The reversible Ca^{2+} -induced permeabilization of rat liver mitochondria

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Rat liver mitochondria became permeabilized to sucrose according to an apparent first-order process after accumulating 35 nmol of Ca^{2+} /mg of protein in the presence of 2.5 mM- P_i , but not in its absence. A fraction (24–32%) of the internal space remains sucrose-inaccessible. The rate constant for permeabilization to sucrose decreases slightly when the pH is decreased from 7.5 to 6.5, whereas the rate of inner-membrane potential ($\Delta\psi$) dissipation is markedly increased, which indicates that H^+ permeation precedes sucrose permeation. Permeabilization does not release mitochondrial proteins. [^{14}C]Sucrose appears to enter permeabilized mitochondria instantaneously. Chelation of Ca^{2+} with EGTA restores $\Delta\psi$ and entraps sucrose in the matrix space. With 20 mM-sucrose at the instant of resealing, about 21 nmol of sucrose/mg of protein becomes entrapped. The amount of sucrose entrapped is proportional to the degree of permeabilization. Entrapped sucrose is not removed by dilution of the mitochondrial suspension. Resealed mitochondria washed three times retain about 74% of the entrapped sucrose. In the presence of Ruthenium Red and Ca^{2+} buffers permeabilized mitochondria reseal only partially with free $[\text{Ca}^{2+}] > 3 \mu\text{M}$. [^{14}C]Sucrose enters partially resealed mitochondria continuously with time, despite maintenance of $\Delta\psi$, in accordance with continued interconversion of permeable and impermeable forms. Kinetic analyses of [^{14}C]sucrose entry indicate two Ca^{2+} -sensitive reactions in permeabilization. This conclusion is supported by the biphasic time courses of resealing and repolarization of permeabilized mitochondria and the acute dependence of the rapid repolarization on the free $[\text{Ca}^{2+}]$. A hypothetical model of permeabilization and resealing is suggested and the potential of the procedure for matrix entrapment of substances is discussed.

INTRODUCTION

It is well known that the excessive accumulation of Ca^{2+} by liver mitochondria (> 50 nmol of Ca^{2+} /mg of protein) is deleterious, leading to dissipation of $\Delta\psi$ and losses of intramitochondrial Mg^{2+} , K^+ , adenine nucleotides and preaccumulated Ca^{2+} [for reviews, see Nicholls & Akerman (1982) and Crompton (1985)]. These effects are promoted by P_i (Wolkowicz & McMillin-Wood, 1980; Beatrice *et al.*, 1980) and a number of other agents including phosphoenolpyruvate (Chudapongse, 1976), atracylate (Asimakis & Sordahl, 1977), oxaloacetate and acetoacetate (Fiskum & Lehninger, 1979) and organic hydroperoxides (Lotscher *et al.*, 1980a). Conversely, other substances protect against the Ca^{2+} -induced destabilization, e.g. ADP and ATP, Mg^{2+} , oligomycin and β -hydroxybutyrate. The opposing actions of oxaloacetate or acetoacetate and β -hydroxybutyrate are explicable by a need for reduced nicotinamide nucleotides for maintenance of $\Delta\psi$ in the presence of high matrix Ca^{2+} (Nicholls & Brand, 1980), which in turn may reflect a requirement for GSH (Beatrice *et al.*, 1984).

There is abundant evidence therefore that excessive matrix Ca^{2+} , particularly in the presence of promoters, induces permeability of the inner membrane to H^+ and small molecules. Studies of this phenomenon are important for two reasons. Firstly, if permeabilization occurred in a reversible manner, then the phenomenon may provide a means of preparing mitochondria with a

matrix space of defined solute composition, of great practical utility in transport studies. In contrast with permeabilization, the question of whether permeabilized mitochondria may be 'resealed' has received scant attention (see the Discussion section). Secondly, there is gathering evidence that excess Ca^{2+} accumulation by a variety of cell types, occurring, for example, during post-ischaemic reoxygenation, severely interrupts mitochondria energy transduction (for a review, see Trump & Berezesky, 1985); the consequent impairment of ATP production in turn may compromise active Ca^{2+} expulsion from the cell, so that the condition becomes irreversible, leading to cell death. There is a clear need, therefore, to identify and characterize the mitochondrial lesion arising under such pathological conditions.

The present study investigates Ca^{2+} -induced permeabilization of rat liver mitochondria and, in particular, whether permeabilized mitochondria may be resealed. The study reveals novel aspects of the mechanism of permeabilization in addition to establishing an entrapment procedure of potential utility.

METHODS

Mitochondrial preparation

Mitochondria were prepared from livers of female Sprague-Dawley rats (250–300 g body wt.) as described previously (Goldstone & Crompton, 1982). The mito-

Abbreviations used: $\Delta\psi$, mitochondrial inner-membrane potential; TPP^+ , tetraphenylphosphonium ion; HEDTA, *N*-(2-hydroxyethyl)ethylene-diamine-*NN'*-tri-acetic acid.

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chondria were suspended finally in a medium containing 210 mM-mannitol, 70 mM-sucrose and 10 mM-Tris/HCl, pH 7.4. Mitochondrial protein was determined by a modified biuret procedure (Kroger & Klingenberg, 1966). In some experiments (Table 1 below) mitochondria were isolated in the same manner from perfused rat liver. The livers were perfused for 20 min with Krebs-Henseleit bicarbonate buffer containing 10 mM-Tris/lactate as described previously (Goldstone *et al.*, 1983). Unless otherwise stated, the mitochondria used were from non-perfused livers.

Mitochondrial incubation conditions

(a) Standard reaction conditions. Mitochondria (1–10 mg of protein/ml) were preincubated for 5 min at 25 °C in standard medium containing 120 mM-KCl, 10 mM-Tris/Hepes, 2.5 mM-phosphate (potassium salt) and rotenone (1 µg/mg of protein); the final pH was 7.0 unless otherwise stated. Under standard permeabilization conditions, 3 mM-succinate (potassium salt) was added and, 1 min later, sufficient CaCl_2 was introduced to give a total (added plus endogenous) of 35 nmol of Ca^{2+} /mg of protein. The mitochondria were subsequently 're-sealed' (see Figs. 3, 4, 6–8 and Table 1 below) by addition of either 2 mM-EGTA or Ca^{2+} buffers (below) 7–9 min after CaCl_2 .

