

## Energy conservation by the plant mitochondrial cyanide-insensitive oxidase

### SOME ADDITIONAL EVIDENCE

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Several measures of energy conservation, namely ADP/O ratio, P/O ratio, ATP/O ratio and phosphorylation detected by continuous assay with purified firefly luciferase and luciferin, all show phosphorylation can occur with mung-bean mitochondria at cyanide concentrations sufficient to inhibit the cytochrome oxidase system. Phosphorylation in the presence of cyanide is uncoupler- oligomycin- and salicylhydroxamate-sensitive. The participation of phosphorylation site 1 is excluded, phosphorylation being attributable to a single phosphorylation site associated with the cyanide-insensitive oxidase. The cyanide-insensitive oxidase has also been shown to support a variety of other energy-linked functions, namely,  $\text{Ca}^{2+}$  uptake, reversed electron transport and the maintenance of a membrane potential detected by the dye probes 8-anilino-naphthalene-1-sulphonate and safranin. High concentrations of cyanide have uncoupler-like activity, decreasing the ADP/O ratio and the  $t_{\frac{1}{2}}$  for the decay of a pH pulse through the mitochondrial membrane. This uncoupler-like effect is most marked with aged mitochondria. The observations of energy conservation attributable to the cyanide-insensitive oxidase are compared with other reports where it is concluded that the alternative oxidase is uncoupled.

Plant mitochondria have a respiratory chain with a normal cytochrome system and also a branch that is insensitive to cyanide and antimycin A and by-passes phosphorylation sites 2 and 3 (Bendall & Bonner, 1971; Henry & Nyns, 1975). The cyanide-insensitive branch probably originates in the ubiquinone region of the electron-transport chain (Solomos, 1977) and terminates in an independent non-cytochrome oxidase. Substituted hydroxamic acids are specific inhibitors of this alternative oxidase system (Schonbaum *et al.*, 1971). Because of the thermogenic role of this oxidase system in aroid plants, it is generally believed that it is uncoupled and incapable of energy conservation. Although several workers have concluded that energy conservation is not associated with the cyanide-insensitive oxidase (Hackett *et al.*, 1960; Storey & Bahr, 1969; Storey, 1971; Passam & Palmer, 1972; Moore, 1978; Moore *et al.*, 1978a), other studies have shown that a variety of energy-linked functions can occur in the presence of cyanide (Bonner & Bendall, 1968; Dawson & Gains, 1969; Wilson, 1970, 1977, 1978a; Wilson & Moore, 1973). However, much of the evidence favouring energy conservation by the cyanide-insensitive oxidase is either indirect or subject to criticism. It is possible that the observed ATP synthesis or other

energy-linked function could be attributed to phosphorylation site 1 with malate derived from the succinate originally supplied as a substrate. Although the comparatively high  $K_m$  for malate oxidation by plant mitochondria, namely 4 mM (Ikuma & Bonner, 1967a), makes the utilization of this product malate unlikely, it cannot be excluded, since there are no inhibitors that can be used to inhibit specifically and completely site 1 in plant mitochondria (Wilson, 1978b).

Many of the experiments described in the present paper use substrates that cannot produce malate in their products and that cannot interact with phosphorylation site 1. The paper provides further evidence that the cyanide-insensitive oxidase of mung-bean (*Phaseolus aureus* L.) mitochondria can support a variety of energy-linked functions, including ATP synthesis. A preliminary report of some of these results has already been presented (Wilson, 1977).

### Materials and methods

#### Materials

Mitochondria were isolated from laboratory-grown etiolated mung-bean hypocotyls by the methods described previously (Wilson, 1978b).

Mitochondria as washed or purified preparations were used only if they had a high proportion of intact membranes and high ADP/O and respiratory-control ratios. All experiments were carried out within 45 min of the completion of the isolation procedure.

The reagents were all of analytical reagent or the best commercially available grade. Enzymes and substrates were obtained from the Sigma Chemical Co., Kingston upon Thames, Surrey, U.K.; compound 1799 [1,1,1,7,7,7-hexafluoro-2,6-dihydroxy-2,6-bis(trifluoromethyl)heptan-4-one; bis-(hexafluoroacetyl)acetone], a potent uncoupler of oxidative phosphorylation, was generously given by Dr. P. Heytler, Du Pont de Nemours, Wilmington, DE, U.S.A.

#### Oxygen uptake

O<sub>2</sub> uptake was measured with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) in a magnetically stirred Perspex [poly(methyl methacrylate)] cuvette of 3 ml capacity. Up to 2 mg of mitochondrial protein was used for experiments where cyanide was present. When samples were required a cuvette with an adjustable lid was used to prevent the ingress of O<sub>2</sub>.

The reaction medium contained (final concentrations) 0.3 M-mannitol, 10 mM-KCl, 5 mM-MgCl<sub>2</sub> and 10 mM-potassium phosphate buffer, pH 7.2, unless otherwise stated; 8.3 mM-succinate, 2–8.3 mM-trimethylquinol, 2 mM-duroquinone or 2 mM-mena-dione reduced with KBH<sub>4</sub> (Lawford & Garland, 1973) was used as substrate. Since high concentrations of oxidized quinones derived from the reduced quinones appeared to be deleterious in some experiments, the quinones were also used at lower concentrations (17 μM) together with 0.3 mM-dithioerythritol to ensure reduction (Taggart & Sanadi, 1972). High concentrations of reduced quinone also gave some non-enzymic O<sub>2</sub> uptake. All the quinones and the dithioerythritol mixture donate electrons at the quinone level of the respiratory chain. The enzymic cyanide-insensitive oxidation of trimethylquinol was unaffected by 1 μM-antimycin A, but was completely inhibited by 1 mM-salicylhydroxamate. The quinones were all added to the reaction mixture in a small volume of ethanolic solution.

#### Assay of phosphorylation

Phosphorylation was assayed by several independent methods, as follows.

(a) *Measurement of the ADP/O ratio as described by Estabrook (1967)*. ADP concentrations were measured spectrophotometrically assuming the molar absorption coefficient to be  $15.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  at 260 nm. O<sub>2</sub> concentrations were calculated from the known solubility of O<sub>2</sub> in

the medium used at the temperature of the experiment (Estabrook, 1967).

