Biochem. J. (1978) **176**, 463–474
Printed in Great Britain

The Regulation of Extramitochondrial Free Calcium Ion Concentration by Rat Liver Mitochondria

By DAVID G. NICHOLLS
Department of Psychiatry, University of Dundee, Ninewells Medical School, Dundee DD1 9SY, Scotland, U.K.

(*Received* 3 *April* 1978)

The mechanism whereby rat liver mitochondria regulate the extramitochondrial concentration of free Ca^{2+} was investigated. At 30°C and pH7.0, mitochondria can maintain a steady-state pCa_o^{2+} (the negative logarithm of the free extramitochondrial Ca^{2+} concentration) of 6.1 (0.8 μM). This represents a true steady state, as slight displacements in pCa_o^{2+} away from 6.1 result in net Ca^{2+} uptake or efflux in order to restore pCa_o^{2+} to its original value. In the absence of added permeant weak acid, the steady-state pCa_o^{2+} is virtually independent of the Ca^{2+} accumulated in the matrix until 60nmol of Ca^{2+}/mg of protein has been taken up. The steady-state pCa_o^{2+} is also independent of the membrane potential, as long as the latter parameter is above a critical value. When the membrane potential is below this value, pCa_o^{2+} is variable and appears to be governed by thermodynamic equilibration of Ca^{2+} across a Ca^{2+} uniport. Permeant weak acids increase, and *N*-ethylmaleimide decreases, the capacity of mitochondria to buffer pCa_o^{2+} in the region of 6 (1 μM-free Ca^{2+}) while accumulating Ca^{2+}. Permeant acids delay the build-up of the transmembrane pH gradient as Ca^{2+} is accumulated, and consequently delay the fall in membrane potential to values insufficient to maintain a pCa_o^{2+} of 6. The steady-state pCa_o^{2+} is affected by temperature, incubation pH and Mg^{2+}. The activity of the Ca^{2+} uniport, rather than that of the respiratory chain, is rate-limiting when pCa_o^{2+} is greater than 5.3 (free Ca^{2+} less than 5 μM). When the Ca^{2+} electrochemical gradient is in excess, the activity of the uniport decreases by 2-fold for every 0.12 increase in pCa_o^{2+} (fall in free Ca^{2+}). At pCa_o^{2+} 6.1, the activity of the Ca^{2+} uniport is kinetically limited to 5nmol of Ca^{2+}/min per mg of protein, even when the Ca^{2+} electrochemical gradient is large. A steady-state cycling of Ca^{2+} through independent influx and efflux pathways provides a model which is kinetically and thermodynamically consistent with the present observations, and which predicts an extremely precise regulation of pCa_o^{2+} by liver mitochondria *in vivo*.

The most probable physiological role played by mitochondrial Ca^{2+} transport is in the regulation of the concentration of free Ca^{2+} in the cytoplasm, and hence in the control of Ca^{2+}-dependent enzymes [for review see Bygrave (1977)]. In contrast with excitable tissues, where the free Ca^{2+} in the cytoplasm can change substantially and rapidly (Ashley & Caldwell, 1974; Douglas, 1974), it is to be expected that the liver operates under conditions in which major deviations from a steady-state distribution of Ca^{2+} between mitochondria and cytoplasm do not occur. It follows that it is those factors that control this steady state that are of prime physiological relevance.

In 1965 it was first suggested that the accumulation of Ca^{2+} by mitochondria might be a dynamic steady state established between a respiration-linked influx and a continuous efflux (Drahota *et al.*, 1965). More recently, evidence has been advanced that, although the predominant pathway of Ca^{2+} transport is an electrophoretic Ca^{2+} uniport (Selwyn *et al.*, 1970; Rottenberg & Scarpa, 1974; Reynafarje & Lehninger, 1977; but see Moyle & Mitchell, 1977*a*,*b*,*c*), an independent efflux mechanism exists whose exact stoicheiometry remains to be established (Vasington *et al.*, 1972; Sordahl, 1974; Stucki & Ineichen, 1974; Crompton *et al.*, 1976; Puskin *et al.*, 1976; Azzone *et al.*, 1977; Nicholls, 1978).

Evidence for an independent efflux mechanism has been advanced on both thermodynamic and kinetic grounds. Thus attempts to estimate the thermodynamic activity of Mn^{2+} and Ca^{2+} in the mitochondrial matrix by e.p.r. techniques (Puskin & Gunter, 1973; Bragadin *et al.*, 1975; Pozzan *et al.*, 1976; Azzone *et al.*, 1976) and osmotic effects respectively (Azzone *et al.*, 1976) have led to the conclusion that the distribution of free Ca^{2+} across the inner membrane can be less than would be predicted from ΔE (the membrane potential across the inner membrane determined from the Nernst

Abbreviations used: ΔE, membrane potential; ΔpH, transmembrane pH gradient; $\Delta\bar{\mu}_{H^+}$, proton electrochemical potential gradient; pCa_o^{2+}, negative logarithm of the free Ca^{2+} concentration in the extramitochondrial compartment; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}ethanesulphonic acid.

distribution of K^+ or Rb^+ in the presence of valinomycin) on the assumption of thermodynamic equilibration via a Ca^{2+} uniporter, but greater than that predicted for a Ca^{2+}/H^+ antiporter, or the thermodynamically equivalent $2Ca^{2+}/P_i^{2-}$ or (Ca monocarboxylate)$^+$ symporters which have been proposed (Moyle & Mitchell, 1977*a*,*b*,*c*). Only under conditions of low ΔE, high Ca^{2+} load and in the presence of permeant anion does the distribution of free Mn^{2+} (Puskin *et al.*, 1976) or total Ca^{2+} (Rottenberg & Scarpa, 1974) approximate to that predicted from electrochemical equilibrium across a bivalent-cation uniport.

It has therefore been proposed (Puskin *et al.*, 1976) that liver mitochondria achieve a steady state in which Ca^{2+} is continuously taken up by a Ca^{2+} uniport, and expelled by an undefined efflux pathway. However, that the Ca^{2+} distribution across the inner membrane of liver mitochondria represents a true steady state, rather than a slow approach to equilibrium, has only been deduced indirectly, by observing net efflux either when pCa_o^{2+} is lowered to 9 by addition of EGTA (Puskin *et al.*, 1976) or when Ruthenium Red is added (Vasington *et al.*, 1972; Sordahl, 1974; Puskin *et al.*, 1976; Crompton *et al.*, 1978). The action of Ruthenium Red has been shown to be complex (Pozzan *et al.*, 1977), and the use of the inhibitor has been avoided in the present work.

Although it is commonly asserted that mitochondria can lower the free extramitochondrial Ca^{2+} concentration to the region of 1 μM, no systematic investigation of the factors that regulate this parameter have been performed. As the effect of independent uptake and efflux mechanisms is that the steady-state Ca^{2+} distribution can be regulated by factors that alter the kinetics or the thermodynamic gradients of either pathway (Puskin *et al.*, 1976; Azzone *et al.*, 1977), the possibilities for regulation are clearly exceedingly complex. The present work sets out firstly to determine the steady-state pCa_o^{2+} maintained by mitochondria under a variety of metabolic conditions, and secondly to interpret these observations by inter-relating kinetic and thermodynamic parameters.

It is concluded that the properties of a steady-state Ca^{2+}-cycling system are such that liver mitochondria can act as almost perfect buffers of extramitochondrial free Ca^{2+} concentration under physiological conditions and that this parameter is capable of extremely precise regulation.

Experimental

Materials

All radioactive isotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Bovine serum albumin (fraction V) was obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K., and was exhaustively dialysed against distilled water before use. The Ca^{2+} content of sucrose stock solutions was minimized by equilibration with Dowex 50W (X8) before addition to media (Heaton & Nicholls, 1976). All other reagents were of analytical grade.

Rat liver mitochondria

Mitochondria were prepared as previously described (Heaton & Nicholls, 1976).

