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The Calcium Conductance of the Inner Membrane of Rat Liver Mitochondria and the Determination of the Calcium Electrochemical Gradient

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1. A method is described for establishing steady-state conditions of calcium transport across the inner membrane of rat liver mitochondria and for determining the current of Ca^{2+} flowing across the membrane, together with the Ca^{2+} electrochemical gradient across the native Ca^{2+} carrier. These parameters were used to quantify the apparent Ca^{2+} conductance of the native carrier. 2. At 23°C and pH7.0, the apparent Ca^{2+} conductance of the carrier is close to 1 nmol of $Ca^{2+} \cdot min^{-1} \cdot mg$ of $protein^{-1} \cdot mV^{-1}$. Proton extrusion by the respiratory chain, rather than the Ca^{2+} carrier itself, may often be rate-limiting in studies of initial rates of Ca^{2+} uptake. 3. Under parallel conditions, the endogenous H^+ conductance of the membrane is 0.3 nmol of $H^+ \cdot min^{-1} \cdot mg$ of $protein^{-1} \cdot mV^{-1}$. 4. Ruthenium Red and La^{3+} both strongly inhibit the Ca^{2+} conductance of the carrier, but are without effect on the H^+ conductance of the membrane. 5. The apparent Ca^{2+} conductance of the carrier shows a sigmoidal dependence on the activity of Ca^{2+} in the medium. At 23°C and pH7.2, half-maximum conductance is obtained at a Ca^{2+} activity of 4.7 μM. 6. The apparent Ca^{2+} conductance and the H^+ conductance of the inner membrane increase fourfold from 23° to 38°C. The apparent Arrhenius activation energy for Ca^{2+} transport is 69 kJ/mol. The H^+ electrochemical gradient maintained in the absence of Ca^{2+} transport does not vary significantly with temperature. 7. The apparent Ca^{2+} conductance increases fivefold on increasing the pH of the medium from 6.8 to 8.0. The H^+ conductance of the membrane does not vary significantly with pH over this range. 8. Mg^{2+} has no effect on the apparent Ca^{2+} conductance when added at concentrations up to 1 mM. 9. Results are compared with classical methods of studying Ca^{2+} transport across the mitochondrial inner membrane.

The transport of Ca^{2+} across the inner membrane of mitochondria has been the subject of extensive study (for reviews, see Chance, 1965; Lehninger *et al.*, 1967; Lehninger, 1970; Carafoli, 1974). In particular, an understanding of the kinetics of Ca^{2+} transport is relevant both to the elucidation of the molecular mechanism of the transport process, and to assessment of the physiological role played by mitochondria in the regulation of cytoplasmic Ca^{2+} concentrations (Lehninger, 1970; Carafoli, 1974). Kinetic studies in the literature have relied universally on following the initial rates of Ca^{2+} uptake from the medium. Reed & Bygrave (1975*a*) recognized two experimental difficulties: first, the need to distinguish between uptake of the cation into the matrix compartment and binding to external sites on the mitochondrion; and second, the need to allow for the presence of chelating species in the incubation medium when calculating the concentration of Ca^{2+} required for half-maximum rates of uptake.

However, even when all experimental precautions are taken, initial-uptake rate studies suffer from inherent theoretical limitations, resulting from the inability to consider the Ca^{2+} electrochemical gradient that is driving the transport process. In any initial-rate study it is virtually impossible to consider either the membrane potential, which undergoes rapid changes on the addition of Ca^{2+} (Mitchell & Moyle, 1969*a*), or the true gradient of Ca^{2+} activity across the native carrier, as this parameter not only changes rapidly as Ca^{2+} enters, but is also not calculable from Ca^{2+} concentration gradients, owing to the extensive and unknown extent of binding of the cation in the matrix.

The necessity for gross electroneutrality in any transport process demands that the translocation of positive charge into the matrix compartment due to Ca^{2+} transport cannot occur faster than the rate at which the respiratory chain or the H^+-translocating adenosine triphosphatase can neutralize this charge by translocating protons out of the matrix. Unless the Ca^{2+} carrier is the rate-limiting step, there is a risk that observed rates of Ca^{2+} transport into the matrix are more relevant to the activity of H^+ translocation than to that of the Ca^{2+} carrier.

This paper represents one attempt to circumvent

the limitations inherent in initial-rate studies. Reed & Lardy (1972) showed that the ionophore A23187 catalysed the electroneutral exchange of one Ca^{2+} for two H^+ across the inner mitochondrial membrane; in respiring mitochondria it established a dissipative cycling of Ca^{2+} across the inner membrane, in concert with the native Ca^{2+} carrier. Thus it is possible to determine the kinetics of Ca^{2+} transport in steady-state conditions. If the ionophore is present in an excess, then the rate of Ca^{2+} cycling will depend on the kinetics of the native Ca^{2+} carrier. As we show, an excess of the ionophore also clamps the ratio of true Ca^{2+} activities across the membrane at a value dependent on the transmembrane pH gradient. As both membrane potential and pH gradient may be determined experimentally (Mitchell & Moyle, 1969*a*; Nicholls, 1974*c*; Rottenberg, 1975), it is possible to determine the electrochemical gradient of Ca^{2+} across the native carrier during this steady-state cycling.

From the rate of flow of Ca^{2+} across the carrier and the Ca^{2+} electrochemical gradient, the apparent Ca^{2+} conductance of the native carrier has been deduced, thus enabling the transport system to be described in terms analogous to those used for the endogenous H^+ conductance of rat liver mitochondria (Mitchell & Moyle, 1967*a*; Nicholls, 1974*c*) or brown-adipose-tissue mitochondria (Nicholls, 1974*a*).

Experimental

Theory of method

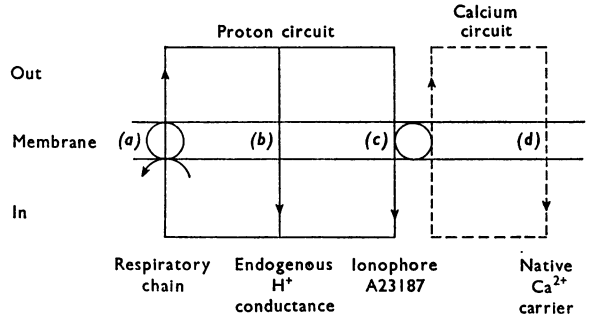
Reed & Lardy (1972) were the first to propose that the stimulation of respiration that was observed on addition of the ionophore A23187 to mitochondrial incubations in the presence of Ca^{2+} was due to the induction of an energy-dissipating cyclic flow of Ca^{2+} across the inner membrane, the cation entering the matrix electrophoretically on the native carrier, and leaving the matrix on the ionophore in exchange for protons. The effect of this cycling is to catalyse the electrical entry of protons into the matrix (Scheme 1). Thus the Ca^{2+} cycling is maintained at the expense of an increase in the effective proton conductance of the membrane, which manifests itself as a decrease in the proton electrochemical gradient (Δp),* and hence in an increase in respiration.

