1982) and some a red shift (von Jagow and Engel, 1981, Gabellini and Hauska, 1983). Among the final group of inhibitors are antimycin and HQNO, which prevent the reoxidation of cytochrome b. The binding of the two inhibitors is mutually exclusive (Nijs, 1967, Eisenbach and Gutman, 1975, Van Ark and Berden, 1977), but additional evidence shows that the effects of binding differ. Both inhibitors prevent cytochrome b reoxidation and so enhance oxidant induced reduction, but the rate of cytochrome b

reduction is also slowed by HQNO, suggesting an additional effect (Eisenbach and Gutman, 1975, Izzo *et al.*, 1978, Papa et al., 1982). The slowing of cytochrome b reduction by HQNO and UHDBT together is additive (Papa *et al.*, 1982). The spectral effects of antimycin and HQNO also differ, as a red shift of 1-2nm is seen in the alpha peak of cytochrome b-562 upon binding of antimycin, but not when HQNO is bound (Brandon *et al.*, 1972, Izzo *et al.*, 1978). Izzo *et al.* (1978) concluded that antimycin and HQNO may bind at different sites.

1.1.5 Cyanide resistant respiration.

Some of the first observations of cyanide insensitive oxygen uptake by plants were made using *Sauromatum guttatum* spadices (van Herk and Badenhuizen, 1934) but it was not until the work of James and Elliot (1955) using spadices from *Arum maculatum* that this activity was shown to be associated with the mitochondria. This pathway has since become known as the cyanide-resistant or alternative pathway (Siedow, 1982), but the components specifically involved remain unidentified.

The pathway is characterised by continued oxygen uptake in the presence of most of the known inhibitors acting on components of the cytochrome pathway between ubiquinol and oxygen. These include cyanide, azide and carbon monoxide which inhibit cytochrome oxidase and prevent electrons from cytochrome c passing to oxygen. In addition most inhibitors of the bc_1 complex, such as antimycin and HQNO are without effect on the alternative pathway, indicating that the two pathways branch near ubiquinone. The precise branchpoint is dealt with more fully below. The affinity of this pathway for oxygen was initially thought to be high, leading to the idea that the oxidase may be a flavoprotein (James and Beevers, 1950). This idea has dropped from favour as subsequent measurements showed an affinity for oxygen different to that expected for a flavoprotein oxidase (see Solomos, 1977).

Because of the implications for the molecular mechanism of the pathway the end product of oxygen reduction was of considerable interest. In intact mitochondria the product is water. What was not clear was whether the pathway catalysed a one electron reduction to form the superoxide anion, a two electron reduction to form hydrogen peroxide or a four electron reduction to produce water directly. In

the case of one electron reduction, water would appear as the end product following the action of superoxide dismutase ($2\ O_2^- + 2\ H^+ \rightarrow H_2O_2$) and catalase ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$) and with two electron reduction catalase activity alone would give water as the end product. Both of these enzymes are present in mitochondrial preparations, but Rich and Bonner (1978a) and Huq and Palmer (1978b) showed that neither the cytochrome nor alternative pathway produced sufficient quantities of superoxide anions to account for the alternative pathway flux. Furthermore the solubilization and partial purification of cyanide insensitive quinol oxidase activity from *Arum maculatum* spadix mitochondria, which produced water as the first detectable product (Huq and Palmer, 1978c, and Rich, 1978) strengthened the current view that the pathway produces water directly (Siedow, 1982, Laties, 1982).

The alternative pathway is found in differing amounts in many types of plant mitochondria. A similar oxidase has also been described in a few animal species, for example pathogenic protozoa and millipedes, and also in some fungi and bacteria such as the poky strain of *Neurospora crassa* and *Candida lipolytica* (Hryniewiecka and Michejda, 1977, Edwards and Lloyd, 1977, Hill, 1977). Among plant species the activity of this pathway varies widely. Mitochondria from *Arum maculatum* spadix contain an alternative pathway ten times more active than the cytochrome pathway and can oxidise exogenous NADH at rates of up to 4000 nmol $O_2.min^{-1}$. mg $protein^{-1}$ (Lance and Chauveau, 1975). In contrast, mitochondria isolated from Jerusalem artichoke (*Helianthus tuberosus*) tubers and fresh potato (*Solanum tuberosum*) tubers are essentially completely cyanide sensitive (Coleman and Palmer, 1972, Hackett *et al.*, 1960, Dizengremel, 1975) although limited cyanide insensitivity is found in mitochondria from aged potato slices (Dizengremel and Lance, 1976). Other types

of plant mitochondria show intermediary levels of cyanide resistance.

There is almost universal agreement that the alternative pathway does not conserve energy as an electrochemical gradient and consequently cannot contribute to ADP phosphorylation. As a result energy is released as heat (thermogenesis). While in thermogenic tissues such as Aroid spadices this heat production is used to release amines that attract flies involved in pollination, the pathway is also present in mitochondria from non-thermogenic tissue and the physiological benefit of the pathway in these cases is less clear. The evidence against energy conservation by the pathway includes the lowering of P:O ratios as the activity of the pathway increases, for example with the developmental stages of *Arum maculatum* spadices Lance et al.1985 . Also,in the presence of cyanide or antimycin and with succinate as the substrate, an absence of phosphorylation was demonstrated in mitochondria from Arum spadices (Passam, 1974) and, with succinate as substrate, an electrochemical potential capable of ATP synthesis was not generated (Moore *et al.*, 1978, Moore and Bonner, 1982). Despite this evidence Wilson (1970, 1978, 1980) has consistently reported some SHAM and oligomycin sensitive ATP production in the presence of adequate concentrations of cyanide to block the cytochrome pathway.

The last redox carrier common to both the cytochrome and alternative pathways is a species of ubiquinone. Resistance to antimycin locates the branchpoint of the two oxidases before the site of cytochrome b reoxidation by ubiquinone. Similarly the observation that P:O ratios in the presence of cyanide dropped to zero for the oxidation of succinate and to near one for the oxidation of malate indicated that the branchpoint should be after the first site of energy conservation (site I). Precise localisation within the flavoprotein-ubiquinone-cytochrome-b region of the chain was more

difficult as this part is still poorly understood, but several observations suggest that ubiquinol that is free within the membrane is the branchpoint, rather than a protein bound semiquinone radical. Storey (1976) showed that ubiquinone was an obligate redox carrier in plant mitochondria. Oxygen pulse experiments showed that reoxidation of the pool of ubiquinol can occur via the alternative pathway in the presence of cyanide (Storey, 1976). Extraction of endogenous ubiquinone with pentane prevented operation of the alternative pathway (von Jagow and Bohrer, 1975, and Huq and Palmer, 1978a) and activity was restored by reincorporation with extracted lipid or pure ubiquinone.

More conclusive evidence of the location of the branchpoint at ubiquinone comes from the demonstration of molecular mobility or "pool behaviour" (see section 1.2.1) between the respiratory dehydrogenases and the alternative oxidase (Cottingham and Moore, 1983) and the ability of partially purified alternative oxidase preparations to oxidise directly the artificial quinols durohydroquinone and menadiol (Huq and Palmer, 1978c, Rich 1978, Huq, 1978, Kay and Palmer, 1985). Moore and Rich (1985) have argued, from the premise that exogenous NADH and succinate reduce ubiquinone on opposite faces of the inner membrane, that since the oxidation of neither substrate via the alternative pathway is electrogenic it follows that the redox donor to the oxidase must carry the electrons and the protons for the reduction of oxygen. If only electrons were donated, and protons were derived specifically from either bulk aqueous phase, then the oxidation of one of the substrates must be electrogenic. From the above it follows that the most likely hydrogen-atom donor to the oxidase is ubiquinol.

There have been several reports of selective solubilization of the oxidase from *Arum maculatum* spadix (Huq and Palmer, 1978c,1980,

1.2 Ubiquinone and respiration.

1.2.1 The organisation of respiratory components.

All naturally occuring ubiquinones are substituted 1,4-benzoquinones and are extremely hydrophobic due to their side chain of six to eleven isoprenoid units (Figure 2, Crane, 1965, Schindler *et al.*, 1984). The redox potential of the 1,4-benzoquinone group is approximately 100mV lower than that of benzoquinone (Morton, 1965) because of the electron donating methoxy and methyl groups substituted onto the ring (Fieser and Fieser, 1961). The potentials for the overall and half reactions, determined in ethanolic solution, are +70mV $E_m(QH_2/Q)$, +380mV $E_m(QH_2/Q^{\cdot -})$ and -240mV $E_m(Q^{\cdot -}/Q)$ (Hauska *et al.*, 1983). A value of +65mV (QH_2/Q) has been reported for the quinone pool of mammalian mitochondria (Urban and Klingenberg, 1969) and of +70mV for the ubiquinone pool of mung bean mitochondria (Storey, 1970). The values for the two half reactions are very different, reflecting the instability of the semiquinone form in solution, but the semiquinone form can be stabilized by factors which increase delocalisation of the unpaired electron such as the conformation of the methoxy and isoprenoid side chains or by interaction with ligands at quinone binding sites (Trumpower, 1981) and also just by binding, as this reduces the rate of semiquinone-semiquinone dismutation. Substitution of the benzoquinone ring at all four positions also prevents reaction with sulphydryl groups in the membrane to form 1-4 thiol ether adducts (Redfearn and Whittaker, 1962).

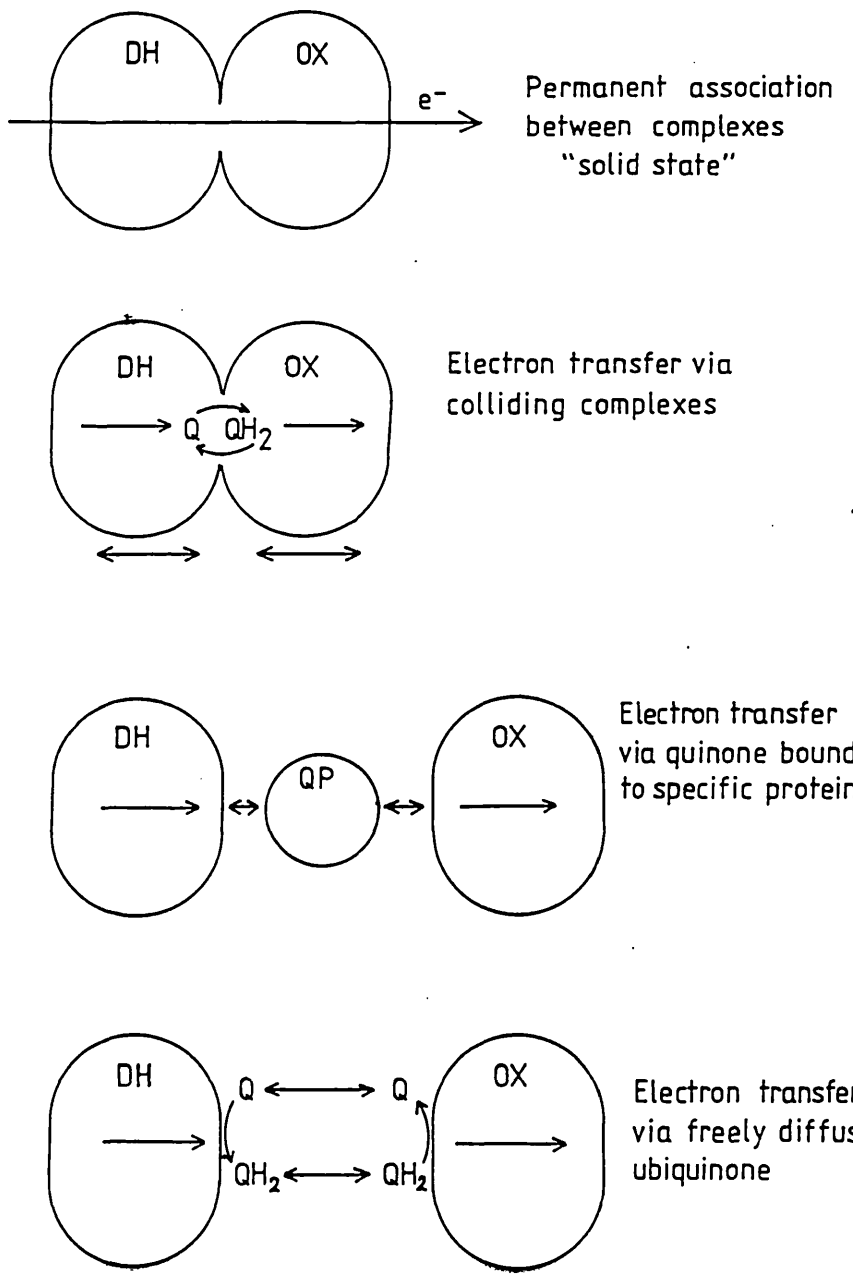
Unsaturation gives the isoprenoid side chain periodic rigidity, somewhat restricting the flexibility of the molecule. Recent studies have indicated that the isoprenoid side chain of ubiquinone is located towards the centre of the membrane and perpendicular to the membrane lipid side-chains. The properties of the methoxy protons of ubiquinone have been studied by NMR (Kingsley and Feigenson, 1981)

and it was concluded that the longer the isoprenoid side chain, the deeper within the membrane the quinone head group resided. The quinol head group lay nearer the aqueous interface, on average, than the quinone form, but both only seldom visited the aqueous phase. In addition, liposomes and monolayers containing ubiquinone have been studied using calorimetric and surface pressure methods (Quinn, 1980, Quinn and Esfahani, 1980, Katsikas and Quinn, 1982), and the results were also consistent with the location of ubiquinone at the centre of the membrane.

As discussed in section 1 ubiquinone mediates electron transfer between the respiratory dehydrogenases and either the bc_1 complex or the alternative oxidase. There are several ways in which this could occur which are shown in Fig.4. One extreme situation would occur if dehydrogenase-bc_1 complex units were permanently associated within the membrane. Tight 1:1 binding of both complex I (Hatefi *et al.*, 1962a, Fowler and Richardson, 1963) and complex II (Yu and Yu, 1980) to the bc_1 complex is found after detergent fractionation, but there is much evidence that in intact mitochondria the complexes are separate such that there is molecular mobility between them. This evidence includes the lack of stoichiometry between dehydrogenases and the bc_1 complex but the ability of NADH to rapidly reduce all the cytochrome c_1 (Kroger and Klingenberg, 1967); the sigmoidal inhibition of oxygen consumption by tightly bound inhibitors such as antimycin (Kroger and Klingenberg, 1973 a,b, Siggel *el al.*, 1972, Cottingham and Moore, 1983) and the combined effects of two loosely bound inhibitors (Moreira *et al.*, 1980). While these observations provide strong evidence of mobility, there are conflicting views as to the nature of the diffusing species. Ragan and co-workers (Ragan and Heron, 1978, Heron *et al.*, 1978) reconstituted NADH-cytochrome c activity from isolated complex I and

Figure 4. Possible mechanisms of electron transfer at the level of ubiquinone.

Four possible mechanisms for the transfer of electrons from dehydrogenase (DH) to ubiquinol oxidising complexes (OX) are depicted. Single-headed arrows represent electron transfer, and double-headed arrows indicate lateral movement of components within the membrane. The role of ubiquinone in electron transfer is discussed in section 1.2.1.



bc_1 complex. By varying the proportion of each complex they were able to test whether the rate of cytochrome c reduction could be predicted by pool functions (see below). In the absence of added phospholipid the different complexes combined in a fixed 1:1 association, did not show pool behaviour and the reduction of cytochrome b was biphasic, corresponding to a rapid reduction of the bc_1 complexes bound to complex I and a slow reduction of unbound complex. However when additional phospholipid was present, pool behaviour was restored and cytochrome b reduction became monotonic (Heron *et al.*, 1978). It was proposed that in the intact membrane there was sufficient phospholipid to allow pool function, but that electron transfer was not mediated by free ubiquinone, but instead by complex I-bc_1 complex units. These dimers would have to associate and dissociate more rapidly than the overall rate of electron transfer in order to give pool kinetics, and, in order to explain the observed kinetics of the ubiquinone-pool (see below), the complexes must be capable of equilibrating with the bulk ubiquinone.

Although not conclusive, the available evidence suggests that it is via free or "pool" ubiquinone that electrons are passed between complexes, rather than through associations of the complexes themselves. By fusing artificial liposomes with the mitochondrial inner membrane Schneider *et al.* (1980) were able to dilute the respiratory components in two dimensions. Increases in average distances between complexes were confirmed by a decrease in particle density in freeze-fractured membranes. The extent of dilution correlated with the decrease in succinate and NADH oxidation by cytochrome c, whereas duroquinol oxidation by oxygen was almost unaffected. This showed that diffusion of components was necessary between the dehydrogenases and bc_1 complexes. Further experiments (Schneider *et al.*, 1982) showed that if ubiquinone was

incorporated with the phospholipid, the inhibition of cytochrome c reduction rate was much less, suggesting that the diffusing component was ubiquinone. When ubiquinone was reincorporated into submitochondrial particles depleted of their endogenous ubiquinone, the rate of electron transfer was found to increase with increasing amounts of incorporated ubiquinone, even at levels above those originally present (Ernster *et al.*, 1978, Heron *et al.* 1978). Further evidence for the involvement and kinetic competence of free ubiquinone has come from the studies of the redox state of the quinone pool in the steady state and pre-steady state (Kroger and Klingenberg, 1973 a,b. see below).

More recently the technique of FRAP (fluorescence recovery after photobleaching) has been used to measure the lateral mobility of the respiratory complexes, cytochrome c and the fluorescent ubiquinone-analogue Q_0C_{10}NBDHA (Figure 2) in the inner mitochondrial membrane of rat liver megamitochondria (Gupte *et al.*, 1984). The complexes and cytochrome c were made fluorescent by binding to specific antibodies conjugated to fluorophores. Complexes I-IV and the ubiquinone analogue all behaved as homogenous mobile pools and cytochrome c as if it were in equilibrium between the membrane surface and the aqueous phase. The diffusion coefficient for the ubiquinone analogue ($D= 3.7 \times 10^{-9}$ $cm^2.sec^{-1}$) is towards the slow end of reported values, but is close to the value ($D= 1 \times 10^{-9}$ $cm^2.sec^{-1}$) proposed by Crofts and Wraight (1983). The diffusion coefficients of the complexes were all close to 4×10^{-10} $cm^2.sec^{-1}$. Gupte *et al.* (1984) were able to calculate the theoretical rates of collision of the various redox partners and so to compare this with the actual rate of electron transfer. It was calculated that the least efficient reactive pair was ubiquinone-complex III which collided 45 times per turnover: with

ubiquinone-complex I the figure was 19 and with ubiquinone-complex II it was 8. Since each pair of redox components could collide several times within their turnover times it was concluded that diffusion-coupling of freely diffusing components could account for observed rates of electron transfer.

1.2.2 Ideal ubiquinone pool behaviour.

The question of the kinetic competence of ubiquinone as an obligatory respiratory component was settled by the extraction/reincorporation work of Ernster *et al.* (1969) and by the work of Kroger and Klingenberg (1967,1970), who took the pool-size, as well as the half-time for oxidation, into account in calculating electron flux through ubiquinone. The idea of Green (1962) of ubiquinone as a mobile redox carrier diffusing in the membrane was first quantitatively tested by Kroger and Klingenberg (1973 a,b). In the first of these studies Kroger and Klingenberg (1973a) correlated steady state rates of succinate and NADH oxidation by beef heart submitochondrial particles with the degree of reduction of ubiquinone, measured after extraction of total ubiquinone into a mixture of methanol and light petroleum. Rates of oxygen consumption were varied by the specific inhibitors malonate, rotenone and antimycin. It was found that, even in the presence of substrate and 1mM KCN, 10-20% of the total ubiquinone remained oxidised and this proportion was therefore considered inactive and ignored in their calculations. Taking just the active ubiquinone (Qa) a linear relation was found between the rate of oxygen uptake and the proportion of Qa in the reduced form (ubiquinol). This pseudo-first order relationship held for titration of either the input into, or output from, ubiquinone. Consequently these two part processes could be represented by pseudo-first order rate constants with respect to the proportion of Qa present in the relevent form (quinone or

quinol): the rate constant for the reduction of ubiquinone was V_{red}/Qa, and for oxidation it was Vox/Qa.

In the steady-state the rates of electron transfer into and out of the ubiquinone-pool are equal, both to each other and to the rate of oxygen uptake.

Therefore:

$$dQox/dt = (Vox.Qred/Qa) - (Vred.Qox/Qa) = 0 \qquad (1)$$

Where: $Qa = Qred + Qox$

Ubiquinone terms can be eliminated from equation (1) to give the overall steady-state rate equation:

$$\text{Rate of oxygen uptake} = v = (Vox.Vred)/(Vox + Vred) \qquad (2)$$

In addition Kroger and Klingenberg analysed the transition from the reduced anaerobic state to the aerobic steady-state both spectrophotometrically and by rapid quenching and extraction of ubiquinone. A good correlation was found between the form of the predicted curve (derived from equation 1) and the values observed.

The first order nature of the reactions of ubiquinone surprised Kroger and Klingenberg. Assuming that a mobile pool of molecules could be expected to saturate the donating and accepting components, the authors expected mixed zero- and first-order reactions. The assumption that ubiquinol in the membrane saturated the quinol oxidising sites was used by the authors to justify the use of saturating levels of DQH_2 to measure Vox. As a possible explanation of the lack of visible saturation the following scheme was suggested, in which component B (possibly cytochrome b) was in rapid equilibrium with the pool of ubiquinone. The subsequent transfer of electrons to component C (possibly the c cytochromes) would be a significantly slower process, and so the first order kinetics of oxidation would reflect the rate at which electrons were passed from B to C, rather than the rate at which collisions occurred

between ubiquinol the bc_1 complex:

Substrate $\longrightarrow$ D $\rightarrow$ Q $\rightleftharpoons$ B $\rightarrow$ C $\longrightarrow$ O_2

This formulation is somewhat similar to the Michaelis-Menton equation in that a rapid equilibrium is set up between a large pool of free ubiquinol molecules (of extended pool size since ubiquinone is constantly re-reduced by the dehydrogenases) and an enzyme intermediate that decays to form the product: in this case an irreversable donation of electrons to molecular oxygen. As such, a near-linear relation between ubiquinol concentration and rate is only expected when ubiquinol concentration is far below its Km, when the apparent first-order rate constant tends towards Vmax/Km. Consequently, if this scheme is correct, it cannot be the case, as asserted by the authors, that ubiquinol saturates acceptor sites, and so the similarity between the values of Vox and the rate at which 1mM duroquinol was oxidised may be coincidental. The failure of the pool ubiquinone to saturate the bc_1 complex is in agreement with observations that increasing the pool above the natural level also increases rates of substrate oxidation (Ernster *et al.*, 1978).

In the second study Kroger and Klingenberg (1973b) further tested these pool-equations using antimycin to inhibit quinol oxidation. It was necessary to show that binding and inhibition by antimycin were stoichiometric in order to be able to calculate inhibition of Vox, particularly since co-operative binding of antimycin, dependent on the redox state of the b cytochromes, had been described (Berden and Slater, 1972). Kroger and Klingenberg were able to show that artificial quinols were oxidised either (a) directly by the bc_1 complex, resulting in linear inhibition by antimycin, or (b) by the bc_1 complex but, in addition, via the ubiquinone-pool, giving hyperbolic inhibition by antimycin. The route of oxidation correlated with the reducing power of the quinol: duroquinol, for which E'_o=

35-60mV, and dimethoxy-dimethylbenzohydroquinone (Eo'= 110mV), are both oxidised via pathway (a) while the more reducing menadiol (E'_o= -1mV) used pathway (b). The involvement of ubiquinone in menadiol oxidation was confirmed by extraction of ubiquinone, when the inhibition profile by antimycin became linear. In addition the steady-state reduction of ubiquinone and the kinetics of ubiquinone oxidation during the transition from anaerobic to aerobic steady-state were also consistent with a stoichiometric inhibition of Vox. It was concluded that the sigmoidal inhibition of NADH and succinate oxidation by antimycin could be quantitatively accounted for by the simple pool kinetics of ubiquinone, together with stoichiometric inhibition of Vox. The deviation at near saturating levels of antimycin was explained by a small, but finite, dissociation of bound antimycin.

Unfortunately not everybody agrees that the binding and effects of antimycin are this straightforward. This is an important point since antimycin cannot be used to test ubiquinone-pool kinetics quantitatively unless the relationship between amount added and inhibitory effect is known. Bryla *et al.* (1969) reported that the effect curve of antimycin, in causing a red shift in the alpha peak of the higher potential cytochrome b (b_{562}), was sigmoidal, even though no turnover of ubiquinone or any other redox component was occurring. In a subsequent study Berden and Slater (1972) used the fluorescence of antimycin to determine that the binding constants were different according to the redox state of cytochrome b. They concluded that co-operative binding of antimycin was needed, in addition to pool kinetics, to explain their results. In their co-operative model two states were postulated: the R state which predominates in oxidised particles, to which antimycin binds more strongly (dissociation constant = 3.2×10^{-11} M) and the T state

which is the conformation of the majority of binding sites in reduced particles (dissociation constant = 3.6 x 10^{-10} M). Binding of antimycin would displace the equilibrium towards the R state, increasing the apparent binding constant. Co-operative binding requires that a number of binding sites interact and in this case the number was estimated to be 7-8. Although a dimer of bc_1 complexes has been postulated as the functional form in the membrane (Weiss and Kolb, 1979, Wikstrom *et al.*, 1981), interaction of 8 binding sites is rather difficult to imagine, given the observed stoichiometry of one binding site per two b cytochromes (Berden and Slater, 1970). Zhu *et al.* (1982) compared HQNO and antimycin titrations as probes of ubiquinone-pool behaviour and found that while HQNO indicated pool kinetics, antimycin did not. They concluded that antimycin should not be used to quantify pool-kinetics. However antimycin was used by De Troostembergh and Nyns (1978) in their investigation of yeast respiration and the distribution of electrons between the cytochrome and alternative pathways (section 1.3.2), to demonstrate that this system closely followed ideal ubiquinone pool kinetics. It was also used to demonstrate ubiquinone pool behaviour in plant mitochondria (Cottingham and Moore, 1983).

1.2.3 The reduction of ubiquinone.

It is clear that the isolated respiratory dehydrogenases are capable of the complete reduction of ubiquinone in the absence of complex III, in contrast to early formulations of the Q-cycle, in which dehydrogenase and complex III cooperate in the reduction of ubiquinone. Two sorts of mechanism for the reduction of ubiquinone can be envisaged: one in which two bound and stabilized semiquinone radicals, generated by one-electron reduction of ubiquinone, subsequently dimutate to produce one quinol and one quinone; another

mechanism may involve two sequential one-electron transfers to a single ubiquinone to produce ubiquinol. In both cases a stabilized semiquinone radical intermediate is expected as it is generally accepted that semiquinone radicals are only likely to be detected if stabilized by binding to specific proteins. Ubisemiquinone was first demonstrated in respiring mitochondria by Backstrom *et al.* (1970).

In this section some of the characteristics of ubiquinone reduction by succinate and NADH dehydrogenases are discussed. Unfortunately most studies have used animal material and so any extrapolation to plant mitochondrial dehydrogenases must allow for the differences between the two types of mitochondria (see sections 1.1.2 and 1.1.3).

King *et al.* (1978) looked for a ubisemiquinone radical generated by isolated mammalian complex I and found an EPR signal at room temperature (g=2.00) that disappeared when the ubiquinone that had co-purified with the complex was removed. Since complex I contains flavin there was a danger of confusion with a flavin semiquinone radical. This radical was therefore generated by photoreduction of the flavin and found to be distinguishable from the suggested ubisemiquinone signal on the basis of linewidth. The view that the signal was ubisemiquinone was strengthened by the finding that it was abolished by rotenone, which acts on the oxygen side of both the flavin and iron-sulphur centres. Rotenone and piericidin A act at the same site (for review see Singer and Gutman, 1971) and piericidin A clearly resembles ubiquinone (Figure 2). The similarity of rotenone to ubiquinone is less obvious but it may resemble the ubichromenol form (Trumpower, 1981 and Figure 2). The reduction of ferricyanide or juglone (5-hydroxy-1,4-napthoquinone) is insensitive to rotenone and piericidin A and so probably occurs at a site prior to the site of reduction of endogenous ubiquinone (Ruzicka and Crane,

1970).

The binding of piericidin A to mammalian complex I is very tight (K_d = (0.3-1) x 10^{-9} M) (Gutman *et al.*, 1971 , Gutman and Kliatchko, 1976) and no exchange of bound radiolabelled inhibitor with unlabelled inhibitor was detected after 30min at 30°C (Gutman and Kliatchko, 1976). Two moles of piericidin A are bound per mole of flavin,with different affinities, resulting in a sigmoidal inhibition curve (Gutman *et al.*, 1970). One site can be blocked by mercurial agents, halving the inhibition titre and leaving a hyperbolic inhibition curve (Gutman *et al.*, 1971), suggesting two independent sites of ubiquinone reduction. Phospholipid is generally thought to be necessary for rotenone sensitive ubiquinone reduction although there are reports of phospholipid-free preparations that do have this activity, but with altered kinetics and less stability (Baugh and King, 1972). Ragan (1978) studied the role of phospholipid in complex I activity and found that at low phospholipid content UQ-1 reduction was rotenone insensitive,and this activity was attributed to "soluble" sites of reduction. At high phospholipid content UQ-1 reduction was rotenone sensitive and UQ-1 was thought to enter the phospholipid and be reduced in a similar way to endogenous UQ-10. The rotenone sensitive pathway had two components: a low Km (0.044μM) low velocity site and a high Km (0.94μM) high velocity site, further supporting the view from inhibitor binding studies that two different sites of rotenone sensitive ubiquinone interaction exist on the complex.

Ubisemiquinone species probably associated with the reduction of ubiquinone by NADH have also been detected in plant mitochondria. Rich and Bonner (1977) reported an unsplit EPR signal (g=2.004) in mung bean mitochondria, observable above 30K (so that the HiPIP signals did not interfere) which the authors attributed to

ubisemiquinone rather than flavosemiquinone on the basis of the high redox potentials determined (+65mV and +180mV). According to the authors the signal displayed heterogenous properties suggesting that other species of ubisemiquinone may also be present. A very similar signal was also observed in both wild type and poky *N.crassa* mitochondria (Rich and Bonner, 1978b) in these cases with apparent midpoint potentials of +75mV and +140mV. The potentials were apparent because the signal appeared and then disappeared with increasing ambient potential without the signal fully developing. As relatively high rates of superoxide anion production have been reported when exogenous NADH is oxidised (Huq and Palmer, 1978a, Rich and Bonner, 1978a, Huq, 1978) it is possible that a ubisemiquinone is involved in this pathway that is accessable to oxidation by molecular oxygen.

In contrast to NADH oxidation the ubisemiquinone generated by succinate oxidation is better characterised in both plant and animal systems. Interest in the ubisemiquinone generated by complex II of plant mitochondria was stimulated by the suggestion that it may be closely involved in the operation of the alternative oxidase (Moore *et al.*, 1976, Rich and Moore, 1976).

Ubisemiquinone associated with centre S3 has been detected in mammalian mitochondria. Ruzicka *et al.* (1975) observed an EPR signal (g=1.99 and 2.04) at temperatures below 20K which they attributed to a ubisemiquinone interacting with a second paramagnetic component. The interaction was of a magnetic dipole-dipole type and on this basis the distance between the components was calculated as 7.7Å. The second component was identified by Ohnishi *et al.* (1977) and Salerno *et al.* (1977) also as ubisemiquinone and it was shown that the pair of radicals were oriented perpendicular to the plane of the membrane (Salerno at *al.*, 1977). The ubisemiquinone signal is dependent of the presence of centre S3 and was destroyed by

extraction of ubiquinone (Ruzicka *et al.*, 1975) and as centre S3 and the ubisemiquinone show identical power saturation characteristics centre S3 may relax the ubisemiquinone at low temperatures (Ingledew *et al.*, 1976). The ubisemiquinone needs to be stabilized in order to enable reduction by the succinate/fumarate couple and values of 110mV ($Q/Q^{\cdot-}$) and 50mV ($Q^{\cdot-}/QH_2$) have been determined at 77K (Ohnishi *et al.*, 1977). This large stabilization is due to binding of ubiquinone to complex II and Yu *et al.* (1977) have isolated a polypeptide tentatively identified as the ubiquinone-binding protein and which is identical to one of the low molecular weight proteins (mw 13.5 kDa) of complex II (see section 1.1.3) that can reconstitute ubiquinone reductase activity to succinate dehydrogenase.

Ubiquinone reductase activity is inhibited by TTFA and carboxin which are competitive inhibitors with respect to ubiquinone analogues (Mowery *et al.*, 1977) and which inhibit the reoxidation but not the reduction of centre S3 (Ackrell *et al.*, 1977b, Singer *et al.*, 1975). The ubisemiquinone-ubisemiquinone EPR signal is abolished, and the signal from centre S3 increased, by TTFA over the same concentration range that inhibits succinate oxidation (Ingledew *et al.*, 1977).

Rapidly relaxing EPR signals (g=2.0), split by a much more slowly relaxing ubisemiquinone, have also been observed in plant mitochondria and seem to differ in their properties from those of the mammalian system. Cammack and Palmer (1977a) observed this signal at 12K (g=2.038, 1.984) in *Arum maculatum* submitochondrial particles. Rich and Bonner (1978c) found the signal to be present in a variety of cyanide sensitive and insensitive mitochondria, but the signal was extremely labile and so was not observed in damaged mitochondria or in submitochondrial particles. In some cases the signal was more

complex than that found in mammalian mitochondria as additional features were present in the spectrum on both the high and low field sides. The additional complexity of the plant mitochondrial signal had been noted by Moore *et al.* (1976) who found that additional features (g= 2.046, 2.033, 1.986, 1.969 and 1.960) were, on the basis of similar temperature dependency and power saturation, associated with the S3 signal. These extra features disappeared in the presence of 235μM SHAM, suggesting a link with the alternative pathway. Subsequently it was shown that hydroxamates perturbed the interaction of ubisemiquinone with centre S3, but at higher concentrations than are necessary for inhibition of the alternative pathway, and without directly affecting centre S3 (Rich and Bonner 1978b). A direct role of centre S3 in cyanide resistant respiration was discounted after it was shown that succinate dehydrogenase could be detached from *Sauromatum guttatum* spadix mitochondria leaving cyanide insensitive oxidation of NADH substantially intact (Rich and Bonner, 1978c).

1.3 Ubiquinone and the branched respiratory chain.

1.3.1 Deviations from ideal pool behaviour in mammalian mitochondria.

The pool equation for oxidation of a single respiratory substrate (section 1.2.2) can be simply extended to cover simultaneous oxidation of two substrates:

$$\text{total rate of oxidation} = v = \frac{V_{ox}.(V_s + V_N)}{(V_{ox} + V_s + V_N)}$$

where V_s and V_n are, for example, the rate constants for ubiquinone reduction by succinate and NADH dehydrogenase respectively and V_{ox} is the rate constant for ubiquinol oxidation. As the equations are symmetrical with respect to inputs to, and outputs from, the ubiquinone pool, they are equally applicable to the situation where one substrate can be oxidised by two possible pathways of ubiquinol oxidation (see section 1.3.2).

Beef heart submitochondrial particles have been used to study the interaction of succinate and NADH oxidase activities (Gutman and Silman, 1972, Gutman 1977). Oxidation rates in the presence of both substrates were found to be significantly different from those predicted by the above equation and the rates of oxidation of each substrate alone.

The total rate of electron transport observed was less than the sum of the rates alone: this is one prediction of the model that is borne out experimentally. But when the contribution of each substrate to the total flux was measured it was found that the rate of oxidation of one substrate was little affected by the oxidation of the other, while the rate of oxidation of the second substrate was inhibited. The substrate preferentially oxidised was the one with the more active dehydrogenase and so the inhibition could be shifted from one substrate to the other by inhibition of the dehydrogenases. If

ubiquinone was removed by pentane extraction such that only low amounts remained, the interaction between substrates was decreased (Gutman and Silman, 1972) and the total rate of oxidation was in excess of that predicted by the above equation. To explain the lack of interaction it was proposed that each dehydrogenase was surrounded by a separate pool of ubiquinone and that equilibration between the separate pools by diffusion of ubiquinone was relatively slow compared to rates of electron transfer. Because of the slow communication, the different pools would not be at redox equilibrium and so, when ubiquinone content was low, the dehydrogenases would behave independently rather than respond to a homogenous pool of ubiquinone. The selective inhibition of one substrate at normal ubiquinone levels, it was suggested, was due to the pools of ubiquinone around the more active dehydrogenase complexes encroaching into the pools surrounding the less active dehydrogenases. The more active dehydrogenases were seen as poaching ubiquinone from the less active dehydrogenase (by reducing it), leading to an inhibition of the less active dehydrogenase greater than otherwise expected.

This explanation relies upon the suggested inability of ubiquinone to diffuse sufficiently quickly such that the redox potential of the total unbound ubiquinone is homogenous under steady-state conditions. The distance a ubiquinol molecule is able to diffuse, and so the number of bc_1 complexes with which it can potentially interact, has been estimated (Rich, 1984) and this is discussed in chapter 7. Until the actual mobility of ubiquinone in the membrane is known more precisely (section 1.2.1) it is difficult to assess whether diffusional limitation occurs, and other explanations of deviation from ideal behaviour have been proposed, such as the patching of respiratory components (Rich, 1984), or more recently modification to the ubiquinone pool model to include rate

limitation at steps other than just the binding of ubiquinone and ubiquinol (Ragan and Cottingham, 1985).

1.3.2 Branching between the cytochrome and alternative pathways.

There are at least three classes of observations of plant mitochondria which suggest that the distribution of electrons into and out of the ubiquinone pool may not occur by straightforward competition among the different donors and acceptors for ubiquinone or ubiquinol. These classes all involve branching of the respiratory chain and relate to (1) the mechanism by which electrons distribute between the alternative and cytochrome pathways, (2) the degree to which different substrates have access to the alternative pathway, and (3) the unequal inhibitions of oxidation rate when two substrates are oxidised together, compared to their rates of oxidation separately.

The regulation of the alternative pathway is of interest since energy is apparently wasted if substrates are oxidised by this route, and the idea that complex III and the alternative oxidase simply compete for electrons (De Troostembergh and Nyns, 1978) is uncomfortable because a short circuit to energy conservation would then always be present. The studies of Bahr and Bonner (1973a,b) and De Troostembergh and Nyns (1978) on the factors determining the flux through the two terminal oxidases have given rise to two fundamentally opposing models of how this is regulated. This problem is made difficult by the lack of specific redox acceptors for the two pathways in intact mitochondria: the contribution of each pathway to the observed rate of oxygen uptake must be determined indirectly. Bahr and Bonner (1973a) used bean hypocotyl and skunk cabbage (*Symplocarpus foetidus*) mitochondria together with the inhibitors cyanide and iodobenzhydroxamate to determine the two oxidase fluxes. Firstly they established that benzhydroxamic acid was

specific for the alternative pathway and then titrated succinate oxidation with benzhydroxamate in state 3, state 4 and in the presence of cyanide. It was argued that the titration in the presence of cyanide gave a function, named g(i), of the maximum possible alternative pathway flux at a given concentration of benzhydroxamate. The authors then made two postulates: that in the absence of cyanide a fraction of g(i) would operate, denoted by p, and having a value between zero and one; and secondly that the cytochrome pathway rate was unaffected by the alternate pathway rate. This second postulate is the fundamental difference between the two proposed models of electron allocation and so the evidence supporting it will be examined below. Given these two premises the total rate of oxygen consumption (V_T) could be expressed by a simple relationship:

$$V_T = p.g(i) + V_{cyt}$$

where V_{cyt} is the flux through the cytochrome pathway and should not be confused with the same symbol used in ubiquinone-pool terminology to denote the rate constant of the cytochrome pathway with respect to ubiquinol. If V_{cyt} really was independent of the alternative pathway flux then a plot of V_T against g(i) would be linear with gradient p. V_{cyt} was determined in the presence of sufficient benzhydroxamate to block the alternative pathway. A linear relationship was found in both state 3 and state 4 and so the assumption of cytochrome pathway independence was apparently corroborated since if V_{cyt} changed with increasing benzhydroxamate concentration "a straight line would not necessarily be obtained". Further support was claimed from the correlation between the proportion of flux along the cytochrome pathway and the ADP:O ratio. It was concluded that the degree of engagement of the alternative pathway (p) was determined soley by the degree of saturation of the cytochrome pathway, being maximal when the cytochrome pathway could

operate no faster. In the second paper (Bahr and Bonner, 1973b) possible mechanisms for this form of regulation were discussed. The one suggested to best fit the data involved equilibration of the ubiquinone pool with the first component of the alternative pathway, possibly a flavin. This component would have a midpoint potential more negative than that of ubiquinone such that only when the ubiquinone-pool was very reduced would the donor to the alternative pathway be reduced and the alternative pathway engaged. The alternative pathway could therefore cope with the oxidation of substrates when the cytochrome pathway was overloaded: as a short-circuit for electrons surplus to requirements.

Does the linearity of the plot of V_T against g(i) provide evidence for the independence of V_{cyt} from the alternative pathway flux? If electrons were diverted to the cytochrome pathway by inhibition of the alternative flux, then the effect of high concentrations of benzhydroxamate on V_T would be less than that predicted by the Bahr-Bonner equation, and the plot would curve upwards at low values of g(i). However, if the effect of a given amount of benzhydroxamate on the alternative flux was greater in the presence of an active cytochrome pathway than in the presence of cyanide then the increase in V_{cyt} could be offset by an additional decrease in the alternative pathway rate. Such an effect is predicted by the ubiquinone pool model. Obviously the increase in the cytochrome pathway flux could not be equal to the inhibition of the alternative pathway, or no decrease in oxygen consumption would occur, but it is shown below (a) that the effect of hydroxamate on the alternative pathway will, according to the ubiquinone pool model, be greater in the presence of the cytochrome pathway than in the presence of cyanide, and (b) that the property described in (a) and the increase in the cytochrome pathway flux upon addition of

benzhydroxamate partially cancel in such a way that a near linear Bahr-Bonner plot results.

According to the ubiquinone pool model the effect of a given concentration of benzhydroxamate on the alternate pathway flux is the product of both the change in alternative oxidase concentration and the change in the degree of reduction of the ubiquinone pool. It can be shown that the redox state of the ubiquinone pool is given by the following expressions:

In the presence of cyanide: $QH_2/Qa = V_{red} / (V_{red} + V_{alt})$

In state 3 or 4: $QH_2/Qa = V_{red} / (V_{red} + V_{alt} + V_{cyt})$

If y is the fraction of alternative oxidase still active after the addition of a given concentration of inhibitor, then the change in the degree of reduction of the ubiquinone pool in the presence and absence of the cytochrome pathway can be found:

$$\frac{QH_2(+\text{cyanide})}{Qa} = \frac{V_{red}}{V_{red} + y.V_{alt}} - \frac{V_{red}}{V_{red} + V_{alt}}$$

$$\frac{QH_2(\text{state } 3,4)}{Qa} = \frac{V_{red}}{V_{red} + V_{cyt} + y.V_{alt}} - \frac{V_{red}}{V_{red} + V_{cyt} + V_{alt}}$$

It follows from this that if all constants are positive then the change in the degree of ubiquinone pool reduction is greater in the absence of the cytochrome pathway. Increasing reduction of the ubiquinone pool will increase the flux through each of the remaining alternative oxidase acceptors.

The changes in the rates of oxidation upon addition of benzhydroxamate are given by:

Change in alternative path flux (+cyanide):

$$= \frac{y.V_{red}.V_{alt}}{y.V_{alt} + V_{red}} - \frac{V_{red}.V_{alt}}{V_{alt} + V_{red}} \qquad \text{(negative)}$$

Change in alternative path flux (state 3,4):

$$= \frac{y.V_{red}.V_{alt}}{y.V_{alt} + V_{red} + V_{cyt}} - \frac{V_{red}.V_{alt}}{V_{alt} + V_{red} + V_{cyt}} \qquad \text{(negative)}$$

Change in cytochrome path flux (state 3,4):

$$= \frac{V_{red}.V_{cyt}}{y.V_{alt} + V_{red} + V_{cyt}} - \frac{V_{red}.V_{cyt}}{V_{alt} + V_{red} + V_{cyt}} \qquad \text{(positive)}$$

Again if all constants are positive then (1) the inhibition of alternative pathway flux is greater in state 3 or 4 that in the presence of cyanide and (2) this increased inhibition of the alternative pathway flux will to some extent offset the increase in the cytochrome pathway rate, giving the appearance that only the alternative pathway rate has changed.

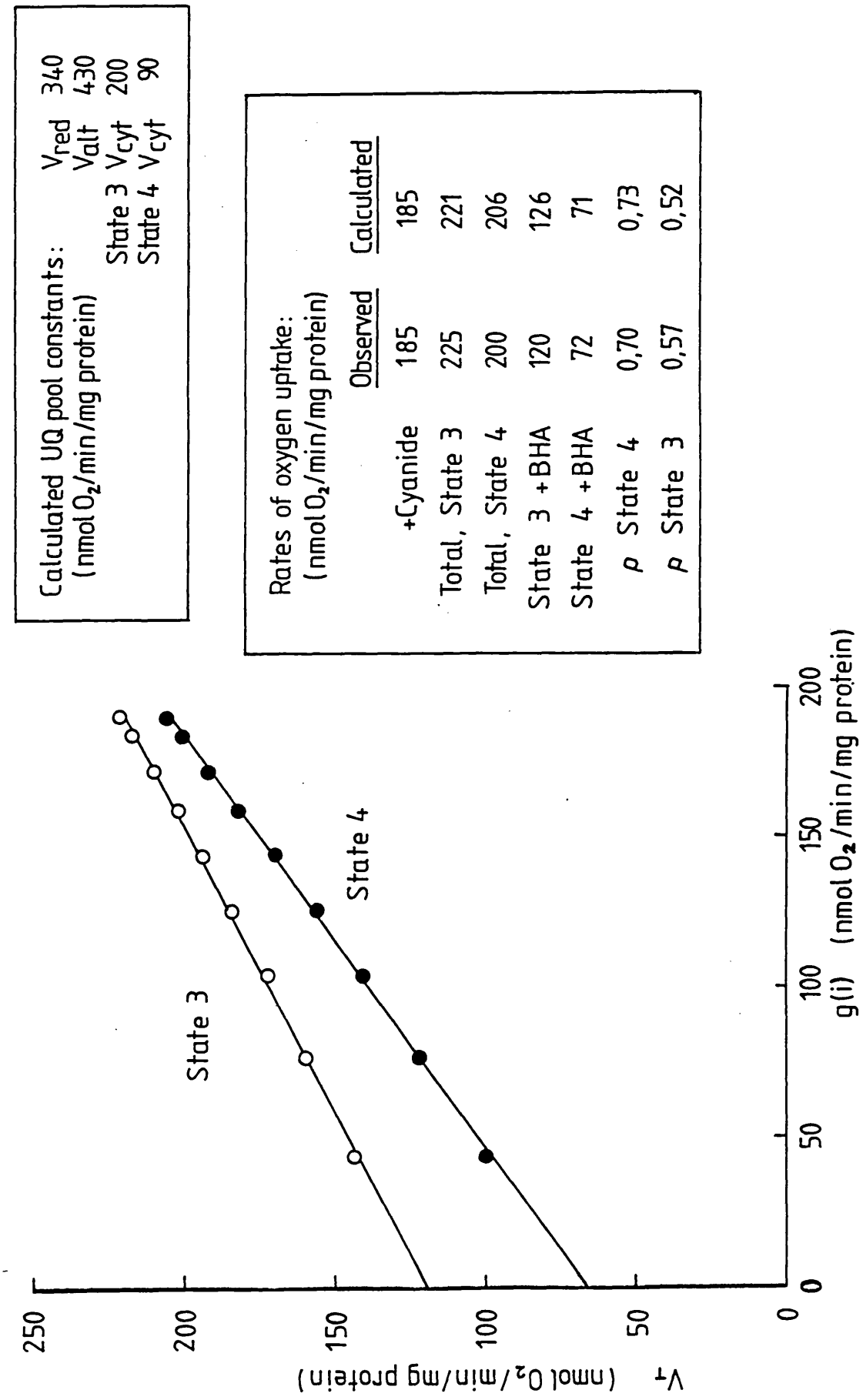
Whether or not these changes lead to a linear Bahr-Bonner plot can be tested using the data, for example, of Table II preparation A from Bahr and Bonner (1973a). If it assumed that only V_{alt} is affected by benzhydroxamate and only V_{cyt} is changed by the transition from state 3 to state 4,then values for V_{alt}, V_{red} and V_{cyt} can be calculated that fit the rates obtained in the presence of cyanide, saturating amounts of benzhydroxamate and in state 3 and state 4 (no inhibitor present). These values are shown in Figure 5.* A titration with benzhydroxamate can then be simulated by progressively

*values are not exact mathematical solutions, but closely match the experimental data.

Figure 5. Simulation of a benzhydroxamate titration according to the ubiquinone-pool model.

Ubiquinone-pool constants were calculated to fit the data of Bahr and Bonner (1973a, Table II) obtained using Skunk cabbage mitochondria, as described in section 1.3.2. A titration with benzhydroxamate was simulated, by progressive decreases in the value of Valt. At each value of Valt the rates of oxidation expected, according to the ubiquinone-pool model, were calculated for state 3, state 4 and in the presence of cyanide (see section 1.3.2). These values were then plotted according to the method of Bahr and Bonner (1973a).

Figure 5. Simulation of a benzhydroxamate titration according to the ubiquinone-pool model.



decreasing V_{alt} and calculating the total rate of oxygen uptake expected. When these results are plotted according to Bahr and Bonner (1973a) the lines for state 3 and state 4 are very nearly (but not quite) linear: probably indistinguishable from straight lines given normal experimental error. Furthermore the apparent p values obtained are also very similar to those obtained by Bahr and Bonner (1973a).

A similar point was made by De Troostembergh and Nyns (1978) who substituted into the Bahr-Bonner equation the ubiquinone-pool functions for V_T and V_{cyt} in order to obtain an expression for p in terms of the ubiquinone-pool constants. By this method:

$$p = \frac{V_{red}.(V_{red} + V_{alt})}{(V_{red} + V_{cyt}).(V_{red} + V_{cyt} + V_{alt})}$$

Taking typical values for the three constants it was found that only a 5% change in the value of p would be predicted for a 50% change in the value of V_{alt}. Significant changes in the value of p during titration of V_{alt} would be expected when $V_{cyt} >> V_{red}$. These authors concluded that the constancy of p was simply a mathematical coincidence and that p may not have any physiological meaning such as the degree of engagement of the alternative pathway. It would seem therefore that the postulate that the cytochrome pathway flux is unaffected by the alternative pathway flux remains to be tested.

In their study, De Troostembergh and Nyns (1978) used mitochondria from the yeast (*S.lipolytica*) in which the alternative pathway had been induced by shaking the cells in a solution containing 1mM $Fe_2(SO_4)_3$. Determination of the rates of oxidation via the alternative and cytochrome pathways separately, and in the absence of inhibitors, allowed calculation of the three ubiquinone-pool

constants. The results of antimycin and benzhydroxamic titrations were consistent with ubiquinone-pool kinetics as only V_{cyt} was decreased by antimycin, and only V_{alt} by benzhydroxamic acid. Furthermore the results of a titration with benzhydroxamate gave a linear Bahr-Bonner plot.

However the model of De Troostembergh and Nyns (1978) has been criticised by Laties (1982). Possibly the most serious objection was that, given the algebraic form of the equation for p in terms of the ubiquinone-pool constants, values of p of zero or one are impossible for positive values of the constants, and yet there are reports of p= 0 and p= 1 in the literature, particularly from studies using tissue slices rather than isolated mitochondria (Theologis and Laties, 1978a,b). In such studies it is often necessary to subtract a rate of oxygen consumption (residual respiration) that is insensitive to inhibitors of both cytochrome and alternative pathways when added together.

Another criticism of the unmodified ubiquinone pool model is that it cannot explain the selective relationship between certain respiratory substrates and the two oxidases. It is often observed that the different respiratory substrates differ in their abilities to feed electrons to the alternative pathway, as judged by the degree of inhibition of state 3 oxidation by cyanide. A number of these reports are gathered in Table 1 (adapted from Lance *et al.*, 1985) and show in particular that exogenous NADH is, except in the case of Aroid mitochondria, much more cyanide sensitive than either malate or succinate oxidation. Cottingham and Moore (1983) showed that titration of the alternative pathway and the cytochrome pathway separately with their specific inhibitors gave inhibition patterns consistent with ubiquinone-pool kinetics for both succinate and exogenous NADH oxidation. When both oxidases operated simultaneously these kinetics

Table 1. The dependence of cyanide inhibition on the substrate oxidised (from Lance *et al.*, 1985).

The figures quoted are the percentage inhibition of oxygen uptake upon addition of cyanide, and are taken from a large number of published reports.

	Substrate oxidised		
Plant species	Malate	Succinate	NADH
Arum maculatum	5-56	0-55	30-55
Symplocarpus foetidus	25-45	17-50	17-50
Sauromatum guttatum	51	0-52	55
Zea mays	10-75	80-100	86-100
Ipomea batatas	39	50-63	87
Triticum aestivum	81	78	96
Cicer arietinum	37	60	-
Acer pseudoplatanus	51	40	90
Phaseolus aureus	75-80	64-85	88
Brassica oleracea	78-90	84-90	90
Helianthus tuberosus	95	95-98	97
Solanum tuberosum			
Fresh slices	78-94	86-99	86-99
Aged slices	47	60-77	89-94

were not seen. This was shown by calculation of the pool constants from oxygen uptake rates of the two pathways separately and together, as all 3 constants were dependent on the substrate being oxidised. If the model was applicable only V_{max} should have changed when the substrate was changed. It should be noted that any three rates of oxygen consumption determined in the presence of cyanide, benzhydroxamate and in the absence of inhibitor will yield values for the ubiquinone pool constants provided (a) that the rate in the absence of inhibitor is higher than either of the other two rates and (b) that the rate in the absence of inhibitor is less than the sum of the two other rates. Calculation of these values cannot alone confirm the kinetic mechanism: it is the way these values change that counts. The preferential association of exogenous NADH oxidation with the cytochrome pathway has not yet been fully explained, but one suggestion has been that substrate specific sub-pools of ubiquinone exist in the membrane which differ in their access to the alternative pathway and communicate only slowly with each other (Rustin *et al.*, 1980). Such a pool of ubiquinone specific to the external NADH dehydrogenase would still have to be in close touch with complex III as exogenous NADH is often the most rapidly oxidised substrate in the absence of cyanide. There is no evidence of compartmentation of complex III: antimycin titres for NADH and succinate oxidation are identical (Cottingham and Moore, 1983). A second suggestion has been of patching of repiratory components so that exogenous NADH dehydrogenases are physically close to complex III (Rich, 1984).

1.3.3 Oxidation of multiple substrates by the plant mitochondria.

Complementary to the distribution of electrons between two oxidases (section 1.3.2) is the situation where two or more respiratory substrates are oxidised simultaneously. The behaviour of plant mitochondria is more complicated than that of mammalian mitochondria under these conditions (section 1.3.1) since the general conclusion drawn by Gutman (1980), that the substrate that is oxidised faster (when the rates are measured separately) will predominate when the two substrates are oxidised together, does not always apply to intact plant mitochondria. However the most basic predictions of ubiquinone-pool behaviour are always observed: that the rate in the presence of two substrates is greater than that of either substrate alone, but is less than the sum of their rates alone.

Day and Wiskich (1977) compared the rates of oxygen consumption of cauliflower (*Brassica oleracea* L.) bud mitochondria when presented with combinations of substrates. Every combination of the substrates succinate, malate and exogenous NADH was tried in both state 3 and state 4. When oxidised alone exogenous NADH was oxidised the most rapidly and malate the most slowly, in both the presence and absence of ADP. The rates of oxygen consumption of combinations of substrates followed the order that would be predicted from the sum of the rates oxidised alone, with one exception: in state 4 the rate of oxidation of (NADH + succinate, 85nmol O_2/min/mg protein) was significantly higher than the rate of oxidation of (NADH + succinate + malate, 64nmol O_2.min/mg/protein). No explanation was offered for this result. The inhibition of NADH oxidation by concurrent oxidation of malate or succinate was measured spectrophotometrically. In state 3,succinate or malate caused equal inhibitions of 20%, rising to 30%

when all three substrates were present. As NADH oxidation rates and oxygen uptake rates were not measured in parallel it was not possible to calculate the inhibition of malate or succinate oxidation by NADH, but the use of rotenone and malonate clearly showed that oxidation of malate or succinate was necessary for inhibition of NADH oxidation. The authors suggested that when these substrates were oxidised separately the "rate-limiting step" of oxidation was on that part of the chain exclusive to each substrate ie. the dehydrogenase, but that when all three substrates were present the cytochrome pathway was saturated and so all substrates shared a common rate-limitation. This conclusion was based on the assertion that the rate of oxidation in state 3 of (malate + succinate + NADH, 173nmol O_2/min/mg protein) was no higher than the rate of oxidation of (NADH + succinate, 167nmol O_2/min/mg protein).

Cowley and Palmer (1980) concentrated specifically on the interaction between the oxidation of succinate and exogenous NADH by Jerusalem artichoke (*Helianthus tuberosus*) tuber mitochondria. These mitochondria are more than 95% sensitive to cyanide or antimycin. External NADH oxidation had been reported to be inhibited in the presence of citrate (Cowley and Palmer, 1978), but this inhibition was apparently due to chelation of Ca^{2+} rather than from competition at the level of the respiratory chain. In contrast Cowley and Palmer (1980), by using malonate to inhibit succinate oxidation, showed that succinate oxidation was necessary for the inhibition of NADH oxidation. Succinate and NADH were oxidised at similar rates when oxidised separately. The inhibition of NADH oxidation by succinate was found to be greater, in percentage terms and as measured by the decrease in rate, in state 4 (51 to 72%), than in state 3 (15 to 20%). When uncoupler was present no inhibition of NADH oxidation by succinate was observed. The most interesting results

were obtained when rates of oxygen uptake and NADH oxidation (followed spectrophotometrically) were measured in tandem. In state 4 the inhibition of NADH by succinate was approximately 75% but the inhibition of succinate by NADH less than 10%. No reduction of external NAD^+ by succinate could be detected, showing that inhibition of NADH oxidation was not due to reverse electron transport. The inhibition by succinate was apparently non-competitive with respect to NADH concentration. Two possible explanations of this interaction were considered by the authors. Firstly that the substrates compete for a step which although not rate-limiting when single substrates are present, becomes so when both are oxidised, and secondly that a change in the spatial organisation of the respiratory components may occur so that the accessability of one or both of the dehydrogenases to the terminal electron pathway changes with respiratory state. The second suggestion followed reports of conformational changes of plant mitochondria dependent on the respiratory state (Gunawardena and Wilson, 1974) and of the relation between increased respiratory rate and mitochondrial contraction (Hanson and Hodges, 1967). With respect to the first suggestion no component of the terminal pathway could be detected that was more reduced in the presence of succinate and NADH together than in the presence of either separately.

It is the asymmetry of the interaction between succinate and NADH oxidation which is puzzling: as both substrates are oxidised at comparable rates a mutually equal inhibition would be predicted by the ubiquinone-pool model. Still more extreme asymmetry of interaction is shown in the oxidation of glycine by mitochondria from photosynthetic tissue (Gardestrom *et al.*, 1980). Glycine is oxidatively decarboxylated to produce serine during the photorespiratory cycle. The process is catalysed by two matrix

enzymes: glycine decarboxylase (EC 2.1.2. 10) which oxidises glycine to CO_2, NH_3 and produces NADH, and serine hydroxymethyltransferase (EC 2.1.2.1) which completes the conversion to serine. The reoxidation of the NADH produced by glycine oxidation is important for the continuation of photorespiration and this may occur either via the respiratory chain or by the reversal of malate dehydrogenase (see Gardestrom and Edwards, 1985). Dry *et al.* (1983) were able to measure the effect of other substrates on the oxidation of glycine by pea (*Pisum sativum*) leaf mitochondria by comparison of oxygen uptake rates with the release of ammonia, measured with an ammonium ion specific electrode. The state 3 rate of glycine oxidation was unaffected by the presence of malate, succinate or NADH. Malate could have been expected to compete with glycine at the respiratory chain level and at the level of endogenous NAD^+, but no evidence for the compartmentation of endogenous NAD^+ was found, since oxaloacetate inhibited the oxidation (oxygen uptake) of all the exogenous NAD^+-linked substrates. The oxidation of malate and NADH were inhibited roughly 30% by the presence of glycine and, as with succinate (see above), oxidation of glycine was necessary for inhibition. The interaction of NADH oxidation with the other substrates was also followed: despite the fact that state 3 NADH oxidation was twice as fast as that of glycine, and faster than any of the other substrates tested, the oxidation of NADH was strongly inhibited by malate (25%), by succinate (27)% and by glycine (27%). The corresponding inhibitions by NADH were: on malate oxidation 7%, on succinate oxidation 30% and on glycine oxidation 2%. To explain the preferential oxidation of glycine Dry *et al.* suggested a structural association between glycine decarboxylase and the inner mitochondrial membrane.

Similar observations of the precedence of glycine oxidation have been made by Bergman and Ericson (1983) using spinach (*Spinacia oleracea*) leaf mitochondria. State 3 rates of NADH and sucinate oxidation were faster than the rate of glycine oxidation, and yet no inhibition of NH_3 release was seen upon addition of other substrates. Instead oxidation of NADH was inhibited by 44%, the oxidation of succinate by 30% and the oxidation of malate by 52%. These authors suggested that a specific endogenous NADH dehydrogenase may be associated with glycine decarboxylase.