Methods

Ca^{2+} activity was measured in mitochondrial incubations by a Ca^{2+}-selective electrode (Radiometer type F2112Ca) with a KCl reference electrode (Radiometer type K801) immersed in an open-topped vessel of 3 ml capacity thermostatically controlled at 30°C unless otherwise stated. The electrodes were connected to a Pye type 292 pH-meter, the output from which was connected to a chart potentiometric recorder across a 100 Ω resistance.

The electrode specifications (catalogue no. 982-647 from Radiometer A/S. Emdrupvej 72, DK-2400 Copenhagen, Denmark) are such that the assembly does not respond accurately to total calcium concentrations below 5 μM. However, in the presence of at least 2 mM-nitrilotriacetate as Ca^{2+} chelator (Reed & Bygrave, 1975), free Ca^{2+} concentrations were linearly recorded down to 0.3 μM ($pCa^{2+} = 6.5$). This was sufficient to cover the range of pCa_o^{2+} encountered in the current investigation. To calibrate the electrode assembly, and to buffer extramitochondrial free Ca^{2+}, Ca^{2+}/nitrilotriacetate buffers were used [apparent stability constants 5.49×10^3 (pH6.8), 8.69×10^3 (pH7.0), 1.38×10^4 (pH7.2) and 2.17×10^4 (pH7.4)]. Because of the greater discrimination between Ca^{2+} and Mg^{2+} afforded by *N'*-(2-hydroxyethyl)ethylenediamine-*NNN'*-triacetate [apparent stability constants at pH 7.0, 2.57×10^5 (Ca^{2+}) and 1.12×10^3 (Mg^{2+})], this chelator was used in experiments in which the effect of Mg^{2+} was investigated. The influence of Mg^{2+} on the chelation of Ca^{2+} was estimated by the method of Portzehl *et al.* (1964). Routinely, the electrode was calibrated by buffers containing 75 mM-KCl, 5 mM-potassium nitrilotriacetate, 50 mM-Tes (potassium salt) and 14 μM-, 44 μM- or 135 μM-$CaCl_2$, giving (at 30°C and pH 7.0) free Ca^{2+} activities of 0.32 μM (pCa^{2+} 6.5), 1 μM (pCa^{2+} 6.0) and 3.2 μM (pCa^{2+} 5.5) respectively. Because of the sensitivity of the electrode to hydrophobic ionophores, it was found to be essential to avoid the use of compounds such as valinomycin and proton translocators. To calculate the matrix Ca^{2+} content from the pCa_o^{2+} determined by the electrode,

the total extramitochondrial Ca^{2+} concentration was calculated by applying the apparent stability constant for nitrilotriacetate, and this was then subtracted from the total Ca^{2+} content of the incubation mixture, including endogenous Ca^{2+}.

The concentration of endogenous Ca^{2+} associated with freshly prepared mitochondria under the present conditions of preparation has been found to be 18nmol/mg of protein (Heaton & Nicholls, 1976). The contamination of incubation media with endogenous Ca^{2+} was determined by atomic absorption spectrophotometry, by using a Corning EEL model 240 mark II atomic absorption spectrophotometer.

$^{45}Ca^{2+}$ association with mitochondria was determined by filtration on 0.6μm-pore-size Sartorius cellulose nitrate filters (Göttingen, West Germany), with the use of ^3H_2O to estimate contamination of the filters with medium as previously described (Heaton & Nicholls, 1976). The components of the proton electrochemical potential gradient ($\Delta\tilde{\mu}_{H^+}$) were determined as previously described (Nicholls, 1974) in incubation media containing 0.5μM-valinomycin, but otherwise identical with the incubations used for the Ca^{2+} electrode.

Results

Mitochondrial incubations are of necessity of short duration, and therefore the apparently constant pCa_o^{2+}, which is usually attained after a few minutes' incubation with Ca^{2+}, could presumably be ascribed not to the achievement of steady-state cycling conditions (Puskin *et al.*, 1976; Azzone *et al.*, 1977) but to the insufficient time to complete a slow approach to equilibrium within the time course of the experiment. Although this apparent steady state can be modulated by inhibition of the Ca^{2+} uniport by Ruthenium Red (Puskin *et al.*, 1976; Crompton *et al.*, 1978), a direct demonstration of a true steady-state pCa_o^{2+} requires that the same pCa_o^{2+} be attained not only by net uptake from a medium but also by a net efflux of matrix Ca^{2+} into a medium whose initial free Ca^{2+} concentration was displaced slightly below the steady-state value.

This was achieved in the experiment shown in Fig. 1. In trace (*a*), mitochondria were allowed to accumulate Ca^{2+} until an apparently constant pCa_o^{2+} of 6.1 was attained (0.8μM-free Ca^{2+}). Nitrilotriacetate (4mM) was present in the incubation medium to buffer extramitochondrial Ca^{2+} (Reed & Bygrave, 1975), and, under these conditions, the mitochondria were capable of accumulating about 50% of the added Ca^{2+}. In trace (*b*), nitrilotriacetate was omitted, and the mitochondria accumulated at least 90% of the added Ca^{2+}. The electrode underestimates pCa_o^{2+} values greater than 5.3 (free Ca^{2+} less than 5μM) in the absence of Ca^{2+}-containing buffer (see the Experimental section). The addition of 4mM-nitrilotriacetate

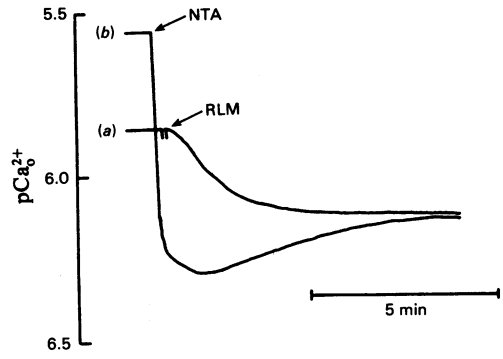

Fig. 1. *Reversible maintenance of a steady-state pCa_o^{2+} by liver mitochondria*

Rat liver mitochondria (1.5mg of protein/ml of incubation mixture) were incubated at 30°C and pH7.0 in an incubation medium containing 75mM-NaCl, 10mM-Tes (sodium salt), 16μM-albumin, 1μM-rotenone, 2mM-sodium succinate, 5mM-sodium acetate and a total Ca^{2+} concentration (including endogenous mitochondrial Ca^{2+}) of 54μM. In trace (*a*) 4mM-sodium nitrilotriacetate was initially present, and mitochondria were added where indicated (RLM). In trace (*b*), mitochondria were added initially and were allowed to accumulate Ca^{2+} for 2min before the addition of 4mM-nitrilotriacetate (NTA). In trace (*b*), pCa_o^{2+} was not accurately recorded before the addition of nitrilotriacetate (see the Experimental section). The traces were corrected for the response time of the electrode assembly.

at this point thus chelates the residual extramitochondrial Ca^{2+} and instantaneously lowers the free Ca^{2+} below the concentration attained in trace (*a*). As an efflux of matrix Ca^{2+} occurs to restore pCa_o^{2+} to precisely the value attained in the first trace, it can be concluded that the maintained pCa_o^{2+} represents a true steady state.

In the light of the steady-state maintenance of Ca^{2+} concentration by mitochondria, it is of physiological relevance to determine the rate at which a steady-state pCa_o^{2+} is regained after a slight displacement to higher or lower pCa_o^{2+}. To this end, the data of Fig. 1 were used to compute rates of uptake and efflux as a function of the displacement (Fig. 2). When pCa_o^{2+} was only 0.1 unit lower than the steady state, there was a net uptake of Ca^{2+} of 5nmol/min per mg of mitochondrial protein. Efflux rates when pCa_o^{2+} was higher than the steady-state value were somewhat lower, but still reached 1.2nmol/min per mg of protein when pCa_o^{2+} was 0.1 unit higher than at steady state. As the traces were not corrected for the response time of the electrode, these are minimal estimates.