(b) *Measurement of the formation of ATP either enzymically or by phosphate uptake after entrapment of the ATP in the presence of 0.16 mM-ADP, 3.3 mM-glucose and 500 μg of hexokinase*. For both methods samples were removed from the oxygen-electrode cuvette at intervals and the reaction was terminated by the addition of ice-cold 5% (w/v) HClO<sub>4</sub> or trichloroacetic acid for the two methods respectively. Precipitated proteins were removed by centrifugation for 5 min in a bench centrifuge. For the phosphate-uptake method the potassium phosphate concentration of the reaction medium was lowered to 2 mM, phosphate being assayed by the method of Lowry & Lopez (1946). ATP was measured by using absorption spectrophotometry at 340 nm with a Unicam SP.1800 spectrophotometer fitted with an automatic sample-changer and temperature control (Pye Unicam, Cambridge, U.K.). The assay with hexokinase, glucose, glucose 6-phosphate dehydrogenase and NADP<sup>+</sup> (Estabrook *et al.*, 1967) was performed after removal of the HClO<sub>4</sub> as the potassium salt. In both assay methods the samples were processed as rapidly as possible and no detectable loss of ATP occurred. In all cases phosphorylation was initiated before the addition of KCN and measurements were started only after the O<sub>2</sub> uptake had remained steady at the inhibited rate for 1–2 min.

(c) *Measurement of ATP production directly in the mitochondrial assay medium with the addition of 10 μg of purified firefly luciferin and 3 μg of purified luciferase (Lemasters & Hackenbrock, 1973)*. Light output was monitored in a specially constructed spectrophotometer (University of Bristol, Medical School Workshops, Bristol, U.K.).

#### Ca<sup>2+</sup> uptake

Ca<sup>2+</sup> uptake was measured by using 50 μM-Arsenazo III, a metallochromic dye, that had been previously purified by two passages through a 50 cm × 1 cm column of Dowex 50-WX (H<sup>+</sup> form) (Scarpa, 1976; Russell & Wilson, 1978). Absorption changes indicating Ca<sup>2+</sup> movements were measured in the 'Bristol spectrophotometer' with the wavelength pair 650 and 700 nm.

#### Membrane potential

Membrane potentials were monitored by using two dye probes, 33 μM-8-anilino-naphthalene-1-sulphonate (Azzi *et al.*, 1969) and 5 μM-safranin (Akerman & Wikström, 1976). The optical changes in these two probes were monitored in the 'Bristol spectrophotometer' by fluorescence at 360 → 470 nm or by absorption with the wavelength pair 524 and 554 nm respectively. Wavelengths for fluorescence were selected by appropriate combinations of

Wratten gelatin filters (Kodak, London W.C.2, U.K.) and interference filters (Balzers of Liechtenstein, via O.E.C. Ltd., Reigate, Surrey, U.K.).

#### Proton permeability

The proton permeability of the mitochondrial membrane was monitored by measuring the decay of

pH after a pH pulse. The buffer concentration in the basal reaction medium was lowered to 1 mM-potassium phosphate and the pH was recorded for 5 min before and after the addition of a pulse of 150 nmol of NaOH or HCl. Since the mitochondria had a very low rate of endogenous respiration, some measurements were made under aerobic conditions; other-

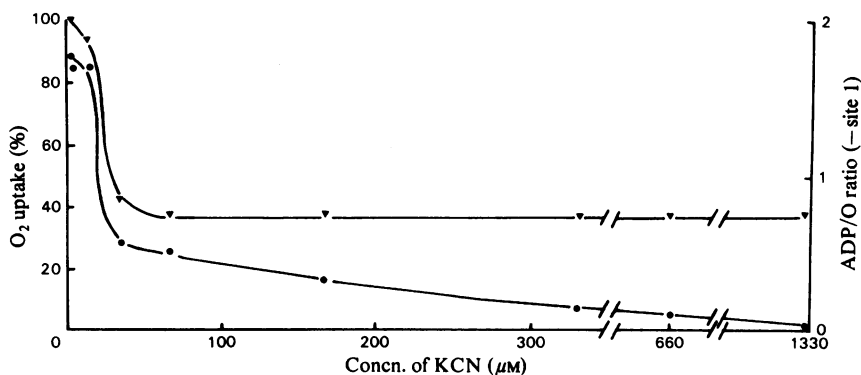


Fig. 1. Effect of cyanide on the  $O_2$ -uptake rate and ADP/O ratio of mung-bean mitochondria oxidizing 16.7 mM-malate. The basal reaction mixture contained 0.3 M-mannitol, 10 mM-KCl and 5 mM-MgCl<sub>2</sub> in 10 mM-potassium phosphate buffer, pH 7.2. ADP/O ratios (●) were measured as described in the text. (Values for the experiment illustrated are shown below in parentheses.) The contribution of site 1 (0.84) calculated from the difference between the ADP/O ratios for succinate (1.84) and malate (2.68) has been deducted from the results.  $O_2$ -uptake rates (▼) measured with an oxygen electrode are plotted as a percentage of the 'state 3' rate. Typical oxidation rates for mung-bean mitochondria are 75 nmol of  $O_2$ /min per mg of protein for malate and 100 nmol of  $O_2$ /min per mg of protein for succinate.