Recently a kinetic explanation for the precedence of glycine oxidation has been proposed (Moore and Rich, 1985) in which the rate constant for the reoxidation of the NADH dehydrogenase,associated with glycine oxidation,by ubiquinone is very fast, but the rate constant for the reduction of the dehydrogenase by glycine is slow. As a consequence the oxidation of glycine becomes insensitive to the presence of respiratory substrates whose dehydrogenases are more limited by their interaction with ubiquinone. A similar model is discussed more fully below (see discussion).

Much of the work presented in this thesis was aimed at finding the factors responsible for the asymmetric interaction of succinate and exogenous NADH oxidation as possibly the most straightforward system for experimental study showing a clear deviation from ideal behaviour.

Chapter 2. Materials and Methods.

2.1 Preparation of Jerusalem artichoke (*Helianthus tuberosus*) mitochondria.

Tubers of Jerusalem artichoke (*Helianthus tuberosus*) were obtained from the Botanical supply unit, University of London, and were stored at 5-10°C until use. All mitochondrial isolation steps were performed at 4°C. The isolation medium contained 0.5M sucrose, 10mM MOPS and 5mM EDTA and the pH of the solution was adjusted to 7.8 using KOH solution. Just prior to use 2mM $Na_2S_2O_5$ and 0.1% BSA (w/v) were added, and the pH readjusted if necessary. For a standard preparation, 200g of peeled tubers were cut into small cubes and homogenised in 300ml of isolation medium for 20 s, using a hand-held mixer (Moulinex 66). The slurry was filtered through two layers of muslin and the solution centrifuged for 2 min at 48,000 g in an SS-34 rotor and a Sorvall RC-2B centrifuge. The pellets were then gently resuspended in fresh wash medium, using a hand operated homogenisor. Wash medium contained 0.4M sucrose, 5mM TES and 0.1% BSA (w/v) and the pH was adjusted to 7.2 using KOH solution. The resuspended material was centrifuged by acceleration to 12,000g followed by immediate deceleration. The pellets were discarded. The supernatant was then centrifuged at 48,000 g for 2 min to recover the mitochondria. The mitochondrial pellets were gently resuspended in the minimum amount fresh wash medium using a pipette, and stored on ice until use. When larger quantities of mitochondria were required, the same proportions of isolation medium and tissue were used, but the initial centrifugation step was changed to 15 min at 10,400 g in a GSA rotor.

2.2 The activation of succinate dehydrogenase.

The ability of freshly isolated mitochondria to oxidise succinate

is impaired due to inactivation of succinate dehydrogenase. Full activity can be restored by incubation at 25°C to 37°C with a variety of activating agents, including ATP, Pi and Mg^{2+} (Singer *et al.*, 1973, Oestreicher *et al.*, 1973). In the present study succinate dehydrogenase was routinely activated when succinate was the substrate to be oxidised. The activation method used was that of Cowley (1977). Mitochondrial samples were incubated in a water bath at 25°C for 5 min, with 5mM $MgCl_2$, ATP (1µmol/mg protein, final concentration) and Pi (potassium salt, 1µmol/mg protein, final concentration). Solutions of activating compounds were prepared in 0.4M sucrose to avoid osmotic damage to the mitochondria, and the pH was adjusted to a value of 7.2 using 10% (w/v) KOH solution. Following activation of succinate dehydrogenase, mitochondria were stored in on ice until used. Longer incubation times, or increased concentrations of ATP, Mg^{2+} or Pi did not increase the rate of oxidation of succinate and so this method was taken to give full activation of succinate dehydrogenase, in agreement with the results of Cowley (1977).

2.3 Polarographic measurement of oxygen consumption.

Rates of oxygen uptake were measured in a total volume of 1ml in a Rank oxygen electrode (Rank Brothers, Bottisham, Cambridge) connected to an Oxygraph recorder (Gilsom Medical Electronics, Wisconsin, USA). The reaction chamber was surrounded by a water jacket, allowing the temperature of the assay to be maintained at 25°C. Unless the legends to figures or tables indicate otherwise, measurements were made in "standard assay medium" containing 0.3M sucrose, 5mM TES, 5mM KH_2PO_4, 2.5mM $MgCl_2$. The pH of the solution was adjusted to 7.2 at room temperature, using 10% (w/v) KOH solution. To obtain maximum uncoupled rates of succinate oxidation, the uncoupler (0.2µM FCCP) was added 1 min after the addition of

succinate. The oxygen electrode was calibrated using crystals of sodium dithionite to remove oxygen from the reaction chamber. The dissolved oxygen concentration of standard assay medium was assumed to be 240μM (Truesdale *et al.*, 1955).

2.4 Assessment of the integrity of the inner mitochondrial membrane.

The latency of malate dehydrogenase activity was used to estimate the the integrity of the inner membrane of mitochondria. The method used was that of Douce *et al.* (1973). The assay medium contained 0.3M sucrose, 5mM $MgCl_2$, 10mM KCl, 10mM potassium phosphate buffer (pH 7.2), 1mM KCN, 0.2mM NADH and 20-50μg mitochondrial protein. The assay was initiated by the addition of 1mM oxaloacetate. The oxidation of NADH was measured spectrophotometrically (section 2.7.2) A similar assay was performed in the presence of 0.02% (w/v) Triton X-100 to rupture the mitochondria. The degree of intactness of the inner membrane was then calculated as:

$$\text{Intactness of inner membrane (\%)} = 100 \times \left(1 - \frac{\text{Rate without Triton}}{\text{Rate with Triton}}\right)$$

2.5 Assessment of the integrity of the outer mitochondrial membrane.

The integrity of the outer membrane of mitochondria, and the membrane polarity of SMP, was determined from the latency of cytochrome oxidase activity using the method of Douce *et al.* (1973). Cytochrome c was reduced by the addition of 2mM sodium ascorbate to a solution of cytochrome c (0.3mM) in 10mM potassium phosphate buffer, pH 7.2. After 10 min at room temperature cytochrome c was separated from ascorbate by passage through a small (8cm x 0.8cm^2) column packed with Sephadex G-25 (superfine) and equilibrated with 10mM potassium phosphate buffer, pH 7.2. The

oxidation of reduced cytochrome c was monitored at 550nm (ϵ_{550} = 21.0 $mM^{-1}.cm^{-1}$) in a medium composed of 0.3M sucrose, 10mM KCl, 10mM potassium phosphate buffer (pH 7.2), 50μM reduced cytochrome c and 0.04-0.10 mg protein. Assays were initiated by the addition of mitochondrial protein and identical assays were performed in the presence and absence of 0.02% (w/v) Triton X-100. The intactness of the mitochondrial outer membrane was calculated in the same way as the intactness of the inner membrane (section 2.4). Where the polarity of SMP preparations was measured, the same calculation gave the percentage of the SMP that were right-side out (Chapter 6).

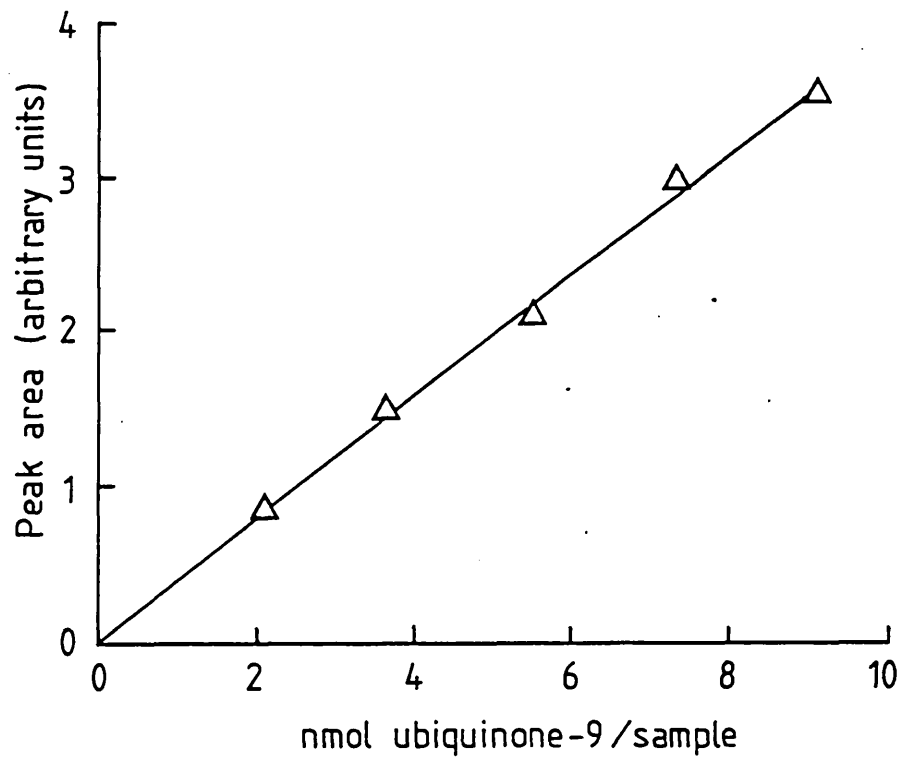
2.6 Characterisation of the ubiquinone content of mitochondria.

The total lipid content of Jerusalem artichoke mitochondria was extracted and then the ubiquinone content identified and quantified by HPLC. Mitochondrial samples (0.8ml, 4.8-6.2mg protein/ml) were added to a mixture of 2.5ml methanol and 1.25ml $CHCl_3$. After thorough shaking the cloudy precipitate was removed by filtration through glass wool. A further 1.25ml of $CHCl_3$ and 1.25ml of 0.88% (w/v) aqueous KCl were added and the mixture again shaken. Upon standing the solution separated into two phases. The bottom phase, containing the ubiquinone, was retained and concentrated by passing a stream of oxygen free nitrogen over the surface of the sample. When this extraction method was applied to mitochondrial samples to which HQNO had been added, complete recovery of HQNO into the lipid extract was achieved (measured by fluorescence, section 2.9).

Lipid samples (100μl) were subjected to HPLC (Kontron Analytic, Kontron, London) using an RP-18 silica column (Spherisorb 5 ODS, 25cm x $0.75cm^2$). The column was run at 30°C, using a two solvent system, with the proportions of each solvent varied automatically during each run. The solvent was varied to rapidly separate the less

Figure 6. Calibration of the of the amount of ubiquinone-9 and the peak area detected at 275nm by HPLC.

Samples of stock solutions ubiquinone-9 were subjected to HPLC as described in section 2.6. The appearance of ubiquinone was measured at 275nm and the peak area measured. This graph was used for the determination of the ubiquinone content of Jerusalem artichoke mitochondria.



hydrophobic lipids from the ubiquinones. Solvent A was methanol:water (93:7, v/v) and solvent B was methanol:ethanol (50:50, v/v). During each run the proportion of each solvent was varied linearly as follows:

0 - 5 min	100% B - 20% B
5 - 20 min	20% B - 0% B
20 min onwards	0% B (ie.100% A)

Methanol and ethanol were of spectroscopic grade and were degassed before use. Ubiquinone was detected at 275nm and the area of the resulting peaks measured using a Hewlitt Packard 3390A integrator. Stock solutions of ubiquinone-9 and ubiquinone-10 were prepared in $CHCl_3$ and bubbled with oxygen free nitrogen to remove oxygen. The concentration of ubiquinone was determined from the reduced and oxidised spectra ($\epsilon_{280-289nm}$ = 8.8mM^{-1}.cm^{-1}, Kroger and Klingenberg, 1973a). Ubiquinone was reduced by the addition of a single crystal of $NaBH_4$. Stock ubiquinone-9 solutions were used to calibrate the amount of ubiquinone added and the peak area detected by HPLC. Within the range used (up to 10nmol ubiquinone/sample) the relationship between the amount of ubiquinone added and peak area was linear, as shown in Figure 6.

2.7 Measurement of the activities of respiratory chain enzymes.

2.7.1 Succinate dehydrogenase.

The activity of succinate dehydrogenase was measured as phenazine methosulphate-mediated reduction of DCPIP, according to the method of Baginsky and Hatefi (1969). The assay medium contained 50mM potassium phosphate (pH 7.4), 20mM succinate, 0.1mM EDTA, 0.1% (w/v) BSA, 70μM DCPIP, 0.2mM ATP (to activate succinate dehydrogenase), 1mM KCN and 0.02% Triton X-100. Mitochondrial samples were incubated in the assay medium for 2 min before the start of each assay to allow full activation of succinate dehydrogenase by ATP to develop. Assays were

initiated by the addition of PMS (1.65mM) and the rate of reduction of DCPIP was measured using a millimolar extinction coefficient of $21mM^{-1}.cm^{-1}$.

2.7.2 NADH:(acceptor) oxidoreductase activity.

The oxidation of NADH was followed by the decrease in absorbance at 340nm (split-beam mode, $\epsilon = 6.22mM^{-1}.cm^{-1}$, Horecker and Kornberg, 1948) or at 340-374nm (dual-beam mode) at 25°C in a total volume of 1ml. Other conditions and protein concentrations are given in the legends relating to individual experiments. The millimolar extinction coefficient used for the measurement of NADH oxidation in dual-beam mode was derived from comparison of the absorbance changes due to NADH oxidation, catalysed by malate dehydrogenase, measured in the two modes. NADH (0-100μM) was added to standard assay medium containing 0.4mM oxaloacetate, in a total volume of 1ml. The oxidation of NADH was initiated by addition of 60 units malate dehydrogenase (Boehringer Mannheim, from pig heart). The rapid and essentially complete oxidation of NADH was followed in both split-beam and dual-beam modes and the millimolar extinction coefficient for the dual-beam mode ($\epsilon = 4.70mM^{-1}.cm^{-1}$) was derived using the value of $6.22mM^{-1}.cm^{-1}$ for oxidation of NADH in the split-beam mode.

Rates of ubiquinone-1 reduction were measured at 420nm using an extinction coefficient of $0.465mM^{-1}.cm^{-1}$. The reduction of cytochrome c (50μM) was monitored at 550nm (ϵ_{550nm}= $21.0mM^{-1}.cm^{-1}$), and 1mM KCN was included in the assay medium to inhibit cytochrome oxidase. Where NADH oxidation was measured in the presence of cytochrome c (Figure 43), a slight adjustment in the monitoring wavelength from 340nm to 336nm (ϵ_{336}= $6.15mM^{-1}.cm^{-1}$) was necessary to avoid spectral interference arising from the reduction of cytochrome c.

2.8 Measurement of membrane potential using safranine O.

The absorbance changes of safranine O were monitored at 511nm (reference 533nm) at room temperature using an Aminco DW-2 spectrophotometer in the dual beam mode. Safranine O was obtained from Sigma and was used without further purification. All measurements were made in a volume of 1 ml using glass cuvettes with an optical path length of 1 cm. Mitochondria were suspended in standard assay medium modified as described in the figure legends. Safranine O was added from stock solutions (2mM) made up in water.

2.9 Measurement of HQNO concentration by fluorescence.

The fluorescence of HQNO was measured at room temperature in a total volume of 3ml using a Perkin-Elmer MPF-3 fluorimeter, according to the method of van Ark and Berden (1977). The wavelengths used to monitor HQNO concentration were those giving maximum fluorescence. The excitation wavelength was 355nm (slit width 6nm) and the emission wavelength was 480nm (slit width 6nm). These conditions made it possible to measure concentrations of HQNO of less than 1μM with accuracy. Mitochondrial suspensions were incubated with HQNO for 5 min at room temperature to allow equilibration of HQNO with its binding sites. The mitochondria were then pelleted by centrifugation (12,000 g for 10 min) and the fluorescence of the supernatant measured. The fluorescence was calibrated with HQNO concentration by the direct addition of HQNO to mitochondrial supernatant. The concentration of stock ethanolic solutions of HQNO were measured, after dilution into standard assay medium, using an extinction coefficient at 348nm of $9.45 mM^{-1}.cm^{-1}$ (Cornforth and James, 1956). Because of the binding of HQNO to BSA (van Ark and Berden, 1977), BSA was omitted from the wash medium used to prepare mitochondria for these experiments.

2.10 Treatment of mitochondria with BAL (2,3-dimercaptopropanol).

Mitochondrial suspensions (approximately 15mg protein/ml) were gently shaken with concentrations of BAL of up to 20mM for 45 min at room temperature. Stock solutions of BAL (1M) were made up in DMSO. After incubation, unreacted BAL was removed from the mitochondria by washing. The mitochondria were pelleted by centrifugation (Eppendorf model 3200 bench top centrifuge) and then resuspended in fresh wash medium. This process was repeated, and then succinate dehydrogenase was activated as described in section 2.2. The samples were kept on ice until use.

2.11 The production of submitochondrial particles (SMP).

2.11.1 Sonication.

Mitochondria were resuspended in wash medium, modified as described in the legends to figures and tables, to a protein concentration of approximately 15mg protein/ml. Mitochondrial samples (4ml) were sonicated in glass tubes for 4 periods of 5 s (50% duty cycle) at power setting 6, using a Branson Model B-30 Sonifier (Branson Sonic Power Co., Connecticut) equipped with a microprobe. During sonication the samples were kept in an ice bath, and 1 min was allowed for cooling after each period of sonication.

2.11.2 French pressing.

Mitochondria were suspended in wash medium, modified as indicated, to a protein concentration of 4-7 mg protein/ml. Samples (20ml) were passed once through a French pressure cell operated at 400 MPa and a flow rate of 5 ml/min. The pressure cell (Aminco) had an internal diameter of 25 mm and was cooled to 4°C before use.

2.11.3 Isolation of SMP.

Samples obtained by either sonication or French pressing were diluted to 160 ml with wash medium. Large membrane fragments were removed by centrifugation at 4°C and 48,000 g for 10 min. The

supernatant was centrifuged at 4°C and 75,000 g (r_{av}) for 60 min in a 70-Ti rotor and a Beckman L5-65B preparative ultracentrifuge. The pellets were rinsed thoroughly with ice-cold 0.3M sucrose to remove any salts present, as these would interfere with the phase partition, and then resuspended in 0.3M sucrose using a hand operated homogenisor.

2.12 Cytochrome measurements and spectra.

Cytochrome determinations were made using an Aminco DW-2 spectrophotometer. Mitochondria were suspended in standard assay medium and added to both the sample and reference cuvettes (0.2 cm optical path length). The samples were then frozen in liquid nitrogen. The baseline was set over the wavelength range of interest, using a bank of 31 potentiometers. The mitochondria were then thawed, and additions made as indicated in the legends to figures. Equal volumes of wash medium were also added to the reference cuvette to avoid artefacts arising from unequal dilution of the samples. Where mitochondria oxidising exogenous NADH were examined, the samples used to set the baseline were replaced with fresh mitochondria from the same suspension, to avoid disruption of the mitochondria by freezing and thawing. The samples were frozen and spectra were recorded using a slow pen response time and a recorder scanning rate of 0.1 nm/sec to minimise noise. The slit width was 1nm. To obtain fully reduced spectra sodium dithionite was added from airtight buffered solutions (0.1M in 1M Tris-HCl, pH 7.2).

Measurements of cytochrome content were made from reduced - oxidised spectra using the absorbance coefficients of Lance and Bonner (1968). These were: cytochrome aa_3, = $16mM^{-1}.cm^{-1}$; cytochromes b, = $20mM^{-1}.cm^{-1}$; cytochromes c, = $19mM^{-1}.cm^{-1}$. Spectra were recorded both at room temperature and at liquid nitrogen temperature to calculate the intensification factor (Lance and

Bonner, 1968) caused by freezing.

2.13 Polyacrylamide gel electrophoresis.

Mitochondrial and SMP fractions were heated to 100°C for 5 min in a solution containing 3% (w/v) SDS, 20% (w/v) glycerol, 7.5% (w/v) 2-mercaptoethanol and 0.01% (w/v) bromphenol blue. Solubilized samples (20-40µg protein) were subjected to electrophoresis in a gradient slab gel (10-17% (w/v) acrylamide). The gel was 20cm long, 14cm wide and 1mm thick and contained 0.1% (w/v) SDS. The concentrating zone (3% (w/v) acrylamide) was 10% of the length of the gel. The gel was run at 4°C and at a constant current of 11 mA. After the run the gel was fixed in 45% (v/v) methanol, 9% (v/v) acetic acid for 30 min, then stained in 0.25% (w/v) Coomassie Brilliant Blue R-250 and destained in 7% (v/v) methanol, 5% (v/v) acetic acid. The gel was scanned at 590nm using a Zeiss KM3 Chromatogram Spectrophotometer.

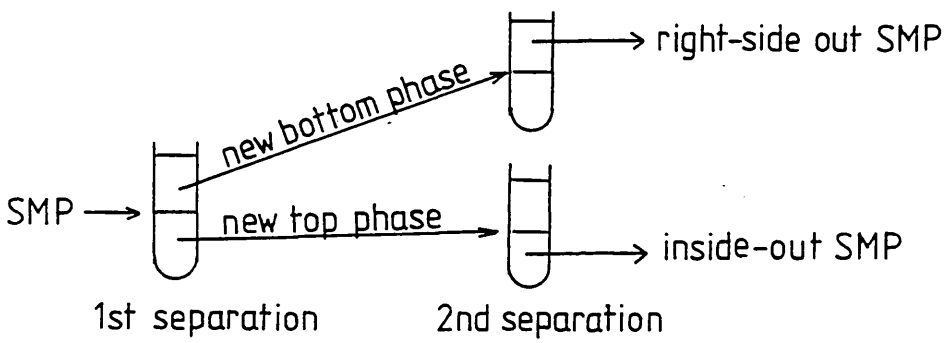
Molecular weight standards were also run to allow the apparent molecular weights of the mitochondrial proteins to be measured, assuming that the mitochondrial proteins behaved as standard proteins. The standard proteins were: phosphorylase b (94kDa), BSA (64kDa), ovalbumin (43kDa), carbonic anhydrase (30kDa), soybean trypsin inhibitor (20.1kDa) and lactalbumin (14.4kDa) (Electrophoresis Calibration LMW Kit, Pharmacia Fine Chemicals).

2.14 Phase partition of SMP.

All phase partition operations were carried out at 4°C. Phase systems were prepared by weight, using concentrated solutions of the components and deionized water. The total weight of the system included the weight of the SMP sample. Solutions of PEG-4000 (40% w/w, Union Carbide, New York) were made with PEG-4000 recrystallised from ethanol. The concentration of dextran T500 (Pharmacia Fine Chemicals) was adjusted to 20% (w/w) using optical rotation

measurements ($[\alpha]_D^{25}$ = +199 degree.ml/g.dm, Albertsson, 1971). Suspensions of SMP were added to phase systems and mixed by 40 gentle inversions. The separation of the two phases was then accelerated by centrifugation at 1,200 g for 2 min using a Christ (Sweden) bench top centrifuge. The top phase could then be separated from the combined bottom phase and interface. For small scale phase partitions the phases were diluted 2 fold with wash medium and assayed directly.

The large scale separation of inside-out from right-side out SMP (section 6.2) was carried out by replacing the top and bottom phases of 40g systems with fresh phases derived from identical systems, to which SMP had not been added, as depicted below:



The final phases were diluted to 120ml with wash medium and the SMP recovered as described in section 2.11.3.

2.15 Determination of protein concentration.

Protein was determined by the method of Lowry *et al.* (1951), with BSA (Fraction V, Sigma) as standard, after solubilization of the mitochondria in 0.5% (w/v) sodium deoxycholate.

2.16 Chemicals and solutions.

Most of the chemicals (buffers, salts, substrates) were obtained from BDH Chemicals Ltd., Poole, Dorset, Hopkins and Williams, Chadwell Heath, Essex, and Sigma (London) Chemical Co., Poole, Dorset and were the highest grade available. NADH, ATP, ADP and malate dehydrogenase were from Boehringer (The Boehringer

Corporation (London) Ltd., Lewes, East Sussex). FCCP was a gift from Dr.P. Heytler, Du Pont Chemicals. Antimycin was from Calbiochem AG, Lucerne, Switzerland. Myxothiazol was a gift from Dr.P.Rich, Department of Biochemistry, University of Cambridge. Ubiquinone-1 and ubiquinone-9 were gifts from Hoffmann-La-Roche. Duroquinol (DQH_2) was from K & K, ICN Pharmaceuticals, Plainview, NY, USA. HQNO, ubiquinone-10, CTC and BAL were from Sigma.

The inhibitors myxothiazol, HQNO and antimycin were dissolved in absolute ethanol. The concentrations of stock solutions were determined spectrophotometrically using absorbance coefficients of $4.8mM^{-1}.cm^{-1}$ at 320nm for antimycin (Strong *et al.*, 1960), of $9.45mM^{-1}.cm^{-1}$ at 348nm for HQNO (Cornforth and James, 1956) and of $10.5mM^{-1}.cm^{-1}$ at 313nm for myxothiazol (Thierbach and Reichenbach, 1981). Solutions of DQH_2 were made up in acidified DMSO and not stored. The concentration of the stock solution was determined spectrophotometrically by causing the DQH_2 to auto-oxidise by addition of 0.1M (final concentration) KOH. An absorbtion coefficient of $12.5mM^{-1}.cm^{-1}$ at 265nm (oxidised - reduced) was used (Lawford and Garland, 1972). Solutions of NADH were made up in carbonate buffer (0.1M, pH 10). All other chemicals were dissolved in distilled water and the pH adjusted where appropriate.

Chapter 3. Succinate and exogenous NADH oxidation.

3.1 Some properties of washed Jerusalem artichoke (*Helianthus tuberosus*) mitochondria.

3.1.1 The oxidation of exogenous NADH and succinate.

Both exogenous NADH (section 1.1.1) and succinate (section 1.1.3) were rapidly oxidised by washed Jerusalem artichoke mitochondria. Rates of oxidation of NADH were slightly faster than those found with succinate as substrate, when measured in state 3, state 4, and in the presence of uncoupler (Table 2). Respiratory states were as defined by Chance and Williams (1956). The addition of uncoupler together with ADP (state 3u) gave no increase in rate over that found in the presence of uncoupler alone (not shown). This indicated that enough FCCP (0.2μM) was present to uncouple electron transport from ATP synthesis such that the presence of ADP did not determine rate. In the absence of uncoupler, the dependence of the rate of oxidation on the presence of ADP is given by the ratio of state 3 to state 4 rates, called the respiratory control ratio. Respiratory control ratios and ADP:O ratios found in this study (Table 2) were comparable to, or rather lower than, those reported previously (Ikuma and Bonner, 1967a, Douce *et al.*, 1972, Moller, 1981, Johnston, 1982).

The ADP:O ratios were similar for succinate and NADH oxidation (Table 2). With both substrates ADP:O ratios of between one and two were found, consistent with the view (Ikuma and Bonner, 1967a, Wilson and Hanson, 1969, Palmer and Passam, 1971, Tomlinson and Moreland, 1975) that electrons from both substrates bypass the first site of phosphorylation, but pass through phosphorylation sites II and III (see Figure 1). The ADP:O ratios observed were less than the theoretical maximum of two, which with some types of plant mitochondria might indicate the involvement of the alternative pathway of oxidation (Bahr and Bonner, 1973a). However succinate and

Table 2. Respiratory activities, membrane integrities and ubiquinone pool size of Jerusalem artichoke (*helianthus tuberosus*) tuber mitochondria.

a. Respiratory activities.

Mitochondria were prepared according to the method described in section 2.1 and succinate dehydrogenase activated by incubation with ATP/$MgCl_2$/Pi (section 2.2). Rates of oxygen uptake were measured at 25°C in standard assay medium as described in section 2.3. Between 0.16 and 0.27 mg of mitochondrial protein were present per ml and 25μM ADP was added to obtain respiratory state 3 (Chance and Williams, 1956). FCCP, to a final concentration of 0.2μM, was added to uncouple respiration. Succinate was added to a final concentration of 20mM and NADH to a final concentration of 1mM. The results represent the average of two preparations.

b. Membrane integrity.

Inner and outer membrane integrities were measured as described in sections 2.4 and 2.5 respectively. Triton X-100 was added to a final concentration of 0.02% (w/v) where indicated. Mitochondrial protein concentration was between 0.014 and 0.022 mg/ml. The results are the mean and standard deviations of five mitochondrial preparations.

c. Ubiquinone content and antimycin titre.

The ubiquinone-9 content of total lipid extracts of washed mitochondrial preparations was measured by HPLC as described in section 2.6. The results are the mean and standard deviations of three mitochondrial preparations. The antimycin titre was determined as shown in the legend to Figure 7 and is taken to be equivalent to the amount of bc_1 complex present. The results are the mean and standard deviation of five mitochondrial preparations.

Table 2a. Respiratory activities.

Rates are: nmol O_2/min/mg protein

Substrate	State 3	State 4	+FCCP	ADP:O	Respiratory Control
Succinate	181	80	206	1.38	2.31
NADH	210	91	225	1.18	2.26

Table 2b. Membrane integrity.

Rates are: nmol NADH/min/mg protein or
nmol cytochrome c/min/mg protein

Enzyme activity	-Triton X-100	+Triton X-100	Integrity (%)
Cytochrome c oxidase	88 ± 13	1901 ± 272	95.3 ± 0.4
Malate dehydrogenase	707 ± 150	12040 ± 2528	94.0 ± 1.3

Table 2c. Ubiquinone content and antimycin titre.

Figures are: nmol/mg protein

Ubiquinone-9	2.71 ± 0.36
Antimycin titre	0.088 ± 0.020
UQ per bc_1 complex	31

NADH oxidation by Jerusalem artichoke mitochondria were both inhibited by antimycin by more than 95% (Figure 7), showing that the contribution of the alternative pathway, if present at all, was negligable using mitochondria from Jeruslaem artichoke tubers.

3.1.2 The integrity of the inner and outer mitochondrial membranes.

The integrity of the outer mitochondrial membrane was estimated from the latency of cytochrome c oxidase activity (Table 2). The assay depends upon the inability of reduced cytochrome c to permeate the outer mitochondrial membrane and so be oxidised by cytochrome oxidase on the outer face of the inner membrane (Wojtczak and Zaluska, 1969, Douce *et al.*, 1973). Full cytochrome oxidase activity is realised by rupture of the outer membrane by detergent. By this method the outer membrane was found to be 95% intact.

A similar degree of integrity was estimated for the inner membrane from measurements of the latency of malate dehydrogenase activity. This assay relies upon the matrix location of malate dehydrogenase and on the impermeability of the inner membrane to NADH. Detergent was again used to determine full enzyme activity and so it was necessary to exclude the possibility that the detergent had a direct effect on the activity of the enzymes measured, as this would lead to erroneous integrity values. A direct effect of Triton X-100 on the activity of either cytochrome oxidase or malate dehydrogenase was not evident for two reasons. Firstly, the activity of the two enzymes developed over very similar detergent concentration ranges (not shown), as has also been demonstrated for cytochrome oxidase and isocitrate dehydrogenase activity of *Arum maculatum* mitochondria (Moller *et al.*, 1981b). The similar concentration of detergent necessary to develop the different maximum enzyme activities is more consistent with the detergent disintegrating the membrane, rather than of a direct effect on the

enzymes themselves. Secondly, as is shown in Chapter 5, when the latency of cytochrome c oxidase activity is used to measure the polarity of submitochondrial particles, almost the entire range of polarities can be observed: if Triton X-100, at the concentration used, directly enhanced or inhibited cytochrome oxidase activity then one or other of the extremes of polarity would not be seen.

Both inner and outer membranes therefore appeared to be highly intact, in agreement with previous reports (Douce *et al.*, 1973, Moller, 1981).

3.1.3 The ubiquinone content of Jerusalem artichoke mitochondria.

The type and quantity of ubiquinone present in plant mitochondria varies with the species tested, and ubiquinones with side chains of between six and eleven isoprenoid units have been found (Crane, 1965, Schindler *et al.*, 1984). The ubiquinone components of total lipid extracts of Jerusalem artichoke mitochondria were resolved and quantified by HPLC (section 2.6). Stock solutions of ubiquinone-9 and ubiquinone-10 were used as standards. These homologues gave rise to peaks with retention times of 32.4 min for ubiquinone-9 and of 37.3 min for ubiquinone-10. Under identical conditions total lipid extracts of mitochondria gave rise to a major peak with retention time of 32.4 min, identical to that obtained using ubiquinone-9. A very much smaller peak (less than 1% of the area of the peak found at 32.4 min), with a retention time of 37 to 38 min, was also detected, probably due to the presence in the mitochondria of low amounts of ubiquinone-10. No other peaks attributable to ubiquinone were detected. The major peak was identified as ubiquinone-9 by two methods. Firstly, mixtures of mitochondrial sample and stock ubiquinone-9 gave only a single peak with a retention time characteristic of stock ubiquinone-9, and secondly, when the mitochondrial peak was collected, the optical absorbtion spectrum

(measured in chloroform, not shown) was identical to that of ubiquinone-9, with a peak at 274nm that was reducible by the addition of a single crystal of $NaBH_4$.

A second solvent extraction of the mitochondrial residue did not yield significant extra ubiquinone, suggesting that a single extraction removed all the ubiquinone, in agreement with the recovery by the same method of the ubiquinone-analogue HQNO from mitochondrial suspensions to which a known amount of HQNO had been added (section 4.2.3). The quantity of ubiquinone-9 present in the mitochondria was found to be 2.71nmol/mg protein (Table 2). Schindler *et al.* (1984) also found ubiquinone-9 to be the major homologue present in Jerusalem artichoke mitochondria, with 2% of the ubiquinone complement as ubiquinone-10. In addition, traces of ubiquinone-8 were found. Ubiquinone-8 was not detected in the present study. The higher content of ubiquinone-9 (4.24nmol/mg protein) determined by Schindler *et al.* (1984) compared to that reported here may be accounted for by the fact that mitochondria purified using a discontinous sucrose gradient were used by Schindler *et al.* (1984). In assessing the size of the ubiquinone-pool it is necessary to find the ratio of ubiquinone to the other respiratory components. For this purpose the titre of antimycin required for total inhibition of electron transport was determined by extrapolation from experiments such as presented in Figure 7. On the assumption of one binding site for antimycin on each bc_1 complex (Berden and Slater, 1970) the ratio of ubiquinone to bc_1 complex of the mitochondria used in the present study was approximately 31:1. Schindler *et al.* (1984) expressed the ubiquinone content of Jerusalem artichoke mitochondria as a ratio of 40 per cytochrome aa_3, and as 17 when related to total cytochromes (b + c). The amount of cytochrome aa_3 present in

Figure 7. The inhibition of succinate, exogenous NADH and DQH_2 oxidation by antimycin.

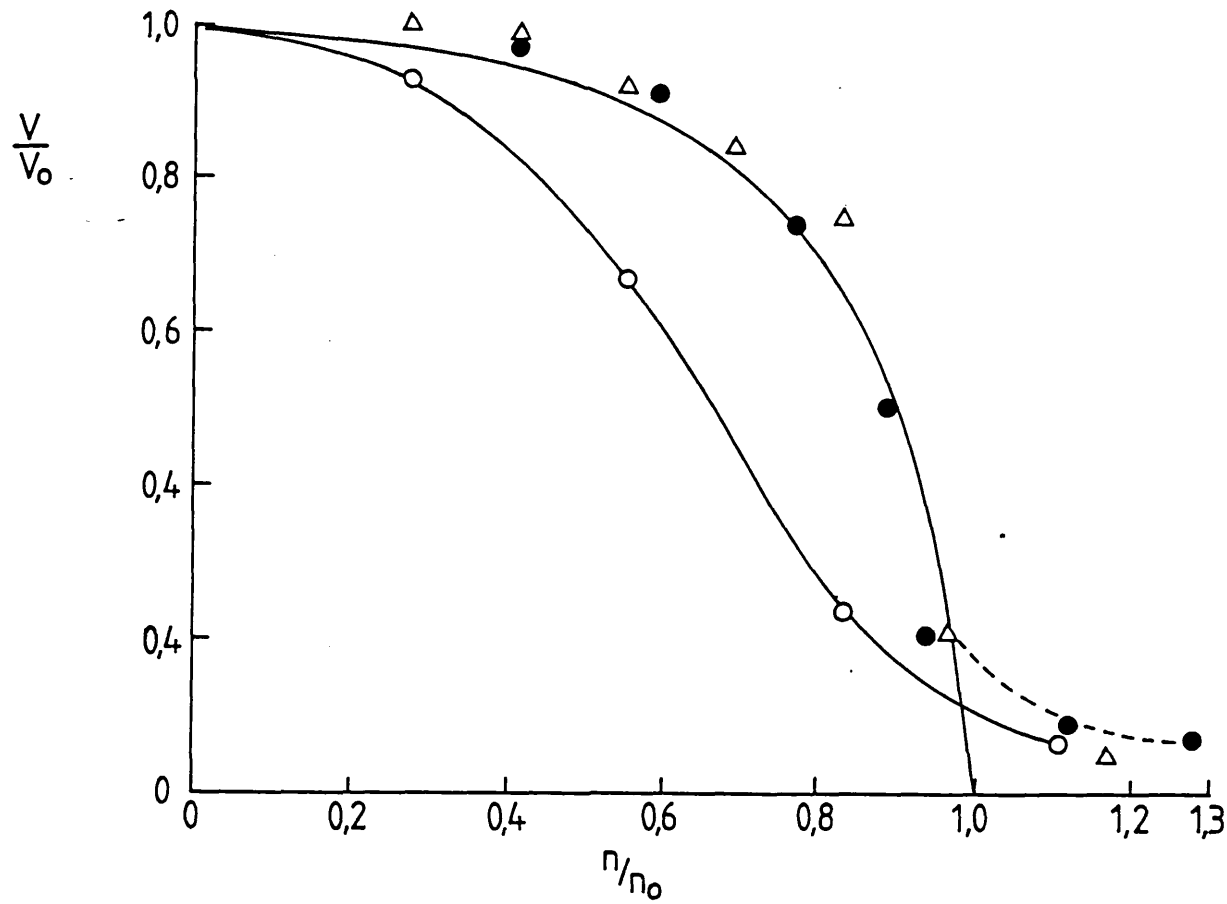
Mitochondria were prepared and succinate dehydrogenase activated as described in sections 2.1 and 2.2 respectively. Rates of oxygen uptake were measured in standard assay medium at 25°C as described in section 2.3. FCCP (0.2μM) was present in all assays to uncouple respiration. Mitochondria (0.37 mg protein/ml) were incubated with antimycin for 2 min at 25°C prior to initiation of the assay by addition of substrate. The final concentrations of substrate used were: succinate, 20mM; NADH, 1mM and DQH_2, 1mM. The uninhibited rates of oxygen uptake were, in nmol O_2/min/mg protein, 176 for succinate, 180 for NADH and 249 for DQH_2. Rates are expressed as a fraction of the uninhibited rate (V/V_o) and antimycin as a fraction of the amount necessary to give full inhibition, determined by extrapolation from the experimental points to zero rate.

△ succinate oxidation

● NADH oxidation

○ DQH_2 oxidation.

Figure 7. The inhibition of succinate, exogenous NADH and DQH_2 oxidation by antimycin.



the mitochondria used for the present study was 0.1 nmol/mg protein (Table 8), resulting in a ratio of ubiquinone per cytochrome aa_3 of 27. The ratio expressed in terms of cytochrome (b + c), using the data presented in Table 8, was 11. The amount of cytochrome (b + c) will clearly be larger than the amount of bc_1 complex, because of the presence of cytochrome c_{550}, and so result in a lower ratio when related to ubiquinone content. Other estimates of ubiquinone pool-size in plant mitochondria have been made by combining separate reported determinations of cytochrome aa_3 and ubiquinone content (Hauska and Hurt, 1982). Ratios of ubiquinone:cytochrome aa_3 were estimated for Arum mitochondria to be 7 (although Schindler *et al.* (1984) reported a value of 30) and for yeast mitochondria to be 36. In general the amount of ubiquinone present in plant mitochondria seems to be greater than that present in mammalian mitochondria: the ratio of active ubiquinone to cytochrome aa_3 was 6-8 as determined by Kroger and Klingenberg (1967, 1973a), and a value of 9 can be calculated from the data presented by Gupte *et al.* (1984). The size of the ubiquinone pool is of interest in assessing the degree to which diffusion of ubiquinone may limit respiration (section 7.2), since the distance ubiquinone is able to move between sites of oxidation and reduction will increase with increasing pool-size for a given rate of electron transport (see Rich, 1984).

3.2 The interaction of succinate oxidase and exogenous NADH oxidase.

3.2.1 Spectrophotometric measurement of NADH oxidation.

The starting point for this study was the observation of Cowley (1977) and Cowley and Palmer (1980) of an asymmetric interaction between succinate and NADH oxidase (section 1.3.3). The rate of succinate oxidation was unaffected (Cowley and Palmer, 1980) or stimulated (Cowley, 1977) by the addition of NADH, but NADH oxidation could be strongly inhibited by the oxidation of succinate. In order to study this interaction it was necessary to be able to determine the rate of oxidation of each substrate in the presence of the other. This was achieved by spectrophotometric measurement of NADH oxidation in either split-beam (340nm) or dual-beam (340nm - 374nm) mode. With both methods the relation between NADH concentration and absorbance was nearly linear up to absorbance values of 0.8 In a cell with an optical path length of 1cm it was therefore possible to accurately monitor NADH concentrations of up to 130μM in split beam mode (ε= 6.22$mM^{-1}.cm^{-1}$, Horecker and Kornberg, 1948) or of up to 170μM in dual beam mode (ε=4.70 $mM^{-1}.cm^{-1}$, see section 2.4). The use of a cuvette of path length 2mm increased the upper concentration limit for linear monitoring of NADH concentration to 0.85mM (dual-beam mode).

The rate of succinate oxidation in the presence of NADH is the only rate that cannot be found directly by measurements of oxygen uptake or by spectrophotometric measurement of NADH oxidation. This rate was determined by measuring the rate of oxygen uptake in the presence of both substrates and the rate of NADH oxidation spectrophotometrically under identical conditions. The rate of succinate oxidation was then found by subtraction.

3.2.2 The initial rapid phase of NADH oxidation.

As shown in Figure 8 an initial rapid phase of NADH oxidation can be resolved spectrophotometrically. This fast phase could be seen using coupled mitochondria in the absence of added ADP (the substrate-state) but not in state 3 or in the presence of uncoupler (Figure 8). Cowley (1977) attributed this rapid phase to the presence of low amounts of ADP added with the mitochondria, formed by hydrolysis of some of the ATP added to activate succinate dehydrogenase (section 2.2). This rapid phase was typically of 15 to 20 s duration, after which a linear rate of oxidation was observed until NADH concentration became limiting. As the duration of the fast phase was similar to the time taken for the electrical component of the membrane potential to reach a steady-state, as indicated using safranine (Figure 11), and as the fast phase persisted even when succinate dehydrogenase had not been activated (not shown), it may be that part of the fast phase was due to the time taken for a protonmotive force to become established. Additions of succinate were made during the slower phase of oxidation, although no difference in the final rate of oxidation was found if succinate was added during the fast phase or prior to the addition of NADH (Figure 8, traces A and D).

3.2.3 The inhibition of NADH oxidation by succinate.

Addition of succinate to mitochondria oxidising NADH caused an inhibition of NADH oxidation, the degree of inhibition depending both on the respiratory state and the concentration of succinate (Figures 9 and 10). There was a larger inhibition of NADH oxidation by 4mM succinate, expressed as percentage inhibition of the rate in the absence of succinate, in the substrate-state (61%) than in state 3 (41%) and succinate had very little effect upon uncoupled mitochondria (7%, Figure 8). These results are in agreement with

Figure 8. The inhibition of exogenous NADH oxidase by succinate.

Mitochondria were prepared and succinate dehydrogenase activated as described in sections 2.1 and 2.2 respectively. Rates of NADH oxidation were measured in a 1cm optical path length cuvette by dual wavelength spectroscopy as described in section 2.7. NADH (150uM) was added to mitochondria (0.18 mg protein/ml) to initiate the assay. (A) Substrate-state (no additions), (B) State 3 (0.25mM ADP), (C) Uncoupled (+ 0.2uM FCCP), (D) Prior addition of succinate. Figures are 0.5 x nmol NADH/min/mg protein to be comparable to rates of oxygen uptake.

Figure 8. The inhibition of exogenous NADH oxidase by succinate.

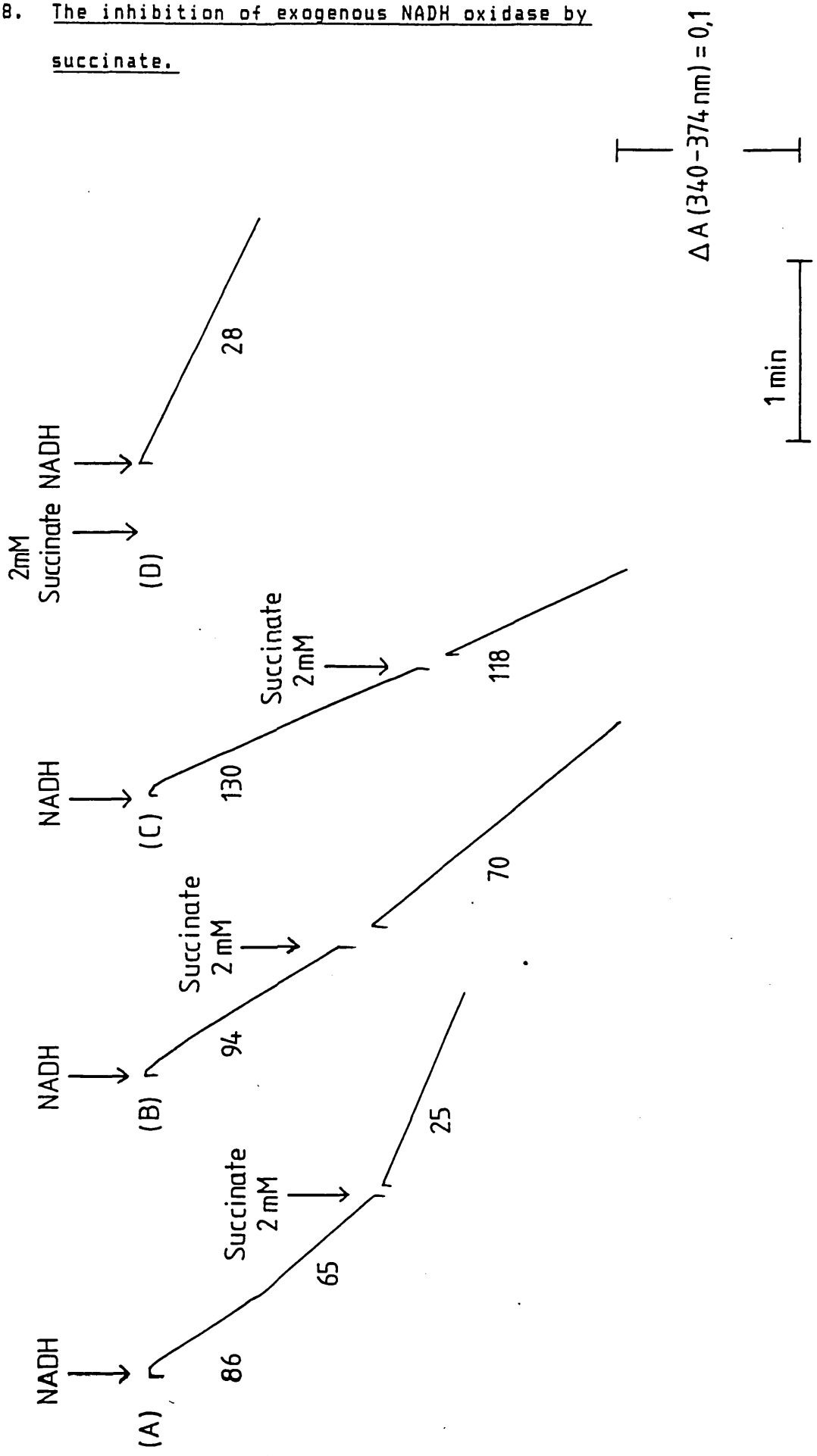
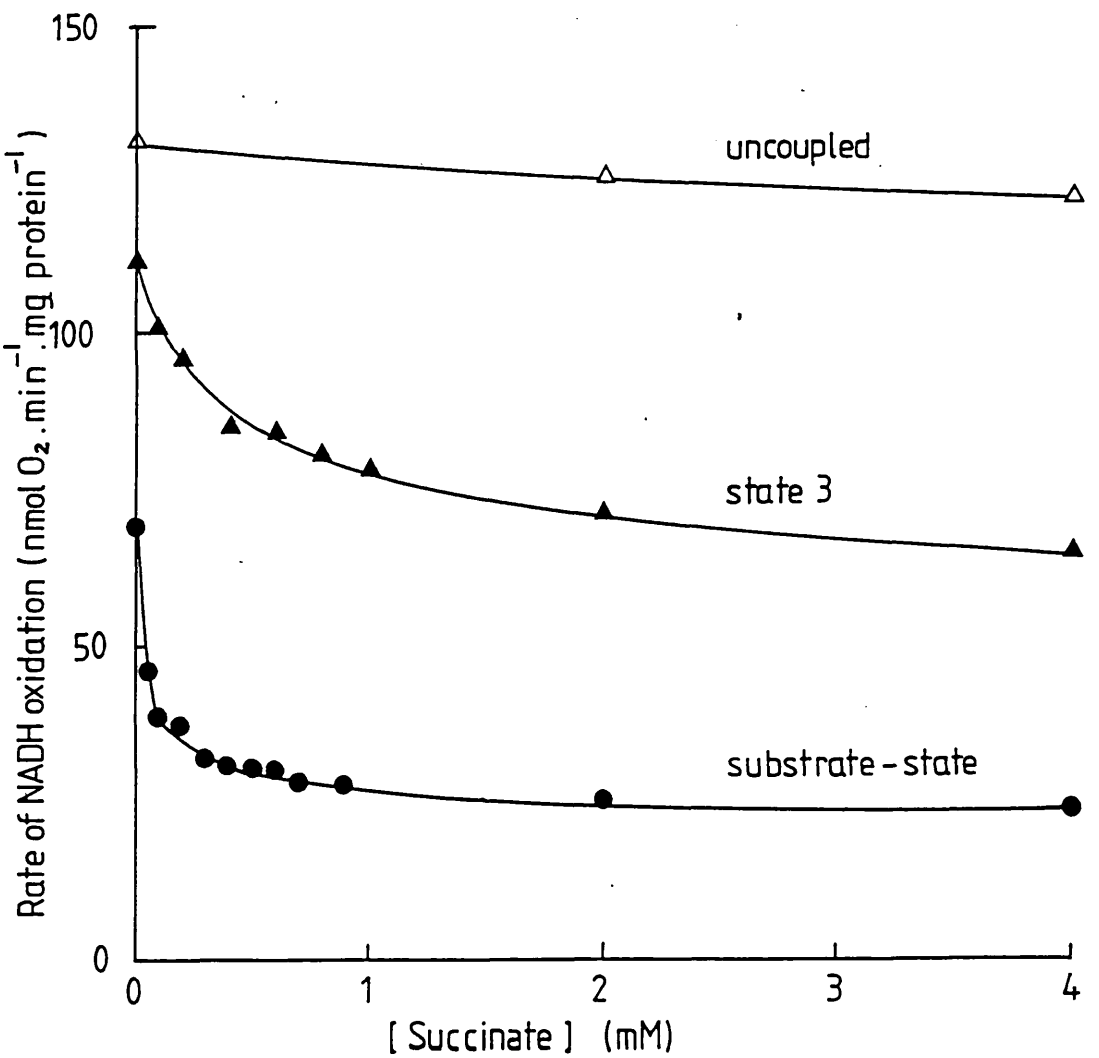


Figure 9. The effect of respiratory state on the inhibition of NADH oxidase by succinate.

The inhibition of the rate of NADH oxidation upon addition of succinate was measured as described in the legend to Figure 8. Additions of succinate were made 1 min after the addition of NADH. The mitochondrial protein concentration was 0.22mg/ml.

- ● Substrate state (no additions),
- ▲ State 3 (+ 0.25mM ADP),
- △ Uncoupled (+ 0.2µM FCCP).



those of Cowley (1977) and Cowley and Palmer (1980), although in these previous studies no inhibition of NADH oxidation by succinate was reported when the mitochondria were uncoupled. It is shown later (Figure 14) that 0.2μM FCCP was sufficient to cause complete uncoupling under these conditions. The concentration of succinate necessary for half-maximal inhibition of NADH oxidation was less in the substrate-state than under state 3 conditions. This suggested that the degree of inhibition of NADH oxidation was related to the capacity of the cytochrome pathway to oxidise ubiquinol, as the activity of the ubiquinol oxidising pathway was greater in state 3 than in the substrate-state. This was because, if inhibition of NADH oxidation was dependent upon rate limitation at the level of ubiquinol oxidation, then a slower rate of ubiquinone reduction by succinate dehydrogenase (and therefore a lower succinate concentration) would be necessary in the substrate state than in state 3 to keep the bulk of the ubiquinone reduced.

3.2.4 The inhibition of succinate oxidase by exogenous NADH.

The effect of NADH on succinate oxidase activity was determined indirectly as described above by combining polarographic and spectrophotometric measurements. As shown in Figure 10, the inhibition of NADH oxidation paralleled the rate of succinate oxidation, showing a close link between succinate oxidase activity and the inhibition of NADH oxidase. This link is in contrast to the inhibition of NADH oxidase by citrate (Coleman and Palmer, 1971) where chelation of Ca^{2+} caused inhibition (section 1.1.1). It has been shown previously that inhibition of NADH oxidase by succinate required oxidation of succinate, as malonate, a competitive inhibitor of succinate dehydrogenase (section 1.1.3), prevented inhibition of NADH oxidase by succinate (Cowley and Palmer, 1980). The maximal decrease of NADH oxidase in the presence of succinate (52nmol

Figure 10. The inhibition of succinate oxidase by NADH oxidase.

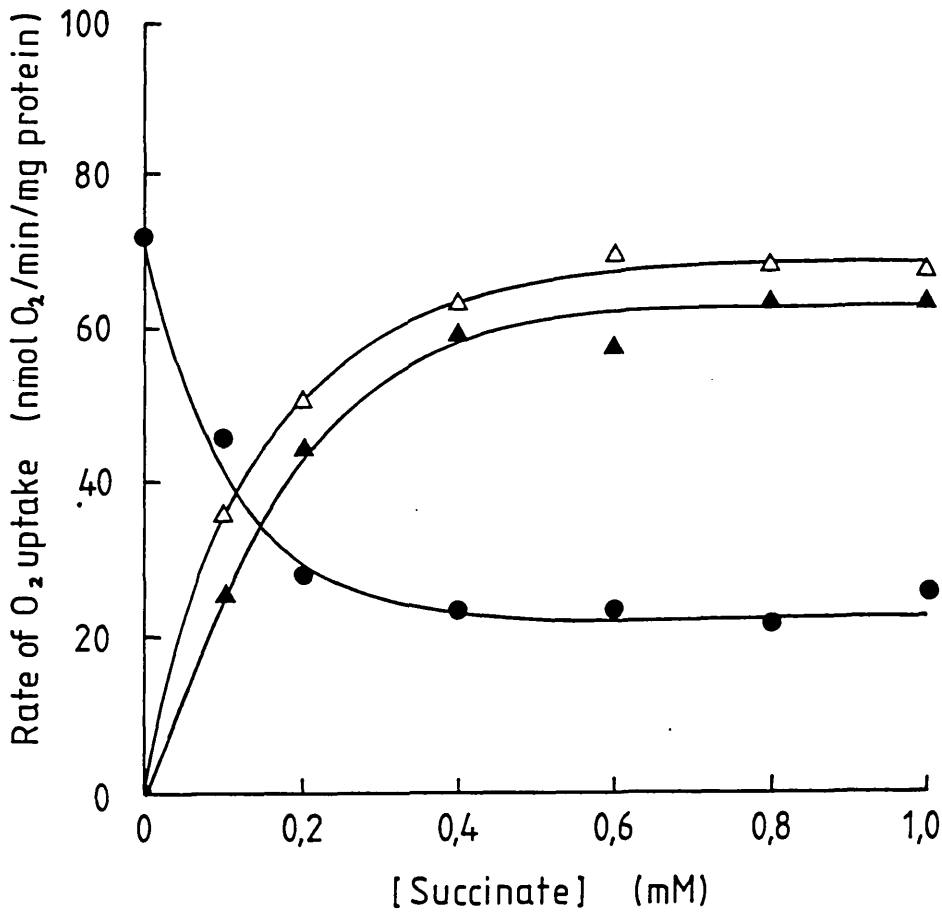
Mitochondria were prepared and succinate dehydrogenase activated as described in sections 2.1 and 2.2 respectively. Parallel measurements of oxygen uptake (section 2.3) and NADH oxidation were made at 25°C in standard assay medium at a mitochondrial protein concentration of 0.16 mg/ml. Rates of NADH oxidation were measured by dual wavelength spectroscopy in a cuvette of optical path length 0.2cm as described in section 2.7. Assays of oxygen uptake and NADH oxidation were initiated by addition of 0.8mM NADH, followed 1 min later by the appropriate concentration of succinate.

△ succinate oxidation

● NADH oxidation in the presence of succinate,

▲ succinate oxidation in the presence of NADH (determined by subtraction: see text).

Figure 10. The inhibition of succinate oxidase by NADH oxidase.



O_2/min/mg protein) was strikingly larger than the corresponding decrease of succinate oxidase in the presence of NADH (4-12nmol O_2/min/mg protein), in agreement with previous findings (Cowley, 1977, Cowley and Palmer, 1980). The asymmetry of the interaction was despite the fact that succinate and NADH oxidase activities were very similar when measured separately (Figure 10). According to the model of ubiquinone function of Kroger and Klingnberg (1973a,b), substrates that are oxidised at equal rates when oxidised alone should mutually inhibit each other equally when oxidised together (see section 1.3). Cowley and Palmer (1980) examined the possibility that succinate could reduce exogenous NAD^+ by reverse electron transport, leading to an apparent inhibition of NADH oxidase, but no succinate-exogenous NAD^+ reductase activity could be detected, nor would this be an energetically favourable reaction.

3.3 Factors determining inhibition of NADH oxidation by succinate oxidase.

As the inhibition of NADH oxidation was dependent on respiratory state (Figure 9) and did not appear to follow ideal ubiquinone pool behaviour (section 1.3.3, Figure 10, and see above), it was possible that a number of factors might be involved, such as membrane potential (since this will influence redox potentials where the centres involved span the dielectric of the membrane), or the presence of adenine nucleotides (cf Sotthubandhu and Palmer, 1975), or that NADH led to an activation of succinate dehydrogenase above that achieved by incubation with ATP (section 2.2) such that full succinate dehydrogenase activity was only expressed in the presence of NADH, or that exogenous NADH dehydrogenase was oxidised by a fundamentally different mechanism to that of succinate (Tomlinson and Moreland, 1975), or finally, that exogenous NADH dehydrogenase was inhibited by a direct interaction with succinate dehydrogenase in the presence of succinate. Stimulation of the activity of plant mitochondrial succinate dehydrogenase by a variety of effectors, including ATP, ADP, NADH, NAD^+-linked substrates and ubiquinol-10 has been reported (Oestreicher *et al.*, 1973). The activation by NADH and NAD^+-linked substrates of succinate oxidation was thought to be mediated by endogenous ubiquinol. In the present study experiments were therefore carried out to determine the factors giving rise to the asymmetric inhibition of NADH oxidation by succinate. In order to test the possible involvement of adenine nucleotides an alternative means of altering the capacity of the cytochrome pathway was neccesary than by the presence or absence of ADP. The capacity of the cytochrome pathway was increased by progressive uncoupling, using low concentrations of FCCP. In this way the correlation between membrane potential and inhibition of NADH

oxidase by succinate could be followed.

3.3.1 The use of safranine to monitor membrane potential.

Safranine is a membrane permeant cation which can enter mitochondria under the influence of a membrane potential ($\Delta\Psi$, positive outside). Entry into the mitochondria is accompanied by binding of the dye to sites on the inner face of the inner membrane. This binding is believed to bring safranine molecules sufficiently close together to give rise to the changes in the absorbtion spectrum and extent of absorbtion which are used to monitor membrane potential (Colonna *et al.*, 1973). This method has been applied to mammalian (Akerman and Wikström, 1976, Zanotti and Azzone, 1980) and plant mitochondria (Moore and Bonner, 1982) and under certain conditions a linear relationship has been reported between membrane potential and safranine response, within the approximate range 50 to 160mV. The membrane potentials maintained in state 4 by a variety of plant mitochondria, measured by the safranine method, are in the region of 130mV (Moore and Bonner, 1982). Typical responses of safranine to energisation of Jerusalem artichoke mitochondria are shown in Figure 11. Following addition of succinate or NADH to mitochondria in the presence of safranine a large signal appeared, taking 10-15 s to reach half maximum size. Once established, the signal remained constant until oxygen became exhausted. Rapid collapse of the signal was seen upon addition of antimycin or FCCP. Valinomycin also abolished the signal, presumably by allowing rapid influx of the K^+ present in the assay medium (the pH of the standard assay medium was adjusted using KOH, section 2.3).

There are two closely related practical problems in the use of safranine. These are the dye to protein ratio to be used, and the calibration of safranine response with a known membrane potential. The effect of dye:protein ratio on the safranine signal is shown in

Figure 11. Monitoring of the mitochondrial membrane potential using safranine.

Mitochondria (0.34 mg protein/ml) were added to standard assay medium containing 10μM safranine. Mitochondrial membrane potential was monitored at 511-533nm (section 2.8). For each trace a membrane potential was induced by addition of substrate: identical signals were observed following addition of either 10mM succinate or 0.5mM NADH. Other additions are as indicated.

