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UBIQUINONE POOL BEHAVIOUR IN PLANT MITOCHONDRIA

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A kinetic analysis of oxygen uptake was carried out in order to investigate the role of ubiquinone pool behaviour in plant mitochondria. The interaction of the external NADH dehydrogenase with either the cytochrome system or the cyanide-insensitive oxidase was examined under various conditions. The involvement of a ubiquinone pool can be deduced from the shape of the titration curve as the appropriate oxidase system is inhibited, by antimycin A for the cytochrome system and salicylhydroxamic acid for the cyanide-insensitive oxidase, at different activities of the NADH dehydrogenase. In the absence of a specific inhibitor, the turnover of the external NADH dehydrogenase was adjusted using a novel NADH-generating system involving the recycling of a low concentration of NAD^+ by added glucose 6-phosphate dehydrogenase in the presence of substrate. The results show that ubiquinone pool behaviour is observed between the external NADH dehydrogenase and either the cytochrome *b*-c_1 complex or the cyanide-insensitive oxidase. However, there is a substantial departure from pool behaviour during the simultaneous operation of both oxidases.

Introduction

There is a considerable amount of evidence to suggest that ubiquinone acts as an obligatory mobile carrier of reducing equivalents between the membrane-bound dehydrogenases and the cytochrome *b*-c_1 complex in the mammalian mitochondrial electron-transfer chain [1–3]. According to the data of Kröger and Klingenberg [4] the behaviour of ubiquinone as a mobile redox carrier can be described by a simple kinetic model in which the rate of oxygen uptake, v, is directly proportional to the redox poise of the ubiquinone pool. The pool function of ubiquinone (Q) can itself be described by the equation:

$$v = \frac{V_{red} \cdot V_{ox}}{V_{red} + V_{ox}} \quad (1)$$

where v is the rate of oxygen uptake, V_{ox} the first-order rate constant for the oxidation of Q and V_{red} the rate constant for the reduction of Q. When antimycin A was used to decrease V_{red}, Kröger and Klingenberg [5] found that the shape of the titration curve could be accurately predicted from a known ratio of V_{red} to V_{ox}. For example, as the ratio of V_{red}/V_{ox} was decreased, the amount of antimycin A needed to give half-maximal inhibition was increased.

Relatively few studies on Q pool behaviour in plant mitochondria have been reported, but data in the literature do suggest that this model is equally applicable [6–8]. For instance, it is generally recognised that ubiquinone, in mung bean

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

mitochondria, is reduced as a single homogeneous pool in the presence of cyanide and an oxidisable substrate [6,9]. However, the mechanism whereby reducing equivalents are transferred from the respiratory chain dehydrogenases to the cyanide-insensitive oxidase is uncertain. Bahr and Bonner [10,11] have suggested that the apportionment of electrons between the cytochrome and alternative pathways is controlled through an unknown modulation mechanism, the net effect of which is to divert electrons to the alternative pathway only when Q is highly reduced. According to this model the fraction of the alternative pathway engaged in respiration can be expressed as:

$$v_t = \rho \cdot g(i) + V_{cyt} \qquad (2)$$

In this equation v_t represents the observed respiration rate, V_{cyt} the respiratory rate through the cytochrome pathway (which is a different meaning to that used by Kröger and Klingenberg) and $g(i)$ the contribution to the observed rate by the alternative pathway as it is gradually inhibited. ρ, which can be vary between zero and one, represents the fraction of the alternative pathway engaged in respiration. This model was based upon the observations that the total respiratory rate with both pathways functioning simultaneously is always inferior to the mathematical sum of the individual rates and that, furthermore, there exists a linear relationship between the total respiratory rate and the alternative pathway rate.

An alternative suggestion, developed by De Troostembergh and Nyns [12] and based upon the Q pool model, suggests that, in *Saccharomycopsis lipolytica* at least, the distribution of electrons between the two pathways is in direct proportion to their capacities. It makes the assumption that, when the two oxidases operate simultaneously, they compete for reducing equivalents from a single homogeneous pool of ubiquinone. Now the activity of the oxidising system, V_{ox}, is simply the sum of the individual rate constants for the cytochrome and alternative pathways. Thus, V_{ox} is the sum of V_{alt} and V_{cyt} and the Q pool equation is transformed into:

$$v_t = \frac{V_{red}\,(V_{alt} + V_{cyt})}{V_{red} + V_{alt} + V_{cyt}} \qquad (3)$$

V_{alt}, the maximum rate through the alternative pathway, can be obtained from the observed rate of oxygen uptake in the presence of cyanide or antimycin A (v_{alt}) whilst V_{cyt}, the maximal rate through the cytochrome pathway, can be obtained from oxygen uptake rates (v_{cyt}) in the presence of hydroxamic acids in a manner similar to that used to verify Eqn. 1. Using two different substrates, De Troostemberg and Nyns [12] found that, as predicted by this model, only V_{red} changed whilst V_{cyt} and V_{alt} remained constant.

