The Influence of Osmolarity on the Reduction of Exogenous Cytochrome *c* and Permeability of the Inner Membrane of Jerusalem Artichoke Mitochondria

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The stimulation of succinate–cytochrome *c* reductase in Jerusalem artichoke mitochondria by lowering osmolarity was found to be associated with conformational changes in the inner membrane rather than with rupture of the outer membrane. This conclusion is based on the following evidence. (1) When the activation of succinate dehydrogenase was measured by using either $K_3Fe(CN)_6$ or exogenous cytochrome *c* as an electron acceptor, electron flow to cytochrome *c* was always 7% of that to $K_3Fe(CN)_6$ throughout the activation process. (2) The rate of exogenous cytochrome *c* reduction by succinate and NADH was directly related to the maximum rate of electron flow as determined by oxygen utilization. These two observations are not consistent with the low rate of succinate–cytochrome *c* reductase being limited by a permeability barrier at the outer membrane. (3) In addition to stimulating the succinate–cytochrome *c* reductase, lowering the osmolarity caused simultaneous changes in the permeability of the inner membrane to ferricyanide and NADH. The data show that lowering the osmolarity results in progressive changes in the permeability of the inner membrane. The first change detected was an increased permeability to $K_3Fe(CN)_6$, then a simultaneous increase in accessibility of the respiratory chain to exogenous cytochrome *c* and an increased permeability to NADH, followed finally by rupture as measured by the release of malate dehydrogenase.

Relatively few investigations of the permeability of the outer mitochondrial membrane have been conducted. These have generally been limited to the assessment of the penetration of very large molecules such as carboxypolyglucose and inulin (Werkheiser & Bartley, 1957) or low-molecular-weight compounds such as inorganic ions, tricarboxylic acid-cycle intermediates and nucleotides (Bücher & Klingenberg, 1958; Harris & van Dam, 1968). The results of these studies indicate that the outer membrane is freely permeable to both charged and uncharged compounds with molecular weights of less than 10000 (Pfaff *et al.*, 1968).

Considerable confusion exists in the literature about the permeability of the outer membrane to cytochrome *c*, whose molecular weight of 12500 approaches the estimated limit for permeability. Observations that endogenous cytochrome *c* could not be removed until the outer membrane was disrupted (Ernster & Kuylenskierna, 1970) suggest that the outer membrane represented a permeability barrier to cytochrome *c*. However, it has been shown that exogenous cytochrome *c* can catalyse the aerobic oxidation of exogenous NADH via a rotenone- and antimycin A-insensitive pathway at normal osmolarity

(Sottocasa *et al.*, 1967). This phenomenon requires the penetration of the outer membrane by cytochrome *c*, in either the oxidized or the reduced form, to gain access to the cytochrome oxidase located on the inner membrane. Ernster & Kuylenskierna (1970) suggest that the release of cytochrome *c* that accompanies the lowering of osmolarity is not entirely due to a change in the permeability of the outer membrane, but may result from the displacement of loosely bound cytochrome *c* from the inner membrane which swells, rupturing the outer membrane under these conditions. Wojtczak & Zaluska (1969) showed that digitonin caused the parallel stimulation of succinate–cytochrome *c* reductase and the release of adenylate kinase, and thus concluded that cytochrome *c* was unable to cross the outer membrane in intact mitochondria. Other observations by Cohen & Fridovich (1971) and Wattiaux-DeConinck & Wattiaux (1971) on the latency of hepatic sulphite oxidase also led to the conclusion that the outer membrane of liver mitochondria is impermeable to cytochrome *c*. Thus it is now generally accepted that the outer membrane of rat liver mitochondria is probably impermeable to cytochrome *c*, whereas in other types of mitochondria, notably ox heart

mitochondria, it appears that cytochrome *c* might readily traverse the outer membrane (Fessenden-Raden & Racker, 1971).

Even fewer investigations of this nature have been conducted on plant mitochondria. The outer membrane of plant mitochondria, on examination with the electron microscope, has been described as pitted (Parsons *et al.*, 1965). The isolation procedures used for plant mitochondria tend to be more vigorous, owing to the requirements for rupture of the tough plant cell wall; hence one might expect, as a result of such methods, that the membranes of isolated plant organelles could suffer damage exhibited by a 'leakiness' or increased permeability. However, Douce *et al.* (1972) have stated that the outer membrane of mung-bean mitochondria is impermeable to cytochrome *c* and that this impermeability is a very sensitive measure of the extent of damage to the mitochondria. This parameter has been exploited to investigate the influence of various reagents on the permeability of the outer membrane (Bonner *et al.*, 1973).