Figure 11. Monitoring the mitochondrial membrane potential using safranine.

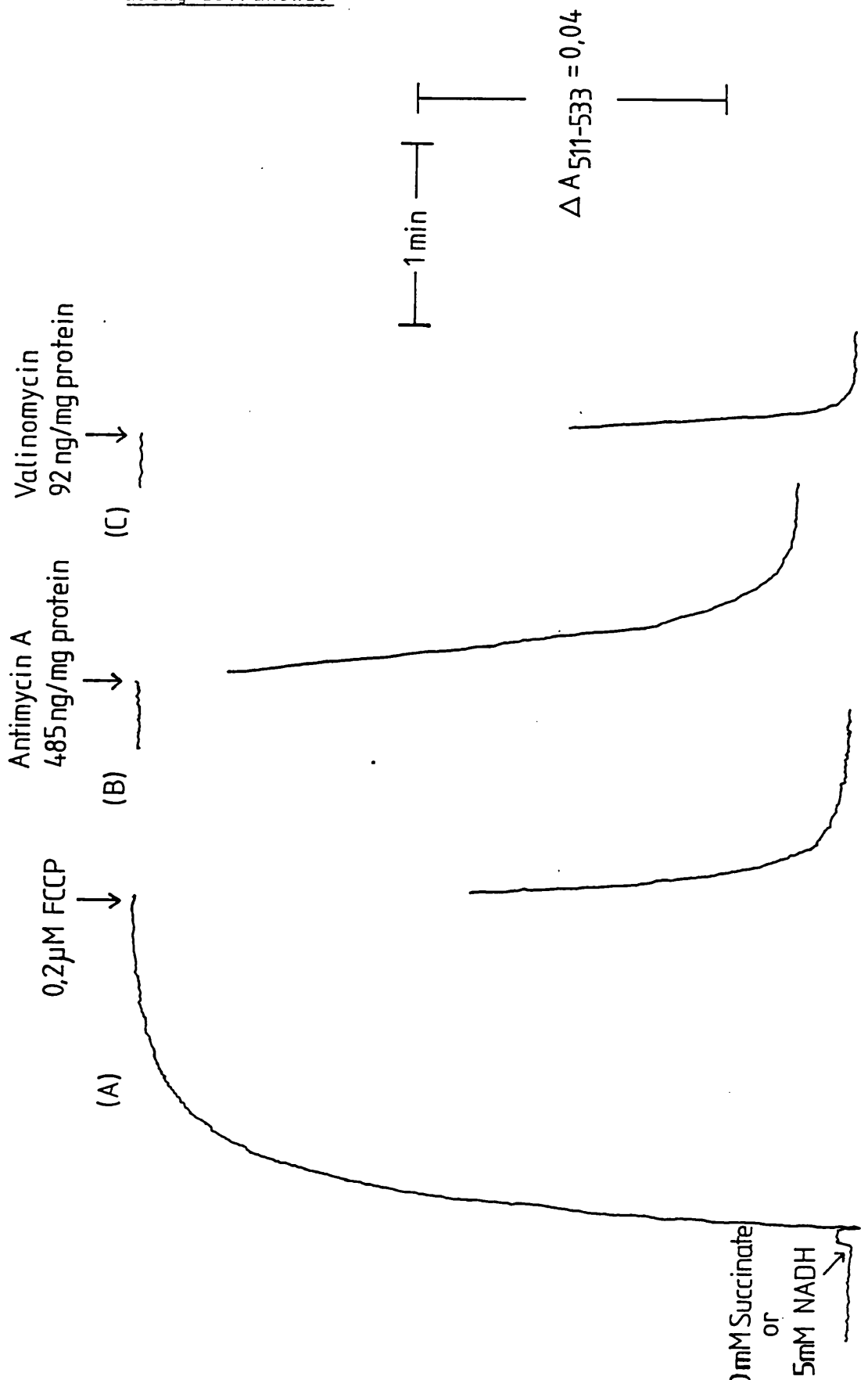


Figure 12. The effect of dye:protein ratio on safranine response.

The maximum absorbance change following addition of 0.5mM NADH to a suspension of mitochondria in standard assay medium was measured as described in the legend to figure 11. Protein concentrations were as indicated.

△ + 5μM safranine

● + 10μM safranine

▲ + 20 μM safranine.

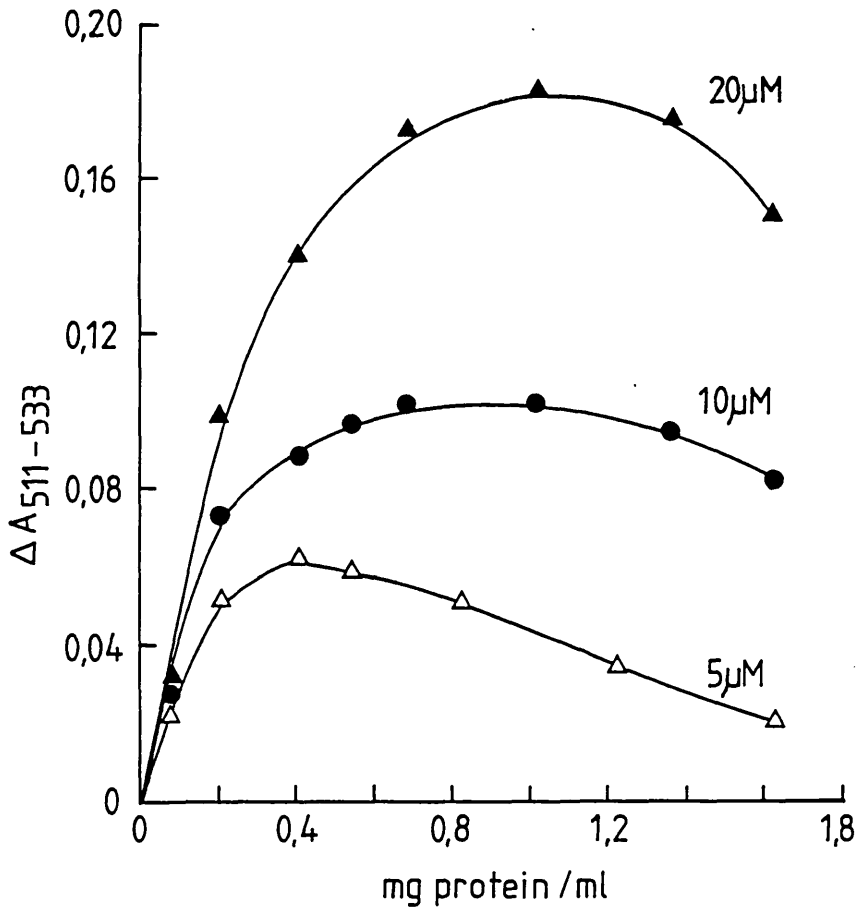


Figure 12. Optimum signal size was obtained at a dye:protein ratio of 15nmol/mg protein, with a rapid decline either side of this value. This pattern is the same as reported by Zanotti and Azzone (1980) and was interpreted as showing that at low protein values the number of potential binding sites is less than the safranine available to bind, while at high protein levels a bound safranine molecule is less likely to be stacked next to another safranine molecule because of the excess of binding sites available, and so does not show the spectral shift being monitored. Calibration of the response of safranine to membrane potential is usually performed by the use of valinomycin, which allows the high matrix concentration of K^+ to move out of the mitochondria in response to its concentration gradient. As accompanying anion movement is not facilitated a membrane potential is established which can be calculated using the Nernst equation if both internal and external K^+ concentrations are known. It is a strength of this method that the matrix K^+ concentration is large compared to the external concentration and so errors in determination of matrix K^+, which involve measurement of matrix volume, have only a small effect on the accuracy of the calculated potential. The potential is then decreased by the addition of known concentrations of KCl to the external medium.

Despite the uncoupling action of valinomycin when external K^+ was present (Figure 11), only very small responses of safranine were observed when valimomycin was added to mitochondria suspended in low K^+ medium (Figure 11, trace C). Further experiments showed that a significant signal appeared upon addition of mitochondria to medium containing safranine, without the need for valinomycin. A rapid decrease in signal, attributable to increased light scatter upon addition of mitochondria, was followed by induction of a large signal with a half time of less than 1 min. This signal was not due to

oxidation of residual substrate as addition of antimycin had no effect (Figure 13, trace A). The signal could be collapsed by the addition of KCl alone showing that these mitochondria had a significant permeability to K^+. The collapse continued steadily as indicated without reaching a new steady-state. Subsequent addition of valinomycin rapidly collapsed the signal, as did FCCP (Figure 13, trace B). The collapse upon addition of FCCP was not dependent on added KCl (not shown). As valinomycin alone caused no additional increase in the signal (Figure 13, trace A), it was concluded that this signal was probably due to a K^+ concentration gradient established without the need for valinomycin. This signal was not seen using standard assay medium because of the high external K^+ concentration. Because of the apparent K^+ leak in these mitochondria it was not possible to calibrate the safranine response using a diffusion potential.

The dye:protein ratio is crucial in obtaining a linear response of safranine to membrane potential (Zanotti and Azzone, 1980, Moore and Bonner, 1982) and generally a more linear response is seen at high dye:protein ratios. Consequently a dye:protein ratio of 20-25:1 was used in this study, rather than the optimum for signal size of 15:1 (Figure 12).

As shown in Figure 14, low concentrations of FCCP progressively decreased the magnitude of the safranine signal during the oxidation of exogenous NADH. Initially this decrease was small, corresponding to increased rates of NADH oxidation, but as the rate of NADH oxidation increased to the maximum rate, the safranine signal decreased linearly with increasing FCCP concentration. The inhibition of NADH oxidation by the addition of succinate was most severe in the absence of uncoupler. The decreasing inhibition of exogenous NADH oxidation by succinate with increasing FCCP concentration correlated

Figure 13. Passive safranine signal in the absence of respiratory substrate.

Mitochondria (0.41 mg protein/ml) were suspended in low K^+ medium (standard assay medium prepared using sodium phosphate and NaOH solution to adjust pH). The response of safranine to membrane potential was monitored at 511-533nm as desribed in section 2.8 and additions were made as indicated.

Figure 13. Passive safranine signal in the absence of respiratory substrates.

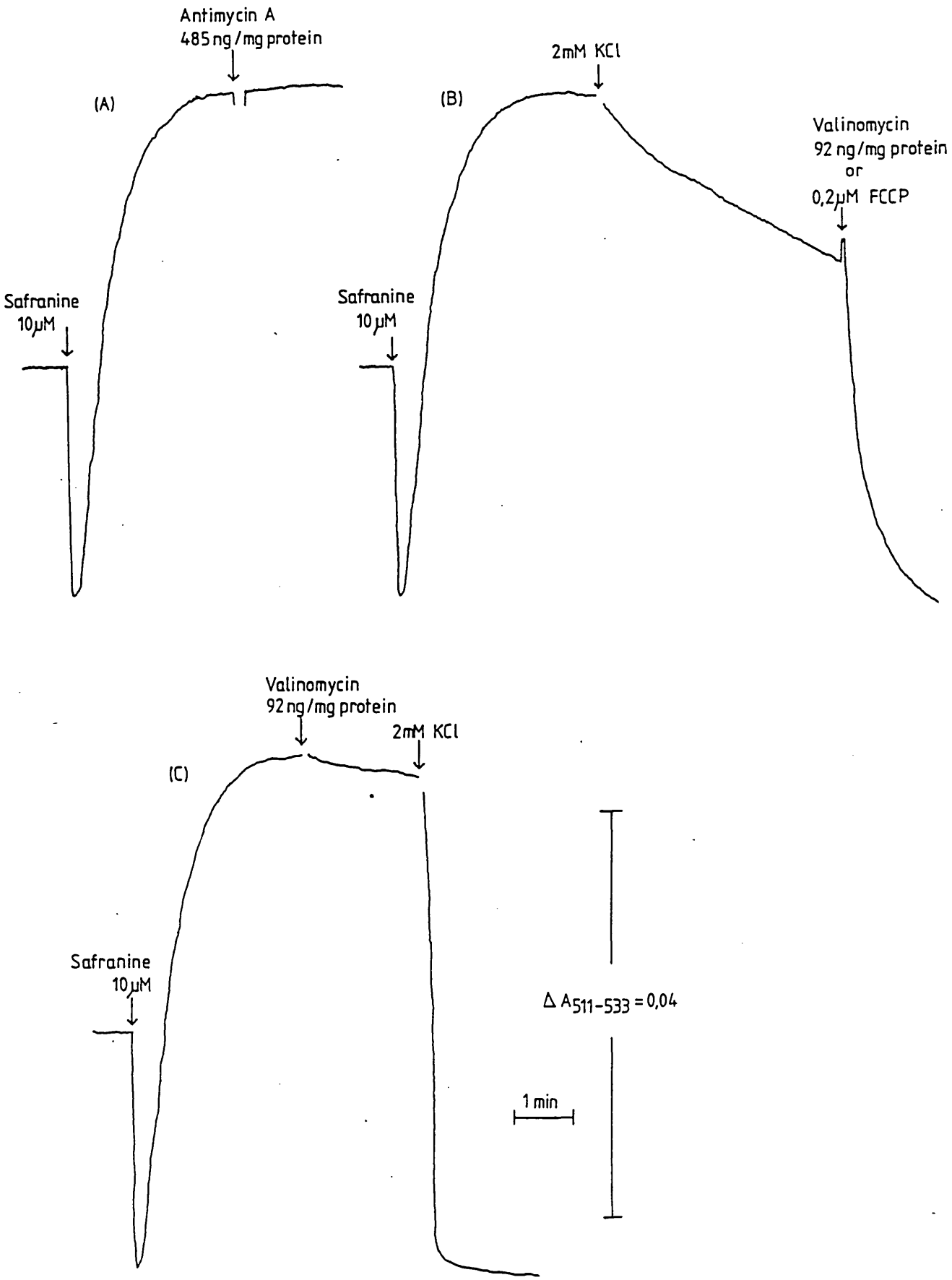


Figure 14. Correlation of membrane potential, NADH oxidation rate and inhibition of NADH oxidation by succinate.

Mitochondria were prepared and succinate dehydrogenase activated as described in sections 2.1 and 2.2 respectively. Mitochondria (0.12 mg protein/ml) were suspended in standard assay medium containing 5μM safranine and concentrations of FCCP as indicated. The rate of oxidation of 0.2mM NADH was monitored spectrophotometrically (section 2.7) in a cuvette with a 1cm optical path length. After 1 min, 10mM succinate was added and the subsequent rate of NADH oxidation recorded. In a parallel experiment the membrane potential generated by NADH oxidation under identical conditions was monitored at 511-533nm (section 2.8).

△ Safranine signal

● Rate of NADH oxidation

▲ % inhibition of NADH oxidation by addition of succinate.

Figure 14. Correlation of membrane potential, NADH oxidation rate and inhibition of NADH oxidation by succinate.

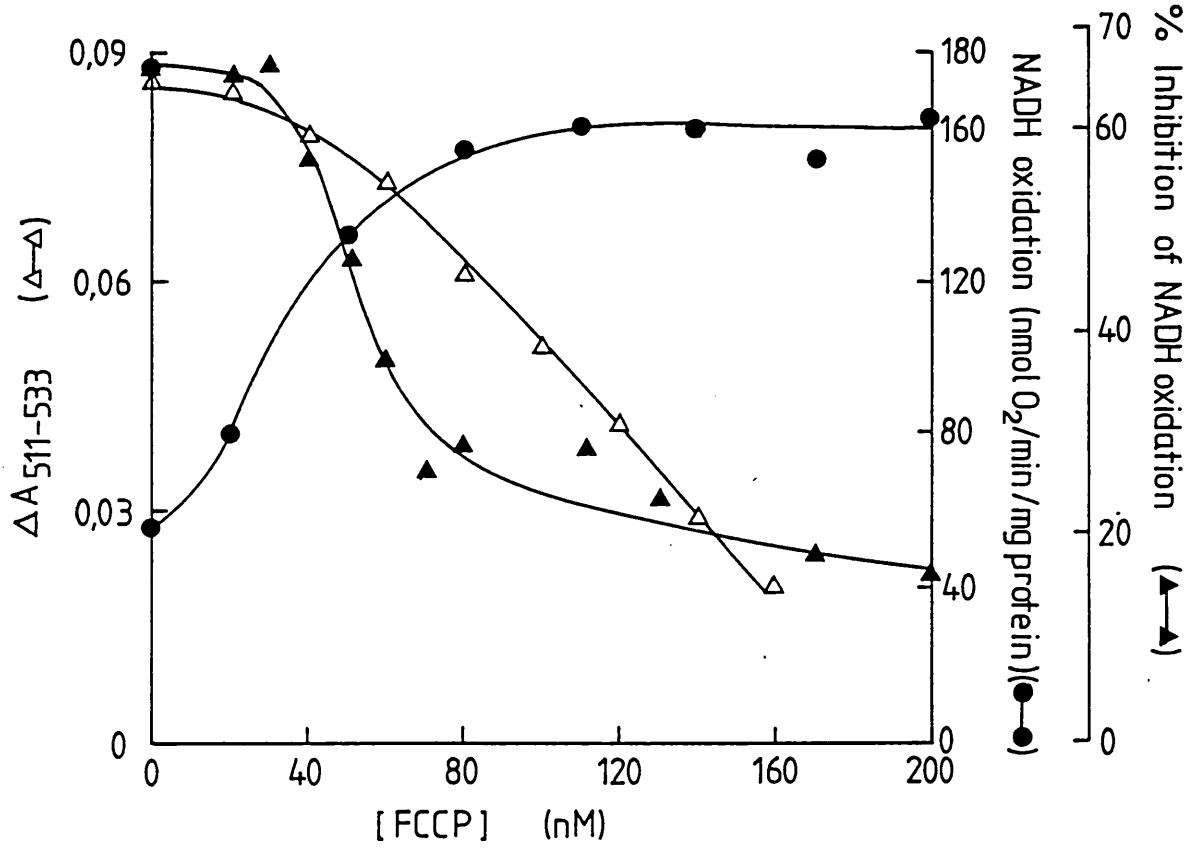
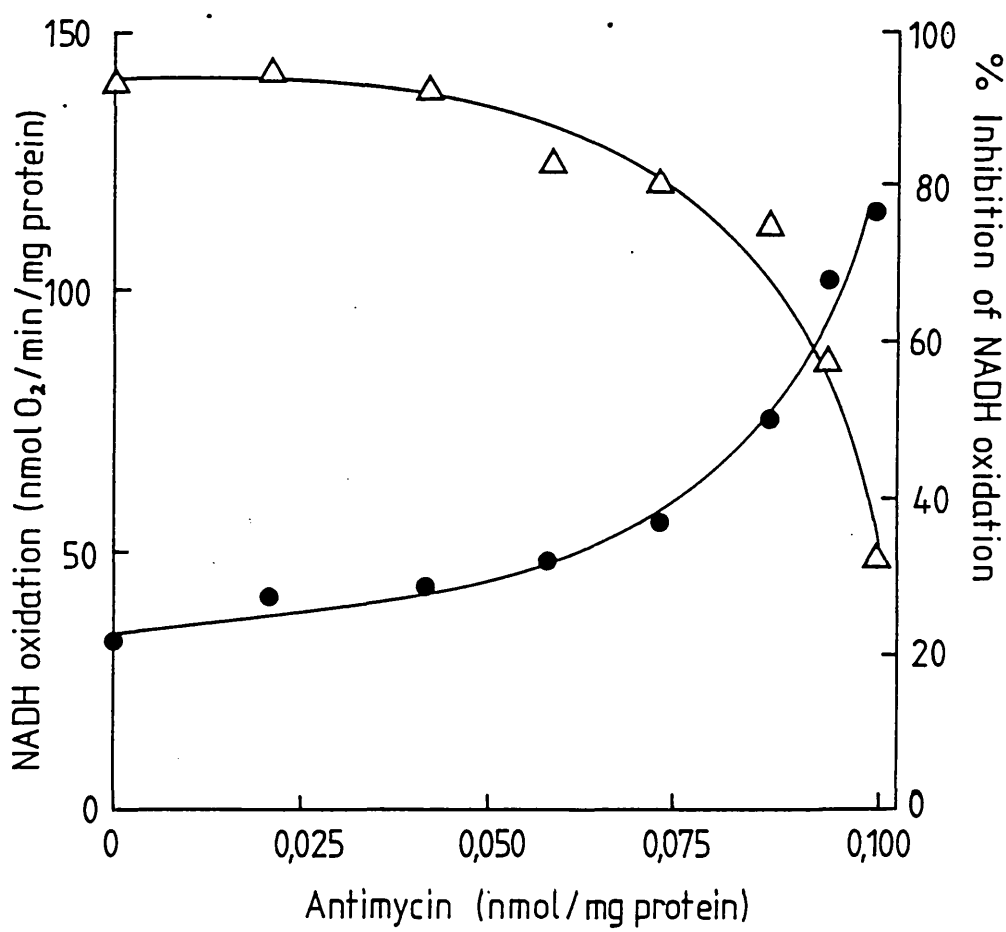


Figure 15. Effect of antimycin on inhibition of NADH oxidation by succinate in uncoupled mitochondria.

Mitochondria were prepared and succinate dehydrogenase activated as described in sections 2.1 and 2.2 respectively. Mitochondria (0.21 mg protein/ml) were suspended in standard assay medium containing 0.2μM FCCP and, where indicated, preincubated with antimycin for 2 min at room temperature. Oxidation of 0.2mM NADH was monitored spectrophotometrically (section 2.7) in a cuvette with a 1cm optical path length. After 1 min 10mM succinate was added and the new rate of NADH oxidation recorded.

△ Rate of NADH oxidation

● % inhibition of NADH oxidation by addition of succinate.



with the increasing rate of NADH oxidation, rather than with the membrane potential, as measured using safranine. It was concluded that adenine nucleotides played no part in the inhibition of NADH oxidation by succinate oxidation as none were added during this experiment, and that the inhibition was not directly related to the membrane potential, but correlated better with the activity of the cytochrome pathway as measured by the uninhibited rate of NADH oxidation. These conclusions were tested by restricting the activity of the cytochrome pathway using antimycin and fully uncoupled mitochondria (Figure 15). An inverse correlation was found between the degree of inhibition of NADH oxidation by succinate and rate of oxidation of NADH in the absence of succinate. This confirmed that a membrane potential was unecessary for the interaction.

The possibility that succinate dehydrogenase itself could directly inhibit the exogenous NADH dehydrogenase was tested by measuring the additivity of succinate and ubiquinone-1 reductase activities (Table 3). The succinate-ubiquinone-1 reductase activity in the presence of NADH was calculated using measurements of NADH oxidation under identical conditions. The rate of ubiquinone-1 reduction in the presence of both substrates was very close to the sum of the rates when the substrates were oxidised alone. There was no evidence of either extra activation of succinate dehydrogenase in the presence of NADH, or of inhibition of NADH dehydrogenase in the presence of succinate. The site of interaction of the two substrates was therefore located between the sites of ubiquinone reduction and the site of antimycin binding: most probably at the level of ubiquinone.

As the interaction was not related to membrane potential, adenine nucleotides or to allosteric interaction between the dehydrogenases, but only to the activities of the ubiquinone reducing and oxidising

components, the kinetic behaviour of the two substrates when oxidised alone was investigated, since the asymmetry of the interaction between them was clearly contrary to the ideal model of ubiquinone pool behavior (section 1.2).

Table 3. Interaction of succinate and NADH dependent UQ-1 reductase activities.

Mitochondria (0.075mg protein/ml) were suspended in standard assay medium containing 1mM KCN and 32μM UQ-1. Either NADH (0.2mM) or succinate (20mM), or both, were added and the rate of UQ-1 reduction measured at 420nm (section 2.7). Identical experiments were performed monitoring NADH oxidation and NADH oxidation in the presence of succinate, at 340-374nm by dual wavelength spectrophotometry (section 2.7). Results are means and standard deviations of four preparations.

(Rates are nmol/min/mg protein)

Substrate(s)	Rate of UQ-1 reduction	Rate of NADH oxidation
NADH	983 ± 37	995 ± 15
Succinate	425 ± 28	-
NADH + Succinate	1474 ± 41	962 ± 21

Chapter 4. The interaction of HQNO with the respiratory chain.

4.1 Inhibition of electron transport by HQNO.

The ubiquinone analogue 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO,Figure 2) is a potent inhibitor of quinol oxidation by the mammalian mitochondrial bc_1 complex (Nijs, 1967, Brandon *et al.*, 1972, Eisenbach and Gutman, 1975, Izzo *et al.*, 1978) and of the b_6f complex of higher plant chloroplasts (Selak and Whitmarsh, 1982). Like antimycin, HQNO inhibits reoxidation of the b cytochromes by quinone, giving rise to the phenomenon of oxidant induced reduction of the b cytochromes (section 1.1.4). The binding of HQNO and antimycin to mammalian submitochondrial particles are mutually exclusive (Nijs, 1967, van Ark and Berden, 1977), but their inhibitory effects are not identical. In addition to inhibiting the oxidation of the b cytochromes, HQNO also slows the rate of reduction of the b cytochromes during oxidant induced reduction (Eisenbach and Gutman, 1975, Izzo *et al.*, 1978, Papa *et al.*, 1982). This suggests that the concerted oxidation of quinol by the Rieske centre and b cytochromes is also inhibited by HQNO, though not by antimycin. A second difference between the effects of the binding of these two inhibitors is that binding of antimycin causes a 1-2nm red shift in the alpha peak of cytochrome b_{562} of the mammalian bc_1 complex, but no spectral shift accompanies the binding of HQNO (Izzo *et al.*, 1978).

The suitability of antimycin as a probe for ideal ubiquinone pool behaviour has been discussed above (Section 1.2.2). A comparison of the use of antimycin and HQNO for this purpose was made by Zhu *et al.* (1982), who concluded that while HQNO could be used to test pool behaviour, antimycin should not. This unsuitability may be due to the complexity of the effects of antimycin, probably involving

comformational changes rather than simple binding and inhibition (see Reiske, 1976 for a discussion).

In this chapter results relating to the sites of HQNO inhibition in the plant respiratory chain are presented and the pathways of oxidation of DQH_2, succinate and exogenous NADH are discussed. In section 4.2 the reason is sought for the resistance of exogenous NADH oxidation to inhibition by HQNO.

The oxidation of succinate, DQH_2, and exogenous NADH by uncoupled Jerusalem artichoke mitochondria differed markedly in their sensitivity to inhibition by HQNO as is shown in Figure 16. Oxidation of DQH_2 was the most inhibited by low concentrations of HQNO, as was also found using antimycin (Figure 7), and as would have been predicted if DQH_2 was oxidised directly by the bc_1 complex rather than via the bulk ubiquinone. Although equally inhibited by low concentrations of HQNO, the oxidation of NADH required a ten fold higher concentration of HQNO for 90% inhibition compared to that necessary for succinate oxidation (Figure 16). Resistance of cyanide sensitive NADH oxidation to HQNO was not reported by Tomlinson and Moreland (1975), probably because the concentration used in their study (10μM) was sufficient to inhibit all substrates equally. The form of inhibition of DQH_2 oxidation by HQNO was found to be non-competitive with respect to DQH_2 (Figure 17), with an apparent Ki of 127nM. This value is half the value for the dissociation constant determined (64nM) for HQNO specifically bound to beef-heart submitochondrial particles, determined by fluorimetric methods (van Ark and Berden, 1977). In view of this difference the binding of HQNO to Jerusalem artichoke mitochondria was studied using a similar method to that used by van Ark and Berden (1977).

Figure 16. Inhibition of oxidation of oxidation of NADH, succinate and DQH_2 by HQNO.

Mitochondria were prepared and succinate dehydrogenase activated as described in sections 2.1 and 2.2 respectively. Mitochondria (0.30mg protein/ml) were suspended in standard assay medium containing 0.2μM FCCP and HQNO concentrations as indicated. After 2 min incubation with inhibitor, substrate was added and oxygen uptake was measured at 25°C (section 2.3).

▲ + 20mM succinate

△ + 1mM NADH

○ + 1mM DQH_2.

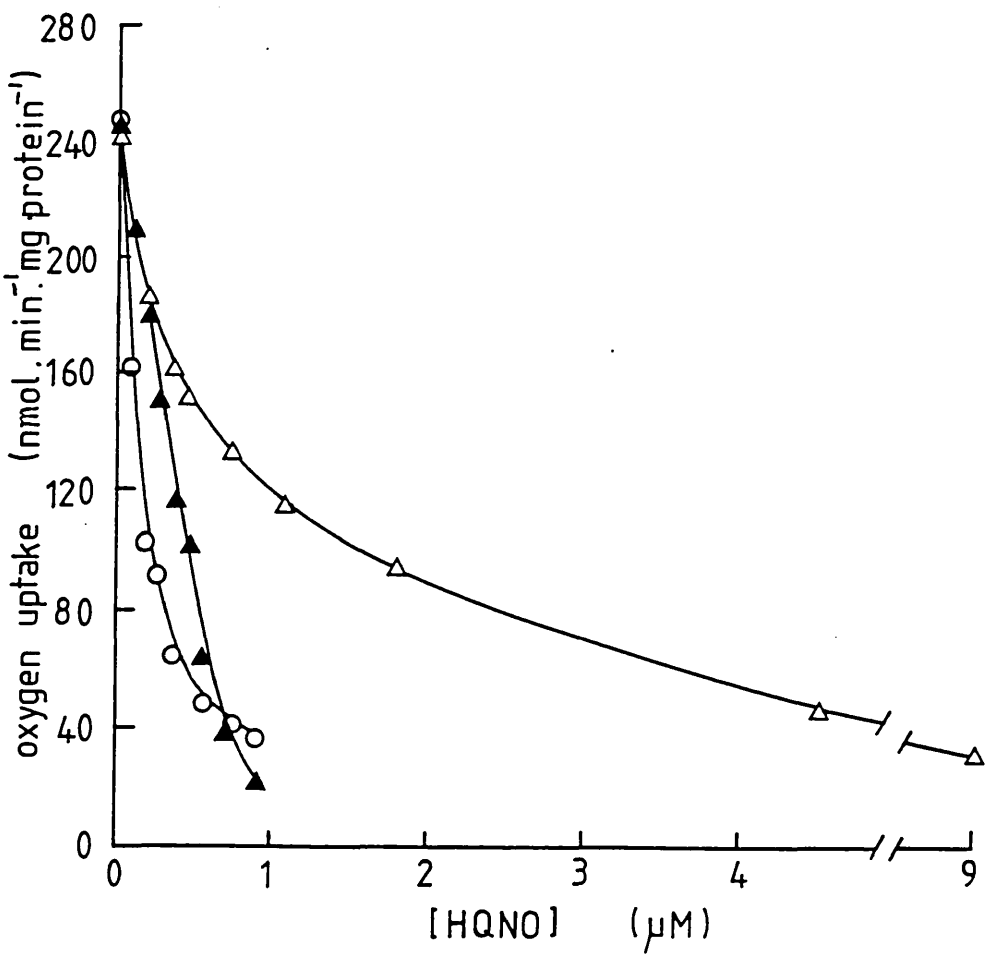


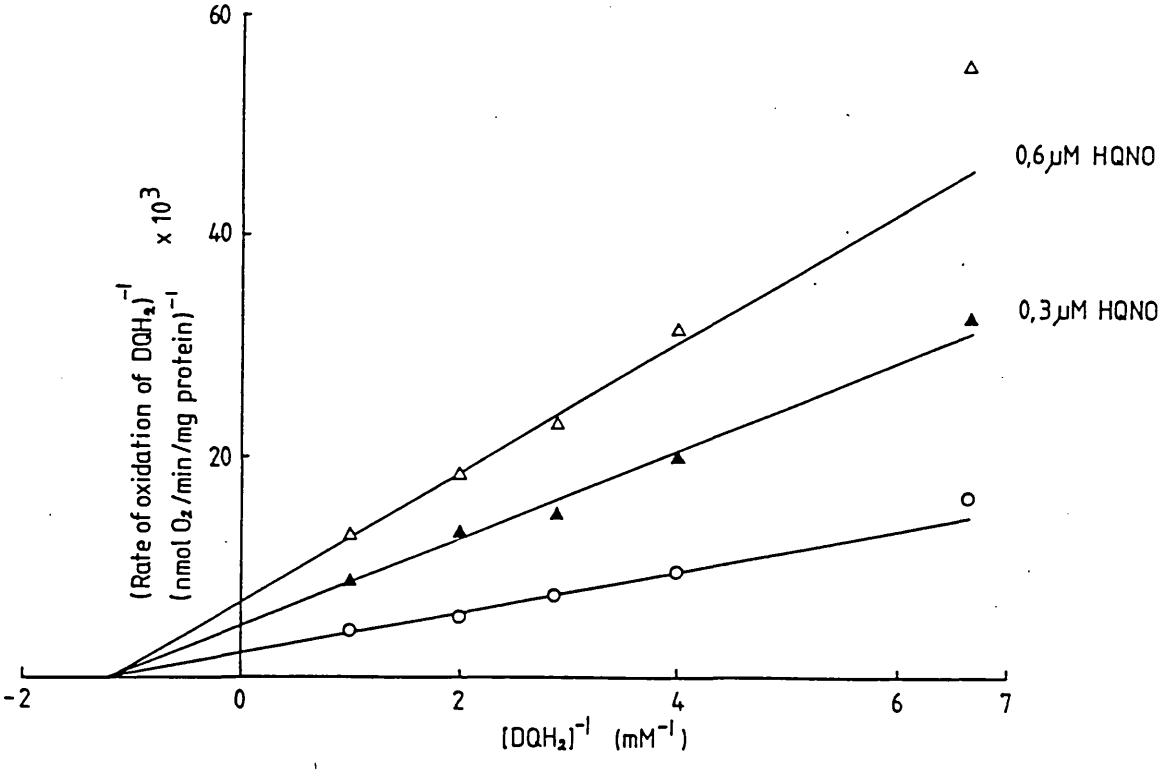
Figure 17. Lineweaver-Burk plot of the inhibition of DQH_2 oxidation by HQNO.

Initial rates of DQH_2 oxidation by uncoupled mitochondrial suspensions (0.65mg protein/ml) were determined as described in the legend to Figure 16. Concentrations of DQH_2 were as indicated and mitochondria were preincubated with HQNO for 2 min before the addition of DQH_2.

○ No HQNO

▲ + 0.3 μM HQNO

△ + 0.6 μM HQNO.



4.1.1 Fluorimetric measurement of HQNO binding.

Varying concentrations of HQNO were incubated with mitochondria for 5 minutes at room temperature to allow equilibration of the inhibitor with binding sites. The mitochondria were pelleted by centrifugation and the concentration of HQNO remaining in the supernatant determined fluorimetrically (section 2.9). The relationship between HQNO concentration and fluorescence was linear, as shown by direct titration of supernatant obtained from mitochondria incubated in the absence of HQNO (Figure 18). In the absence of antimycin, specific binding of HQNO was indicated by non-linearity, particularly at low HQNO concentrations. As these sites were progressively saturated the relationship between HQNO in the supernatant and that added, became linear. In the presence of 1μM antimycin no specific binding of HQNO was detected indicating that antimycin and HQNO binding are mutually exclusive, as found by van Ark and Berden (1977) using beef heart submitochondrial particles. The gradients of the lines of Figure 18 determined in the presence of antimycin and by direct titration are different indicating a partition of HQNO, between the aqueous phase and the mitochondria, of 0.61. This was lower than the value obtained by van Ark and Berden (1977) of 1.21 at a similar protein concentration. This difference may reflect the higher lipid:protein ratio expected with submitochondrial particles compared to intact mitochondria.

The number of specific binding sites for HQNO in Jerusalem artichoke mitochondria was estimated from a Scatchard plot (Figure 19) derived from the data of Figure 18. The values obtained in the absence of antimycin were graphically corrected for non-specific binding using the value 0.61 (see above) by the method of Weder *et al.* (1974). By this method the contribution of non-specific binding is equivalent to the part of the line joining each point with the origin that lies

Figure 18. Fluorescent determination of the binding of HQNO to Jerusalem artichoke mitochondria.

Mitochondria were suspended (7.47 mg protein/ml) in standard assay medium not containing BSA and incubated for 5 min at room temperature with concentrations of HQNO and antimycin as indicated. Mitochondria were removed by centrifugation and the concentration of HQNO remaining in the supernatant determined by the intrinsic fluorescence of HQNO (excitation wavelength 355nm, emission wavelength 480nm, slit widths 6nm) as described in section 2.9. The fluorescence of HQNO was calibrated by direct addition of HQNO to supernatant obtained from a mitochondrial suspension to which no inhibitors had been added.

△ Direct titration of supernatant

○ HQNO only

● HQNO + 1μg antimycin.

Figure 18. Fluorescent determination of the binding of HQNO to Jerusalem artichoke mitochondria.

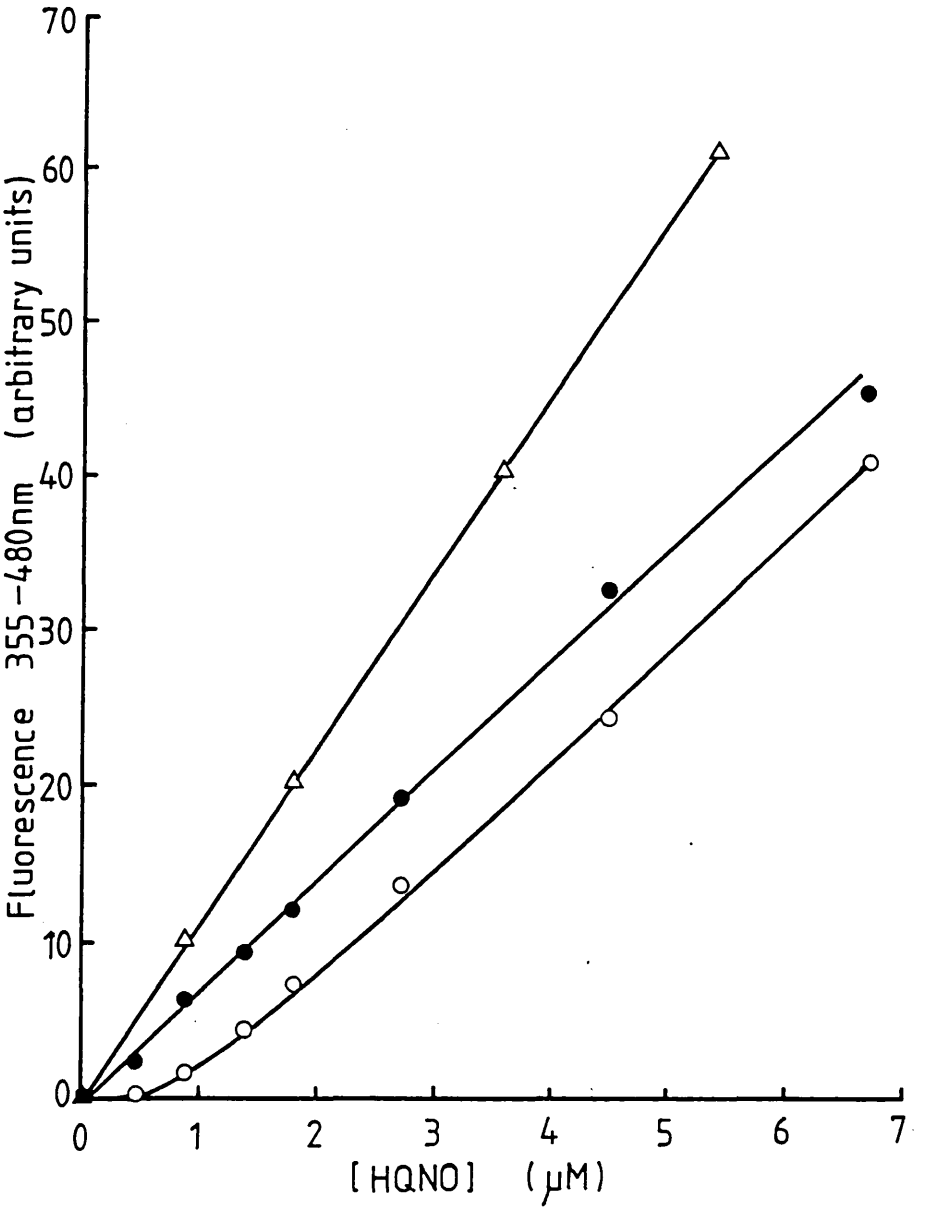


Figure 19. Scatchard plot of the binding of HQNO to Jerusalem artichoke mitochondria.

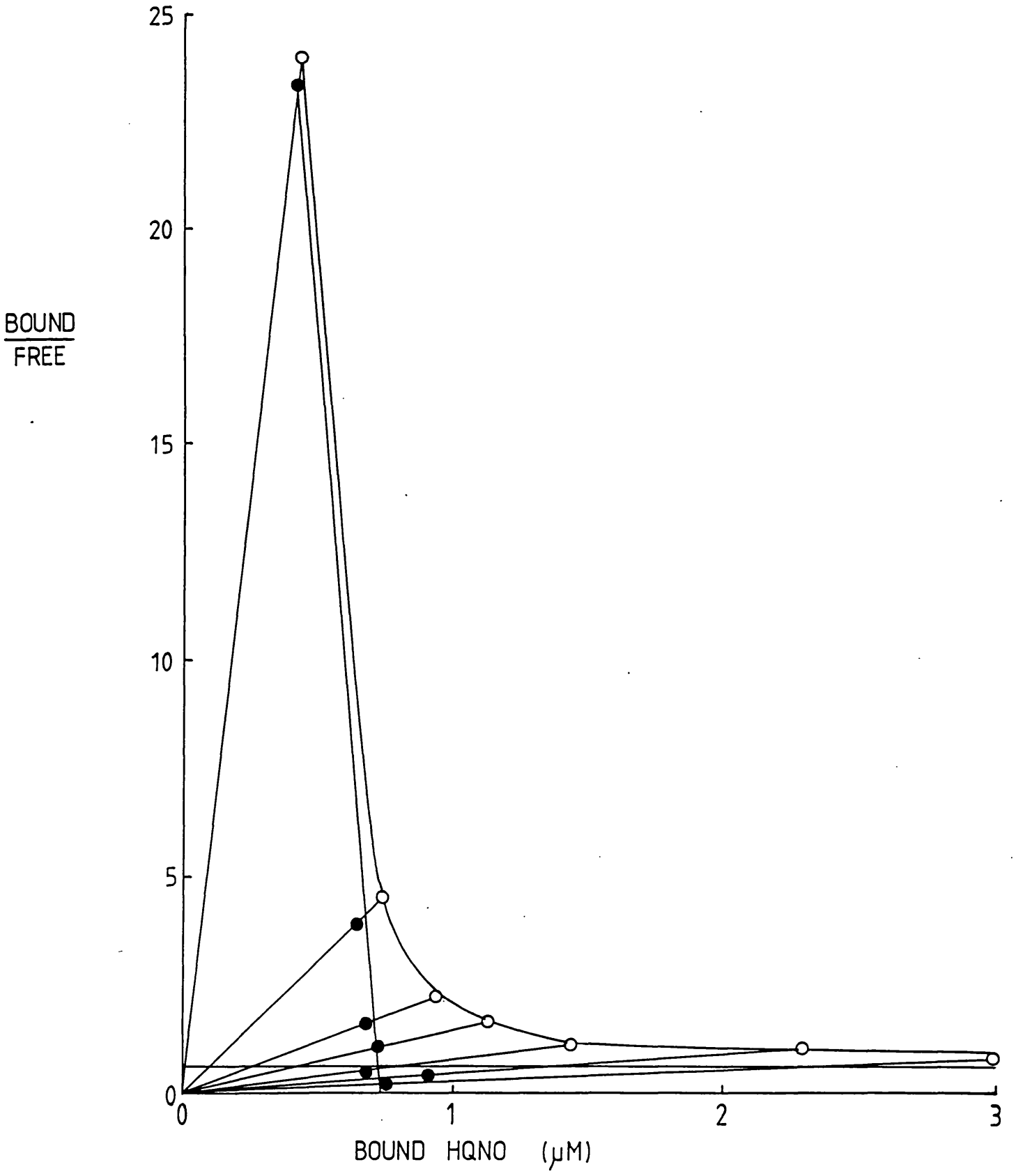
The points representing total binding of HQNO were obtained from the experimental points presented in Figure 18 for direct titration of supernatant and for incubation with HQNO only. Graphical correction for non-specific binding of HQNO was made according to the method of Weder *et al.* (1974) using a partition coefficient for HQNO between supernatant and mitochondria of 0.61 (see text).

○ Total binding of HQNO

● Specific binding of HQNO

From this plot the concentration of specific HQNO binding sites was found to be 0.096 nmol/mg protein.

Figure 19. Scatchard plot of the binding of HQNO to Jerusalem artichoke mitochondria.



below the line denoting non-specific binding. By moving each point towards the origin by this length, only the contribution of the specific binding remains. The concentration of binding sites was 0.72μM, which, at a protein concentration of 7.47mg/ml, corresponded to 0.096nmol/mg protein, in good agreement with the titre for antimycin (Table 2) measured by inhibition of oxygen uptake.

Inclusion of 5mM succinate and 1mM KCN in the mitochondrial suspension had no effect on the binding of HQNO, showing that binding was not dependent on the state of reduction of the bc_1 complex (results not shown). Unfortunately it was not possible to carry out a similar experiment using mitochondria reduced by NADH because of interference by NADH and NAD^+ with fluorescent measurement of HQNO concentration. Further evidence for the similarity of the inhibitory effects of antimycin and HQNO can be seen from reduced-oxidised spectra of the cytochromes recorded at 77K (Figure 20). With saturating amounts of either antimycin or HQNO present cytochromes c, c_1, a and a_3 were almost totally oxidised, but the b cytochromes were largely reduced, consistent with inhibition of cytochrome b reoxidation. In the presence of antimycin, but not HQNO, a bathochromic shift of 1-2nm in the alpha peak of cytochrome b_{563} was discernable, in agreement with a previous report of an antimycin induced peak shift in Jerusalem artichoke mitochondria (Passam *et al.*, 1973). It was of interest that no spectral differences could be found between mitochondria in which succinate oxidation had been completely inhibited by the addition of 2.5μM HQNO, and mitochondria still oxidising NADH despite the presence of 2.5μM HQNO. This would imply that the resistance of NADH oxidation to HQNO is not mediated by the degree of reduction of the b cytochromes. Apart from the resistance of NADH oxidation to HQNO, which is further examined in section 4.2, these results show that the binding to, and inhibition

Figure 20. Effects of antimycin and HQNO upon the degree of reduction of the cytochromes, during the oxidation of succinate and NADH.

Mitochondrial samples (2.17mg protein/ml) were incubated in standard assay medium containing 0.2μM FCCP with either 1mM NADH or 20mM succinate for 15 s before being frozen to 77K by immersion in liquid nitrogen. This short period of incubation ensured that the sample did not become anaerobic (this was tested using an oxygen electrode). The cytochrome spectra of each sample was then recorded at 77K as described in section 2.12. Where inhibitors were used a period of 2 min was allowed for binding before the addition of substrate.

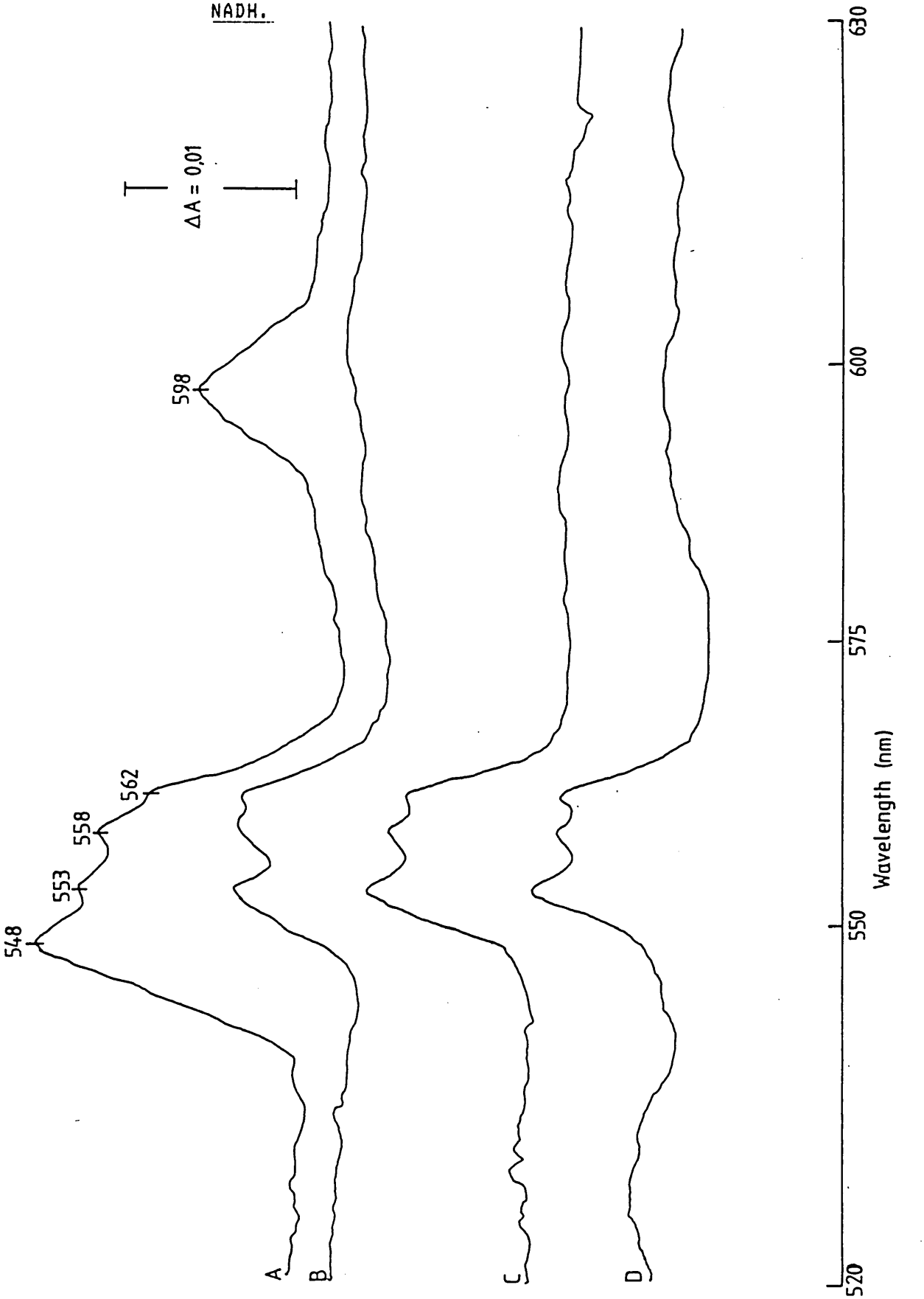
A. Complete reduced-oxidised spectrum obtained using dithionite.

B. + Succinate and 0.5μM antimycin.

C. + Succinate and 2.5μM HQNO.

D. + NADH and 2.5μM HQNO.

Figure 20. Effects of antimycin and HQNO upon the degree of reduction of the cytochromes, during the oxidation of succinate and NADH.



of, the respiratory chain by HQNO is similar in Jerusalem artichoke and mammalian mitochondria.

4.1.2 Relationship between saturation with HQNO and inhibition of respiration.

The non-competitive nature of HQNO inhibition of DQH_2 oxidation (Figure 17) allowed calculation of the degree of saturation of the specific binding sites for a given HQNO concentration ($\bar{Y}$), using the previously determined Ki of 127nM. When the rate of DQH_2 oxidation was plotted as a function of saturation with HQNO, a linear plot was obtained (Figure 21). This was evidence that DQH_2 was oxidised directly by the bc_1 complex in intact mitochondria, rather than via the bulk ubiquinone, as for the latter mechanism a hyperbolic relation between saturation and rate would have been expected (Kroger and Klingenberg, 1973b). Direct oxidation of DQH_2 has been reported by Kroger and Klingenberg (1973a) and is consistent with the failure of lipid dilution of the mitochondrial membrane to affect the specific rate of DQH_2 oxidation (Schneider *et al.*, 1982). The linear inhibition observed with HQNO was in contrast to the sigmoidal pattern seen using antimycin (Figure 7). It is likely that the same pathway of DQH_2 oxidation operates in each case and therefore this difference was consistent with the reported complex binding and inhibition behaviour of antimycin (section 1.2.2). It was concluded that antimycin could not be used directly to quantify pool kinetics in these mitochondria (Zhu *et al.*, 1982). However the direct oxidation mechanism meant that the rate of DQH_2 oxidation could be used to measure the degree of inhibition of the quinol oxidase pathway (see Chapter 5).

The relationship between inhibitor saturation and succinate oxidation was essentially hyperbolic (Figure 22), as expected from

Figure 21. Relationship between binding of HQNO and inhibition of DQH_2 oxidation.

Mitochondria were prepared as described in section 2.1. Mitochondria (0.54-0.61 mg protein/ml) were incubated in standard assay medium containng 0.2μM FCCP for 2 min at 25°C with HQNO concentrations as indicated. The rate of oxygen consumption was measured following addition of 1mM DQH_2 as described in section 2.3. Saturation of binding sites ($\bar{Y}$) was calculated using the value for Ki of 127nM determined from figure 17. Results represent the means and standard deviations of two preparations. The uninhibited rate of DQH_2 oxidation was 247 ± 6 nmol O_2/min/mg protein.

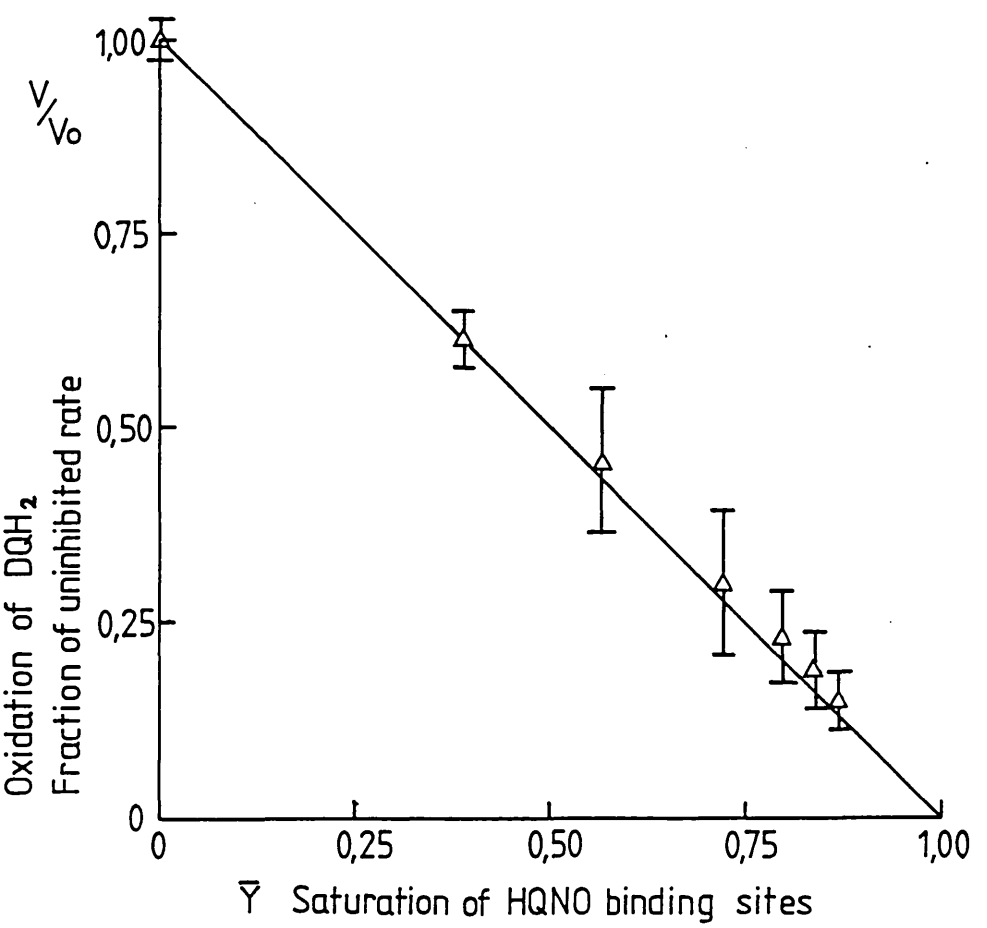
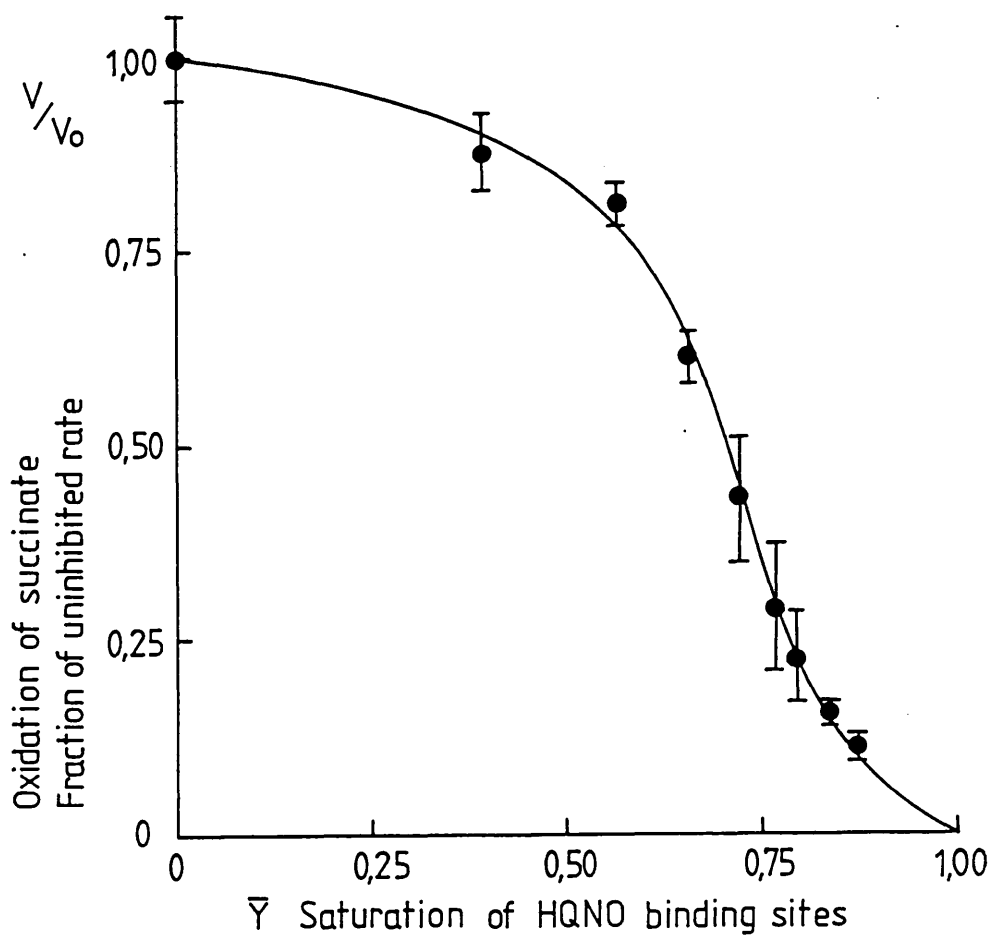


Figure 22. Relationship between binding of HQNO and inhibition of succinate oxidation.

Rates of oxygen consumption were measured as described in the legend to Figure 21 with the exception that 20mM succinate was the substrate used and succinate dehydrogenase was activated as described in section 2.2. Results represent the means and standard deviations of 4 preparations. Mitochondrial protein concentration were between 0.29 and 0.43 mg/ml. The uninhibited rate of succinate oxidation was 188 ± 11 nmol O_2/min/mg protein.



molecular mobility between succinate dehydrogenase and the bc_1 complex, and in qualitative agreement with the antimycin inhibition profile (Figure 7) and previous reports (Cottingham and Moore, 1983). However a significant departure from ideal behaviour was apparent at high levels of HQNO saturation, resulting in a sigmoidal rather than hyperbolic plot. This deviation seemed not to be due to experimental error in the determination of oxygen uptake rate, as indicated by the error bars, nor can it be accounted for by a small error in the determination of the Ki value used to calculate saturation. For example in order for the last point on the graph ($\bar{Y}$=0.88) to fit a hyperbolic plot ($\bar{Y}$=0.95) the true Ki value would have had to have been 47nM rather than the experimentally determined value of 127nM.