Although these rates are low in comparison with

a rate of Ca^{2+} cycling of 450nmol/min per mg of mitochondrial protein, which can be attained at 30°C with high external Ca^{2+} concentrations in the presence of excess ionophore A23187 [Fig. 12; see also Heaton & Nicholls (1976), Hutson *et al.* (1976) and Hutson (1977)], in the intact hepatocyte, with 60mg of mitochondrial protein/g wet wt., displacements of ±0.1 in cytoplasmic pCa_o^{2+} would be countered by rates of uptake or efflux by the mitochondria of respectively 5 and 1.2nmol of Ca^{2+}/s per g wet wt. at 30°C.

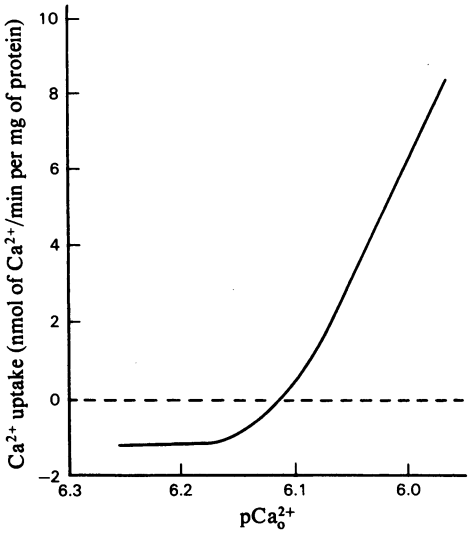

Fig. 2. *Net rates of Ca^{2+} movement between mitochondrial matrix and extramitochondrial space as a function of displacement from the steady-state pCa_o^{2+}*
Results were computed from the data of Fig. 1. Total extramitochondrial Ca^{2+} was calculated as a function of pCa_o^{2+} (see the Experimental section).

Dependence of pCa_o^{2+} on matrix Ca^{2+} content

If the distribution of Ca^{2+} across the inner membrane were governed primarily by the electrochemical equilibrium of the Ca^{2+} uniport, then it would be predicted, to a first approximation, that the extramitochondrial Ca^{2+} activity would increase in proportion to the Ca^{2+} content of the matrix. Fig. 3 shows that, in contrast, the steady-state pCa_o^{2+}, attained after addition of up to 50nmol of Ca^{2+}/mg of protein, was essentially independent of the amount of added Ca^{2+}. Only with the largest addition (100nmol/mg of protein) did the steady-state pCa_o^{2+} fall below 6 (i.e. free Ca^{2+} exceeds 1 μM). The addition of 10mM-acetate as a permeant anion had no significant effect on the steady-state pCa_o^{2+}, except with the largest addition of Ca^{2+}. Phosphate (2mM) produced the same results as acetate (not shown) and had no effect on the pCa_o^{2+} maintained after small additions of Ca^{2+}.

To investigate how pCa_o^{2+} varies as a function of the Ca^{2+} accumulated in the matrix, rather than the Ca^{2+} added, parallel experiments were performed with both the Ca^{2+}-selective electrode and $^{45}Ca^{2+}$ (Fig. 4). In the electrode experiment, the Ca^{2+} content of the matrix was calculated from pCa_o^{2+}

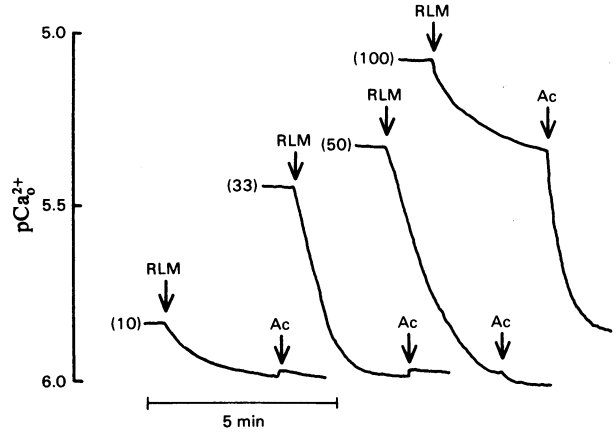

Fig. 3. *pCa_o^{2+} attained by mitochondria after various additions of Ca^{2+}*
Mitochondria (1.0mg of protein/ml of incubation mixture) were incubated at pH7.0 and 30°C in a medium containing 75mM-KCl, 10mM-Tes (potassium salt), 16μM-albumin, 0.5μM-rotenone, 2mM-potassium succinate and 2mM-potassium nitrilotriacetate. $CaCl_2$ (10–100nmol/mg of mitochondrial protein) was additionally present (amounts in parentheses). Mitochondria (RLM) and 10mM-potassium acetate (Ac) were added where indicated.

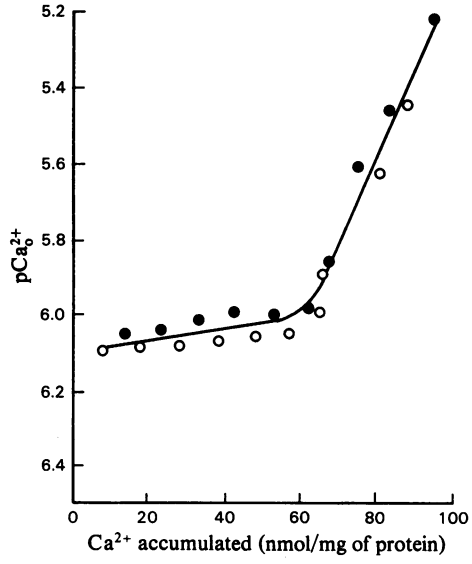

Fig. 4. *Steady-state pCa_o^{2+} as a function of Ca^{2+} accumulated in the matrix in the absence of added permeant weak acid*

Mitochondria (1.5mg of protein/ml of incubation) were incubated at 30°C and pH7.0 in a medium containing 75mM-NaCl, 10mM-Tes (sodium salt), 16μM-albumin, 1μM-rotenone, 2mM-sodium succinate and 2mM-sodium nitrilotriacetate, with various additions of $CaCl_2$ from 0 to 250μM final concentration. ○, pCa_o^{2+} was measured with the Ca^{2+}-selective electrode, and Ca^{2+} accumulation was calculated as described in the Experimental section. ●, Ca^{2+} accumulation was determined isotopically (see the Experimental section) and pCa_o^{2+} calculated from the residual extramitochondrial Ca^{2+} by using the apparent stability constant for nitrilotriacetate.

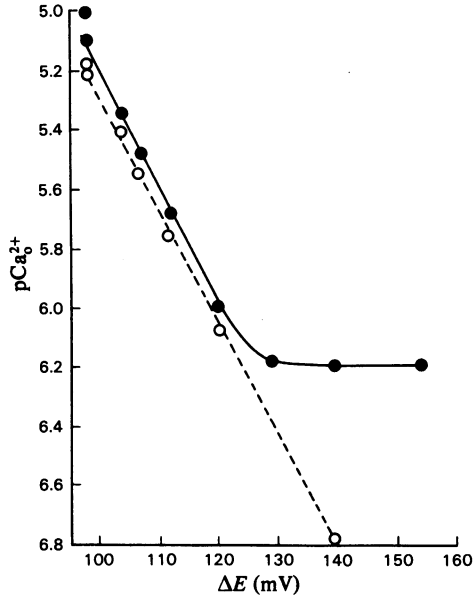

Fig. 5. *Steady-state pCa_o^{2+} as a function of ΔE when the latter is varied by increasing Ca^{2+} in the matrix*