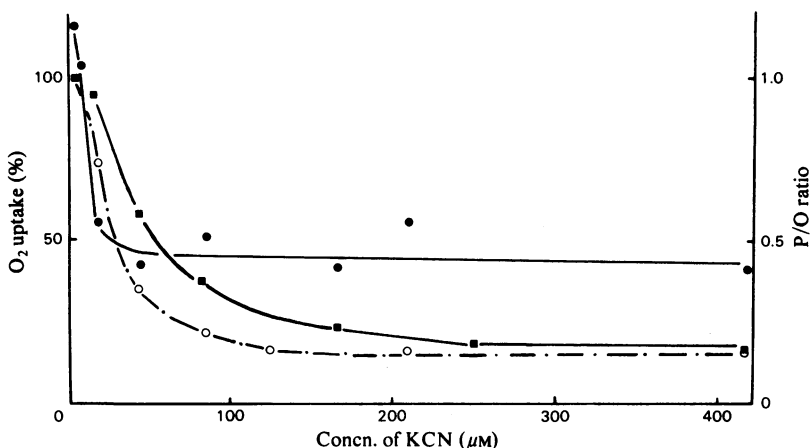


Fig. 2. Effect of cyanide on the  $O_2$ -uptake rate and P/O ratio of purified mung-bean mitochondria oxidizing 8.3 mM-trimethylquinol

The basal reaction mixture described for Fig. 1 was used with the phosphate buffer concentration lowered to 2 mM; 0.16 mM-ADP, 3.3 mM-glucose and 500  $\mu$ g of hexokinase were included as an ATP trap. Mitochondria were allowed to phosphorylate before the addition of cyanide, and sampling was not started until the cyanide-inhibited rate had remained linear for at least 1 min. Phosphate was determined on samples removed from the oxygen-electrode cuvette by the methods described in the text (●, P/O ratio).  $O_2$ -uptake rates (■) were measured with an oxygen electrode and are plotted as a percentage of the uninhibited rate in the presence of 0.8 mM-ADP. A typical  $O_2$ -uptake rate with trimethylquinol is 40 nmol of  $O_2$ /min per mg of protein. Also plotted is the effect of cyanide on the  $O_2$ -uptake rate of mitochondria utilizing 10 mM-ascorbate + 1 mM-*NNN'*-tetramethylene-*p*-phenylenediamine mixture (○).

wise reagents were made anaerobic by bubbling with  $O_2$ -free  $N_2$  (Mitchell & Moyle, 1967; Moore & Wilson, 1978). Similar results were obtained under both aerobic and anaerobic conditions. Traces were corrected manually for the low rate of drift observed before and after the completion of the pulse.

#### Redox changes in NAD

The redox poise of the endogenous NAD pools of the mitochondria were monitored as a measure of reversed electron transport by fluorescence by using the wavelengths 374–420 nm and the fluorescence attachment of the 'Bristol spectrophotometer'. Wavelengths were selected by the appropriate combination of interference and gelatin filters as described above.

### Results

#### Formation of ATP in the presence of cyanide

Since the branch point for the alternative oxidase system is in the quinone region of the respiratory chain, the ADP/O ratio obtained for cyanide-inhibited malate oxidation is composed of the contribution attributable to the activity of phosphorylation site 1 plus any contribution due to the activities of the cyanide-insensitive oxidase. Fig. 1 shows the results of an experiment when the ADP/O ratio for malate oxidation was measured over a range of cyanide concentrations and the contribution of the ADP/O ratio for site 1 was deduced from the results. The contribution due to the activity of phosphorylation site 1 was measured from the difference between the ADP/O ratios obtained for succinate and malate oxidation in the absence of cyanide. Although the measurement of ADP/O ratios at high cyanide concentrations is difficult because the respiratory-control ratio declines markedly, the results clearly show that the ADP/O ratio is approximately 0.5 at a cyanide concentration that maximally inhibits  $O_2$  uptake. This value for the ADP/O ratio is similar to that (0.7–0.8) observed for the operation of a single phosphorylation site in plant mitochondria and is equivalent to other observations of phosphorylation by the alternative oxidase (Wilson, 1970, 1978b). Increasing the cyanide concentrations above this minimum value causes a further decline in the ADP/O ratio and the respiratory-control ratio (results not shown) without any further change in the  $O_2$  consumption, suggesting that cyanide is uncoupling the system. Salicylhydroxamate (1 mM) inhibits the KCN-resistant respiration and in the absence of KCN raises the ADP/O ratio slightly as shown previously (Schonbaum *et al.*, 1971).

Because this measurement of the ADP/O ratio for the cyanide-insensitive oxidase is indirect, it was

decided to measure the P/O ratio by the uptake of  $P_i$  in the presence of a glucose + hexokinase trap system. Trimethylquinol was used as substrate, since it is oxidized rapidly and can only donate electrons at the quinone level of the respiratory chain. Fig. 2 shows that the P/O ratio for trimethylquinol oxidation also has a plateau value of 0.4–0.5 over a range of cyanide concentrations that give no further change in the  $O_2$ -uptake rate. The P/O ratio is, however, a minimum value, since there is some non-enzymic oxidation of the quinone. Since this non-enzymic oxidation rate probably varies during the course of the experiment, it is impossible fully to assess its effect on the P/O ratio. Assuming that the non-enzymic rate remains that observed in the absence of mitochondria (3.5 nmol of  $O_2$ /min per ml), the P/O ratio would be decreased by 9–25%, depending on the cyanide concentrations. The corrected P/O ratio at cyanide concentrations that maximally inhibit  $O_2$  uptake is then approx. 0.6. The inhibition of oxidation with the cytochrome *c*-linked substrate mixture ascorbate + *NNN'*-tetrameth-

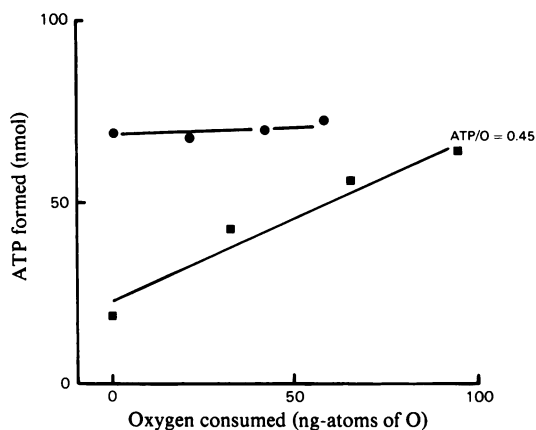


Fig. 3. Stoichiometry of ATP formed and  $O_2$  consumption by mung-bean mitochondria oxidizing 8.3 mM-succinate in the presence of 330  $\mu$ M-KCN and the presence and absence of oligomycin

Mitochondria were allowed to phosphorylate before the addition of the inhibitors, and sampling was not started until the inhibited rate had been linear for 1–2 min. ATP was determined by assaying the ATP formed via glucose 6-phosphate as described in the text. The basal reaction mixture was as described for Fig. 1 but also containing 0.8 mM-ADP.  $O_2$  consumption was measured with an oxygen electrode. The higher initial value for ATP in the presence of 2  $\mu$ g of oligomycin (●) than in its absence (■) is due to a longer period of phosphorylation before the addition of inhibitor and the start of the experiment.

ylene-*p*-phenylenediamine shows that the cytochrome oxidase is completely inhibited by the cyanide concentrations that give the plateau values of ADP/O ratio and P/O ratio observed in Figs. 1 and 2. The low rate of O<sub>2</sub> uptake seen with the ascorbate substrate mixture at high cyanide concentrations has been shown to be due to the reversed electron transport through phosphorylation site 2 to the branch point and oxidation via the cyanide-insensitive oxidase (Wilson, 1978*b*).