The inhibition of succinate oxidation was plotted using the linear form of the pool equation (Figure 23):

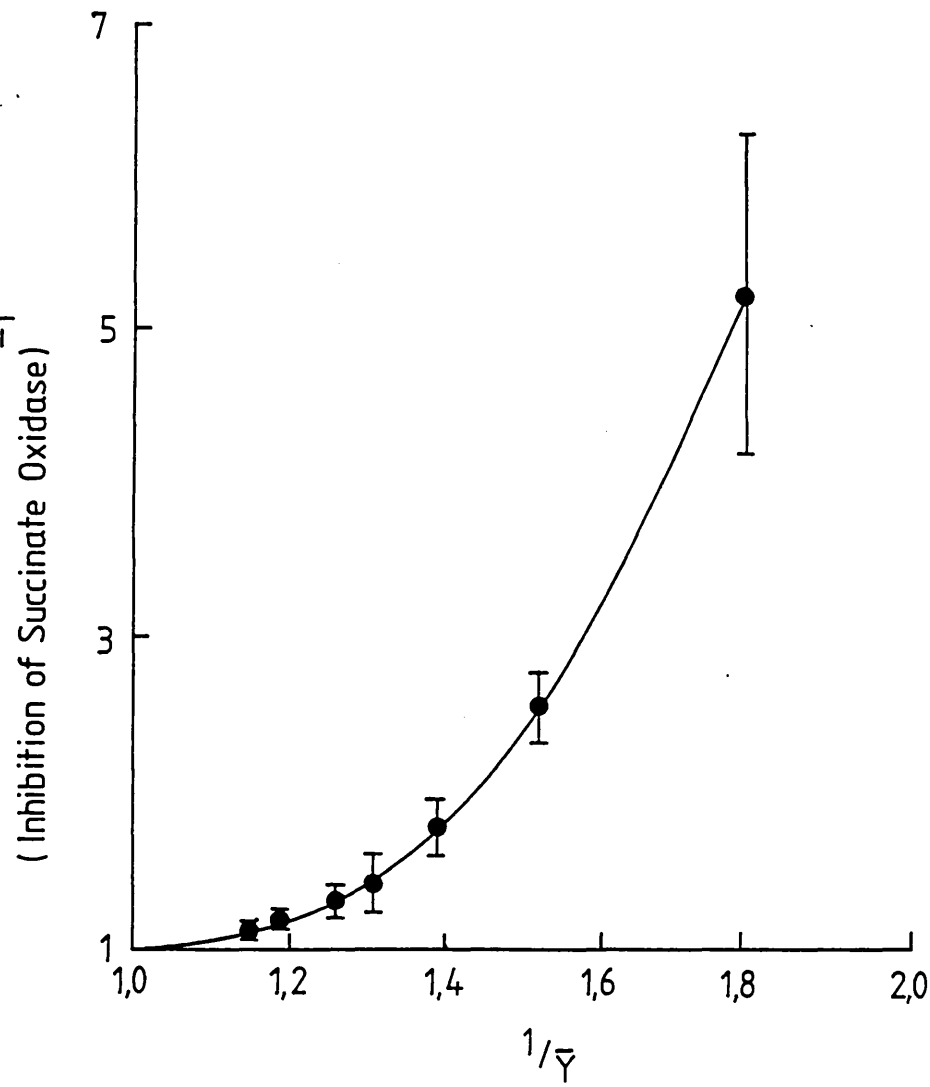
$$1/\text{inhibition} = 1/\bar{Y}.(Vox/Vred + 1) - Vox/Vred$$ (Zhu *et al.*, 1982)

The obvious non-linearity of this plot (Figure 23) is in contrast to the results obtained by Zhu *et al.* (1982) using beef heart submitochondrial particles, although the data presented by these authors covered a more restricted range of saturation (0.8 - 1.0).

Since this non-ideal behaviour was possibly relevent to the asymmetric interaction of succinate and NADH oxidase, confirmation was sought using other means of restricting ubiquinol oxidation (Chapter 5). HQNO was apparently useless in testing the pool kinetics of NADH oxidation, because NADH oxidation was not fully inhibited by concentrations of HQNO that should have virtually completely saturated the HQNO binding sites on the bc_1 complex, although the reason for this failiure to fully inhibit was not known. In section 4.2 experiments are presented that were aimed at discrimination between

Figure 23. Succinate oxidation and ideal ubiquinone pool behaviour, tested using HQNO.

The data of Figure 22 is plotted according to a linear form of the equation describing ideal ubiquinone pool behaviour (Zhu *et al.*, 1982, see text).



possible reasons for the resistance of exogenous NADH oxidation to inhibition by HQNO.

4.2 The resistance of NADH oxidation to inhibition by HQNO.

As is shown in Figure 16, much higher concentrations of HQNO were required to fully inhibit NADH oxidation compared to succinate or DQH_2 oxidation. As HQNO inhibits the bc_1 complex it was of interest to explore how the difference in sensitivity arose, since it was not obvious how HQNO and the bc_1 complex could discriminate the origin of the ubiquinol being oxidised.

Two other inhibitors, UHDBT (Cook and Cammack, 1985) and dibutylchloromethyl tin chloride (Moore *et al.*, 1980), have been reported to be less effective at blocking exogenous NADH oxidation than the oxidation of other substrates. DBCT strongly inhibited malate and succinate oxidation via the cytochrome pathway but not via the alternative oxidase, suggesting a site of inhibition after the dehydrogenases on the cytochrome pathway. However the oxidation of exogenous NADH was not strongly inhibited at these concentrations of DBCT. To reconcile these observations with the idea that the cytochrome and alternative pathways branch at ubiquinol it was suggested that some form of functional compartmentation of the ubiquinone pool occurred and that DBCT selectively interacted with the ubiquinone pool involved in the oxidation of succinate or malate (Moore *et al.*, 1980).

Ubiquinol oxidation by the Rieske centre and the b cytochromes of the bc_1 complex is inhibited by UHDBT (section 1.2.4). The partial resistance of NADH oxidation to UHDBT, shown by Jerusalem artichoke or white potato (*Solanum tuberosus*) tuber mitochondria (Cook and Cammack, 1985) had the following characteristics. A slow recovery of NADH oxidation in the presence of UHDBT took place over 1-2 minutes. In addition NADH oxidation facilitated succinate oxidation in the

presence of UHDBT: the rate of oxidation of succinate in the presence of UHDBT and NADH was greater than the rate of succinate oxidation in the presence of UHDBT alone. As UHDBT is itself redox active (E_m QH_2/Q = -40mV, Rich and Bendall, 1980) it cannot at present be ruled out that reduction of UHDBT may be the cause of the apparent resistance of NADH oxidation to this inhibitor.

The following experiments were aimed at distinguishing between several possible causes of the resistance of NADH oxidation to HQNO. In view of the repeated suggestion of a preferential relationship between exogenous NADH oxidation and the cytochrome, rather than alternative, pathway (Pomeroy, 1975, Tomlinson and Moreland, 1975, Huq and Palmer, 1978a) one possibility was that the exogenous NADH dehydrogenase does not pass electrons to the bc_1 complex through the bulk ubiquinone, but somehow more directly: with exogenous NADH as substrate Storey (1970) reported that cytochrome b reduction preceded reduction of the ubiquinone pool, while the reverse order of reduction was seen with succinate as substrate. A second possibility was that the low redox potential of the NADH/NAD^+ couple was transmitted through ubiquinone to the bc_1 complex lowering the efficiency of HQNO binding or inhibition. Thirdly there was the possibility that HQNO was chemically altered during NADH oxidation.

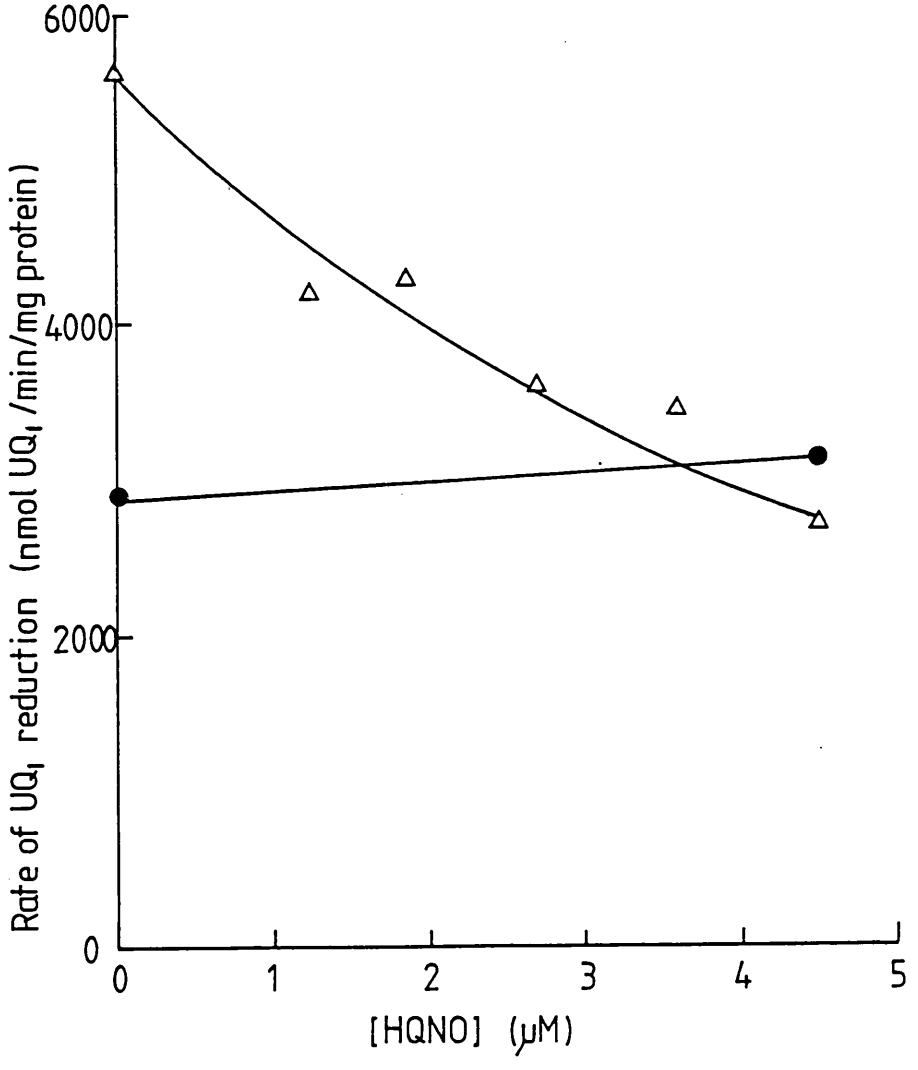
4.2.1 Inhibition of NADH-ubiquinone-1 reductase activity by HQNO.

In the presence of KCN to prevent reoxidation of ubiquinol-1, the reduction of ubiquinone-1 by NADH was inhibited approximately 50% by 4.5μM HQNO. Succinate-ubiquinone-1 reductase activity was unaffected by this concentration of HQNO (Figure 24). The inhibition of NADH-ubiquinone-1 reductase activity was less severe than the inhibition of oxygen uptake, but was of interest as it further disqualified HQNO as a probe for the study of pool kinetics of NADH oxidation. The mode of inhibition of ubiquinone-1 reductase activity

Figure 24. Inhibition of NADH-ubiquinone-1 reductase activity by HQNO.

Mitochondria were prepared and succinate dehydrogenase activated as described in sections 2.1 and 2.2 respectively. Mitochondria (0.12 mg protein/ml) were suspended in standard assay medium containing 1mM KCN, 0.54mM ubiquinone-1 and HQNO as indicated. After 2 minutes either 20mM succinate or 0.5mM NADH was added and the rate of ubiquinone-1 reduction monitored at 420nm (section 2.10).

△ NADH (1mM), ● Succinate (20mM)



by HQNO did not appear to be of a simple form (results not shown).

The rate of reduction of ubiquinone-1 by NADH was identical whether ubiquinol-1 reoxidation was inhibited by KCN or antimycin. This would seem to rule out the possibility that HQNO inhibited reduction of ubiquinone-1 at the antimycin site of inhibition ie. that electrons were passed directly to the bc_1 complex and subsequently reduced ubiquinone. The observation of Cook and Cammack (1985) that UHDBT was without effect on NADH-ubiquinone-1 reductase activity and the recent reports of isolation of the exogenous NADH dehydrogenase (Cook and Cammack, 1984, Cottingham and Moore, 1984, Klein and Burke, 1984) which are capable of direct reduction of quinone, are convincing evidence that the pathway of exogenous NADH oxidation involves reduction of ubiquinone prior to reduction of the bc_1 complex.

4.2.2 Combined inhibitory effects of HQNO and antimycin.

It was not possible to determine directly the effect on the binding of HQNO of reduction of the respiratory chain by NADH, because of interference by NADH in the fluorimetric assay of HQNO concentration. Another method was therefore needed to try to distinguish whether HQNO binding was prevented by NADH oxidation or whether NADH oxidation continued despite bound HQNO. Antimycin titrations of both succinate and NADH oxidation were carried out in the presence of various concentrations of HQNO (Figure 25). By restricting ubiquinol oxidation, increasing concentrations of HQNO would be expected to make the antimycin inhibition profile less hyperbolic (Kroger and Klingenberg, 1973b). This was the case with both succinate and NADH oxidation (Figure 25) indicating that the main site of inhibition for both substrates was ubiquinol oxidation. The two substrates differed, however, in the amount of antimycin required for full inhibition of oxygen uptake. In the presence of increasing concentrations of HQNO increasing amounts of antimycin were required

Figure 25. Inhibition of succinate and NADH oxidation by combinations of HQNO and antimycin.

Mitochondria were prepared and succinate dehydrogenase activated as described in sections 2.1 and 2.2. Mitochondria were suspended in standard assay medium including 0.2μM FCCP and antimycin and HQNO as indicated, for 2 min prior to addition of substrate. Rates of oxygen uptake were measured at 25°C as described in section 2.3.
Rates of oxidation in the absence of antimycin are in parentheses (nmol O_2/min/mg protein).

(A) 20mM succinate as substrate. Mitochondrial protein 0.4mg/ml.

△ No HQNO (166)

● + 0.24μM HQNO (96)

○ + 0.72μM HQNO (44)

(B) 1mM NADH as substrate. Mitochondrial protein 0.4mg/ml.

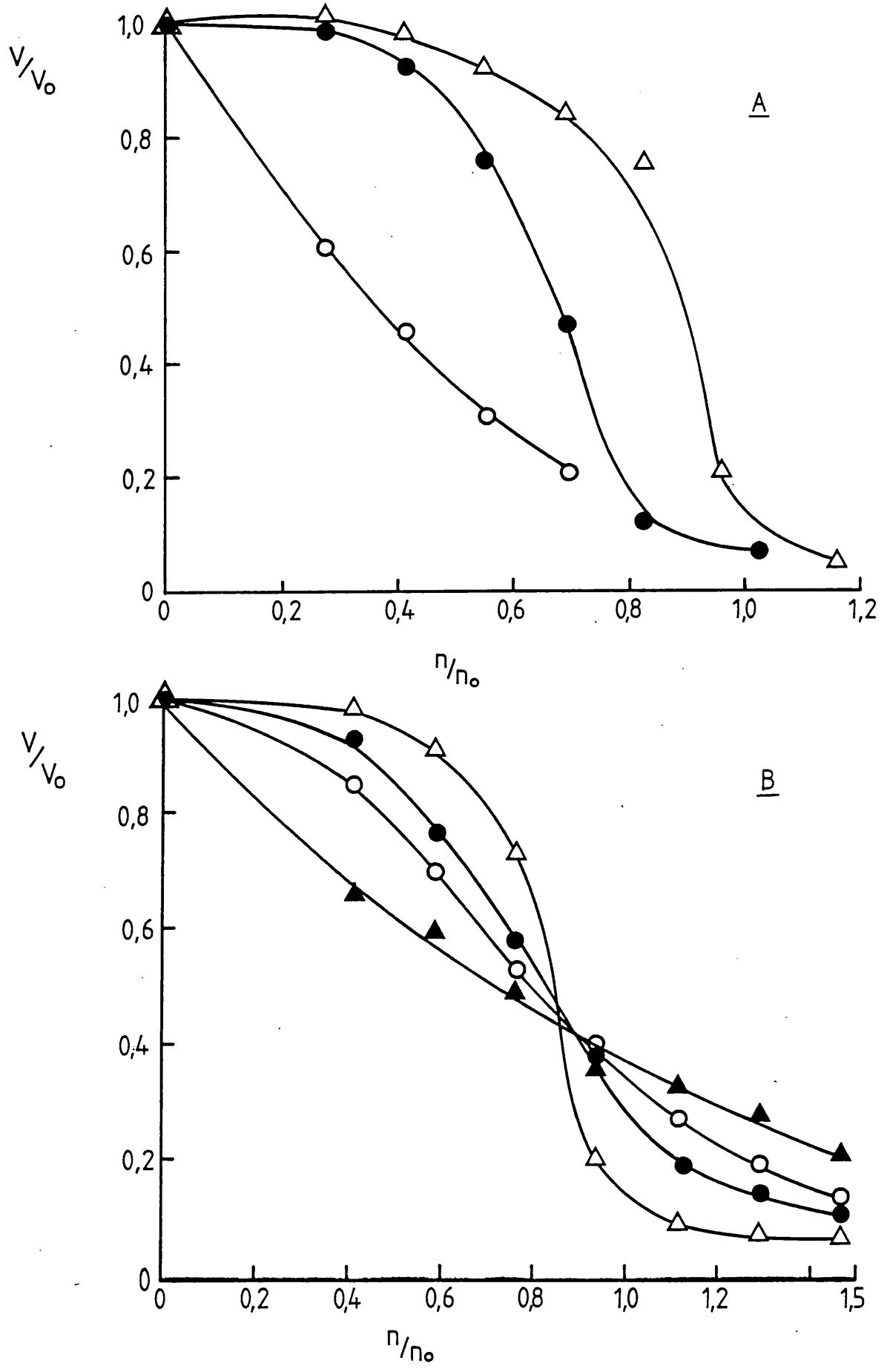
△ No HQNO (187)

● + 0.27μM HQNO (152)

○ + 0.63μM HQNO (115)

▲ + 1.80μM HQNO (72)

Figure 25. Inhibition of succinate and NADH oxidation by combinations of HQNO and antimycin.

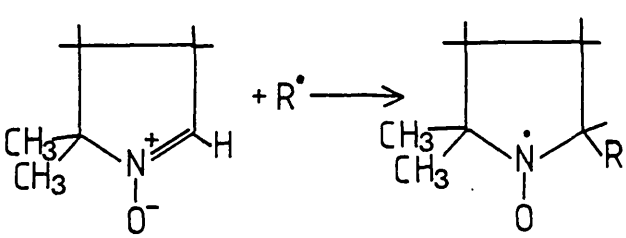


to stop NADH oxidation, but no increase in antimycin titre was found for succinate oxidation. As HQNO and antimycin binding are mutually exclusive (Figure 18) either HQNO or antimycin binding inhibited succinate oxidation. The increased antimycin titre for NADH oxidation in the presence of HQNO could be explained in two ways. Either binding of HQNO does not fully inhibit operation of the bc_1 complex or, once bound, unbinding is promoted, perhaps by a chemical change in HQNO, leaving a functional bc_1 complex until a new molecule HQNO or antimycin binds. It is commonly observed that antimycin binding is a slow process. In this study two minutes incubation with mitochondria at room temperature was adequate for maximal inhibition by antimycin, but much longer preincubations have been reported (Cottingham and Moore, 1983).

If binding of HQNO led to only partial inactivation of the bc_1 complex then a maximum degree of inhibition of NADH oxidation should have been observed, corresponding to saturation of the HQNO binding sites. In fact total inhibition of oxygen uptake was seen at sufficiently high HQNO concentrations (Figure 16), apparently supporting the second possibility of unbinding of HQNO. Evidence was therefore sought for possible chemical alteration of HQNO during the oxidation of NADH.

4.2.3 Possible chemical alteration of HQNO during NADH oxidation.

The chemical structure of HQNO (Figure 2) is related to 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) which has been used as a spin-trapping agent for the detection of free radicals:



(from Harbour and Bolton, 1975)

The relative stability of the resulting free radical has been used to detect production of superoxide anions in chloroplasts (Harbour *et al.*, 1974, Harbour and Bolton, 1975). Significant superoxide production occurs during exogenous NADH oxidation, but not succinate oxidation, by plant mitochondria (Huq and Palmer, 1978b, Rich and Bonner, 1978a, Huq, 1978). To test the involvement of superoxide anions in the resistance of NADH oxidation to HQNO, rates of oxidation of NADH were compared in the presence and absence of added superoxide dismutase (Table 4). Superoxide dismutase did not affect the rate of NADH oxidation in the absence of HQNO, but in the presence of 1.35μM HQNO, significant additional inhibition of NADH oxidation occurred upon addition of superoxide dismutase. This was consistent with superoxide anions decreasing the effectiveness of HQNO as an inhibitor.

Attempts were made to detect destruction of HQNO during NADH oxidation. Complete recovery of HQNO, measured by fluorescence (section 2.9), from mitochondrial suspensions was possible using the same lipid extraction method used in the determination of ubiquinone content (section 2.6). No decrease in HQNO recovery occurred after oxidation of NADH or succinate, or in the presence of succinate or NADH, with or without antimycin. Neither was any change in the optical or fluorescent spectra detectable under these conditions (results not shown). This could have been because no chemical changes in HQNO had occurred, or because the extent of chemical change was too small or localised to detect, or because any changes that did occur did not alter the spectral properties of the molecule. These possibilities were not investigated further in the present study as it seemed unlikely from the preceding results that this would give useful information about the pathway of NADH oxidation.

Table 4. Effect of superoxide dismutase on inhibition of NADH oxidation by HQNO.

Mitochondria (0.35mg protein/ml) were suspended in standard assay medium containing 0.2μM FCCP, in the presence or absence of 1.35μM HQNO. Superoxide dismutase (SOD, 2800 units/mg protein) was added (150 and 500 μg/ml) and, after 2 min, oxygen consumption started by addition of 1mM NADH. The rate of oxygen consumption was measured as described in section 2.3. Results are means and standard deviations for three preparations.

Rates are: nmol O_2/min/mg protein

[HQNO] (uM)	- SOD	+ SOD	% inhibition due to SOD
0	209 ± 6	206 ± 9	1.5
1.35	65 ± 5	52 ± 6	19.2

4.2.4 Simultaneous oxidation of NADH and succinate in the presence of HQNO.

The oxidation of NADH was reported to facilitate the oxidation of succinate in the presence of UHDBT (Cook and Cammack, 1985, see above). The data presented in Figure 26 shows that this was not the case with HQNO. Irrespective of the order of addition of the substrates, in the presence of concentrations of HQNO greater than 0.3μM the combined rate of oxidation was less than that found with NADH alone. Even very low rates of succinate oxidation were able to cause inhibitions in the rate of oxidation of NADH larger than the rate of oxidation of succinate alone. This observation, as well as showing that NADH oxidation does not significantly facilitate succinate oxidation, is of particular interest because the ability of succinate oxidation to inhibit NADH oxidation under these conditions is a severe requirement of any model proposed to account for the interaction of succinate and NADH oxidation. These constraints are discussed in chapter 7.

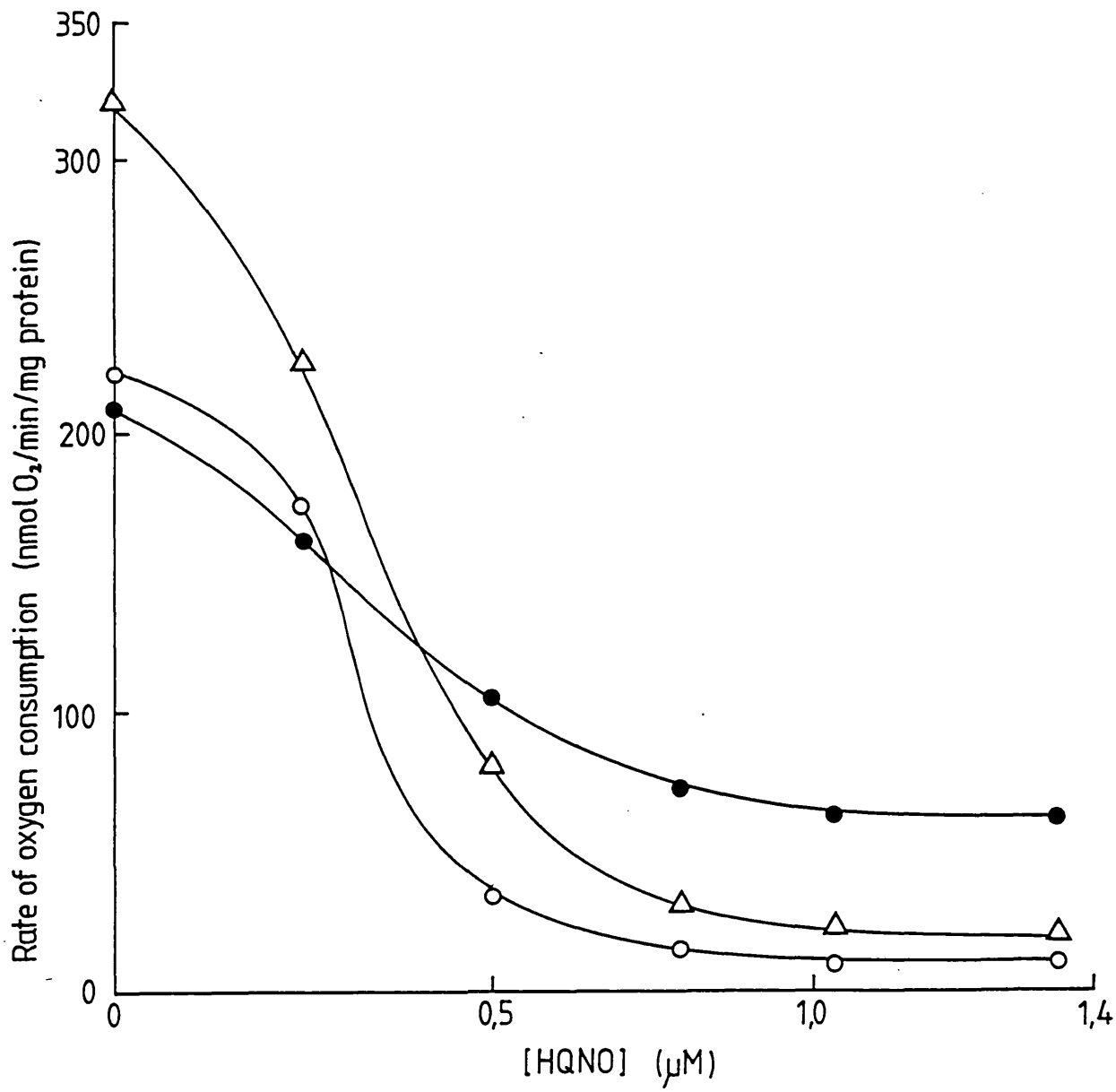
Figure 26. Interaction of succinate and NADH oxidation in the presence of HQNO.

Mitochondria were prepared and succinate dehydrogenase activated as described in sections 2.1 and 2.2. HQNO concentrations as indicated were added to mitochondrial suspensions (0.24mg protein/ml) in standard assay medium containing 0.2μM FCCP. After 2 min NADH (1mM) was added, followed 1 min later by 20mM succinate. Rates of oxygen uptake (nmol O_2/min/mg protein) were recorded as described in section 2.3.

● NADH (1mM), ○ Succinate(20mM)

Δ NADH and succinate together.

Figure 26. Interaction of succinate and NADH oxidation in the presence of HQNO.



Chapter 5. Inhibition of electron transport by myxothiazol, BAL and CTC.

Results presented in Chapter 4 showed that HQNO could be used to test how closely the oxidation of succinate followed the model of ubiquinone function developed by Kroger and Klingenberg (1973a,b, section 1.2). The oxidation of succinate did not follow this simple model completely. As shown in Figures 22 and 23, the inhibition of succinate oxidation seemed to be characterised by lower than expected inhibition at low concentrations of HQNO, and higher than expected inhibition as the specific binding sites for HQNO neared saturation. However it was not possible to test NADH oxidation in this way for comparison with succinate oxidation, because of the apparent resistance of exogenous NADH oxidation to inhibition by HQNO. In this chapter results are presented of experiments using myxothiazol (Figure 2) and BAL (British anti-lewisite: 2,3-dimercaptopropanol) to specifically inhibit ubiquinol oxidation, and CTC (chlorotetracycline) to specifically inhibit the oxidation of exogenous NADH.

5.1 Inhibition of electron transport by myxothiazol.

Myxothiazol specifically inhibits the reduction of the b cytochromes of mammalian bc_1 complex (Becker *et al.*, 1981, von Jagow and Engel, 1981) and of the bc_1 complex of *Rhodopseudomonas sphaeroides* (Meinhardt and Crofts, 1982, Gabellini and Hauska, 1983). It is one of several inhibitors, called moa inhibitors, which have a common structural element in E-β-methoxyacrylate (Becker *et al.*, 1981). The binding of myxothiazol displaces bound UHDBT from the Reiske centre (Meinhardt and Crofts, 1982), but the Reiske centre is not essential for myxothiazol binding, at least in the case of bc_1 complex from *Rhodopseudomonas sphaeroides* (Von Jagow and Engel, 1981). Spectral shifts in the low potential form of cytochrome

b (b_{556}) have been reported that are independent of those induced by antimycin (von Jagow and Engel, 1981, Gabellini and Hauska, 1983). Taken together these reports suggest that myxothiazol inhibits the reduction of the low potential b cytochrome by ubisemiquinone (section 1.1.4).

Long periods of incubation of myxothiazol with Jerusalem artichoke mitochondria were found necessary for the full development of inhibition of oxygen uptake. Even after incubation at 4°C for one hour, non-linear rates of oxygen uptake were obtained upon addition of substrate. Full inhibition could be obtained after about 10 min at room temperature and binding was not dependent upon the presence of substrate (Figure 27). FCCP was added in this case to ensure rapid substrate oxidation followed by anaerobiosis. No change in the final rate of NADH oxidation, measured without inhibitor, was caused by this period of incubation (Figure 27). The slow binding of myxothiazol, and the dependence of the time taken for full inhibition to develop upon temperature suggest a high activation energy for the binding of myxothiazol to these mitochondria. In the following experiments myxothiazol was incubated with mitochondria for 10 min before the addition of substrate. The effect of myxothiazol on the oxidation of DQH_2 is shown in Figure 28. Only very low amounts of myxothiazol were necessary to inhibit oxygen uptake. Because myxothiazol binding was nearly stoichiometric with the bc_1 content of the mitochondria, the degree of inhibition depended on the ratio of inhibitor to protein over a range of protein concentrations (0.21-0.51mg/ml, Figure 28), rather than depending only on the concentration of inhibitor present. This is because amounts of inhibitor comparable to the number of binding sites are used when binding is very tight. Consequently the amount of inhibitor left unbound is significantly lower than the amount that was added, unlike

Figure 27. Dependence upon time and temperature of the development of inhibition by myxothiazol.

Mitochondrial samples (0.2ml, 20mg protein/ml) were incubated with myxothiazol for different lengths of time and at different temperatures as indicated. Following incubation, mitochondria (0.3mg protein/ml) were added to standard assay medium containing 0.2μM FCCP and the rate of oxidation of 200μM NADH monitored at 25°C by dual-wavelength spectroscopy as described in section 2.7.

● 25°C, no myxothiazol

▲ 4°C, 0.10 nmol myxothiazol/mg protein

○ 25°C, 0.05 nmol myxothiazol/mg protein

X 25°C, 0.10 nmol myxothiazol/mg protein

△ 25°C, 0.10 nmol myxothiazol/mg protein preincubated with 1mM NADH and 0.2μM FCCP

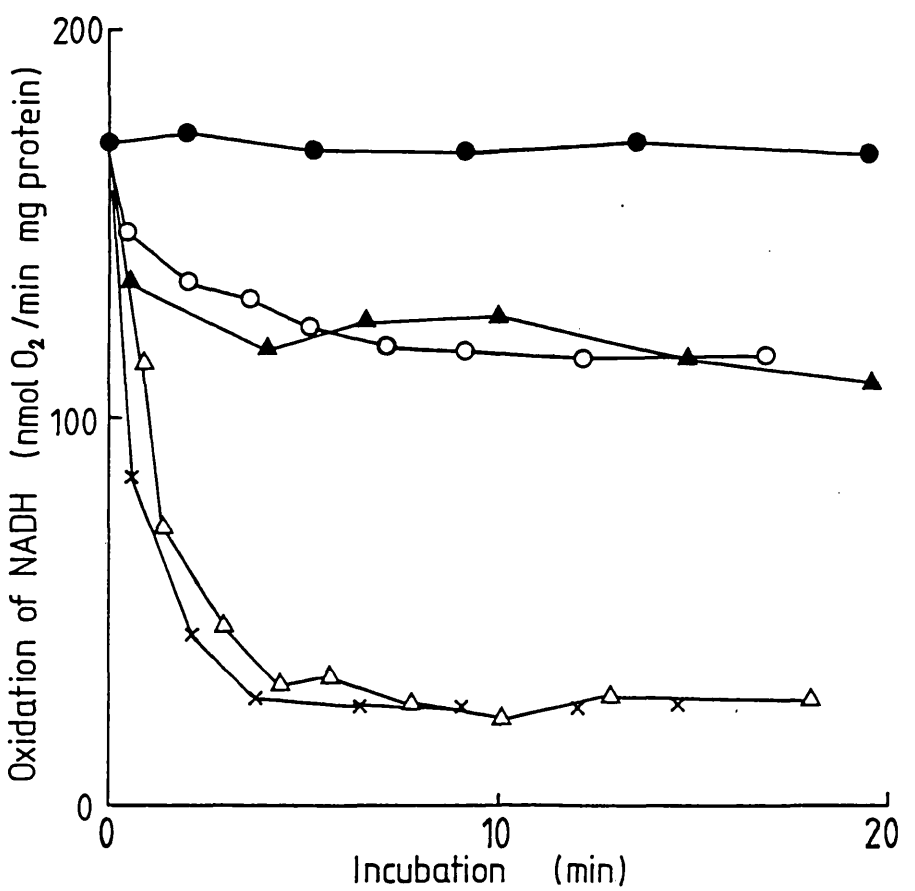
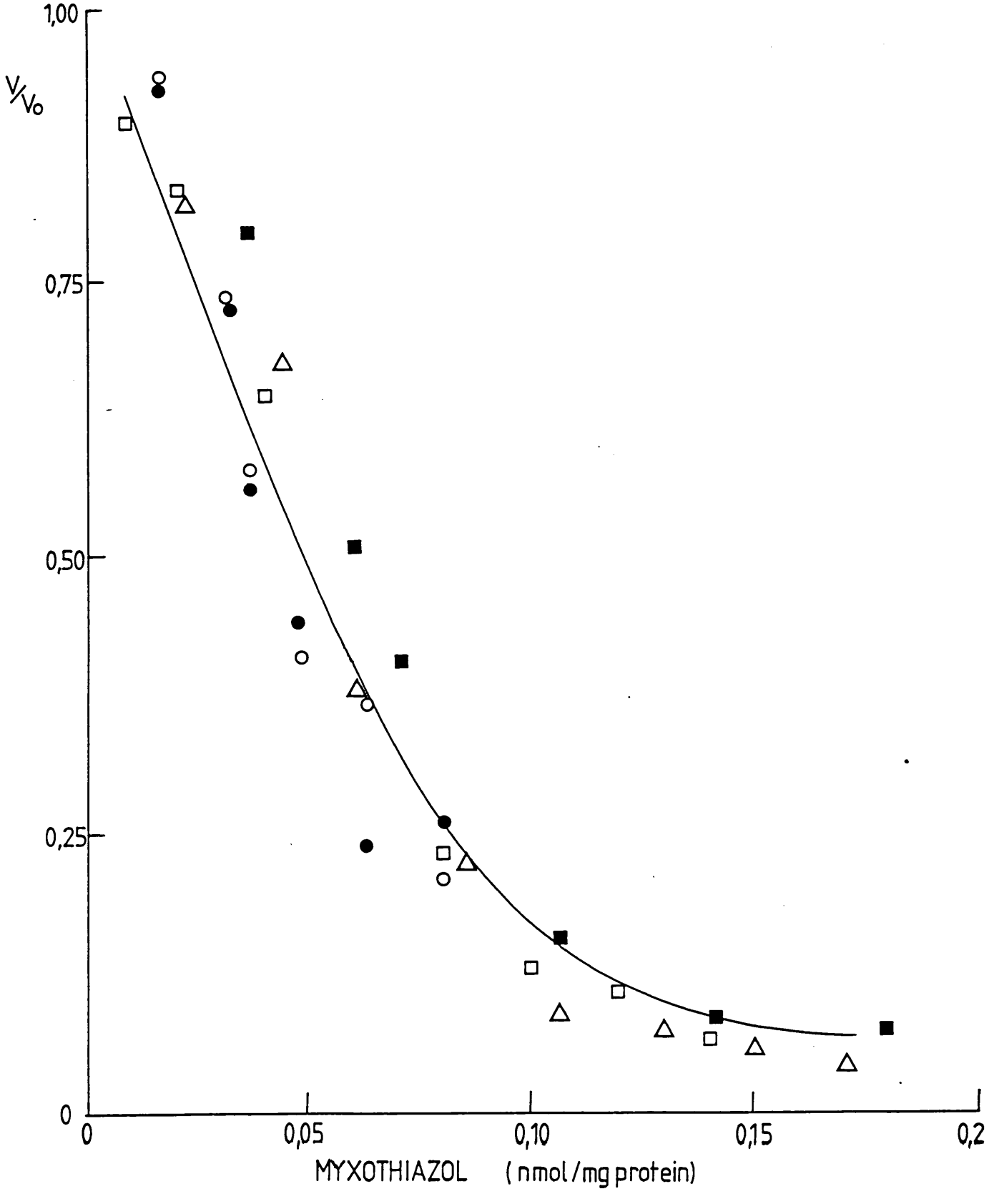


Figure 28. Inhibition of uncoupled DQH_2 oxidation by myxothiazol.

Mitochondria were incubated with amounts of myxothiazol as indicated, for 10min at room 25°C in standard assay medium containing 0.2µM FCCP. Samples (1ml) were then transferred to the cell of an oxygen electrode and the rate of DQH_2 (1mM) oxidation measured as described in section 2.3. The combined results from 5 preparations are presented (0.21-0.51mg protein/ml, each type of symbol represents a different preparation). The rates are expressed as the proportion of the uninhibited rate (V/Vo). The uninhibited rate of oxygen uptake was 245 $\pm$ 26 nmol O_2/min/mg protein (mean and standard deviation from 5 preparations). The concentration of binding sites was calculated using the mean protein concentration (0.33mg protein/ml) and the antimycin titre (0.088nmol/mg protein, Table 2). The line drawn through the experimental points was calculated using this value and a value for myxothiazol dissociation (Kd) of 2nM. A value of Kd of 2nM was used in subsequent experiments to calculate saturation of myxothiazol binding sites.

Figure 28. Inhibition of uncoupled DQH_2 oxidation by myxothiazol.



the case of a weakly bound inhibitor. Extrapolation of the initial part of the inhibition curve gave an estimate of 0.09 nmol/mg protein for the concentration of binding sites present, in close agreement with the antimycin titre (0.088nmol/mg protein, Table 2). The curved relationship between V/Vo and myxothiazol concentration shown in Figure 28 indicated that a finite dissociation of myxothiazol occured, and this was most clearly seen at high concentrations of myxothiazol. To be able to predict the degree of inhibition of ubiquinol oxidation expected from a given addition of myxothiazol, it was necessary to have an estimate of the value of the dissociation constant. The results presented in Figure 28 are from five separate preparations of mitochondria and the line drawn through the points was the expected pattern of inhibition calculated using the antimycin titre (Table 2) of 0.088 nmol of binding sites/mg protein, the average protein concentration of 0.33mg/ml and a value for the dissociation constant of myxothiazol (Kd) of 2×10^{-9}M. As this value of Kd fitted the data it was subsequently used to calculate the degree of saturation of binding sites with myxothiazol.

Unlike HQNO, myxothiazol inhibited the oxidation of succinate and NADH equally effectively over an identical range of myxothiazol concentrations. The relationship between the rate of NADH oxidation and the extent of saturation of myxothiazol binding sites was hyperbolic (Figure 29) as has been shown using antimycin (Cottingham and Moore, 1983, and Figure 7). When the results presented in Figure 29 were plotted according to a linear form of the ubiquinone pool equation (Zhu *et al.*, 1982, see section 4.1.3) a straight line was obtained that passed through the point (1,1). This showed that the oxidation of exogenous NADH could be described using the ideal ubiquinone pool equation of Kroger and Klingenberg (1973a,b). The gradient of the plot presented in Figure 30 is equal to (Vox/Vred +

Figure 29. Relationship between saturation of myxothiazol binding sites and inhibition of exogenous NADH oxidation.

Mitochondria were incubated with myxothiazol in standard assay medium containing 0.2μM FCCP for 10 min at 25°C. Samples (1ml) were then transferred to the cell of an oxygen electrode and the rate of oxygen uptake measured upon addition of 1mM NADH, as described in section 2.3. The combined results from 5 preparations (0.21-0.51mg protein/ml) are presented and each type of symbol represents a different preparation. The degree of saturation of myxothiazol binding sites was calculated using the protein concentration in each case, the antimycin titre of 0.088nmol/mg protein shown in Table 2 and a value for Kd of 2nM (Figure 28). The uninhibited rate of NADH oxidation was 167 ± 40 nmol O_2/min/mg protein (mean and standard deviation from 5 preparations).

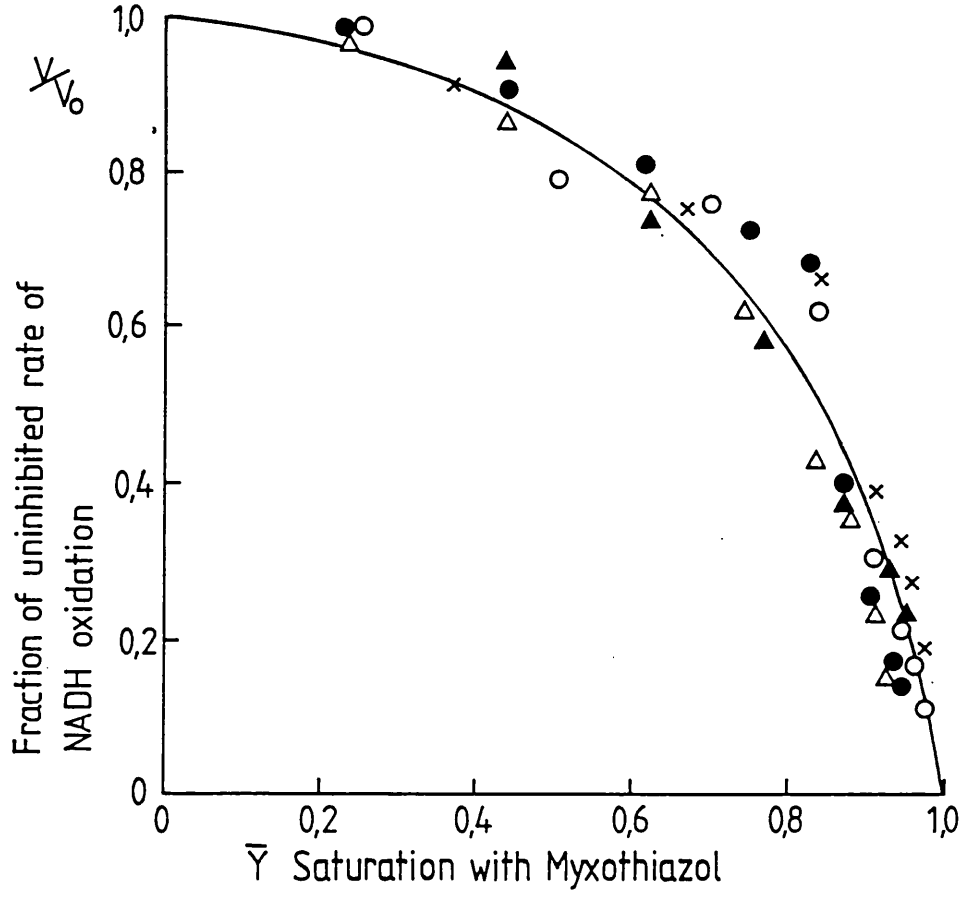


Figure 30. Relationship between NADH oxidation and ideal ubiquinone pool behaviour as tested using myxothiazol.

Results presented in Figure 29 were plotted according to a linear form of the ideal ubiquinone-pool equation (Zhu *et al.*, 1982, see section 4.1.3). In this plot consistency with ubiquinone pool behaviour is indicated by a straight line of gradient (Vox/Vred + 1) passing through the point (1,1) corresponding to full inhibition of oxidation at full saturation with myxothiazol. The regressed line fitted to the data gives a value for Vox/Vred of 4.4.

Figure 30. Relationship between NADH oxidation and ideal ubiquinone-pool behaviour as tested using myxothiazol.

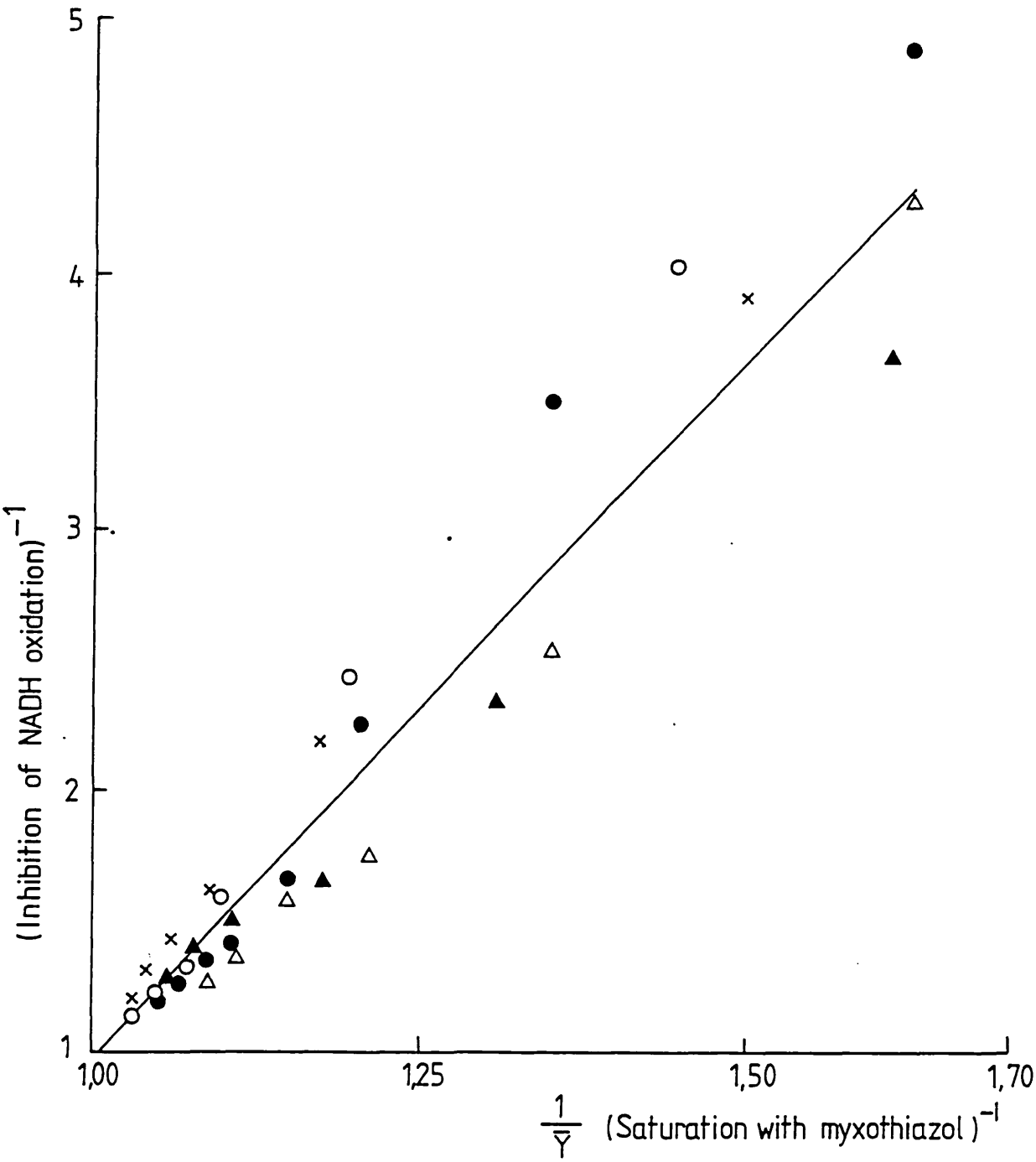


Figure 31. Relationship between saturation of myxothiazol binding sites and inhibition of succinate oxidation.

Mitochondria were prepared and succinate dehydrogenase activated as described in sections 2.1 and 2.2 respectively. Mitochondria (0.35-0.51mg protein/ml) were incubated with myxothiazol in standard assay medium for 10min at 25°C. Samples (1ml) were then transferred to the cell of an oxygen electrode and 20mM succinate was added. After 1min, 0.2µM FCCP was added and the rate of oxygen uptake recorded as described in section 2.3. The combined results of 3 preparations are presented, each symbol represents a different preparation. The degree of saturation with myxothiazol was calculated as described in the legend to Figure 31. The uninhibited rate of succinate oxidation was 173 ± 41 nmol O_2/min/ng protein (mean and standard deviation of three preparations). The line plotted is included for comparison with NADH oxidation, and represents ideal behaviour corresponding to a value of Vox/Vred of 4.4, derived from Figure 32.

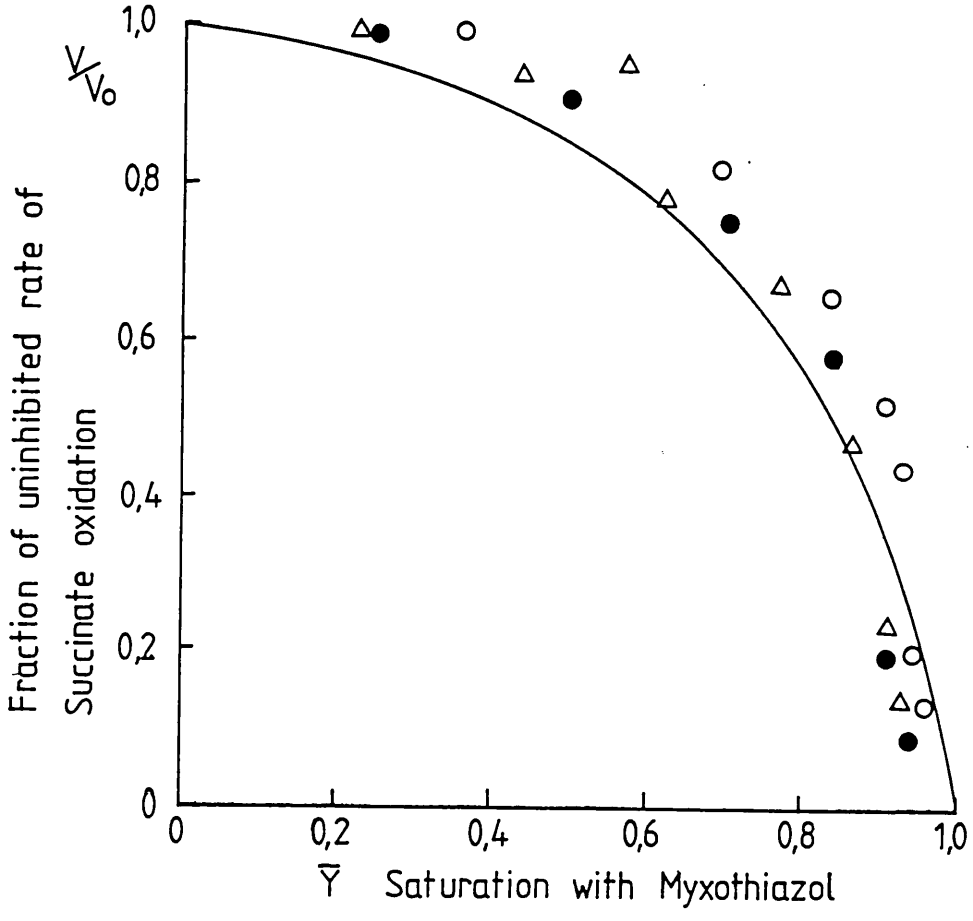
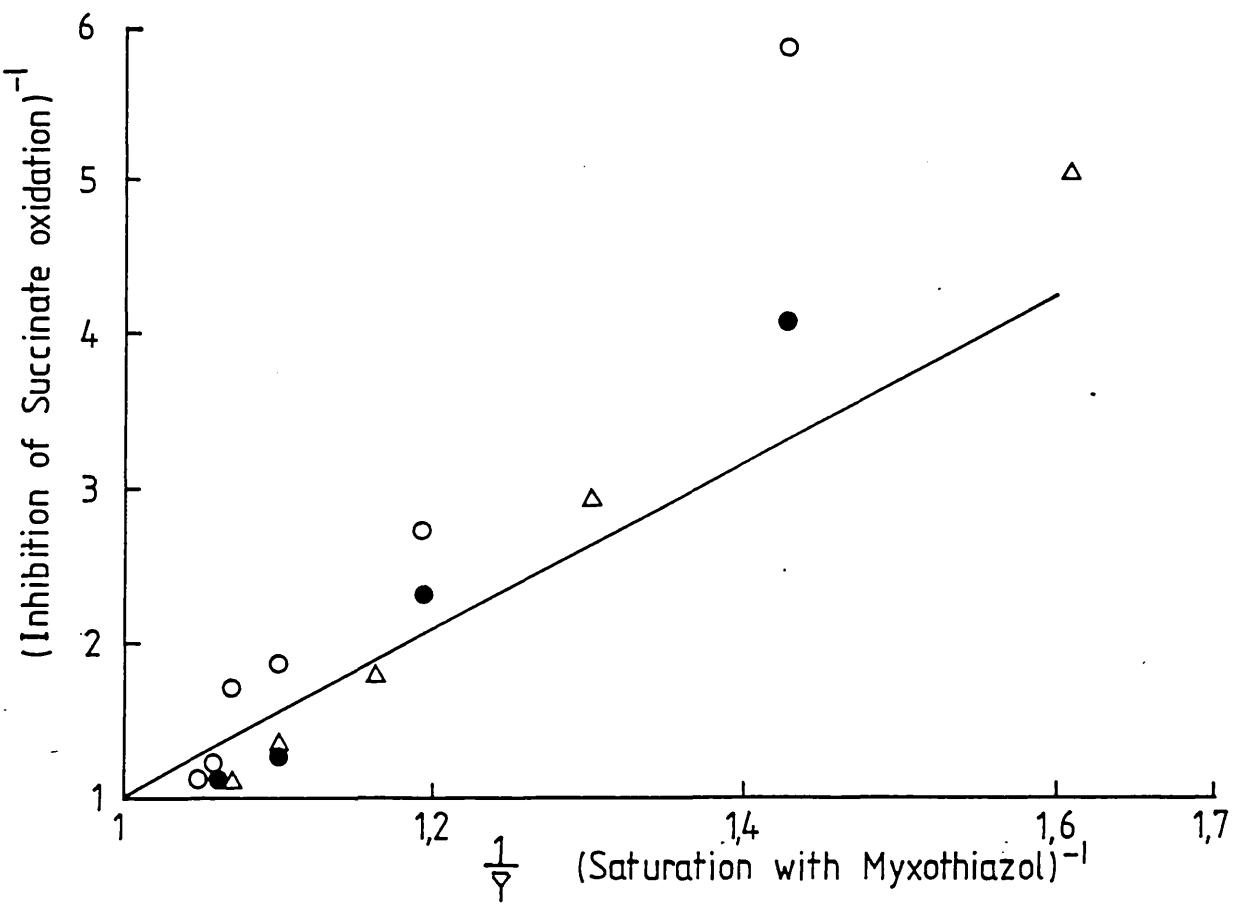


Figure 32. Relationship between succinate oxidation and ideal ubiquinone-pool behaviour as measured using myxothiazol.

The results presented in Figure 31 were plotted according to a linear form of the ubiquinone-pool equation (Zhu *et al.*, 1982, see section 4.1.3) as described in the legend to Figure 30. For comparison the line denoting Vox/Vred equal to 4.4 is included, this being the value that was determined for NADH oxidation (Figure 30).



1) and gave a value for Vox/Vred of 4.4. The line plotted through the data of Figure 29 is therefore the theoretical relationship between rate and saturation of binding sites calculated using this value for Vox/Vred and the equation given in section 4.1.3.

When the same theoretical line was drawn through the points relating succinate oxidation to saturation with myxothiazol it was found that the fit was poor (Figure 31). In particular, low saturation with myxothiazol resulted in less inhibition of succinate oxidation than would have been predicted from the results obtained for oxidation of NADH by the same preparations of mitochondria. Also, a slightly more rapid falling off in the rate of oxidation was apparent at high levels of saturation. These deviations from ideal behaviour for the oxidation of succinate were of the same type as those found using HQNO (section 4.1.3), as can be seen by comparing Figures 32 and 23, which are both clearly non-linear. The straight line drawn through Figure 32 is included for comparison and corresponds to the value of Vox/Vred of 4.4 obtained for NADH oxidation.

5.2 Inhibition of electron transport by BAL.

Slater (1948) reported that the succinate oxidase activity of beef heart mitochondria could be extensively inactivated by gentle shaking with BAL (2,3-dimercaptopropanol). Oxygen is required for this reaction and the site of inhibition was identified by spectroscopy as between cytochromes b and c. More recently the BAL labile factor has been identified as the Rieske centre by EPR measurements (Slater and de Vries, 1980). Incubation with BAL caused little inactivation of succinate dehydrogenase or of cytochrome oxidase (Slater, 1948) and therefore was potentially of use in the present study as another means of selectively inhibiting ubiquinol oxidation. The presence of the Rieske centre in higher plant

mitochondria has only recently been confirmed. Usually no EPR signal attributable to the Rieske centre can be detected in higher plant mitochondria, but the signal can be made EPR visible by the binding of UHDBT (Prince *et al.*, 1981, Bonner and Prince, 1984).

When alliquots (1ml) of mitochondria were gently shaken in air at 20-24°C with concentrations of BAL of up to 20mM, inhibitions of up to 70% were found in the rates of NADH, succinate and DQH_2 oxidation (Figure 33). The maximum degree of inhibition developed after 45-50 mins incubation (results not shown). At the end of this time BAL was removed by washing the mitochondria. The selectivity of BAL as an inhibitor in these mitochondria was tested by assaying PMS mediated DCPIP reduction by succinate (section 2.7) to measure succinate dehydrogenase activity, NADH dependent duroquinone reductase activity as a measure of the external NADH dehydrogenase activity (see section 5.3), and DQH_2 oxidase activity as a measure of ubiquinol oxidase activity (Figure 34). During the period of incubation typically 25% of NADH and succinate dehydrogenase activity was lost, but the presence of BAL did not increase the loss of activity (Figure 34). Inhibition by BAL was selective for DQH_2 oxidase activity. Following incubation with BAL all the cytochromes were more oxidised than those of mitochondria incubated in the absence of BAL, when samples were frozen during NADH oxidation in the aerobic steady state (Figure 35). This would suggest a similar site of inhibition to myxothiazol, in the presence of which similar oxidation of the cytochromes occured (Figure 35). No decrease in cytochrome oxidase activity due to BAL could be detected, nor did the outer membrane become any less intact (Table 2, results not shown). The similarity of the pattern of inhibition in these and mammalian mitochondria provides supporting evidence for the presence of the Reiske centre in Jerusalem artichoke mitochondria.

Figure 33. Inhibition of NADH, succinate and DQH_2 oxidation by treatment with BAL.

Mitochondrial samples (1ml, 5.4mg protein/ml) were treated with concentrations of BAL (2,3-dimercaptopropanol) as described in section 2.10. After the BAL had been removed by washing, the mitochondria were resuspended in standard suspension medium and succinate dehydrogenase activated (section 2.2). The uncoupled (+0.2μM FCCP) rates of oxygen consumption following addition of 1mM NADH, 1mM DQH_2 or 20mM succinate were measured at 25°C (section 2.3).

△ +20mM succinate (uninhibited rate 127 nmol O_2/min/mg protein)

○ +1mM NADH (uninhibited rate 109 nmol O_2/min/mg protein)

● +1mM DQH_2 (uninhibited rate 130 nmol O_2/min/mg protein)

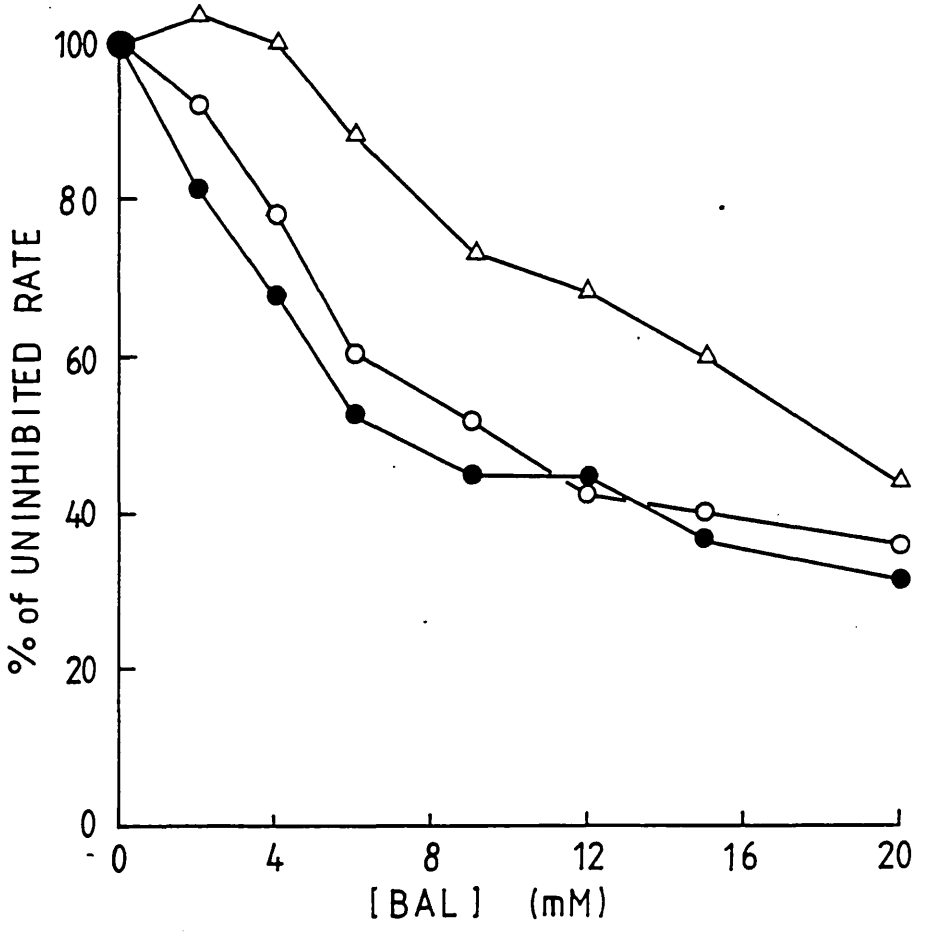


Figure 34. Specificity of inhibition by BAL for quinol oxidation.