Mitochondria were incubated under the conditions of Fig. 4, with the addition of 0.5μM-valinomycin, 0.2mM-KCl and $CaCl_2$ varying from 0 to 250μM. In one series of experiments, ^3H_2O (0.5μCi/ml) and 10μM-^{86}RbCl (0.05μCi/ml) were included to determine ΔE, while in a second series ^3H_2O (0.5μCi/ml) and $^{45}CaCl_2$ (0.1μCi/ml) were included to enable pCa_o^{2+} to be calculated. ●, Experimental points. ○, Theoretical pCa_o^{2+} predicted for thermodynamic equilibrium of a Ca^{2+} uniport assuming a matrix activity coefficient for Ca^{2+} of 0.1 (see the text).

and the apparent stability constants for nitrilotriacetate (see the Experimental section), whereas in the isotopic experiment pCa_o^{2+} was calculated from the extramitochondrial $^{45}Ca^{2+}$. Good agreement was obtained between the two methods. The steady-state pCa_o^{2+} varied by less than 0.1 when matrix Ca^{2+} increased from 10 to 60nmol/mg of mitochondrial protein. Although more than 60nmol/mg of mitochondrial protein can be accumulated in the matrix (Fig. 4), the steady-state extramitochondrial free Ca^{2+} concentration maintained by the mitochondria also rises. Thus, even when pCa_o^{2+} is 5, less than 100nmol of Ca^{2+}/mg is accumulated in the matrix in the absence of added permeant weak acid (Lehninger *et al.*, 1967; Lehninger, 1974).

Dependence of pCa_o^{2+} on ΔE

Net accumulation of Ca^{2+} by mitochondria decreases ΔE and increases $-60\Delta pH$ (Gear *et al.*, 1967; Mitchell & Moyle, 1969). That 60nmol of Ca^{2+}/mg of mitochondrial protein can be accumulated by mitochondria without a rise in the steady-state pCa_o^{2+} (Fig. 4) implies that the steady state is unaffected by any changes in ΔE resulting from the uptake of this amount of Ca^{2+}. This was investigated in the experiment shown in Fig. 5. Increasing amounts of $^{45}Ca^{2+}$ were added to incubations of mitochondria in the presence of valinomycin and 0.2mM-KCl. Ca^{2+} accumulation and ΔE were determined in parallel. The presence of valinomycin did not affect the relation between pCa_o^{2+} and Ca^{2+} accumulated. A distinctly biphasic relationship between pCa_o^{2+} and ΔE was obtained. Additions of up to 50nmol of Ca^{2+}/mg of mitochondrial protein, which did not significantly decrease pCa_o^{2+} (Fig. 4), nevertheless lowered ΔE from 155 to 130mV.

The lowered pCa_o^{2+} (increased free Ca^{2+}) maintained when more than 50nmol of Ca^{2+}/mg of

protein was accumulated within the matrix was linearly correlated with ΔE, with a slope of 26mV/pCa_o^{2+}. This suggests that in this region pCa_o^{2+} is governed by thermodynamic considerations. The broken line in Fig. 5 depicts the pCa_o^{2+} that would be predicted from ΔE and the accumulation of Ca^{2+} in the matrix if the cation distribution were governed by thermodynamic equilibrium across a Ca^{2+} uniport on the assumption of a matrix activity coefficient for Ca^{2+} of 0.1. The actual activity coefficient for Ca^{2+} is, of course, unknown in this experiment; however, e.p.r. studies of Mn^{2+} accumulation indicate an activity coefficient for matrix Mn^{2+} that increases from 0.017 to 0.32 as the concentration of permeant weak acid increases to 50mM (Puskin *et al.*, 1976). Somewhat higher activity coefficients for Ca^{2+} can be deduced from the osmotic data of Azzone *et al.* (1976). Therefore the assumption of an activity coefficient of 0.1 for Ca^{2+} in the matrix compartment is unlikely to be greatly in error. The thermodynamic correlation breaks down when pCa_o^{2+} reaches 6, when pCa_o^{2+} becomes independent of ΔE, even though thermodynamic equilibration via a Ca^{2+} uniport would predict a pCa_o^{2+} of 6.8 when ΔE is 140mV.

In an experiment identical in other respects with that presented in Fig. 5, ΔpH was additionally determined. It has been suggested (Moyle & Mitchell, 1977*a*,*b*,*c*) that the pathway for Ca^{2+} uptake by mitochondria functions as a $2Ca^{2+}/P_i^{2-}$ symport or a (Ca monocarboxylate)$^+$ symport. As the equilibrium distribution of P_i or monocarboxylic acids across the inner membrane is governed by ΔpH, these are both thermodynamically equivalent to a Ca^{2+}/H^+ antiport (Moyle & Mitchell, 1977*b*). Over the range of high matrix Ca^{2+} content when ΔE decreases from 120 to 98mV (Fig. 5), ΔpH changed from −1.49 to −1.61. As the equilibrium distribution of Ca^{2+} across a Ca^{2+}/H^+ antiport is given by:

$$pCa_o^{2+} - pCa_i^{2+} = \frac{\Delta E}{60} + \Delta pH$$

where pCa_i^{2+} is the negative logarithm of the thermodynamic activity of Ca^{2+} in the matrix (Moyle & Mitchell, 1977*b*; Azzone *et al.*, 1977), it is possible to calculate a maximum activity coefficient for the matrix Ca^{2+} if it were accumulated by a Ca^{2+}/H^+ antiport. Calculated values vary from 3.5×10^{-5} to 8.3×10^{-5} as matrix Ca^{2+} increases. The activity coefficient would need to be even lower if account were taken of a steady-state cycling involving an independent efflux mechanism. It is difficult to see what matrix component could so effectively chelate up to 100nmol of Ca^{2+}/mg of protein (Fig. 5) and yet release 90% of the accumulated Ca^{2+} into a KCl medium containing no Ca^{2+} chelator within 10s of the addition of valinomycin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to abolish ΔE (D. G. Nicholls, unpublished observation). There are thus severe thermodynamic problems with the acceptance of a Ca^{2+}/H^+ antiport rather than a Ca^{2+} uniport mechanism.

It is of physiological significance to determine not only the effect of varying matrix Ca^{2+} on pCa_o^{2+} (Fig. 5), but also to determine the influence of metabolically induced modulations in ΔE on this parameter. Thus with isolated rat liver mitochondria, the transition from State 4 to State 3 (Chance & Williams, 1956) is accompanied by a decrease of some 20mV in ΔE from 150 to 130mV (Nicholls, 1974). To determine the effect of varying ΔE when the total Ca^{2+} content of the incubation is constant, the experiment depicted in Fig. 6 was performed. ΔE was varied from 155 to 56mV by increasing KCl concentrations in the presence of valinomycin. Osmotic equilibrium was assumed, with K^+ contributing maximally 50% of the osmotic support of the matrix (Nicholls, 1974).

When ΔE was less than 80mV (Fig. 6), the

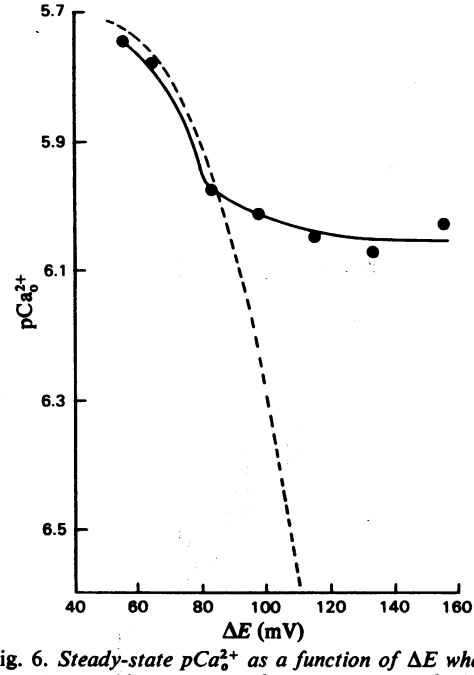

Fig. 6. *Steady-state pCa_o^{2+} as a function of ΔE when the latter is varied by increasing the concentration of K^+ in the presence of valinomycin*

Mitochondria were incubated under the conditions of Fig. 4 with the inclusion of 37.5μM-$CaCl_2$ (including endogenous mitochondrial Ca^{2+}) and KCl varying from 0.2 to 10mM. Further additions were as described in the legend to Fig. 5. ●, Experimental points. ----, Theoretical pCa_o^{2+} predicted for thermodynamic equilibrium of a Ca^{2+} uniport assuming a matrix activity coefficient for Ca^{2+} of 0.1.

steady-state pCa_o^{2+} adhered closely to the theoretical curve calculated for thermodynamic equilibrium of a Ca^{2+} uniport, assuming as before an activity coefficient of 0.1 for Ca^{2+} within the matrix. However, with increasing ΔE, the theoretical and experimental curves diverged, and pCa_o^{2+} again became invariant at about 6.05.