The phosphorylation observed in these experiments is oligomycin-sensitive. Fig. 3 shows that, with succinate as substrate and sufficient cyanide to inhibit maximally the cytochrome system, phosphorylation is related to the O<sub>2</sub> uptake with an ATP/O ratio of 0.45, whereas in the presence of oligomycin there is negligible phosphorylation. This experiment, conducted in the absence of the hexokinase trap system, also shows that phosphorylation can occur even if the ATP concentration is allowed to rise, increasing the phosphate potential.

Since the assay of phosphorylation used does not provide proof that the product of these reactions is ATP, it was decided to use a completely independent approach. Experiments were performed with the firefly luciferin + luciferase assay system directly coupled to the phosphorylation process, with AMP

present to exclude the possibility of adenylate kinase being the source of ATP. Fig. 4 shows the result of a typical experiment. When the substrate mixture of trimethylquinol + dithioerythritol and cyanide is added to the mitochondria there is an initial burst of light, indicative of phosphorylation. This phosphorylation is due to the activity of the cytochrome system, since in the presence of cyanide (but not in its absence) it rapidly ceases and the ATP concentration starts to decline rapidly. This decline in ATP concentration is followed by a steady increase, which continues for some time. This latter phase of ATP production can be inhibited by oligomycin, salicylhydroxamate (at the low concentrations required to inhibit the cyanide-insensitive oxidase) and by the uncoupler compound 1799 (results not shown). Similar results were obtained with menadione, with duroquinone, with higher concentrations of all the quinones reduced by borohydride and with succinate as the substrate.

#### Other energy-linked functions

ATP formation is a particularly labile function in the presence of cyanide, and additionally is not the only energy-linked function of mitochondria. Experiments were therefore conducted to study a variety of other energy-linked functions directly energized by

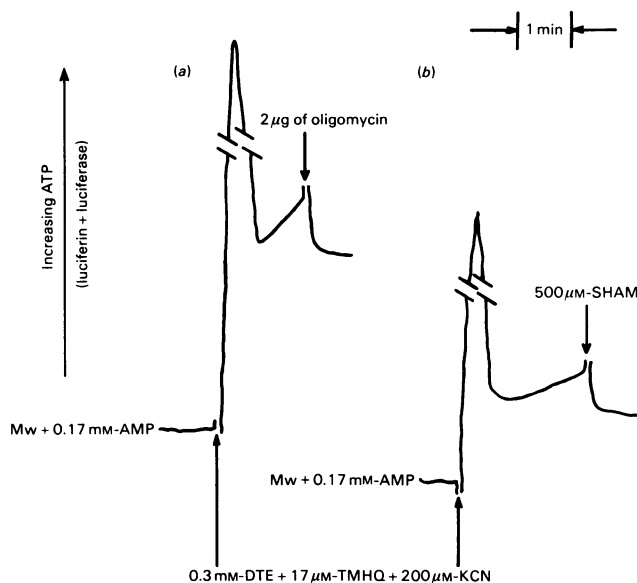


Fig. 4. Formation of ATP by washed mung-bean mitochondria (*Mw*) in the presence of cyanide assayed continuously by purified firefly luciferase + luciferin incorporated into the reaction medium as described in the text

The basal reaction medium was as described for Fig. 1 with the addition of 0.17 mM-AMP. The reaction was started by the simultaneous addition of trimethylquinol (TMHQ), dithioerythritol (DTE) and KCN as indicated. All other additions are shown [oligomycin, AMP and salicylhydroxamate (SHAM)]. ATP production is indicated by light-emission, shown by an upward deflexion of the trace. Measurements were made in a specially constructed spectrophotometer in the absence of any other light-source. (a) Effect of oligomycin; (b) effect of salicylhydroxamate.

substrate oxidations in the presence of cyanide where the ATPase and ATP were not involved.

#### Reversed electron transport through phosphorylation site 1

Reversed electron transport from the quinone level to  $\text{NAD}^+$  through phosphorylation site 1 can be used as an index of energy conservation. Observation of the redox state of NAD also serves to confirm if the quinone/substrate mixtures interact on the substrate side of site 1. Fig. 5(a) shows that the NAD of uncoupled mitochondria remains fully oxidized in the presence of the quinone/substrate mixture, cyanide and salicylhydroxamate, confirming that there is no interaction with the substrate side of site 1. Fig. 5(b) shows that in coupled mitochondria the NAD pool becomes reduced in the presence of the quinone/substrate mixture when  $\text{O}_2$  utilization is blocked by cyanide, salicylhydrox-

amate causing a partial oxidation. Use of endogenous ATP is excluded by the presence of oligomycin. If salicylhydroxamate is added first (Fig. 5c), then the effect of KCN on the redox state of the NAD pool is small.