Calcium electrochemical gradient across the native carrier. The equation for the electrochemical gradient across the native carrier depends on the ionic species transported. There is some ambiguity in the literature as to whether Ca^{2+} is transported by the native carrier as a Ca^{2+} uniport (Selwyn *et al.*, 1970; Rottenberg & Scarpa, 1974) or as a partially charge-compensated Ca^{2+}/H^+ antiport (Reed & Bygrave, 1975*a*). These two modes of Ca^{2+} entry predict very different equilibrium distributions of the cation. The electrochemical gradient of calcium across a Ca^{2+} uniport is given by eqn. (1), and that across a Ca^{2+}/H^+ antiport is given by eqn. (2):

$$\Delta\mu Ca^{2+} = 2\Delta E - 59\log\frac{[Ca^{2+}]\text{in}}{[Ca^{2+}]\text{out}} \quad (1)$$

$$\Delta\mu' Ca^{2+} = \Delta E - 59\log\frac{[Ca^{2+}]\text{in}\,[H^+]\text{out}}{[Ca^{2+}]\text{out}\,[H^+]\text{in}} \quad (2)$$

* Abbreviations: Δp, proton electrochemical potential gradient (protonmotive force); EGTA, ethanedioxybis-(ethylamine)tetra-acetic acid; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulphonate; ΔE, membrane potential; ΔpH, transmembrane pH gradient; JH^+, proton current; C_MH^+, effective proton conductance of the membrane; $\Delta\mu Ca^{2+}$, electrochemical potential gradient of Ca^{2+}; JCa^{2+}, calcium current; C_MCa^{2+}, apparent calcium conductance of the membrane.



Scheme 1. *Diagrammatic representation of the flow of H^+ and Ca^{2+} across the mitochondrial inner membrane in the presence of the ionophore A23187*

The endogenous proton conductance is shown as a uniport (*b*), the ionophore is shown catalysing a $Ca^{2+}/2H^+$ antiport (*c*), and the native calcium carrier is depicted as a Ca^{2+} uniport (*d*).

Table 1. *Accumulation of $^{45}Ca^{2+}$ in the matrix of mitochondria in relation to that predicted due to a Ca^{2+} uniport or a Ca^{2+}/H^+ antiport*

Rat liver mitochondria (1.27 mg of protein/ml of medium) were incubated for 4½ min at 23°C in a medium containing 250 mM-sucrose, 10 mM-Tes, 10 mM-choline chloride, 2 mM-succinate, 0.5 μM-valinomycin, 0.5 mM-RbCl and $CaCl_2$ (87 nmol/mg of protein), pH 7.2. Parallel experiments were performed in which accumulation of $^{45}Ca^{2+}$ and the magnitude of the components of Δp were determined as described in the Experimental section. The volume of the mitochondrial matrix was taken to be 0.73 μl/mg of protein.

Observed	
ΔE (mV)	91
-59ΔpH (mV)	+103
$^{45}Ca^{2+}$ accumulated (nmol/mg of protein)	42
$[^{45}Ca^{2+}]$ in the matrix/$[^{45}Ca^{2+}]$ in the medium	1007
Predicted	
$[^{45}Ca^{2+}]$ in the matrix/$[^{45}Ca^{2+}]$ in the medium (at electrochemical equilibrium)	
(*a*) for Ca^{2+} uniport (eqn. 1)	1200
(*b*) for Ca^{2+}/H^+ antiport (eqn. 2)	0.63

Osmotic-swelling studies (Selwyn *et al.*, 1970) favour a Ca^{2+} uniport, as does the ability to obtain a $2K^+/Ca^{2+}$ exchange in non-respiring mitochondria in the presence of valinomycin (Rossi *et al.*, 1967). Although a Ca^{2+} uniport predicts a 10-fold accumulation of free Ca^{2+} in the matrix for every 30 mV of membrane potential (eqn. 1), the accumulation at equilibrium predicted by a Ca^{2+}/H^+ antiport is a function of the difference between ΔE and -59ΔpH. Table 1 shows the membrane-potential and pH-gradient components of the proton electrochemical gradient across the inner membrane of mitochondria that have been allowed to accumulate Ca^{2+}. From the observed Ca^{2+} accumulation by the mitochondria the ratio of Ca^{2+} concentrations in the matrix and medium compartments can be estimated approximately. Although the unknown extent of binding of the cation renders any attempt to convert this concentration ratio into an activity ratio impossible, it is clear that the observed distribution far more closely approximates to that predicted for a uniport, than to that for a Ca^{2+}/H^+ antiport. A Ca^{2+} uniport will thus be assumed for this study, although it should be noted that only a simple correction factor is required for the Ca^{2+}-conductance values obtained to be relevant to a Ca^{2+}/H^+ antiport (see below).

As shown by eqn. (1), calculation of the Ca^{2+} electrochemical gradient requires knowledge of both the membrane potential and the activity ratio of free calcium ($[Ca^{2+}]$in/$[Ca^{2+}]$out) across the membrane. Although the membrane potential may be calculated from the Nernst distribution of $^{86}Rb^+$ in the presence of valinomycin (Nicholls, 1974*c*; Rottenberg & Scarpa, 1974), the distribution of $^{45}Ca^{2+}$ across the membrane bears little resemblance to $[Ca^{2+}]$in/$[Ca^{2+}]$out, owing to the extensive, variable and essentially unknown extent of binding of the cation in the matrix compartment.