(b) Use of radioisotopes. [^{14}C]Sucrose and either [6,6'(n)- ^3H]sucrose or $^3\text{H}_2\text{O}$ (approx. 0.5 µCi/ml) were added as stated in the Figure legends. Samples of the incubation mixture (containing 1–2 mg of protein) were centrifuged in an Eppendorf bench centrifuge for 2 min. The ^{14}C and ^3H contents of the pellets and supernatants were used to calculate either the sucrose-inaccessible matrix space ($^3\text{H}_2\text{O}$ minus [^{14}C]sucrose) or the amount of [^{14}C]sucrose in excess of [^3H]sucrose in the mitochondria ([^{14}C]sucrose minus [^3H]sucrose) by standard techniques (e.g. Crompton & Heid, 1978).

In the experiments of Figs. 3(b) and 4 below, the incubation mixture was diluted up to 40-fold with standard medium (as described above, minus rotenone) before centrifugation. In some of the experiments of Table 1 (designated 'washed') mitochondria, containing 90–100 mg of protein, were preincubated, permeabilized and 'resealed' in 10 ml of standard medium. At 3 min after the addition of EGTA, the mitochondria were diluted with 80 ml of medium containing 210 mM-mannitol, 70 mM-sucrose and 10 mM-Tris/HCl, pH 7.4. The mitochondria were sedimented and the pellet was washed twice, each time by resuspension in 90 ml of the same medium followed by resedimentation, according to the standard mitochondrial preparation procedure. In the experiments of Figs. 1 and 8, where centrifugation in the Eppendorf bench centrifuge was used to stop the progress of the reaction, it was necessary to know the effective time after beginning centrifugation that the mitochondria were sedimented. Measurements were made of the fraction of mitochondria unsedimented [assayed by succinate dehydrogenase activity (Ackrell *et al.*, 1978)] and centrifugation time, and the relationship was represented graphically. The area below this curve, together with the measured time-dependencies of acceleration and deceleration, indicated that the reactions were effectively stopped 14 s after centrifugation was begun.

(c) $\Delta\psi$ measurements. Inner-membrane potentials were estimated routinely from the accumulation of TPP^+ , which was measured with an electrode constructed as described by Kamo *et al.* (1979); the 90% response time was < 1 s. TPP^+ at 10 µM was added to the incubation media as indicated in the legends. $\Delta\psi$ was calculated from the Nernst relation modified as described by Rottenberg (1984) to take account of TPP^+ binding to the inner and outer faces of the inner membrane. In those experiments including Ruthenium Red, which could only be removed completely between experiments with acid washes (which caused electrode drift), $\Delta\psi$ was monitored spectrophotometrically with 10 µM-safranin as described by Akerman & Wikstrom (1976). Safranin uptake was measured at 511–533 nm with a Perkin-Elmer model 356 dual-wavelength spectrophotometer.

(d) Ca^{2+} buffers. In the experiments of Figs. 7 and 8, Ca^{2+} /EGTA and Ca^{2+} /HEDTA buffers were introduced to yield a final [ligand] of 11 mM. The free [Ca^{2+}] established with each buffer was determined in standard medium (above) with a Ca^{2+} -selective electrode (Philips IS 561-Ca). The buffers were calibrated from a standard curve of the mV-log [Ca^{2+}] relationship obtained over the range 30 µM–1 mM- Ca^{2+} (slope 29.5 at 25 °C) and extrapolated to the mV range required.

(e) Respiration. Mitochondrial respiration was measured with a Clark-type O_2 electrode in standard reaction medium (above) minus rotenone and containing 5 mM-glutamate, 5 mM-malate and 50 µM-cytochrome c. Other additions are given in the legends.

Protein analysis

Mitochondria (5 mg of protein/ml) were incubated under standard conditions (above) with 3 mM-succinate and 175 µM- Ca^{2+} (total); 2 mM-EGTA was added 8 min after CaCl_2 . Samples (1 ml) of the incubation mixture were withdrawn immediately before the addition of succinate and 3 min after the addition of EGTA, and centrifuged for 4 min in an Eppendorf bench centrifuge. The proteins of the supernatant fluids obtained were analysed by SDS/polyacrylamide-gel electrophoresis as described by Weber & Osborn (1969) with 300 µg of protein/tube and 5 mA/tube for 7 h. The gels were stained with Coomassie Brilliant Blue (Chrombach *et al.*, 1967) and analysed with a Joyce-Loebl u.v. scanner. The test gels were run in parallel with M_r standards (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Mitochondrial proteins released by osmotic lysis were obtained by suspending mitochondria (55 µl, containing 2 mg of protein) in 2 ml of water for either 5 min or 15 min. The mitochondria were sedimented as described above and the supernatant analysed.

RESULTS

Mitochondrial permeabilization

In the present study, permeability to low- M_r solutes was judged from the permeability to sucrose. Permeabilization under standard conditions is assessed in Fig. 1; 95% of the Ca^{2+} added at zero time was accumulated within 10 s (results not shown). After the addition of Ca^{2+} , the sucrose-inaccessible space decreased with time

Fig. 1. Ca²⁺-induced decrease in sucrose-inaccessible space and TPP⁺ accumulation

Mitochondria (5 mg of protein/ml) were incubated in standard medium containing 10 μ M-TPP⁺, succinate, [¹⁴C]sucrose and ³H₂O. CaCl₂ giving 35 nmol of total Ca²⁺/mg of protein was added at zero time. Samples were withdrawn immediately before addition of CaCl₂ (zero time) and at intervals thereafter, and centrifuged (see the Methods section). Symbols: ●, sucrose-inaccessible matrix space (means $\pm$ S.E.M.; six determination); ○, TPP⁺ accumulation. The inset (▲) is a semilogarithmic plot of the fractional decrease in sucrose-inaccessible space calculated from the spaces at time zero (S_0), time t (S_t) and the residual space (S_r) after 5 min (0.16 μ l/mg of protein). The broken line gives the same curve displaced 14 s to the right to take account of the time delay in mitochondrial sedimentation (see the Methods section).

and attained a minimal value (designated 'residual space') after 5 min.