#### $\text{Ca}^{2+}$ uptake in the presence of cyanide

$\text{Ca}^{2+}$  uptake is an energy-linked function of plant mitochondria similar to that in mammalian mitochondria except that the rates of uptake observed are very much lower (Russell & Wilson, 1978). Monitoring with the dye Arsenazo III demonstrates that  $\text{Ca}^{2+}$  uptake by mung-bean mitochondria can occur at concentrations of KCN sufficient to inhibit completely the cytochrome system with either succinate (Figs. 6a and 6b) or trimethylquinol + dithioerythritol (Fig. 6c) as substrate. In the presence of a low concentration of salicylhydrox-

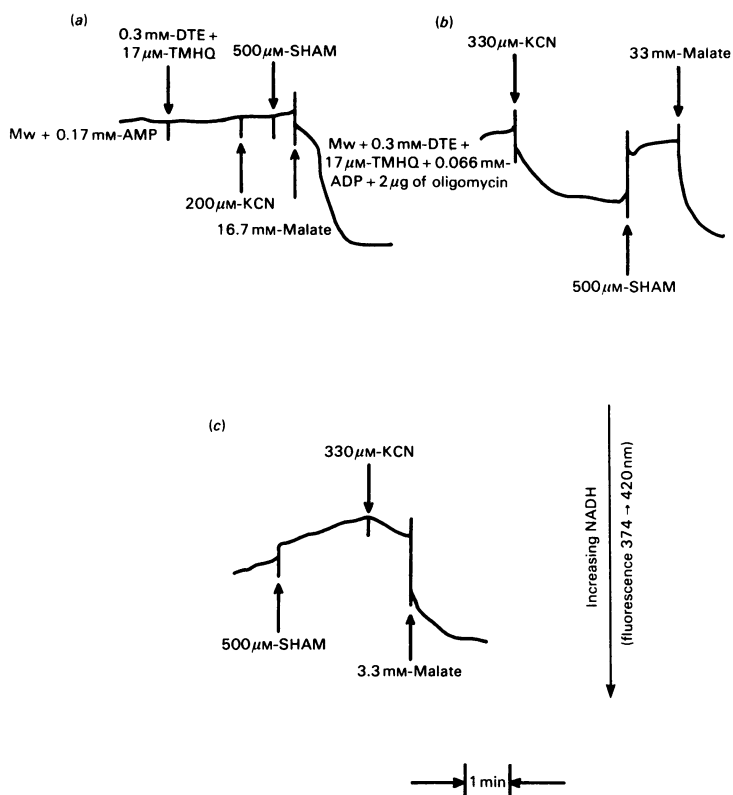


Fig. 5. Effects of cyanide on reversed electron transport from the quinone level to  $\text{NAD}^+$  in mung-bean mitochondria. For (b) and (c) mitochondria were preincubated for 2 min with 0.066 mM-ADP in the basal reaction mixture described for Fig. 1 to oxidize the NAD pool before the addition of the other reagents. Cyanide (KCN), salicylhydroxamate (SHAM), ADP, AMP and malate were added where indicated. For (a) mitochondria (Mw) were preincubated for 10 min with 7  $\mu\text{M}$ -compound 1799 instead of ADP, and 2  $\mu\text{g}$  of oligomycin was added before the start of the experiment. NADH concentrations were monitored by fluorescence at 374  $\rightarrow$  420 nm as described in the text. The substrate was dithioerythritol (DTE) + trimethylquinol (TMHQ). Reduction is indicated by a downwards deflexion of the trace.

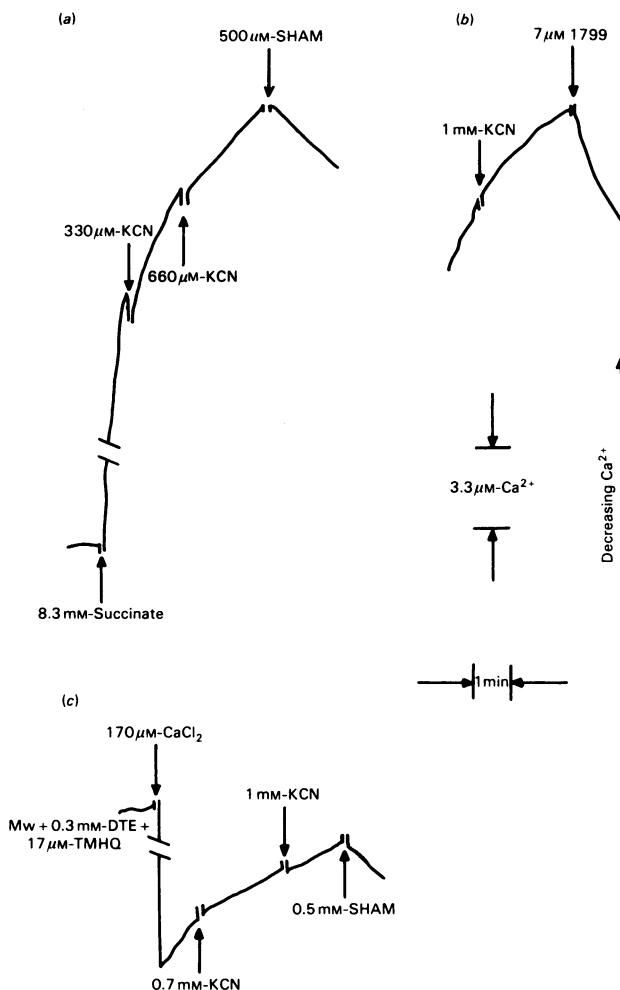


Fig. 6. Effects of cyanide and salicylhydroxamate on the uptake of  $\text{Ca}^{2+}$  by mung-bean mitochondria (*Mw*) measured by the Arsenazo III technique described in the text

The reaction medium was as described for Fig. 1 but also containing  $50 \mu\text{M}$ -Arsenazo III. For (a) and (b) the medium contained  $33 \mu\text{M}$ - $\text{CaCl}_2$  and  $8.3 \text{ mM}$ -succinate as substrate. Succinate was prepared from A.R.-grade succinic acid adjusted to pH 7.0 with A.R.-grade KOH. For (c) the substrate was trimethylquinol (TMHQ) + dithioerythritol (DTE), a different batch of mitochondria was used and the sensitivity was one-fifth of that for (a) and (b).  $\text{CaCl}_2$ , cyanide (KCN), salicylhydroxamate (SHAM) and compound 1799 were added where indicated.  $\text{Ca}^{2+}$ -uptake is indicated by an upwards movement of the trace.

amate or the uncoupler compound 1799,  $\text{Ca}^{2+}$  uptake is completely inhibited and efflux occurs.