In an attempt to discriminate between these two models we have investigated the kinetic behaviour of ubiquinone, in a variety of plant mitochondria, during the transfer of reducing equivalents from the respiratory chain dehydrogenases to the cytochrome b-c_1 complex and the cyanide-insensitive pathway. In this paper we demonstrate that Q pool behaviour is observed between the external NADH dehydrogenase and either the cytochrome or the cyanide-insensitive pathway. However, when both pathways are operating simultaneously, Q pool behaviour is not observed.

Materials and Methods

The following enzymes and chemicals were purchased from the sources indicated: glucose 6-phosphate, Hepes, Mops, tetramethyl-*p*-benzoquinone (duroquinol), FCCP, glucose-6-phosphate dehydrogenase (from *Leuconostoc mesentroides*, 6150 U/ml at 30°C using β-NAD$^+$) and bovine serum albumin from Sigma London Chemical Co. Ltd.; salicylhydroxamic acid from Aldrich Chemical Co. Ltd.; and antimycin A from Boehringer Mannheim. All other reagents were of a suitably high purity.

Plant materials were obtained as follows: etiolated mung beans (*Phaseolus aureus*) were grown over tap water for 5 days at 30°C; Jerusalem artichokes (*Helianthus tuberosum*) and sweet potato (*Ipomea batatas*) were purchased locally; and *Arum maculatum* spadices were collected last season (mitochondria were prepared the same day and frozen in liquid nitrogen until required). In all cases the mitochondria were prepared by the method of Moore and Proudlove [13].

Duroquinol was prepared by shaking a solution

of duroquinone in diethyl ether with aqueous sodium dithionite as described by Rich [24]. It was used immediately or stored as an acidic solution in dimethyl sulphoxide at −20°C for, at most, a few days. The concentration of duroquinol was estimated from the oxygen uptake caused by the addition of a measured volume of quinone to a large excess of mitochondria (based on the assumption that 1 mol quinone is completely oxidised by 16 gatom oxygen). Salicylhydroxamic acid, antimycin A and FCCP were dissolved in absolute ethanol. All other reagents were made up in glass-distilled water and adjusted to pH 7.4 before use.

Oxidase activities were measured polarigraphically using a Rank oxygen electrode (Rank Bros. Ltd., Bottisham, Cambridge) in a closed 2 ml reaction vessel mantained at 22°C. The standard assay medium contained 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$ and 10 mM Hepes and was adjusted to pH 7.4 with KOH. The concentration of oxygen in air-saturated medium was assumed to be 240 μM. When measuring succinate oxidase activity the dehydrogenase was activated by the addition of 1 mM ATP prior to the addition of the substrate [14]. In the presence of salicylhydroxamic acid linear rates of oxygen uptake were obtained only if the mitochondria were preincubated with the inhibitor for at least 1 min before the addition of substrate. Rates of oxygen uptake are quoted as nmol oxygen consumed/min per mg protein. Protein concentrations were determined by the method of Lowry et al. [15] using bovine serum albumin as standard.

A stock solution of antimycin A was standardized by measuring the absorbance in ethanol at 320 nm using an extinction coefficient of 4800 $1 \cdot M^{-1} \cdot cm^{-1}$ [16]. Antimycin A titrations were performed by mixing samples of mitochondria (100 μl at 10 mg protein/ml) with a constant volume (5 μl) of ethanol containing the appropriate amount of inhibitor. Oxidase activities were measured after incubating for 1 h on ice to allow the antimycin A to equilibrate with its binding sites.

Before use a measured volume of glucose 6-phosphate dehydrogenase was pelleted from ammonium sulphate suspension by centrifugation at $10000 \times g$ for 10 min and resuspended to the original volume in assay buffer. The enzymic generation of NADH was initiated by the addition of appropriate volumes of diluted glucose-6-phosphate dehydrogenase to 2 ml of assay medium which contained NAD^+ (200 nmol) and glucose 6-phosphate (4 μmol). The activity of the dehydrogenase is quoted in units where one unit is the amount of enzyme which generates 1 μmol NADH/min under the assay conditions specified by the supplier.

Results

Generation of NADH by glucose-6-phosphate dehydrogenase

In order to determine the conditions under which NADH generation is directly related to the amount of glucose-6-phosphate dehydrogenase added the following experiment was performed: NADH oxidase activity was measured, under standard assay conditions, in the presence of a large excess of mitochondria from *A. maculatum*, following the addition of increasing amounts of glucose-6-phosphate dehydrogenase (Fig. 1). The relationship between the rate of oxygen uptake and the number of units of dehydrogenase added is linear up to a maximum of 40 nmol/min per assay. This is the maximum useful rate for inhibitor studies and the amount of mitochondria in any one assay was adjusted accordingly. The assay is