Mitochondrial samples were treated with BAL as described in section 2.10 and then succinate dehydrogenase was activated (section 2.2). The rate of oxygen consumption following addition of 1mM DQH_2 was measured in standard assay medium containing 0.2μM FCCP (section 2.3). Succinate dehydrogenase activity was measured as reduction of DCPIP, and exogenous NADH dehydrogenase by duroquinone reduction (section 2.7).

△ NADH-duroquinone reductase activity (0.75mM NADH, 0.5mM duroquinone 200ng antimycin/mg protein, 0.2cm path length cuvette)

○ Succinate-DCPIP reductase activity

● DQH_2 oxidase activity

Figure 34. Specificity of inhibition by BAL for quinol oxidation.

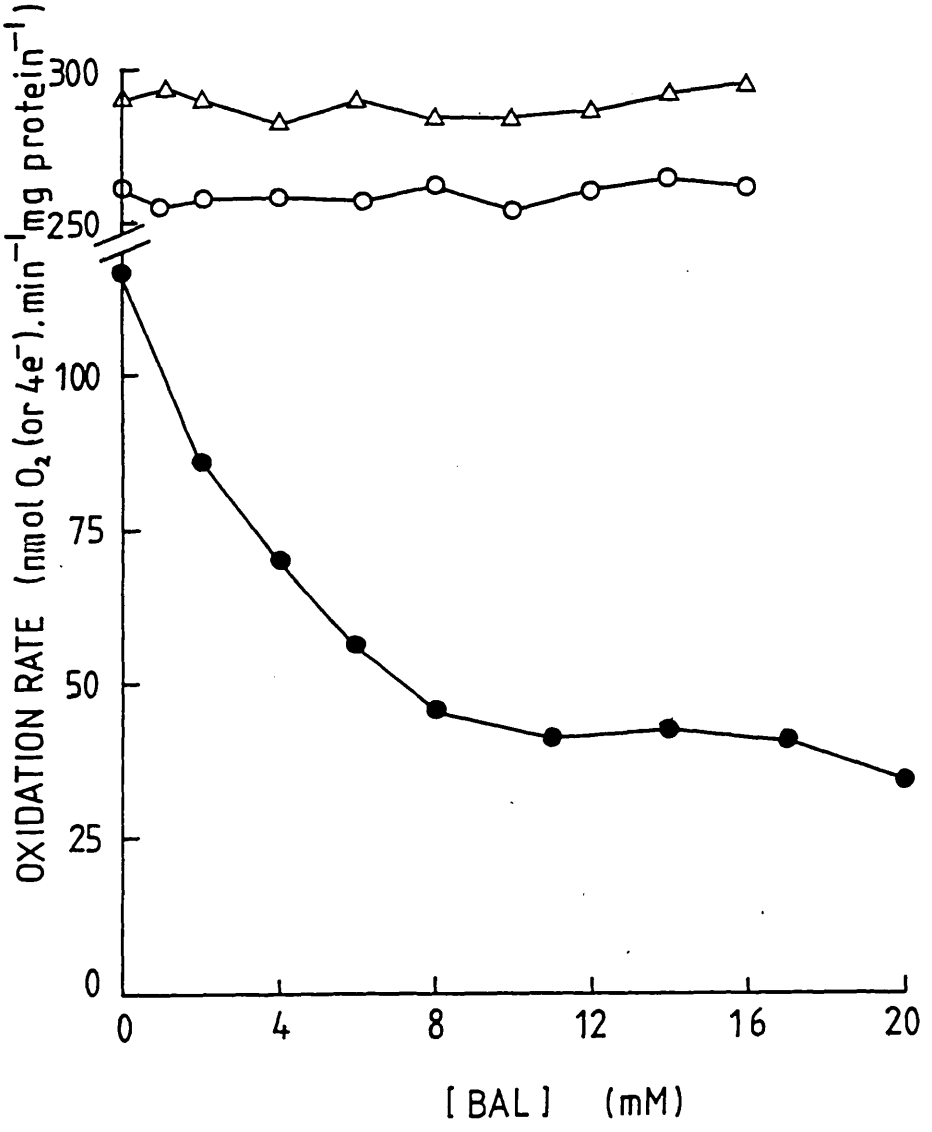


Figure 35. Effect of myxothiazol and treatment with BAL upon the degree of reduction of the cytochromes during NADH oxidation.

Mitochondrial samples (1.82mg protein/ml) were incubated in standard assay medium containing 0.2µM FCCP with 1mM NADH for 15 s before being frozen to 77K by immersion in liquid nitrogen. This short period of incubation ensured that the sample did not become anaerobic (this was tested using an oxygen electrode). The cytochrome spectra of each sample was then recorded at 77K as described in section 2.12.

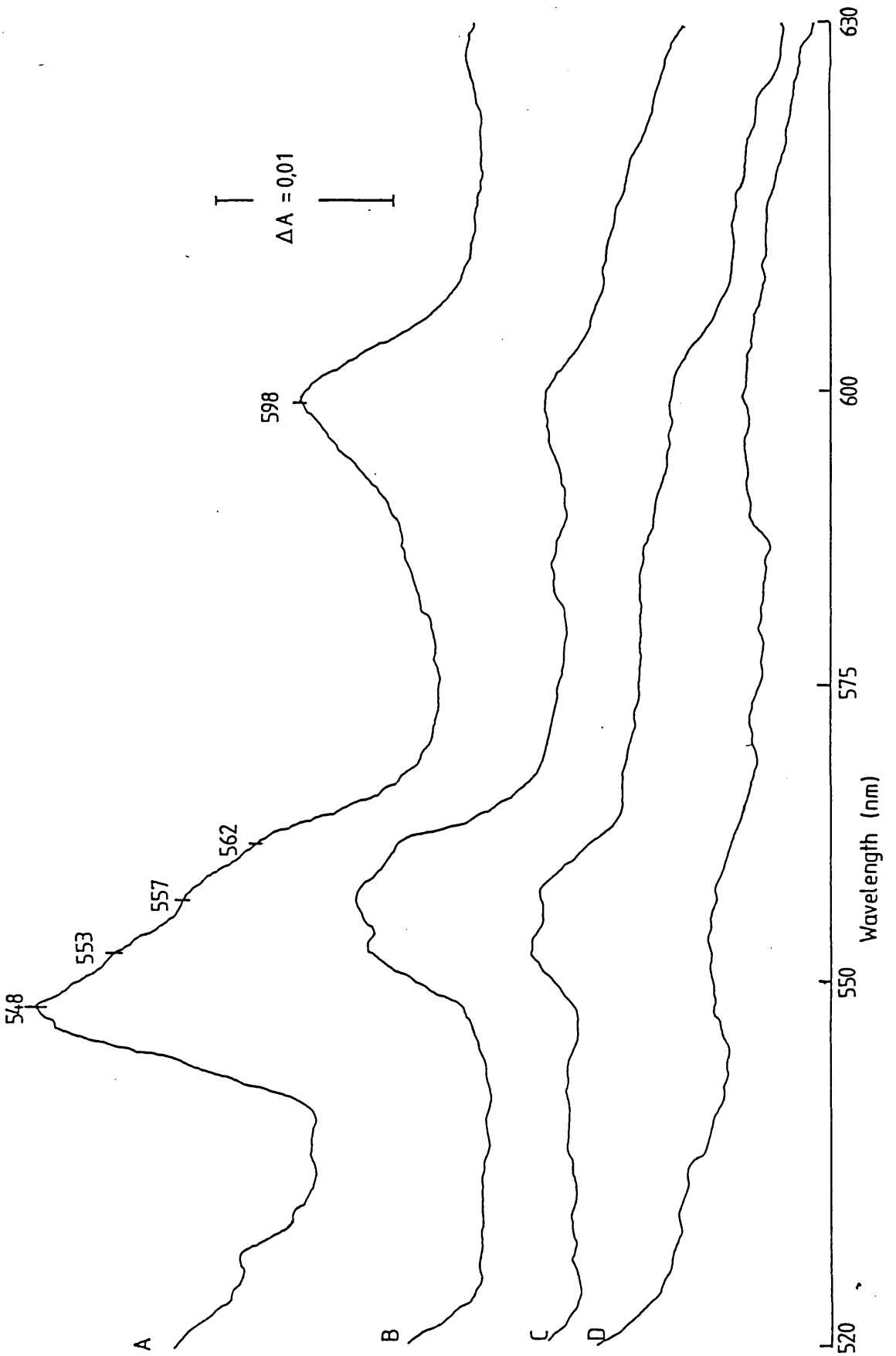
A. Complete reduced-oxidised spectrum obtained using dithionite.

B. Frozen during NADH oxidation.

C. As for B, after treatment with 10mM BAL (section 2.10).

D. As for B, including 0.9nmol myxothiazol/mg protein.

Figure 35. Effect of myxothiazol and treatment with BAL upon the degree of reduction of the cytochromes during NADH oxidation.



Following incubation with varying concentrations of BAL, it was possible to correlate the rate of quinol oxidation with the rates of oxidation of succinate and exogenous NADH. However, because BAL is an irreversable inhibitor, the degree of inhibition cannot be predicted from the amount added, as was possible using myxothiazol. Instead the following method was used to correlate DQH_2 oxidase activity, measured for each sample, with the rates of succinate and NADH oxidation:

According to the model of Kroger and Klingenberg (1973a):

rate of oxidation = Vred.Vox/(Vred + Vox)

If it is assumed that the rate of DQH_2 oxidation is linearly related to Vox (see Figure 21) by an unknown factor X, then:

rate of oxygen uptake = $Vred.V_{DQH}.X/(Vred + V_{DQH}.X)$

and by inversion:

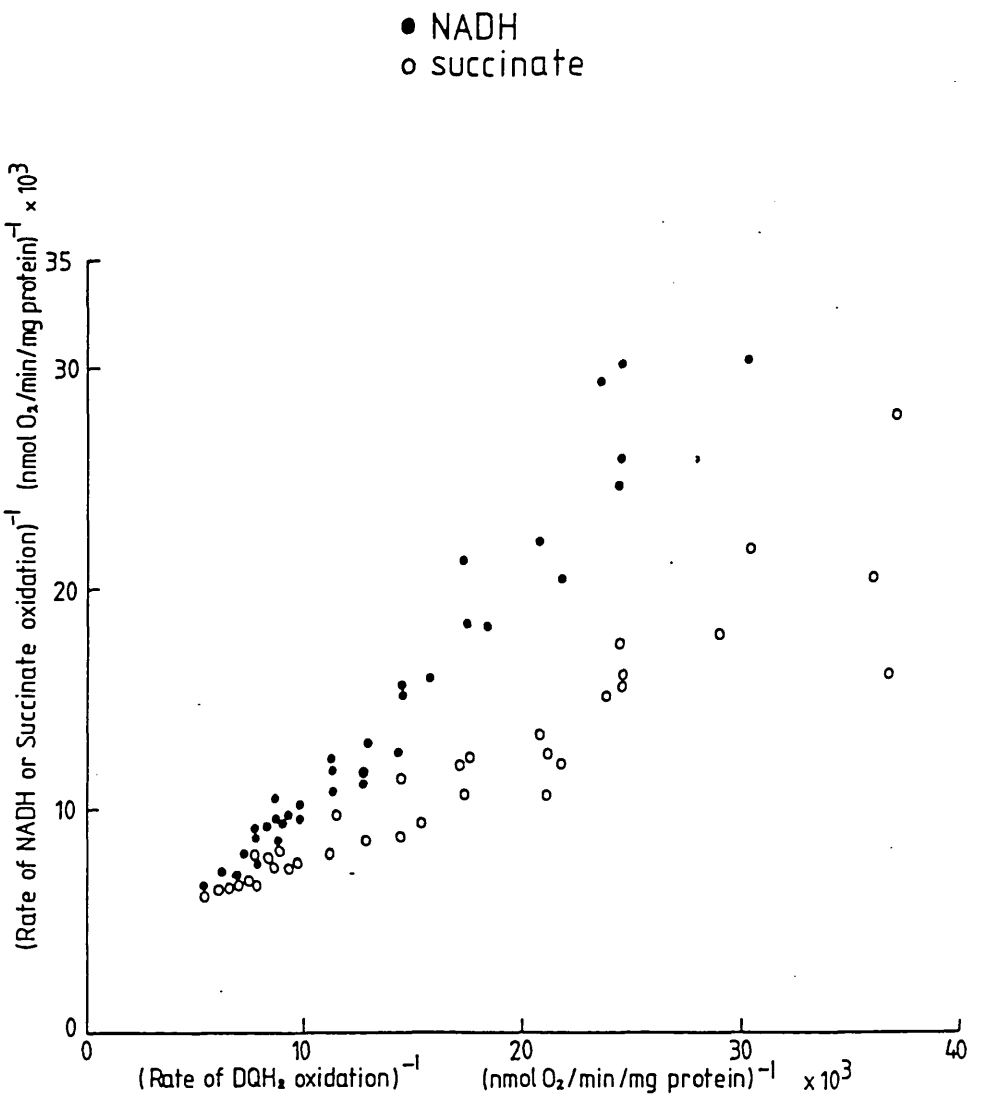
$1/\text{rate of oxygen uptake} = 1/Vred + 1/V_{DQH}.X$

where V_{DQH} is the measured rate of oxidation of DQH_2.

Consequently a graph of the reciprocal of the rate of NADH or succinate oxidation plotted against the reciprocal of the rate of DQH_2 oxidation should give a straight line of gradient 1/X and intercept 1/Vred if the model is obeyed. Ideally the value of Vox should also be independent of the substrate used and consequently the lines expected for succinate and NADH oxidation should be parallel. Data from six mitochondrial preparations are presented in Figure 36, and confirm the deviation from ideal behaviour already observed using HQNO and myxothiazol. The gradients of the lines for NADH and succinate oxidation are clearly different. The steeper gradient found with NADH as substrate indicates a closer dependence of the rate of NADH oxidation on the capacity for quinol oxidation than was found with succinate oxidation. This pattern was also found using myxothiazol (Figures 30 and 32). The plots for both substrates show a

Figure 36. The use of BAL to test ideal ubiquinone-pool behaviour of succinate and NADH oxidation.

Mitochondria were treated with BAL, succinate dehydrogenase activated and succinate, NADH and DQH_2 oxidation rates measured as described in the legend to Figure 33. The results presented are the combined data from 6 preparations (protein concentrations at assay 0.31-0.43 mg protein/ml). This plot is derived from a different linear form of the ubiquinone-pool equation to that presented in Figures 30 and 32. The derivation and meaning of the plot are discussed in section 5.2.



tendency to curve upwards. This was more marked with succinate, although this is not immediately obvious owing to the inherent distribution of errors in a double reciprocal plot. This non-linearity indicated a rapid decrease in the rate of oxidation at low quinol oxidase activities, by preparations treated with high concentrations of BAL. The same trend was shown previously using HQNO or myxothiazol to inhibit succinate oxidation. Because the plots were both non-linear and not parallel, meaningful measurement of Vred and Vox were not possible.

Three different means of restricting quinol oxidation had led to similar conclusions about how closely the oxidation of NADH and succinate conformed to the ideal pattern. The relationship of these deviations to the interaction between succinate and exogenous NADH oxidase is discussed in Chapter 7. An additional approach to the same problem was to vary the ubiquinone reducing capacity by regulating the activity of the dehydrogenases, for example by the use of glucose-6-phosphate dehydrogenase to control the rate of production of NADH (Cottingham and Moore, 1983). In the following section the use of duroquinone reductase activity as a measure of the activity of the exogenous NADH dehydrogenase is examined and the inhibition of NADH oxidation by CTC and Ca^{2+} together (Moller *et al.*, 1983) tested as a means of regulating the activity of the external NADH dehydrogenase.

Figure 37. Lineweaver-Burk plot of the reduction of duroquinone by exogenous NADH.

Initial rates of NADH oxidation were measured at 340-374nm by dual wavelength spectroscopy (section 2.7). Mitochondria (0.12mg protein/ml) were suspended in standard assay medium and incubated with 200ng antimycin/mg protein (to prevent oxidation of DQH_2) for 2 min prior to initiation of the assay by addition of NADH. The concentration of NADH was varied between 18 and 133 μM. From these plots the Km for NADH was 36μM.

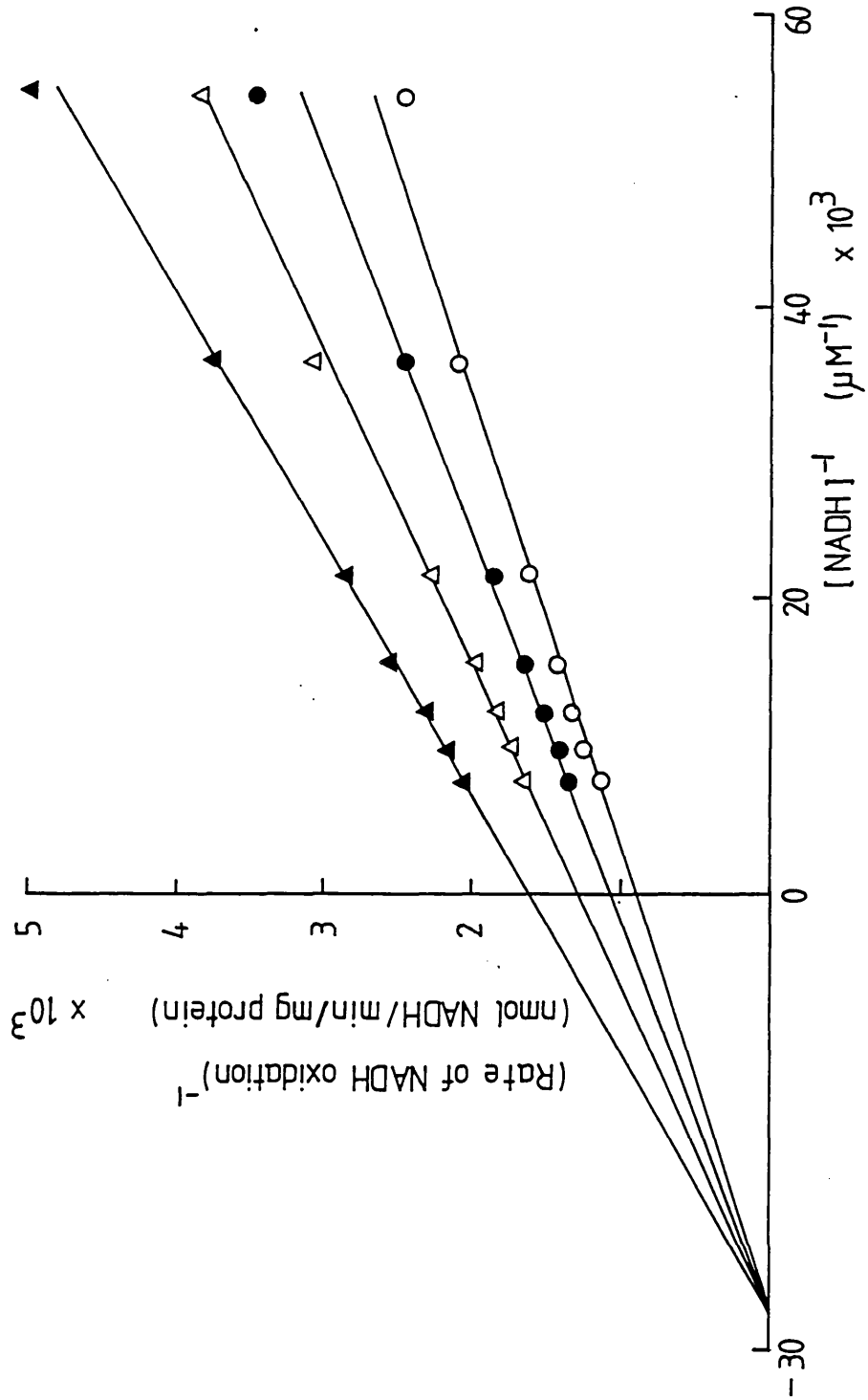
▲ 75μM duroquinone

△ 150μM duroquinone

● 300μM duroqunone

○ 500μM duroquinone

Figure 37. Lineweaver-Burk plot of the reduction of duroquinone by exogenous NADH.



5.3 NADH dependent duroquinone reduction.

It was assumed in sections 4.2 and 5.2 that NADH dependent duroquinone or UQ-1 reductase activity was due only to the exogenous NADH dehydrogenase of the inner mitochondrial membrane. In addition to this dehydrogenase plant mitochondria possess a second exogenous NADH dehydrogenase located in the outer mitochondrial membrane and capable of reducing added cytochrome c in the presence of antimycin (Douce et al., 1973). In this section results are presented which show that the exogenous NADH dehydrogenase of the outer mitochondrial membrane does not reduce duroquinone at a significant rate. The suitability of chlorotetracycline (CTC) complexed with Ca^{2+} as a specific inhibitor of the exogenous NADH dehydrogenase was examined as another possible method for testing the ubiquinone pool kinetics of NADH oxidation, in addition to the methods already discussed involving inhibition of ubiquinol oxidation (Chapters 3 and 4).

When the concentrations of NADH and duroquinone were varied, NADH dependent duroquinone reductase activity was found to have characteristics consistent with the operation of a single enzyme species (Figure 37). A single value for the Km for NADH (36μM) was found over a range of duroquinone concentrations, and at each constant duroquinone concentration the resulting Lineweaver-Burk plot was linear. The independence of the duroquinone concentration and the Km for NADH (Figure 37) suggested that the sites of NADH oxidation and of duroquinone reduction were different. Similarly the Km for duroquinone (150μM) was not dependent on the NADH concentration (results not shown). The apparent Km for NADH oxidation by intact plant mitochondria is very dependent upon the ionic composition of the assay medium. This is probably due in part to screening of fixed negative charges on the inner mitochondrial membrane, facilitating the approach of negatively charged NADH to the NADH binding site,

thus lowering the apparent Km for NADH (section 1.1.1). As well as the lowering of the Km for NADH, the presence of screening cations also increased the apparent Vm for NADH oxidation (Moller *et al.*, 1984). In contrast to NADH oxidase activity, there was very little difference in the Vm for duroquinone reductase activity between measurements made in low cation containing medium (Km 180μM, Vm 593 nmol NADH/min/mg protein) and the value obtained in the presence of the organic divalent cation NNNN'N'N'-hexamethyldecane-1,10-diamine bromide ((DM)Br_2) (Km 51μM, Vm 676 nmol NADH/min/mg protein, Figure 38). Changes in surface potential have often been reported to change Km values for membrane bound enzymes (Wojtczak and Nalecz, 1979, Nalecz *et al.*, 1980). Since the change in Vm for NADH oxidase activity could be attributed to increased quinol oxidising capacity in the presence of the screening cation (DM)Br_2 (Moller *et al.*, 1984), the change in Km only for duroquinone reductase activity provided supporting evidence that only the activity of the exogenous NADH dehydrogenase was being measured. This view was strengthened by subsequent reports of solubilization and isolation of the exogenous NADH dehydrogenase, capable of reducing duroquinone directly (Cook and Cammack, 1984, Klein and Burke, 1984).

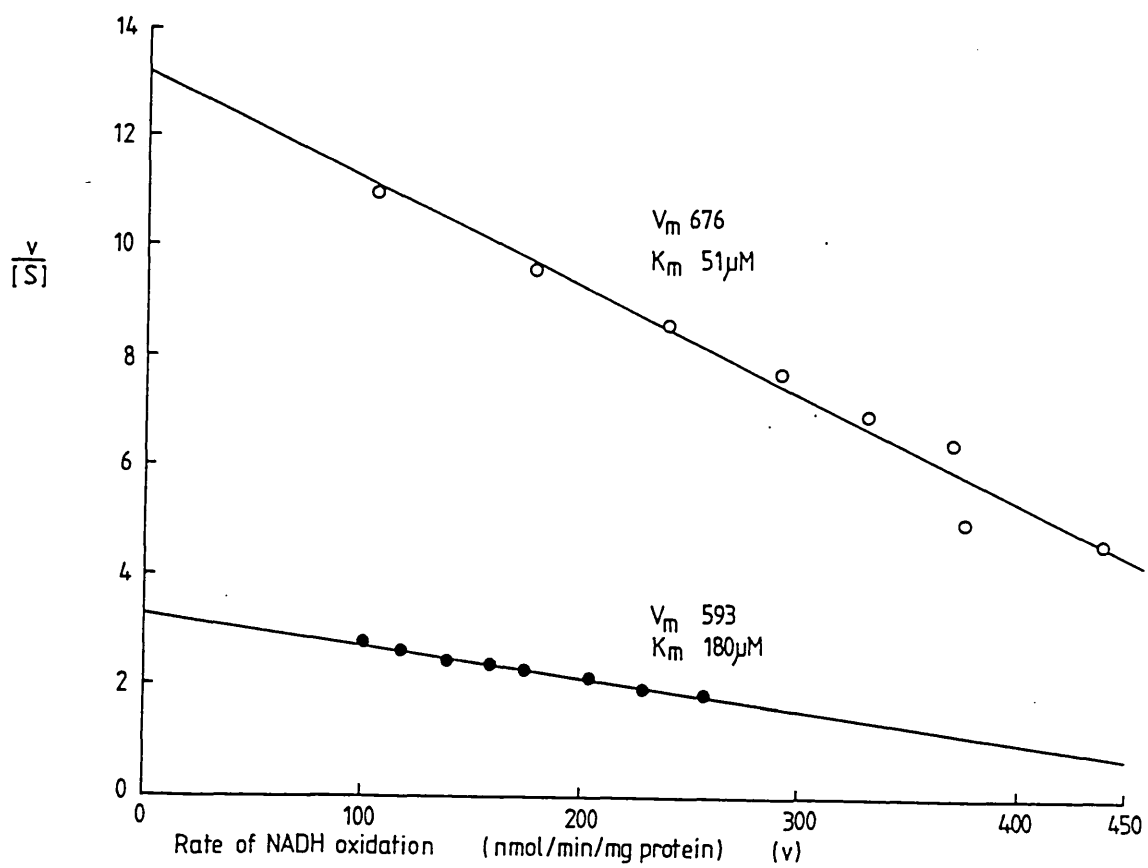
In the absence of a specific inhibitor for the outer membrane exogenous NADH dehydrogenase, possible reduction of duroquinone by this enzyme was tested indirectly. Chlorotetracycline (CTC) is a potent inhibitor of exogenous NADH oxidation by Jerusalem artichoke mitochondria when Ca^{2+} is present. The oxidation of succinate was inhibited only marginally under identical conditions (Moller *et al.*, 1983). It would seem that inhibition by CTC and Ca^{2+} is predominantly at the level of the NADH dehydrogenase as succinate and NADH oxidation share a common pathway from ubiquinone to oxygen (Figure 1). The effects of CTC in the presence of 2mM $CaCl_2$ on NADH

Figure 38. Eadie-Hofstee plot of NADH oxidation by duroquinone at low and high concentrations of cations.

Rates of NADH oxidation were measured (section 2.7) in a medium containing 0.3M sucrose, 5mM Mops buffer, pH7.2, 0.2µM FCCP, 0.36µM antimycin and 0.18mg protein/ml. The concentration of NADH was varied between 10 and 140µM and the assay was initiated by addition of 300µM duroquinone. The units of Vm are nmol NADH/min/mg protein.

● Assay medium as specified

○ + 2mM $(DM)Br_2$



dependent duroquinone reductase activity and NADH dependent cytochrome c reduction were compared (Table 5). CTC (25μM) had only a small inhibitory effect (20%) on antimycin-insensitive cytochrome c reductase activity, yet inhibited duroquinone reductase activity by 83%. Since the outer membrane dehydrogenase was much less inhibited than was duroquinone reductase activity, it was concluded that the outer membrane dehydrogenase probably did not significantly contribute to duroquinone reduction.

To determine whether CTC + Ca^{2+} could be used as a specific inhibitor of the external NADH dehydrogenase, NADH oxidase and duroquinone reductase activities were titrated with CTC in the presence of Ca^{2+} (Figure 39). CTC inhibited oxygen uptake more effectively than duroquinone reductase activity. This suggested that there were additional inhibitory effects of CTC upon the respiratory chain that would make CTC/Ca^{2+} unsuitable as a specific inhibitor of exogenous NADH dehydrogenase. Additional inhibitory effects of CTC are consistent with the 25% inhibition of the oxidation of succinate in the presence of CTC and Ca^{2+} (Moller *et al.*, 1983), as it has been shown in the present study that ubiquinol oxidase activity can be significantly inhibited without greatly inhibiting succinate oxidation. Such inhibition may be related to effects of the CTC/Ca^{2+} complex upon the properties of the membrane itself, rather than to interaction with specific inhibitory sites (Pershadsingh *et al.*, 1982).

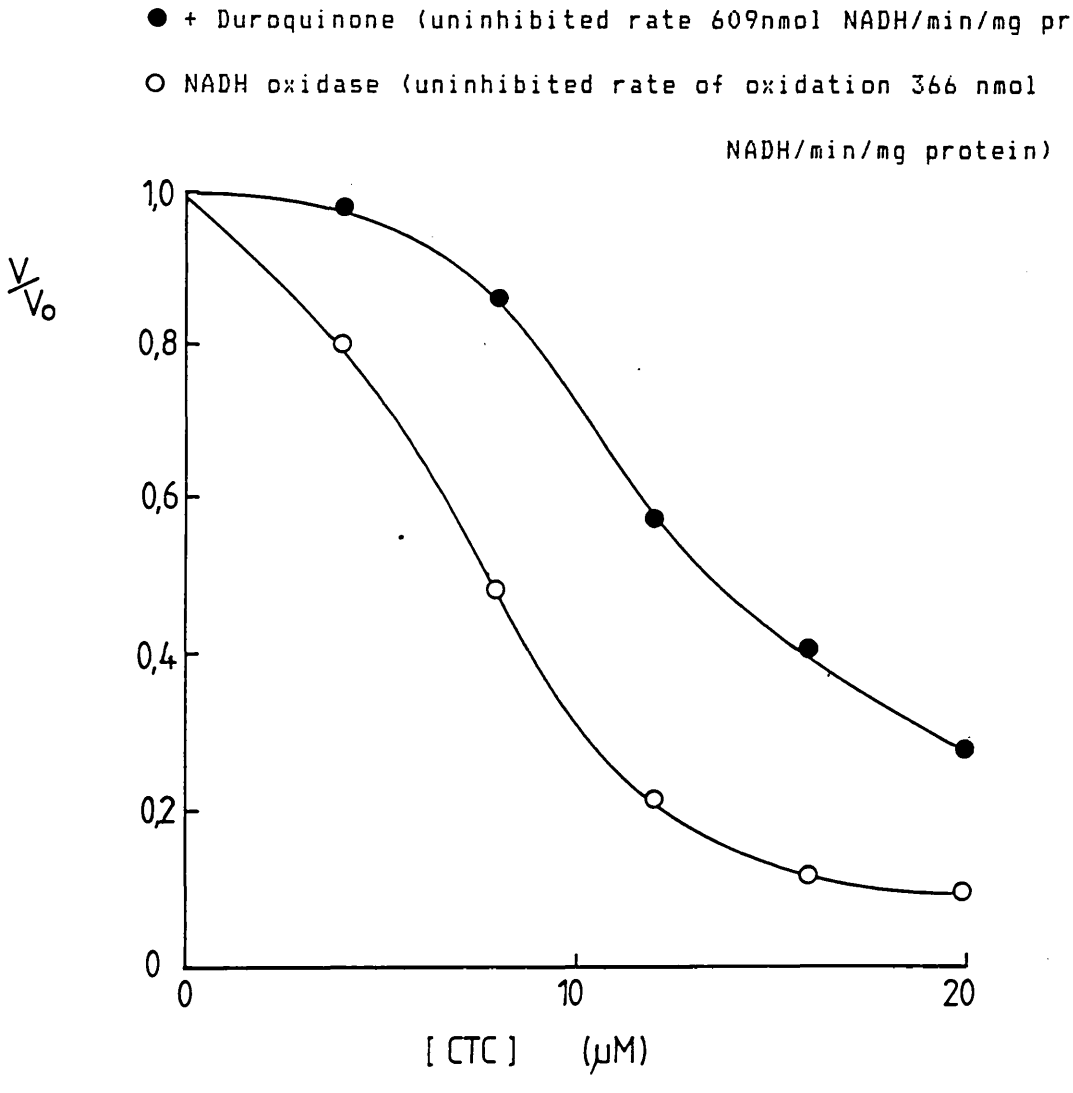
Table 5. Inhibition of antimycin-insensitive NADH-cytochrome c reductase and NADH-duroquinone reductase activities by CTC in the presence of Ca^{2+}.

Rates of NADH oxidation were measured as described in section 2.7 in standard assay medium containing 200ng antimycin/mg protein and 2mM $CaCl_2$. Protein concentrations were 0.09mg/ml and 0.15mg/ml for the measurement of cytochrome c reduction and duroquinone reduction respectively. Cytochrome c concentration was 50μM and duroquinone 300μM. Assays were initiated by addition of 200μM NADH. Results are expressed as nmol NADH/min/mg protein.

	Antimycin-insensitive	
Additions	Cytochrome c reduction	Duroquinone reduction
-	88	654
25uM CTC	70	111

Figure 39. Comparison of inhibition by CTC/Ca^{2+} of NADH oxidation by duroquinone and oxygen.

Rates of NADH oxidation were measured (section 2.7) in standard assay medium containing 0.2μM FCCP, 2mM $CaCl_2$ 0.21mg protein/ml and, for measurement of duroquinone reduction, 200ng antimycin/mg protein and 300μM duroquinone. Assays were initiated by addition of 200μM NADH.



Chapter 6. Production of SMP and subsequent separation of particles according to membrane polarity.

With the exception of Figure 43, results presented in this chapter were obtained in collaboration with Dr.I.Ericson at the Department of Biochemistry, University of Umea, Sweden.

The ability to produce submitochondrial particles that are either inside-out or right-side out with respect to the orientation of the inner membrane of intact mitochondria would be useful in the study of the organisation of respiratory components within the membrane and of the mechanisms that determine electron flux between phosphorylating and non-phosphorylating pathways. Of particular relevance to the present study was the possibility of showing that the interaction between succinate and NADH oxidase remained assymetric (Figure 10) when tested using submitochondrial particles. This would indicate that ultrastructural organisation of the mitochondria, for example into cristae and non-cristae regions was not essential to the interaction. Removal of the outer membrane by disruption of the mitochondria would also allow measurement of electron transport rates using cytochrome c as the acceptor instead of oxygen. This would permit the determination of all respiratory fluxes during the interaction between NADH and succinate oxidation by spectrophotometric measurements of NADH oxidation and cytochrome c reduction under strictly identical conditions, rather than relying on independent measurements of oxygen uptake and NADH oxidation. Similarly, removal of the outer membrane would allow cytochrome c to be used as a specific acceptor for the cytochrome pathway in the presence of the alternative oxidase. By this method the diversion of electrons between the two terminal pathways could be followed with greater precision than is currently possible.

The experiments presented in this chapter were aimed firstly at finding conditions for disruption of mitochondria that would yield submitochondrial particles that were predominantly either inside-out or right-side out, so that the respiratory activities associated with each face of the inner membrane could be studied. Secondly it was hoped that suitable conditions could be found for the separation of submitochondrial particles according to membrane polarity from a mixed population, by the use of phase-partitioning. The second objective was of interest as it was thought that different regions of the mitochondrial membrane may give rise to particles of different membrane polarity: cristae regions were thought more likely to give rise to inverted particles than non-cristae regions, by analogy with fractionation of the thylakoid membrane, which yields mainly inverted vesicles from regions where the membranes are "stacked" (Akerlund *et al.*, 1976). If this were the case with plant mitochondria it would be possible to test the composition of inner membrane particles originating from different regions of the membrane for signs of lateral separation of respiratory components. Patching of respiratory components within the inner membrane has been suggested as a possible cause of the unequal access of different respiratory substrates, and in particular exogenous NADH, to the alternative pathway of plant mitochondria (Rich, 1984).

6.1 Influence of the method of disruption, ionic and osmotic conditions on the polarity of SMP.

Two methods of mitochondrial disruption were used for the production of submitochondrial particles (SMP). Mitochondrial samples were either sonicated, or disrupted by the use of a French press. In order to test the idea that the polarity of the resulting particles would be related to the conformation of the inner membrane a variety of ionic and osmotic conditions were used. Magnesium ions promote

aggregation of the inner membrane of mammalian mitochondria (Stoner and Sirak, 1978) and a large variety of divalent and trivalent cations have been shown to induce contraction of both inner and outer membranes of non-energised corn mitochondria (Earnshaw, 1978). As can be seen from Table 6, the presence of $MgCl_2$ had a large influence on the polarity of the SMP produced. For each method of disruption, and at constant sucrose concentration, $MgCl_2$ caused increased inversion of the resulting SMP. Following sonication in standard suspending medium SMP were approximately 40% right-side out, as assayed by the latency of cytochrome oxidase activity. In the presence of 2mM $MgCl_2$ the latency of cytochrome oxidase activity increased, indicating that approximately 20% of the particles were right-side out. Increasing the concentration of $MgCl_2$ to 20mM caused only slight further decrease in the proportion of inside-out particles, suggesting that sites of interaction of the membrane with Mg^{2+} were nearly saturated at a concentration of 2mM. Earnshaw (1978) found that the binding of Ca^{2+} associated with mitochondrial contraction had a Kd of 0.3mM. Addition of EDTA in the absence of added $MgCl_2$ increased the proportion of right-side out particles, presumably by removing divalent cations bound to the outer surface of the inner membrane following isolation of the mitochondria. The effects of $MgCl_2$ and EDTA on the polarity of SMP produced were consistent with inside-out particles coming from aggregated or cristae regions since the same ionic conditions that produced inside-out particles were also those that cause mitochondrial contraction. In contrast to the large influence ionic conditions had on the polarity of particles produced, variation of the osmotic strength of the suspending medium by the use of sucrose concentrations in the range 0.15-0.6M had little effect (Table 6).

The same response of the polarity to ionic conditions was

Table 6. The influence of method of disruption, ionic and osmotic conditions on the polarity of submitochondrial particles.

Submitochondrial particles were prepared as described in section 2.11 by either sonication (S) or French pressing (Fp) as indicated. The medium in which the mitochondria were disrupted was standard suspension medium (section 2.1) modified as shown in the table. The polarity of the SMP was measured by the latency of cytochrome c oxidase activity as described in section 2.5. %OP is the proportion of the total activity measured in the absence of detergent and is taken to represent the proportion of SMP retaining the same membrane polarity as intact mitochondria (see text). The rate of cytochrome c oxidase activity quoted was the total activity measured in the presence of 0.02% Triton X-100. Rates are given as μmol cytochrome c oxidised/min/mg protein and where errors are quoted the results are the means and standard deviations of the number of preparations shown in parentheses.

Table 6.

Treatment	Ionic conditions	[Sucrose] (M)	%OP	Cytochrome c oxidase activity
S	-	0.3	43 ± 4 (11)	2.23 ± 0.98 (11)
S	+5mM $MgCl_2$	0.3	22 ± 1 (2)	1.95 ± 0.90 (2)
S	+20mM $MgCl_2$	0.3	18	0.95
S	+5mM EDTA	0.3	53	1.34
Fp	-	0.3	81 ± 8 (2)	0.94 ± 0.20 (2)
Fp	+5mM $MgCl_2$	0.3	66 ± 1 (2)	0.90 ± 0.28 (2)
Fp	+20mM $MgCl_2$	0.3	52	0.36
Fp	+5mM EDTA	0.3	97	1.76
Fp	-	0.15	87	2.53
Fp	-	0.6	90	1.40
Fp	+5mM $MgCl_2$	0.15	71	1.44
Fp	+5mM $MgCl_2$	0.6	89	1.58

observed when mitochondria were disrupted using a French press, except that the range of polarities produced were different. Preparations of SMP with polarities from 18 to 53% right-side out could be produced by sonication, depending on the ionic composition of the medium in which the mitochondria were disrupted (Table 6). Under identical conditions, French pressing generated a higher proportion of right-side out particles (52-97%) than did sonication. The two disruption methods differed in that mitochondria were sonicated for a total of 10secs (4 x 5 secs, 50% duty cycle) during which time vesicles may been broken and have resealed a number of times. Mitochondria were passed through the French press only once, and so would experience only a very short period of disruption. Some indication of the degree of disruption caused by each method was sought by following each treatment with the other, noting the extent to which the second treatment altered the polarity of the SMP (Table 7). It was found that French pressing SMP generated by sonication resulted in large changes in polarity, approaching the polarity values expected for French pressing alone. In contrast, sonication of SMP prepared by French pressing caused very little change in polarity. This suggested that French pressing may generate small particles, less prone to further disruption by sonication than were membrane fragments produced by sonication.

The SMP obtained by sonication in the presence of 5mM $MgCl_2$ were rather less right-side out (22%) than those made under very similar conditions by Moller and Palmer (1982), who reported that 51% of the SMP were right-side out. The reason for this discrepency is not known, but it may be that small differences in membrane composition have a large influence on the polarity with which the vesicles reseal, since identical procedures yielded Arum SMP that were 84% inside-out (Moller *et al.*, 1981b).

Table 7. The effect of order of method of disruption on the polarity of SMP.

Mitochondria were prepared as described in section 2.1, except that the preparation was split after the final centrifugation step. Half the mitochondria were resuspended in standard suspension medium containing 20mM $MgCl_2$ (5.28mg protein/ml) and the other half in standard suspension medium containing 5mM EDTA (7.48mg protein/ml). Both suspensions were split into two and one half disrupted by sonication and the other half by French pressing (four samples). Following centrifugation to remove large membrane fragments (section 2.11) half of each supernatant was again disrupted but by the alternative method. SMP were then isolated from the resulting 8 samples and the polarity of the SMP measured by the latency of cytochrome oxidase activity, as described in section 2.5 and the legend to Table 6. Values given are the percentage of the SMP that were right-side out.

	Disruption 1		Disruption 2	
Ionic conditions	Fp	S	S	Fp
+5mM EDTA	97	-	96	-
+5mM EDTA	-	53	-	76
+20mM $MgCl_2$	51	-	52	-
+20mM $MgCl_2$	-	18	-	52

6.2 Separation of SMP according to membrane polarity by phase-partition.

Phase partitioning between aqueous mixtures of dextran and polyethylene glycol (PEG) can be used to separate materials according to their surface properties (Albertsson, 1971). This method has been applied to the purification of inside-out SMP from Arum mitochondria, resulting in an improvement in purity of the inside-out SMP from 86% to 95% inside-out (Moller *et al.*, 1981b). In the present study Jerusalem artichoke SMP prepared by sonication in standard suspension medium (43% right-side out, Table 2) were used to try to find a composition of phase-system that would be capable of separating the SMP present in this mixed population, according to the polarity of the membrane. The concentration of the polymers (PEG and dextran), pH and ionic composition are all critical in determining the resolving power of phase partition. As the effects of these variables interact it was not possible to simply determine the best value for each, and then combine them. By trial and error a useful system was found in which inside-out particles partitioned into the lower dextran-rich phase and the right-side out particles into the top, PEG-rich phase.

This composition of phase system was used to purify inside-out SMP from those prepared by sonication in the presence of 20mM $MgCl_2$ (Figure 40). The SMP applied to the phase system were 83% inside-out but after phase partition the polarity of the particles in the bottom phase had improved to 92% inside-out. Only the first phase-separation was effective in removing right-side out SMP: no improvement was found following subsequent extractions of the bottom phase with fresh top phase. By this method SMP 92% inside-out were prepared. A similar degree of purification (95% inside-out), achieved using a similar method, was reported for SMP made from Arum mitochondria, (Moller *et al.*, 1981b).

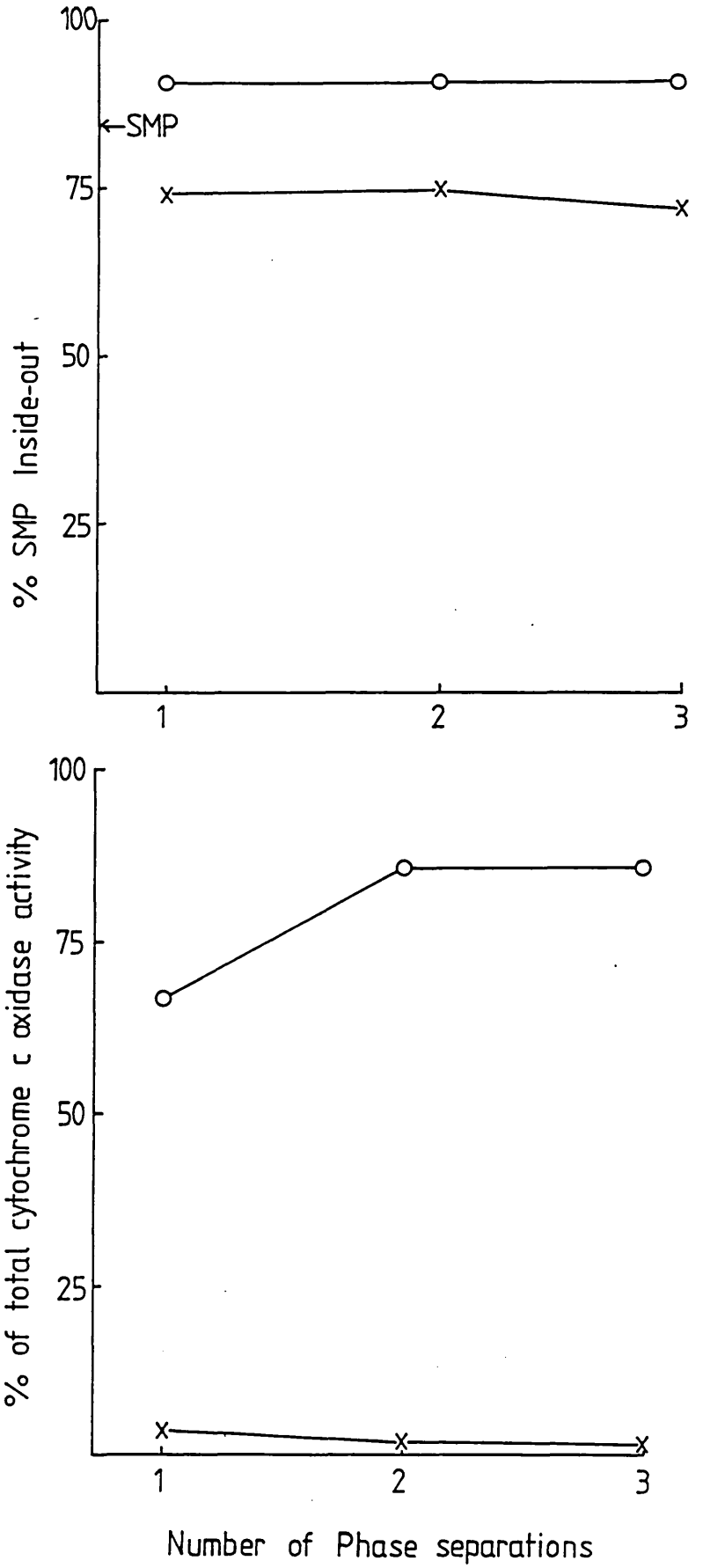
Figure 40. Purification of inside-out SMP by phase partition.

SMP were prepared by sonication in standard suspension medium containing 20mM $MgCl_2$, as described in section 2.11. SMP (4.9mg protein) were applied to a 4g (total weight) phase system containing 6.6% (w/w) of both dextran and PEG-4000, 0.3M sucrose and 10mM potassium phosphate buffer, pH 8.0. The top phase was discarded and the bottom phase re-partitioned with new top phase taken from an identical phase system prepared without SMP. This process was repeated to give three phase separations in all. Samples were removed from each top and bottom phase and assayed for polarity using the latency of cytochrome c oxidase activity (section 2.5). The percentage of total cytochrome c oxidase activity was calculated from the activity of the SMP before phase partition, assayed in the presence of 0.2% Triton X-100.

X Top phase

O Bottom phase

Figure 40. Purification of inside-out SMP by phase partition.



Replacement of a part of the PEG with PEG to which hexamethylene diamine had been covalently attached (HMDA-PEG) improved the resolving power of the phase system by increasing the amount of material in the upper phase and by slightly increasing selectivity for polarity (Figure 41). Substitution of 10% of the PEG with HMDA-PEG gave maximal improvement in the performance of the phase-system and this system was used to separate inside-out and right-side out SMP from the mixed population produced by sonication in standard suspension medium by the method described in section 2.14. The resulting SMP were assayed for cytochrome c oxidase, succinate dehydrogenase and latent malate dehydrogenase activity. Also the cytochrome content was measured at 77K (Table 8). Good separation of SMP of different polarities was achieved as measured by the cytochrome c oxidase assay, and the accuracy of this method of polarity determination was supported by measurements of the activity of malate dehydrogenase released upon addition of 0.02% (w/v) Triton X-100, since approximately three times more activity was trapped in the SMP from the top phase than was trapped in those from the bottom phase. The specific activities for both cytochrome c oxidase and succinate dehydrogenase activity were higher in the inside-out than in the right-side out SMP. This difference was also reflected in the cytochrome content, although the ratio of cytochrome c in top and bottom phases was slightly lower than the ratios for the other cytochromes, suggesting loss of cytochrome c from the right-side out particles, as would be expected from the location of cytochrome c on the outer face of the inner membrane.

The difference in the specific activities and cytochrome contents of the material from top and bottom phases had two possible explanations. Either the inside-out and right-side out particles differed in their protein composition indicating lateral separation

Figure 41. Effect of HMDA-PEG 6000 on the partition of SMP.

SMP were prepared by sonication in standard assay medium as described in section 2.11. SMP (1.5mg protein) were added to 2g (total weight) phase systems containing 6.6% (w/w) of both dextran and PEG-4000, 0.3M sucrose and 10mM potassium phosphate buffer, pH 8.0. In each system a proportion of the PEG-4000 was replaced by HMDA-PEG 6000. The specific cytochrome c oxidase activity of the SMP was 1.21µmol cytochrome c/min/mg protein measured in the presence of 0.02% Triton X-100 (section 2.5). The amount of activity that had collected at the interface of the two phases was calculated from the difference between the activity added and that recovered in the two phases.

X Top phase

O Bottom phase

● Interface

Figure 41. Effect of HMDA-PEG 6000 on the partition of SMP.

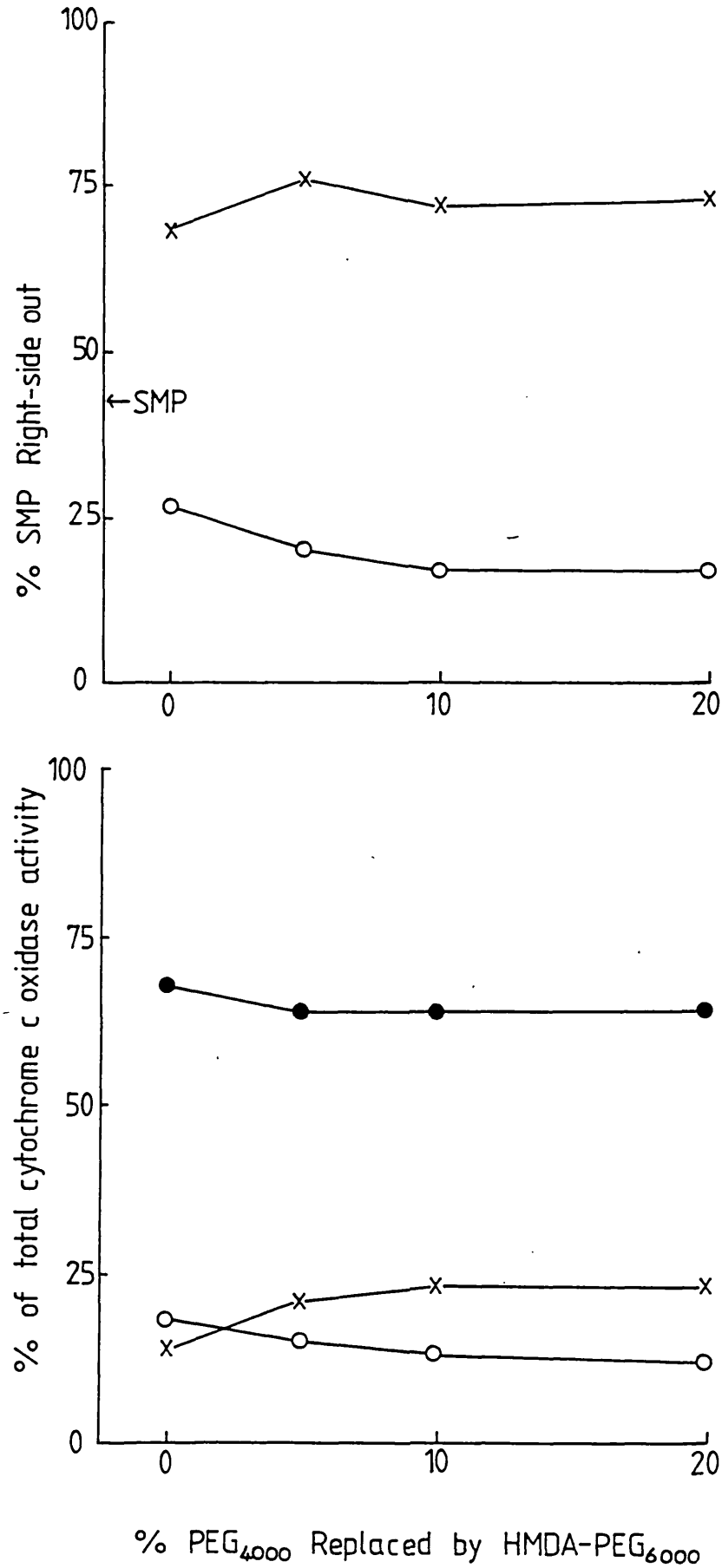


Table 8. Properties of right-side out and inside-out SMP separated from a mixed population by phase partition.

SMP were prepared by sonication in standard suspension medium as described in section 2.11 and inside-out and right-side out particles separated by phase partition as described in section 2.14. Succinate dehydrogenase (SDH) activity was measured by the rate of DCPIP reduction (section 2.7), after activation of succinate dehydrogenase by incubation with ATP/$MgCl_2$/Pi (section 2.2). The units of succinate dehydrogenase activity are nmol DCPIP/min/mg protein. The polarity of the particles was measured by latency of cytochrome oxidase activity (section 2.5 and the legend to Table 5). Rates of cytochrome c oxidase activity quoted were obtained in the presence of 0.02% Triton X-100 and the units are μmol cytochrome c/min/mg protein. The latency of malate dehydrogenase (MDH) was measured as described in section 2.4 and the units are μmol NADH oxidised/min/mg protein. Cytochrome content was measured by dual-wavelength spectroscopy using the wavelength-pairs of Lance and Bonner (1968) (section 2.12) and the units are nmol cytochrome/mg protein. Results presented are, with the exception of mitochondrial cytochrome content, the means and standard deviations from 3 preparations.

Table 8.

	Mitochondria	SMP	Inside-out SMP	Right-side out SMP
% Right-side out	-	45 ± 3	26 ± 1	76 ± 7
Cytochrome oxidase	2.02 ± 0.1	2.30 ± 0.7	3.28 ± 0.3	2.15 ± 0.7
SDH	66 ± 23	140 ± 25	186 ± 38	118 ± 41
Latent MDH	16.2 ± 2.6	4.50 ± 0.9	3.69 ± 0.6	9.70 ± 3.8

	Mitochondria	Inside-out SMP	Right-side out SMP
Cytochromes a	0.101	0.259 ± 0.023	0.164 ± 0.037
Cytochromes b	0.084	0.211 ± 0.014	0.122 ± 0.038
Cytochromes c	0.161	0.348 ± 0.044	0.183 ± 0.032

of proteins within the membrane, or some unidentified contaminating proteins had preferentially partitioned into the top-phase. When the protein content of the samples was examined by polyacrylamide gel electrophoresis (Figure 42) significant differences were found between material from the top and bottom phases. Polypeptides of molecular weights 15.5, 18.5 and 19.6 kDa were present in the SMP preparation but partitioned almost entirely into the top phase when compared to the major protein bands of molecular weights 53.1 and 29.8 kDa. In addition several minor differences could be seen between material from the top and bottom phases, particularly in the high molecular weight region of the gel (above 60kDa). The identities of the polypeptides of molecular weights 15.5, 18.5 and 19.6 kDa are not known, but it may be significant that the degree of difference between the amounts of these proteins between top and bottom phases appears somewhat greater than the degree of separation of the SMP according to their polarity (Table 8). This would imply that these proteins may not be associated with the mitochondrial inner membrane. Clearly much more work is necessary to establish the differences in membrane composition that may exist between inside-out and right-side out particles, but from the work presented no evidence could be found for uneven distribution of the cytochromes or of succinate dehydrogenase activity.

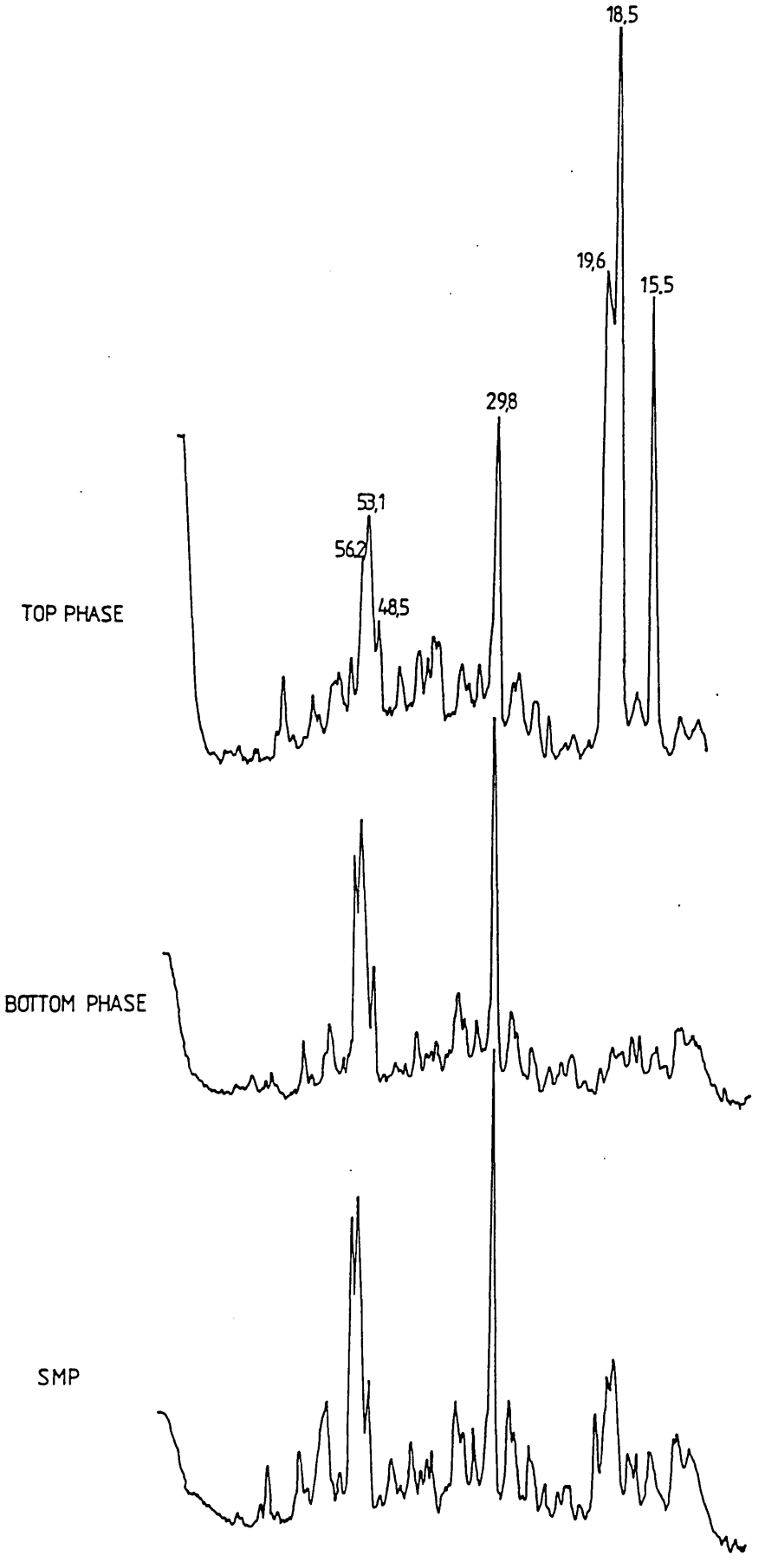
6.3 The interaction of succinate and NADH oxidase activity in SMP.

Cowley (1977) was unable to find significant inhibition of NADH oxidation by succinate oxidation using Jerusalem artichoke SMP. This was probably because the SMP had little respiratory control. From the results presented in Chapter 3 it was clear that the role of respiratory control in the interaction between the two substrates was only to restrict the activity of the cytochrome pathway. Consequently it was possible, by the use of inhibitors of the cytochrome pathway,

Figure 42. Polypeptide composition of inside-out and right-side out SMP purified by phase partition.