Role of permeant weak acids

It is well established that the capacity of liver mitochondria to take up Ca^{2+} is increased in the presence of anions that can permeate the inner membrane electroneutrally (Lehninger *et al.*, 1967; Lehninger, 1974). In Fig. 7, the steady-state pCa_o^{2+} maintained by liver mitochondria is plotted as a function of the accumulation of Ca^{2+} in the matrix under control conditions, in the presence of *N*-ethylmaleimide to inhibit movement of endogenous phosphate (Johnson & Chappell, 1973) and in the presence of 5mM-acetate. In each case the mitochondria were capable of accumulating 20nmol of Ca^{2+}/mg of protein while maintaining a pCa_o^{2+} of 6.1. However, with *N*-ethylmaleimide, pCa_o^{2+} fell immediately the matrix Ca^{2+} concentration was increased above this amount, thus effectively limiting the Ca^{2+} accumulated to 40nmol/mg of protein. In contrast, the presence of acetate, although not influencing pCa_o^{2+} at low Ca^{2+} accumulations, extended the capacity of the mitochondria to accumulate Ca^{2+} before the precipitous decrease in pCa_o^{2+} occurred.

The ratio of Ca^{2+} accumulated to net H^+ extrusion by the respiratory chain depends on the ability of electroneutrally permeable anions to redistribute across the membrane and lower ΔpH (Brand *et al.*, 1976*a*,*b*). It would thus be expected that ΔpH would increase more rapidly as Ca^{2+} is accumulated, if compensatory anion movements are prevented. The experiment depicted in Fig. 8, which was performed under conditions identical with those of Fig. 7 except for the inclusion of 10μM-[^{3}H]acetate to indicate ΔpH, confirmed this prediction. The increase in mitochondrial matrix volume during Ca^{2+} accumulation in the presence of 5mM-acetate (Lehninger *et al.*, 1967) was allowed for by assuming that the volume increased with accumulated acetate to maintain osmotic equilibrium.

In Fig. 9, the results of the two previous experiments are plotted to show the variation of the steady-state pCa_o^{2+} with transmembrane pH gradient. Regardless of the availability of electroneutrally permeable weak acid, the experimental points follow a common trend, with a steady-state pCa_o^{2+} of about 6.0 maintained until $-60\Delta pH$ rises to 95mV, above which pCa_o^{2+} falls rapidly.

Valinomycin was excluded from these experiments (cf. Figs. 5 and 6) in order to eliminate complexities

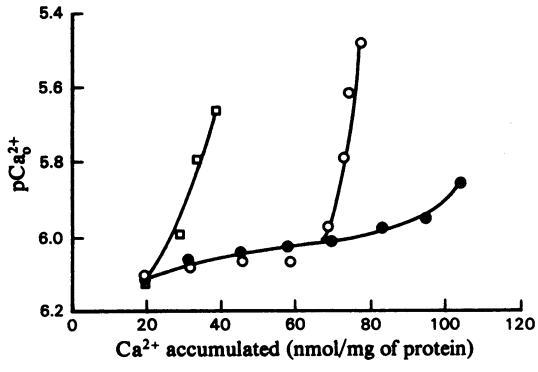

Fig. 7. *Steady-state pCa_o^{2+} as a function of Ca^{2+} accumulated in the matrix: effect of permeant weak acid*
Mitochondria (1.5mg of protein/ml of incubation mixture) were incubated at 30°C and pH7.0 in a medium containing 75mM-KCl, 10mM-Tes (potassium salt), 2mM-potassium succinate, 2mM-potassium nitrilotriacetate, 16μM-albumin, 1μM-rotenone, 1μg of oligomycin/ml, $^{45}CaCl_2$ (0.1μCi/ml of incubation mixture) varying from 0 to 140μM and ^3H_2O (0.5μCi/ml). Further additions: ○, none (control); □, 100μM-*N*-ethylmaleimide; ●, 5mM-potassium acetate.

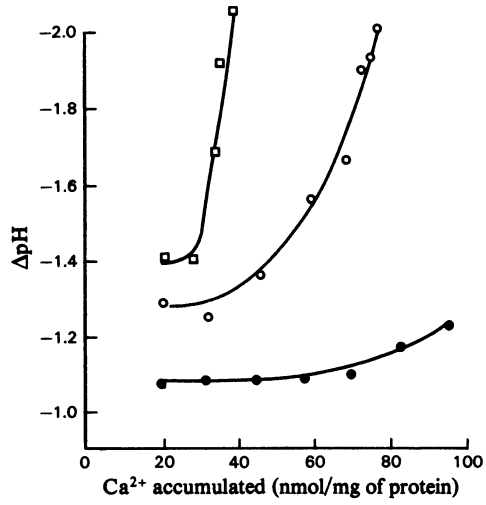

Fig. 8. *ΔpH as a function of Ca^{2+} accumulated in the matrix: effect of permeant weak acid*
Mitochondria were incubated under the conditions of Fig. 7. In addition to $^{45}Ca^{2+}$ distribution to determine pCa_o^{2+}, a parallel experiment was performed with the addition of 10μM-[^{3}H]acetate (0.5μCi/ml) and [^{14}C]sucrose (0.1μCi/ml) to determine ΔpH. ○, Control; □, +100μM-*N*-ethylmaleimide; ●, +5mM-potassium acetate.

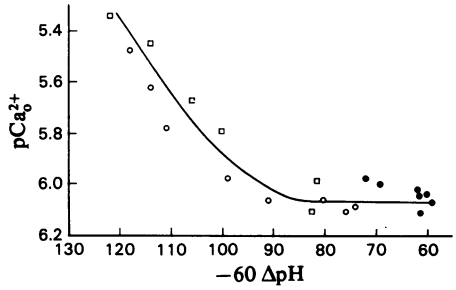

Fig. 9. *Steady-state pCa_o^{2+} as a function of ΔpH: lack of an effect of permeant weak acid*
The data of Figs. 7 and 8 are replotted. ○, Control; □, +100 μM-*N*-ethylmaleimide; ●, +5 mM-potassium acetate.

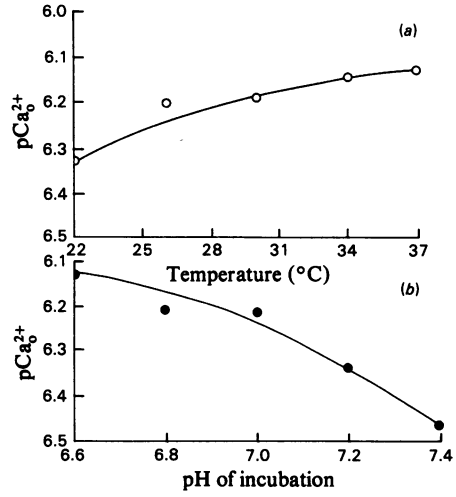

Fig. 10. *Steady-state pCa_o^{2+}: effect of temperature and extramitochondrial pH*
Mitochondria (1.5 mg of protein/ml of incubation mixture) were incubated in the medium described in the legend to Fig. 3 with the addition of 20 μM-$CaCl_2$. (*a*) Incubation temperature was varied, the pH being re-adjusted to 7.0 at each temperature. (*b*) pH was varied and the temperature was kept at 30°C.

arising from exchange of Ca^{2+} with K^+ (Scarpa & Azzone, 1970). However, if it is assumed that in the steady-state, $\Delta\tilde{\mu}_{H+}$ is maintained in the region of 220 mV (Mitchell & Moyle, 1969; Nicholls, 1974), then the increase in −60ΔpH implies a proportionate fall in ΔE. Comparison of Figs. 5 and 9 shows that a sufficient explanation for the effects of electroneutrally permeant weak acid or *N*-ethylmaleimide on the capacity of liver mitochondria to accumulate Ca^{2+} lies in the ability of weak acid to diminish the build-up of ΔpH, and consequently to aid the retention of a sufficient ΔE to maintain pCa_o^{2+} in the region of 6.1 (Figs. 5 and 6), and that it is not necessary to postulate a more direct action of weak acids on Ca^{2+} transport (Harris & Zaba, 1977; Bygrave *et al.*, 1977). In the presence of *N*-ethylmaleimide, steady-state conditions were retained for at least 5 min.