As reported in Table 1 (Expt. 2), the residual space was not decreased when the Ca²⁺ was increased from 35 to 70 nmol Ca²⁺/mg. Some decrease in residual space was attained when [P_i] was increased. However, an increase in [P_i] above 2.5 mM led to less sucrose entrapment and retention after 'resealing' (discussed below). Conversely, in the absence of added P_i (Table 1, Expt. 1), there was no significant decrease in sucrose-inaccessible space or entrapment of sucrose. Accordingly, 2.5 mM-P_i was adopted in the standard medium. The nature of the residual space, i.e. non-mitochondrial or resistant mitochondria, is not known. The residual spaces of mitochondrial preparations from perfused liver were no less than in normal mitochondria (Table 1, Expts. 7 and 8), which seemingly excludes contaminating erythrocytes as a significant contributor.

Fig. 1 (inset) shows that the sucrose-inaccessible space, corrected for residual space, decreased according to the first-order relation:

$$-dS/dt = k_{\text{perm}} \cdot S$$

(where S = space, k_{perm} is the rate constant for permeabilization and t = time), when account is taken of the fact that the mitochondria do not sediment instantaneously (broken line). In four such experiments

with different mitochondrial preparations the mean value of k_{perm} ($\pm$ S.E.M.) was $0.47 \pm 0.04 \text{ min}^{-1}$.

Permeabilization is associated with uncoupling and dissipation of $\Delta\psi$ and, since earlier studies by Hunter *et al.* (1976) concluded that these parameters are an accurate index of permeabilization, their utility as a more convenient assay was investigated. Fig. 1 shows the changes in $\Delta\psi$ -dependent accumulation of TPP⁺ measured in parallel with the sucrose-inaccessible space. If it is assumed that the residual space is mitochondrial and that $\Delta\psi$ (118 mV at zero time) is maintained at 118 mV in the non-permeabilized fraction of mitochondria, then a TPP⁺ uptake of 1.54 nmol of TPP⁺/mg of protein at 1 min would be predicted. Alternatively, if the residual space is taken to be non-mitochondrial (with no contribution to $\Delta\psi$), and that $\Delta\psi$ (119 mV at zero time) is maintained unimpaired in the non-permeabilized fraction, then a TPP uptake of 1.48 nmol of TPP⁺/mg of protein at 1 min would be expected. In either case, the values predicted greatly exceed the value actually measured at 1 min (0.14 nmol of TPP⁺/mg). It may be concluded that $\Delta\psi$ is dissipated before mitochondria are fully permeabilized to sucrose and that $\Delta\psi$ -dependent parameters are not reliable indices of the degree of permeabilization to sucrose.

This conclusion is reinforced by comparing the effect of pH on k_{perm} and on the rate of Ca²⁺-induced TPP⁺

Table 1. Permeabilization of mitochondria and the entrapment and retention of sucrose by resealed mitochondria under different experimental conditions

Mitochondria were prepared in the standard manner (S) except in Expts. 7 and 8, where they were isolated from perfused liver (P). Sucrose-inaccessible spaces were measured as in Fig. 1; the residual space is that remaining after 8 min permeabilization. [^{14}C]sucrose entrapment in 'unwashed' resealed mitochondria was measured as in Fig. 4, with no dilution; the permeabilization period was 8 min. [^{14}C]sucrose entrapment and retention in 'washed' mitochondria was measured in the same way, except that the resealed mitochondria were washed three times as stated in the Methods section. Results are means $\pm$ S.E.M. for n determinations, with either the same mitochondrial preparation (*; Expts. 1 and 2) or different mitochondrial preparations (Expts. 3–13). The incubation media contained 20 mM-sucrose.

Expt. no.	Incubation conditions			Sucrose inaccessible space ($\mu\text{l}/\text{mg}$)		Sucrose entrapped (nmol/mg of protein)			
	Mitochondria	Added P_i (mM)	Ca^{2+} (nmol/mg of protein)	Before permeabilization	Residual space	Unwashed	Washed; permeabilization period		n
							3 min	8 min	
1	S	0	35	0.81 ± 0.08	0.76 ± 0.05	—	—	0.1 ± 0.1	4*
2	S	2.5	35	0.96 ± 0.04	0.31 ± 0.04	—	—	14.2 ± 0.5	6*
2	S	2.5	70	0.96 ± 0.04	0.31 ± 0.03	—	—	—	6*
2	S	10	35	0.96 ± 0.04	0.20 ± 0.05	—	—	9.1 ± 0.5	6*
2	S	20	35	0.96 ± 0.04	0.18 ± 0.04	—	—	—	6*
3–6	S	2.5	35	0.75 ± 0.06	0.18 ± 0.01	—	6.4 ± 0.1	13.6 ± 1.2	4
7, 8	P	2.5	35	$0.89, 0.77$	$0.31, 0.28$	—	—	—	2
9–13	S	2.5	35	—	—	21.2 ± 2.1	—	15.0 ± 0.6	5

Fig. 2. Effect of pH on the Ca^{2+} -induced decrease in TPP^+ accumulation and sucrose permeation

Mitochondria (containing 5 mg of protein and 45 nmol of Ca^{2+}) were incubated in standard medium plus 10 μM - TPP^+ adjusted to either pH 6.5 (a), pH 7.0 (b) or pH 7.5 (c). Succinate (at the relevant pH) and 130 nmol of CaCl_2 were added as indicated (succinate additions are not shown for curves b and c). Ruthenium Red (10 nmol) was introduced in (c) (broken line). The experiments were repeated with $^3\text{H}_2\text{O}$ and [^{14}C]sucrose to determine the rate constants (k_{perm}) as in Fig. 1. The values of S_0 (Fig. 1) did not differ significantly with pH ($0.74 \pm 0.03 \mu\text{l}/\text{mg}$ of protein). The values in parentheses by each curve are the times (in min) after addition of CaCl_2 at which 30% of the accumulated TPP^+ had been released. The values of k_{perm} for each experiment are given below each curve.

release (Fig. 2). The rate of TPP^+ loss was progressively diminished (about 4.4-fold) by increase in pH over the range 6.5–7.5, whereas k_{perm} was increased to a small extent (1.3-fold).