While using this method to assess the damage in our mitochondrial preparations, observations were made which indicate that the osmotic treatment which was believed to alter the permeability of the outer membrane was also altering the permeability of the inner membrane.

Materials and Methods

Chemicals

NADH and cytochrome *c* (horse heart) were obtained from C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany; antimycin A was from Calbiochem Ltd., London W.1, U.K.; *cis*-oxaloacetic acid, bovine serum albumin (fraction V) and ADP were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; sucrose, EDTA, KCN, $K_3Fe(CN)_6$, $MgSO_4$ and succinic acid were from BDH Chemicals Ltd., Poole, Dorset, U.K., and Mops [3-(*N*-morpholino)propanesulphonic acid], Tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid], Tris buffers and KH_2PO_4 were from Hopkin and Williams, Chadwell Heath, Essex, U.K.

Isolation of mitochondria

Mitochondria were isolated from 200 g of tubers of the Jerusalem artichoke. The peeled tubers were grated by hand in 300 ml of an ice-cold solution containing 0.5 M-sucrose, 10 mM-Mops, 1 mM-EDTA and 0.1% (w/v) bovine serum albumin at pH 7.8. The homogenate was then filtered through three layers of muslin and centrifuged for 2 min at 48000 g in a Sorvall RC2-B centrifuge, rotor no.

SS-34. The pellets thus obtained were combined and resuspended in 50 ml of a washing medium containing 0.4 M-sucrose, 5 mM-Tes and 0.1% bovine serum albumin at pH 7.2 and centrifuged for 5 min at 480 g. The supernatant was then centrifuged for 15 min at 9750 g. The final pellet was resuspended in a small amount of washing medium to give the desired protein concentration of 20–30 mg/ml. All steps of the isolation procedure were carried out at 0–5°C.

Oxygen consumption

O₂ uptake by mitochondrial suspensions was measured in a Rank O₂-electrode assembly (Rank Bros., Bottisham, Cambridge, U.K.) at 25°C. The reaction medium consisted of 0.5 ml of 0.4 M-sucrose, 5 mM- $MgSO_4$, 1 mM-ADP, 2 mM-potassium phosphate, 4 mM-Tes, at pH 7.2, plus 10 mM-potassium succinate or 600 μ M-NADH.

Osmotic treatment of mitochondria

In order to subject mitochondria to varying degrees of osmotic shock, a 12.5 μ l sample of mitochondria was added to 5 ml of a series of sucrose solutions ranging in concentration from 0 to 0.4 M and containing 200 μ M-ATP. In the assay for malate dehydrogenase (EC 1.1.1.37) activation the mitochondrial suspension was diluted 10-fold with 0.4 M-sucrose before use. The mixture was left for 6 min and then 2.5 ml was used for the assays described below. When the release of malate dehydrogenase was to be measured, the mixture was centrifuged for 10 min at 10000 g before the assay, and 2.5 ml of the supernatant was used.

Enzyme assays

Succinate- and NADH-cytochrome *c* reductase. The activities of succinate- and NADH-cytochrome *c* reductase of mitochondria were measured by following the reduction of cytochrome *c* in an Aminco DW-2 dual-wavelength spectrophotometer at 550 nm, with the reference wavelength at 540 nm. The assays were done in 2.5 ml of a medium which contained sucrose (0–0.4 M), 4 mM-Tes, 2 mM-potassium phosphate, 0.5 mM-KCN, 0.05 mM-cytochrome *c*, 5 mM- $MgSO_4$ and 1 mM-ADP at pH 7.2, plus 10 mM-potassium succinate or 600 μ M-NADH. The antimycin A-insensitive activity was determined in the presence of 500 ng of antimycin A/mg of mitochondrial protein, added before initiation of the assay.

Succinate-ferricyanide reductase. The activity of succinate-ferricyanide reductase was measured by following the reduction of ferricyanide at 420 nm, with the reference wavelength at 490 nm. The assay

medium was identical with that used for succinate-cytochrome *c* reductase, except that cytochrome *c* was replaced by 0.5 mM-K₃Fe(CN)₆. Antimycin A was included where indicated. All rates were corrected by subtracting endogenous rates of ferricyanide reduction.

Malate dehydrogenase. The activity of malate dehydrogenase was measured with a Perkin-Elmer fluorescence spectrophotometer, model MPF-3, by following the decrease in fluorescence of NADH during its oxidation. The nicotinamide-adenine dinucleotide fluorescence was excited at 340 nm and measured at 460 nm. The assay medium was the same as those described above except that in place of cytochrome *c* or K₃Fe(CN)₆, 400 μ M-potassium oxaloacetate and 100 μ M-NADH were added. The KCN concentration was increased to 1 mM in this assay.