If the ionophore A23187 is added in a sufficient excess, then the distribution of proton and calcium activities across the membrane must approach the electrochemical equilibrium for the electroneutral exchange (Reed & Lardy, 1972; Pfeiffer *et al.*, 1974) which the ionophore catalyses. In this case eqn. (3) will hold:

$$\Delta\mu''Ca^{2+} = 59\log\frac{[Ca^{2+}]\text{out}\,[H^+]^2\text{in}}{[Ca^{2+}]\text{in}\,[H^+]^2\text{out}} = 0 \qquad (3)$$

$[Ca^{2+}]$in/$[Ca^{2+}]$out will then be governed by, and calculable from, the pH gradient as shown in eqn. (4):

$$\frac{[Ca^{2+}]\text{in}}{[Ca^{2+}]\text{out}} = \frac{[H^+]^2\text{in}}{[H^+]^2\text{out}} \qquad (4)$$

Thus, under the conditions of calcium cycling in the presence of an excess of ionophore, the electrochemical gradient of Ca^{2+} across the native carrier simplifies (from eqns. 1 and 4) to:

$$\Delta\mu Ca^{2+} = 2\Delta E - 59\log\frac{[H^+]^2\text{in}}{[H^+]^2\text{out}} \qquad (5)$$

or:

$$\Delta\mu Ca^{2+} = 2\Delta p \qquad (6)$$

Thus the Ca^{2+} electrochemical gradient across the native carrier may be calculated from the experimentally determinable proton electrochemical gradient across the inner membrane during calcium cycling in the presence of an excess of ionophore.

Calcium current through the native carrier. The proton circuit across the mitochondrial inner membrane, and its coupling to the calcium circuit via the ionophore A23187, is shown in Scheme 1. The stoicheiometry of the electroneutral exchange induced by the ionophore results in the cycling of one Ca^{2+} for every two H^+ entering via the ionophore (Reed & Lardy, 1972). However, although the total current of protons expelled by the respiratory chain (*a* in Scheme 1) may be directly calculated from the respiratory rate and the stoicheiometry of proton extrusion by the respiratory chain (Mitchell & Moyle, 1967*b*), the low but significant proton conductance of the membrane (*b* in Scheme 1) (Mitchell & Moyle, 1967*a*; Nicholls, 1974*c*) allows some of this total proton current to leak back into the matrix as an electrical uniport (*b* in Scheme 1). We quantified the calcium current by using the respiratory rate in the presence of the ionophore A23187 to calculate the total proton current, subtracting the proportion of this proton current that we estimate leaks back into the matrix by electrical uniport (*b* in Scheme 1), and

hence calculating the current of protons that enters in exchange for Ca^{2+} via the ionophore. To calculate this proton leakage, the respiratory rate and proton electrochemical gradient are determined in the absence of the ionophore, i.e. when the sole pathway of proton re-entry is via the electrical uniport. The proton current through the uniport will change on adding the ionophore A23187, as the proton electrochemical gradient decreases, owing to the additional conductance resulting from the initiation of Ca^{2+} cycling. To estimate the extent of the H^+ current through the electrical uniport in the presence of the ionophore, some assumption must be made as to the variation of H^+ current with Δp. The simplest assumption, and that made in this paper, is that the proton current via the electrical uniport is directly proportional to Δp, i.e. that the conductance is ohmic, in which case the conductance of the uniport in the absence of the ionophore A23187 may be used together with the value of Δp in the presence of the ionophore to calculate the extent of the proton leak during Ca^{2+} cycling. In this way the net proton current through the ionophore can be calculated by difference. In practice, the endogenous proton conductance deviates from ohmic behaviour (Nicholls, 1974*c*), such that the leak at decreased values of Δp is less than that estimated by this interpolation. However, as the leak through the proton uniport is usually considerably less than the proton current through the ionophore (see Table 2), we did not consider it essential to obtain detailed information on the extent of the deviation from non-ohmic behaviour of the proton uniport for the variety of incubation conditions examined.

The Ca^{2+} current is calculated as follows: if in the absence of the ionophore A23187 the steady-state respiratory rate is R^- nmol of $O \cdot min^{-1} \cdot mg$ of $protein^{-1}$, and the proton electrochemical gradient is Δp^- mV, then the proton conductance of the membrane, assuming a stoicheiometry of proton extrusion by the respiratory chain for succinate of $4H^+/O$ (Mitchell & Moyle, 1967*b*) is:

$$C_M H^+ = \frac{4 \cdot R^-}{\Delta p^-} \quad (7)$$

If the ionophore is now added, and respiration accelerates to a new value R^+ nmol of $O \cdot min^{-1} \cdot mg$ of $protein^{-1}$, and the proton electrochemical gradient falls to Δp^+ mV, then the leak through the endogenous proton conductance is now given by eqn. (8):

$$\text{Proton leak} = \frac{4 \cdot R^- \cdot \Delta p^+}{\Delta p^-} \quad (8)$$

As the total proton current is now $4 \cdot R^+$, the net proton current through the ionophore is:

$$4 \cdot R^+ - \frac{4 \cdot R^- \cdot \Delta p^+}{\Delta p^-} \quad (9)$$

Thus the calcium current, which is determined by the stoicheiometry of the ionophore, is given by eqn. (10):

$$JCa^{2+} = 2 \cdot R^+ - \frac{2 \cdot R^- \cdot \Delta p^+}{\Delta p^-} \quad (10)$$

As both the calcium current and the calcium electrochemical gradient (eqn. 6) can thus be determined by experiment, an apparent calcium conductance of the membrane can be calculated ($C_M Ca^{2+}$), with the units of nmol of $Ca^{2+} \cdot min^{-1} \cdot mg$ of $protein^{-1} \cdot mV^{-1}$. The apparent calcium conductance of the native carrier is thus given by eqn. (11) from (eqns. 6 and 10):

$$C_M Ca^{2+} = \frac{R^+}{\Delta p^+} - \frac{R^-}{\Delta p^-} \quad (11)$$

The conductance is apparent as, in the absence of detailed information on the relation between potential and flow, the calcium carrier cannot be assumed to have an ohmic conductance for the ion.

Effect of alternative stoicheiometries. The overall stoicheiometry relating respiration to proton translocation, and hence to the rate of proton cycling, is such that the passage of two reducing equivalents from succinate to oxygen is associated with the initial extrusion of four H^+ (Mitchell & Moyle, 1967*b*), and that through the intermediacy of ionophore A23187, this is coupled to the cycling of 2 Ca^{2+} across the membrane. It should be noted that complete charge neutralization is required for sustained transport under steady-state conditions such as those described in this paper, and that this should not be confused with initial-uptake studies where the parallel movement of unspecified ions leads to ratios of H^+ extruded to Ca^{2+} accumulated of close to 1, instead of the 2 required for electroneutrality (Rossi & Azzone, 1965; Rossi *et al.*, 1967; Gear *et al.*, 1967; Lehninger *et al.*, 1967; Gear & Lehninger, 1968; Scarpa & Azzone, 1968; Carafoli & Lehninger, 1971).