Other investigators have suggested that Ca^{2+} cycling dissipates $\Delta\psi$ significantly (Lotscher *et al.*, 1980a). However, this is not supported by the fact that Ruthenium Red, which blocks Ca^{2+} uptake, has little effect on TPP^+ distribution and that $\Delta\psi$ may be essentially fully restored immediately after Ca^{2+} uptake (Fig. 2c).

Reversal of permeabilization; sucrose entrapment in the matrix space

In the above experiments it has been assumed that the Ca^{2+} -induced decrease in sucrose-inaccessible space reflects an increase in sucrose permeability rather than mere shrinkage of the matrix compartment. This assumption was tested in the study reported in Fig. 3. Mitochondria were treated in the standard manner and EGTA was added at 8 min, when the supposed permeabilization was complete (Fig. 3a). EGTA restored $\Delta\psi$ and, presumably, low H^+ permeability, completely. It is evident that repolarization by EGTA occurs in two phases: an initial rapid partial repolarization followed by a slower phase (see the Discussion section).

If the phase from 5 to 8 min genuinely involves high permeability to sucrose, then [^{14}C]sucrose added during this phase would be entrapped in the matrix after the addition of EGTA. Possible complications arising from external binding of sucrose (to the outer membrane and the outer surface of the inner membrane) were allowed for by introducing [^3H]sucrose 2 min after EGTA. The samples were then diluted, centrifuged, and the difference between the pellet contents of [^{14}C]sucrose and [^3H]sucrose were calculated.

Fig. 3(b) shows that 10 nmol of [^{14}C]sucrose/mg of

Fig. 3. EGTA-induced restoration of $\Delta\psi$ and entrapment of sucrose in permeabilized mitochondria

(a) Mitochondria were incubated as in Fig. 2(b) with the addition of succinate and CaCl₂ at 0 min and 1 min respectively. Further additions as indicated were [¹⁴C]sucrose (1), 2 mM-EGTA (2) and [³H]sucrose (3). EGTA was not added in the case of the trace represented by the broken line. (b) Samples were withdrawn from the incubations 1 min and 9 min after additions of [³H]sucrose (as indicated by 4 and 5 in a). These samples were immediately diluted 5-fold with 120 mM-KCl/10 mM-Tris/Hepes, pH 7.0. Portions of the diluted samples were then centrifuged at the times indicated in (b) and the amounts of [¹⁴C]sucrose in excess of [³H]sucrose in the mitochondrial pellets were determined (see the Methods section). Symbols: ●, ○, diluted at 11 min and 19 min respectively, +EGTA; ▲, △, diluted at 11 min and 19 min respectively, -EGTA. The medium [sucrose] was 13 mM.

Fig. 4. Effect of dilution on sucrose retention after resealing permeabilized mitochondria

Mitochondria were incubated as in Fig. 3(b) (+EGTA), except that the degree of dilution of the incubation mixture was varied over the range 0–40-fold. The incubations were diluted at 11 min (refer to Fig. 3a). The medium [sucrose] was 12 mM. The continuous line was obtained by linear regression.

indicates negligible efflux of entrapped [¹⁴C]sucrose during this period. Fig. 3(b) also shows that comparable yields of [¹⁴C]sucrose in excess of [³H]sucrose were obtained when the samples were diluted 9 min after introduction of [³H]sucrose, which indicates that negligible influx of [³H]sucrose occurred. In contrast, when EGTA was not added (Fig. 3a, broken line), the same procedures gave negligible sucrose entrapment (Fig. 3b).

Further experiments were conducted in which the degree of dilution after [³H]sucrose addition was varied (Fig. 4). It is evident that the quantity of [¹⁴C]sucrose retained in excess of [³H]sucrose is essentially constant, within error, irrespective of the degree of dilution. The capacity of 'resealed' mitochondria to retain entrapped sucrose when washed (as in normal mitochondrial-preparation procedures) was also tested (Table 1, Expts. 9–13). About 26% of the sucrose originally entrapped was lost during washing.

It would be expected that the amount of sucrose entrapped and retained by 'resealed' mitochondria would depend on the degree of permeabilization. Although this question was not pursued rigorously, such a dependence is evident. Thus mitochondria subjected to Ca²⁺ in the absence of added P_i entrap no sucrose (Table 1, Expt. 1), in line with the absence of permeabilization under these conditions. Mitochondria subjected to an incomplete period of permeabilization (3 min) retained only about half the amount of sucrose retained by fully permeabilized mitochondria (8 min; Table 1, Expts. 3–6). On the other hand, as noted previously, increased [P_i] during permeabilization, which might facilitate the

protein in excess of [³H]sucrose was retained by mitochondria diluted 1 min after [³H]sucrose addition. Moreover, the degree of retention was not significantly decreased with time after dilution (0.6–6.3 min), which

Fig. 5. Protein analysis of the supernatants remaining after sedimentation of non-permeabilized and permeabilized-and-resealed mitochondria

U.v. absorbance traces of SDS/polyacrylamide electrophoresis gels were obtained as described in the Methods section; M_r standards (abscissa) were separated in parallel. For reference the supernatant proteins were analysed at times corresponding to -0.5 min (trace a) and 11 min (trace b) of Fig. 3(a). The total supernatant protein of traces a and b amounted to 10.5 and 10.6% respectively of total mitochondrial protein. The total supernatant protein after water lysis of mitochondria for 5 min and 15 min amounted to 35.3 and 36.3% of total mitochondrial protein respectively (mitochondria prepared as described in the Methods section).

process, decreased subsequent sucrose retention (Table 1, Expt. 2).