As H_2CO_3 can function as an electroneutrally permeant weak acid (Elder & Lehninger, 1973), and as no attempts were made to eliminate CO_2 from the incubation medium, it is possible that, even in the presence of *N*-ethylmaleimide, some movement of permeant weak acid into the matrix occurred during Ca^{2+} uptake, and that elimination of entry of weak acid would still further increase the rate at which ΔpH builds up as Ca^{2+} enters.

Effect of temperature and incubation pH on pCa_o^{2+}

The activity of the Ca^{2+} uniport of rat liver mitochondria is highly dependent on both the temperature and the pH of the incubation medium. Thus the Ca^{2+} conductance of the inner membrane, as determined in the presence of excess ionophore A23187, increased 4-fold over the temperature range 23–38°C, and the conductance increases 5.7-fold when the pH of the medium is raised from 6.8 to 8.0 (Heaton & Nicholls, 1976). It should be emphasized that studies of Ca^{2+} cycling in the presence of ionophore A23187 (Heaton & Nicholls, 1976; Hutson *et al.*, 1976; Hutson, 1977) determine the absolute rate of Ca^{2+} entry via the uniport, in contrast with studies of initial uptake rates and the steady-state pCa_o^{2+} investigated here, which are both dependent on the resultant of uptake and efflux pathways. Any factor that influences the activity of uptake and efflux pathways unequally should thus influence pCa_o^{2+}.

Fig. 10 shows the effects of temperature and pH of the incubation mixture on the steady-state pCa_o^{2+}. Increasing the temperature from 22 to 37°C decreased pCa_o^{2+} from 6.33 to 6.13, reflecting a 1.5-fold increase in free Ca^{2+}. An increase in external pH decreased the steady-state free Ca^{2+} concentration, such that over the pH range 7–7.4 pCa_o^{2+} increased by 0.65 unit/pH unit. In view of the increase in activity of the uniport with pH (Heaton & Nicholls, 1976), it is likely that activation of the uniport, rather than inhibition of the efflux pathway, is responsible for the altered steady state.

Effect of Mg^{2+} on the steady-state pCa_o^{2+}

The presence of Mg^{2+} in the incubation medium decreases the initial rate of Ca^{2+} uptake by liver mitochondria at a given pCa_o^{2+} (Hutson *et al.*, 1976; Hutson, 1977; Åkerman *et al.*, 1977) and increases

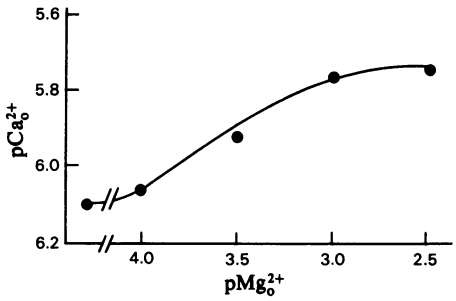

Fig. 11. *Steady-state* pCa_o^{2+}: *effect of* Mg^{2+}
Mitochondria (1.5mg of protein/ml of incubation mixture) were incubated at 30°C and pH7.0 in a medium containing 75mM-NaCl, 10mM-Tes (sodium salt), 16μM-albumin, 1μM-rotenone, 2mM-sodium succinate, 5mM-sodium acetate, 0.1mM-sodium *N'*-(2-hydroxyethyl)ethylenediamine-*NNN'*-triacetate, 50μM-$^{45}CaCl_2$ (0.1μCi/ml), ^3H_2O (0.5μCi/ml) and $MgCl_2$ varying from 0 to 2mM. pCa_o^{2+} and pMg_o^{2+} were calculated by the reiterative method of Portzehl *et al.* (1964).

the apparently sigmoidal relation between initial rates of uptake and free external Ca^{2+} concentration when the latter is plotted on a linear scale. Mg^{2+} would thus be expected to lower the pCa_o^{2+} at which the rate of uptake via the uniport balances the rate of efflux, and hence alter the steady state. The magnitude of this effect of Mg^{2+} is depicted in Fig. 11. In order to allow the co-existence of millimolar Mg^{2+} and micromolar free Ca^{2+} concentrations, *N'*-(2-hydroxyethyl)-ethylenediamine-*NNN'*-triacetate was used, and free Mg^{2+} concentrations in the steady state were calculated by the reiterative method of Portzehl *et al.* (1964). pCa_o^{2+} was determined isotopically, the selectivity of the ion-selective electrode being insufficient. Increasing pMg_o^{2+} to 2.5 (3mM) decreased pCa_o^{2+} at the steady state from 6.1 in the absence of added Mg^{2+} to 5.75.

Discussion

The steady-state cytoplasmic free Ca^{2+} concentration maintained by mitochondria represents a parameter of great importance for the regulation of metabolism in the cell. The findings in this paper, that mitochondria possess a mechanism that allows a precise regulation of pCa_o^{2+} even if ΔE or matrix accumulation varies, are clearly of significance in assessing the physiological role of mitochondrial Ca^{2+} transport.

Nature of the efflux pathway

The earliest suggestions for an independent efflux pathway (Drahota *et al.*, 1965; Vasington *et al.*, 1972; Sordahl, 1974) were not within the framework of the chemiosmotic theory, but instead envisaged a leak pathway competing against a chemically coupled Ca^{2+} pump. Puskin *et al.* (1976) introduced chemiosmotic concepts by proposing competition between an efflux pathway and a Ca^{2+} uniport, and suggesting that Ca^{2+} cycling could rationalize the conflicting demands of a high ΔE for oxidative phosphorylation while at the same time preventing excessive raising of pCa_o^{2+} by a Ca^{2+} uniport. This interpretation was modified by Pozzan *et al.* (1977), who proposed that uptake and efflux both occurred through the Ca^{2+} uniport, efflux occurring through carriers located in regions of the membrane experiencing low local electrical fields. The present paper provides no evidence for such local field heterogeneity as only bulk-phase gradients were determined. However, it is difficult to see why the apparent Nernst equilibrium for Rb^+ in the presence of valinomycin, from which ΔE is calculated, would not be equally distorted by any local heterogeneity.

Steady-state conditions are, of course, attained when the rates of uptake and efflux are equal (Puskin *et al.*, 1976; Azzone *et al.*, 1977). Little evidence is available as to the nature of the efflux pathway. In heart and brain mitochondria, the activity of the efflux pathway is activated by Na^+ (Crompton *et al.*, 1976, 1977, 1978; Nicholls, 1978), and it has been suggested that efflux occurs as a Na^+-for-Ca^{2+} antiport. However, the efflux in liver is not Na^+-dependent (Crompton *et al.*, 1978), and the alternative hypothesis of a $Ca^{2+}/2H^+$ antiport should be investigated. The activity of the efflux pathway determined by either Ruthenium Red addition (Stucki & Ineichen, 1974; Puskin *et al.*, 1976; Crompton *et al.*, 1978) or raising pCa_o^{2+} (Fig. 2; Puskin *et al.*, 1976) falls in the range 1.2–5nmol of Ca^{2+}/min per mg of protein, depending on the conditions used. The energy dissipation inherent in this cycling is not excessive; a State-4 respiration of 2nmol of O_2/min per mg of protein would be sufficient to maintain Ca^{2+} cycling via a Ca^{2+} uniport and a $Ca^{2+}/2H^+$ antiport at a rate of 5nmol of Ca^{2+}/min per mg of protein.