Protein

The protein content of suspensions of mitochondria was measured by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin (fraction V) as the standard.

Results and Discussion

Douce *et al.* (1972) claimed that since the outer membrane cannot be penetrated by cytochrome *c*, an assay of succinate-cytochrome *c* reductase activity was a means of assessing the intactness of this membrane. However, in mitochondria prepared by conventional techniques the succinate dehydro-

genase (EC 1.3.99.1) is often inhibited (Kearney, 1957) and requires activation before the maximal rates of succinate oxidation can be observed. Activation can be brought about by incubating the mitochondria in the presence of either succinate (Kearney, 1957; Nordenbrand & Ernster, 1971) or ATP (Wiskich & Bonner, 1963). In the present investigation the slow activation of succinate dehydrogenase resulted in interesting observations during attempts to use the succinate-cytochrome *c* reductase assay to measure the permeability of the outer membrane to exogenous cytochrome *c*. Data in Table 1 show the activation of succinate dehydrogenase in the presence of a number of electron acceptors.

The reduction of ferricyanide was inhibited 90% by antimycin A, indicating that this electron acceptor did not penetrate the inner membrane and thus could only accept electrons from endogenous cytochrome *c* located on the outer face of the inner membrane. Therefore it can be assumed that the rate of ferricyanide reduction measures the maximum rate of electron transport to the endogenous cytochrome *c*. Since there is no rate-limiting step in the electron-transport system between cytochrome *c* and O₂ in the presence of excess of ADP, the rates of ferricyanide reduction and oxygen utilization were expected to be similar. This was true after approx. 60 s; however, during the early phase the rate of O₂ uptake was much slower. Part of this lag was due to the response time of the O₂ electrode, which was fitted with a standard Yellow Springs Instrument electrode membrane. A similar lag in the rate of

Table 1. Rate of activation of electron flow from succinate to various electron acceptors

The assays were carried out as described in the Materials and Methods section, except that ATP was omitted during the osmotic treatment of the mitochondria. All the assays were carried out by using 1 mg of mitochondrial protein, except for succinate-cytochrome *c* reductase in water; this reaction was very rapid and only 0.2 mg of protein was used. The values, quoted as ng electron equivalents, are the rates of electron flow to the various acceptors. For cytochrome *c* and ferricyanide this is identical with the rate of reduction of the acceptor expressed as nmol, whereas for O₂ the rate of electron flow was obtained by multiplying the μ mol of O₂ used by a factor of 4.

Acceptor	Average rate of electron transport for the given time-interval (ng electron equivalents · min ⁻¹ · mg of protein ⁻¹)			
	0-5 s	20-25 s	90-95 s	120-125 s
O ₂	—	37.1	196	266
Cytochrome <i>c</i> (in 0.4 M-sucrose)	4.4	6.2	14.2	16.5
K ₃ Fe(CN) ₆ (in 0.4 M-sucrose)	59.8	78	197	266
Cytochrome <i>c</i> (in water)	123	149	195	195
K ₃ Fe(CN) ₆ (in water)	132	155	223	223
Rate of cytochrome <i>c</i> reduction as % of K ₃ Fe(CN) ₆ reduction (in 0.4 M-sucrose)	7.4	7.9	7.2	6.2

Fig. 1. Comparison of the initial rates of NADH and succinate oxidation with either cytochrome *c* or O_2 as the terminal electron acceptor

The rate of O_2 uptake was measured polarographically and the rate of cytochrome *c* reduction was measured spectrophotometrically in the presence of 0.5 mM-KCN as described in the Materials and Methods section. The succinate-cytochrome *c* reductase was completely sensitive to antimycin A whereas the NADH-cytochrome *c* reductase was only 80% inhibited by antimycin A. The antimycin A-sensitive component of the NADH-cytochrome *c* reductase plotted in this Figure was obtained by subtracting the antimycin A-resistant rate, obtained in the presence of 500 ng of antimycin A/mg of mitochondrial protein, from the total rate. ●, Antimycin A-sensitive NADH-cytochrome *c* reductase; ▲, succinate-cytochrome *c* reductase; ○, NADH oxidase; △, succinate oxidase.

O_2 uptake was observed when NADH was used as the substrate (Fig. 1). It is therefore concluded that of these assays the rate of ferricyanide reduction was the best measure of the potential ability of succinate dehydrogenase to donate electrons to the respiratory chain.