SMP were prepared by sonication in standard assay medium as described in section 2.11. Inside-out and right-side out particles were separated by phase partition as described in section 2.14. The polypeptide composition was analysed by SDS-polyacrylamide gel electrophoresis (section 2.13). The apparent molecular weights of the major peaks are given in kDa.

Figure 42. Polypeptide composition of inside-out and right-side out SMP purified by phase partition.

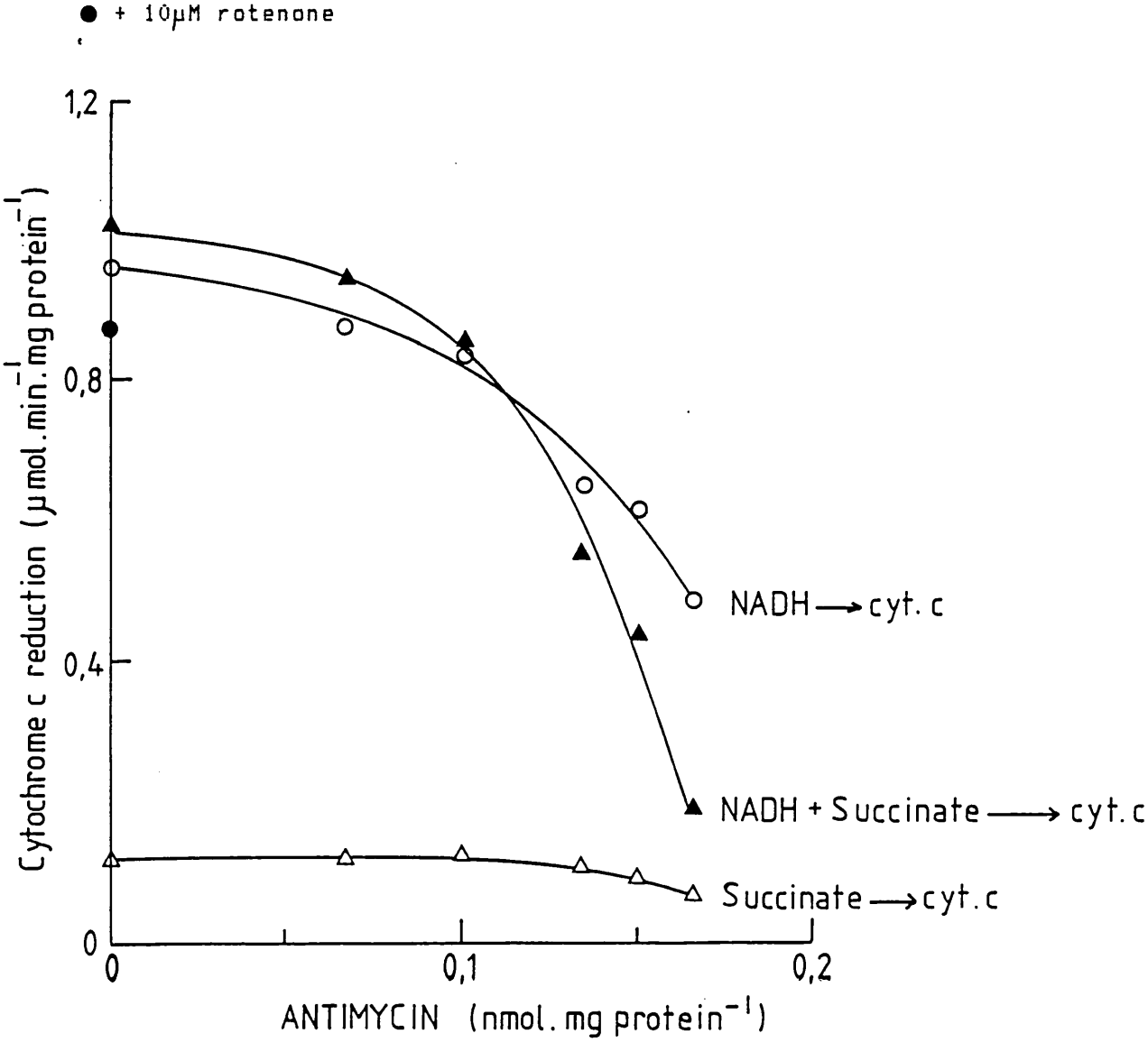


to induce the inhibition of NADH oxidation by succinate even in uncoupled mitochondria (Figure 15). This method made it possible to determine whether the asymmetry of the interaction was preserved following disruption of the mitochondria to form SMP. If the inhibition were not still present in SMP then it could be concluded that some essential feature of the organisation of the respiratory components had been lost during disruption, but if it persisted then the interaction was probably due to intrinsic properties of the respiratory components themselves. The use of SMP had the advantage that the outer membrane was no longer a barrier to the use of cytochrome c as an electron acceptor. Therefore cytochrome c oxidase was inhibited by addition of 1mM KCN to the assay medium and the rates of NADH oxidation and of the reduction of added cytochrome c were measured. For this experiment SMP prepared by French pressing in the presence of 5mM EDTA were used as this method gave SMP that were predominantly right-side out (Table 6). The use of cytochrome c as the terminal electron acceptor had an additional advantage over the use of oxygen, apart from the ability to monitor total electron transport spectrophotometrically: the specificity of cytochrome c reduction for the outer face of the inner membrane meant that only right-side out SMP would be able to reduce exogenous cytochrome c, consequently only NADH oxidation via the exogenous NADH dehydrogenase would be measured. This was confirmed by the observation that rotenone failed to significantly inhibit NADH oxidation in this system (Figure 43), indicating that the membrane had not become permeable to NADH during SMP preparation, nor had mixed polarity vesicles been formed containing regions of inside-out and right-side out membrane. The formation of mixed vesicles would in any case not have shown such a high right-side out polarity (Table 6).

To determine whether the asymmetric interaction between succinate

Figure 43. Inhibition of NADH oxidation by succinate oxidation using SMP.

SMP were prepared by French pressing in standard assay medium containing 5mM EDTA (section 2.11), and succinate dehydrogenase was activated according to the method given in section 2.2. Rates of cytochrome c reduction and NADH (150μM) oxidation were measured in standard assay medium (section 2.7) containing 1mM KCN, 50μM cytochrome c, 0.2μM FCCP, 0.08mg SMP protein/ml and antimycin as indicated.



and NADH oxidation persisted after membrane disruption, SMP produced by French-pressing in the presence of EDTA were used as these were predominantly right-side out (Figure 43). To completely uncouple the SMP, FCCP was included in the assay. Low levels of antimycin were used to restrict the cytochrome pathway, although similar results were obtained using myxothiazol instead of antimycin (results not shown). No non-enzymatic rate of cytochrome c reduction by NADH or succinate could be detected.

The production of SMP from mitochondria resulted in a doubling of the bc_1 complex content (on a protein basis), as shown by the increased antimycin titre of the SMP (Figure 43). Taking into account the doubling of the antimycin titre the rates of exogenous NADH oxidation shown in Figure 43 are equivalent to a rate of NADH oxidation in intact mitochondria of 250nmol NADH/min/mg mitochondrial protein, showing that little inactivation had occured during SMP preparation. In contrast the rate of succinate oxidation found with these SMP was less than 15% of that expected on the same basis. The susceptibility of succinate oxidation to inactivation during the production of SMP was also noted by Moller *et al.* (1981b). This imbalance in the rates of oxidation of the two substrates led to a very interesting observation. At low concentrations of antimycin the rate of cytochrome c reduction in the presence of both succinate and NADH was nearly additive, but as the cytochrome pathway was progressively inhibited, addition of succinate actually gave a lower total electron flux to cytochrome c than was obtained in the presence of NADH alone. Obviously this result shows that the assymetry of the interaction is not affected by disruption of the mitochondria. A relatively low rate of succinate oxidation was capable of inhibiting NADH oxidation to a great extent: the inhibition was in fact larger than the rate of succinate oxidation causing it. The control that

succinate oxidation can exert upon the oxidation of cytosolic NADH is potentially an important mechanism for the regulation of exogenous NADH oxidation, particularly when energy demand is low. Possible explanations for these effects are the subject of Chapter 7.

Chapter 7. Discussion.

Electron transport along the mitochondrial respiratory chain is diffusion coupled at two points: at the level of cytochrome c and at the level of ubiquinone. One advantage of this arrangement over stoichiometric association of complexes is that it allows branching between different pathways of oxidation. Cytochrome c no longer fulfils this role in mitochondrial electron transport, but it still behaves as a mobile pool (Froud and Ragan, 1984, Gupte *et al.*, 1984). This property could be an evolutionary remnant from bacteria as the electron transport pathways of some bacteria, from which mitochondria may have evolved, still branch between different oxidases at cytochrome c (Froud and Ragan, 1984). Branching at ubiquinone is more complicated in the plant respiratory chain than in mammalian mitochondria because of the additional ubiquinone reducing and ubiquinol oxidising components present in the membrane (section 1.1). With the ability to branch comes the possibility of regulation of electron distribution, and it would be remarkable if this potential were not developed by evolution to the advantage of the plant. The simplest means of regulation is that proposed by Kroger and Klingenberg (1973a,b) where the rates of oxidation of different substrates are determined only by the activities of the enzymes reducing and oxidising the pool of ubiquinone. Simple competition between different substrates is then predicted in the situation where more than one oxidisable substrate is present. However, this simple behaviour is hardly ever observed (one exception is the report of De Troostembergh and Nyns, 1978), either in mammalian mitochondria (Gutman, 1980) or in the case of plant mitochondria (section 1.3).

In the present study one example of the complex behaviour of branching in the plant respiratory chain was chosen for study. This was the asymmetric mutual inhibition of exogenous NADH oxidation by

succinate oxidation. This was only one of many examples of the odd behaviour of branches in plant mitochondria, but was chosen as the most straightfoward to study as it was not complicated either by the presence of the alternative oxidase, the nature of which is still controversial, nor by the complexities of malate oxidation (section 1.1.2). The system was amenable to kinetic analysis and it was hoped that results obtained from the study of the interaction of succinate and exogenous NADH oxidation may be applicable to these other, more complex examples. The reports of Cowley (1977) and Cowley and Palmer (1980) showed that oxidation of succinate and exogenous NADH together did not show simple behaviour, since succinate oxidase, which is generally less active, or equally active, compared to exogenous NADH oxidase, greatly inhibited exogenous NADH oxidase. It was therefore of interest to know how closely the oxidation of either substrate alone conformed to the ideal model of ubiquinone pool behaviour (Kroger and Klingenberg, 1973a,b) and which factors were important in the interaction of the two substrates. The inhibition of exogenous NADH by the oxidation of endogenous NADH or by succinate oxidation may be an important method of regulation of exogenous NADH oxidation, particularly when energy demand is low. The oxidation of exogenous NADH is only coupled to two sites of energy conservation and is therefore energetically wasteful when compared to rotenone sensitive endogenous NADH oxidation. For this reason one might expect the oxidation of exogenous NADH to be regulated. The extent of the interaction is dependent on the respiratory state such that exogenous NADH oxidation is inhibited when the concentration of ADP is low (state 4). This study has shown that the interaction has an additional property that is useful in control: this was the ability of low succinate oxidase activities to cause a large inhibition of a much higher rate of NADH oxidation (Figures 43).

Some discussion of the results has already been included. It is the purpose in this section to discuss the results with respect to the mechanism of oxidation of the two substrates, the way the pattern of oxidation of each substrate when oxidised alone may relate to the interaction between them when both are present, and the possible relevance of this study to other examples of branching of the plant respiratory chain.

7.1 The pathway of exogenous NADH oxidation.

The involvement of all or part of the ubiquinone pool in the oxidation of exogenous NADH has been brought into question by certain observations. The relative inability of exogenous NADH to be oxidised via the alternative pathway of sweet potato mitochondria led Tomlinson and Moreland (1975) to suggest that exogenous NADH dehydrogenase may donate electrons directly to the bc_1 complex, rather than directly to the ubiquinone pool. This was consistent with the previous report of Storey (1971) that the reduction of ubiquinone was preceded by reduction of the b cytochromes when exogenous NADH was added to mitochondria, but that the reverse order of reduction was found using succinate. A more recent suggestion, made to account for the resistance of exogenous NADH oxidation to inhibition by UHDBT, has been that the exogenous NADH dehydroenase reduces ubiquinone only as far as the ubisemiquinone which may then reduce cytochrome b, so bypassing the site of UHDBT inhibition (Cook, 1984). For exogenous NADH oxidation to be maintained by such a pathway it was also necessary to suggest that the bc_1 complex operated according to a branched-linear mechanism, rather than according to a Q- or b-cycle. An alternative suggestion has been that the ubiquinone pool is compartmentalized such that different sub-pools selectively connect certain dehydrogenases with one of the two oxidases (Palmer,

1979, Moore and Rich, 1980). Lance *et al.* (1985) distinguished three separate sub-pools of ubiquinone: one associated with the exogenous NADH dehydrogenase, one preferentially accepting electrons from complex I and succinate dehydrogenase, and a third pool reduced by the rotenone insensitive endogenous NADH dehydrogenase. Similarly, the resistance of exogenous NADH oxidation to the inhibitor DBCT (Moore *et al.* , 1980) has also been explained on the basis of subdivisions of the ubiquinone pool. Clearly the mechanism and factors regulating exogenous NADH oxidation are not properly understood, and it is far from clear how ubiquinone may effectively be compartmented within the membrane given both the high mobility values reported for ubiquinone within the membrane and the large amount of ubiquinone present, especially in Jerusalem artichoke mitochondria (Table 2 and section 7.2).

The results of the present study allow some conclusions to be drawn about the pathway of exogenous NADH oxidation. The amounts of myxothiazol or antimycin necessary for complete inhibition of either succinate or exogenous NADH oxidation were identical, showing that the same complement of bc_1 complexes were passing electrons from both substrates. Therefore there was no evidence of compartmentation of the bc_1 complexes. Any mechanism proposed to explain the asymmetric interaction of succinate and exogenous NADH oxidation, based on ubiquinone compartmentation, would have to take into account the ability of each dehydrogenase to communicate with the same bc_1 complexes. The pattern of inhibition of exogenous NADH oxidation by antimycin, BAL and myxothiazol were all essentially hyperbolic with respect to inhibition of quinol oxidation. Qualitatively this is evidence of molecular mobility between the dehydrogenase and the bc_1 complex, presumably mediated by ubiquinone (section 1.2). This property of exogenous NADH oxidation has been noted previously using

antimycin (Cottingham and Moore, 1983). The use of duroquinone as an acceptor for the exogenous NADH dehydrogenase was shown to be free from interference by the outer membrane NADH dehydrogenase, and to behave as a single enzyme type with respect to substrate concentration and electrostatic screening of the membrane surface. The possibility that the exogenous NADH reduced the bc_1 complex directly, without mediation of free ubiquinone was also considered as an explanation of the resistance of exogenous NADH oxidation to HQNO, particularly as ubiquinone reductase activity was partially sensitive to HQNO. This possibility could be excluded as antimycin was without effect on quinone reductase activity, but completely inhibited exogenous NADH oxidase activity. The resistance of NADH oxidation to HQNO was probably related to the production of superoxide radicals during exogenous NADH oxidation (Huq and Palmer, 1978b, Rich and Bonner, 1978a, Huq, 1978), rather than to an intrinsic difference between the pathways of oxidation of NADH and succinate. In this respect it is of interest that organotin compounds, of which DBCT is an example, are also susceptible to breakdown by free radical attack, including attack by superoxide anions (Neumann,1970, Rehorek and Janzen, 1984). Taken together with the recent reports of isolation of the exogenous NADH dehydrogenase of plant mitochondria, which can reduce ubiquinone directly (Cook and Cammack, 1984, Cottingham and Moore, 1984, Klein and Burke, 1984) these results strongly suggest that exogenous NADH oxidation occurs as shown in Figure 1, and the participation of free ubiquinone in the oxidation of exogenous NADH is assumed in the following discussion.

7.2 The kinetics of oxidation of succinate and exogenous NADH.

The extent to which the oxidation of succinate and exogenous NADH followed the ideal kinetics of Kroger and Klingenberg (1973a,b) was assessed using three different methods of partially restricting the oxidation of ubiquinol oxidation of uncoupled mitochondria. The degree of inhibition of ubiquinol oxidase activity that was caused by these inhibitors was determined using duroquinol as an artificial donor to the bc_1 complex. In the cases of HQNO and myxothiazol, duroquinol oxidase activity was used to determine inhibition constants which could then be used to calculate saturation of binding sites. Treatment with BAL probably resulted in partial destruction of the Rieske centre, and the extent of inhibition was measured using duroquinol, once the remaining BAL had been removed from the mitochondria. The similarity of the inhibitor sensitivities of duroquinol oxidation and (with the exception of NADH oxidation inhibited by HQNO) oxidation of exogenous NADH and succinate are evidence that duroquinol is oxidised by the same pathway as endogenous ubiquinol and so can be used as a measure of ubiquinol oxidase activity. Direct oxidation of DQH_2 was reported by Kroger and Klingenberg (1973a,b) and is consistent with the results of Schneider *et al.* (1980), who found that the rate of DQH_2 oxidation was unaffected by dilution of the membrane, and so of the ubiquinone pool, by incorporation of phospholipid. However, Zhu *et al.*, (1982) found a requirement for ubiquinone for the oxidation of DQH_2. The reoxidation, but not the reduction, of cytochrome b by DQH_2 required low concentrations of ubiquinone, and it was concluded that ubiquinone may be required at the antimycin sensitive site of the bc_1 complex because duroquinone was insufficiently stable in the semiquinone form to act as the oxidant for cytochrome b. The oxidation of DQH_2 in the present study did not appear to

involve the pool of ubiquinone. This could be deduced from the linear relationship between inhibition of oxidation and the degree of saturation of specific HQNO binding sites (Figure 21), and from the pattern of inhibition by myxothiazol which was consistent with a simple equilibrium between the myxothiazol and its binding sites (Figure 28).

The oxidation of exogenous NADH could be accurately described by the ubiquinone pool equation (section 1.2) when tested using myxothiazol or BAL. The oxidation of succinate differed from this pattern, although a diffusion step at the level of ubiquinone was evident. When succinate oxidation was compared to the oxidation of NADH a rapid drop in activity was seen at high levels of saturation, while at low levels of saturation (up to 50%) rather less inhibition of oxygen consumption was seen than would have been predicted from the results obtained using NADH. According to the model of Kroger and Klingenberg (1973a,b) the rate of electron transfer through the ubiquinone pool is governed only by the rates at which reactive dehydrogenase-ubiquinone and bc_1 complex-ubiquinol complexes form. The extent to which the lateral diffusion of ubiquinone limits these processes is still contentious. The rate of collision between the reaction partners will be determined by two factors: the rate of diffusion of ubiquinone within the membrane and the effective concentration of ubiquinone (the influence of the motion of the respiratory complexes is ignored). A reaction is said to be diffusion controlled when the rate of overall reaction is the same as the rate of collision of the reacting species: if the number of collisions exceeds the number that result in reaction, then the process is diffusion coupled rather than diffusion controlled (Gupte *et al.*, 1984). For ideal kinetics to be followed it is important that diffusion between sites of reduction and oxidation is rapid so that

ubiquinone can potentially interact with a great many respiratory complexes during its lifetime as ubiquinone or as ubiquinol. In this way electron transfer between dehydrogenases and the bc_1 complexes behaves as if in homogenous solution. From the data presented in the present study it is possible to estimate some of the important factors in this process. The concentrations of some of the respiratory components and their rates of turnover under optimal conditions are shown in Table 9. It can be seen that the approximate turnover time for ubiquinone (n=2) is 362msec under uncoupled conditions and this is very much slower than that of either the bc_1 complex or of cytochrome oxidase. This is a consequence of the large excess of ubiquinone relative to the respiratory complexes and will affect how far the ubiquinone or ubiquinol can travel during a single cycle. The maximum rate that the cytochrome pathway could be expected to pass electrons can be estimated from the Vm measured for the oxidation of DQH_2 under identical conditions. From Table 9 rates of transfer from DQH_2 and through the bc_1 complex and cytochrome oxidase would be expected to be about twice as high as the rates measured with NADH as substrate, if saturation of the quinol oxidising site could be achieved. This suggests (a) that the effective concentration of ubiquinol in the membrane is some way short of saturation and (b) that the pathway from ubiquinol onwards does not significantly determine rate during the oxidation of succinate or exogenous NADH by uncoupled mitochondria. The distance that ubiquinone could be expected to travel during its lifetime within the inner membrane of mammalian mitochondria has been estimated by Rich (1984) using a diffusion coefficient for ubiquinone of $10^{-8} cm^2.sec^{-1}$ (by analogy with phospholipid diffusion) and a random walk model of ubiquinone movement. The ubiquinone:bc_1 complex ratio of 31 found in the present study was much higher than

Table 9. Concentrations of redox components of Jerusalem artichoke mitochondria and approximate rates of turnover.

Rates of oxidation and the content of redox components are taken from Tables 2 and 8. The Vm for DQH_2 oxidation (444nmol O_2/min/mg protein) was determined from the results presented in Figure 17. The rates of turnover are calculated assuming that the full complement of each component participates in electron transport.

	Redox carrier	Substrate		
	(nmol/mg protein)	NADH	Succinate	Vm DQH_2
Uncoupled rate of oxidation (nmol O_2/min/mg protein)	-	225	206	444
		(electrons/sec/carrier)		
Ubiquinone-9	2.71	5.53	5.05	-
Cytochrome oxidase	0.101	150	137	296
bc_1 complex	0.088	170	156	336

the values of 5 and 10 used in the calculations of Rich (1984). At a ubiquinone:bc_1 ratio of 5 and a rate of electron transport through the bc_1 complex of 200electrons/sec/bc_1 monomer, Rich (1984) calculated that ubiquinol could travel a distance of approximately 224nm and potentially interact with 80 bc_1 dimers (see section 1.1.4 regarding possible bc_1 complex dimer formation). Applying the same method and assumptions, ubiquinol in uncoupled Jerusalem artichoke mitochondria could be expected to travel 600nm, comparable to the state 4 situation calculated by Rich (1984) in which ubiquinol could interact with 800 bc_1 dimers during its lifetime. Clearly the larger ubiquinone pool size of Jerusalem artichoke mitochondria makes it unlikely that electron transport would be limited either by diffusion along or across the membrane under uncoupled and uninhibited conditions. These calculations are necessarily approximate because of uncertainty about the actual diffusion coefficient of ubiquinone in the membrane. Gupte *et al.* (1984) approached the same problem slightly differently, by measuring the diffusion rate of a ubiquinone analogue in the mitochondrial membrane (see section 1.2.1) and then calculating from this that approximately 45 collisions occur between each ubiquinol and bc_1 complex for each turnover. Therefore electron transport was diffusion coupled rather than diffusion limited. The ratio of ubiquinone:bc_1 monomers can be calculated from the data presented by Gupte *et al.* (1984) as 21, rather than the value of 8 that can be calculated from the results of Kroger and Klingenberg (1973b).

It is possible that diffusion limitation may become increasingly important as the concentration of active bc_1 complexes is decreased, for example by titration with antimycin, HQNO or myxothiazol, as the path length between dehydrogenases and active bc_1 complexes will increase. This is a different situation to the

transition from state 3 to state 4 used by Rich (1984) in his calculation of the number of bc_1 complexes within range of a ubiquinol molecule, since the concentration of active bc_1 complexes remains constant during this transition. The reduced number of active bc_1 complexes in the membrane at high levels of inhibitor saturation may explain the tendency observed during the present study for the rates of succinate and NADH oxidation to drop sharply as the few remaining active bc_1 complexes became increasingly separated within the membrane.

7.3 The simultaneous oxidation of succinate and exogenous NADH.

From the preceding section it can be seen that the results of this study tend to confirm the view that both succinate and exogenous NADH oxidation broadly follow the ideal model (Kroger and Klingenberg, 1973a) of ubiquinone pool behaviour. The deviations from ideal behaviour were apparently comparatively minor. It was therefore of interest to find out how such large deviations from ideal behaviour could arise when the two substrates were oxidised together. The oxidation of exogenous NADH has been found to be inhibited by both glycine oxidation and endogenous NADH oxidation (section 1.3), and so the mechanism and properties of this process are important in assessing the extent to which the oxidation of substrates within the matrix and the energy demand may regulate exogenous NADH oxidation. The properties of the interaction as found from the experiments presented in the present study can be summarised:

1) The rates of oxidation of succinate and exogenous NADH were similar, measured under state 3, state 4 or uncoupled conditions: in fact the oxidation of succinate tended to be slightly slower.

2) When the rate of oxidation of each substrate, measured in the presence of the other, is compared to the rate of oxidation of each

alone, it can be seen that the decrease in the rate of oxidation of exogenous NADH is much more than the decrease in the rate of succinate oxidation. This is not predicted by the ubiquinone pool model, and is unlike the situation observed using mammalian SMP where the more active dehydrogenase predominates (Gutman, 1980).

3) The interaction is not dependent upon the presence of a membrane potential or adenine nucleotides: it is soley dependent upon the activity of the ubiquinol oxidase pathway and became more pronounced as ubiquinol oxidation was restricted.

4) The interaction is not caused by activation of succinate dehydrogenase by the oxidation of exogenous NADH, nor is there direct inhibition of the exogenous NADH dehydrogenase by succinate dehydrogenase.

5) The interaction is at the level of the ubiquinone pool, as it occurs between the dehydrogenases and the site of BAL or myxothiazol inhibition.

6) Low levels of succinate oxidase activity were able to cause comparatively large inhibitions in the oxidation of exogenous NADH. This was apparent when SMP were used (Figure 43): The inhibition of exogenous NADH oxidation could be greater than the rate of succinate oxidation causing it.

7) Disruption of the mitochondria to form SMP does not stop the very asymmetric mutual inhibition from occurring. It was concluded that organisation of the mitochondria into cristae and non-cristae regions was not essential to the interaction.

These properties are similar to those determined in studies of mammalian mitochondria, with the exception of numbers 2 and 6. The ability of the more active dehydrogenase to predominate (Gutman and Silman, 1972) is not the case with exogenous NADH oxidation in the presence of succinate (Cowley and Palmer, 1980), malate (Day and

Wiskich, 1977) or glycine (Bergman and Ericson, 1983, Dry *et al.*, 1983). Gutman (1980) proposed a model to explain the observations made with mammalian mitochondria in which the more active dehydrogenase poached ubiquinone from the less active dehydrogenase when ubiquinone concentration was low (section 1.3). The high concentration of ubiquinone present in Jerusalem artichoke mitochondria (Table 2) and the failure of the exogenous NADH dehydrogenase to predominate suggests that the explanation advanced by Gutman (1980) may not be applicable in this case.

7.4 Extensions to the ubiquinone pool model.

Since the ubiquinone pool model of Kroger and Klingenberg (1973a) only considers the rate at which reactive collisions occur between ubiquinone and the dehydrogenases, and the bc_1 complex and ubiquinol, it represents an extreme case in which the other steps in electron transport do not determine rate. The work of Kacser and Burns (1973,1979) on the control of metabolic processes in general, and the application of their analysis to mitochondrial electron tranport (Groen *et al.*, 1982) show that it is only in the most extreme cases that the control of electron transport would be expected to be exerted by only two steps: control is usually shared between a large number of individual processes, with the result that the overall flux is little affected by small changes in the activity of each stage. As the ubiquinone pool model fails to account quantitatively for a large number of observations of the behaviour of plant mitochondria, in particular the inhibition of exogenous NADH oxidation by succinate, the consequences of extending the model to include other potential rate controlling steps are examined.

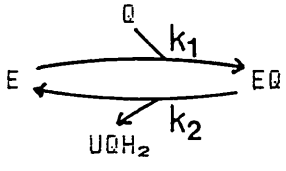
Recently Ragan and Cottingham (1985) have discussed the theory of the kinetics of ubiquinone reactions in detail. By considering

particular simplified examples of a very generalised model, they were able to show that a variety of mechanisms of electron transfer could give rise to the first order kinetics, with respect to ubiquinone, that were observed by Kroger and Klingenberg (1973a) and that it was not true that the rate of electron transfer, in systems which followed this ideal model, was necessarily limited by the binding of ubiquinone or ubiquinol. In addition to ubiquinone binding, the consequences of slow reduction of the dehydrogenase by substrate, of dissociation of potentially reactive ubiquinone from its binding sites, and of non-productive binding of the inappropriate redox form of ubiquinone were all included in the extended model.

In considering whether the interaction of succinate and exogenous NADH reported by Cowley and Palmer (1980) represented a significant deviation from the ideal model of ubiquinone behaviour, Ragan and Cottingham (1985) took the experimentally determined rates of oxygen uptake obtained for succinate oxidation, exogenous NADH oxidation and both substrates together, under both state 3 and state 4 conditions, and determined,from these,values for the three ubiquinone pool constants: Vs, V_N and Vox. They searched for sets of solutions for these constants such that only Vox changed upon transition from state 3 to state 4. Although the exact solutions for the experimentally obtained rates did not fit this model (since all three constants changed between state 3 and state 4) solutions could be found provided certain combinations of experimental error, less than 5% in each determination, were accepted. It was concluded that the deviation from the ideal model was not sufficiently extreme to necessitate modification of the ubiquinone pool model. However, although these calculations took account of the combined rate of oxygen uptake in the presence of both substrates, it took no account of the asymmetry of the interaction (section 1.3.1, Cowley and

Palmer, 1980, Figure 10 of this thesis). The precedence that succinate oxidation takes over NADH oxidation when both substrates are present cannot be explained on the basis of experimental error.

One recently suggested explanation of the precedence of the oxidation of glycine over the oxidation of other respiratory substrates (Dry *et al.*, 1983, Bergman and Ericson, 1983, section 1.3) was that the reduction of ubiquinone by glycine was not limited by collision with ubiquinone, but by the rate of reduction of the ubiquinone reducing enzyme by glycine (Moore and Rich, 1985). A similar model is considered in this section, which represents a very simple extension to the ideal ubiquinone pool model, according to which the operation of each dehydrogenase is defined by two rate constants: one for the binding of ubiquinone to the dehydrogenase, and one meant to include all other processes during the operation of the dehydrogenase, such as reduction of ubiquinone by the dehydrogenase and the subsequent release of ubiquinol from the enzyme. Ubiquinol is treated as a single pool regardless of the substrate oxidised, the reoxidation of ubiquinol is left as a first order process characterised by Vox. The reduction of ubiquinone by a dehydrogenase may be described as follows:



where EQ and E represent dehydrogenase to which ubiquinone has bound or not bound, respectively. It can be shown (appendix A) that the degree of reduction of the ubiquinone pool in the steady state (Q/Qa) and the overall rate of electron transfer (v) are related by:

$$Q/Qa = v.k_2/(Eo.k_1.k_2 - v.k_1) \qquad (7.1)$$

where Eo is the total concentration of dehydrogenase in the membrane. If k_2 is very much larger than k_1 then the relationship between

rate (v) and Q/Qa will become linear, as in the ubiquinone pool model of Kroger and Klingenberg (1973a), and Vred will be equal to (k_1.Eo). If k_2 is comparable to or less than k_1 then the relationship between rate and Q/Qa is hyperbolic. The relationship becomes more hyperbolic as the ratio of k_1/k_2 increases, as shown in Figure 44. The actual rate of electron transport (v) can be found most simply (especially when two different dehydrogenases operate simultaneously, see below) as the solution for a single value of Q/Qa of two simultaneous equations: those for ubiquinone reduction and ubiquinol oxidation:

$$v = Vox.Q/Qa = (Eo.k_1.k_2.Q/Qa) / (k_2 + k_1.Q/Qa) \qquad (7.2)$$

To make realistic comparison between this model and the experimentally observed behaviour of Jerusalem artichoke mitochondria it was necessary to estimate values for the variables used. From the data presented in Figure 29 (ie. the ratio Vox/Vred = 4.4 and the uninhibited rate of oxidation was 167nmol O_2/min/mg protein) a value of 902nmol O_2/min/mg protein was calculated for Vox during uncoupled exogenous NADH oxidation and similarly a value of 205 nmol O_2/min/mg protein calculated for Vred. The definition of Vred, according to Kroger and Klingenberg (1973a,b), is the rate of electron transfer that would be sustained if Q/Qa were equal to one. Therefore for each chosen ratio of k_1/k_2 values for these constants could be obtained from equation 7.1. In the absence of published values for the plant mitochondrial content of exogenous NADH dehydrogenase and succinate dehydrogenase, values of 0.03nmol/mg protein were used, by analogy with the ratios of complex I, II and the bc_1 complex found in mammalian mitochondria (Capaldi 1982). Equation 7.2 was solved by iteration (Appendix A) and the value of Vox progressively decreased to simulate an inhibitor titration at

Figure 44. Dependence of the rate of ubiquinone reduction upon the degree of reduction of the ubiquinone pool, according to the extended ubiquinone pool model.

These lines were calculated according to equation 7.2 and were drawn for a variety of ratios of k_1/k_2. They represent the dependence of the rate of ubiquinone reduction upon the degree of reduction of the ubiquinone pool. According to the model of Kroger and Klingenberg (1973a,b) this relationship is linear (first-order), similar to that calculated at a ratio of k_1/k_2 of 0.01. Ubiquinol oxidation is treated as first-order in the extended model, as shown by the line representing Vox.

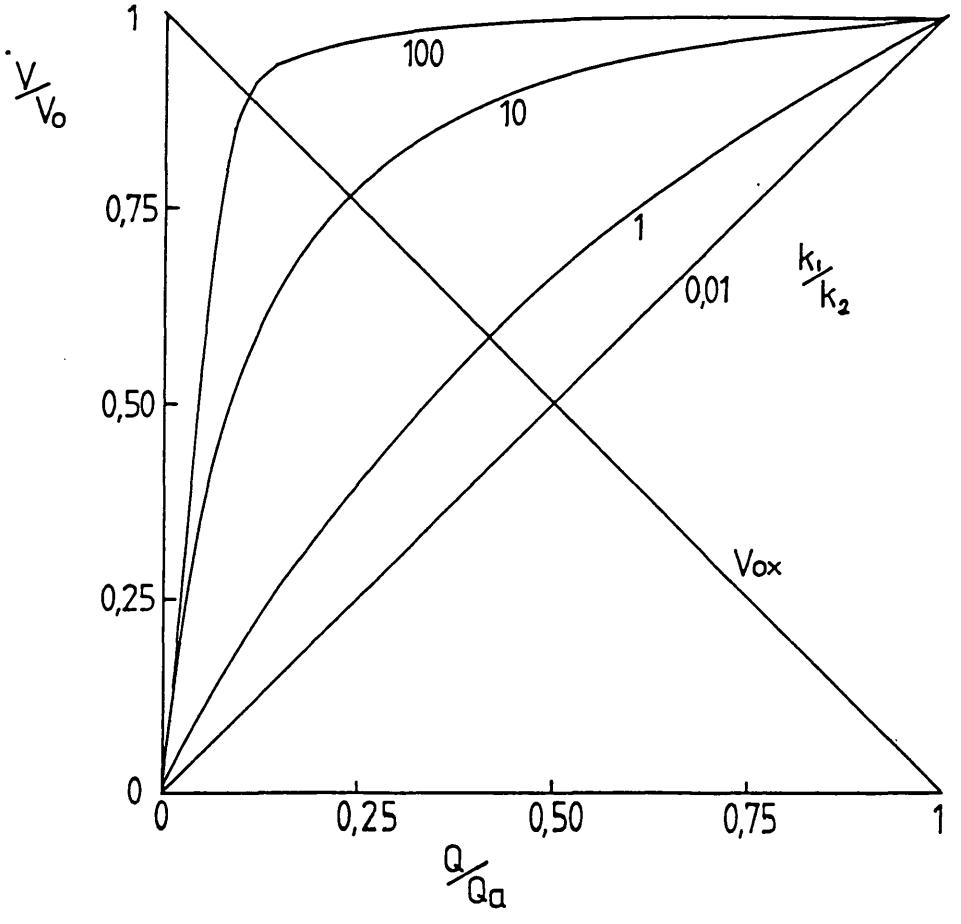
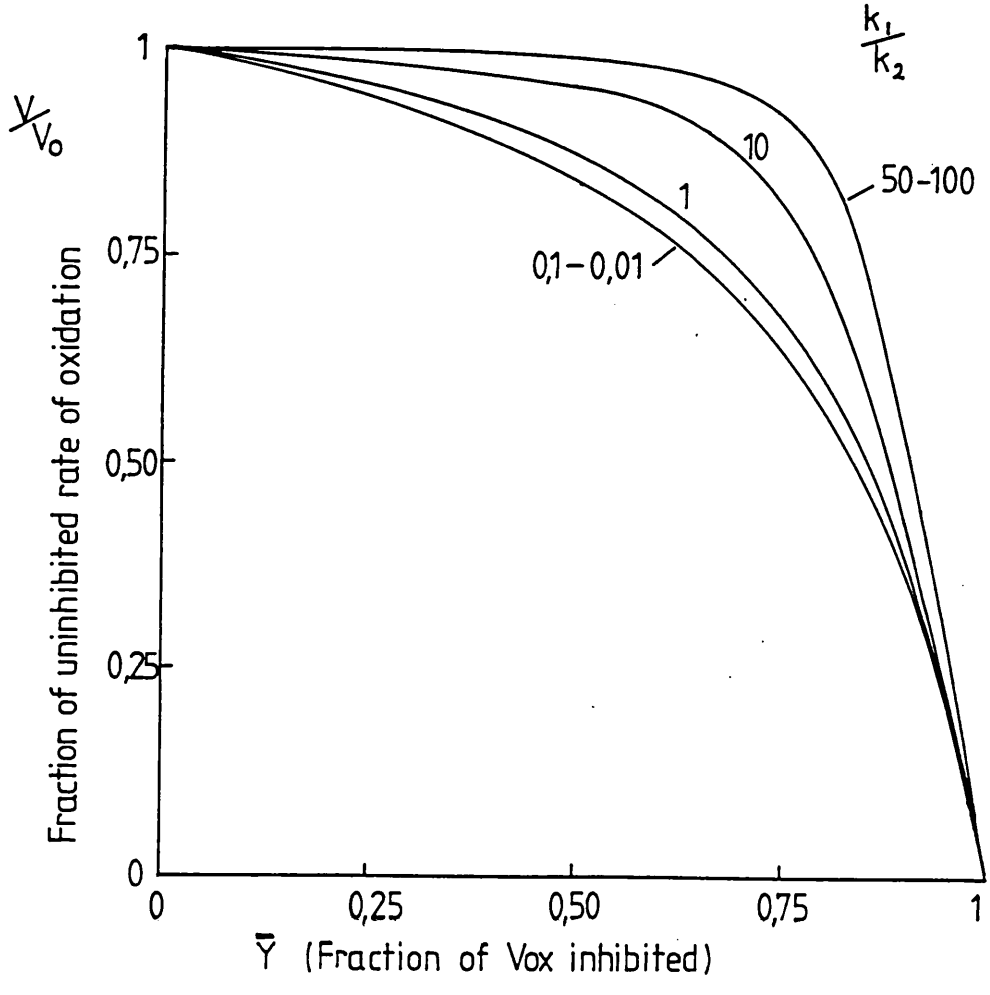


Figure 45. Simulation of the relationship between rate of oxidation and degree of inhibition of ubiquinol oxidase activity.

These lines were calculated using equation 7.2, at a variety of ratios of k_1/k_2 as described in appendix A. The values of the variables were chosen to be as close as possible to those determined from the pattern of inhibition of exogenous NADH oxidation caused by myxothiazol (Figure 29). From the data presented in Figure 29 a value of 410nmol ubiquinone/min/mg protein was determined for Vred and 1804nmol ubiquinol/min/mg protein for Vox. The value for Vred was used to calculate the values for the rate constants k_1 and k_2 as described in the text. Eo was estimated as 0.03nmol/mg protein.



different ratios of k_1/k_2 (Figure 45). It can be seen that ratios of k_1/k_2 of less than one would be difficult to distinguish from ideal ubiquinone pool behaviour, but that as k_2 becomes more rate controlling (k_1/k_2 increasing) the inhibition curve becomes more hyperbolic. In practise this too would be indistinguishable from ideal ubiquinone pool behaviour as it closely resembles the expected pattern for a high value of Vox/Vred.

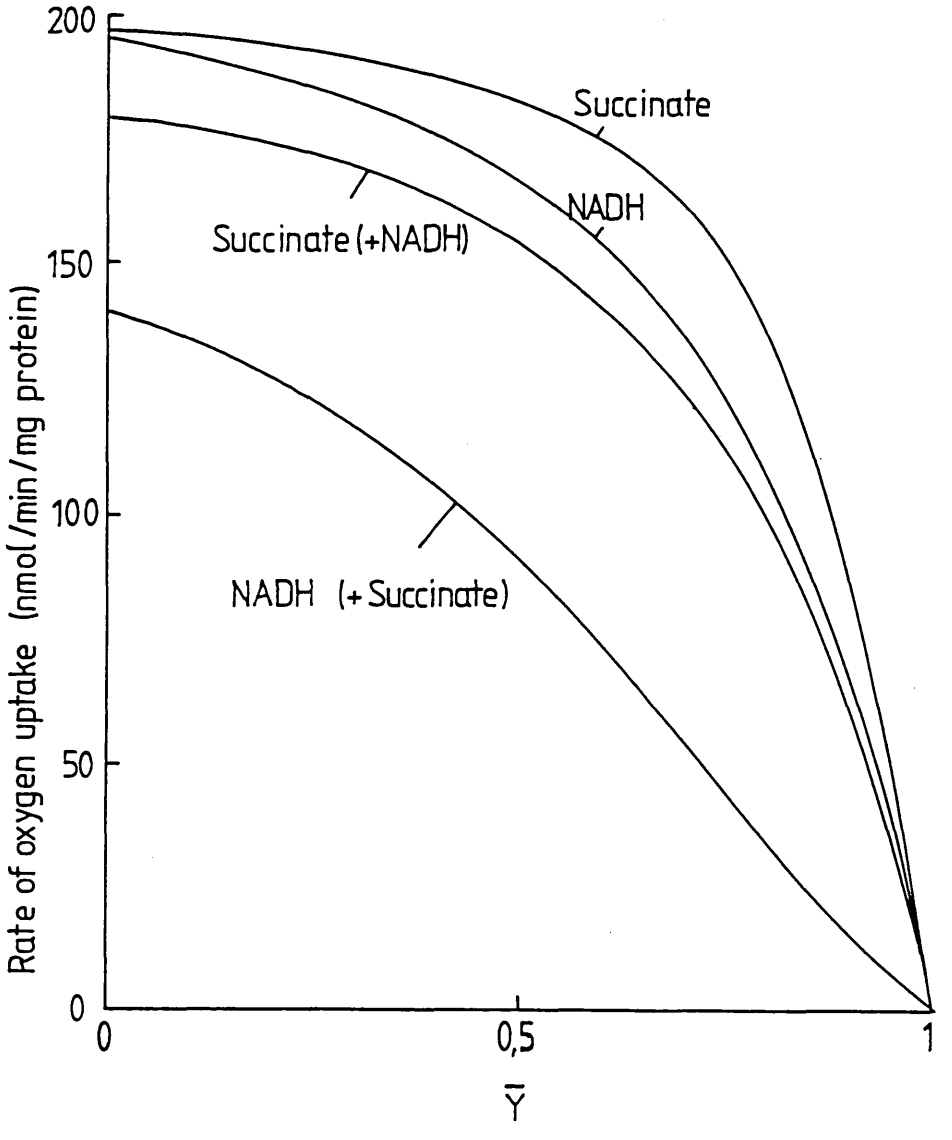
Comparison of the inhibition profiles for exogenous NADH and succinate oxidation obtained in the present study (Figures 30 and 32) suggested that exogenous NADH oxidation appeared, by this extended model, to be that expected for a dehydrogenase that followed the ideal model of Kroger and Klingenberg (1973a) more closely that did the oxidation of succinate. Succinate oxidation resembled the case where k_2 is significantly rate controlling. This can be deduced from the tendency of succinate oxidation to remain uninhibited relative to exogenous NADH oxidation until Vox is very much decreased. It was therefore of interest to find out to what extent this model could account for the asymmetric mutual inhibitions observed when two substrates were oxidised together. In the presence of a second substrate equation 7.2 needs to be modified to include the equation for the new dehydrogenase:

$$v = Vox.Q/Qa = (Eo.k_1.k_2.Q/Qa)/(k_2 + k_1.Q/Qa) + (Es.k_3.k_4.Q/Qa)/(k_4 + k_3.Q/Qa)$$

where k_3, k_4 and Es are the constants describing the second dehydrogenase, corresponding to k_1, k_2 and Eo respectively. This new equation was then solved for a unique value of Q/Qa (by iteration, Appendix A) to give the total rate of electron transfer. The contribution of each substrate to the total electron flux could then be found using the value for Q/Qa obtained and equation 7.1. The results of such a simulation are shown in Figure 46. Ratios of

Figure 46. The interaction between succinate and exogenous NADH oxidation as predicted by the extended ubiquinone pool model.

The relationship between the rate of oxygen consumption and degree of inhibition of ubiquinol oxidase activity was calculated as described in the legend to Figure 45. For NADH oxidation a ratio of k_1/k_2 of 0.01 was used (close to ideal ubiquinone pool kinetics) and for succinate oxidation a ratio of 5 was used. The rates of oxidation of each substrate in the presence of the other were calculated as described in Appendix A using equation 7.3.



k_1/k_2 were chosen which were consistent with the hyperbolic plots experimentally obtained by titration with myxothiazol (Figures 30 and 32). These were 0.01 for exogenous NADH oxidation, approximating to ideal ubiquinone pool behaviour (Figure 44) and 5 for succinate oxidation. The other values used are shown in the legend to Figure 44. The result of this modification to the ubiquinone pool model was the prediction that at all values of Vox, the inhibition of exogenous NADH by the oxidation of succinate would be larger than the inhibition of succinate oxidation by NADH. Furthermore the extent of the inhibition, expressed as a percentage of the rate when oxidised alone went up sharply as Vox was decreased, as was found experimentally when the interaction was measured using uncoupled mitochondria and antimycin to vary Vo (Figure 15). By choosing more extreme values of k_1/k_2 for succinate oxidation, the mutual inhibition could be made even more asymmetric. The more extreme asymmetry of interaction that can be obtained may explain the failure of other substrates to affect the rate of oxidation of glycine, as was originally suggested by Moore and Rich (1985).

Evidence has been presented that the resistance of exogenous NADH oxidation to inhibition by HQNO was due to the well documented production of superoxide during exogenous NADH oxidation. It was also shown that in the presence of high concentrations of HQNO the addition of succinate reduced the total rate of oxygen uptake such that it was lower than the rate obtained in the presence of NADH alone (Figure 26). This phenomenon is easily explained by the extended pool model presented in this section if it is assumed, by analogy with the production of superoxide by the bc_1 complex (Boveris *et al.*, 1972, 1976), that the exogenous NADH dehydrogenase produces superoxide from the oxidation of ubisemiquinone by molecular oxygen. According to the model presented here, succinate

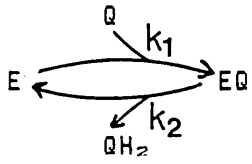
dehydrogenase is able to preferentially scavenge ubiquinone, particularly when the ubiquinone pool is largely reduced. This will have the effect of depriving exogenous NADH of the means for the generation of superoxide, and, since succinate oxidation produces much lower amounts of superoxide (Huq, 1978), the cause of the resistance to inhibition by HQNO is largely removed and inhibition is re-established. Similarly, the large inhibition of NADH oxidation caused by succinate oxidation, seen using SMP, may also be related to the preferential scavenging of low levels of ubiquinone by succinate dehydrogenase.

The model may also be relevant to the low rates of cyanide insensitive oxidation reported with exogenous NADH as the substrate, when these rates are contrasted with the cyanide sensitivity of other substrates. This tends to be most pronounced when the activity of the alternative pathway is low compared to the cytochrome pathway (Table 1). From Figure 45 it can be seen that upon going from a high level of Vox to a lower one, the extent of inhibition of oxidation that is predicted depends on the ratio of k_1/k_2. Therefore addition of cyanide to mitochondria with low alternative pathway activity would tend to bring Vox into the range where the rate of oxidation of the substrate which most nearly follows ideal ubiquinone pool behaviour would be inhibited significantly more than that of a substrate (eg. succinate) which behaved as if the the ratio k_1/k_2 were high.

The inhibition of exogenous NADH oxidation by the oxidation of matrix substrates is a potential mechanism for the regulation of the oxidation of cytosolic NADH. This is because the extent of inhibition is dependent upon the respiratory state of the mitochondria (through the activity of ubiquinol oxidase) and because the oxidation of the matrix substrates are little affected by the presence of exogenous NADH. The different respiratory activities articulate at ubiquinone and the extended ubiquinone pool model analysed shows that preferred pathways of oxidation could be established by variations in the rates of ubiquinone binding and turnover of the dehydrogenases. This would occur through changes in the sensitivity of the rate of ubiquinone reduction to the degree of reduction of the ubiquinone pool, as shown in Figure 44. It is this relationship that could be the subject of further study. The model may also be applicable to the question of the different degrees of access that different substrates have to the alternative oxidase. The ability to produce relatively pure SMP of either membrane polarity will make it possible to study the properties of endogenous NADH oxidation directly, how these activities interact with succinate, and how electron flux between the cytochrome and alternative pathways is regulated.

Appendix A. Details of extended ubiquinone pool model.

If the operation of each dehydrogenase is described by two constants:



Where [Q] represents the degree of oxidation of ubiquinone (Q/Qa in the main text).

Then in the steady-state: $\frac{d[EQ]}{dt} = k_1[E][Q] - k_2[EQ] = 0$ (1)

and rate of ubiquinone reduction = $v = k_2[EQ]$ (2)

Let $Eo = [E] + [EQ]$ = total dehydrogenase concentration. (3)

From (1) and (3): $(Eo - [EQ])/[EQ] = k_2/k_1[Q]$

$\Rightarrow$ $Eo = k_2[EQ]/k_1[Q] + [EQ]$

Substituting for equation (2): $Eo = v/k_1[Q] + v/k_2$

From which $[Q] = v.k_2/(Eo.k_1.k_2 - v.k_1)$ (7.1)

or $v = (Eo.k_1.k_2.[Q])/(k_2 + k_1.[Q])$ (7.2)

To obtain the predicted rate of oxygen uptake when ubiquinol oxidation was also occuring, the Newton-Raphson iteration was used to solve the two simultaneous equations:

$[Q] = v.k_2/(Eo.k_1.k_2 - v.k_1) = Vox$

Solutions for a single value for [Q] between 0 and 1 were found to the required level of accuracy. An identical method was used in the presence of two substrates (see main text for extended equation), and the single value of [Q] obtained was then used to calculate the rate of oxidation of each substrate in the presence of the other, using equation (7.2).

REFERENCES

ACKRELL, B.A.C., BALL, M.B. & KEARNEY, E.B. (1980) Peptides from complex II active in reconstitution of succinate-ubiquinone oxidoreductase. J. Biol. Chem. 255 , 2761-2769.

ACKRELL, B.A.C., KEARNEY, E.B. & COLES, C.J. (1977a) Isolation of reconstitutively active succinate dehydrogenase in highly purified state. J. Biol. Chem. 252 , 6963-6965.

ACKRELL, B.A.C., KEARNEY, E.B., COLES, C.J., SINGER, T.P., BEINERT, H., WAN, Y.-P. & FOLKERS, K. (1977b) Kinetics of the reoxidation of succinate dehydrogenase. Arch. Biochem. Biophys. 182 , 107-117.

AKERLUND, H.-E., ANDERSSON, B., ALBERTSSON, P.-A. (1976) Isolation of photosystem II enriched membrane vesicles from spinach chloroplasts by phase partition. Biochim. Biophys. Acta. 449 , 525-535.

AKERMANN, K.E.O. & WIKSTROM, M.K.F. (1976) Safranine as a probe of the mitochondrial membrane potential. FEBS Lett. 68 , 191-197.

ALBERTSSON, P.-Å. (1971) Partition of cell particles and macromolecules, 2nd edn, Almquist and Wiksell, Stockholm.

ALBRACHT, S.P.J. (1974). Some new paramagnetic centres in submitochondrial particles detectable by EPR. Biochim. Biophys. Acta 347 , 183-192.

ALBRACHT, S.P.J., DOOIJEWAARD, J., LEEUWERIK, F.J. & VAN SWOL, B. (1977) EPR signals of NADH:Q oxidoreductase: shape and intensity. Biochim. Biophys. Acta 459 , 300-317.

ALBRACHT, S.P.J., LEEUWERIK, F.J. & VAN SWOL, B. (1979) The stoichiometry of the iron-sulphur clusters 1a, 1b and 2 of NADH :Q oxidoreductase as present in beef heart submitochondrial particles. FEBS Lett. 104 , 197-200.

ARRON, G.P. & EDWARDS, G.E. (1979) Oxidation of reduced nicotinamide adenine dinucleotide phosphate by plant mitochondria. Can. J. Biochem. 57 , 1392-1399.

ARRON, G.P. & EDWARDS, G.E. (1980) Oxidation of reduced nicotinamide adenine dinucleotide phosphate by potato mitochondria. Inhibition by sulfhydryl reagents. Plant Physiol. 65 , 591-594.

BACKSTROM, D., NORLING, B., EHRENBERG, A. & ERNSTER, L. (1970) Electron spin resonance measurements on ubiquinone-depleted and ubiquinone-replenished submitochondrial particles. Biochim. Biophys. Acta 197 , 108-111.

BAGINSKY, M.L. & HATEFI, Y. (1969) Reconstitution of succinate -coenzyme Q reductase (complex II) and succinate oxidase activities by a highly purified, reactivated succinate dehydrogenase. J. Biol. Chem. 244 , 5313-5319.

BAHR, J.T. & BONNER, W.D. (1973a) Cyanide-insensitive respiration. I. The steady states of skunk cabbage spadix and bean hypocotyl mitochondria. J. Biol. Chem. 248 , 3441-3445.

BAHR, J.T. & BONNER, W.D. (1973b) Cyanide-insensitive respiration. II. Control of the alternative pathway. J. Biol. Chem. 248 , 3446-3450.

BAUGH, R.F. & KING, T.E. (1972) Purification, properties and reconstitutive activity of a DPNH dehydrogenase. Biochem. Biophys. Res. Commun. 49 , 1165-1173.

BAUM, H., RIESKE, J.S., SILMAN, H.I. & LIPTON, S.H. (1967) On the mechanism of electron transfer in complex III of the electron transfer chain. Proc. Natl. Acad. Sci. USA 57 , 798-805.

BECKER, W.F., VON JAGOW, G., ANKE, T. & STEGLICH, W. (1981) Oudemansin, strobilurin A, strobilurin B and myxothiazol: new inhibitors of the bc_1 segment of the respiratory chain

with an E-β-methoxyacrylate system as common structural element. FEBS Lett. 132 , 329-333.

BEINERT, H. & ALBRACHT, S.P.J. (1982) New insights, ideas and unanswered questions concerning iron-sulphur clusters in mitochondria. Biochim. Biophys. Acta 683 , 245-277.

BERDEN, J.A. & SLATER, E.C. (1970) The reactions of antimycin with a cytochrome b preparation active in reconstitution of the respiratory chain. Biochim. Biophys. Acta 216 , 237-249.

BERDEN, J.A. & SLATER, E.C. (1972) The allosteric binding of antimycin to cytochrome b in the mitochondrial membrane. Biochim. Biophys. Acta 256 , 199-215.

BERGMAN, A. & ERICSON, I. (1983) Effects of pH, NADH, succinate and malate on the oxidation of glycine in spinach leaf mitochondria. Physiol. Plant. 59 , 421-427.

BONNER, W.D. & PRINCE, R. (1984) The Rieske iron-sulphur cluster of plant mitochondria. FEBS Lett. 177 , 47-50.

BONNER, W.D. & RICH, P.R. (1977) The nature and location of cyanide and antimycin resistant respiration in higher plants. Proc. 11th Febs meeting, 1977 Vol.49 B6-1.

BONNER, W.D., CLARKE, S.D. & RICH, P.R. (1985) Purification and partial characterisation of the quinol oxidase activity of *Arum maculatum* mitochondria. (submitted)

BOVERIS, A., OHNISHI, N. & CHANCE, B. (1972) The cellular production of hydrogen peroxide. Biochem. J. 128 , 617-630.

BOVERIS, A., CADENAS, E. & STOPPANI, A.O.M. (1976) Role of ubiquinone in the mitochondrial generation peroxide. Biochem. J. 156 , 435-444.

BRANDON, J.R., BROCKLEHURST, J.R. & LEE, C.P. (1972) Effect of antimycin A and 2-heptyl-4-hydroxyquinoline-*N*-oxide on the respiratory chain of submitochondrial particles of beef heart. Biochemistry 11 , 1150-1154.

BRUNTON, C.J. & PALMER, J.M. (1973) Pathways for the oxidation of malate and reduced pyridine nucleotide by wheat mitochondria. Eur. J. Biochem. 39 , 283-291.

BRYLA, J., KANIUGA, Z. & SLATER, E.C. (1969) Studies on the mechanism of inhibition of the electron transport chain by antimycin. Biochim. Biophys. Acta 189 , 317-326.

BURKE, J.J., SIEDOW, J.N. & MORELAND, D.E. (1982) Succinate dehydrogenase. A partial purification from mung bean hypocotyls and soybean cotyledons. Plant Physiol. 70 , 1577-1588.

CAMMACK, R. & PALMER, J.M. (1977a) Iron-sulphur centres in mitochondria from *Arum maculatum* spadix with very high rates of cyanide-resistant respiration. Biochem. J. 166 , 347-355.

CAMMACK, R. & PALMER, J.M. (1977b) EPR studies of iron-sulphur proteins of plant mitochondria. Ann. NY Acad. Sci. 222 , 816-822.

CAPALDI, R.A. (1982) Arrangement of proteins in the mitochondrial inner membrane. Biochim. Biophys. Acta 694 , 291-306.

CAPALDI, R.A., SWEETLAND, J. & MERLI, A. (1977) Polypeptides in the succinate-coenzyme Q reductase segment of the respiratory chain. Biochemistry 16 , 5707-5710.

CHANCE, B., ERNSTER, L., GARLAND, P.B., LEE, C.-P., LIGHT, P.A., OHNISHI, T, RAGAN, C.I. & WONG, D. (1967) Flavoproteins of the mitochondrial respiratory chain. Proc. Natl. Acad. Sci. USA 57 , 1498-1505.

CHANCE, B. & WILLIAMS, G.R. (1956) The respiratory chain and oxidative phosphorylation. Adv. Enz. 17 , 65-134.

COLEMAN, J.O.D. & PALMER, J.M. (1971) Role of Ca^{2+} in the oxidation of exogenous NADH by plant mitochondria. FEBS Lett. 17 , 203-208.

COLEMAN, J.O.D. & PALMER, J.M. (1972) The oxidation of malate by isolated plant mitochondria. Eur. J. Biochem. 26 , 499-509.

COLONNA, R., MASSARI, S. & AZZONE, G.F. (1973) The problem of cation binding sites in the energised membrane of intact mitochondria. Eur. J. Biochem. 34 , 577-585.

COOK, N.D. (1984) Studies on electron transport in higher plant mitochondria. Ph.D. thesis, University of London, 200pp.

COOK, N.D. & CAMMACK, R. (1984) Purification and characterisation of the rotenone-insensitive NADH dehydrogenase of mitochondria from *Arum maculatum*. Eur. J. Biochem. 141 , 573-577.

COOK, N.D. & CAMMACK, R. (1985) The inhibition of plant mitochondrial respiration by the synthetic analogue of ubiquinone 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT). Arch. Biochem. Biophys. (in the press).

CORNFORTH, J.W. & JAMES, A.T. (1956) Structure of a naturally occuring antagonist of dihydrostreptomycin. Biochem. J. 63 , 124-130.

COTTINGHAM, I.R. & MOORE, A.L. (1983) Ubiquinone pool behaviour in plant mitochondria. Biochim. Biophys. Acta 724 , 191-200.

COTTINGHAM, I.R. & MOORE, A.L. (1984) Partial purification and properties of the external NADH dehydrogenase from Cuckoo-pint (*Arum maculatum*) mitochondria. Biochem. J. 224 , 171-179.

COWLEY, R.C. (1977) The interaction of branched electron transport pathways in plant mitochondria. Ph.D. thesis, University of

London, 300pp.

COWLEY, R.C. & PALMER, J.M. (1978) The interaction of citrate and calcium in regulating the oxidation of exogenous NADH in plant mitochondria. Plant Sci. Lett. 11 , 345-350.

COWLEY, R.C. & PALMER, J.M. (1980) The interaction between exogenous NADH oxidase and succinate oxidase in Jerusalem artichoke *Helianthus tuberosus* mitochondria. J. Exp. Bot. 31 , 199-207.

CRANE, F.L. (1965) Distribution of ubiquinones. In: Biochemistry of quinones (Morton, R.A. ed), Academic Press, New York, pp 183-206.

CROFTS, A.R. & WRAIGHT, C.A. (1983) The electrochemical domain of photosynthesis. Biochim. Biophys. Acta 726 , 149-185.

DAVIS, K.A. & HATEFI, Y. (1971) Succinate dehydrogenase I. Purification, molecular properties and substructure. Biochemistry 10 , 2509-2516.

DAVIS, K.A., HATEFI, Y., POFF, K.L. & BUTLER, W.L. (1973) The b-type cytochromes of bovine heart mitochondria: absorbtion spectra, enzymatic properties , and distribution in the electron transfer complexes. Biochim. Biophys. Acta 325 , 341-356.

DAY, D.A., RAYNER, J.R. & WISKICH, J.T. (1976) Characteristics of exogenous NADH oxidation by beetroot mitochondria. Plant Physiol. 58 , 38-42.