The criticism has been raised (Pozzan *et al.*, 1977) that, although most evidence for an independent efflux pathway stems from the use of Ruthenium Red, the effects of the inhibitor are highly complex. It should be emphasized that in the present work use of the inhibitor is avoided, and the existence of an independent efflux pathway is deduced from the regulation of pCa_o^{2+} under more closely physiological conditions.

Activity of the Ca^{2+} *uniport*

The activity of the Ca^{2+} uniport is highly dependent on pCa_o^{2+}. By introducing ionophore A23187 to

provide a $Ca^{2+}/2H^+$ (Reed & Lardy, 1972) efflux pathway in excess of the activity of the native Ca^{2+} uniport (Heaton & Nicholls, 1976; Hutson *et al.*, 1976; Hutson, 1977), it is possible to study the uniport in isolation, under conditions where a considerable Ca^{2+} electrochemical potential gradient is maintained across the uniport (Heaton & Nicholls, 1976). In Fig. 12, the rate of Ca^{2+} cycling in the presence of ionophore A23187 is plotted as a function of pCa_o^{2+} for the conditions adopted in this paper (30°C, pH7.0). As has been shown (Heaton & Nicholls, 1976, Hutson *et al.*, 1976; Hutson, 1977), the rate of Ca^{2+} cycling is highly dependent on pCa_o^{2+}. When pCa_o^{2+} was less than 5.3, the rate of Ca^{2+} cycling appeared to be limited by the rate at which the respiratory chain can expel protons, rather than by any characteristic of the uniporter itself (Heaton & Nicholls, 1976; Hutson, 1977). As the steady-state pCa_o^{2+} was increased above 5.3, the rate of cycling became rapidly inhibited. Also included in Fig. 12 are experimental points calculated, in the absence of ionophore A23187, for the activity of the uniport close to steady-state conditions. These results were obtained from Figs. 1 and 3. The activity of the uniport decreased continuously by 2-fold for each 0.12 unit increase in pCa_o^{2+} (1.32-fold decrease in free Ca^{2+}) over the range 5.3–6.3.

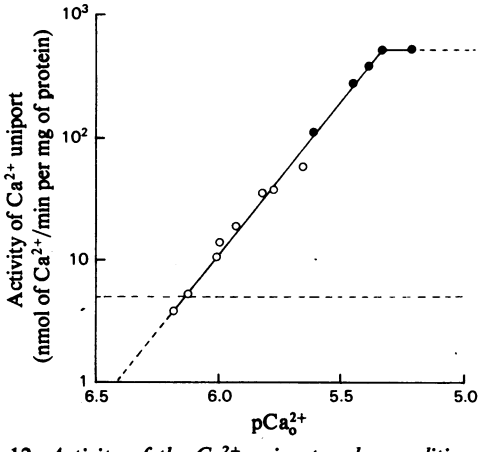

Fig. 12. *Activity of the Ca^{2+} uniport under conditions of excess Ca^{2+} electrochemical potential gradient, as a function of pCa_o^{2+}*

●, Mitochondria (1.5mg of protein/ml of incubation mixture) were incubated at 30°C and pH7.0 in a medium containing 75mM-KCl, 10mM-Tes (potassium salt), 2mM-potassium succinate, 2mM-potassium nitrilotriacetate, 16μM-albumin, 1μM-rotenone and 1μg of oligomycin/ml with the addition of $CaCl_2$ varying from 0 to 60μM. Respiration was measured before and after addition of 4.6μM-ionophore A23187 and the rate of Ca^{2+} cycling calculated as previously described (Heaton & Nicholls, 1976). For the calculation of pCa_o^{2+}, endogenous mitochondrial Ca^{2+} was assumed to contribute to the extramitochondrial pool. ○, Activity of the Ca^{2+} uniport was calculated from the net uptake rates obtained from Ca^{2+}-selective-electrode experiments under the conditions of Figs. 1 and 2, with the assumption of a constant efflux of 5nmol of Ca^{2+}/min per mg of mitochondrial protein (given by the horizontal broken line).

Kinetic regulation of pCa_o^{2+}

In order to rationalize the kinetic behaviour of the Ca^{2+} uniport and the empirical regulation of pCa_o^{2+} described in this paper, it is necessary merely to superimpose a constant efflux rate in the region of 5nmol of Ca^{2+}/min per mg of protein (the horizontal broken line in Fig. 12). As efflux in the presence of Ruthenium Red appears to be linear with time (Puskin *et al.*, 1976; Crompton *et al.*, 1978) and therefore largely independent of matrix and extramitochondrial free Ca^{2+} concentrations, it is reasonable to assume that efflux is unaffected by variations in pCa_o^{2+} over the range studied.

To illustrate how pCa_o^{2+} may be regulated, consider the case of mitochondria, under the conditions of Fig. 12, which are instantaneously exposed to 4μM-free Ca^{2+} ($pCa_o^{2+} = 5.4$). As long as ΔE remains above about 130mV (Fig. 5), Ca^{2+} uptake will be limited kinetically rather than thermodynamically. Thus there will be an initial uptake rate through the uniport in the region of 300nmol of Ca^{2+}/min per mg of protein, a rate that will fall very rapidly as pCa_o^{2+} increases. As pCa_o^{2+} approaches 6.1, rates of uptake and efflux become closer, until steady-state conditions are attained with Ca^{2+} cycling at 5nmol/min per mg of protein. If the free Ca^{2+} were lowered below the steady-state concentration ($pCa_o^{2+} > 6.1$), uptake through the uniport would be less than the activity of the efflux pathway and a net efflux would occur (e.g. Fig. 1) until the same steady state was regained. The rate of steady-state cycling is kinetically limited by the rate at which external Ca^{2+} interacts with the uniporter, and is therefore independent of the Ca^{2+} electrochemical gradient across the uniporter, as long as this is in excess (Azzone *et al.*, 1977). The mitochondria are thus able to regulate pCa_o^{2+} with extreme precision, even though matrix Ca^{2+} and ΔE might vary widely (Figs. 4, 5, 6 and 7).

A fine control of pCa_o^{2+} might be obtained by regulating the activity of the efflux pathway: thus a doubling in efflux would lead to a decrease of 0.12 in pCa_o^{2+} (Fig. 12). It is significant that, in heart and brain, Na^+ appears to perform such a regulatory role (Crompton *et al.*, 1976, 1978; Nicholls, 1978).

A cycling system rationalizes the differing abilities of Ca^{2+} chelators and proton translocators to induce Ca^{2+} efflux from mitochondria. When ΔE is in the region of 160mV, equilibrium distribution of Ca^{2+}

across a Ca^{2+} uniport would predict a pCa_o^{2+} in the region of 8 (Figs. 5 and 6). This is compatible with the pCa^{2+} achieved by chelating 50 μM-Ca^{2+} with 1 mM-EGTA at pH 7.0. When EGTA is added to respiring mitochondria, there is therefore only a slow efflux of Ca^{2+} (Puskin *et al.*, 1976) because the Ca^{2+} uniporter is thermodynamically unable to reverse, and all efflux still occurs only through the slow efflux pathway. In contrast, a sufficient concentration of proton translocator induces extremely rapid efflux of Ca^{2+} (Puskin *et al.*, 1976) by lowering ΔE to values that allow thermodynamic reversal of the highly active Ca^{2+} uniport, and hence efflux through both pathways.