Similarly, as much of the evidence for a stoicheiometry of proton extrusion by the respiratory chain in excess of $2\ H^+/2e^-$ per energy-conserving site comes from calcium-uptake experiments (Carafoli *et al.*, 1965, 1966, 1967; Rossi & Azzone, 1965; Lehninger *et al.*, 1967; Reynafarje & Lehninger, 1974*a*,*b*), and as their significance has been called in doubt by the finding (Brand & Lehninger, 1975) that hydrolysis of matrix ATP under these conditions contributes to proton extrusion but not to O_2 uptake, there is at present insufficient evidence for discarding the stoicheiometries of Mitchell & Moyle (1967*b*). The effect of an authentic super-stoicheiometry on the calculations of conductance would be simply to increase the proton and calcium currents sustained by a given rate of respiration.

Evidence has been advanced that the native calcium carrier is unlikely to operate as a Ca^{2+}/H^+ antiporter (Table 1). The effect of such a stoicheiometry would be to double the calcium current circulating for a given rate of proton re-entry on ionophore A23187 (Scheme 1). Similarly, under conditions where ionophore A23187 was present in an excess, the calcium electrochemical gradient given by eqn. (2) becomes identical with, rather than equal to twice, the proton electrochemical gradient. It would thus simply be necessary to multiply the conductances obtained, assuming a Ca^{2+} uniport, by a factor of four, in order to obtain the conductance of a supposed Ca^{2+}/H^+ antiporter.

Materials

Reagents. All radioactive isotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The ionophore A23187 was a generous gift from Dr. R. L. Hamill, Eli Lilly and Co., Indianapolis, IN, U.S.A. Ruthenium Red was obtained from Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. and was recrystallized before use (Fletcher *et al.*, 1961). All other reagents were of analytical grade. Unless specifically stated, all anions were added as Na^+ salts. Sucrose and NaCl, the major components of the preparation and incubation media respectively were of AnalaR grade and obtained from BDH, Poole, Dorset, U.K. The calcium content of sucrose stock solutions was minimized by equilibration with Dowex 50W(XB) before addition to media.

Mitochondria. Mitochondria were prepared from the livers of male Wistar rats (150–250g), which had been maintained at 23°C with free access to food and water. Livers were homogenized in 250mM-sucrose/5mM-Tris/HCl (pH7.2)/1mM-EGTA, at 0°C. The homogenate was centrifuged for 10min and 650*g* ($r_{av.}$ 8cm). Bovine serum albumin (fraction V, 5mg/ml) was added to the supernatant, which was centrifuged for 10min at 6500*g*. The pellet was resuspended in 250mM-sucrose/5mM-Tris/HCl, pH7.2, and centrifuged for 5min at 650*g*. The supernatant was again centrifuged at 6500*g* for 10min, washed once in the same medium, and finally resuspended in 250mM-sucrose/5mM-Tris/HCl, pH7.2. Mitochondria were stored at 0°C for not more than 4h before use. Mitochondrial protein was determined by the biuret (Layne, 1952) method.

Experimental design

The basic incubation medium for all experiments contained 75mM-NaCl, 10mM-Tes (sodium salt), 1mM-KCl, 2mM-succinate, 2μM-rotenone, 0.5μM-valinomycin and bovine serum albumin (fraction V, 16μM). The temperature and pH of the medium was adjusted as described in the text. The medium was equilibrated with air and then divided into portions for the determination of the proton electrochemical gradient, respiratory rates and where indicated, for the determination of calcium associated with the mitochondria.

To compensate for the endogenous proton conductance of the membrane it is necessary to determine respiration and Δp both in the presence and the absence of the ionophore A23187 (eqn. 11), and this was done in parallel experiments. All samples were preincubated in the basic medium for 2min in the presence of the appropriate concentrations of $CaCl_2$ and further additions as detailed in the Results section. Respiratory rates were determined in this medium by a Clark-type oxygen electrode in a chamber with a capacity of 1.2ml. The components of the proton electrochemical gradient were determined in an identical medium, with the further additions of 50μM-^{86}RbCl (0.05μCi/ml), 100μM-[^{14}C]methylamine (0.1μCi/ml) and 100μM-[^{3}H]acetate (1μCi/ml), as previously described (Nicholls, 1974*c*). The distribution of the isotopes between the matrix and medium was determined after rapid filtration of the mitochondria through Sartorius (Gottingen, West Germany) 0.6μm-pore cellulose nitrate filters, and membrane potentials and transmembrane pH gradients were determined exactly as previously described (Nicholls, 1974*c*).

The [^{14}C]sucrose-impermeable and ^3H_2O-permeable space of mitochondrial incubations was determined in a medium containing 75mM-NaCl, 10mM-Tes (sodium salt), pH7.2, 1mM-KCl, 2mM-succinate, 1μCi of ^3H_2O/ml and 0.1μCi of [^{14}C]sucrose/ml. Mitochondria were separated by centrifugation for 2min in a bench microcentrifuge. A mean matrix volume (16 determinations) of $0.73 \pm 0.17$$\mu$l/mg of protein was obtained. Although this represents a value for the anaerobic mitochondrial pellet after centrifugation, it would be expected that the matrix volume of mitochondria supporting a proton electrochemical gradient would be significantly smaller, as Na^+ will tend to be expelled, on the Na^+/H^+ antiporter (Mitchell & Moyle, 1969*b*), as will Cl^-, owing to the significant Cl^- uniport of liver mitochondria (Mitchell & Moyle, 1969*b*). The proton electrochemical gradient thus tends to decrease the matrix volume (Nicholls, 1974*b*), with the result that values for the proton electrochemical gradient may be slightly underestimated.

Mitochondrial calcium association was determined in incubations containing $^{45}Ca^{2+}$ and ^3H_2O. Mitochondria were filtered through Sartorius 0.6μm cellulose nitrate membrane filters, and counted for radioactivity by the same technique as for the proton electrochemical gradient determination (Nicholls, 1974*c*).

Buffers of calcium activity were prepared with nitrilotriacetate as described by Reed & Bygrave (1975*b*).