The rate of reduction of cytochrome *c* was very much lower than the rate of reduction of ferricyanide. This low rate has been considered to be due to the inability of exogenous cytochrome *c* to cross the outer membrane to reach the respiratory chain (Douce *et al.*, 1972). On the other hand, it is

also possible that cytochrome *c* crosses the outer membrane but is unable to react with the appropriate donor in the chain because of its orientation within the inner membrane. Although the rate of cytochrome *c* reduction was low it did reflect the activation of the succinate dehydrogenase. A constant proportion of the total electron flux (as measured by the reduction of ferricyanide) reached cytochrome *c*, as can be seen when the rate of cytochrome reduction is expressed as a percentage of the rate of ferricyanide reduction. This observation is inconsistent with the view that the rate-limiting factor of cytochrome *c* reduction is the rate of its penetration through the outer membrane. A linear rate of cytochrome *c* reduction would be expected unless the permeability of the outer membrane was continuously changing. If the permeability of the outer membrane was not rate-limiting and the low rate of cytochrome reduction was due to its ability to interact with only a restricted number of electron-transport chains, then one would expect that the rate of reduction would reflect the rate of electron flux along the chain. The data presented in Table 1 are consistent with this alternative view, and show that exogenous cytochrome *c* can only interact with 7–8% of the electron-transport chains. When the mitochondria were suspended in water instead of 0.4 M-sucrose, there was a dramatic increase in the initial rate of cytochrome *c* reduction, which now equalled the rate of ferricyanide reduction. This stimulation could result from the osmotic damage to the membranes causing either the loss of a permeability barrier or the changing organization of the inner membrane, unmasking the site at which exogenous cytochrome *c* can accept electrons. The stimulation of the rate of ferricyanide reduction that was observed under hypo-osmotic conditions was unexpected and resulted from a partial activation of the succinate dehydrogenase rather than from an increased permeability to the acceptor. The activation of the succinate dehydrogenase in intact mitochondria by pretreatment with ATP resulted in a similar increase in the activity of succinate-ferricyanide reductase.

The view that the reduction of exogenous cytochrome *c* was limited by its inability to interact with the physiological donor in the inner membrane rather than the permeability of the outer membrane was further strengthened by the observation that the initial rate of the antimycin A-sensitive component of the NADH-cytochrome *c* reductase was much more rapid than the succinate-cytochrome *c* reductase (Fig. 1). Since in both assays the exogenous cytochrome *c* interacts at the same position in the electron-transport chain, the rate of succinate-cytochrome *c* reductase could not have been limited by the rate at which cytochrome *c* crossed the outer membrane. The rate of cytochrome *c* reduction is

apparently related to the total rate of electron transport, as measured in these assays by O_2 consumption. The data in Fig. 1 have been plotted to show this relationship. The rates of O_2 uptake and cytochrome *c* reduction are parallel for each donor and the relationship between the two rates shows that approx. 5% of the respiratory chains can donate electrons to exogenous cytochrome *c*.

A similar conclusion can also be drawn from data presented by Douce *et al.* (1972): it appears from their Figs. 5 and 6 that under iso-osmotic conditions the rate of succinate-cytochrome *c*

reductase was $6 \text{ nmol of cytochrome } c \text{ reduced} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$, whereas the antimycin A-sensitive component of the NADH-cytochrome *c* reductase was $9 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$; thus it seems that a permeability factor was not limiting the velocity of the succinate-cytochrome *c* reductase. These rates obtained under iso-osmotic conditions also bear a relationship to the maximal rates obtained under extremely hypo-osmotic conditions, which were 200 and $300 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$ for succinate-cytochrome *c* reductase and NADH-cytochrome *c* reductase respectively.

Fig. 2. Effect of osmolarity on succinate-cytochrome *c* reductase, antimycin A-insensitive succinate-ferricyanide reductase and malate dehydrogenase

The 6 min osmotic treatment and the enzyme assays were carried out as described in the Materials and Methods section. Mitochondrial protein was added as follows: Δ , succinate-cytochrome *c* reductase, 0.13 mg; $\circ$, antimycin A-insensitive succinate-ferricyanide reductase, 0.26 mg; $\square$, malate dehydrogenase, 0.3 mg. The succinate-ferricyanide reductase and succinate-cytochrome *c* reductase activities are plotted on the same axis.