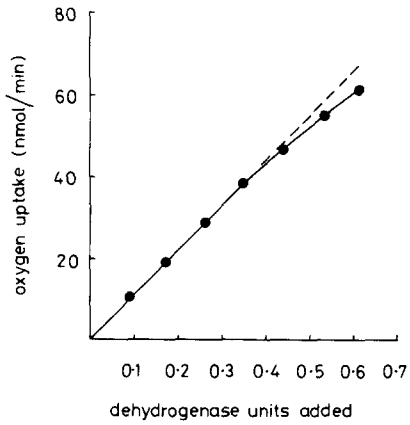

Fig. 1. The effect of glucose-6-phosphate dehydrogenase concentration on the rate of NADH generation. Serial additions (1 μl) of 70-fold diluted glucose-6-phosphate dehydrogenase were added to 2 ml of assay medium which contained *A. maculatum* mitochondria (0.4 mg protein) and NADH and glucose 6-phosphate as described in Materials and Methods. The rate of oxygen uptake is expressed as nmol/min per assay.

operated near the K_m of the glucose-6-phosphate dehydrogenase for NAD^+ which was calculated as 90 μM from a double-reciprocal plot (not shown). This means that the departure from a linear relationship above 40 nmol/min per assay is due to the failure of the mitochondrial dehydrogenase to recycle the NAD^+ fast enough. It is not due to the reversal of the NADH-generating reaction (which is greatly in favour of NADH formation anyway [17]) or to the removal of glucose 6-phosphate (which would only decrease from 2 to 1.8 mM during the course of a 10 min assay). This has the useful consequence that the oxygen consumption remains linear at 40 nmol/min for at least 10 min because only glucose 6-phosphate is being used due to the rapid recycling of the NAD^+.

Maximum possible rates of NADH oxidation could be measured in the same assay by adding saturating amounts of NADH (1.25 mM) after recording the glucose-6-phosphate dehydrogenase-dependent rates. This is possible because the low concentration of NAD^+ (100 μM) in the recycling assay is insufficient to cause inhibition of the mitochondrial dehydrogenase. Curiously, concentrations of glucose 6-phosphate much greater than 2 mM inhibit maximum rates of NADH oxidation.

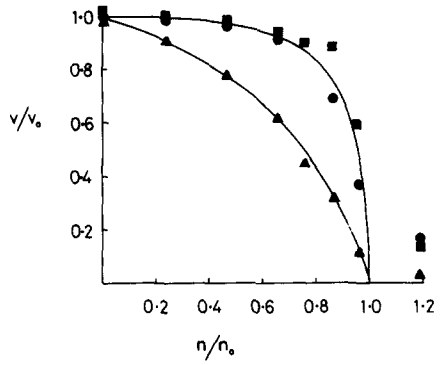

Fig. 2. Antimycin A titration curves for NADH and succinate oxidation by mung bean mitochondria. All rates were measured in the presence of 1 mM salicylhydroxamic acid, 0.1 μM FCCP and 0.75 mg mitochondrial protein. NADH oxidation was measured initially after the addition of 10 μl of 150-fold diluted glucose-6-phosphate dehydrogenase and again after the addition of excess NADH (0.9 mM). Succinate oxidase activity was measured as described in Materials and Methods. The maximum rates in the absence of antimycin A, v_0, were 24 (●) and 135 (▲) nmol/min per mg protein with NADH and 26 nmol/min per mg protein (■) with succinate. The rates measured in the presence of antimycin A are expressed as v. The ratio n/n_0 is the ratio of the number of antimycin A-binding sites occupied to that of the total number of sites. In this experiment n_0 was measured as 0.035 nmol antimycin A/mg protein from a plot of rate of oxygen uptake against amount of antimycin A (not shown). The normalized plot shown in this figure is drawn through the predicted end-point (where $n/n_0 = 1$) rather than through the experimentally measured points.

Inhibition by antimycin A

The degree of inhibition of NADH oxidation by antimycin A in mung bean mitochondria, in the presence of an inhibitor of the cyanide-insensitive oxidase, is dependent on the turnover of the dehydrogenase (Fig. 2). When the dehydrogenase is operating at its maximum rate (135 nmol/min per mg protein, ▲) it is inhibited half-maximally when 75% of the antimycin A-binding sites are occupied. However, when the turnover of the dehydrogenase is limited using the NADH-generating system (24 nmol/min per mg protein, ●), half-maximal inhibition needs 95% of the sites to the occupied. The maximum rate of succinate oxidation is similar to the slower rate of NADH oxidation (26 nmol/min per mg protein, ■) and shows a similar sensitivity to antimycin A. These observations are consistent with the prediction of the Q pool model where the overall oxidase activity is only inhibited when the total oxidase activity of the cytochrome b-c_1 complex becomes rate limiting. If a stoicheiometric relationship existed between each dehydrogenase and a corresponding cytochrome b-c_1 complex then half-maximal inhibition would always occur at the same antimycin A titre.