Retention of mitochondrial protein and oxidative phosphorylation capacity during permeabilization and resealing

Experiments were carried out to determine whether permeabilization and resealing caused any loss of mitochondrial protein. As reported in Fig. 5, the protein not sedimented after permeabilization and resealing did not differ significantly from that not sedimented before permeabilization; about 10.5% of total protein was not sedimented in both cases (see the legend). Moreover, SDS/polyacrylamide-gel-electrophoretic analysis did not expose any obvious selective loss of any mitochondrial protein. Since osmotic lysis did release considerable protein (see the legend), it may be concluded that

negligible osmotic breakage occurs during permeabilization.

In addition, mitochondria permeabilized and resealed in the presence of NAD, but not in its absence, exhibited coupled respiration with glutamate plus malate as substrate (Table 2) which approached that of intact mitochondria. ADP stimulation of respiration was abolished with oligomycin. This indicates that glutamate dehydrogenase and malate dehydrogenase remain active in permeabilized and resealed mitochondria, that NAD is lost during permeabilization but may be reincorporated during resealing and that permeabilized and resealed mitochondria are capable of oxidative phosphorylation. Since resealing in the absence of NAD led to a 95% loss of respiratory capacity, the results also suggest that the residual space (Table 1) was non-mitochondrial.

Time courses of sucrose entry and the resealing of permeabilized mitochondria

It was particularly important to clarify, firstly, the rate at which sucrose, for example, enters permeabilized mitochondria and, secondly, the time period required for complete resealing. Fig. 6(b) reports the time course of resealing after addition of EGTA. In this experiment, [14 C]sucrose was added to permeabilized mitochondria either immediately before EGTA (zero time point) or at various time intervals after EGTA. [3 H]Sucrose was introduced later, when resealing was complete, to allow measurement of entrapped [14 C]sucrose. The data confirm that about 90 s is required for completion of resealing. A semilogarithmic plot of the fractional decrease in entrapped sucrose with time (Fig. 6c) reveals that resealing is a biphasic process with an initial rapid phase that is complete within 1 s (rate constant, $k'_r > 25 \text{ min}^{-1}$) and a much slower subsequent phase ($k_r = 2.0 \text{ min}^{-1}$).

The rate at which sucrose enters permeabilized mitochondria was investigated as reported in Fig. 6(a). [14 C]Sucrose was added to fully permeabilized mitochondria either together with EGTA (zero time point) or at various time intervals ($-\Delta t$) before 'resealing' with EGTA. Abbreviation of the time interval between [14 C]sucrose addition and resealing did not decrease significantly the amount of [14 C]sucrose entrapped.

The biphasic time course of 'resealing' (Fig. 6b) may be interpreted in terms of two mitochondrial fractions: one that reseals rapidly and another more slowly. According to this interpretation, the data of Fig. 6(a) indicate that sucrose entry into the rapidly resealing

Table 2. Effect of permeabilization and resealing on respiration and respiratory control with glutamate and malate

Mitochondria (1 mg of protein/ml) were permeabilized according to the standard procedure except that rotenone and succinate were omitted and glutamate and malate (both 5 mM) were added. The mitochondria were resealed with 1 mM-EGTA plus 1 mM-MgCl₂, 0.5 mM-ATP and, as indicated, 1 mM-NAD added simultaneously. Assays were conducted in the presence of 5 μ g of oligomycin/mg of protein ('+ olig') when indicated. Results are means $\pm$ S.E.M. (three expts.).

Preparation ...	Respiration (ng-atom of O/min per mg of protein)		
	Mitochondria	Permeabilized and resealed mitochondria	
		Resealed - NAD	Resealed + NAD
-ADP	21 $\pm$ 3	4 $\pm$ 1	24 $\pm$ 4; + olig, 23 $\pm$ 3
+ADP	85 $\pm$ 3	4 $\pm$ 2	74 $\pm$ 5; + olig, 23 $\pm$ 4

Fig. 6. Time courses of sucrose entry into permeabilized mitochondria before (a) and after (b) resealing with EGTA

In both (a) and (b) mitochondria were incubated in standard medium with succinate and 35 nmol of Ca²⁺/mg of protein as in Fig. 2(b); EGTA and [³H]sucrose were added 9 min and 12 min respectively after CaCl₂. In both (a) and (b), zero time denotes the time of EGTA addition. In (a), [¹⁴C]sucrose was added at various times (0–120 s) before EGTA (–Δt). In (b) the addition of [¹⁴C]sucrose was made at various times (0–90 s) after EGTA (Δt). In both (a) and (b) the incubations were diluted 5-fold and immediately centrifuged 1 min after [³H]sucrose (as in Fig. 3a). The amounts of [¹⁴C]sucrose in excess of [³H]sucrose in the mitochondrial pellets were calculated (ordinate). The medium [sucrose] was 6 mM. The data are expressed as means ± S.E.M. of six determinations with the same mitochondrial preparation. Error bars are not shown when less than the height of the symbol. For clarity a scheme of the procedures following CaCl₂ addition is given below:

The inset (c) is a semilogarithmic plot of the results in (b). The subscripts 0 and *t* refer to time zero and *t* = 1–60 s respectively.