Since it appeared that the permeability of the outer membrane is not a rate-limiting factor, an investigation was made to correlate the stimulation of succinate-cytochrome *c* reductase with other changes associated with the organization of the inner membrane. The mitochondria were preincubated in 200 μ M-ATP to activate the succinate dehydrogenase before the assay. Two parameters related to the integrity of the inner membrane were measured: the antimycin A-insensitive succinate-ferricyanide reductase, which gives a measure of the ability of ferricyanide to cross the inner membrane and interact directly with the flavoprotein of the succinate dehydrogenase, and the oxaloacetate-dependent oxidation of exogenous NADH, which measures the ability of NADH to cross the inner membrane and reach the malate dehydrogenase situated in the matrix (Douce & Bonner, 1972).

Fig. 2 shows that lowering the osmolarity enhanced the activity of both malate dehydrogenase, by in-

Fig. 3. Effect of osmolarity on the 'activation' and release of malate dehydrogenase

The 6 min osmotic treatment and the enzyme assays were carried out as described in the Materials and Methods section. In the 'activation' assay the malate dehydrogenase was measured in the complete mitochondrial preparation, thus measuring the total malate dehydrogenase accessible to exogenous NADH. To measure the release of the malate dehydrogenase the assay was conducted on the supernatant only after the membranes had been removed by centrifugation, and measures the ability of the enzyme to diffuse out of the matrix of the mitochondrion. Amounts of mitochondrial protein used were as follows: 'activation' assay (●), 0.034 mg; release assay (○), 0.27 mg.

creasing the ability of NADH to permeate the inner membrane, and succinate-cytochrome *c* reductase in a very similar manner. The permeability of the inner membrane to ferricyanide, as measured by the antimycin A-resistant succinate-ferricyanide reductase, proved to be even more sensitive to changes in osmolarity. These results therefore confirm that changes in the permeability of the inner membrane occur simultaneously with the activation of the succinate-cytochrome *c* reductase. The main activation occurred when the osmolarity was lowered to 100 mosm, which is considerably higher than that at which Douce *et al.* (1972) reported damage to the inner membrane. They, however, used the release of malate dehydrogenase as their criterion for permeability changes in the inner membrane. The results in Fig. 3 confirm that the 'activation' of malate dehydrogenase was more sensitive to changes in osmolarity than was the release of the malate dehydrogenase to the medium.

In Jerusalem artichoke mitochondria osmotic changes produce a fairly continuous change in the permeability of the inner membrane. This can be detected in its earliest stages by an increased penetration of ferricyanide, followed by an increased permeability to exogenous NADH and an increased ability of exogenous cytochrome *c* to interact with the respiratory chain. The final stage is detected by the release of malate dehydrogenase resulting from the rupture of the inner membrane.

Bonner *et al.* (1973), by using the activation of succinate-cytochrome *c* reductase as an assay for the integrity of the outer membrane, have reported that the presence of EDTA in the suspending medium makes the outer membrane more susceptible to osmotic damage. We have repeated these experiments and confirm that EDTA does increase the osmotic damage. However, the effect is not restricted to succinate-cytochrome *c* reductase but is also observed on the activity of the malate dehydrogenase (Fig. 4). This indicates that the locus of EDTA action is more likely to be the inner rather than the outer membrane. EDTA is known to affect the permeability of the inner membrane of animal mitochondria (Settlemyre *et al.*, 1968).

Thus in Jerusalem artichoke mitochondria, succinate-cytochrome *c* reductase does not necessarily depend on the integrity of the outer membrane, since activation occurs as a result of changes in the inner membrane that may allow exogenous cytochrome *c* to interact more readily with the respiratory chain. Lowering the osmolarity causes a progressive change in permeability of the inner membrane. We did not see a discrete rupture of the membrane that results in the simultaneous change in permeability to both NADH and malate dehydrogenase as reported by Douce *et al.* (1973) in mung-bean (*Phaseolus aureus*) mitochondria.

Fig. 4. Effect of EDTA on the increase in the rate of succinate-cytochrome *c* reductase and malate dehydrogenase induced by lowering the osmolarity

The 6 min osmotic treatment and the enzyme assays were carried out as described in the Materials and Methods section. The assay for malate dehydrogenase was identical with that described for the 'activation' assay in the legend to Fig. 3. EDTA (170 μM) was added to the sucrose solution before the beginning of the osmotic treatment. Mitochondrial protein was added as follows: succinate-cytochrome *c* reductase, ○ (control) or ● (plus 170 μM-EDTA), 0.137 mg; malate dehydrogenase, □ (control) or ■ (plus 170 μM-EDTA), 0.03 mg.

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