Two other features of these titration profiles need explaining: firstly, the failure of antimycin A to inhibit completely oxidase activities is due to the small but finite dissociation constant of the enzyme-inhibitor complex [18]; secondly, as a consequence of this, it is necessary to determine the end-point of the titration by extrapolating back to the axis. This information is then used to plot the normalized curves shown in Fig. 2.

In mitochondria from certain plant tissues it has been reported that reducing equivalents from the external NADH dehydrogenase are preferentially passed to the cytochrome b-c_1 complex rather than to the cyanide-insensitive oxidase [19–21]. For instance, NADH oxidation in mitochondria from sweet potato (*I. batatas*) (maximum rate 71

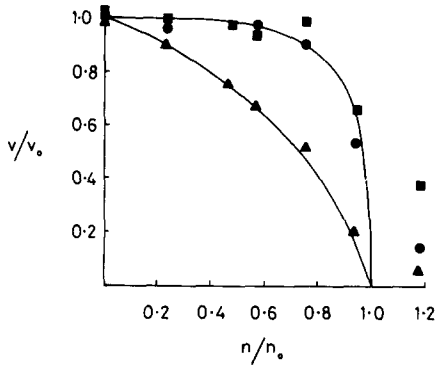

Fig. 3. Antimycin A titration curves for NADH and succinate oxidation by sweet potato mitochondria. Rates were measured as described in the legend to Fig. 2 except that 1.4 mg mitochondrial protein were added and the slower rate of NADH oxidation was measured after the addition of 4 μl of 150-fold diluted glucose-6-phosphate dehydrogenase. The maximum rates were 16.3 μmol/min per mg protein (●) and 43.4 nmol/min per mg protein (▲) for NADH and 14.2 nmol/min per mg protein (■) for succinate. The maximum antimycin A titre (n_0) is 0.04 nmol/mg protein.

nmol/min per mg protein) is 87% inhibited by antimycin A whilst succinate (maximum rate 25 nmol/min per mg protein) is only inhibited by 53%. Similar results have been reported elsewhere for sweet potato [19] and also for cassava (*Manihot esculenta* [20]) and spinach (*Spinacea oleracea* [21]). If there exists a specific interaction between the NADH dehydrogenase and the cytochrome b-c_1 complex in sweet potato mitochondria, then it would be predicted that the antimycin A sensitivity of the NADH oxidase activity would be independent of the turnover of the dehydrogenase. The antimycin titration profiles at different rates of turnover of the external NADH dehydrogenase in sweet potato mitochondria show that this is clearly not the case. The maximum rate of NADH oxidation (Fig. 3, ▲) is half-maximally inhibited when about 70% of the antimycin A-binding sites are occupied but when the dehydrogenase is slowed down (Fig. 3, ●) the same degree of inhibition requires that over 95% of the sites are occupied. This is identical to the behaviour seen with mung bean mitochondria (Fig. 2) and requires another explanation for the strange behaviour of sweet potato mitochondria other than a stoicheiometric interaction between the external dehydrogenase and the cytochrome b-c_1 complex.

The antimycin A titrations shown (Figs. 2 and 3) were performed in the presence of salicylhydroxamic acid to inhibit cyanide-insensitive oxidase activity. In order to check that this does not interfere, the same titrations were performed in the absence of salicylhydroxamic acid on Jerusalem artichoke mitochondria, which lack a cyanide-insensitive oxidase, and similar inhibition profiles were obtained at comparable rates of NADH oxidation (not shown).

Inhibition by salicylhydroxamic acid

The salicylhydroxamic acid sensitivity of NADH oxidase activity in mitochondria from *A. maculatum*, in the presence of an inhibitor of the cytochrome system, is dependent on the turnover of the dehydrogenase (Fig. 4). As before, the dehydrogenase activity was regulated using the NADH-generating system. Half-maximal inhibition of the maximum rate of NADH oxidation (260 μmol/min per mg protein) occurs at about 20 μM salicylhydroxamic acid but the lowest rate (76 μmol/min per mg protein) requires 320 μM to

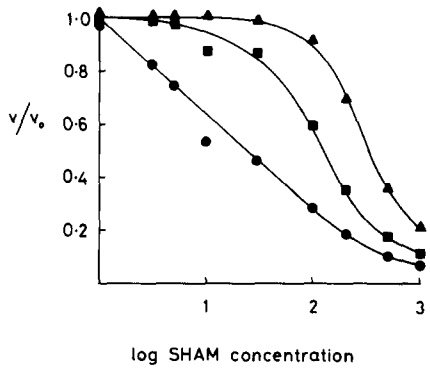

Fig. 4. Salicylhydroxamic acid titration curves for the oxidation of NADH by *A. maculatum* mitochondria. The three different rates of NADH were each measured in the same assay after two individual additions of glucose-6-phosphate dehydrogenase (5 μl, 30-fold diluted) and then a single addition of NADH (to a final concentration of 0.9 mM). Each assay contained 0.1 μM FCCP, 1 nmol antimycin A/mg protein, and 0.65 mg mitochondrial protein. Salicylhydroxamic acid was added 2 min before the reaction was initiated by the addition of substrate. The non-inhibited rates of NADH oxidation, v_0, were 82 (●), 153 (■) and 366 (▲) nmol/min per mg protein. As before, v is the rate measured in the presence of inhibitor. The relative rate, v/v_0, is plotted against the log of the salicylhydroxamic acid (SHAM) concentration (μM).

give the same degree of inhibition. These results show that, in a way already described for the cytochrome b-c_1 complex, the transfer of reducing equivalents from the external NADH dehydrogenase to the cyanide-insensitive oxidase occurs via a mobile pool of ubiquinone.