fraction is complete within 1 s, since this population reseals with 1 s. Sucrose entry into the more slowly resealing fraction need not necessarily be equally rapid. Although the zero time point of Fig. 6(a) does not differ significantly from the line of best fit of the other data, it is informative to consider the minimal rate of sucrose entry that could be accommodated within the limits of error of the zero time point. Assuming that sucrose permeation is a first-order process, then:

$$d[\text{Sucrose}]_i/dt = k_{\text{suc}}[\text{sucrose}]_e - k_{\text{suc}}[\text{sucrose}]_i$$

where k_{suc} is the rate constant for sucrose permeation into the permeable form of the slowly resealing fraction, *i* and *e* refer to intramitochondrial and extramitochondrial respectively, and *t* refers to time. [Sucrose]_e may be replaced by [sucrose]_{i∞}, the maximal [sucrose]_i attainable. Since the available space into which sucrose enters decreases exponentially with time (Fig. 6c), then:

$$d[\text{Sucrose}]_i/dt = k_{\text{suc}}([\text{sucrose}]_{i\infty} - [\text{sucrose}]_i)e^{-k_r t}$$

where $k_r = 2$ (Fig. 6c). If [sucrose]_{i∞} for the slowly resealing fraction is taken to be 3.5 nmol/mg and the actual [sucrose]_i attained (when added simultaneously with EGTA) is assumed to be 0.4 nmol/mg less than this (from the error bar of the zero-time value of Fig. 6a; i.e. 3.1 nmol/mg), then integration of the above equation shows that the data could be accounted for with $k_{\text{suc}} \geq 4.3 \text{ min}^{-1}$ (i.e. $t_{1/2} \leq 10 \text{ s}$). In other words, although rapid sucrose entry into the rapid resealing fraction is evident, a similarly rapid entry into the slowly resealing fraction cannot be assumed.

Dependence of permeabilization and resealing on free [Ca²⁺]

Fig. 7(a) shows the repolarization of the inner membrane on addition of EGTA or Ca²⁺/HEDTA buffers to permeabilized mitochondria as indicated by safranin absorbance. Whereas EGTA repolarized almost completely, Ca²⁺/HEDTA buffers yielding 10–24 μM free Ca²⁺ repolarized only partially, and 42 μM

Fig. 7. Dependence of inner-membrane repolarization and resealing on the free $[Ca^{2+}]$

In (a) mitochondria (1 mg/ml) were incubated under standard conditions plus 10 μM -safranin with succinate and $CaCl_2$. Ruthenium Red (RR, 5 nmol/mg) and, 10 s later, either 11 mM-EGTA or Ca^{2+} /HEDTA (11 mM) buffer were added as indicated. The free $[Ca^{2+}]$ (0–42 μM) values are given in parentheses alongside the respective curves. In (b) mitochondria were incubated as in (a), except that $[^{14}C]sucrose$ was added at 6 min and $[^3H]sucrose$ at 10 min. The samples were centrifuged at 10.7 min and the $[^{14}C]sucrose$ in excess of $[^3H]sucrose$ in the mitochondria determined. This was repeated with Ca^{2+} /EGTA and Ca^{2+} /HEDTA buffers yielding the free $[Ca^{2+}]$ stated. The medium $[sucrose]$ was 1.4 mM. The data are means $\pm$ S.E.M. for three preparations of mitochondria. Symbols: ●, $[^{14}C]sucrose - [^3H]sucrose$; ○, maximal increase in safranin absorbance on addition of Ruthenium Red/ Ca^{2+} buffer.

free Ca^{2+} gave no repolarization. It is important to stress that these experiments were conducted in the presence of sufficient Ruthenium Red to block Ca^{2+} uptake. This point was investigated with permeabilized mitochondria that had been resealed with EGTA and washed three times (as in Table 1). The initial rate of succinate-supported Ca^{2+} uptake by these mitochondria, 10.4 nmol of Ca^{2+} /s per mg of protein (with 35–41 μM extramitochondrial free Ca^{2+}), was decreased to < 1 pmol/s per mg of protein by 5 nmol of Ruthenium Red/mg of protein. If it is assumed that HEDTA permeates as rapidly as sucrose, then the internal $[HEDTA]$ would be about the same as that in the medium. This would correspond to about 11 nmol of internal HEDTA/mg of protein, sufficient to maintain internal free $[Ca^{2+}]$ constant for a prolonged period. This was confirmed by the fact that resealing with lower $[HEDTA]$ (3 mM) gave the same results.

This experiment was repeated over a range of free $[Ca^{2+}]$ with three mitochondrial preparations (Fig. 7b). Repolarization was impaired with $> 3 \mu M$ free Ca^{2+} . $[^{14}C]sucrose$ and $[^3H]sucrose$ was included to permit determination of the $[^3H]sucrose$ -inaccessible space after resealing. Like $\Delta\psi$, this space decreased with increased free $[Ca^{2+}]$ above 3 μM .

Fig. 7 reveals an important feature of sucrose permeation. Thus $[^3H]sucrose$ enters with 10 μM and 24 μM free Ca^{2+} (Fig. 7b) during the time that $\Delta\psi$ is maintained constant (Fig. 7a); $\Delta\psi$ was maintained

constant for a period of at least 5 min in all six experiments performed. This may be explained by assuming either (a) that mitochondria are heterogeneous in their sensitivity to Ca^{2+} , that the permeable fraction constitutes the most sensitive, and that $\Delta\psi$ is maintained by the more resistant fraction, which remains impermeable to sucrose with time, or (b), that the permeable fraction at any point in time represents that fraction in continual steady-state interconversion with the impermeable fraction and that increase in free $[Ca^{2+}]$ displaces this steady state to the permeable form; in this case, the fraction that is impermeable at any point in time would be effectively permeable over a period of time via reversible transition to the permeable form.