DAY, D.A. & WISKICH, J.T. (1974) The oxidation of malate and exogenous reduced nicotinamide adenine dinucleotide by isolated plant mitochondria. Plant Physiol. 53 , 104-109.

DAY, D.A. & WISKICH, J.T. (1975) Isolation and properties of the outer membrane of plant mitochondria. Arch. Biochem. Biophys. 171 , 117-123.

DAY, D.A. & WISKICH, J.T. (1977) Factors limiting respiration by

isolated cauliflower mitochondria. Phytochemistry 16 , 1499-1502.

DE TROOSTEMBERGH, J. & NYNS, E. (1978) Kinetics of the respiration of cyanide-insensitive mitochondria from the yeast *Saccharomycopsis lipolytica*. Eur. J. Biochem. 85 , 423-432.

DE VRIES, S., ALBRACHT, S.P.J., BERDEN, J.A. & SLATER, E.C. (1981) A new species of bound ubisemiquinone anion in QH_2:cytochrome c oxidoreductase. J. Biol. Chem. 256 , 11996-11998.

DE VRIES, S., ALBRACHT, S.P.J., BERDEN, J.A. & SLATER, E.C. (1982) The pathway of electrons through QH_2:cytochrome c oxidoreductase studied by pre-steady-state kinetics. Biochim. Biophys. Acta 681 , 41-53.

DE VRIES, S., BERDEN, J.A. & SLATER, E.C. (1980) Properties of a semiquinone anion located in the QH_2:cytochrome c oxidoreductase segment of the mitochondrial respiratory chain. FEBS Lett. 122 , 143-148.

DERVARTANIAN, D.V. & VEEGER, C. (1964) Studies on succinate dehydrogenase I. Spectral properties of the purified enzyme and formation of enzyme-competitive inhibitor complexes. Biochim. Biophys. Acta 92 , 233-247.

DIZENGREMEL, P. (1975) La voie d'oxydation insensible au cyanure dans les mitochondries de tranches de pommes de terre (*Solanum tuberosum* L) maintenues en survie. Physiol. Veg. 13 , 39-54.

DIZENGREMEL, P. & LANCE, C. (1976) Control of changes in mitochondrial activites during ageing of potato slices. Plant Physiol. 58 , 147-151.

DOUCE, R., CHRISTENSEN, E.L. & BONNER W.D. (1972) Preparation of

intact plant mitochondria. Biochim. Biophys. Acta 275 , 148-160.

DOUCE, R., MANNELLA, C.A. & BONNER, W.D. (1973). The external NADH dehydrogenases of intact plant mitochondria. Biochim. Biophys. Acta 292 , 105-116.

DRY, I.B., DAY, D.A. & WISKICH, J.T. (1983) Preferential oxidation of glycine by the respiratory chain of pea leaf mitochondria. FEBS Lett. 158 , 154-158.

DUCET, G. & DIANO, M. (1978) On the dissociation of the cytochrome bc_1 of potato mitochondria. Plant Sci. Lett. 11 , 217-226.

DUTTON, P.L. & STOREY, B.T. (1971) The respiratory chain of plant mitochondria IX. Oxidation-reduction potentials of the cytochromes of mung bean mitochondria. Plant Physiol. 47 , 282-288.

EARNSHAW, M.J. (1978) The calcium-induced contraction in non-energised corn mitochondria. In "Plant Mitochondria" (G. Ducet & C. Lance eds.), pp. 159-166, Elsevier/North-Holland Biomedical Press.

EDWARDS, S.W. & LLOYD, D. (1977) Cyanide-insensitive respiration in *Acanthamoeba castellanii*. Proc. 11th Febs meeting 1977 Vol. 49 B6-1, 307.

EISENBACH, M. & GUTMAN, M. (1975) Characterization of the component, which controls the transformation between the kinetic forms of the b cytochromes. Eur. J. Biochem. 59 , 223-230.

ERECINSKA, M., WILSON, D.F., MUKAI, Y. CHANCE, B. (1970) Oxidation-reduction potentials of the mitochondrial flavoproteins. Biochem. Biophys. Res. Commun. 41 , 386-392.

ERNSTER, L., GLASER, E. & NORLING, B. (1978) Extraction and reincorporation of ubiquinone in submitochondrial particles.

Methods Enzymol. LIII [55].

ERNSTER, L., LEE, I.Y., NORLING, B. & PERSSON, B. (1969) Studies with ubiquinone-depleted submitochondrial particles. Eur. J. Biochem. 9 , 299-310.

FIESER, L.F. & FIESER, M. (1961) Advanced organic chemistry. Rheinold, New York.

FOWLER, L.R. & RICHARDSON, S.H. (1963) Studies on the electron transfer systems L. On the mechanism of reconstitution of the mitochondrial electron transfer system. J. Biol. Chem. 238 , 456-463.

FROUD, R.J. & RAGAN, C.I. (1984) Cytochrome c-mediated electron transfer between ubiquinone-cytochrome c reductase and cytochrome c oxidase. Biochem. J. 217 , 551-560.

GABELLINI, N. & HAUSKA, G. (1983) Characterisation of cytochrome b in the isolated ubiquinol-cytochrome c_2 oxidoreductase from *Rhodopseudomonas sphaeroides* GA. FEBS Lett. 153 , 146-150.

GARDESTROM, P., BERGMAN, A. & ERICSON, I. (1980) Oxidation of glycine via the respiratory chain in mitochondria prepared from different parts of spinach. Plant Physiol. 65 , 389-391.

GARDESTROM, P. & EDWARDS, G.E. (1985) Leaf mitochondria (C_3 + C_4 + CAM). Encyclopedia of plant physiology (R. Douce & D.A.Day, eds.), Springer-Verlag, Berlin. 18 , 314-346.

GREEN, D.E. (1962) Structure and function of subcellular particles. Comp. Biochem. Physiol. 4 , 81-122.

GROEN, A.K., WANDERS, R.J.A., WESTERHOFF, H.V., VAN DER MEER, R. & TAGER, J.M. (1982) Quantification of the contribution of various steps to the control of mitochondrial respiration. J. Biol. Chem. 257 , 2754-2757.

GUNAWARDENA, H. & WILSON, S.B. (1974) Configurational changes in isolated turnip (*Brassica napus* L) mitochondria. Biochem.

Soc. Trans. 2 , 199-200.

GUPTE, S., WU, E., HOECHLI, L., HOECHLI, M., JACOBSON, K., SOWERS, A.E. & HACKENBROCK, C.R. (1984) Relationship between lateral diffusion, collision frequency, and electron transfer of mitochondrial inner membrane oxidation-reduction components. Proc. Natl. Acad. Sci. USA 81 , 2606-2610.

GUTMAN, M. (1977) in Bioenergetics of Membranes (Packer, L., Papageorgiou, G.C. and Trebst, A., eds.), p.165, Elsevier, Amsterdam.

GUTMAN, M. (1980) Electron flux through the mitochondrial ubiquinone. Biochim. Biophys. Acta 594 , 53-84.

GUTMAN, M., COLES, C.J., SINGER, T.P. & CASIDA, J.E. (1971) On the functional organisation of the respiratory chain at the dehydrogenase-coenzyme Q junction. Biochemistry 10 , 2036-2045.

GUTMAN, M. & KLIATCHKO, S. (1976) Mechanism of inhibition by ubicidin: inhibitor with piericidin ring structure and ubiquinone side chain. FEBS Lett. 67 , 348-353.

GUTMAN, M. & SILMAN, N. (1972) Mutual inhibition between NADH oxidase and succinoxidase activities in respiring submitochondrial particles. FEBS Lett. 26 , 207-210.

GUTMAN, M., SINGER, T.P. & CASIDA, J.E. (1970) Studies on the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase. XVII Reaction sites with piericidin A and rotenone.

HACKETT, D.P., HAAS, D.W., GRIFFITHS, S.K. & NIEDERPRUENS D.J. (1960) Studies on the development of cyanide-resistant respiration in potato tuber slices. Plant Physiol. 35 , 8-19.

HALL, D.O., CAMMACK, R. & RAO, K.K. (1974) In "Iron in Biochemistry and Medicine" (A.Jacobs & M.Warwood, eds.), pp. 279-334,

Academic Press, London.

HANSON, J.B. & HODGES, T.K. (1967) Energy-linked reactions of plant mitochondria. Curr. Top. Bioenerg. 2 , 65-98.

HARBOUR, J.R. & BOLTON, J.R. (1975) Superoxide formation in spinach chloroplasts: electron spin resonance detection by spin trapping. Biochem. Biophys. Res. Commun. 64 , 803-807.

HARBOUR, J.R., CHOW, V. & BOLTON, J.R. (1974) An electron spin resonance of the spin adducts of OH and HO_2 radicals with nitrones in the ultraviolet photolysis of aqueous hydrogen peroxide solutions. Can. J. Chem. 52 , 3549-3553.

HATEFI, Y. & GALANTE, Y.M. (1980) Isolation of cytochrome b_{560} from complex II (succinate-ubiquinone oxidoreductase) and its reconstitution with succinate dehydrogenase. J. Biol. Chem. 255 , 5530-5537.

HATEFI, Y., HAAVIK, A.G., FOWLER, L.R. & GRIFFITHS D.E. (1962a) Studies on the electron transfer system. XLII Reconstitution of the electron transfer system. J. Biol. Chem. 237 , 2661-2669.

HATEFI, Y., HAAVIK, A.G., GRIFFITHS, D.E. (1962b) Studies on the electron transfer system XL. Preparation and properties of mitochondrial DPNH-coenzyme Q reductase. J. Biol. Chem. 237 , 1676-1680.

HATEFI, Y. & RIESKE, J.S. (1967) Preparation and properties of DPNH-coenzyme Q reductase (complex I of the respiratory chain). Methods Enzymol. 10 , 235-239.

HATEFI, Y. & STIGGALL, D.L. (1976) Metal containing flavoprotein dehydrogenase. In "The enzymes" Vol.XIII (ed. P.Boyer), Third edition, pp. 175-279. Academic Press.

HATTORI, T. & ASAHI, T. (1982) The presence of two forms of succinate dehydrogenase in sweet potato root mitochondria. Plant and

Cell Phys. 23 , 515-523.

HAUSKA, G. & HURT, E. (1982) Pool function behaviour and mobility of isoprenoid quinones. In: "Function of Quinones in Energy Conserving Systems" (Trumpower, B.L. ed.), pp. 87-109, Academic Press, New York.

HAUSKA, G., HURT, E., GABELLINI, N. & LOCKAU, W. (1983) Comparative aspects of quinol-cytochrome c/plastocyanin oxidoreductases. Biochim. Biophys. Acta 726 , 97-133.

HEDERSTEDT, L. & RUTBERG, L. (1981) Succinate dehydrogenase: a comparative review. Microbiological Reviews 45 , 542-555.

HEMMERICH, P., EHRENBERG, A., WALKER, W.H., ERIKSON, L.E.G., SALACH, J., BADER, P. & SINGER, P. (1969) On the structure of succinic dehydrogenase flavocoenzyme. FEBS Lett. 3 , 37.

HERON, C., CORINA, D. & RAGAN, C.I. (1977) The phospholipid annulus of mitochondrial NADH-ubiquinone reductase - A dual phospholipid requirement for enzymatic activity. FEBS Lett. 79 , 399-403.

HERON, C., GORE, M.G. & RAGAN, C.I. (1979a) Effects of lipid phase transitions on the interactions of mitochondrial NADH-ubiquinone oxidoreductase with ubiquinol-cytochrome c oxidoreductase. Biochem. J. 178 , 415-426.

HERON, C., RAGAN, C.I. & TRUMPOWER, B.L. (1978) The interaction between mitochondrial NADH-ubiquinone oxidoreductase and ubiquinol-cytochrome c oxidoreductase. Restoration of ubiquinone-pool behaviour. Biochem. J. 174 , 791-800.

HERON, C., SMITH, S. & RAGAN, C.I. (1979b) An analysis of the polypeptide composition of bovine heart mitochondrial NADH-ubiquinone oxidoreductase by two dimensional polyacrylamide-gel electrophoresis. Biochem. J. 181 , 435-443.

HILL, G.C. (1977) Characterisation of the electron transport systems

present during differentiation of Africam trypanosomes. Proc. 11th Febs meeting 1977 Vol.49 B6-1 L3.

HORECKER, B.L. & KORNBERG, A. (1948) The extinction coefficients of the reduced band of pyridine nucleotides. J. Biol. Chem. 175 , 385-390.

HRYNIEWIECKA, L. & MICHEJDA, J.W. (1977) Cyanide-insensitive respiration in amoeba, *Acanthamoeba castellanii*. Proc. 11th Febs meeting 1977 Vol.49 B6-1 304.

HUMPHREYS, T.E. & CONN, E.E. (1956) Oxidation of reduced diphosphopyridine nucleotide by lupin mitochondria. Arch. Biochem. Biophys. 60 , 226-243.

HUQ, S. (1978) Cyanide resistant respiration in Cassave and other plant mitochondria. PhD thesis, Unversity of London.

HUQ, S. & PALMER, J.M. (1978a) The involvement and possible role of quinone in cyanide-resistant respiration. In "Plant Mitochondria" (G. Ducet & C. Lance, eds.), pp. 225-232, Elsevier/North-Holland Biomedical Press.

HUQ, S. & PALMER, J.M. (1978b) Superoxide and hydrogen peroxide production in cyanide resistant *Arum maculatum* mitochondria. Plant Sci. Lett. 11 , 351-358.

HUQ, S. & PALMER, J.M. (1978c) Isolation of a cyanide-resistant duroquinol oxidase from *Arum maculatum* mitochondria. FEBS Lett. 95 , 217-220.

HUQ, S. & PALMER, J.M. (1980) Isolation and characterisation of the cyanide-resistant oxidase from the spadices of *Arum maculatum*. In: "Cyanide in Biology" (Vennesland *et al.*, eds.), pp.451-460, Academic Press, London.

IKUMA, H. & BONNER, W.D. (1967a) Properties of higher plant mitochondria: I. Isolation and some characteristics of coupled mitochondria from dark-grown mung bean hypocotyls.

Plant Physiol. 42 , 67-75.

IKUMA, H. & BONNER, W.D. (1967b) Properties of higher plant mitochondria: III. Effects of respiratory inhibitors. Plant Physiol. 42 , 1535-1544.

INGLEDEW, W.J., SALERNO, J.C. & OHNISHI, T. (1976) Studies on electron paramagnetic resonance spectra manifested by a respiratory chain hydrogen carrier. Arch. Biochem. Biophys. 177 , 176-184.

INGLEDEW, W.J., SALERNO, J.C. & OHNISHI, T. (1977) A paramagnetic interaction of ubisemiquinone and the probable site of action of tenoyltrifluoroacetone. Fed. Proc. 36 , 901.

IZZO, G., GUERRIERI, F. & PAPA, S. (1978) On the mechanism of inhibition of the respiratory chain by 2-heptyl-4-hydroxyquinoline-*N*-oxide. FEBS Lett. 93 , 320-322.

JAMES, W.O. & BEEVERS, H. (1950) The respiration of Arum spadix. A rapid respiration, resistant to cyanide. New Phytol. 49 , 353-374.

JAMES, W.O. & ELLIOT, D.C. (1955) Cyanide-resistant mitochondria from the spadix of an Arum. Nature (London) 175 , 89.

JENG, M., HALL, C., CRANE, F.L., TAKAHASHI, N., TAMURA, S. & FOLKERS, K. (1968) Inhibition of mitochondrial electron transport by piericidin A and related compounds. Biochemistry 7 , 1311-1322.

JOHNSTON (1982) The role of calcium in the regulation of NADH in Jerusalem artichoke tubers. PhD thesis, University of London.

JUNG, D.W. & HANSON, J.B. (1975) Activation of 2,4-dinitrophenol-stimulated ATPase activity in cauliflower and corn mitochondria. Arch. Biochem. Biophys. 168 ,

358-368.

KACSER, H. & BURNS, J.A. (1973) The control of flux. SEB Symposia XXVII , 65-104.

KACSER, H. & BURNS, J.A. (1979) Molecular democracy: who shares the controls? Biochem. Soc. Trans. 7 , 1149-1160.

KATSIKAS, H. & QUINN. P.J. (1982) The distribution of UQ-10 in phospholipid bilayers. (A study using differential scanning calorimetry). Eur. J. Biochem. 124 , 165-169.

KAY, C.J. & PALMER, J.M. (1985) Solubilization of the alternative oxidase of cuckoo-pint (*Arum maculatum*) mitochondria. Stimulation by high concentrations of ions and effects of specific inhibitors. Biochem. J. 228 , 309-318.

KENNY, W.C., MOWERY, P.C., SENG, R.L. & SINGER, T.P. (1976) Localization of the substrate and oxaloacetate binding site of succinate dehydrogenase. J. Biol. Chem. 251 , 2367-2373.

KING, T.E. (1963) Reconstitution of respiratory chain enzyme systems XII Some observations on the reconstitution of the succinate oxidase system from heart muscle. J. Biol. Chem. 238 , 4037-4051.

KING, T.E., YU, L., NAGOAKA, S., WIDGER, W.R. & YU, C.-A. (1978) In "Frontiers of Biological Energetics" (L.Dutton, J.Leigh & T.Scarpa, eds.), 1 , 174-182, Academic Press, New York.

KINGSLEY, D.B. & FEIGENSON, G.W. (1981) ^{1}H-NMR study of the location and motion of ubiquinones in perdeuterated phosphatidyl-choline bilayers. Biochim. Biophys. Acta 635 , 602-618.

KLEIN, R.R. & BURKE, J.J. (1984) Separation procedure and partial characterisation of two NAD(P)H dehydrogenases from cauliflower mitochondria. Plant Physiol. 76 , 436-441.

KLINGENBERG, M. (1970) Localization of the glycerol-phosphate

dehydrogenase in the outer phase of the mitochondrial inner membrane. Eur. J. Biochem. 13 , 247-252.

KROGER, A. & KLINGENBERG, M. (1967) On the role of ubiquinone. Curr. Top. Bioenerg. 2 , 151-193.

KROGER, A. & KLINGENBERG, M. (1970) In "Vitamins and Hormones" (Harries, R.S. *et al.*, eds.) 28 , 533-573, Academic Press, New York.

KROGER, A. & KLINGENBERG, M. (1973a) The kinetics of the redox reactions of ubiquinone related to the electron-transport activity in the respiratory chain. Eur. J. Biochem. 34 , 358-368.

KROGER, A. & KLINGENBERG, M. (1973b) Further evidence for the pool function of ubiquinone as derived from the inhibition of the electron transport by antimycin. Eur. J. Biochem. 39 , 313-323.

LAMBOWITZ, A.M. & BONNER, W.D. (1974a) The b cytochromes of plant mitochondria. In: Dynamics of energy-transducing membranes. (L.Ernster, R.W.Estabrook & E.C.Slater eds.), Elsevier, Amsterdam, pp77-92.

LAMBOWITZ, A.M. & BONNER, W.D. (1974b) The b cyrochromes of plant mitochondria. A spectrophotometric and potentiometric study. J. Biol. Chem. 249 , 2428-2440.

LANCE, C. & BONNER, W.D. (1968) The respiratory chain components of higher plant mitochondria. Plant Physiol. 43 , 756-766.

LANCE, C. & CHAUVEAU, M. (1975) Evolution des activities oxydatives et phosphorylantes des mitochondries de l'*Arum maculatum* L. au cours du developpment de l'inflorescence. Physiol. Veg. 13 , 83-94.

LANCE, C., CHAUVEAU, M. & DIZENGREMEL, P. (1985) The cyanide-resistant pathway of plant mitochondria. Encyclopedia of plant

physiology (R.Douce & D.A.Day, eds.), Springer-Verlag, Berlin. 18 , 202-247.

LANCE, C., HOBSON, G.E., YOUNG, R.E. & BIALE, J.E. (1967) Metabolic processes in cytoplasmic particles of the avacado fruits. IX. The oxidation of pyruvate and malate during the climacteric cycle. Plant Physiol. 42 , 471-478.

LATIES, G.G. (1982) The cyanide-resistant alternative pathway in higher plant respiration. Annu. Rev. Plant Physiol. 33 , 519-555.

LAWFORD, H.G. & GARLAND, P.B. (1972) Proton translocation coupled to quinone reduction by reduced nicotinamide-adenine dinucleotide in rat liver and ox-heart mitochondria. Biochem. J. 130 , 1029-1044.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 , 265-275.

MACHINIST, J.M. & SINGER, T.P. (1965) Studies on the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase. IX. Reactions with coenzyme Q. J. Biol. Chem. 240 , 3182-3190.

MACRAE, A.R. & MOORHOUSE, R. (1970) The oxidation of malate by mitochondria isolated from cauliflower buds. Eur. J. Biochem. 16 , 96-102.

MAESHIMA, M. & ASAHI, T. (1979) Sweet potato cytochrome c oxidase: its properties and biosynthesis in wounded root tissue. In: Cytochrome oxidase (T.E.King *et al.*, eds.), Elsevier/North Holland Biomedical Press, Amsterdam, New York, pp.375-382.

MARX, R. & BRINKMAN, K. (1978) Characteristics of rotenone-insensitive oxidation of matrix-NADH by broad bean mitochondria. Planta 142 , 83-90.

MATSUOKA, M., MAESHIMA, M. & ASAHI, T. (1981) The subunit composition of pea cytochrome c oxidase. J. Biochem. 90 , 649-655.

MEINHARDT, S.W. & CROFTS, A.R. (1982) The site and action of myxothiazol as an inhibitor of electron transfer in *Rhodopseudomonas sphaeroides*. FEBS Lett. 149 , 217-222.

MERLI, A., CAPALDI, R.A., ACKRELL, B.A.C. & KEARNEY, E.B. (1979) Arrangement of complex II (succinate-ubiquinone reductase) in the mitochondrial inner membrane. Biochemistry 18 , 1393-1400.

METZLER, D.E. (1977) In "Biochemistry: the Chemical Reactions of Life" p.173, Academic Press, London.

MITCHELL, P. (1976) Possible molecular mechanisms of the protonmotive function of cytochrome systems. J. Theor. Biol. 62 , 327-367.

MOLLER, I.M. (1981) The effect of cations on the oxidation of NAD(P)H by plant mitochondria. PhD thesis, University of London.

MOLLER, I.M., BERGMAN, A., GARDESTROM, P., ERICSON, I. & PALMER, J.M. (1981b) Characterisation and purification of inside-out submitochondrial particles obtained from plant mitochondria. FEBS Lett. 126 , 13-17.

MOLLER, I.M., JOHNSTON, S.P. & PALMER, J.M. (1981a) A specific role for Ca^{2+} in the oxidation of exogenous NADH by Jerusalem artichoke (*Helianthus tuberosus*) mitochondria. Biochem. J. 194 , 487-495.

MOLLER, I.M., KAY, C.J. & PALMER, J.M. (1984) Electrostatic screening stimulates rate-limiting steps in mitochondrial electron transport. Biochem. J. 223 , 761-767.

MOLLER, I.M. & PALMER, J.M. (1981a) The inhibition of exogenous NAD(P)H oxidation by chelators and mersalyl as a function of pH. Physiol. Plant. 53 , 413-420.

MOLLER, I.M. & PALMER, J.M. (1981b) Charge screening by cations

affects the conformation of the mitochondrial inner membrane. A study of exogenous NAD(P)H oxidation in plant mitochondria. Biochem. J. 195 , 583-588.

MOLLER, I.M. & PALMER, J.M. (1982) Direct evidence for the presence of a rotenone-resistant NADH dehydrogenase on the inner face of the inner membrane of plant mitochondria. Physiol. Plant. 54 , 267-274.

MOLLER, I.M., PALMER, J.M. & JOHNSTON, S.P. (1983) Inhibition of exogenous NADH oxidation in plant mitochondria by chlorotetracycline in the presence of calcium ions. Biochim. Biophys. Acta 125 , 289-291.

MOORE, A.L. & BONNER, W.D. (1982) Measurements of membrane potentials in plant mitochondria with the safranine method. Plant Physiol. 70 , 1271-1276.

MOORE, A.L., BONNER, W.D. & RICH, P.R. (1978) The determination of the proton motive force during cyanide-insensitive respiration in plant mitochondria. Arch. Biochem. Biophys. 186 , 298-306.

MOORE, A.L., LINNETT, P.E. & BEECHEY, R.B. (1980) Dibutylchloromethyltin chloride, a potent inhibitor of electron transport in plant mitochondria. J. Bioenerg. Biomem. 12 , 309-323.

MOORE, A.L. & RICH, P.R. (1980) The bioenergetics of plant mitochondria. Trends Biochem. Sci. 5 , 284-288.

MOORE, A.L. & RICH, P.R. (1985) Organisation of the respiratory chain and oxidative phosphorylation. Encyclopedia of plant physiology (R.Douce & D.A.Day, eds.), Springer-Verlag, Berlin. 18 , 134-172.

MOORE, A.L., RICH, P.R., INGLEDEW, W.J. & BONNER, W.D. (1976) A complex EPR signal in mung bean mitochondria and its possible relation to the alternative pathway. Biochem. Biophys. Res.

Commun. 72 , 1099-1107.

MOREIRA, M.T.F., RICH, P.R. & BENDALL, D.S. (1980) A method for demonstrating the involvement of a mobile quinone pool in an electron transport chain by the use of loosely bound inhibitors. 1st EBEC Short Reports, pp.61-62, Patron Editore.

MORTON, R.A. (1965) In "Biochemistry of Quinones" (R.A.Morton, ed.), Academic Press, New York, pp.1-21.

MOWERY, P.C., STEENKAMP, D.J., ACKRELL, B.A.C., SINGER, T.P. & WHITE, G.A. (1977) Inhibition of mammalian succinate dehydrogease by carboxin. Arch. Biochem. Biophys. 178 , 495-506.

NALECZ, M.J., ZBOROWSI, J., FAMULSKI, K.S. & WOJTCZAK, L. (1980) Effect of phospholipid composition on the surface potential of liposomes and the activity of enzymes incorporated into liposomes. Eur. J. Biochem. 112 , 75-80.

NASH, D. & WISKICH, J.T. (1983) Properties of substantially chlorophyll-free pea leaf mitochondria prepared by sucrose density gradient separation. Plant Physiol. 71 , 627-634.

NELSON, B.D. & GELLERFORS, P. (1974) The redox properties of the cytochromes of purified complex III. Biochim. Biophys. Acta 357 , 358-364.

NEUBERGER, M. & DOUCE, R. (1978) Transport of NAD^+ through the inner membrane of plant mitochondria. In "Plant Mitochondria" (G.Ducet & C.Lance, eds.), pp. 109-116, Elsevier/North Holland Biomedical Press, Amsterdam.

NEUMANN, W.P. (1970) The organic chemistry of tin. Wiley, New York, pp38-40.

NIJS, P. (1967) On the nature of electron and energy transport in the mitochondria. I Multiple inhibition of mitochondrial respiration. Biochim. Biophys. Acta 143 , 454-461.

OESTREICHER, G., HOGUE, P. & SINGER, T.P. (1973) Regulation of succinate dehydrogenase in higher plants II Activation by

substrates, reduced coenzyme Q, nucleotides, and anions. Plant Physiol. 52 , 622-626.

OHNISHI, T. (1973) Mechanism of electron transport and energy conservation in the site I region of the respiratory chain. Biochim. Biophys. Acta 301 , 105-128.

OHNISHI, T. (1979) In "Membrane Proteins in Energy Transduction" (R.A. Capaldi, ed.) pp.1-87, Dekker, New York.

OHNISHI, T., LEIGH, J.S., RAGAN, C.I. & RACKER, E. (1974) Low temperature electron paramagnetic resonance studies on iron-sulphur centres in cardiac NADH dehydrogenase. Biochem. Biophys. Res. Commun. 56 , 775-782.

OHNISHI, T. & SALERNO, J.C. (1982) Iron-sulphur clusters in the mitochondrial electron-transport chain. In "Iron-sulphur Proteins" (T.G.Spiro, ed.) Vol.IV, pp.285-326, John Wiley, New York.

OHNISHI, T., SALERNO, J.C., BLUM, H., LEIGH, J.S. & INGLEDEW, W.J. (1977) In "Bioenergetics of membranes" (L.Packer, G.C.Papageorgiou & A.Trebst, eds.), pp.209-216, Elsevier/North Holland, Amsterdam.

OHNISHI, T. & TRUMPOWER, B.L. (1980) Differential effects of antimycin on ubisemiquinone bound in different environments in isolated succinate:cytochrome c oxidoreductase. J. Biol. Chem. 255 , 3278-3284.

ORME-JOHNSON, N.R., HANSEN, R.E. & BEINERT, H. (1974) Electron paramagnetic resonance-detectable electron acceptors in beef-heart mitochondria. J. Biol. Chem. 249 , 1922-1927.

PALMER, J.M. (1979) The uniqueness of plant mitochondria. Biochem. Soc. Trans. 7 , 246-252.

PALMER, J.M. (1980) The reduction of nicotinamide adenine dinucleotide by mitochondria isolated from *Helianthus tuberosus*. J. Exp. Bot. 31 , 1497-1508.

PALMER, J.M. (1984) The mechanism and regulation of malate oxidation in isolated plant mitochondria. Physiol. Veg. 22 , 665-673.

PALMER, J.M. & ARRON, G.P. (1976) The influence of exogenous nicotinamide adenine dinucleotide on the oxidation of malate by Jerusalem artichoke mitochondria. J. Exp. Bot. 27 , 418-430.

PALMER J.M. & COLEMAN, J.O.D. (1974) Multiple pathways of NADH oxidation in the mitochondrion. Horizons Biochem. Biophys. 1 , 220-260.

PALMER, J.M., COWLEY, R.C. & AL-SANE, N.A. (1978) The inhibition of malate oxidation by oxaloacetate in jerusalem artichoke mitochondria. In "Plant Mitochondria" (G.Ducet & C.Lance, eds.) pp.117-124, Elsevier/North-Holland Biomedical Press, Amsterdam.

PALMER, J.M. & MOLLER, I.M. (1982) Regulation of NAD(P)H dehydrogenases in plant mitochondria. Trends Biochem. Sci. 7 , 258-261.

PALMER, J.M. & KIRK, B.I (1974) The influence of osmolarity on the reduction of exogenous cytochrome c and permeability of the inner membrane of Jerusalem artichoke mitochondria. Biochem. J. 140 , 79-86.

PALMER, J.M. & PASSAM, H.C. (1971) Oxidation of exogenous reduced nicotinamide-adenine dinucleotide by plant mitochondria. Biochem. J. 122 , 16-17P.

PAPA, S., IZZO, G. & GUERRIERI, F. (1982) On the inhibition of the

bc_1 segment of the mitochondrial respiratory chain by quinone analogues and hydroxyquinoline derivatives. FEBS Lett. 145 , 93-98.

PASSAM, H.C. (1974) The oxidative phosphorylation and ATPase activity of Arum spadix mitochondria in relation to heat generation. J. Exp. Bot. 25 , 653-657.

PASSAM, H.C., BERDEN, J.A. & SLATER, E.C. (1973) The cytochrome b spectrum of plant mitochondria and its response to ATP and antimycin. Biochim. Biophys. Acta 325 , 54-61.

PERSHADSINGH, H.A., MARTIN, A.P., VORBECK, M.L., LONG, J.W. & STUBBS, E.B. (1982) Ca^{2+}-dependent depolarisation of energised mitochondrial membrane potential by chlortetracycline (Aureomycin). J. Biol. Chem. 257 , 12481-12484.

POMEROY, M.K. (1975) The effects of nucleotides and inhibitors on respiration in isolated wheat mitochondria. Plant Physiol. 55 , 51-58.

PRINCE, R.C., BONNER, W.D. & BERSHAK, P.A. (1981) On the occurence of the Rieske iron-sulphur cluster in plant mitochondria. Fed. Proc. 40 , 1667.

QUINN, P.J. (1980) The interactions of coenzyme Q with phospholipid monolayers. Biochem. Int. 1 , 77-88.

QUINN, P.J. & ESFAHANI, M.A. (1980) Ubiquinones have surface active properties suited to transport electrons and protons across membranes. Biochem. J. 185 , 715-722.

RAGAN, C.I. (1976) NADH-ubiquinone oxidoreductase. Biochim. Biophys. Acta 456 , 249-290.

RAGAN, C.I. (1978) The role of phospholipids in the reduction of ubiquinone analogs by the mitochondrial reduced nicotinamide -adenine dinucleotide-ubiquinone oxidoreductase complex. Biochem. J. 172 , 539-547.

RAGAN, C.I. (1980) The molecular organisation of NADH dehydrogenase. In "Subcellular Biochemistry" 7 ,267-307, Plenum Press, New York.

RAGAN, C.I. & COTTINGHAM, I.R. (1985) The kinetics of quinone pools on electron transport. Biochim. Biophys. Acta 811 , 13-31.

RAGAN, C.I. & HERON, C. (1978) The interaction between mitochondrial NADH-ubiquinone oxidoreductase and ubiquinol-cytochrome c oxidoreductase - Evidence for a stoicheiometric association. Biochem. J. 174 , 783-790.

RAGAN, C.I. & HINKLE, P.C. (1975) Ion transport and repiratory control in vesicles formed from reduced nicotinamide adenine dinucleotide coenzyme Q reductase and phospholipids. J. Biol. Chem. 250 , 8472-8476.

REDFEARN, E.R. & WHITTAKER, P.A. (1962) The inhibitory effects of quinones on the succinic oxidase system of the respiratory chain. Biochim. Biophys. Acta 56 , 440-444.

REHOREK, D. & JANZEN, E.G. (1984) Spin trapping of radicals generated by ultrasonic decomposition of organotin compounds. J. Organo. Chem. 268 , 135-139.

RICH, P.R. (1978) Quinol oxidation in *Arum maculatum* mitochondria and its application to the assay, solubilization and partial purification of the alternative oxidase. FEBS Lett. 96 , 252-256

RICH, P.R. (1983) Electron transfer through the isolated mitochondrial cytochrome bc_1 complex. Biochim. Biophys. Acta 722 , 271-280.

RICH, P.R. (1984) Electron and proton transfers through quinones and cytochrome bc_1 complexes. Biochim. Biophys. Acta 768 , 53-79.

RICH, P.R. & BENDALL, D.S. (1980) The kinetics and thermodynamics of the reduction of cytochrome c by substituted *p*-benzoquinols in solution. Biochim. Biophys. Acta 592 , 506-518.

RICH, P.R. & BONNER, W.D. (1977) Nature and location of cyanide and

antimycin resistant respiration in higher plants. Proc. 11th FEBS meeting 1977, Vol.49 B6.

RICH, P.R. & BONNER, W.D. (1978a) The sites of superoxide anion generation in higher plant mitochondriä. Arch. Biochem. Biophys. 188 , 206-213.

RICH, P.R. & BONNER, W.D. (1978b) An EPR analysis of cyanide-resistant mitochondria isolated from the mutant poky strain of *Neurospora crassa*. Biochim. Biophys. Acta 504 , 345-363.

RICH, P.R. & BONNER, W.D. (1978c) EPR studies of higher plant mitochondria. II Center S3 of succinate dehydrogenase and its relation to alternative respiratory oxidations. Biochim. Biophys. Acta 501 , 381-395.

RICH, P.R. & MOORE, A.L. (1976) The involvement of the protonmotive ubiquinone cycle in the respiratory chain of higher plants and its relation to the branchpoint of the alternative pathway. FEBS Lett. 65 , 339-344.

RIESKE, J.S. (1976) Composition, structure, and function of complex III of the respiratory chain. Biochim. Biophys. Acta 456 , 195-247.

RUSTIN, P., MOREAU, F. & LANCE, C. (1980) Malate oxidation in plant mitochondria via malic enzyme and the cyanide-insensitive electron transport pathway. Plant Physiol. 66 , 457-462.

RUZICKA, F.J., BEINERT, H., SCHEPLER, K.L., DUNHAM, W.R. & SANDS, R.H. (1975) Interaction of ubisemiquinone with a paramagnetic component in heart tissue. Proc. Natl. Acad. Sci. USA 72 , 2886-2890.

RUZICKA, F.J. & CRANE F.L. (1970) Quinone interaction with the respiratory chain-linked NADH dehydrogenase of beef heart mitochondria. I Juglone reductase activity. Biochim. Biophys. Acta 223 , 71-85.

SALERNO, J.C., HARMAN, H.J., BLUM, H., LEIGH, J.S. & OHNISHI, T. (1977) A transmembrane quinone pair in the succinate dehydrogenase-cytochrome b region. FEBS Lett. 82 , 179-182.

SCHINDLER, S., LICHTENTHALER, H.K., DIZENGREMEL, P., RUSTIN, P. & LANCE, C. (1984) Distribution and significance of different ubiquinone homologues in purified mitochondria and in intact plant tissue. In "Structure, Function and Metabolism of Plant Lipids" (P.-A.Siegenthaler & W.Eichenberger, eds.), pp.267-272, Elsevier, Amsterdam.

SCHNEIDER, H., LEMASTERS, J.J. & HACKENBROCK, C.R. (1982) In "Functions of Quinones in Energy Conserving Systems" (B.L.Trumpower, ed.), pp.125-129, Academic Press, New York.

SCHNEIDER, H., LEMASTERS, J.J., HOCHLI, M. & HACKENBROCK, C.R. (1980) Liposome-mitochondrial inner membrane fusion. Lateral diffusion of integral electron transfer components. J. Biol. Chem. 255 , 3748-3756.

SELAK, M.A. & WHTMARSH, J. (1982) Kinetics of the electrogenic step and cytochrome b_6 and f redox changes in chloroplasts: evidence for a Q cycle. FEBS Lett. 150 , 286-292.

SIEDOW, J.N. (1982) The nature of the cyanide-resistant pathway in plant mitochondria. Rec. Adv. Phytochem. 16 , 47-84.

SIGGEL, U., RENGER, G., STIEL, H.H. & RUMBERG, B. (1972) Evidence for electronic and ionic interaction between electron transport chains in chloroplasts. Biochim. Biophys. Acta 256 , 328-335.

SINGER, T.P., BEINERT, H., ACKRELL, B.A.C. & KEARNEY, E.B. (1975) Proc. 10th FEBS meeting, Paris (Y.Raol, ed.), pp. 173-185, Associated Scientific, Amsterdam.

SINGER, T.P. & GUTMAN, M. (1971) The DPNH dehydrogenase of the mitochondrial respiratory chain. Adv. Enzymol. 34 , 79-153.

SINGER, T.P. & GUTMAN, M. (1974) The quest for coupling site I. Horizons Biochem. Biophys. 1 , 261-302.

SINGER, T.P., OESTREICHER, G., HOGUE, P., CONTREIRAS, J. & BRANDAO, I. (1973) Regulation of succinate dehydrogenase in higher plants I. Some general characteristics of the membrane bound enzyme. Plant Physiol. 52 , 616-621.

SLATER, E.C. (1948) A factor in heart muscle required for the reduction of cytochrome c by cytochrome b. Nature 161 , 405-406.

SLATER, E.C. & DE VRIES, S. (1980) Identification of the BAL-labile factor. Nature 228 , 717-718.

SMITH, S. & RAGAN, C.I. (1978) Mitochondrial reduced nicotinamide-adenine dinucleotide dehydrogenase is transmembranous. Biochem. Soc. Trans. 6 , 1349-1352.

SOLOMOS, T. (1977) Cyanide-resistant respiration in higher plants. Annu. Rev. Plant Physiol. 28 , 279-297.

SOTTHIBANDHU, R. & PALMER, J.M. (1975) The activation of non-phosphorylating electron transport by adenine nucleotides in Jerusalem artichoke (*Helianthus tuberosus*) mitochondria. Biochem. J. 152 , 637-645.

STONER, C.D. & SIRAK, H.D. (1978) Magnesium-induced inner membrane aggregation in heart mitochondria. Prevention and reversal by carboxyatractyloside and bongkrekic acid. J. Cell Biol. 77 , 417-426.

STOREY, B.T. (1970) The respiratory chain of plant mitochondria. VI Flavoprotein components of the respiratory chain of mung bean mitochondria. Plant Physiol. 46 , 13-20.

STOREY, B.T. (1971) The respiratory chain of plant mitochondria. X Oxidation-reduction potentials of the flavoproteins of Skunk cabbage mitochondria. Plant Physiol. 48 , 493-497.

STOREY, B.T. (1976) Respiratory chain of plant mitochondria. XVIII Point of interaction of the alternate oxidase with the respiratory chain. Plant Physiol. 58 , 521-525.

STOREY, B.T. (1980) Electron transport and energy coupling in plant mitochondria. In: The biochemistry of plants, a comprehensive treatise" (D.Davies, ed.) Vol.II pp.113-195, Academic Press, London, New York.

STRONG, F.M., DICKIE, J.P., LOOMANS, M.E., VAN TAMELEN, E.E. & DEWEY, R.S. (1960) The chemistry of antimycin A. IX Structure of the antimycins. J. Am. Chem. Soc. 82 , 1513-1514.

THEOLOGIS, A. & LATIES, G.G. (1978a) Relative contribution of cytochrome-mediated and cyanide-resistant electron transport in fresh and aged potato slices. Plant Physiol. 62 , 232-237.

THEOLOGIS, A. & LATIES, G.G. (1978b) Antimycin-insensitive cytochrome-mediated respiration in fresh and aged potato slices. Plant Physiol. 62 , 238-242.

THEORELL, H. (1935) Reines cytochrom c. Vorlaufige mitteilung. Biochem. Z. 279 , 463-464.

THIERBACH, G. & RIECHENBACH, H. (1981) Myxothiazol, a new inhibitor of the bc_1 segment of the respiratory chain. Biochim. Biophys. Acta 638 , 282-289.

TISSUT, M., RAVANEL, P. & MACHEREL, D. (1984) Study of some natural and synthetic compounds inhibiting the electron transfer in plant mitochondria. Physiol. Veg. 22 , 607-614.

TOBIN, A., DJERDJOUR, B., JOURNET, E., NEUBERGER, M. & DOUCE, R. (1980) Effect of NAD^+ on malate oxidation in intact plant mitochondria. Plant Physiol. 66 , 225-229.

TOMLINSON, P.F. & MORELAND, D.E. (1975) Cyanide-resistant respiration of sweet potato mitochondria. Plant Physiol. 55 , 365-369.

TRUESDALE, G.A., DOWNING, A.L. & LOWDEN, G.F. (1955) The solubility of oxygen in pure water and sea-water. J. Appl. Chem. 5 , 53-62.

TRUMPOWER, B.L. (1981) New concepts on the role of ubiquinone in the mitochondrial respiratory chain. J. Bioenerg. Biomem. 13 , 1-24.

URBAN, P.F. & KLINGENBERG, M. (1969) On the redox potentials of ubiquinone and cytochrome b in the respiratory chain. Eur. J. Biochem. 9 , 519-525.

VAN ARK, G. & BERDEN, J.A. (1977) Binding of HQNO to beef-heart sub-mitochondrial particles. Biochim. Biophys. Acta 459 , 119-137.

VAN HERK, A.W.H. & BADENHUIZEN, N.P. (1934) Uber die atmung und katalasewirkung im *Saurematum*-kolben. Proc. Kon. Akad. Wetensch. 37 , 99-105.

VON JAGOW, G. & BOHRER, C. (1975) Inhibition of electron transfer from ferrocytochrome b to ubiquinone, cytochrome c_1, and duroquinone by antimycin. Biochim. Biophys. Acta 387 , 409-424.

VON JAGOW, G. & ENGEL, W.D. (1981) Complete inhibition of electron transfer from ubiquinol to cytochrome b by the combined action of antimycin and myxothiazol. FEBS Lett. 136 , 19-24.

VON JAGOW, G. & KLINGENBERG, M. (1970) Pathways of hydrogen in mitochondria of *Saccharomyces carlsbergensis*. Eur. J. Biochem. 12 , 583-592.

WEDER, H.G., SCHILDKNECHT, J., LUTZ, R.A. & KESSELRING, P. (1974) Determination of binding parameters from Scatchard plots: theoretical and practical considerations. Eur. J. Biochem. 42 , 475-481.

WEISS, H. & KOLB, H.J. (1979) Isolation of mitochondrial

succinate:ubiquinone reductase, cytochrome c reductase and cytochrome c oxidase from *Neurospora crassa* using non-ionic detergent. Eur. J. Biochem. 99 , 139-149.

WEISS, H., VON JAGOW, G., KLINGENBERG, M. & BUCHEN, T. (1970) Characterisation of *Neurospora crassa* mitochondria prepared with a grind-mill. Eur. J. Biochem. 14 , 75-82.

WIKSTROM, K. & BERDEN, J.A. (1972) Oxidoreduction of cytochrome b in the presence of antimycin. Biochim. Biophys. Acta 283 , 403-420.

WIKSTROM, M. & KRAB, K. (1980) Respiration-linked H^+ translocation in mitochondria: stoichiometry and mechanism. Curr. Top. Bioeneg. 10 , 51-101.

WIKSTROM, M., KRAB, K. & SARASTE, M. (1981) Proton translocating cytochrome complexes. Annu. Rev. Biochem. 50 , 623-655.

WILSON, S.B. (1970) Energy conservation associated with cyanide-insensitive respiration in plant mitochondria. Biochim. Biophys. Acta 223 , 383-387.

WILSON, S.B. (1978) Cyanide-insensitive oxidation of ascorbate + NNN'N'-tetramethyl-*p*-phenylamine mixture by mung bean (*Phaseolus aureus*) mitochondria. An energy linked function. Biochem. J. 176 , 129-136.

WILSON, S.B. (1980) Energy conservation by the plant mitochondrial cyanide-insensitive oxidase. Some additional evidence. Biochem. J. 190 , 349-360.

WILSON, R.H. & HANSON, J.B. (1969) The effect of respiratory inhibitors on NADH, succinate and malate oxidation in corn mitochondria. Plant Physiol. 44 , 1335-1341.

WOJTCZAK, L. & NALECZ, M.J. (1979) Surface charge of biological membranes as a possible regulator of membrane-bound enzymes. Biochim. Biophys. Acta 193 , 64-72.

WOJTCZAK, L. & ZALUSKA, H. (1969) On the impermeability of the outer mitochondrial membrane to cytochrome c. I. Studies on whole mitochondria. Biochim. Biophys. Acta 193 , 64-72.

YU, G.A. & YU, L. (1980) Resolution and reconstitution of succinate-cytochrome c reductase. Biochim. Biophys. Acta 591 , 409-420.

YU, G.A., YU, L. & KING, T.E. (1977) The existance of a ubiquinone binding protein in the reconstitutively active bc_1 complex. Biochem. Biophys. Res. Commun. 78 , 259-265.

ZANOTTI, A. & AZZONE, G.F. (1980) Safranine as membrane potential probe in rat liver mitochondria. Arch. Biochem. Biophys. 201 , 255-265.

ZHU, Q.S., BERDEN, J.A., DE VRIES, S. & SLATER, E.C. (1982) On the role of ubiquinone in the respiratory chain. Biochim. Biophys. Acta 680 , 69-79.

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Solubilization of the alternative oxidase of cuckoo-pint (*Arum maculatum*) mitochondria

Stimulation by high concentrations of ions and effects of specific inhibitors

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1. Selective solubilization of cyanide- and antimycin-insensitive duroquinol oxidase activity from cuckoo-pint (*Arum maculatum*) mitochondria was achieved using taurocholate. Inhibitor-sensitivities and water-forming DQH_2 (tetramethyl-*p*-hydroquinone, reduced form):O_2 stoichiometry were the same for the alternative oxidase of intact *Arum* mitochondria. 2. Cyanide-insensitive oxidation of DQH_2 by intact and solubilized mitochondria was stimulated by up to four-fold by high concentrations of anions high in the Hofmeister series, such as phosphate, sulphate or citrate. Optimal (0.7M) sodium citrate increased $V_{max.}$ for DQH_2 oxidation by the solubilized preparation from 450 to 2400nmol of $O_2 \cdot min^{-1} \cdot mg$ of protein^{-1} and decreased the apparent K_m for DQH_2 from 0.53 to 0.38mM. 3. Inhibition of solubilized DQH_2 oxidase activity by CLAM (*m*-chlorobenzhydroxamic acid) and SHAM (salicylhydroxamic acid) was mixed competitive/non-competitive, with apparent inhibition constants for CLAM of 25μM (K_i) and 81μM (K_I) and for SHAM of 53μM (K_i) and 490μM (K_I). Propyl gallate and UHDBT were non-competitive inhibitors with respect to DQH_2 (apparent $K_i = 0.3\mu$M and 12nM respectively). 4. Low concentrations of C_{18} fatty acids selectively inhibited cyanide-insensitive oxidation by intact and solubilized mitochondria, and inhibition was reversed by 1%(w/v) bovine serum albumin. Inhibition was competitive with DQH_2, suggesting that fatty acids interfere reversably with the binding of DQH_2 to the oxidase. 5. These results tend to support the view that quinol oxidation by the alternative pathway of *Arum maculatum* mitochondria is catalysed by a quinol oxidase protein, rather than by a non-enzymic mechanism involving fatty acid peroxidative reactions [Rustin, Dupont & Lance (1983) *Trends Biochem. Sci.* **8**, 155–157; (1983) *Arch. Biochem. Biophys.* **225**, 630–639].

The nature of the non-phosphorylating cyanide- and antimycin A-insensitive oxidase present in many higher-plant mitochondria remains poorly understood. There is, however, general agreement that the product of dioxygen reduction by the oxidase is water (Huq & Palmer, 1978*a*,*c*; Rich, 1978). Operation of this oxidase, which branches from the cytochrome pathway at the level of ubiquinone (Huq & Palmer, 1978*b*), seems not to generate specific e.p.r. (Cammack & Palmer, 1977; Rich *et al.*, 1977) or optical signals (Bendall & Bonner, 1971). Consequently the oxidase tends to be identified by the sensitivity of oxygen uptake to inhibitors such as benzhydroxamic acids, for example SHAM and CLAM (Schonbaum *et al.*, 1971), propyl gallate (Siedow & Girvin, 1980), chloroquine (James & Spencer, 1982) or dibromo-

Abbreviations used: CLAM, *m*-chlorobenzhydroxamic acid; DCPIP, 2,6-dichlorophenol-indophenol; DQ, tetramethyl-*p*-benzoquinone (oxidized form); DQH_2, tetramethyl-*p*-hydroquinone (reduced form); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; SHAM, salicylhydroxamic acid; TMPD, *NNN'N'*-tetramethyl-*p*-phenylenediamine; UHDBT, 5-(n-undecyl)-6-hydroxy-4,7-dioxobenzothiazole.

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thymoquinone (Siedow *et al.*, 1978) and insensitivity to inhibitors of the cytochrome pathway such as cyanide or antimycin A. Fairly recently Cook & Cammack (1983) have reported that UHDBT, a quinone analogue inhibitor of both mitochondrial bc_1 and photosynthetic b_6f complexes (for a review, see Hauska *et al.*, 1983), is also a very potent inhibitor of the alternative pathway of cuckoo-pint (*Arum maculatum*) mitochondria.

Mitochondria from mature *Arum* spadices are capable of very rapid rates of cyanide-insensitive oxidation. Rates of exogenous NADH oxidation of 3000nmol of $O_2 \cdot min^{-1} \cdot mg$ of $protein^{-1}$ have been reported (Cammack & Palmer, 1977), and duroquinol, ubiquinol-1 and menadiol are also rapidly oxidized. Quinol oxidase activity with similar inhibitor-sensitivities to the alternative pathway has been solubilized from *Arum* mitochondria with deoxycholate (Rich, 1978) and Lubrol (Huq & Palmer, 1978*c*, 1981). Characterization of the oxidase has been rather slow because of its thermolability (Lance *et al.*, 1978) and the short seasonal availability of *Arum* spadices, but a preparation free of cytochrome and e.p.r.-detectable iron–sulphur clusters, but containing significant amounts of copper and flavoprotein, has been reported (Huq & Palmer, 1978*c*), and Bonner & Rich (1983) have reported that e.p.r.-silent spin-coupled copper may be involved in operation of the oxidase.

However, a new hypothesis has fairly recently been proposed that questions the participation of an enzyme in the oxidation of ubiquinol by the alternative pathway (Rustin *et al.*, 1983*a*). In this model, quinol oxidation is catalysed by free radicals of fatty acids, arising from lipid peroxidation, which cycle between oxygenated peroxy and deoxygenated radical forms. In support of this scheme, duroquinol was reported to be co-oxidized with linoleic acid in a cyclic process in the presence of lipoxygenase. This system showed similar inhibitor-sensitivity to the alternative pathway and had a quinol-to-oxygen stoichiometry consistent with water as the end product (Rustin *et al.*, 1983*b*).

In the present paper we report results obtained when we used a detergent-solubilized preparation of *Arum maculatum* mitochondria to follow the kinetics of duroquinol oxidation and inhibition by SHAM, CLAM, n-propyl gallate, UHDBT and non-esterified fatty acids. We also report the effects of high concentrations of ions of the Hofmeister series on the stability and activity of this solubilized cyanide-insensitive DQH_2 oxidase preparation. Results of both of these studies support the view that in *Arum* mitochondria quinol oxidation is catalysed by a highly active quinol oxidase protein.

Materials and methods

Isolation of mitochondria

Mitochondria were isolated, from mature spadices of *Arum maculatum* collected from the wild, by the method of Cammack & Palmer (1977). Isolated mitochondria subsequently treated with detergent were stored in liquid N_2 (77K) until use. Storage in this way did not cause significant loss of KCN-insensitive respiratory activity over a period of 1 month. Protein was determined, after solubilization in 10% (w/v) sodium deoxycholate, by the method of Lowry *et al.* (1951). Bovine serum albumin (fraction V, Sigma) was the standard used.

Solubilization of mitochondria

All operations were carried out at 4°C. Mitochondria were diluted to 16–18mg of $protein \cdot ml^{-1}$ with 10mM-KH_2PO_4/KOH buffer, pH7.0, and sodium taurocholate was added to a final detergent/protein ratio of 3:5 (w/w). After gentle mixing for 5min the solution was centrifuged at 100000*g* for 45min at 4°C in a Spinco model L centrifuge. The resulting clear yellow supernatant was retained and kept on ice until use.

Substrates and inhibitors

Fatty acids were emulsified in 20mM-Hepes buffer, pH7.0, containing 0.66% (v/v) Tween-80, by sonication for 30s with a Dawe Soniprobe model 7530A tuned to an output of 3–4A at setting 3. Samples were immersed in an ice bath during sonication.

Other inhibitors were added from solutions in ethanol or dimethyl sulphoxide such that the final concentration of solvent did not exceed 1% (v/v). In the absence of a published absorption coefficient for UHDBT, solutions of this compound were prepared by weight.

Duroquinol solutions were prepared in acidified dimethyl sulphoxide and not stored. The concentration of the stock solution was determined spectrophotometrically by causing the duroquinol to auto-oxidize by addition of 0.1M (final concn.)-KOH. An absorption coefficient of 12500$litre \cdot mol^{-1} \cdot cm^{-1}$ (oxidized − reduced) at 265nm was used (Lawford & Garland, 1972).

Measurement of oxidation rates

Oxygen consumption was measured in a total volume of 1ml at 25°C in a Rank oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.). Assay media are as specified in the legends to the Figures, and inhibitors were added before the addition of substrate to ensure maximum inhibition.

The concentration of oxygen in each of the

media used was measured with an oxygen electrode by titration with an airtight solution of 10mM (approx. concn.) $Na_2S_2O_4$ in 1M-Tris, pH9.0. The exact concentration of $Na_2S_2O_4$ was determined by an identical titration of 10mM-potassium phosphate buffer, pH7.0, an oxygen concentration of 240μM being assumed.

The stoichiometry of duroquinol oxidation to oxygen uptake was measured by comparison of oxygen uptake due to operation of the oxidase, in the presence of 1mM-KCN, with that due to autooxidation of duroquinol at pH9.5 in the presence of catalase (0.5mg·ml^{-1}; Sigma).

Succinate dehydrogenase activity was measured as phenazine methosulphate-mediated DCPIP reduction by the method of Baginsky & Hatefi (1969), with the addition of 0.04% (v/v, final concn.) Triton X-100. Samples were activated by preincubation with 0.15mM-ATP for 5min to relieve inhibition of succinate dehydrogenase.

Chemicals

UHDBT and CLAM were kindly given by Dr. N. Packham, Imperial College of Science and Technology, London, U.K., and Dr. I. Ericson, University of Umeå, Umeå, Sweden, respectively. DCPIP (sodium salt), n-propyl gallate, linoleic acid (sodium salt), linolenic acid (non-esterified acid), oleic acid (sodium salt) and taurocholic acid (sodium salt) were from Sigma. DQH_2 was from K and K Laboratories, Plainview, NY, U.S.A. SHAM was from Ralph N. Emanuel, Wembley, Middx., U.K.

Other chemicals were from BDH Chemicals, Poole, Dorset, U.K., Hopkin and Williams, Chadwell Heath, Essex, U.K., or Sigma Chemical Co., Poole, Dorset, U.K., and were of the highest grade available.

Results

Properties of the solubilized preparation

Treatment of *Arum maculatum* mitochondria with taurocholate yielded a clear yellow fraction (9–13mg of protein·ml^{-1}) containing increased specific DQH_2 oxidase activity over that found in intact mitochondria (Table 1). This activity was stable for several hours if the preparation was kept on ice. Addition of 0.5mM-DQ to mediate electron transfer between dehydrogenase and oxidase indicated the presence of NADH dehydrogenase and some succinate dehydrogenase in the solubilized fraction. The presence of succinate dehydrogenase was confirmed by DCPIP reduction, because the assay conditions used to optimize KCN-insensitive DQH_2 oxidation (Fig. 1 and Table 2 below) caused an inhibition of succinate oxidase activity. Oxidation of NADH or succinate in the absence of DQ was slow, presumably because detergent treatment caused separation of the oxidase from dehydrogenase complexes, so preventing ubiquinol-mediated electron transfer. The preparation contained very little cytochrome oxidase activity and less of the *b*- and *c*-type cytochromes than in intact mitochrondria (Table 1). The properties of the taurocholate-solubilized preparation are similar to those reported by Rich (1978) after treatment with deoxycholate at a detergent/protein ratio of 4:1 (w/w).

Table 1. *Properties of intact and solubilized Arum mitochondria*

(*a*) Oxygen uptake was measured in a medium consisting of 0.7M-sodium citrate and 0.2μM-FCCP, pH7.0. Rates are nmol of O_2·min^{-1}·mg of protein^{-1} or per 4 reducing equivalents. (*b*) Cytochrome was measured by using the wavelength pairs of Lance & Bonner (1968).

(*a*) Oxygen uptake

	Oxygen uptake rate	
Substrate	Mitochondria	Solubilized preparation
1mM-DQH_2+1mM-KCN	1762	2390
0.75mM-NADH +1mM-KCN	691	69
0.75mM-NADH +0.5mM-DQ +1mM-KCN	693	642
10mM-Succinate +1mM-KCN	62 (see the text)	0
10mM-Sucinate +0.5mM-DQ +1mM-KCN	61	10
Succinate dehydrogenase	510	195
5mM-Ascorbate +0.5mM-TMPD	75	2

(*b*) Cytochrome content

	Content (nmol·mg of protein^{-1})	
Cytochrome	Mitochondria	Solubilized preparation
b	0.301	0.105
c	0.194	0.045

The stoichiometry of oxygen uptake was unaffected by addition of 1mM-cyanide (to inhibit residual catalase activity) and was consistent with water being the end product, in agreement with the reports of Huq & Palmer (1978*c*) and Rich (1978).

We have previously experienced difficulty in obtaining reliable kinetic measurements of DQH_2 oxidation by intact *Arum* mitochondria. This may be due to problems of partition of DQH_2 between

aqueous and membrane phases. We therefore used the soluble preparation for kinetic measurements, since oxidation of DQH_2 by solubilized and intact mitochondria showed similar inhibitor-sensitivities, stoichiometry and specific activity.

Effects of high concentrations of ions on DQH_2 oxidation

High concentrations of sodium citrate, up to an optimum of 0.7M, were found to enhance cyanide-insensitive DQH_2 oxidation by both intact and solubilized mitochondria (Fig. 1) while retaining complete sensitivity to 1 μM-UHDBT and $DQH_2:O_2$ stoichiometry consistent with water production. As DQH_2 oxidation by both intact and solubilized mitochondria was stimulated, it is unlikely that the effect is due to changes in the solubilizing properties of taurocholate (critical micelle concentration and micelle size) known to occur with increasing ionic strength (Carey & Small, 1969). This view was supported by the observation that inclusion of 1% (w/v) sodium taurocholate in the assay of the solubilized preparation did not affect the oxidation rate.

The effect of sodium citrate on the kinetics of DQH_2 oxidation (Fig. 2) was simultaneously to increase $V_{max.}$ (from 450 to 2400nmol of $O_2 \cdot min^{-1} \cdot mg$ of $protein^{-1}$) and decrease K_m (from 0.53 to 0.38mM).

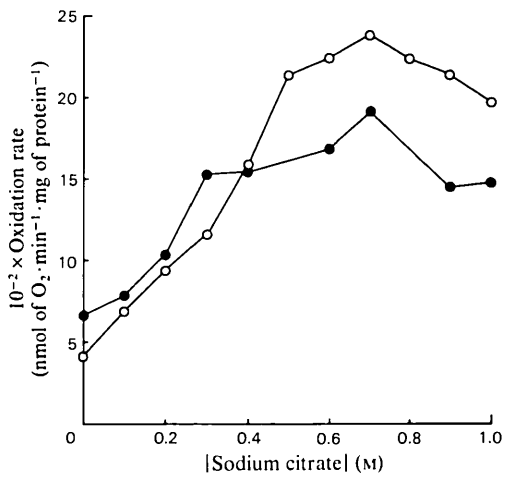

Fig. 1. *Effect of high concentration of sodium citrate on DQH_2 oxidation by intact and solubilized mitochondria*
Initial rates of oxidation on addition of 1mM-DQH_2 were measured at 25°C in buffers of increasing sodium citrate concentration at pH7.0. For zero citrate concentration, 10mM-sodium phosphate, pH7.0, was the buffer used. ○, Solubilized preparation (0.5mg of protein·ml^{-1}); ●, non-solubilized mitochondria (0.34mg of protein·ml^{-1})+1mM-KCN.