Results

For the distribution of calcium activities across the mitochondrial inner membrane to reflect ΔpH, and hence be calculable, ionophore A23187 must be present in a sufficient excess. In Fig. 1, the effect of increasing the ionophore concentration on the respiratory rate, calcium distribution and components of the proton electrochemical gradient, are plotted as a function of the concentration of ionophore A23187 present in the incubation medium. The initial incubation medium contained calcium at a concentration of 50 μM. A clearly defined crossover is apparent when the rate of calcium transport by the native carrier becomes rate-limiting, manifested by a decrease in the $^{45}Ca^{2+}$ associated with the mitochondria (Fig. 1*b*). At the same time, respiration increases to a maximum value, whereas the components of the proton electrochemical gradient decrease (Fig. 1*a*). Under the conditions of this experiment, the highest concentration of ionophore allows a ΔpH of +0.25 unit to be maintained across the membrane, which at thermodynamic equilibrium (eqn. 4) implies a ratio of $[Ca^{2+}]in/[Ca^{2+}]out$ across the membrane of only 3.2:1, concentrated in the matrix. Fig. 1(*b*) demonstrates that very little calcium is associated with the mitochondria under these conditions, as would be predicted. In subsequent experiments, the maintenance of a calcium association with the mitochondria compatible with the measured pH gradient was taken as evidence that the ionophore was present in excess. The concentration of ionophore A23187 used in subsequent experiments was 1.3 nmol/mg of protein. It should be emphasized that the action of ionophore A23187 is clearly distinguishable from that of a proton translocator ('uncoupler') such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Once ionophore A23187 is present in an excess over the native calcium carrier, no further increase in the total proton conductance of the membrane occurs. Thus respiration and the proton electrochemical gradient reach a plateau. In contrast, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone causes an increase in proton conductance of the mitochondrial inner membrane which is directly proportional to the translocator added, and which never appears to saturate (Nicholls, 1974*a*).

Table 2 shows the calculation of the apparent calcium conductance in the experiment depicted in Fig. 1. Under these conditions, a conductance of 0.86 nmol·min^{-1}·mg^{-1}·mV^{-1} was obtained, which should be compared with the value for the endogenous proton conductance of 0.3 nmol of H^+·min^{-1}·mg^{-1}·mV^{-1}.

It is significant that calcium cycling is capable of lowering the proton electrochemical gradient to less than 100 mV. As might be expected (Nicholls, 1974*a*), this gradient is insufficient to control the rate of

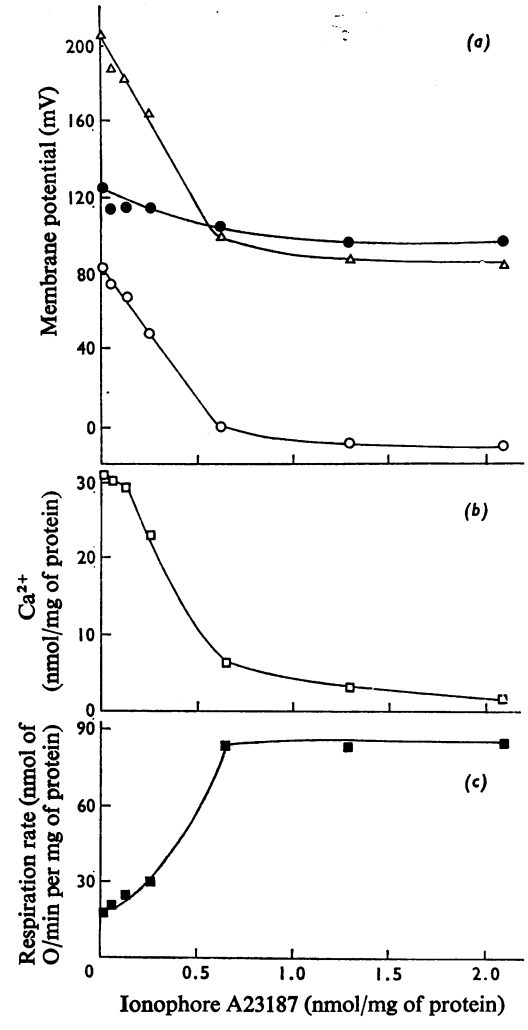


Fig. 1. *Influence of ionophore A23187 on the components of the proton electrochemical gradient, on Ca^{2+} association with and on the respiration of mitochondria respiring in the presence of Ca^{2+}*

The incubation medium contained 75 mM-NaCl, 10 mM-Tes, 2 mM-sodium succinate, 2 μM-rotenone, 0.5 μM-valinomycin, 1 mM-KCl, 50 μM-$CaCl_2$ and 16 μM-albumin, pH 7.0; temperature 23°C. (*a*) Membrane potential (●), $-59\Delta pH$ (○) and Δp (△) after 2 min incubation in the presence of various concentrations of the ionophore A23187. The components of Δp were determined as described in the Experimental section. (*b*) Association of $^{45}Ca^{2+}$ with mitochondria after 2 min preincubation with ionophore A23187. (*c*) Respiration of mitochondria 2 min after the addition of ionophore A23187. The mitochondrial concentration in each case was 1.5 mg of protein/ml of medium.

Table 2. *Calculation of the endogenous proton conductance and the apparent calcium conductance of the mitochondrial inner membrane*

The incubation medium contained 75mM-NaCl, 10mM-Tes, 2mM-sodium succinate, 1μM-rotenone, 0.5μM-valinomycin, 1mM-KCl, 50μM-$CaCl_2$ and 16μM-albumin. Mitochondria were incubated for 2min in the presence or the absence of 1.3nmol/mg of protein of ionophore A23187. In parallel experiments, respiratory rate and the components of the proton electrochemical gradient were determined.

Function		−Ionophore A23187	+Ionophore A23187
Respiration	(nmol of $O \cdot min^{-1} \cdot mg$ of $protein^{-1}$)	17.2 (R^-)	84.4 (R^+)
Total JH^+	(nmol of $H^+ \cdot min^{-1} \cdot mg$ of $protein^{-1}$)	69	338
ΔE	(mV)	133	106
$-59\Delta pH$	(mV)	+92	−15
Δp	(mV)	225 (Δp^-)	91 (Δp^+)
$C_M H^+$	(nmol of $H^+ \cdot min^{-1} \cdot mg$ of $protein^{-1} \cdot mV^{-1}$)	0.31	—
$C_M Ca^{2+}$	(nmol of $Ca^{2+} \cdot min^{-1} \cdot mg$ of $protein^{-1} \cdot mV^{-1}$)	—	0.86

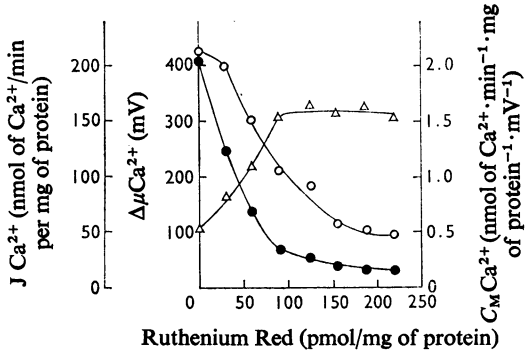


Fig. 2. *Influence of Ruthenium Red on the calcium conductance of the mitochondrial inner membrane*