#### Membrane potentials in the presence of cyanide

Membrane potentials can be measured either by the distribution of cations or by the use of dye probes. The latter technique offers the advantage that it provides a continuous read-out, unlike the cation distribution method, which indicates potential at the time of sampling. Dye probes have been shown to respond to membrane potential in plant

mitochondria, although it has only been possible to quantify the method by comparison with the cation-distribution methods (Howard, 1978). With the use of 8-anilinoanthralene-1-sulphonate as a probe, Figs. 7(a) and 7(b) show that the collapse in membrane potential reported by the dye is greater when cyanide is added in the presence of salicylhydroxamate than in its absence. The effect of the uncoupler compound 1799 on the resultant potential is therefore correspondingly greater in the absence of salicylhydroxamate than in its presence.

Fig. 7(c) confirms that the 8-anilino-naphthalene-1-sulphonate responds in the expected manner when a small pulse of ADP or oligomycin is added.

The result with 8-anilino-naphthalene-1-sulphonate indicates that a salicylhydroxamate-sensitive membrane potential is maintained in the presence of cyanide. However, the use of 8-anilino-naphthalene-1-sulphonate has been criticized on the basis that it may also report the state of the ATPase (Ferguson *et al.*, 1976). To confirm the above results, experiments have also been per-

formed with safranin as a probe to indicate membrane potential. Fig. 8 shows the results of an experiment with the use of safranin with succinate as the substrate in the absence (Fig. 8a) or presence of oligomycin (Fig. 8b). As observed with 8-anilino-naphthalene-1-sulphonate, in the presence of oligomycin (to prevent the utilization of endogenous ATP), salicylhydroxamate further decreases the indicated membrane potential in the presence of cyanide. No such decrease of the membrane potential occurs in the absence of oligomycin.

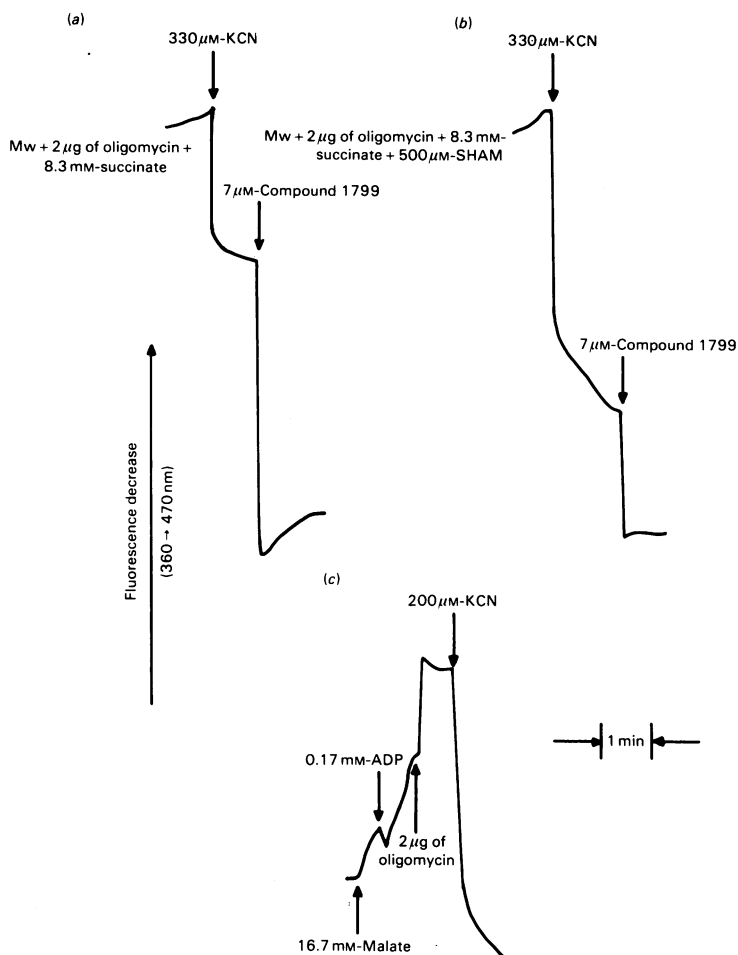


Fig. 7. Effect of cyanide and salicylhydroxamate on the fluorescence of 8-anilino-naphthalene-1-sulphonate with mung-bean mitochondria (*Mw*)

Fluorescence was measured at 360→470 nm as described in the text. For (a) mitochondria were suspended in the basal reaction medium used for Fig. 1 but also containing 33 μM-8-anilino-naphthalene-1-sulphonate, 2 μg of oligomycin and 8.3 mM-succinate. For (b) the starting medium also contained 500 μM-salicylhydroxamate (SHAM). For (c) succinate was omitted from the starting medium. Other additions of cyanide (KCN), ADP and compound 1799 are indicated on the traces. Energization is shown by an upward deflexion of the trace.



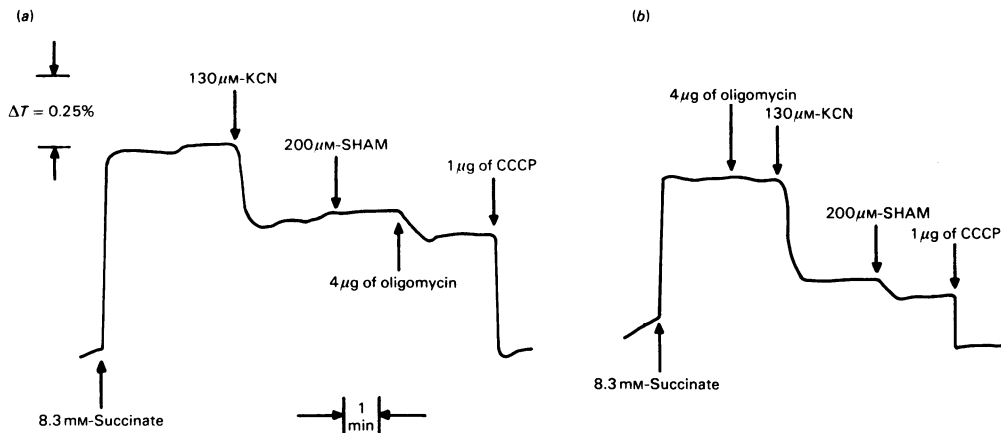


Fig. 8. *Effect of inhibitors on the absorption of safranine and mung-bean mitochondria measured at 524–544 nm* Mitochondria were suspended in the basal reaction medium used for Fig. 1 but also containing 5 μM-safranine. All other additions are shown. Energization is shown by an upwards deflexion of the trace. (a) Effect of adding cyanide (KCN) and salicylhydroxamate (SHAM) in the absence of oligomycin; (b) as (a) but oligomycin is added first. CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