In order to normalise the salicylhydroxamic acid titration curve with respect to the proportion of occupied inhibitor-binding sites, as done before with the antimycin A titration curves, it is necessary to make an accurate measurement of the K_i for the inhibitor-enzyme complex. However, when the data from the titration curves in Fig. 4 were plotted according to Dixon [22], three biphasic curves resulted (Fig. 5). The horizontal components obtained by titrating sub-maximal rates of NADH oxidation are a consequence of Q pool behaviour. This is because inhibition of the overall oxidase activity only occurs when sufficient salicylhydroxamic acid has been added to make the activity of the cyanide-insensitive oxidase rate limiting. The inhibition of the higher of the two sub-maximal rates occurs at a lower concentration of salicylhydroxamic acid (200 μM; ■ in Fig. 5) than does that of the lower rate (500 μM; ▲) which is consistent with this explanation. If the upper point of the titration curve of the middle rate (■) is extrapolated to cut the horizontal axis a K_i value of 275 μM is obtained. In contrast, when the maximum rate of NADH oxidation is titrated with salicylhydroxamic acid (Fig. 5; ●) there is no horizontal part. However, the titration profile is initially linear (Fig. 5, insert) and can be extrapolated to give a second K_i value of 8 μM. The existence of two K_i values may indicate an effect of the redox poise of the Q pool on the binding of salicylhydroxamic acid to the cyanide-insensitive oxidase [23]: the low K_i is obtained when the ubiquinone is mostly oxidised; the high K_i results from the high degree of reduction of ubiquinone which competes with the salicylhydroxamic acid at its binding site.

Duroquinol is oxidised by *A. maculatum* mitochondria in a salicylhydroxamic acid-sensitive reaction [24]. In yeast the oxidation of duroquinol by the cyanide-insensitive oxidase is dependent on the participation of the cytochrome b-c_1 complex [25]. The explanation given was that the reduction of the endogenous ubiquinone occurred because the duroquinol reduced the b-type cytochromes which then equilibrated reducing equivalents with ubiquinone. This was demonstrated by the observation that cyanide-insensitive oxidation only occurred in the presence of cyanide and not in the presence of antimycin A which prevented electron

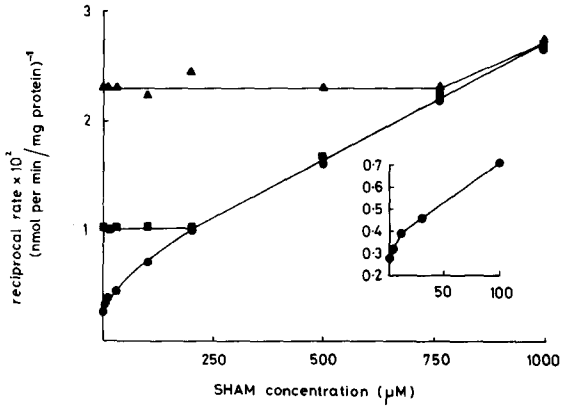

Fig. 5. Dixon plot of salicylhydroxamic inhibition at different rates of NADH oxidation by *A. maculatum* mitochondria. The data used here were obtained from the same experiment as that shown in Fig. 4 and the symbols are explained in the legend to that figure. The inset shows a magnification of the intercept from the maximum rate showing that initially the relationship is linear. The intercept of this line with the horizontal axis was used to calculate the second K_i value mentioned in the text.

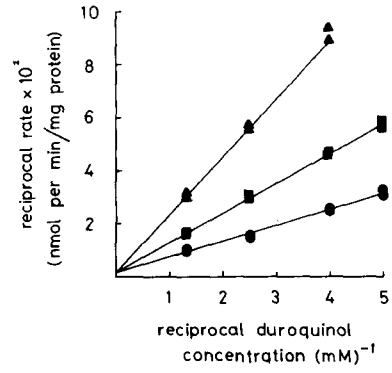

Fig. 6. Double-reciprocal plot showing the inhibition of duroquinol oxidation in *A. maculatum* mitochondria by increasing concentrations of salicylhydroxamic acid. Duroquinol oxidation was measured using 0.65 mg mitochondrial protein in the standard assay medium to which 0.1 μM FCCP, 1 mM EDTA and 1 nmol antimycin A/mg protein had been added. The three plots are: in the absence of inhibitor (●); plus 10 μM salicylhydroxamic acid (■); and plus 50 μM salicylhydroxamic acid (▲).