The incubation medium was as described in Fig. 1, pH7.0, temperature 23°C. The following parameters were calculated as described in the Experimental section: JCa^{2+}, the current of Ca^{2+} across the membrane (○); $\Delta\mu Ca^{2+}$, the electrochemical gradient of calcium across the native Ca^{2+} carrier (△); $C_M Ca^{2+}$, the apparent calcium conductance (●). Mitochondria (1.5mg of protein/ml of medium) were preincubated for 2min in the presence of the indicated concentrations of Ruthenium Red.

respiration, as additions of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone did not stimulate mitochondrial respiration in the presence of an excess of ionophore A23187 (results not shown). It is therefore apparent that the rate-limiting step under the conditions of Table 2 is the rate of proton translocation by the respiratory chain, rather than the activity of the calcium carrier, which if rate-limiting would result in a build-up of a high Δp with the induction of respiratory control. Thus it is of doubtful validity to refer to the 'maximal velocity' of calcium transport from uptake experiments in the presence of substrates such as succinate, as the rates obtained are more applicable to the maximum rates of respiratory-chain-linked proton extrusion. This further emphasizes the relevance of determining both the calcium current and the electrochemical gradient.

Influence of Ruthenium Red and lanthanum on the calcium conductance

Ruthenium Red is firmly established as an inhibitor of calcium transport into the mitochondrial matrix (Moore, 1971; Vasington *et al.*, 1972; Reed & Bygrave, 1974*b*); Fig. 2 shows its effect on the apparent calcium conductance. As the Ruthenium Red concentration is increased, the calcium electrochemical gradient increases, and the current of Ca^{2+} decreases. Thus the apparent calcium conductance of the membrane decreases rapidly with increasing inhibitor. Maximum inhibition of the conductance requires about 150pmol of the recrystallized inhibitor/mg of protein (Fletcher *et al.*, 1961) and the extent of inhibition is greater than 90%.

These concentrations of Ruthenium Red did not significantly inhibit the endogenous proton conductance of the membrane as determined in the absence of ionophore A23187. Stucki & Ineichen (1974) have reported that a slow apparent cycling of calcium in the absence of ionophore A23187 contributes to the respiratory rate, and hence to what is measured in the present work as the endogenous proton conductance of the membrane. At 37°C and pH7.4, this cycling was found by the above authors to amount to a calcium current of 3.5nmol $\cdot min^{-1} \cdot mg$ of prote in^{-1}, which is some two orders of magnitude below that obtained in the present work in the presence of the ionophore (Fig. 2). Thus the error induced by ignoring any ionophore-independent calcium cycling is very small.

Lanthanides inhibit initial rates of calcium uptake by mitochondria (Mela, 1968; Lehninger & Carafoli, 1971; Reed & Bygrave, 1974*a*,*b*). The effect of preincubating with La^{3+} on the calcium conductance is depicted in Fig. 3. As with Ruthenium Red, an inhibition of the calcium current, together with an increased calcium electrochemical gradient across the

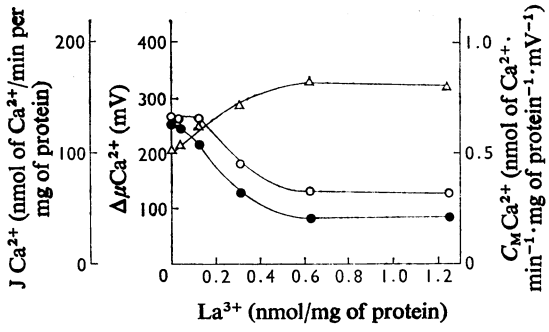


Fig. 3. *Influence of $LaCl_3$ on the calcium conductance of the mitochondrial inner membrane*

The incubation medium was as described in Fig. 1. Mitochondria (1.5mg of protein/ml of medium) were preincubated for 2min in the presence of the indicated concentrations of $LaCl_3$. ○, JCa^{2+}; △, $\Delta\mu Ca^{2+}$; ●, $C_M Ca^{2+}$.

carrier, indicate an inhibition of the calcium conductance. The apparent sigmoid inhibition of the conductance most probably relates to the slow uptake of La^{3+} that has been observed (Reed & Bygrave, 1974*a*,*b*), with the result that low La^{3+} concentrations in the matrix are depleted during the 2min preincubation period before the addition of ionophore A23187.

As La^{3+} is very firmly chelated by ionophore A23187 (Pfeiffer *et al.*, 1974; Puskin & Gunter, 1975), and thus presumably lowers the concentration of ionophore available to transport Ca^{2+} out of the matrix, it was important to establish that La^{3+} was inhibiting the native calcium carrier, rather than the ionophore. This was confirmed by determining the amounts of $^{45}Ca^{2+}$ associated with the mitochondria at each concentration of La^{3+}. As this association remained low, the ionophore was not catalysing the rate-limiting step (see Fig. 1).

Influence of Mg^{2+} on the calcium conductance

Mg^{2+} is not transported by the native calcium carrier in liver mitochondria (Lehninger *et al.*, 1967). Up to 1mM Mg^{2+} had no effect on either the endogenous proton conductance or the apparent calcium conductance. As ionophore A23187 can chelate and transport Mg^{2+} (Reed & Lardy, 1972), controls were performed with $^{45}Ca^{2+}$ which confirmed that Mg^{2+} concentrations up to 1mM did not prevent the ionophore from maintaining a low mitochondrial calcium concentration.

Ionophore A23187 induces a Mg^{2+} efflux from mitochondria analogous to that for Ca^{2+} (Reed & Lardy, 1972). It is therefore significant that the inclusion of Mg^{2+} in the medium, which limits matrix depletion of the cation, does not influence the ion conductances. Thus the changes in respiration and proton electrochemical gradient are unlikely to be artifacts resulting from alterations in the concentration of matrix Mg^{2+}.

Dependence of the proton and calcium conductances on the calcium activity in the medium

Mitochondria *in situ* are exposed to calcium activities in the region of 1μM. It is therefore of most significance to establish the conductance of the membrane to calcium when mitochondria are exposed to such concentrations of the cation. As discussed by Reed & Bygrave (1975*a*,*b*), true calcium activities in the micromolar range can only be maintained in the presence of suitable buffers, as failure to correct for chelation has led to variations in published K_m values from 1μM to 100μM (Chance, 1965; Drahota *et al.*, 1965; Chance & Schoener, 1966; Scarpa & Azzone, 1970; Bygrave *et al.*, 1971; Carafoli & Azzi, 1972; Spencer & Bygrave, 1973; Vinogradov & Scarpa, 1973; Scarpa, 1974; Sordahl, 1974; Wohlrab, 1974; Bygrave *et al.*, 1975; Reed & Bygrave, 1975*a*).