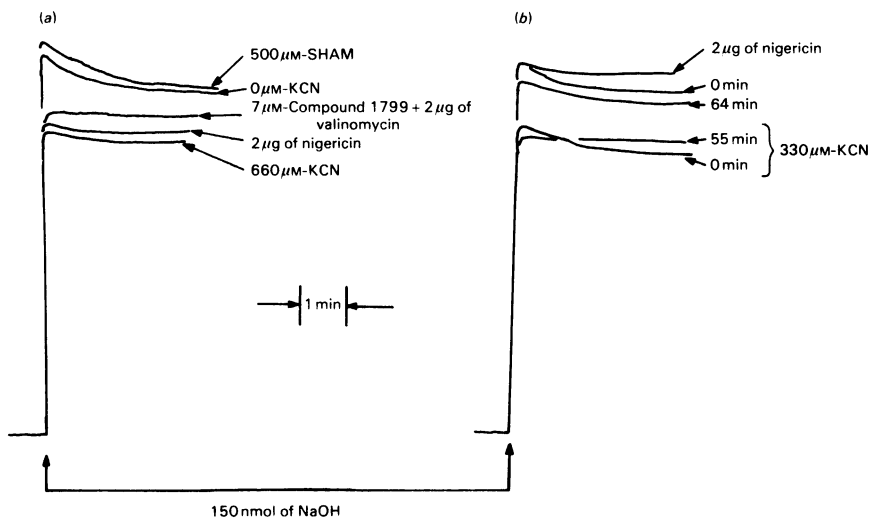


Fig. 9. *Time course of the pH changes during pulsed alkali titrations of mung-bean mitochondria at 20°C* The reaction medium was as described for Fig. 1 except that the phosphate concentration was 1.0 mM; 150 nmol of NaOH was added where indicated. All other reagents present for the individual traces are indicated [cyanide (KCN), salicylhydroxamate (SHAM)] For (a) NaOH was not added until the drift rate had become uniform after the addition of the mitochondria, KCN and any other reagents to the reaction medium. Normally this occurred within about 5 min. For (b) mitochondria were stored at 0°C until the experiment was started by adding them to the reaction medium and treating them as described under (a).

*Effect of cyanide on the permeability of the membrane to protons*

Since high concentrations of KCN apparently cause a partial uncoupling of the mitochondria, giving a low ADP/O ratio, it was decided to obtain

further evidence on this point. An index of uncoupling can be obtained from the ability of the compound in question to catalyse the movement of protons through the mitochondrial membrane. Thus the classical uncouplers lower the  $t_{\frac{1}{2}}$  for the decay of

a pH pulse added to plant mitochondria apparently by catalysing the movement of protons or  $\text{OH}^-$  ions through the membrane (Moore & Wilson, 1978). Fig. 9(a) shows that  $660\ \mu\text{M}$ -cyanide diminishes the response to an alkaline pulse, the slow decay attributable to the permeation of the membrane by protons (or  $\text{OH}^-$  ions) being accelerated in the presence of cyanide. A similar trace is obtained with nigericin, whereas a combination of the uncoupler compound 1799 and valinomycin completely abolishes the slow permeation process. Salicylhydroxamate, however, has no effect on the decay of a pH pulse. Under anaerobic conditions the  $t_{\frac{1}{2}}$  for this slow decay of the pH pulse was decreased to 30 s by  $660\ \mu\text{M}$ -cyanide, compared with 46 s recorded in the absence of inhibitor. The latter value, obtained with washed mitochondria, is comparable with the 54 s reported for purified turnip mitochondria under similar conditions (Moore & Wilson, 1978). A decrease in the  $t_{\frac{1}{2}}$  value can also be observed for antimycin A (S. B. Wilson, unpublished work). The effect of cyanide is also dependent on the age of the mitochondria relative to the time of completion of the isolation procedure. Fig. 9(b) shows that the response to a pH pulse is further diminished when the mitochondria have been stored before the addition of the inhibitor. These results confirm that high concentrations of cyanide have some uncoupler-like activity and limit the observation of energy conservation to freshly isolated mitochondria. The loss of the ability to conserve energy in the presence of cyanide is not accompanied by any change in the respiratory control ratio exhibited in the absence of the inhibitor.

## Discussion

The experiments reported in the present paper clearly show that energy can be conserved as ATP as a result of the activity of the cyanide-insensitive oxidase of mung-bean mitochondria. The assay of the ADP/O ratio after the deduction of the contribution of site 1 and the use of quinone substrates specifically exclude the involvement of phosphorylation site 1 either directly or via site-1-linked substrates produced by the primary oxidation process. Other sources of ATP such as adenylate kinase are excluded by the use of AMP in some experiments. There seems little doubt that the product measured was ATP since both the assay via glucose 6-phosphate and that with the highly specific firefly luciferase demonstrated that phosphorylation was occurring. The use of the inhibitors oligomycin and salicylhydroxamate indicates that the ATP produced is formed by the  $F_1$  ATPase and that the cyanide-insensitive oxidase is involved in the process. The observed ADP (ATP or P)/O ratio in these experiments of approx. 0.5 is very little less

than that observed for the operation of a single phosphorylation site in the absence of inhibitors in washed plant mitochondria (S. B. Wilson, unpublished work). Consequently it can be concluded that only a single phosphorylation site can be operating in the presence of cyanide in these experiments. This result confirms earlier conclusions (Wilson, 1970, 1978a) that there is only a single phosphorylation site associated with the cyanide-insensitive oxidase in plant mitochondria.