TABLE I

SIMULTANEOUS OPERATION OF TWO OXIDASES IN *A. MACULATUM* MITOCHONDRIA

All rates of oxygen uptake are expressed as nmol O_2 consumed/min per mg protein. The constants are in nmol/min per mg protein. The buffer used was standard assay medium to which FCCP (0.1 μM) had been added. Each assay was initiated by the addition of substrate and contained 0.12 mg mitochondrial protein. When antimycin A was used it was preincubated with the mitochondria for 1 h at 4°C at a ratio of 2 nmol/mg protein. Salicylhydroxamic acid (1 mM) was preincubated with the mitochondria for at least 1 min before the addition of substrate. When succinate was used as substrate the mitochondria were preincubated with ATP as described in Materials and Methods. The rate constants were calculated from the appropriate rates of oxygen uptake, as discussed in the Introduction.

Substrate	Inhibitor	Rate of oxygen uptake		Rate constant	
NADH	antimycin A	v_{alt}	510	V_n	1780
	salicylhydroxamic acid	v_{cyt}	150	V_{cyt}	164
	none	v_t	617	V_{alt}	715
Succinate	antimycin A	v_{alt}	115	V_s	147
	salicylhydroxamic acid	v_{cyt}	115	V_{cyt}	528
	none	v_t	140	V_{alt}	528

transfer from the *b*-type cytochromes.

In contrast, the rate of duroquinol oxidation in plant mitochondria is the same in the presence of either cyanide or antimycin A. This was so with mitochondria from both *P. aureus* or *A. maculatum* (Cottingham and Moore, unpublished observation) showing that, unlike yeast, in these tissues duroquinol is directly oxidized by the cyanide-insensitive oxidase.

The interaction of duroquinol with the cyanide-insensitive oxidase of *A. maculatum* mitochondria was investigated by adding increasing amounts of duroquinol in the presence of different salicylhydroxamic acid concentrations. When the results are plotted as a double-reciprocal plot (Fig. 6) a common intercept on the *y*-axis is obtained, suggesting simple competitive inhibition. An average value for the K_i was calculated as 16 μM which is comparable to the lower of the two K_i values calculated for NADH oxidation.

Q pool behaviour during the simultaneous operation of both oxidases

Oxygen uptake rates by *A. maculatum* mitochondria were measured for two different substrates, succinate and NADH, after the addition of the inhibitors antimycin A or salicylhydroxamic acid or in the absence of inhibitors (Table I). The results were analysed, using the equations of De Troostemberg and Nyns [12], to calculate the values of the various donor and acceptor rate constants. In accordance with the Q pool theory the values of V_{cyt} and V_{alt} should be independent of the substrate used whilst only the value of V_{red} (V_n for NADH as substrate and V_s for succinate) should alter. This was not observed with *A. maculatum* mitochondria (Table I), with mung bean or sweet potato mitochondria (not shown) showing that, unlike mitochondria from *S. lipolytica*, plant mitochondria do not behave as predicted by the Q pool model during the simultaneous operation of two oxidases.

Discussion

The data presented in this report suggest that Q pool behaviour is exhibited when external NADH is oxidised either by the cytochrome or the cyanide-insensitive pathway. In these experiments the recycling NADH-generating system was used to control the rate of NADH production. This was necessary, since there are no specific inhibitors of this dehydrogenase [7] and the K_m for NADH (72 μM [26]) is so low that the rates obtained with sub-saturating substrate concentrations are too slow for accurate oxygen consumption measurements.

The data obtained with this system are good evidence in favour of the idea that plant mitochondria, like their mammalian counterparts,

use a mobile quinone pool as a carrier of reducing equivalents in the respiratory chain. The involvement of a Q pool between the external NADH dehydrogenase and the cytochrome system was demonstrated in all of the tissues tested. This is of particular interest with respect to sweet potato mitochondria, since it means that reducing equivalents pass through a Q pool from dehydrogenase to the cytochrome system yet appear to be inaccessible to the cyanide-insensitive oxidase. Hence, the idea of a fixed stoicheiometric interaction between these two respiratory chain enzymes is unlikely unless ubiquinone can move freely between, as well as within, multi-enzyme complexes. The nature of the barrier which separates the cyanide-insensitive oxidase from a partially reduced pool of substrate is difficult to imagine in view of the high lateral mobility reported for proteins within the inner mitochondrial membrane [27].