Fig. 4 depicts the effect of varying the initial free calcium concentration of the medium on the endogenous proton conductance of the membrane, and on the calcium electrochemical gradient, current and conductance. The endogenous proton conductance itself was found to vary with the initial free calcium concentration in the medium (Fig. 4). An increase in proton conductance was also found when free calcium concentrations were lowered with EGTA (results not shown). The calcium activities in the medium were calculated on the assumption that the addition of mitochondria will not significantly influence the calcium content of the medium, as ionophore A23187 prevents calcium accumulation by the mitochondria. The endogenous Ca^{2+} content of mitochondria prepared in the initial presence of EDTA was found to be 18nmol of Ca^{2+}/mg of protein by atomic absorption spectrophotometry of HCl extracts of mitochondria. This is somewhat higher than previously reported values in the literature (Chance & Mela, 1966; Carafoli & Lehninger, 1971). If all this Ca^{2+} is assumed to contribute to the pool of circulating cation, this would have the effect of raising the free Ca^{2+} concentration in equilibrium with nitrilotriacetate by 0.2μM. This correction has not been made in Fig. 4.

Fig. 4 demonstrates that there is a fivefold increase in the apparent Ca^{2+} conductance on increasing the activity of free Ca^{2+} in the medium from 0.5 to 10μM. The apparent Ca^{2+} conductance does not tend to zero even at very low free calcium concentrations. However, the same residual conductance persists both

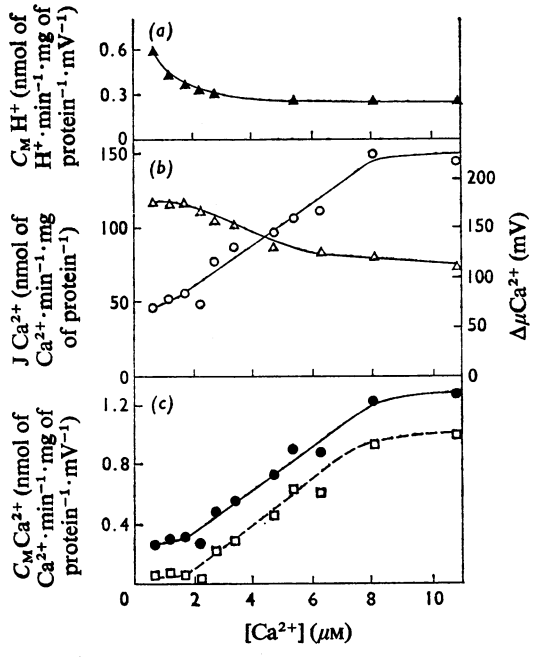


Fig. 4. *Influence of medium calcium activity on the calcium and proton conductances of the mitochondrial inner membrane*

The incubation medium contained 75mM-NaCl, 10mM-Tes, 2mM-succinate, 2μM-rotenone, 0.5μM-valinomycin, 1mM-KCl, 16μM-albumin, 5mM-sodium nitrilotriacetate, with the further addition of $CaCl_2$ to give final free Ca^{2+} concentrations in the range 0.5–10μM. Mitochondria were preincubated for 2min at 23°C, pH7.2, before determination of the proton and calcium conductances. ○, J Ca^{2+}; △, $\Delta\mu Ca^{2+}$; ▲, $C_M H^+$; ●, $C_M Ca^{2+}$ (calculated as described in the Experimental section); □, $C_M Ca^{2+}$ (calculated after subtracting the Ruthenium Red-insensitive component, see the text).

in the presence of sufficient EGTA to decrease the free Ca^{2+} to 1nM, and also after the addition of Ruthenium Red (Fig. 2). When this Ruthenium Red-insensitive component is subtracted, a sigmoidal dependence of the apparent calcium conductance on the free calcium activity is obtained (given by the broken line in Fig. 4), half-maximum conductance being achieved with 4.7μM-free calcium. Initial-rate studies on mitochondria from a variety of sources have shown sigmoidal relationships similar to that shown in Fig. 4 (Bygrave *et al.*, 1971; Scarpa & Grazzioti, 1973; Vinogradov & Scarpa, 1973; Bygrave *et al.*, 1975; Reed & Bygrave, 1975*a*), a possible exception being mitochondria prepared from smooth muscle (Wikström *et al.*, 1975).

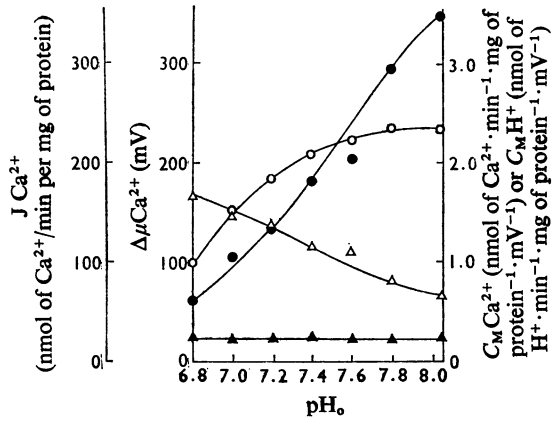


Fig. 5. *Influence of medium pH on the proton and calcium conductances of the mitochondrial inner membrane*

The incubation medium was as described in Fig. 1. The initial pH of the medium was varied from 6.8 to 8.05. Conductances were determined after a 2min preincubation. ○, J Ca^{2+}; △, $\Delta\mu Ca^{2+}$; ▲, $C_M H^+$; ●, $C_M Ca^{2+}$.

Dependence of the endogenous calcium and proton conductances on the pH of the medium

The weak-acid and weak-base indicators of ΔpH, acetate and methylamine have been shown to retain the ability to indicate ΔpH over a range of medium pH from 6.8 to 8.2 (Nicholls, 1974*a*). Thus the electrochemical ion gradients, and hence the ion conductances, can be determined over this range of pH (Fig. 5). Although the endogenous proton conductance is completely independent of the medium pH over the range examined, the calcium conductance shows a high pH-sensitivity, with a 3.5-fold increase as the pH is raised from 7.0 to 8.0. This increase is manifested not only in an increase in the calcium current through the native carrier under the experimental conditions, which, as discussed above, could depend on the maximum activity of the respiratory chain, but also in a decrease in the calcium electrochemical gradient required to drive this current. Control experiments with $^{45}Ca^{2+}$ confirmed that the ionophore remained in excess at each pH value.