Thermodynamic regulation of pCa_o^{2+}

Under most physiological conditions it is likely that the Ca^{2+} electrochemical gradient across the Ca^{2+} uniport is sufficiently in excess to maintain the extramitochondrial Ca^{2+} buffered at a pCa_o^{2+} of 6. However, *in vitro* it is possible to lower ΔE to regions where thermodynamic equilibrium of the uniport would predict an extramitochondrial free Ca^{2+} concentration higher than 1 μM (pCa_o^{2+} less than 6) (Figs. 5 and 6). Clearly, under these conditions there are no means by which pCa_o^{2+} can be maintained at 6. As the conductance of the Ca^{2+} uniport increases extremely rapidly as pCa_o^{2+} falls below 6 (Fig. 12; Heaton & Nicholls, 1976), such that at a pCa_o^{2+} of 5.4 the maximal activity of the uniport is 100 times greater than that of the efflux pathway, the steady-state distribution approaches asymptotically to the predicted thermodynamic equilibrium of the Ca^{2+} uniport (Rottenberg & Scarpa, 1974). In this context, the effects of *N*-ethylmaleimide and permeant weak acids on Ca^{2+} accumulation are seen to be entirely secondary to their effects on the relative magnitudes of ΔE and $-60\,pH$ (Figs. 7–9).

In conclusion, it has long been suggested that Ca^{2+} continuously cycles across the inner mitochondrial membrane by independent influx and efflux pathways (Drahota *et al.*, 1965; Vasington *et al.*, 1972; Stucki & Ineichen, 1974; Sordahl, 1974; Puskin *et al.*, 1976). However, it is apparent that Ca^{2+} cycling is not merely a means of resolving the dilemma inherent in maintaining a high ΔE for oxidative phosphorylation while at the same time maintaining a sufficiently low pCa_o^{2+} for cytoplasmic metabolism (Puskin *et al.*, 1976). It would appear that the fine regulation of pCa_o^{2+} by mitochondria is analogous to the fine regulation of carbohydrate metabolism by substrate cycling (for review see Newsholme, 1976), where sensitive control is attained at the expense of metabolic energy to maintain the dissipative cycling. That mitochondria have evolved such a complex mechanism supports the importance of their role as regulators of free cytoplasmic Ca^{2+} concentration (Bygrave, 1977).

This work is supported by a grant from the Medical Research Council. The expert technical assistance of Mr. C. Adam is gratefully acknowledged.

References

Åkerman, K. E. O., Wikström, M. K. F. & Saris, N.-E. (1977) *Biochim. Biophys. Acta* **464**, 287–294
Ashley, C. C. & Caldwell, P. C. (1974) *Biochem. Soc. Symp.* **39**, 29–50
Azzone, G. F., Bragadin, M., Pozzan, T. & Dell'Antone, P. (1976) *Biochim. Biophys. Acta* **459**, 96–109
Azzone, G. F., Pozzan, T., Massari, S., Bragadin, M. & Dell'Antone, P. (1977) *FEBS Lett.* **78**, 21–24
Bragadin, M., Dell'Antone, P., Pozzan, T., Volpato, O. & Azzone, G. F. (1975) *FEBS Lett.* **60**, 354–358
Brand, M. D., Reynafarje, B. & Lehninger, A. L. (1976*a*) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 437–441
Brand, M. D., Chen, C.-H. & Lehninger, A. L. (1976*b*) *J. Biol. Chem.* **251**, 968–974
Bygrave, F. L. (1977) *Curr. Top. Bioenerg.* **6**, 259–318
Bygrave, F. L., Ramachandran, C. & Smith, R. L. (1977) *FEBS Lett.* **83**, 155–158
Chance, B. & Williams, G. R. (1956) *Adv. Enzymol. Relat. Subj. Biochem.* **17**, 65–134
Crompton, M., Capano, M. & Carafoli, E. (1976) *Eur. J. Biochem.* **69**, 453–462
Crompton, M., Kunzi, M. & Carafoli, E. (1977) *Eur. J. Biochem.* **79**, 549–558
Crompton, M., Moser, R., Lüdi, H. & Carafoli, E. (1978) *Eur. J. Biochem.* **82**, 25–31
Douglas, W. W. (1974) *Biochem. Soc. Symp.* **39**, 1–28
Drahota, Z., Carafoli, E., Rossi, C. S., Gamble, R. L. & Lehninger, A. L. (1965) *J. Biol. Chem.* **240**, 2712–2720
Elder, J. A. & Lehninger, A. L. (1973) in *Mechanisms in Bioenergetics* (Azzone, G. F., Ernster, L., Papa, S., Quagliariello, E. & Siliprandi, N., eds.), pp. 513–526, Academic Press, New York
Gear, A. R. L., Rossi, C. S., Reynafarje, B. & Lehninger, A. L. (1967) *J. Biol. Chem.* **242**, 3403–3413
Harris, E. J. & Zaba, B. (1977) *FEBS Lett.* **79**, 284–290
Heaton, G. M. & Nicholls, D. G. (1976) *Biochem. J.* **156**, 635–646
Hutson, S. M. (1977) *J. Biol. Chem.* **252**, 4539–4545
Hutson, S. M., Pfeiffer, D. R. & Lardy, H. A. (1976) *J. Biol. Chem.* **251**, 5251–5258
Johnson, R. N. & Chappell, J. B. (1973) *Biochem. J.* **134**, 769–774
Lehninger, A. L. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1520–1524
Lehninger, A. L., Carafoli, E. & Rossi, C. S. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol.* **29**, 259–320
Mitchell, P. & Moyle, J. (1969) *Eur. J. Biochem.* **7**, 471–484
Moyle, J. & Mitchell, P. (1977*a*) *FEBS Lett.* **73**, 131–136
Moyle, J. & Mitchell, P. (1977*b*) *FEBS Lett.* **77**, 136–140
Moyle, J. & Mitchell, P. (1977*c*) *FEBS Lett.* **84**, 135–140
Newsholme, E. A. (1976) *Biochem. Soc. Symp.* **41**, 61–109
Nicholls, D. G. (1974) *Eur. J. Biochem.* **50**, 305–315
Nicholls, D. G. (1978) *Biochem. J.* **170**, 511–522

Portzehl, H., Caldwell, P. C. & Ruegg, J. C. (1964) *Biochim. Biophys. Acta* **79**, 581–591
Pozzan, T., Bragadin, M. & Azzone, G. F. (1976) *Eur. J. Biochem.* **71**, 93–99
Pozzan, T., Bragadin, M. & Azzone, G. F. (1977) *Biochemistry* **16**, 5618–5625
Puskin, J. S. & Gunter, T. E. (1973) *Biochem. Biophys. Res. Commun.* **51**, 797–803
Puskin, J. S., Gunter, T. E., Gunter, K. K. & Russell, P. R. (1976) *Biochemistry* **15**, 3834–3842
Reed, K. C. & Bygrave, F. L. (1975) *Eur. J. Biochem.* **55**, 497–504
Reed, P. W. & Lardy, H. A. (1972) *J. Biol. Chem.* **247**, 6970–6977
Reynafarje, B. & Lehninger, A. L. (1977) *Biochem. Biophys. Res. Commun.* **77**, 1273–1279
Rottenberg, H. & Scarpa, A. (1974) *Biochemistry* **13**, 4811–4817
Scarpa, A. & Azzone, G. F. (1970) *Eur. J. Biochem.* **12**, 328–335
Selwyn, M. J., Dawson, A. P. & Dunnett, S. J. (1970) *FEBS Lett.* **10**, 1–5
Sordahl, L. A. (1974) *Arch. Biochem. Biophys.* **167**, 104–115
Stucki, J. W. & Ineichen, E. A. (1974) *Eur. J. Biochem.* **48**, 365–375
Vasington, F. D., Gazzotti, P., Tiozzo, R. & Carafoli, E. (1972) *Biochim. Biophys. Acta* **256**, 43–54