With intact mitochondria, only KCN-insensitive oxidation of DQH_2 was stimulated by the presence of high citrate concentrations (Table 2). Oxidation of succinate, NADH and DQH_2+SHAM were inhibited to different extents by sodium citrate. Inhibition of NADH dehydrogenase may have been by chelation of bivalent cations by citrate, particularly Ca^{2+} (Palmer & Coleman,

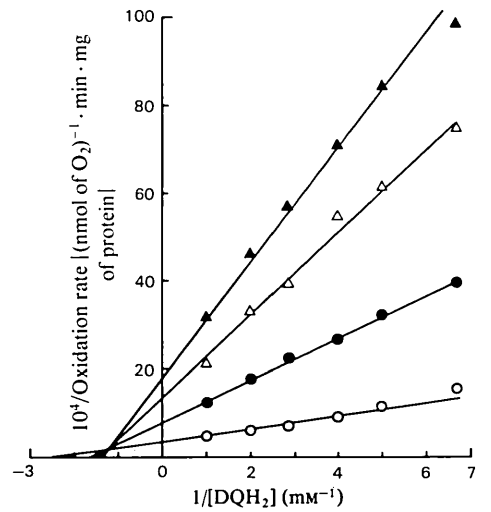

Fig. 2. *Effect of high sodium citrate concentrations on the kinetics of cyanide-insensitive DQH_2 oxidation*
Assay conditions were as shown in Fig. 3, but with sodium citrate concentrations as indicated: ▲, 0.1M; △, 0.2M; ●, 0.4M; ○, 0.7M.

Table 2. *Substrate oxidation by Arum mitochondria in the presence of 0.7M-sodium citrate*
Linear rates of oxygen uptake were measured in (i) standard medium (0.3M-sucrose/10mM-Tris/KOH/5mM-KH_2PO_4/5mM-$MgCl_2$/0.2μM-FCCP, pH7.2), or (ii) 0.7M-sodium citrate/0.2μM-FCCP, pH7.0. Inhibitor concentrations were 1mM.

Substrate	Medium	Rate (nmol of $O_2 \cdot min^{-1} \cdot mg$ of $protein^{-1}$)	
		Standard	Citrate
NADH (0.75mM)		872	792
	+KCN	872	824
	+SHAM	214	103
Succinate (10mM)		722	179
	+KCN	668	179
	+SHAM	450	97
DQH (1mM)		765	1763
	+KCN	388	1704
	+SHAM	186	124

1974), but the cause of the large inhibition of succinate dehydrogenase is not known. Thus, in intact mitochondria, limitation at the dehydrogenase level may obscure increases in the activity of the oxidase caused by citrate. Oxidation of citrate itself was not detected under these conditions.

Oxidation rates obtained with the solubilized preparation in the presence of high concentrations of other ions are shown in Table 3. Of the anions tested, citrate, phosphate and sulphate were particularly stimulatory: the rate when assayed in 1 M-sodium citrate was four times higher than that found in 10 mM-potassium phosphate. Tartrate also increased the oxidation rate, but acetate and chloride had little effect. Cations were less effective at increasing the rate, K^+ and Na^+ stimulating more than Li^+ or Mg^{2+}. The specificity of this effect for different ionic species indicates that these observations are not simply due to high ionic strength or osmolarity.

High concentrations of both cations and anions have been known for many years to have marked effects on conformational stability and solubility of biopolymers [see reviews by Franks & Eagland (1975) and Von-Hippel & Schliech (1969)]. The effectiveness of different ions in promoting these transitions is often similar to the empirical Hofmeister series, originally derived from studies on the effectiveness of cations and anions in the precipitation of serum globulin proteins. In order of increasing effectiveness as precipitants, the ions used in Table 3 are ranked in the Hofmeister series:

$$\text{Cations: } Li^+ > Na^+ > K^+ > Mg^{2+}$$

$$\text{Anions: sulphate} > \text{phosphate} > \text{acetate} > \text{citrate} > \text{tartrate} > \text{chloride}$$

It can be seen that the order of ions in this series is similar to, but not identical with, the degree to which cyanide-insensitive DQH_2 oxidation was stimulated (Table 2). Specific differences were that Li^+ and acetate had less effect than would be predicted from the Hofmeister series.

High ionic concentrations also affect the solubility of many small molecules (Long & McDevitt, 1952). However, since the effect of ions high in the Hofmeister series is to decrease the solubility of hydroquinone and ubiquinone (Linderstrom-Lang, 1923) and the maximum concentration of DQH_2 used in the present study was 1 mM, it is unlikely that changes in solubility of DQH_2 are relevant to the effects described here.

An increase in rate in the presence of high concentrations of anions has also been reported for O_2 evolution by isolated spinach (*Spinacia oleracea*) chloroplasts (Stewart, 1982), but, in contrast with that report, sodium citrate (0.7 M) was not found to protect DQH_2 oxidase activity from denaturation by heat or by concentrations of the chaotropic agent $NaNO_3$ of up to 1 M (results not shown).

Table 3. *Effects of ions on KCN-insensitive oxidation of DQH_2*

Initial rates of oxidation of 1 mM-DQH_2 by solubilized *Arum* mitochondria (0.3–0.4 mg of protein·ml^{-1}) were measured at pH 7.0. All assay media contained 10 mM-potassium phosphate as buffer.

Addition to 10 mM-potassium phosphate	Concn. (M)	$[O_2]$ (μM)	Rate (nmol of O_2·min^{-1}·mg of protein^{-1})
Control	0	240	550
Sodium sulphate	0.5	154	822
	1.0	119	1856
Potassium phosphate	0.5	167	901
	1.0	130	1811
Sodium acetate	0.5	183	432
	1.0	147	425
Sodium citrate	0.5	142	2148
	1.0	95	1973
Sodium potassium tartrate	0.5	162	770
	1.0	103	1046
LiCl	0.5	194	665
	1.0	158	632
NaCl	0.5	201	725
	1.0	159	848
	2.0	107	899
KCl	0.5	199	696
	1.0	168	723
	2.0	118	865
$MgCl_2$	0.5	214	537
	1.0	166	464

Effects of inhibitors

(*1*) *Inhibition by SHAM and CLAM.* Benzhydroxamic acids were introduced as inhibitors of the alternative pathway by Schonbaum *et al.* (1971). The inhibition by both CLAM and SHAM of DQH_2 oxidation by the solubilized preparation was found to be mixed competitive/non-competitive in type (Fig. 3). Secondary plots gave values for apparent inhibition constants for CLAM of 25 μM (K_i) and 81 μM (K_I), and for SHAM of 53 μM (K_i) and 490 μM (K_I). It was of interest that both DQH_2 oxidation and its inhibition by all compounds studied showed saturation kinetics consistent with binding of substrates and inhibitors to specific sites. These results suggest that both SHAM and CLAM inhibit by binding to either the free enzyme (K_i) or the enzyme–DQH_2 complex (K_I), though with both inhibitors binding to the enzyme–quinol complex was less tight than to the enzyme alone.

(*2*) *Inhibition by n-propyl gallate and UHDBT.* In contrast with SHAM and CLAM, propyl gallate was found to inhibit DQH_2 oxidation non-competitively with respect to DQH_2, with an apparent K_i of 0.3 μM (Fig. 4*a*). As with SHAM and CLAM, this result suggests that propyl gallate is able to bind at a separate site from DQH_2, but unlike SHAM and CLAM the presence of bound DQH_2 does not affect the binding of propyl gallate. This strengthens the view that separate inhibitor- and substrate-binding sites exist on the oxidase.

The quinone analogue inhibitor UHDBT, reported by Cook & Cammack (1983) to inhibit succinate and NADH oxidation by *Arum* mitochondria at very low concentrations, was found to inhibit DQH_2 oxidation by the solubilized preparation non-competitively (apparent $K_i = 12$ nM; Fig. 4*b*). This result is rather surprising, since a quinone analogue inhibitor might have been expected to inhibit competitively with respect to a quinol substrate.

(*3*) *Inhibition by non-esterified fatty acids.* A near doubling of NADH and succinate oxidation by mung bean (*Phaseolus aureus*) mitochondria has been reported to occur in the presence of bovine serum albumin (Rich & Bonner, 1978). Similarly, non-esterified fatty acids were found to inhibit the operation of the alternative pathway (Rustin *et al.*, 1984). In view of the recent suggestion that quinol oxidation via the alternative pathway is catalysed by lipid-peroxidative processes (Rustin *et al.*, 1983*a*), the effects of non-esterified fatty acids on solubilized and intact mitochondria were compared. The effect of linoleic acid on DQH_2 oxidation by intact mitochondria via both the cytochrome and alternative pathways is shown in Fig. 5. Young *Arum* spadices of the α-β stage (James & Beevers, 1950) were used in this experiment, since these mitochondria had cytochrome and alternative pathways of similar activity. At

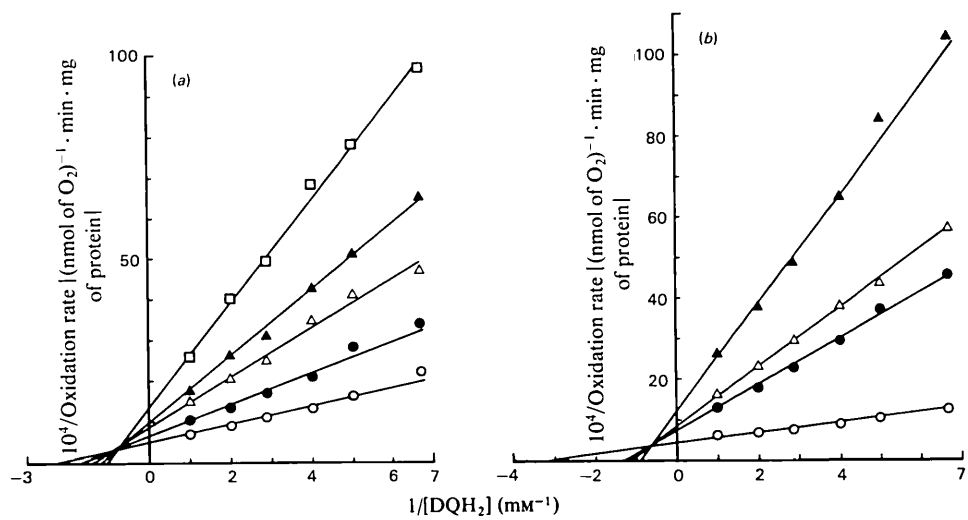

Fig. 3. *Inhibition of cyanide-insensitive DQH_2 oxidation by (a) CLAM and (b) SHAM*
Initial rates of oxidation by a taurocholate-solubilized mitochondrial preparation (0.30–0.4 mg of protein·ml^{-1}) were measured in a medium consisting of 0.7 M-sodium citrate, pH 7.0, at 25°C, at a range of DQH_2 and inhibitor concentrations. (*a*) ○, No inhibitor; CLAM at: ●, 20 μM; △, 50 μM; ▲, 100 μM; □, 200 μM. (*b*) ○, No inhibitor; SHAM at: ●, 100 μM; △, 200 μM; ▲, 400 μM.

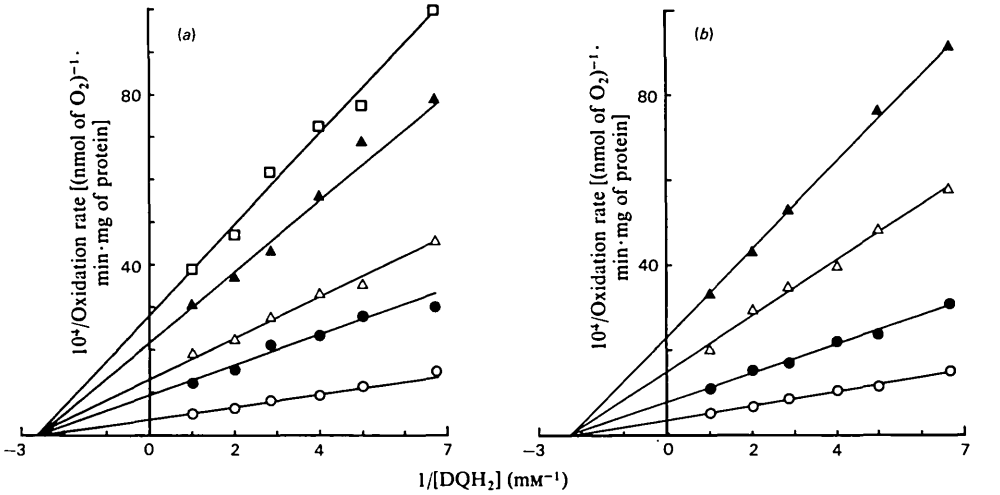

Fig. 4. *Inhibition of cyanide-insensitive DQH_2 oxidation by (a) n-propyl gallate and (b) UHDBT*
Assay conditions were as shown in Fig. 3. (*a*) ○, No inhibitor; n-propyl gallate at: ●, 0.1 μM; △, 0.2 μM; ▲, 0.5 μM; □, 1 μM. (*b*) ○, No inhibitor; UHDBT at: ●, 68 nM; △, 101 nM; ▲, 135 nM.

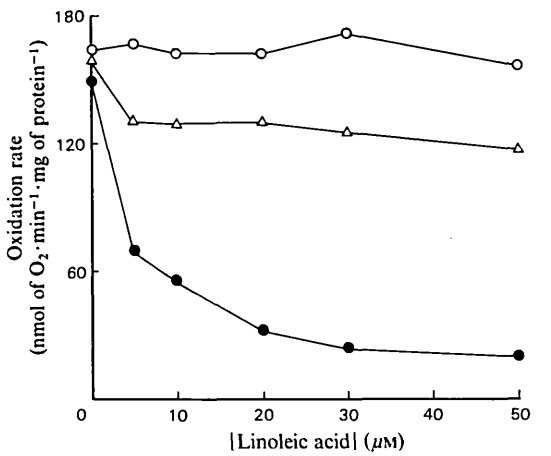

Fig. 5. *Inhibition by linoleic acid of DQH_2 oxidation by α-β-stage Arum mitochondria*
Initial rates of oxidation of 1 mM-DQH by *Arum* mitochondria of the α-β stage (James & Beevers, 1950) were measured in the standard assay medium of Table 2. ●, +1 mM-KCN; ○, +1 mM-SHAM; △, +1 mM-KCN +1% (w/v) bovine serum albumin.

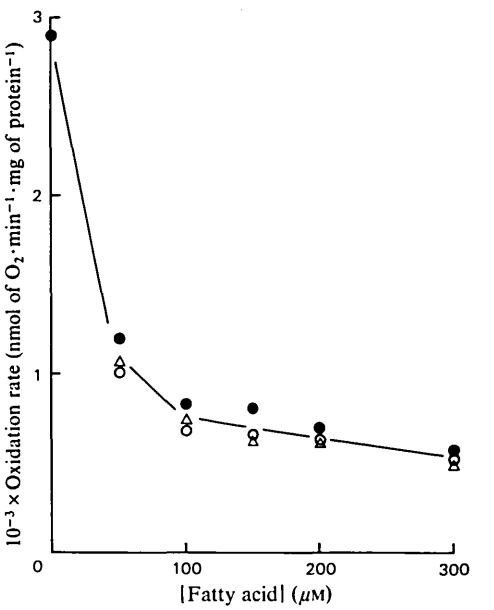

Fig. 6. *Inhibition of cyanide-insensitive DQH_2 oxidation by C_{18} fatty acids*
Initial rates of oxidation on addition of 1 mM-DQH_2, by a solubilized preparation (0.43 mg of protein · ml^{-1}), was measured at 25°C in 0.7 M-sodium citrate, pH 7.0, in the presence of increasing fatty acid concentrations. ○, Linolenic acid ($C_{18:3}$); ●, linoleic acid ($C_{18:2}$); △, oleic acid ($C_{18:1}$).

these concentrations (up to 50 μM, or 0.125 μmol of fatty acid · mg of protein^{-1}), linoleic acid selectively inhibited cyanide-insensitive DQH_2 oxidation without causing significant inhibition of KCN-sensitive DQH_2 oxidation. Inhibition was largely reversed by the presence of 1% (w/v) bovine serum albumin. Oxidation of succinate or NADH by intact mitochondria via the alternative, but not the cytochrome, pathway was similarly inhibited by linoleic acid (results not shown). Since the alternative and cytochrome pathways branch at the level of ubiquinone, these results show selective inhibition by linoleic acid of quinol oxidation via the alternative pathway. It was found that

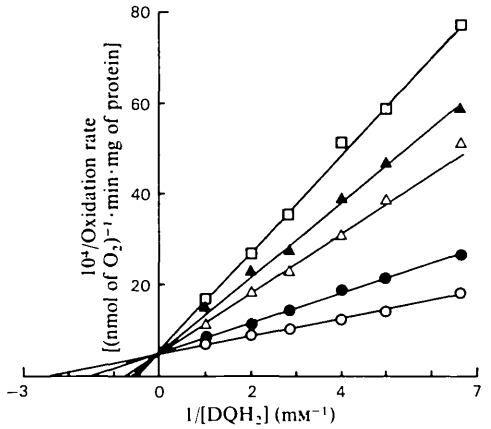

Fig. 7. *Inhibition of cyanide-insensitive DQH_2 oxidation by linoleic acid*

Assay conditions were as shown in Fig. 3. ○, No inhibitor; linoleic acid at: ●, 10 μM; △, 50 μM; ▲, 100 μM; □, 200 μM.

oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids were equally effective inhibitors of DQH_2 oxidation by the solubilized preparation (Fig. 6) and that inhibition by linoleic acid was essentially competitive with respect to DQH_2 (Fig. 7). The lack of inhibition of DQH_2 oxidation via the cytochrome pathway and the small amount of fatty acid required for inhibition (in comparison with the concentrations of DQH_2 added as substrate) make it unlikely that fatty acids inhibit by sequestering DQH_2 away from the oxidase into mixed fatty acid–DQH_2 micelles.

Since intact and solubilized mitochondria were similarly inhibited, it seems that non-esterified fatty acid can interfere reversibly with the interaction of quinol with the oxidase.

Discussion

The present results show that the selective solubilization of *Arum maculatum* mitochondria by taurocholate produces a suitable soluble system for study of the kinetics of the alternative oxidase in the absence of cytochrome oxidase-mediated quinol oxidation. Our interest in inhibitors of the alternative oxidase was firstly to attempt to distinguish between quinol oxidation catalysed by a protein oxidase and that catalysed by a cycling free-radical system (Rustin *et al.*, 1983*a*,*b*). It was thought that the free-radical system would be unlikely to give the simple inhibition patterns expected for an enzymic system, because of the lack of inhibitor- or substrate-binding sites. Our results showed simple enzymic-inhibition patterns for all inhibitors tested and therefore tend to support the enzymic hypothesis of the alternative oxidase.

Benzhydroxamic acids inhibit several other enzymes, such as mushroom tyrosinase (Rich *et al.*, 1978), peroxidase (Schonbaum, 1973) and lipoxygenase (Siedow & Girvin, 1979). Inhibition of tyrosinase was reported to be competitive with reducing substrate, but not with O_2, and possible competition between hydroxamate and ubiquinol for binding sites was suggested (Rich *et al.*, 1978) to explain the dependence of apparent K_i for inhibition of cyanide-insensitive respiration by SHAM on electron flux (Tomlinson & Moreland, 1975). Cottingham & Moore (1983) have reported competitive inhibition by SHAM of DQH_2 oxidation by intact *Arum* mitochondria. However, our results with both SHAM and CLAM, obtained with a solubilized system, are more consistent with mixed competitive/non-competitive than simple competitive inhibition. Mixed inhibition may provide an alternative explanation for the non-linear Dixon plot reported by Cottingham & Moore (1983) for SHAM inhibition of NADH oxidation by *Arum* mitochondria. From this plot, two limiting apparent K_i values were derived of 8 μM and 275 μM at low and high SHAM concentrations respectively. Values of 53 μM (apparent K_i) and 490 μM (apparent K_I) were obtained in the present study, suggesting that, at low SHAM concentration when the ubiquinone pool is relatively oxidized, SHAM will bind predominantly to the free enzyme (K_i), with high affinity. At high SHAM concentration the ubiquinone pool will become reduced, and SHAM will bind to the enzyme–substrate form (K_I), with low affinity. The lower affinities for SHAM reported here compared with those of Cottingham & Moore (1983) may be due to partition of hydroxamate into the membrane phase of intact mitochondria.

The mechanism of hydroxamate binding to, and inhibition of, the alternative oxidase is not clear, but metal-ion chelation (Anderegg *et al.*, 1963) has been discounted (Rich *et al.*, 1977). Other suggestions have been polyfunctional hydrogen-bonding (Schonbaum, 1973) or formation of a 'charge-transfer complex' between hydroxamate and an acceptor group on the enzyme (Rich *et al.*, 1978). The latter theory derives from the ability of hydroxamates to undergo one-electron oxidation to form short-lived radicals (Ramsbottom & Waters, 1966).

Grover & Laties (1978) reported that CLAM and SHAM binding are mutually exclusive in sweet-potato (*Ipomoea batatas*) mitochondria, suggesting that both compounds compete for identical binding sites. The finding of mixed inhibition for both SHAM and CLAM is consistent with an identical binding site, but not with the

suggestion that these compounds act as general competitors with quinol (Rich *et al.*, 1978), since benzhydroxamate and DQH_2 binding is not mutually exclusive and so probably occur at different sites.

The finding that UHDBT, a quinone analogue, was non-competitive inhibitor with respect to DQH_2 was surprising. However, it may be significant that both UHDBT and dibromothymoquinone are redox-active inhibitors of the alternative pathway, capable of forming relatively stable semiquinones [$E'_m(Q^{-\cdot}/Q) = -70$ and $+70$mV respectively] (Rich & Bendall, 1980). Inhibition may therefore, as suggested for benzhydroxamates, be due to the ability of UHDBT and dibromothymoquinone to form a charge-transfer complex with an as-yet-unidentified centre of the enzyme, rather than acting simply as a competitive quinone-analogue inhibitor. Whatever the mode of inhibition, it is apparent that sites of hydroximate, propyl gallate and UHDBT inhibition are different from the site of DQH_2 binding.

Inhibition by linoleic acid of the alternative pathway of substrate oxidation and reversal of inhibition by bovine serum albumin have been reported for intact *Arum* mitochondria (Rustin *et al.*, 1984). The reversal of inhibition is due to the well-known binding of fatty acids to bovine serum albumin. Rustin *et al.* (1984) suggested that, according to the non-enzymic hypothesis of the alternative oxidase (Rustin *et al.*, 1983*a*), fatty acids may inhibit the alternative pathway by causing fluidity changes in the mitochondrial inner membrane. These changes would disrupt the oxidative interaction of the lipid free radicals with ubiquinol. The inhibition of the oxidase in the solubilized form and the finding of competitive inhibition with respect to DQH_2 reported here suggest the alternative explanation, namely that fatty acids bind reversably to the quinol-binding site of the oxidase.

High concentrations of anions such as phosphate, sulphate and citrate were found to enhance DQH_2 oxidation by up to 400% compared with the rate when assayed in 10mM-potassium phosphate. The increased rate was mediated by the alternative oxidase, since correct inhibitor-sensitivities and DQH_2:O_2 stoichiometries were preserved. Although it is unlikely to be of physiological significance, this stimulation of rate was of interest (*a*) in order to optimize assay conditions for the further purification of the oxidase, and (*b*) since the stimulation provides further evidence for the existence of a protein oxidase, by analogy with other proteins similarly affected. Unfortunately one of the problems with isolation of the oxidase is its lability (Lance *et al.*, 1978), and 0.7M-sodium citrate did not significantly protect against denaturation by heat or by high concentrations of the chaotrope $NaNO_3$.

The order of effectiveness of ions in stimulating the oxidase paralleled closely the Hofmeister series, with some differences (noted above). The effects of electrolytes high in the Hofmeister series on soluble proteins is often to stabilize native conformation, as with ribonuclease (Jencks, 1969). Applications of these effects to membrane proteins include the stabilization of mammalian mitochondrial complex II by phosphate, sulphate, citrate and acetate (Jencks, 1969; Davis & Hatefi, 1972) and the stimulation and stabilization of O_2 evolution by spinach chloroplasts in the presence of ions high in the Hofmeister series (Stewart, 1982). At the low end of the series, washing with 2M-NaBr has been reported to detach coupling factor from thylakoid membranes (Nelson, 1980).

The molecular basis for the effects of these electrolytes is not fully understood. However, most explanations involve modification of the organization of solvent around potentially exposed groups on the protein, increasing or decreasing the free energy of transfer of these groups to the exterior, through effects on local water structure (Von-Hippel & Schliech, 1969). Franks (1977) has described three solvent regions around a solvated protein: 'A'-shell water, or the primary hydration sphere of the protein; 'C'-phase or bulk-phase water, and an intermediate 'B'-shell in which water structure reflects perturbations resulting from the proximity of the amphipathic protein molecule. It was suggested that effects of electrolytes on protein conformational stability are mediated through effects on the structure of the 'B'-shell water. The increase in $V_{max.}$ and decrease in K_m of the oxidase suggests that increasing citrate concentration stabilizes a protein conformation of increased catalytic efficiency and alters the microenvironment of the DQH_2-binding site such that interaction of DQH_2 with the oxidase is promoted. Further purification of the oxidase will be needed to determine whether the conformational stability being altered is that of a single protein or of a protein complex, but it seems that factors affecting thermal stability of the oxidase, perhaps loss of bound copper (Bonner & Rich, 1983), are not modified by high ion concentrations.

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References

Anderegg, G., L'Eplattenier, F. & Schwarzenbach, G. (1963) *Helv. Chim. Acta* **46**, 1409–1422

Baginsky, M. L. & Hatefi, Y. (1969) *J. Biol. Chem.* **244**, 5313–5319

Bendall, D. S. & Bonner, W. D., Jr. (1971) *Plant Physiol.* **47**, 236–245
Bonner, W. D., Jr. & Rich, P. R. (1983) *Plant Physiol.* **72** (1, *suppl.*) 19
Cammack, R. & Palmer, J. M. (1977) *Biochem. J.* **166**, 347–355
Carey, M. C. & Small, D. M. (1969) *J. Colloid Interface Sci.* **31**, 382–396
Cook, N. D. & Cammack, R. (1983) *Biochem. Soc. Trans.* **11**, 785
Cottingham, I. R. & Moore, A. L. (1983) *Biochim. Biophys. Acta.* **724**, 191–200
Davis, K. A. & Hatefi, Y. (1972) *Arch. Biochem. Biophys.* **149**, 505–512
Franks, F. (1977) *Philos. Trans. R. Soc. London Ser. B* **278**, 89–95
Franks, F. & Eagland, D. (1975) *Crit. Rev. Biochem.* **3**, 165–219
Grover, S. D. & Laties, G. C. (1978) in *Plant Mitochondria* (Ducet, G. & Lance, C., eds.), pp. 259–266, Elsevier/North-Holland Biomedical Press, Amsterdam
Hauska, G., Hurt, E., Gabellini, N. & Lockau, W. (1983) *Biochim. Biophys. Acta* **726**, 97–133
Huq, S. & Palmer, J. M. (1978*a*) *Plant Sci. Lett.* **11**, 351–358
Huq, S. & Palmer, J. M. (1978*b*) in *Plant Mitochondria* (Ducet, G. & Lance, C., eds.), pp. 225–232, Elsevier/North-Holland Biomedical Press, Amsterdam
Huq, S. & Palmer, J. M. (1978*c*) *FEBS Lett.* **95**, 217–220
Huq, S. & Palmer, J. M. (1981) in *Cyanide in Biology* (Vennesland, B., Conn, E. E., Knowles, C. J., Westley, J. & Wissing, F., eds.), pp. 451–460, Academic Press, London
James, T. W. & Spencer, M. S. (1982) *Plant Physiol.* **69**, 1113–1115
James, W. O. & Beevers, H. (1950) *New Phytol.* **49**, 353–374
Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp. 351–392, McGraw-Hill, New York
Lance, C. & Bonner, W. D., Jr. (1968) *Plant Physiol.* **43**, 756–766
Lance, C., Dizengremel, P. & Chauveau, M. (1978) in *Functions of Alternative Terminal Oxidases* (Degn, H., Lloyd, D. & Hill, G. C., eds.), pp. 133–139, Pergamon Press, Oxford
Lawford, H. G. & Garland, P. B. (1972) *Biochem. J.* **130**, 1029–1044
Linderstrom-Lang, K. (1923) *C. R. Trav. Lab. Carlsberg* **15**, (4)
Long, F. A. & McDevitt, W. F. (1952) *Chem. Rev.* **51**, 119–154
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Nelson, N. (1980) *Methods Enzymol.* **69**, 301–312
Palmer, J. M. & Coleman, J. O. D. (1974) *Horizons Biochem. Biophys.* **1**, 220–261
Ramsbottom, J. C. & Waters, W. A. (1966) *J. Chem. Soc. B* 132–134
Rich, P. R. (1978) *FEBS Lett.* **96**, 252–256
Rich, P. R. & Bendall, D. S. (1980) *Biochim. Biophys. Acta* **592**, 506–518
Rich, P. R. & Bonner, W. D., Jr. (1978) *Arch. Biochem. Biophys.* **188**, 206–213
Rich, P. R., Moore, A. L., Ingledew, W. J. & Bonner, W. D. Jr. (1977) *Biochim. Biophys. Acta* **462**, 501–514
Rich, P. R., Wiegand, N. K., Blum, H., Moore, A. L. & Bonner, W. D. Jr. (1978) *Biochim. Biophys. Acta* **525**, 325–337
Rustin, P., Dupont, J. & Lance, C. (1983*a*) *Trends Biochem. Sci.* **8**, 155–157
Rustin, P., Dupont, J. & Lance, C. (1983*b*) *Arch. Bioch. Biophys.* **225**, 630–639
Rustin, P., Dupont, J. & Lance, C. (1984) *Physiol. Veg.* **22**, 643–663
Schonbaum, G. R. (1973) *J. Biol. Chem.* **248**, 502–511
Schonbaum, G. R., Bonner, W. D., Storey, B. T. & Bahr, J. T. (1971) *Plant Physiol.* **47**, 124–128
Siedow, J. N. & Girvin, M. E. (1979) *Plant Physiol.* **63**, 5
Siedow, J. N. & Girvin, M. E. (1980) *Plant Physiol.* **65**, 669–674
Siedow, J. N., Huber, S. C. & Moreland, D. E. (1978) in *Plant Mitochondria* (Ducet, G. & Lance, C., eds.), pp. 233–240, Elsevier/North-Holland Biomedical Press, Amsterdam
Stewart, A. C. (1982) *Biochem. J.* **204**, 705–712
Tomlinson, P. F. & Moreland, D. E. (1975) *Plant Physiol.* **55**, 365–369
Von-Hippel, P. H. & Schliech, T. (1969) *Acc. Chem. Res.* **2**, 257–265

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Electrostatic screening stimulates rate-limiting steps in mitochondrial electron transport

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The effect of electrostatic screening of fixed negative charges on uncoupled mitochondrial electron transport was investigated with substrates with different charge and different sites of donation of electrons to the electron-transport chain of Jerusalem-artichoke (*Helianthus tuberosus* L.) mitochondria. Duroquinol (neutral substrate) was oxidized with a pH optimum of 7.6–7.8. The addition of cations caused a doubling of $V_{max.}$ (order of efficiency $C^{3+} > C^{2+} > C^{+}$) through electrostatic screening, whereas the K_m was unaffected. Screening stimulated (by 150%) the $V_{max.}$ for the oxidation of reduced cytochrome *c* (positive substrate; to O_2), but in this case the K_m doubled. The $V_{max.}$ of the oxidation of exogenous NADH (negative substrate) was also stimulated by screening when the acceptor was O_2, but unaffected when duroquinone was the acceptor. In both cases, the K_m for NADH was considerably decreased. The effect of screening on the K_m for the different substrates can be explained by the changes in the effective concentration of substrate near the active site due to the lowering in the size of the surface potential. The effect of screening on the $V_{max.}$ of the different partial processes indicates that increasing the salt concentration of the medium enhances the maximal activity of cytochrome *c* oxidase. However, the results also point at the existence of other rate-limiting steps, which are affected by screening and may involve ubiquinone, in electron transport in plant mitochondria.

Schneider *et al.* (1980, 1982) have pin-pointed two rate-limiting steps in the electron-transport system of mammalian mitochondria. By fusing mitoplasts with liposomes they obtained several populations of mitoplasts that differed in their concentration of respiratory complexes. The partial reactions of electron transport from NADH to O_2 most affected by this dilution of complexes were those involving ubiquinone and cytochrome *c*. These reactions were therefore proposed to be diffusion rate-limiting in electron transport (Schneider *et al.*, 1980, 1982).

Abbreviations used: (DM)Br_2, *NNNN'N'N'*-hexamethyldecane-1,10-diamine bromide; duroquinol, tetramethyl-*p*-benzoquinol; duroquinone, tetramethyl-*p*-benzoquinone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mops, 4-morpholinepropanesulphonic acid.

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In plant mitochondria the problem of rate-limiting steps has been approached from an entirely different angle. Added NADH is oxidized by these mitochondria and by mitochondria from fungi and other micro-organisms by a dehydrogenase located on the external surface of the inner membrane (see Palmer & Møller, 1982, for a brief review). A strong stimulation of this oxidation (to O_2) is observed as the cation content of the medium is increased. The stimulation is due to electrostatic screening of fixed negative charges on the mitochondrial membrane (Hackett, 1961; Johnston *et al.*, 1979; Møller & Palmer, 1981*a*; Møller *et al.*, 1982). We originally thought that the decreased negative surface potential (Møller *et al.*, 1981*a*) facilitated the approach of the negatively charged NADH to the membrane-bound enzyme and thus decreased the apparent K_m for NADH (Johnston *et al.*, 1979). Effects of changes in surface potentials on the K_m of membrane-bound enzymes have been reported repeatedly (see, e.g.,

Wojtczak & Nałecz, 1979; Nałecz *et al.*, 1980; Wojtczak *et al.*, 1982). However, increased screening affected not only the K_m of the reaction NADH–O_2 in plant mitochondria but also the $V_{max.}$. The rate of oxidation along the cytochrome *c* oxidase pathway was stimulated in *Arum* mitochondria, whereas the alternative oxidase pathway was unaffected (Møller & Palmer, 1981*a*). Since the two pathways are assumed to diverge at ubiquinone, a step between ubiquinone and O_2 seemed to be involved in changing the $V_{max.}$ (Henry & Nyns, 1975; Storey, 1976).

In the present work we have attempted to locate the partial reaction in mitochondrial electron transport affected by electrostatic screening. The $V_{max.}$ of the oxidation of NADH (negative substrate), duroquinol (neutral substrate) and cytochrome *c* (positive substrate) are all strongly increased by screening when the receptor is O_2. The $V_{max.}$ of the reaction NADH–duroquinone is unchanged by the salt concentration of the medium. It is concluded that cytochrome *c* oxidase is stimulated by electrostatic screening, but that other rate-limiting steps are involved when longer segments of the chain are used.

Materials and methods

Isolation of mitochondria

Mitochondria were prepared from Jerusalem-artichoke (*Helianthus tuberosus* L.) tubers in a low-salt medium containing 0.3M-sucrose as described by Møller *et al.* (1981*a*). When these mitochondria were suspended in a medium containing only 0.02M-sucrose to rupture the outer membrane (Douce *et al.*, 1973), the rate of cytochrome *c* oxidation was 15-fold higher than in 0.3M-sucrose, indicating 93% integrity of the outer membrane.

Duroquinol oxidation

The oxidation of duroquinol (0.1–2.0mM as indicated) was measured in a Rank Brothers oxygen electrode at 25°C in a total volume of 1.0ml. The medium is described in the legends to Figures and Tables.

Cytochrome c oxidation

The oxidation of reduced cytochrome *c* (3–75 μM, prepared as described by Møller & Palmer, 1982) was measured in the presence of only 0.02M-sucrose (to rupture the outer membrane, see above) at 550–540nm in an Aminco DW2 dual-wavelength spectrophotometer at room temperature in a total volume of 1.0ml. The total composition of the medium is described in the legends to the Figures.

NADH oxidation

The rate of NADH oxidation by duroquinone was monitored spectrophotometrically at 340nm at room temperature in a total volume of 1.0ml. The concentration of NADH was varied and the reaction was started by the addition of 0.3mM-duroquinone (final concentration). The total composition of the medium is described in the legend to Fig. 5.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, after solubilizing the mitochondria in 0.5% (w/v) sodium deoxycholate.

Chemicals

Duroquinol from K & K, ICN Pharmaceuticals, Plainview, NY, U.S.A., was dissolved in dimethyl sulphoxide just before use (except for Fig. 1; see the main text). The concentration of the stock solution (about 100mM) was determined by increasing the pH by the addition of 0.1M-KOH (final concentration), which causes the duroquinol to auto-oxidize. An absorption coefficient of $12.5\,mM^{-1}\cdot cm^{-1}$ for $A_{265(red.)} - A_{265(ox.)}$ was used (Lawford & Garland, 1972).

Cytochrome *c* was from Boehringer; EGTA, $(DM)Br_2$, mersalyl, rotenone and chlortetracycline were from Sigma (London) Chemical Co.; tris-(ethylenediamine)Co(III) chloride was from Alfa Products, Danvers, MA, U.S.A. (U.K. distributors: Lancaster Synthesis, Lancaster, U.K.); antimycin A was from Calbiochem, Lucerne, Switzerland; FCCP was a gift from Dr. P. G. Heytler, Du Pont Chemicals, and *m*-chlorobenzhydroxamic acid a gift from Dr. A. Bergman and Dr. I. Ericson, Department of Biochemistry, University of Umeå, Sweden.

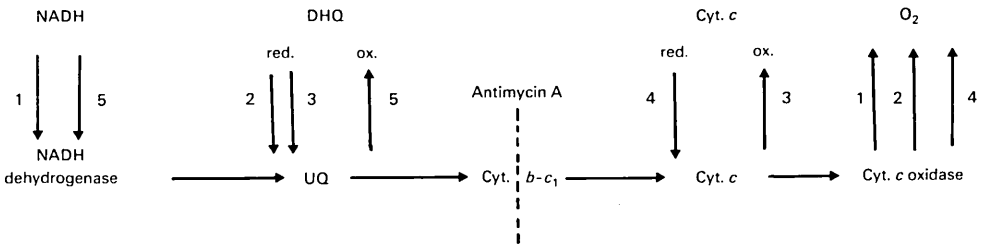
The rest of the chemicals were from BDH Chemicals, Poole, Dorset, U.K., Hopkin and Williams, Chadwell Heath, Essex, U.K., or Sigma Chemical Co. and were of the highest grade available.

Results and discussion

The partial reactions investigated were (1) NADH–O_2 (Johnston *et al.*, 1979; Møller & Palmer, 1981*a*), (2) duroquinol–O_2, (3) duroquinol–oxidized cytochrome *c*, (4) reduced cytochrome *c*–O_2 and (5) NADH–duroquinone (numbers refer to schematic drawing in Scheme 1 and the results are presented in this order).

Duroquinol oxidation

Before the use of duroquinol oxidation (reaction 2, Scheme 1) in kinetic measurements, the pH optimum was determined. After reaching an optimum at pH 7.8 the rate of duroquinol oxidation decreased at pH 8.0, only to increase dramatically



Scheme 1. *Schematic representation of the different partial reactions investigated in the electron-transport chain of Jerusalem-artichoke mitochondria*

We do not know the exact sites of interaction of reduced and oxidized duroquinone (DHQ). For convenience, we let both of them interact with the ubiquinone pool (UQ). Other abbreviation: Cyt., cytochrome.

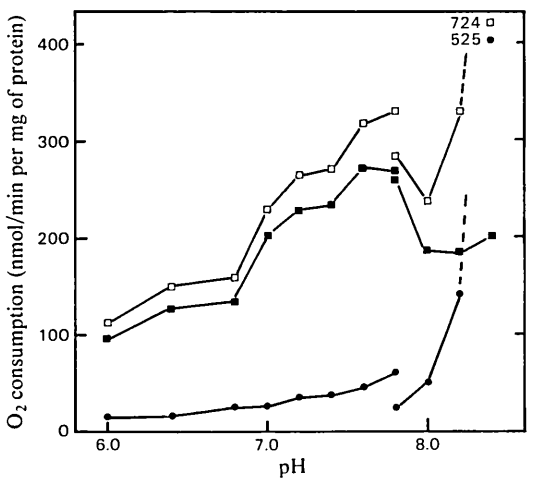


Fig. 1. *pH-dependence of duroquinol oxidation by Jerusalem-artichoke mitochondria*

The oxidation was measured by suspending mitochondria at 0.30mg of protein/ml in medium containing 0.3M-sucrose, 0.1M-Mops buffer, 2mM-(DM)Br_2 and 0.2μM-FCCP with (●) or without (□) 1.9μM-antimycin A at the pH indicated and measuring the rate of O_2 consumption upon addition of 2mM-duroquinol (final concentration). The antimycin A-sensitive part of the oxidation is also shown (■). A 1–2h-old duroquinol solution was used at pH6.0–7.8 and a fresh one at pH7.8–8.4 (see the text).

at pH8.2 and 8.4 (Fig. 1). In the presence of antimycin A to inhibit mitochondrial electron transport, duroquinol oxidation increased exponentially with increasing pH. The antimycin A-insensitive oxidation of duroquinol was also observed in the absence of mitochondria (results not shown), and was thus due to chemical reduction of O_2 by duroquinol at alkaline pH. The pH optimum of the antimycin A-sensitive oxidation of duroquinol was at pH7.6–7.8 (Fig. 1). At pH7.2 that rate was 85% of the maximum, and, since this pH has been used in our previous investigations (Johnston *et al.*, 1979; Møller & Palmer, 1981*a*) and the interference from chemical oxidation of duroquinol was minimal, pH7.2 was used in subsequent experiments. Fig. 1 also illustrates the importance of using freshly prepared solutions of duroquinol, as a 1–2h-old solution gave much higher antimycin A-insensitive oxidation than did a freshly prepared one.

Duroquinol oxidation was insensitive to EGTA, EDTA, chlortetracycline+$CaCl_2$ and mersalyl, all of which inhibit NADH oxidation strongly (Table 1; Coleman & Palmer, 1971; Cowley & Palmer, 1978; Møller & Palmer, 1981*b*; Møller *et al.*, 1981*b*; Palmer & Møller, 1982; Møller *et al.*, 1983). Antimycin A inhibited the oxidation of both substrates completely, whereas rotenone had only a marginal effect (Table 1). The results show that duroquinol donated electrons either to the quinone pool or directly to the cytochrome b–c_1 complex but before the antimycin A block. The partial inhibition of duroquinol oxidation by *m*-chlorobenzhydroxamic acid is consistent with the suggestion by Rich *et al.* (1978) that substituted benzhydroxamic acids can act as competitive inhibitors in enzyme reactions involving quinones.

The addition of cations to mitochondria suspended in a low-salt medium strongly stimulated duroquinol oxidation (Fig. 2). The concentration at which uni-, bi- and ter-valent cations were efficient (10–100μM, 0.1–5mM and 10–100mM respectively) is the same as that observed with NADH oxidation (Johnston *et al.*, 1979; Møller *et al.*, 1981*a*, 1982) and is indicative of electrostatic screening. Cations stimulated the oxidation by 100–200% (Fig. 2), and this was the result of an increased $V_{max.}$ (Fig. 3). The K_m was 0.53 and 0.45mM in the presence and in the absence of 100mM-KCl respectively (Fig. 3).

An attempt to measure mitochondrial electron transport from duroquinol to oxidized cytochrome *c* (reaction 3, Scheme 1) at pH7.2 was unsuccessful.

Table 1. *Effect of inhibitors on NADH and duroquinol oxidation by Jerusalem-artichoke mitochondria*
The reaction medium contained 0.3M-sucrose, 0.1M-Mops/KOH buffer, pH7.2, 2mM-(DM)Br_2, 0.2μM-FCCP and 0.3–0.5mg of mitochondrial protein/ml plus inhibitors as indicated. The reaction was started by the addition of 1mM-NADH or 1mM-duroquinol (final concentration). No correction was made for antimycin A-insensitive duroquinol oxidation. Control rates were 172 and 237nmol of O_2/min per mg for NADH oxidation and 188, 208 and 382nmol of O_2/min per mg for duroquinol oxidation. Values are means ± S.D. (numbers of independent preparations in parentheses).

Inhibitor	Inhibition (%)	
	Of NADH oxidation	Of duroquinol oxidation
EGTA (1mM)	88 (1)	4±4 (3)
EDTA (1mM)	91 (1)	8±3 (3)
Chlortetracycline (50μM)	9 (1)	12±8 (2)
Chlortetracycline (50μM)+$CaCl_2$ (2mM)	82 (1)	15±8 (3)
Mersalyl (20μM)	80±6 (2)	26±4 (2)
Rotenone (20μM)	17±6 (2)	15±6 (2)
m-Chlorobenzhydroxamic acid (1mM)	5±4 (2)	31±8 (2)
Antimycin A (0.36μM)	>97 (1)	>95 (2)

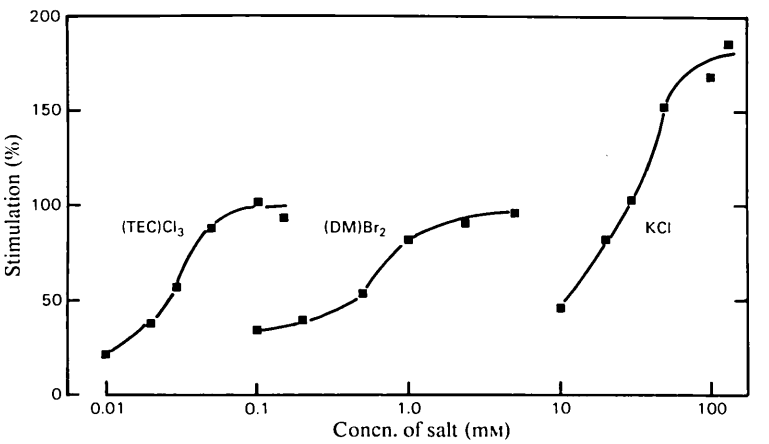


Fig. 2. *Stimulation of duroquinol oxidation by Jerusalem-artichoke mitochondria through addition of salts*
The reaction medium contained 0.3M-sucrose, 0.1M-Mops/KOH buffer, pH7.2, 2mM-(DM)Br_2, 0.2μM-FCCP and of mitochondrial protein/ml, 1.0mM-duroquinol and salts as indicated. Abbreviation: (TEC)Cl_3, tris(ethylenediamine)Co(III) chloride. The control rate in the absence of added salts was 134nmol/min per mg.

A very rapid antimycin A-insensitive cytochrome *c* reduction was observed. This is consistent with the observation by Rich & Bendall (1980) that duroquinol will reduce cytochrome *c* non-enzymically at neutral pH.

Oxidation of reduced cytochrome c

The oxidation of reduced cytochrome *c* (reaction 4, Scheme 1) was also stimulated by cations through an increased $V_{max.}$, but, unlike the results with duroquinol, the K_m for cytochrome *c* was increased by screening (Fig. 4). The increase in $V_{max.}$ with increased salt concentration is in contrast with results obtained with cytochrome *c* oxidase from mammalian tissues, where either no effect (Smith *et al.*, 1981) or a decrease (Douzou & Maurel, 1976) has been reported at neutral pH. The reason for this difference between plant and mammalian cytochrome *c* oxidases is not known, as we have very little information about the enzyme from plant sources (Bomhoff & Spencer, 1977).

Oxidation of NADH

When O_2 is the ultimate electron acceptor (reaction 1, Scheme 1), the $V_{max.}$ of NADH oxidation is strongly stimulated by screening, whereas the K_m is decreased (Johnston *et al.*, 1979; Møller & Palmer, 1981*a*). When the acceptor is duroquinone, the K_m for NADH is also decreased by screening. However, in this case the $V_{max.}$ is practically unaffected (Fig. 5).

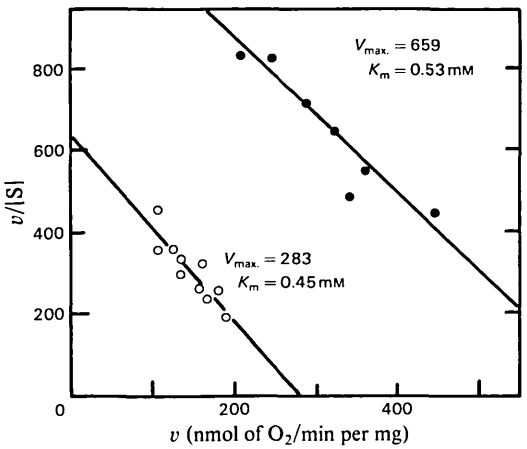


Fig. 3. *Eadie–Hofstee plot of the kinetics of duroquinol oxidation by Jerusalem-artichoke mitochondria at low and high concentrations of cations*

The medium contained 0.3M-sucrose, 5mM-Mops buffer, pH7.2, 0.2μM-FCCP and 0.28mg of mitochondrial protein/ml with (●) or without (○) 100mM-KCl. The reaction was started by the addition of 0.25–2.0mM-duroquinol (final concentration). The lines are fitted by linear-regression analysis. $V_{max.}$ is given in nmol of O_2/min per mg.

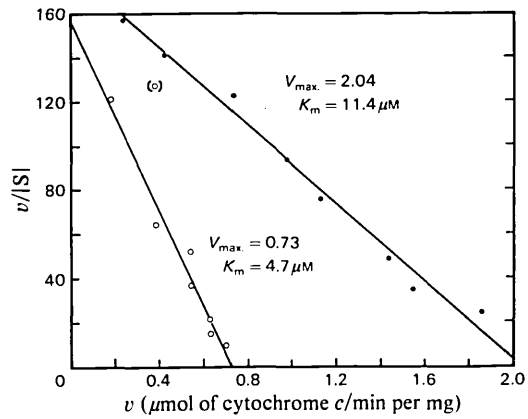


Fig. 4. *Eadie–Hofstee plot of the kinetics of cytochrome c oxidation by Jerusalem-artichoke mitochondria at low and high concentrations of cations*

The medium contained 0.02M-sucrose (see the Materials and methods section), 5mM-Mops buffer, pH7.2, and 0.2μM-FCCP with (●; 9.2μg of protein/ml) or without (○; 18μg of protein/ml) 100mM-KCl. The reaction was started by the addition of 1.5–75μM-reduced cytochrome *c* (final concentration) and the initial rate was determined. $V_{max.}$ is given in nmol of cytochrome *c*/min per mg.

Effect of screening on K_m of membrane-bound enzymes

Cations decrease the size of the negative surface potential through screening of the negative surface charges on biological membranes (Johnston *et al.*, 1979; Barber, 1980, 1982; Chow & Barber, 1980; Møller & Palmer, 1981*a*). The concentration of charged substrates near the membrane surface changes with changes in the surface potential, and the effect on the kinetic parameters of membrane-bound enzymes can be predicted (Douzou & Maurel, 1976; Maurel & Douzou, 1976; Wojtczak & Nałecz, 1979; Nałecz *et al.*, 1980). For enzymes with a positively charged substrate one should observe an increased K_m as the size of the surface potential is decreased, whereas a decreased K_m is the result for enzymes with a negatively charged substrate. The K_m for enzymes with a neutral substrate should be unaffected. The results for Jerusalem-artichoke mitochondria are summarized in Table 2, and there is an excellent agreement with the above predictions.

Effect of screening on $V_{max.}$ of membrane-bound enzymes

The $V_{max.}$ of single enzymes is normally not affected by changes in the surface potential (Wojtczak & Nałecz, 1979; Nałecz *et al.*, 1980). In

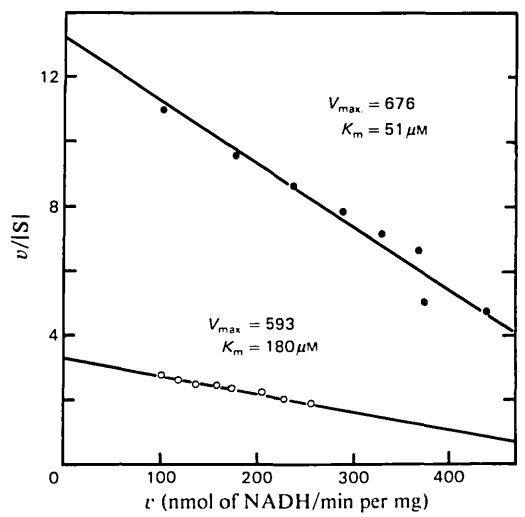


Fig. 5. *Eadie–Hofstee plot of NADH oxidation by duroquinone in Jerusalem-artichoke mitochondria at low and high concentrations of cations*

The medium contained 0.3M-sucrose, 5mM-Mops buffer, pH7.2, 0.2μM-FCCP, 0.36μM-antimycin A, 0.18mg of protein/ml and 10–140μM-NADH with (●) or without (○) 2mM-(DM)Br_2. The reaction was initiated by the addition of 0.3mM-duroquinone (final concentration). $V_{max.}$ is given in nmol of NADH/min per mg.

Table 2. *Effect of electrostatic screening on the kinetics of oxidation of substrates with different charge by Jerusalem-artichoke mitochondria*

High-cation conditions were obtained with 100mM-KCl, except for NADH oxidation (both to O_2 and to duroquinone), where 2mM-(DM)Br_2 was used. Results for the NADH–O_2 reaction (K_m and $V_{max.}$ under low-cation conditions 91 μM and 395nmol of NADH/min per mg respectively) are from Møller & Palmer (1981*a*); for the duroquinol–O_2 reaction they are the average for three independent preparations of mitochondria (including Fig. 3), for the cytochrome *c*–O_2 reaction for two preparations (including Fig. 4) and one preparation for the NADH–duroquinone reaction (Fig. 5).

Parameter	Part reaction ...	NADH–duroquinone	NADH–O_2	Duroquinol–O_2	Cytochrome *c*–O_2
Charge of substrate at pH7.2		Negative	Negative	Neutral	Positive
		Effect of cations (% change from value under low-cation conditions)			
Apparent K_m		−72%	−41%	−13%	+120%
$V_{max.}$		+14%	+41%	+92%	+164%

contrast with this, the $V_{max.}$ for the multistep electron transfers from NADH (Møller & Palmer, 1981*a*) and duroquinol (Fig. 2 and Table 2) is strongly increased by electrostatic screening. This prompted us to suggest that diminished repulsion between different respiratory complexes and/or between complexes and charged lipids due to the lowered surface potential might result in an increased lateral mobility of the respiratory complexes and thus in an increased collision frequency and higher rates of oxidation (Møller & Palmer, 1981*a*; Palmer & Møller, 1982). From previous experiments we knew that the rate-limiting step(s) involved was probably located between ubiquinone and O_2 (Møller & Palmer, 1981*a*), in agreement with the results obtained by Hackenbrock and co-workers (Schneider *et al.*, 1980, 1982). The absence of an increase in $V_{max.}$ upon addition of cations for the NADH–duroquinone reaction (reaction 5 in Scheme 1; Fig. 5 and Table 2) is consistent with this view.

Cytochrome *c* oxidase constitutes one of the rate-limiting steps in the respiration of mammalian mitochondria (Groen *et al.*, 1982), although it does not appear to be stimulated by cations (see above). In contrast with this and with what is normally observed on single enzymes (see above), electrostatic screening strongly enhanced the $V_{max.}$ for cytochrome *c* oxidase in Jerusalem-artichoke mitochondria (Fig. 4 and Table 2). It would appear as if this enzyme might be responsible for part of the increased electron flow through the respiratory chain of plant mitochondria upon electrostatic screening. However, the stimulation of respiration by cations decreased substantially as longer segments of the respiratory chain were involved: cations only stimulated the duroquinol–O_2 reaction by one-half as much and the NADH–O_2 reaction by one-quarter as much as they stimulated the cytochrome *c*–O_2 reaction (Table 2). This suggests that cytochrome oxidase is not rate-limiting when longer portions of the chain are involved and that there are other rate-limiting steps earlier in the chain, possibly involving ubiquinone, that are stimulated by electrostatic screening.

Other electron-transport systems

Electrostatic effects on thylakoid structure and function, including electron transport, are well documented (for reviews see Barber, 1980, 1982). However, plant membranes other than mitochondria and chloroplasts are charged and contain electron-transport chains/complexes. Thus antimycin A-insensitive NAD(P)H oxidation to cytochrome *c* can be observed with crude microsomal preparation and with purified plasma membranes (Lundborg *et al.*, 1981; Widell & Larsson, 1983). The negatively charged plasma membrane in plant roots (Gibrat & Grignon, 1982; Møller *et al.*, 1984; Körner *et al.*, 1984) is exposed to the variable ionic environment of the soil solution. It would be interesting to see to what extent the electron-transport properties of the root plasma membrane are affected by cation binding and electrostatic screening.

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References

Barber, J. (1980) *Biochim. Biophys. Acta* **594**, 253–308
Barber, J. (1982) *Annu. Rev. Plant Physiol.* **33**, 261–295
Bomhoff, G. H. & Spencer, M. (1977) *Can. J. Biochem.* **55**, 1114–1117

Chow, W. S. & Barber, J. (1980) *J. Biochem. Biophys. Methods* **3**, 173–185
Coleman, J. O. D. & Palmer, J. M. (1971) *FEBS Lett.* **17**, 203–208
Cowley, R. C. & Palmer, J. M. (1978) *Plant Sci. Lett.* **11**, 345–350
Douce, R., Mannella, C. A. & Bonner, W. D., Jr. (1973) *Biochim. Biophys. Acta* **292**, 105–116
Douzou, P. & Maurel, P. (1976) *C. R. Hebd. Séances Acad. Sci. Sér. D* **282**, 2107–2110
Gibrat, R. & Grignon, C. (1982) *Biochim. Biophys. Acta* **692**, 462–468
Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., van der Meer, R. & Tager, J. M. (1982) *J. Biol. Chem.* **257**, 2754–2757
Hackett, D. P. (1961) *Plant Physiol.* **36**, 445–452
Henry, M. F. & Nyns, E. J. (1975) *Subcell. Biochem.* **4**, 1–65
Johnston, S. P., Møller, I. M. & Palmer, J. M. (1979) *FEBS Lett.* **108**, 28–32
Körner, L., Møller, I. M., Kjellbom, P. & Larsson, C. (1984) *Abstr. Congr. Fed. Eur. Soc. Plant Physiol. 4th* 514–515
Lawford, H. G. & Garland, P. B. (1972) *Biochem. J.* **130**, 1029–1044
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Lundborg, T., Widell, S. & Larsson, C. (1981) *Physiol. Plant.* **52**, 89–95
Maurel, P. & Douzou, P. (1976) *J. Mol. Biol.* **102**, 253–264
Møller, I. M. & Palmer, J. M. (1981*a*) *Biochem. J.* **195**, 583–588
Møller, I. M. & Palmer, J. M. (1981*b*) *Physiol. Plant.* **53**, 413–420
Møller, I. M. & Palmer, J. M. (1982) *Physiol. Plant.* **54**, 267–274
Møller, I. M., Chow, W. S., Palmer, J. M. & Barber, J. (1981*a*) *Biochem. J.* **193**, 37–46
Møller, I. M., Johnston, S. P. & Palmer, J. M. (1981*b*) *Biochem. J.* **194**, 487–495
Møller, I. M., Schwitzguébel, J.-P. & Palmer, J. M. (1982) *Eur. J. Biochem.* **123**, 81–88
Møller, I. M., Palmer, J. M. & Johnston, S. P. (1983) *Biochim. Biophys. Acta* **735**, 289–297
Møller, I. M., Lundborg, T. & Bérczi, A. (1984) *FEBS Lett.* **167**, 181–185
Nałecz, M. J., Zborowski, J., Famułski, K. S. & Wojtczak, L. (1980) *Eur. J. Biochem.* **112**, 75–80
Palmer, J. M. & Møller, I. M. (1982) *Trends Biochem. Sci.* **7**, 258–261
Rich, P. R. & Bendall, D. S. (1980) *Biochim. Biophys. Acta* **592**, 506–518
Rich, P. R., Wiegand, N. K., Blum, H., Moore, A. L. & Bonner, W. D., Jr. (1978) *Biochim. Biophys. Acta* **525**, 325–337
Schneider, H., Lemasters, J. J., Hochli, M. & Hackenbrock, C. R. (1980) *J. Biol. Chem.* **255**, 3748–3756
Schneider, H., Lemasters, J. J. & Hackenbrock, C. R. (1982) *J. Biol. Chem.* **257**, 10789–10793
Smith, H. T., Ahmed, A. J. & Millett, F. (1981) *J. Biol. Chem.* **256**, 4984–4990
Storey, B. T. (1976) *Plant Physiol.* **58**, 521–525
Widell, S. & Larsson, C. (1983) *Physiol. Plant.* **57**, 196–202
Wojtczak, L. & Nałecz, M. J. (1979) *Eur. J. Biochem.* **94**, 99–107
Wojtczak, L., Famułski, K. S., Nałecz, M. J. & Zborowski, J. (1982) *FEBS Lett.* **139**, 221–224