This question was approached by investigating whether $[^3H]sucrose$ enters partially polarized mitochondria with time; $[^3H]sucrose$ entry was measured as a decrease in the $[^{14}C]sucrose - [^3H]sucrose$ space as shown in Fig. 8(a). The data clearly show that $[^3H]sucrose$ does enter with time in the presence of 24 μM free Ca^{2+} (lower broken line). Moreover, these data give a good fit to a single first-order process over the time span 28–120 s, in line with slow sucrose entry into a single homogeneous population over this period. With 1.7 μM free Ca^{2+} , $[^3H]sucrose$ entry with time is scarcely detectable (upper broken line). These data, therefore, may be accounted for far more readily by assumption (b) [above; rather than assumption (a)], in which Ca^{2+} increases the rate constant for the impermeable $\rightarrow$ per-

Fig. 8. Time courses of sucrose entry into incompletely resealed mitochondria at different free [Ca²⁺] values

The experiments of (a) were carried out as in Fig. 7(b), except that the samples were centrifuged at various times (30–120 s) after addition of [³H]sucrose; these times are indicated on the abscissa. Each data point is the mean $\pm$ S.E.M. from six samples of the same incubation; error bars are not shown when their heights were less than that of the symbol. The free [Ca²⁺] obtained with the buffers (1.7–24 μM) are given alongside the respective curves. The Figure shows data from two separate mitochondrial preparations as indicated by the solid and broken lines. The ordinate is positioned at -14 s to account for the effective time at which mitochondria were sedimented. (b) Gives the rate constants (k_p) obtained from the slopes of the continuous lines of (a). The value of k_p at zero free Ca²⁺ (11 mM-EGTA) was from other experiments with three separate mitochondrial preparations ($k_p = 0.013 \pm 0.004$; mean $\pm$ S.E.M.).

meable transition:

At any particular free [Ca²⁺], the impermeable fraction, and therefore $\Delta\psi$, would be constant with time, even though sucrose would eventually enter the entire population. According to this interpretation, the rate constant for the rate-limiting step of sucrose entry may be tentatively designated k_p , where the subscript p denotes permeabilization; k_p is thus analogous to (and may be the same as) the rate constant for the permeabilization of mitochondria as isolated (k_{perm} ; Fig. 1).

The dependence of k_p on free [Ca²⁺] was investigated further (continuous lines, Fig. 8a) and the relation derived is given in Fig. 8(b). This relation is linear over the range 0–10 μM free Ca²⁺ and is far from saturation at 24 μM free Ca²⁺. Ca²⁺ at 24 μM increased the value of k_p about 40-fold.

DISCUSSION

The present study provides firm evidence that permeabilization to sucrose is in fact reversible, and fully corroborates the conclusion drawn much earlier by Hunter *et al.* (1976) from studies with heart mitochondria, the implications of which have been poorly appreciated. The latter work employed changes in the total (³H₂O

minus [¹⁴C]sucrose) space of mitochondrial pellets as an index of permeabilization. In the present study, the problem was approached in two parts. Firstly, permeabilization conditions were obtained under which the mitochondria were indeed freely permeable to sucrose, at least as judged by the fact that sucrose entry was complete within the time resolution of the technique employed (Fig. 6a). Secondly, it was shown that mitochondria thus permeabilized entrap the sucrose in a manner quite resistant to dilution when free Ca²⁺ is removed. This aspect was investigated with a dual-isotope technique designed to exclude rigorously any contribution by external binding of sucrose or by entry of sucrose in the presence of an applied centrifugal force as suggested by Sitaramam & Janardana Sarma (1981). There are no previous unequivocal indications that liver mitochondria permeabilized to such a degree may be fully resealed. Although EGTA-induced restoration (in large part) of $\Delta\psi$ in Ca²⁺-treated liver mitochondria has been demonstrated (Lotscher *et al.*, 1980b), this observation is ambiguous, since $\Delta\psi$ is not a reliable index of permeability to species other than H⁺ (Figs. 1 and 2). Broekemeier *et al.* (1985) have suggested that an H⁺-specific permeability increase precedes the non-specific permeability increase, and our data are consistent with that.

The present data indicate that the rate of mitochondrial permeabilization is directly proportional to the fraction unpermeabilized at any time (Fig. 1). This simple view differs from that of Beatrice *et al.* (1982), who proposed

that the non-synchronous permeabilization of liver mitochondria reflected heterogeneity of the mitochondrial population, so that mitochondria with the lowest stability were permeabilized first, followed by permeabilization of the next stability range, and so on. Although mitochondria once permeabilized may not respond homogeneously, as discussed below, it seems unnecessary to apply this concept to mitochondria as isolated.

A notable feature of the present work was that sucrose-permeabilized mitochondria were resealed throughout merely by removing free Ca^{2+} . It seems to us that this carries certain implications for the mechanisms of permeabilization and resealing. There is irrefutable evidence that Ca^{2+} -induced permeabilization, at least to H^+ , is associated with Ca^{2+} -induced activation of phospholipase A_2 , and it has been proposed that this is a key event in the process (Pfeiffer *et al.*, 1979; Beatrice *et al.*, 1980, 1984). Although phospholipid hydrolysis may indeed be necessary, it follows that, if phospholipid hydrolysis were linked directly to sucrose permeability as suggested (Broekemeier *et al.*, 1985), then reacylation of phospholipids would be necessary for the restoration of sucrose-impermeability. Yet our own data demonstrate resealing under conditions in which (it seems reasonable to assume) essential cofactors for reacylation (ATP, CoA) would be released from the matrix space and available only at extremely low concentrations: perhaps one-thousandth of normal, since we have not detected any decrease in resealing efficiency when mitochondrial protein was decreased from 10 mg/ml to 1 mg/ml. This point is most evident with the rapidly resealing fraction, which is freely permeable to sucrose (entry within 1 s) and yet reseals within 1 s. Thus although phospholipid hydrolysis may be a prerequisite for sucrose permeabilization, perhaps in a 'priming' capacity, sucrose permeation *per se* may reflect a more direct action of Ca^{2+} , conceivably via a Ca^{2+} -activated pore.