In contrast to the results from *S. lipolytica*, a deviation from the Q pool model is observed during the simultaneous oxidation of two substrates by plant mitochondria. This is so with *A. maculatum* mitochondria and with mung bean mitochondria (Moore and Cottingham, unpublished observation). Since these results are contrary to those of others [28], we have tested the Q pool model using published data from a third laboratory [29] to avoid any experimental bias (Table II). The equations derived from the Q pool theory to describe the simultaneous operation of two oxidases in *S. lipolytica* [12] are easily adapted for two substrates with one oxidase. A departure from theory is demonstrated by increases in both V_n and V_s when ADP is added because Q pool theory predicts that only V_{ox} should change under these conditions (Table II). A similar deviation has been reported during the simultaneous oxidation of NADH and succinate by bovine heart submitochondrial particles [30], showing that this behaviour is not unique to the external NADH dehydrogenase or to plant mitochondria.

The departure from Q pool theory when two dehydrogenases or two oxidases operate simultaneously appears to be widespread [2]. It is not a peculiarity of plant mitochondria or of the cyanide-insensitive oxidase. Thus, it is not necessary to invoke the model of Bahr and Bonner [11] to explain the behaviour of the cyanide-insensitive oxidase as an alternative to the Q pool theory. We would like to propose a simple model to explain the observation that, in mung bean mitochondria at least, the cytochrome pathway always appears to operate at its maximum rate whilst the cyanide-insensitive pathway only operates when the input of reducing equivalents exceeds the capacity of the cytochrome system, such as in State 4. Bahr and Bonner (Ref. 11, and see also Ref. 31) dismiss the idea that the two oxidases compete for a limited quantity of reducing equivalents because the cyanide-insensitive pathway in mung bean mitochondria appears to be completely inactive in State 3. In our opinion this does not eliminate simple competition but indicates that the two oxidases have consideraly different K_m values for their substrates. If the cytochrome system has a lower K_m value than the cyanide-insensitive oxidase it will always operate faster at any particular substrate concentration. The difference in the K_m values for duroquinol with the two oxidases supports this idea; the K_m for the cyanide-insensitive oxidase in the presence of antimycin A is 3.3 mM (Fig. 6) which is considerably higher than the value of 28 μM reported for the cytochrome pathway of bovine heart mitochondria [32]. The suggestion by Bahr and Bonner [11] that the un-

TABLE II

SIMULTANEOUS OXIDATION OF TWO SUBSTRATES IN JERUSALEM ARTICHOKE MITOCHONDRIA

The rates of oxygen uptake are expressed as nmol/min per mg protein. Rates were measured under similar conditions to those described in Materials and Methods (for exact conditions see original report [29]) in the presence of 0.4 mg mitochondrial protein isolated from Jerusalem artichoke. Succinate oxidation was measured after activation of the dehydrogenase and the State 3 rate was measured in the presence of 0.5 mM ATP. The initial concentrations of NADH and succinate were 0.85 and 15 mM, respectively. The values of the rate constants V_{ox}, V_n and V_s are given in nmol oxygen consumed/min per mg protein and were calculated as described in the text.

Substrate	No ADP	Plus ADP
NADH (v_n)	77.4	172.1
Succinate (v_s)	84.6	172.3
Both (v_t)	99.3	236.7
V_{ox}	127.6	378.5
V_n	196.7	315.6
V_s	251.0	316.3

known modulator is a flavoprotein also seems unlikely in view of the fact that the partially purified preparations of the cyanide-insensitive oxidase possess quinol oxidase activity but do not contain appreciable amounts of flavin [20,24].

It is difficult to understand how the Q pool model, which is consistent with the idea that the mitochondrial membrane is highly fluid and acts as a solvent for mobile ubiquinone, works only with a single pathway for the transfer of reducing equivalents. The most common explanation for the departure from the Q pool behaviour is that multiple kinetically distinguishable pools can be created [33,34]. This view is difficult to reconcile with the observation that all of the redox-active ubiquinone is rapidly reduced when substrate is added to bovine heart submitochondrial particles [4] or mung bean mitochondria [9] in the presence of cyanide. If it is accepted, however, that multiple pools can exist, various explanations can be postulated. One possibility is that the transmembraneous transfer of reducing equivalents may become rate limiting if two competing acceptors are on different sides. There is good evidence to suggest, for example, that the rapid relaxation of ubisemiquinone EPR signals, thought to be associated with the cyanide-insensitive oxidase, is due to the close proximity of the centre S-3 of succinate dehydrogenase [35,36]. This raises the possibility that the cyanide-insensitive oxidase may have its quinone-binding site on the opposite side to the cytochrome system. Another possibility is that the membrane is not homogeneous with respect to the distribution of respiratory enzymes and that clumping occurs. This would be analogous to the way in which acetylcholine receptors self-associate in the post-synthetic membrane in nerve cells [37].

In conclusion, it is evident that the full picture of how ubiquinone operates as a mobile carrier within the plant mitochondrial membrane is far from clear. Further progress in resolving this complex but fundamental process will require either experiments to measure the redox poise of ubiquinone during the simultaneous operation of two dehydrogenases or two oxidases by methods similar to those of Kröger and Klingenberg [4,5]; or an investigation of a three-way reconstitution of purified respiratory chain complexes in a controlled lipid and ubiquinone environment similar to those already done for two-way reconstitutions [38–40].