This present paper also shows that energy-linked functions that do not involve the ATPase, namely reversed electron transport,  $\text{Ca}^{2+}$  uptake and the maintenance of a membrane potential, can be supported by the operation of the cyanide-insensitive oxidase. In all these cases the energy-linked function was easily observable at comparatively high cyanide concentrations, where observations of the formation of ATP was difficult. This observation is in agreement with those reported elsewhere (Wilson & Bonner, 1970) showing that other energy-linked functions of plant mitochondria are also relatively resistant to uncouplers when driven by substrate oxidations rather than ATP. The absence of complete inhibition of these functions by salicylhydroxamate is attributable to the concentrations of this inhibitor being too low for complete inhibition of the alternative oxidase. The use of low concentrations of salicylhydroxamate, however, helps to eliminate possible non-specific side effects attributable to high concentrations of the inhibitor.

Since these conclusions are contrary to many of those reported in the literature, it is necessary to consider the reasons for some of the discrepancies. The observations of membrane potential reported by Moore (1978) and Moore *et al.* (1978a) failed to demonstrate any response of the membrane potential to the addition of salicylhydroxamate, albeit to antimycin A-inhibited mitochondria. However, their experiments were conducted with mitochondria either in 'State 3' or in 'State 4' in the absence of oligomycin. Under these conditions the mitochondria are able to utilize any ATP that may be present to support the membrane potential (Moore *et al.*, 1978b; Howard, 1978). As shown in Fig. 8(a), the absence of oligomycin prevents the salicylhydroxamate-induced decrease of membrane potential in cyanide-inhibited mitochondria. The absence of this response is then attributable to the ability of the ATP to support the membrane potential and not to the lack of energy conservation. Nevertheless cyanide appears to decrease the membrane potential, but in the absence of a suitable calibration for the dye-probe method and suitable pH measurements the magnitude of the protonmotive force under these conditions cannot be calculated.

In the experiments reported in the present paper use has been made of the technique of titration with

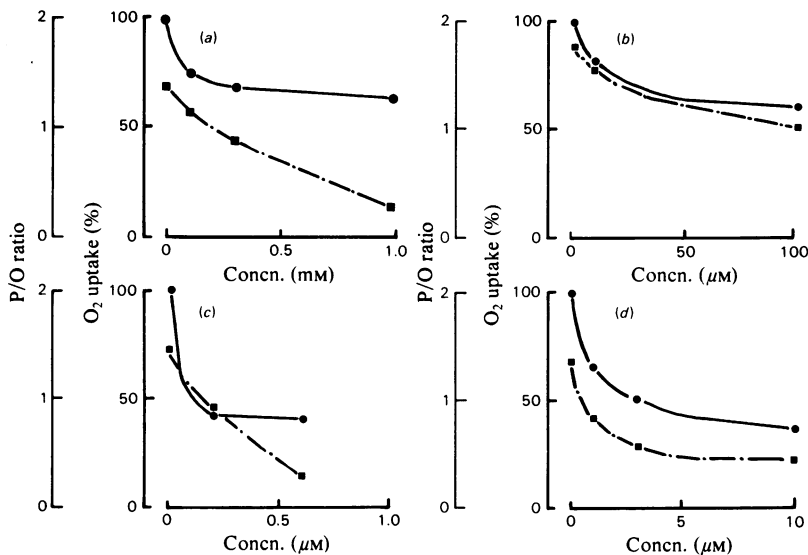


Fig. 10. Effect of the inhibitor concentration on the P/O ratios obtained with sweet-potato mitochondria supplied with succinate as substrate

Data are from Hackett *et al.* (1960). ●, O<sub>2</sub> uptake; ■, P/O ratio. Respiration is expressed as a percentage of the control (no inhibitor) rate. The P/O ratio was obtained by measuring the uptake of P<sub>i</sub>. The inhibitors used were azide (a), cyanide (b), antimycin A (c) and 2-heptyl-4-hydroxyquinoline *N*-oxide (d). Measurements were made manometrically in a reaction mixture containing 60 μmol of succinate, 1325 μmol of sucrose, 60 μmol of glucose, 20 μmol of MgSO<sub>4</sub>, 0.2 μmol of thiamin pyrophosphate, 1.2 μmol of NAD<sup>+</sup>, 80 μmol of phosphate buffer (pH 7.0), 12 μmol of Tris buffer (pH 7.0), 3 μmol of ADP, 1 mg of hexokinase, 2 mg of mitochondrial protein in a final volume of 3 ml. The incubation time was 60 min at 30°C.

the inhibitor to demonstrate effects at the cyanide concentration that just inhibits the cytochrome system. Under these conditions it is possible to demonstrate that cyanide apparently uncouples the energy-conservation system without altering the O<sub>2</sub> uptake. Such an observation, confirmed by the observations of proton permeability and the continuing decline in respiratory-control ratio as the cyanide concentration is increased, explains why energy conservation is often not observed. Such an uncoupling action with plant mitochondria has been reported elsewhere (Ikuma & Bonner, 1967*b*). Many studies of energy conservation in the presence of cyanide are performed at a single high inhibitor concentration, where the uncoupling action is more serious, particularly with aged mitochondria, in agreement with the observation that many of the experiments reported in the present paper show energy conservation only if carried out with very fresh mitochondria. If the data in the classical paper of Hackett *et al.* (1960) are plotted on the same basis as that used for the present study (Fig. 10), it can be seen that phosphorylation probably occurred at limiting concentrations of all the inhibitors employed, with a subsequent fall in P/O ratio at high inhibitor concentrations. This observation, con-

firms that obtained for the present paper, is, however, contrary to the conclusion reached by Hackett *et al.* (1960). The data of Hackett *et al.* (1960) are also compatible with the conclusion that a single phosphorylation site is associated with the cyanide-insensitive oxidase.

Although the present paper deals only with the cyanide-insensitive oxidase of plant mitochondria, similar oxidases occur widely (Henry & Nyns, 1975), and lack of energy conservation has been reported for some of these other systems, e.g. by Akimoto *et al.* (1979). It is tempting to speculate that some at least of these other alternative oxidase systems may also be capable of conserving energy.

Although the pathway for the plant mitochondrial alternative cyanide-insensitive oxidase is not known, any proposals must accommodate the observations that in the presence of cyanide a single phosphorylation site is active in the ubiquinone-to-O<sub>2</sub> segment of the respiratory chain.

#### Note added in proof (received 6 March 1980)

Since this paper was written, a paper by Dousière *et al.* (1979) has provided evidence that the

cyanide-insensitive oxidase of *Paramecium* can also be capable of energy conservation.

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