The utility of the phenomenon for the matrix entrapment of substances of interest has been assessed from a number of aspects. The amount of sucrose entrapped seems reasonable assuming sucrose equilibration in permeabilized mitochondria. Thus, with 20 mM external sucrose, approx. 21 nmol of sucrose/mg of protein became entrapped (Table 1, Expts. 9–13). If account is taken of the residual space that resists permeabilization (25–32% of the total space), then the sucrose content of the permeabilized and resealed fraction would amount to about 30 nmol/mg of protein. This is compatible with sucrose equilibration into a matrix space of about 1.5 μl /mg of protein, a value that seems reasonable in view of electron-microscopic evidence (Hunter *et al.*, 1976) that Ca^{2+} -treated mitochondria are swollen with respect to non-permeabilized mitochondria (matrix volume, 0.76–0.91 μl /mg in the present study). Although depletion of low- M_r solutes in resealed mitochondria would be expected, we were unable to detect any loss of protein. Some losses of entrapped sucrose (about 28%) did occur when the resealed mitochondria were washed three times. In this connection, Sitaramam & Jarnardana Sarma (1981) suggested that mitochondria may become permeabilized to sucrose during centrifugation. Other experiments (not shown) revealed that these losses were not decreased by inclusion of EGTA in the washing medium, and the losses are not attributable to Ca^{2+} -induced repermeabilization during washing. Further experiments investigated the capacity

of permeabilized and resealed mitochondria, subsequently washed three times, to generate $\Delta\psi$ (in standard reaction medium without P_i and with 3 mM-succinate, 10 μM -TPP⁺ and 2 mM-EGTA). $\Delta\psi$ values of 160 mV and 146 mV were obtained with two separate preparations (matrix volumes 0.51 and 0.47 μl /mg) when corrections were applied for TPP⁺ binding to the membrane (see the Methods section). Other studies (not shown) indicate that the maximal amount of Ca^{2+} that resealed mitochondria may accumulate without rapid repermeabilization is about equivalent (± 1 nmol/mg of protein) to the amount of entrapped EGTA. Thus permeabilized mitochondria may well lose much of their endogenous capacity to buffer matrix Ca^{2+} . In spite of this the novel technique of permeabilization and resealing described here would appear to offer a powerful means of investigating matrix reactions via the entrapment of substances of interest, e.g. specific metabolites, Ca^{2+} -buffers, and indicators (pH, Ca^{2+} etc.).

Although the simplest conceivable model of permeabilization and resealing (see the Results section) is consistent with much of the data reported here, it is inadequate in other respects. According to this model, the ordinate intercepts in Fig. 8(a) would represent the instantaneously impermeable fraction. The consistency of the model can thus be cross-checked by comparing the values derived from the intercepts with the values calculated from the measured rate constants (instantaneously impermeable fraction = k_p/k_r). Experiments with three different mitochondrial preparations yielded a measured (intercepts) mean instantaneously impermeable fraction ($\pm$ S.E.M.) of 0.73 ± 0.03 at 24 μM free Ca^{2+} (letting the measured instantaneously impermeable fraction with 1.7 μM free Ca^{2+} equal 1). The measured value of $k_p \pm$ S.E.M. was 0.46 ± 0.06 in the same experiments. If the value of k_r is taken to be 2 (from the slowly resealing fraction, Fig. 6c), then the calculated instantaneously impermeable fraction (as a fraction of the total) becomes 0.81, in fair agreement with that measured. The model therefore is reasonably consistent so far.

An instantaneously impermeable fraction of 0.9 with 10.5 μM - Ca^{2+} may be calculated in the same way (using the k_p value from the continuous line of Fig. 8a); the intercept, however, indicates a value not significantly different from 1. The same inconsistency is apparent during repolarization (Fig. 7). Repolarization with zero free Ca^{2+} and with 10.5 μM free Ca^{2+} is clearly biphasic, with an initial rapid phase. The magnitude of this initial rapid repolarization was the same with zero free Ca^{2+} and with 10.5 μM free Ca^{2+} in six such experiments with different mitochondrial preparations. In the same six experiments, there was no detectable initial rapid repolarization with 24 μM - Ca^{2+} . It seems then that the initial rapid repolarization depends critically on the free $[\text{Ca}^{2+}]$, being undetectable with 24 μM - Ca^{2+} and fully developed with 10.5 μM - Ca^{2+} .

It is tempting to correlate the fraction responsible for the initial rapid repolarization with the instantaneously permeable fraction, which constituted 0.27 of the total (i.e. $1 - 0.73$) with 24 μM - Ca^{2+} , and was undetectable with 10.5 μM free Ca^{2+} . In view of the time resolution of the isotope technique employed, 'instantaneously' permeable could imply either complete displacement towards a permeable form at 24 μM - Ca^{2+} or rapid equilibration with such a form. In addition, it seems

reasonable to correlate the initial rapid repolarization with the rapidly resealing fraction (Figs. 6*b* and 6*c*), which comprised 0.3 of the total.

The simple model, therefore, is inadequate in that it fails to account for the rapidly resealing fraction, the rapidly repolarizing fraction and the absence of an instantaneously permeable fraction at 10.5 μM -Ca²⁺, all of which, in principle, may be common. Moreover, the rapidly repolarizing fraction and the instantaneously permeable fraction displayed an acute sensitivity to [Ca²⁺] over the range 10–24 μM that cannot be related to k_p , which has a linear dependence on [Ca²⁺] over this range, and which was derived for the slowly permeabilizing fraction. Accordingly it seems necessary to postulate two distinct actions of Ca²⁺: one that activates a relatively slow process in a linear manner (up to 24 μM -Ca²⁺) and another that controls a more rapid event in a much more sensitive manner such that this event is minimal (or nearly so) at 10 μM -Ca²⁺ and maximal (or nearly so) at 24 μM -Ca²⁺. As a working hypothesis the following scheme may be suggested:

Where the reaction Impermeable $\rightarrow$ Permeable is slow, and Permeable $\rightarrow$ Permeable $\cdot n\text{Ca}^{2+}$ represents the rapid stabilization of a pore with $n\text{Ca}^{2+}$ ions. The latter reaction could involve intramitochondrial and/or extra-mitochondrial Ca²⁺. Conceivably, phospholipase A₂ may provide the Ca²⁺-sensitivity of the slow process.

Elucidation of the molecular events underlying the disruption of mitochondrial energy transduction by excess Ca²⁺ is essential to the general question of Ca²⁺ as a mediator of cell death (see the Introduction section). It seems particularly important to identify specifically the processes that are sensitive to Ca²⁺ with a view to developing effective pharmacological interventions. The scheme may offer a suitable basis for further studies of this topic.

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