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References

1 Crane, F.L. (1977) Annu. Rev. Biochem. 46, 439–469
2 Gutman, M. (1980) Biochim. Biophys. Acta 594, 53–84
3 Trumpower, B.L. (1981) J. Bioenerg. Biomembranes 13, 1–24
4 Kröger, A. and Klingenberg, M. (1973) Eur. J. Biochem. 34, 358–368
5 Kröger, A. and Klingenberg, M. (1973) Eur. J. Biochem. 39, 313–323
6 Storey, B.T. (1980) in The Biochemistry of Plants (Conn, E. and Stumpff, P.K., eds.), Vol. 2, pp. 125–195. Academic Press, New York
7 Palmer, J.M. (1983) The Physiology and Biochemistry of Plant Respiration, Cambridge University Press, Cambridge, in the press
8 Moore, A.L. and Cottingham, I.R. (1983) in Uptake and Utilization of Metals by Plants. Proceedings of The Phytochemical Society of Europe (Robb, D.A. and Pierpoint, W.S., eds.), pp. 169–204, Academic Press, New York
9 Storey, B.T. (1974) BBA Library 13, 93–102
10 Bahr, J.T. and Bonner, W.D., Jr. (1973) J. Biol. Chem. 248, 3441–3445
11 Bahr, J.T. and Bonner, W.D., Jr. (1973) J. Biol. Chem. 248, 3446–3450
12 De Troostembergh, J.-C. and Nyns, E.-J. (1978) Eur. J. Biochem. 85, 423–432
13 Moore, A.L. and Proudlove, M.O. (1983) in Isolation of Membranes and Organelles from Plant Cells (Hall, J.L. and Moore, A.L., eds.), pp. 153–184. Academic Press, London.
14 Singer, T.P., Oestreicher, G., Hogue, P., Contreiras, J. and Brandao, I. (1973) Plant Physiol. 52, 616–621
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
16 Van Ark, G. and Berden, J.A. (1977) Biochim. Biophys. Acta 459, 119–137
17 Glaser, L. and Brown, D.H. (1955) J. Biol. Chem. 216, 67–73
18 Berden, J.A. and Slater, E.C. (1972) Biochim. Biophys. Acta 256, 199–215
19 Tomlinson, P.F. and Moreland, D.E. (1975) Plant Physiol. 55, 365–369
20 Huq, S. and Palmer, J.M. (1978) in Plant Mitochondria (Ducet, G. and Lance C., eds.), pp. 225–232, Elsevier, Amsterdam
21 Douce, R., Moore, A.L. and Neuberger, M. (1977) Plant Physiol. 60, 625–628
22 Dixon, M. (1953) Biochem. J. 55, 170–171

23 Rich, P.R., Wiegand, N.K., Blum, M., Moore, A.L. and Bonner, W.D., Jr. (1978). Biochem. Biophys. Acta 525, 325–337
24 Rich, P.R. (1978) FEBS Lett. 96, 252–256
25 Von Jagow, G. and Bohrer, C. (1973) Biochim. Biophys. Acta 387, 409–424
26 Ikuma, H. and Bonner, W.D., Jr. (1967) Plant Physiol. 42, 1534–1544
27 Hackenbrock, C.R. (1981) Trends Biochem. Sci. 6, 151–154
28 Moreira, M.T.F., Rich, P.R. and Bendall, D.S. (1980) EBEC 1, 61–62
29 Cowley, R.C. and Palmer, J.M. (1980) J. Exp. Biol. 31, 199–207
30 Gutman, M. and Silman, N. (1972) FEBS Lett. 26, 207–210
31 Laties, G.G. (1982) Annu. Rev. Plant Physiol. 33, 519–555
32 Lawford, H.G. and Garland, P.B. (1973) Biochem. J. 136, 711–720
33 Moore, A.L., Linnett, P.E. and Beechey, R.B. (1980) J. Bioenerg. Biomembranes 12, 309–323
34 Rustin, P., Moreau, F. and Lance, C. (1980) Plant Physiol. 66. 457–462
35 Moore, A.L. and Rupp, H. (1978) FEBS Lett. 93, 73–77
36 Moore, A.L., Hall, D.O. and Rupp, H. (1978) in Frontiers of Biological Energetics (Dutton, P.L., Leigh, J.S. and Scarpa, A., eds.), Vol. 1, pp. 210–217. Academic Press, New York
37 Axelrod, D., Raudin, P., Koppel, D.E., Schlessinger, J., Webb, W.W., Elson, E.L. and Podlesk, T.R. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4594–4598
38 Ragan, C.I. and Heron, C. (1978) Biochem. J. 174, 783–790
39 Heron, C., Ragan, C.I. and Trumpower, B.L. (1978) Biochem. J. 174, 791–800
40 Cottingham, I.R. and Ragan, C.I. (1980) Biochem. J. 192, 19–31