Temperature-sensitivity of the endogenous proton and calcium conductances

The endogenous proton conductance of the membrane is depicted as a function of temperature in Fig. 6. It is significant that the magnitude of the proton electrochemical gradient (Δp) appears to be largely independent of temperature over the range 23–36°C. At higher temperatures there is a slight fall in Δp, but this can be accounted for by the need to sustain

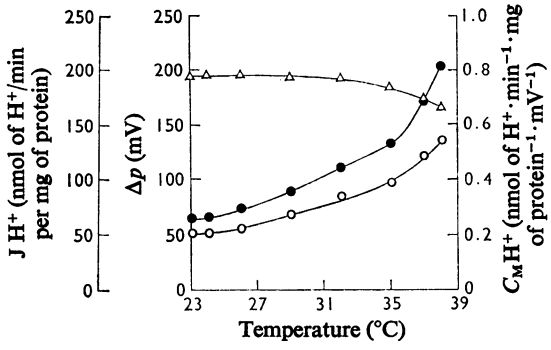


Fig. 6. *Influence of temperature on the endogenous proton conductance of the mitochondrial inner membrane*

The incubation medium was as described in Fig. 1. The incubation temperature was varied from 23° to 38°C, and the conductance was determined after a 2min preincubation. ○, JH^+; △, Δp; ●, C_MH^+.

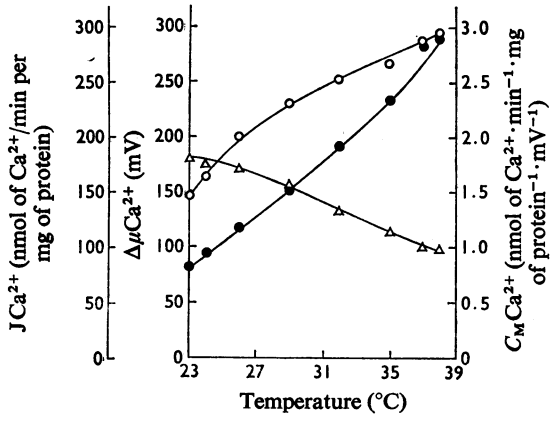


Fig. 7. *Influence of temperature on the calcium conductance of the mitochondrial inner membrane*

The incubation medium was as described in Fig. 1. The incubation temperature was varied from 23° to 38°C, and the conductance was determined after a 2min preincubation. ○, JCa^{2+}; △, $\Delta\mu Ca^{2+}$; ●, C_MCa^{2+}.

an increased proton current, because of the increased proton conductance of the membrane. It may be noted that the endogenous proton conductance shows an enhanced temperature-sensitivity over physiological temperature ranges.

Fig. 7 depicts the temperature-dependence of the calcium current, the calcium electrochemical gradient and the apparent calcium conductance for mitochondria oxidizing succinate at pH7.0. Over the temperature range studied, the calcium current doubles, whereas the calcium electrochemical gradient decreases to half, corresponding to a fourfold increase in the apparent calcium conductance of the membrane. Although this temperature coefficient corresponds to an Arrhenius activation energy of 69kJ/mol, this value should be treated with reservation, as a significant deviation from ohmic behaviour by the calcium carrier would make the absolute value for the conductance dependent on the calcium current, which, as discussed above, is likely to be a function of the maximum activity of the respiratory chain.

Discussion

The rate of transport of an ion across a biological membrane depends on the existence of a pathway for the ion and on the magnitude of the electrochemical gradient across the carrier. Although numerous studies have been made of the rates of calcium transport across the mitochondrial inner membrane, there have been few, if any, attempts to quantify the electrochemical gradient of Ca^{2+} driving calcium transport. Since Mitchell (1966) postulated the identity of the high-energy state with a proton electrochemical gradient across the inner membrane, there has been a growing consensus that calcium accumulation by mitochondria is a consequence of an electrophoretic transport of the ion into the matrix in response to a membrane potential maintained by a net extrusion of protons by the respiratory chain or the proton-translocating adenosine triphosphatase.

As the rate of calcium uptake is far more amenable to measurement than the magnitude of the calcium electrochemical gradient, there is a tendency to ignore the role of the driving force in the study of calcium uptake. As discussed above, this is due to the virtually insurmountable problem of determining a time-dependent membrane potential and ratio of free calcium activities (see eqn. 1). It has been generally assumed (e.g. Reed & Bygrave, 1975*a*) that the initial rate of calcium uptake is limited by neither the rate of charge-compensating proton extrusion by the respiratory chain nor the magnitude of the membrane potential. The current study, however, demonstrates that the calcium conductance of liver mitochondria is sufficient to decrease the steady-state proton electrochemical gradient to about 100mV (Table 1), a value at which no respiratory control is observed (Nicholls, 1974*a*). Thus the rate-limiting step for calcium uptake can easily be the rate of proton translocation by the respiratory chain, rather than at a site directly related to the calcium carrier. The technique described in this paper is still subject to this rate-limitation, as the rate at which calcium can cycle across the membrane (Scheme 1) cannot be greater than one-half of the rate at which respiration-dependent proton translocation

is occurring. However, this paper does measure the magnitude of the electrochemical gradient needed to maintain this rate of calcium transport. If the conductance of calcium is ohmic (i.e. if the rate of calcium transport was directly proportional to the calcium electrochemical gradient) then the value obtained for the conductance would be independent of the activity of the respiratory chain, a lowering of respiratory rate, and hence of calcium cycling (Scheme 1), being matched by a decrease in the calcium electrochemical gradient. As preliminary observations (G. M. Heaton & D. G. Nicholls, unpublished work) suggest significant deviations from ohmic behaviour for the calcium carrier, then the rate of respiration will influence somewhat the value of $C_M Ca^{2+}$, which has thus been termed the apparent calcium conductance.

The values obtained for the apparent calcium conductance are not dramatically high, being only one order of magnitude greater than the leakage conductance of the membrane to protons, and substantially less than the purine-nucleotide-sensitive proton conductance of brown-adipose-tissue mitochondria (Nicholls, 1974*a*).

The present paper has been concerned mainly with providing sufficient controls to establish beyond reasonable doubt that the parameter measured is the apparent calcium conductance of the inner membrane. Once this system is established it is clear that it may be used to investigate aspects of the behaviour of the native calcium carrier which could not be observed by using uptake studies alone.

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